PREDICTING THE RELATIVE SENSITIVITY OF STURGEONS TO ARYL HYDROCARBON RECEPTOR AGONISTS

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By

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ABSTRACT

Along with overexploitation and habitat loss, pollution is one cause for decreases in populations of fishes. One class of pollutants of particular global environmental concern to fishes are dioxin-like compounds (DLCs). DLCs elicit their toxicity through activation of the aryl hydrocarbon receptor (AHR). Despite this common mechanism of all DLCs, dramatic differences in sensitivity exist among fishes. Sturgeons (Acipenseridae) are an ancient family of fishes in which most species are endangered. It is hypothesized that pollutants, including DLCs, might be contributing to the observed declines in populations because sturgeons have a unique life-style that makes them susceptible to exposure to bioaccumulative chemicals. However, determining sensitivities of sturgeons to DLCs through traditional *in vivo* toxicity testing is not feasible for practical and ethical reasons. Therefore, the aim of this research was to develop a mechanism-based biological model capable of predicting the relative sensitivity of sturgeons to DLCs. This mechanism-based biological model was developed through investigations into the AHR and AHR-mediated molecular and biochemical responses of white sturgeon (*Acipenser transmontanus*) relative to teleost fishes and another species of sturgeon. White sturgeon responded to activation of the AHR in a manner that is consistent with responses of teleost fishes (induction of cytochrome P450 1A). Two AHRs with similar levels of expression were identified in white sturgeon, an AHR1 that resembles AHR1s of tetrapods and an AHR2 that resembles AHR2s of other fishes. Both AHR1 and AHR2 of white sturgeon were activated by exposure to five selected DLCs *in vitro* with effect concentrations less than any other AHR tested to date. These findings were suggestive that white sturgeon might be among the most sensitive species of fish to exposure to DLCs. These findings raised the question as to whether other members of the

Acipenseridae are similarly sensitive to exposure to DLCs. Therefore, AHR1 and AHR2 were identified in a second species of sturgeon, the lake sturgeon (*Acipenser fulvescens*). AHR1 of lake sturgeon had the same *in vitro* sensitivity to activation by the five selected DLCs as AHR1 of white sturgeon, while AHR2 of lake sturgeon was 10-fold less sensitive to activation by the five selected DLCs relative to AHR2 of white sturgeon. AHR2 has been demonstrated to drive adverse effects of DLCs in other fishes, while AHR1 has no known role in mediating toxicities in fishes. Therefore, it was hypothesized that white sturgeon are 10-fold more sensitive to DLCs relative to lake sturgeon *in vivo*. However, there were uncertainties in whether differences in activation of the AHR are representative of differences at higher levels of biological organization. Therefore, whole transcriptome and whole proteome responses were investigated following exposure to equipotent concentrations of three agonists of the AHR. Equal activation of the AHR of white sturgeon resulted in similar global responses and magnitude of responses across levels of biological organization. This supports the hypothesis that activation of the AHR is predictive of apical level adverse effects of regulatory relevance, such as mortality of embryos. In order to test this hypothesis, AHR1s and AHR2s from seven species of fish of known sensitivity were investigated and the relationship between *in vitro* and *in vivo* sensitivities were characterized for the model DLC, 2,3,7,8-TCDD. All AHR1s and AHR2s were activated *in vitro* by 2,3,7,8-TCDD. There was no significant linear relationship between *in vitro* sensitivity of AHR1 and *in vivo* sensitivity among the seven species. However, there was a highly significant linear relationship between *in vitro* sensitivity of the AHR2 and *in vivo* sensitivity. The equation of this relationship enables the prediction of the *in vivo* sensitivity of any species of fish based on *in vitro* sensitivity of the AHR2. This predictive model could be essential in guiding more objective risk assessments of DLCs to fishes, including endangered species such as sturgeons.

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LIST OF ABBREVIATIONS

°C – degree Celsius

< D – undetectable

Å – angstrom

α – alpha

β – beta

βNF – β-naphthoflavone

δ – delta

γ – gamma

μg – microgram

μL – microliter

μM – micromolar

3-D – three dimensional

ADH – aldehyde dehydrogenases

ADME – adsorption, distribution, metabolism, elimination

AHR – aryl hydrocarbon receptor

AHR2α – aryl hydrocarbon receptor, family 2, α isoform

AHRR – aryl hydrocarbon receptor repressor

Ala – Alanine

ANOVA – analysis of variance

ANCOVA – analysis of covariance

AO – adverse outcome

AOP – adverse outcome pathway

AR – androgen receptor

ARNT – aryl hydrocarbon receptor nuclear translocator

ARNT2 – aryl hydrocarbon receptor nuclear translocator, family 2

ATRF – Aquatic Toxicology Research Facility

BaP – benzo[a]pyrene

BC – British Columbia

BCA – bicinchoninic acid

B.C.E. – before the common era

bHLH – basic/helix-loop-helix

bm – body mass

BMP – bone morphometric protein

bp – base pair

BSA – bovine serum albumin

bw – body weight

cDNA – complementary deoxyribonucleic acid

Castp – Computed Atlas of Surface Topography of Proteins

CIA – coinertia analysis

cm – centimeter

COS-7 – African green monkey kidney fibroblast cells

COX – cytochrome c oxidase

CPM – count per million

CYP1A – cytochrome P450, family 1, subfamily A

CYP7A1A – cholesterol 7-alpha-monooxygenase, family 1, subfamily a

DLC – dioxin-like compound

DRE – dioxin response element

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EC_{20} – concentration of chemicals causing 20 % effect

EC_{50} – concentration of chemicals causing 50 % effect

EC_{80} – concentration of chemicals causing 80 % effect

ER – estrogen receptorr

EROD – ethoxyresorufin *O*-deethylase

FAT – fatty acid translocase

FBP1A - fructose-1,6-bisphosphatase, family 1, subfamily a

FBS – fetal bovine serum

FDR – false discovery rate

FOX – forkhead box

g – gram

GEO – Gene Expression Omnibus

GO – gene ontology

GST – glutathione S-transferases

h or hr or hrs – hour(s)

HAH – halogenated aromatic hydrocarbon

HC – HEPES-Cortland

HIF1α – hypoxia inducible factor, family 1, α isoform

His – histidine

IHH – Indian hedgehog

i.p. – intraperitoneal injection

kDa – kilodaltons

KEGG – Kyoto Encyclopedia of Genes and Genomes

kg – kilogram

L – litre

LBD – ligand binding domain

LD_{50} – dose causing 50 % lethality

Leu – leucine

LOEC – lowest observed effect concentration

LRG – luciferase reporter gene assay

LS – lake sturgeon

M – molar

mM – millimolar

mm – millimeter

mg – milligram

MIE – molecular initiating event

min or mins – minute(s)

mo or mos – month(s)

mRNA – messenger ribonucleic acid

MROD – methoxyresorufin *O*-deethylase

MS-222 – tricaine mechanesulfonate

n – sample size

NA – not analyzed

NADPH – nicotinamide adenine dinucleotide phosphate

NCBI – National Center for Biotechology Information

ng - nanogram

nM – nanomolar

nm – nanometer

NQO – NAD(P)H quinone oxidoreductases

NSERC – Natural Sciences and Engineering Research Council of Canada

p – sample proportion

PAH – polycyclic aromatic hydrocarbon

PAS – Per-Arnt-Sim

PCB – polychlorinated biphenyl

PCB 77 – 3,3’,4,4’-tetrachlorobiphenyl

PCB 105 – 2,3,3’,4,4’-pentachlorobiphenyl

PCB 126 – 3,3’,4,4’,5-pentachlorobiphenyl

PCB 169 – 3,3’,4,4’,5,5’-hexachlorobphenyl

PCDD – polychlorinated dibenzo-*p*-dioxin

PCDF – polychlorinated dibenzofuran

PCR – polymerase chain reaction

PDB – Protein Data Bank

PeCDF – 2,3,4,7,8-pentachloro-dibenzofuran

pg - picogram

pmol – picomolar

PPAR – peroxisome proliferation-activated receptor

PRIDE – Proteomics Identifications

PROD – pentoxyresorufin *O*-deethylase

PXR – pregnane X receptor

GC-MS – gas chromatography and mass spectrometry

qPCR or qRT-PCR – quantitative polymerase chain reaction

RACE-PCR – rapid amplification of cDNA ends polymerase chain reaction

ReP – relative potency

ReS – relative sensitivity

RIN – RNA integrity number

RNA – ribonucleaic acid

rpm – revolutions per minute

RT – rainbow trout

S.D. – standard deviation

S.E. or S.E.M – standard error of the mean

SOD – superoxide dismutase

SOX9 – sex determining region Y-box, family 9

TCDD – 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin

TCDD-EQ – 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin equivelents

TCDF – 2,3,7,8-tetrachloro-dibenzofuran

TEF – toxic equivalency factor

$TEF_{WHO\text{-}Birds}$ – WHO TEFs developed for birds

$TEF_{WHO\text{-}Fish}$ – WHO TEFs developed for fishes

$TEF_{WHO\text{-}Mammals}$ – WHO TEFs deceloped for mammals

TEQ – toxic equivalency quotient

Thr – threonine

Tyr – tyrosine

UGT – UDP-glucuronosyltransferases

US or USA – United States of America

v/v – volume to volume

Val – valine

WHO – World Health Organization

WNT – wingless-type MMTV integrated site family

WS – white sturgeon

ww – wet weight

xg – times gravity

yr or yrs – year(s)

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 8 contains a general discussion and overall conclusion. Chapters 2, 3, 4, 5, 6, and 7 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. A condensed version of Chapter 1 was published as a review article in Environmental Science and Pollution Research, Chapter 2 and Chapter 3 have been published in Aquatic Toxicology, Chapter 4, Chapter 5, and Chapter 6 have been published in Environmental Science & Technology, Chapter 7 is in preparation for submission for publication. Full citations for the published review and research papers and a description of author contributions are provided following the preface of each chapter. As a result of the manuscript-style format, there is some repetition of material in the introduction and material and methods sections of the thesis. The tables, figures, supporting information, and references cited in each chapter have been reformatted here to a consistent thesis style. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with research chapters are presented in the Appendix section at the end of this thesis as Cx.Sy format, where 'Cx' indicates chapter number; 'Sy' indicates figure or table number.

CHAPTER 1

1 GENERAL INTRODUCTION

PREFACE

Chapter 1 is a general introduction and literature review of the topics of sturgeons, dioxin-like compounds, the aryl hydrocarbon receptor, and application of predictive toxicology. Chapter 1 includes the overall goals and objectives of the project in general and each study in particular, and includes null hypotheses.

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Author contributions:

Jon A. Doering (University of Saskatchewan) developed the concept, reviewed the literature, and drafted the manuscript.

Drs. John P. Giesy, Steve Wiseman, and Markus Hecker (all University of Saskatchewan) provided inspiration for, commented on, and edited the manuscript.

1.1 Sturgeons of North America

For as long as 65 million years, fish similar to modern day sturgeons have lived in the waters of what is now North America (Hochleithner and Gessner, 2001; Wilimovsky, 1956). These ancient fishes have an almost entirely cartilaginous skeleton and a shark-like body covered in five separate rows of large scutes (Moyle and Cech, 2004). Sturgeons are among the largest freshwater fish, with white sturgeon (*Acipenser transmontanus*) being able to reach upwards of 6 m in length and weigh over 800 kg (Hochleithner and Gessner, 2001). Sturgeons are extremely long-lived and can reach more than 100 yrs of age, and they might not reach sexual maturity until they are 20 yrs of age or older (Kardong, 2006). Only one family of sturgeons is found in North America, the Acipenseridae, which is divided into two genera, *Acipenser* and *Scaphirhynchus* (LeBreton et al., 2004). Within these two genera, a total of eight species and one sub-species exist: the shortnose sturgeon, *Acipenser brevirostrum*; lake sturgeon, *A. fluvescens*; green sturgeon, *A. medirostris*; Atlantic sturgeon, *A. oxyrinchus oxyrinchus*, gulf sturgeon, *A. oxyrinchus desotoi*; white sturgeon, *A. transmontanus*; pallid sturgeon, *Scaphirhynchus albus*; shovelnose sturgeon, *S. platorynchus*; and Alabama sturgeon, *S. suttkusi* (LeBreton et al., 2004). An additional fifteen extant species of sturgeons are known from Eurasia (Hochleithner and Gessner, 2001).

Sturgeon are widely distributed throughout the northern hemisphere and inhabit large lakes and river systems as well as coastal marine habitats, with some species known to migrate between freshwater and marine systems (Hochleithner and Gessner, 2001). In North America, sturgeon can be found in major drainage systems, including the St. Lawrence and Columbia Rivers, the Great Lakes, and south in the Ohio, Missouri, and Mississippi river systems

(LeBreton et al., 2004). Anadromous species of sturgeon can be found along both the Atlantic and Pacific coasts from Canada and Alaska to the southern United States (LeBreton et al., 2004). Armed with their large size and protective scutes, sturgeons have few known predators beyond their juvenile life-stages (Helfman et al., 2006). Sturgeon spend the majority of their time moving along the sediment in search of prey such as benthic invertebrates and dead or young fish (Kardong, 2006). Vision plays a minor role in locating prey, with touch and chemoreception by use of four barbells located on the ventral surface in front of the mouth likely being most important (Helfman, et al., 2006). Electrolocation by use of rostral ampullary organs, similar to those of elasmobranchs, might also aid sturgeons in the locating prey (Helfman, et al., 2006).

Historically, sturgeons have been of great economic importance everywhere they were found. Prior to European influences, tribal groups in North America were dependant on sturgeon for their meat, oil, roe, skin, and "isinglass" – a preservative and beer fining ingredient, which has made sturgeons an important part of aboriginal culture (LeBreton et al., 2004). Once European descendants discovered their economic value, sturgeon were almost completely fished to extinction from the waterways of North America within a short period of time (LeBreton et al., 2004). Similarly, sturgeon have been prized in Eurasia with historic and archaeological evidence of sturgeon fisheries dating back as far as 3,500 B.C.E. in the Danube area and 1,104 B.C.E. in China (Hochleithner and Gessner, 2001). Over the last 100 yrs most sturgeon fisheries world-wide have been reduced to a fraction of what they once were (LeBreton et al., 2004). The declines in sturgeon populations have been attributed to several activities of humans, including overharvesting, alteration of habitats, and pollution. However, only during the last 30 yrs has there been an increase in the scientific interest in the conservation of sturgeons in North America (LeBreton et al., 2004).

1.2 Dioxin-like compounds

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are anthropogenic pollutants of potential concern to fishes, including sturgeons. PCDDs, PCDFs, and PCBs are structurally related compounds that are collectively known as dioxin-like compounds (DLCs) (Figure 1.1). These chemicals are lipophilic environmental pollutants that, under certain conditions, can be highly persistent and bioaccumulative. DLCs can be produced through activities of humans as unwanted by-products during the manufacture of certain chemicals, such as herbicides and industrial products, through combustion of organic material, or through intentional production as dielectric and coolant fluids (Lohmann and Jones, 1998). DLCs can therefore be released into the aquatic environment through municipal and industrial wastes, contaminated surface runoff, industrial emissions, automobile exhaust, herbicide application, as well as through some natural sources, such as forest fires and volcanic eruptions (Freeman and de Tejada, 2002; Lohmann and Jones, 1998). Due to their potential for persistence, release of DLCs into the environment can result in legacy contamination that can span decades. Detectable concentrations of DLCs have been found in sediments of water bodies world-wide, including in China, Korea, Japan, Canada, the United States, and throughout Europe (Gabos et al., 2001; Hilscherova et al., 2003; Marvin et al., 2002; Naile et al., 2011; Wade et al., 2008). Extirpation of lake trout (*Salvelinus namayrush*) from Lake Ontario in the 1960's has been largely attributed to mortality of sac fry as a result of exposure to mixtures of DLCs (Cook et al., 2003). Since eliminating production of DLCs as part of the 2001 Stockholm Convention, environmental concentrations of DLCs have declined in most areas world-wide; however, a few locations still have sediment concentrations of DLCs that

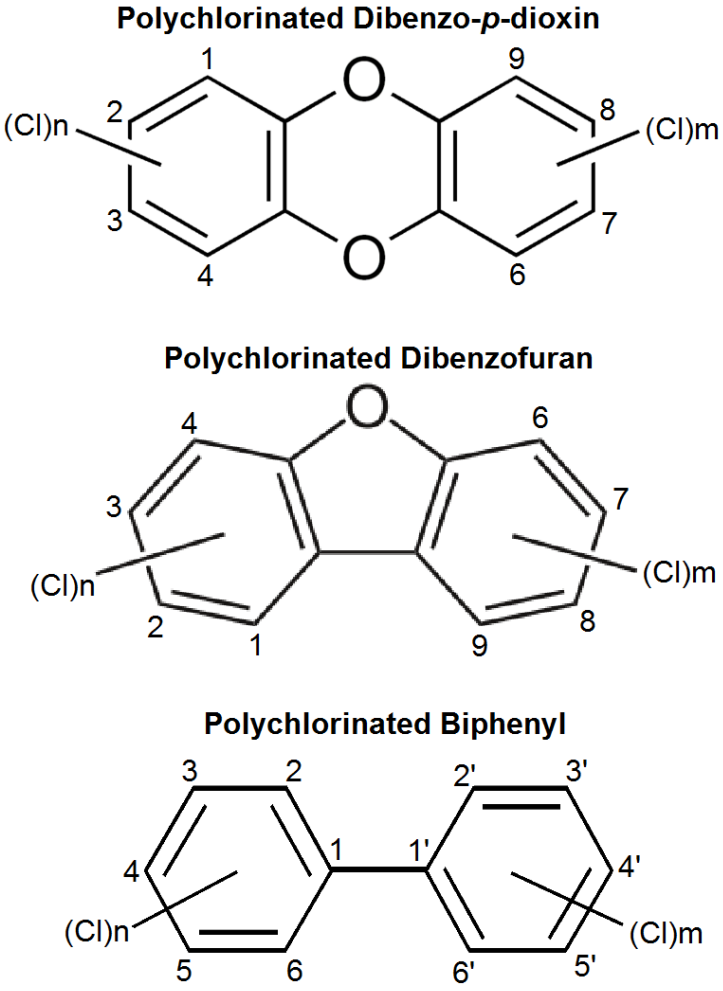

Figure 1.1. Generic structure of polychlorinated dibenzo-p-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), and polychlorinated biphenyl (PCB).

exceed sediment quality guidelines (Weber et al., 2008). Despite a general trend of decreasing concentrations in the environment, DLCs continue to be of ongoing environmental concern due to their widespread distribution and persistence.

1.3 Aryl Hydrocarbon Receptor

Of the possible 75 PCDD, 135 PCDF, and 209 PCB congeners, a total of seven PCDDs, ten PCDFs, and twelve PCBs are considered "dioxin-like" in that they share a planar structure, which allows them to bind with relatively great affinity to the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor that mediates the expression of a suite of biotransformation enzymes and regulates most, if not all, adverse effects of exposure to DLCs in vertebrates (Okey, 2007). Over time the AHR has undergone gene duplication and diversification in vertebrates, which has resulted in multiple AHR clades, namely AHR1, AHR2, and AHR3 (Hahn, 2002). Birds and fishes express AHR1s and AHR2s, while humans and other mammals express a single AHR that is homologous to the AHR1 (Hahn, 2002; Hahn, 2006). AHR1s have been identified in amphibians and reptiles; however, little is known about diversity of AHRs in these taxa (Hahn, 2002). A third clade, AHR3, has been identified in some cartilaginous fishes (Hahn, 2002). Although homologes of the AHR have been identified in invertebrates, these proteins do not bind DLCs (Hahn et al., 1994).

The AHR is expressed in most cell types where it is found in the cytoplasm as part of an inactive complex of co-factors (Pongratz et al., 1992). Upon binding a ligand, AHRs shed the associated co-factors and translocate to the nucleaus where they heterodimerize with the aryl hydrocarbon nuclear translocator (ARNT) (Figure 1.2) (Okey, 2007). This heterodimer complex is able to interact with dioxin-responsive elements (DREs) on the DNA causing the up-regulation

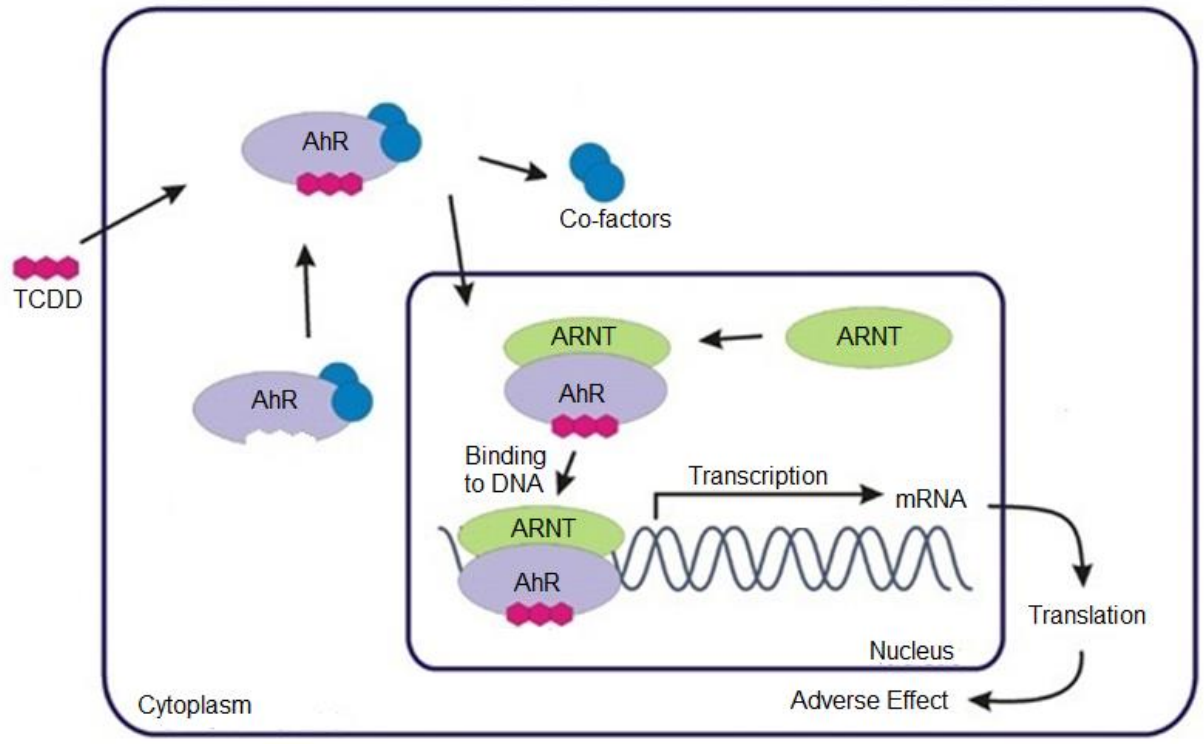

Figure 1.2. Simplified diagram showing activation of the aryl hydrocarbon receptor (AHR) by a ligand (TCDD) leading to an adverse effect on the organism.

in expression of a wide range of dioxin-responsive genes (Figure 1.2) (Whitlock et al., 1996). The AHR is also able to down-regulate expression of certain genes through mechanisms that are not fully understood (Riddick et al., 2004). Despite a clear role in response to exposure to DLCs, the endogenous physiological role of the AHR is not fully understood to date. Investigations of early AHR homologes and use of AHR knockdown approaches suggest the AHR to have evolutionarily functioned in regulation of the cell cycle, cellular proliferation and differentiation, and cell to cell communication; however, functions in angiogenesis, immune regulation, neuronal effects, metabolism, development of the heart and other organ systems, and detoxification have evolved in vertebrates (Duncan et al., 1998; Lahvis and Bradfield, 1998; Emmons et al., 1999). Because of this wide range of functions, activation of the AHR and dysregulation of AHR-responsive genes have been shown to cause a range of adverse effects in vertebrates, including hepatotoxicity, suppression of immune and reproductive functions, teratogenicity, carcinogenicity, anorexia, and death (Kawajiri and Fujii-Kuriyama, 2007).

DLCs are typically found in the environment in complex mixtures of different PCDD, PCDF, and PCB congeners of varying potency (Van den Berg et al., 1998). The most potent DLC for most endpoints and species is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is up to 2,500-fold more potent to embryos of rainbow trout (*Oncorhynchus mykiss*) than is the dioxin-like 3,3′,4,4′,5,5′-hexachlorobphenyl (PCB 169) (Zabel et al., 1995). It is known that this differential potency between DLCs is largely related to the pattern of chlorination, which can either facilitate or interfere with how the ligand binds with the AHR (Van den Berg et al., 1998; Whyte et al., 2000)). Planar DLCs exhibiting chlorine patterns in the lateral positions (i.e. 2,3,7,8) generally have greater AHR affinity, and therefore, greater potency relative to DLCs exhibiting chlorines in ortho positions (Figure 1.1) (Whyte et al., 2000). The common and

specific mechanism of all DLCs means toxicity of a mixture of DLCs is approximately additive (Van den Berg et al., 1998). This additivity has allowed for the development of toxic equivalency quotients (TEQs) or TCDD equivalents based on toxic equivalency factors (TEFs) or relative potencies (RePs) (Van den Berg et al., 1998). This approach has greatly facilitated the assessment of risk posed by complex environmental mixtures containing numerous DLCs with dramatically different potencies through calculating total potency of the mixture.

1.4 Relative Sensitivity to Dioxin-like Compounds

Similarly, great differences in potency of DLCs exist both within and among vertebrate classes. Among birds, a 46-fold difference in sensitivity of embryos to lethality of TCDD was observed between species with the greatest and least sensitivity (Cohen-Barnhouse et al, 2010). And a more than 1,000-fold difference in sensitivity is known among species of mammals (Ema et al., 1993; Wang et al., 2013). However, differences in sensitivity among species of amphibians and reptiles is almost unknown (Jung and Walker., 1997). Embryos of the least sensitive known species of fish, the shovelnose sturgeon, are 200-fold less sensitive to the effects of TCDD than are embryos of the most sensitive known species of fish, the lake trout (Figure 1.3) (Buckler et al., 2015; Walker et al., 1991). There are at least 30,000 species of fish, as compared to approximately 15,000 species of reptiles and amphibians, 10,000 species of birds, and 5,000 species of mammals; making the diversity among fishes greater than that of any other vertebrate class. Of these species, the sensitivity to DLCs of only a few has been well characterized (Walker et al., 1992; Zabel et al., 1995), and only a handful of the remaining species have been investigated (Figure 1.3). Due to differences in relative sensitivity to DLCs among fishes that

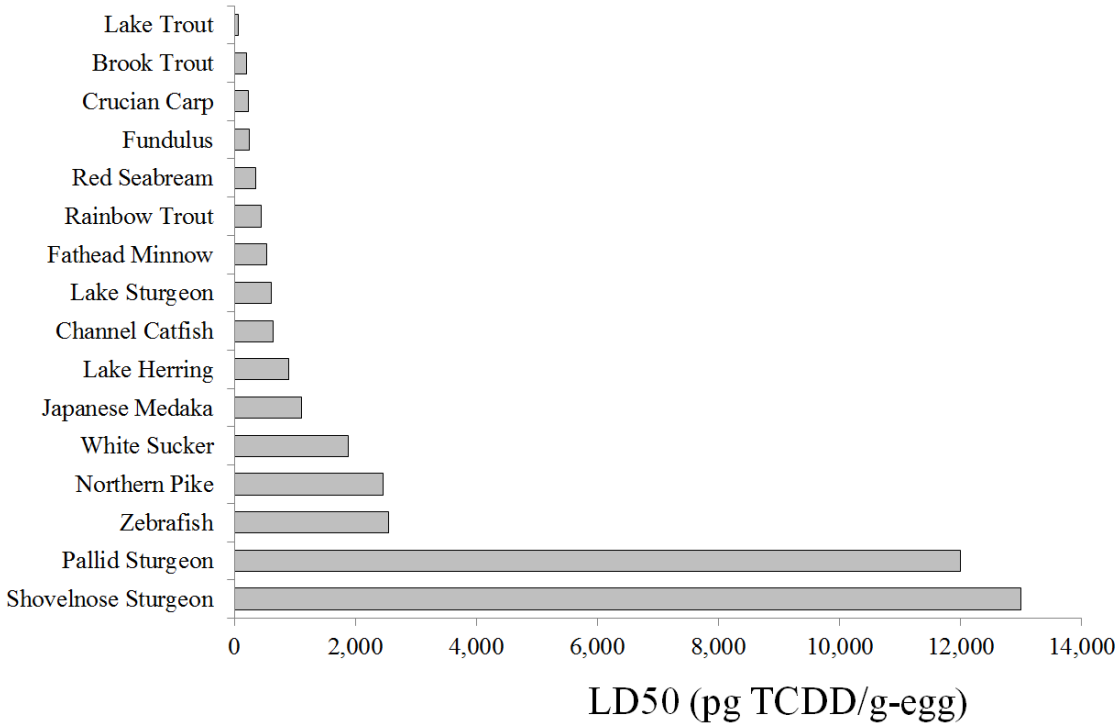

Figure 1.3. Relative sensitivity of fishes to the effects of embryo-lethality (dose to cause 50 % mortality; LD_{50}) following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Buckler et al., 2015; Elonen et al., 1998; Guiney et al., 2000; Henry et al., 1997; Park et al., 2014; Tillitt et al., 2016; Toomey et al., 2001; Walker et al., 1991; 1992; 1994; Yamauchi et al., 2006).

have been investigated, there is great uncertainty in the risk assessment of DLCs to fishes (Elonen et al., 1998; Walker et al., 1994). Considering the relevance of native species of fish as indicators for aquatic ecosystem health, there is need for further understanding as to why there is this observed difference in sensitivity among species, and ultimately to develop models that will allow prediction of the sensitivity of any species to DLCs, particularly species of special concern, such as endangered species.

1.5 Predicting the toxic potency of any dioxin-like compound to any avian species

The specific, molecular mechanism through which the AHR regulates sensitivity among species is well established for birds, although less is known about mechanisms for differences in sensitivity among species of mammals, amphibians, reptiles, and fishes. Differences in sensitivity among species of birds have been demonstated to result from differences among species in affinity of AHR1 for DLCs (Karchner et al., 2006). It was further demonstrated that these differencs in affinity result from functional differences in the ligand binding domain (LBD) (Karchner et al., 2006; Head et al., 2011). In particular, it was demonstrated that substitutions of critical amino acid residues at positions 324 and 380 within the LBD of the AHR1 confer differences in binding affinity for DLCs (Karchner et al., 2006; Farmahin et al., 2012). These studies found that sensitive birds, such as the chicken (*Gallus gallus*) have an isoleucine and serine genotype at positions 324 and 380, moderately sensitive birds, such as the ring-necked pheasant (*Phasianus colchicus*), have an isoleucine and alanine genotype, and insensitive birds, such as the Japanese quail (*Coturnix japonica*), have a valine and alanine genotype (Head et al., 2008; Farmahin et al., 2012). These structural differences in the LBD of the avian AHR1 are

transferable across all species of birds tested thus far and are predictive of both affinity of binding of DLCs to the AHR1 and sensitivities of embryos to adverse effects across all PCDDs, PCDFs, and PCBs (Farmahin et al., 2012; 2013; Manning et al., 2012). This discovery has allowed the use of AHR1 genotyping as a genetic screen for predicting species sensitivity among any species of bird for application to the risk assessment of environments contaminated by DLCs. Similarly, differences in binding affinity of the AHR have been demonstated to explain differences in sensitivity among sensitive and tolerant strains of mice (*Mus musculus*) and these differences in binding are the result of amino acid subsitutions in the LBD of the AHR (Bisson et al., 2009; Ema et al., 1993). Further, the tolerance of most amphibians to exposure to DLCs is attributed, at least in part, to low binding affinity of the AHR1, which has also been demonstrated to result from amino acid substitutions in the LBD (Shoots et al., 2015). However, in contrast to birds, multiple, significant mechanisms could exist among species of fish that contribute towards differences in sensitivity to DLCs and complicate prediction of sensitivity to DLCs among fishes, particularly through use of a genetic screen based on amino acids substitutions of the LBD.

1.6 Aryl hydrocarbon receptor dynamics within the piscine system

As in birds, adverse effects of exposure to DLCs in fishes are mediated primarily through activation of the AHR (Clark et al., 2010; Prasch et al., 2003). However, numerous differences in the AHR-pathway exist between these distantly related groups of vertebrates. General structural properties of the AHR are broadly conserved among classes of vertebrates, but subtle differences in structure can result in distinct differences in function and alter sensitivity *in vivo*

(Bisson et al., 2009; Ema et al., 1993; Hahn, 2002; Karchner et al., 2006; Shoots et al., 2015). Further, where the AHR1 or its homologous AHR drives toxicity of DLCs in birds, amphibians, and mammals, the AHR2 has been demonstrated to drive toxicity of DLCs in fishes based on knockdown studies with zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*), while a role of the AHR1 in toxicity has not been demonstrated (Clark et al., 2010; Incardona et al., 2006; Prasch et al., 2003; Scott et al., 2011; Teraoka et al., 2003; 2010; Van Tiem and Di Giulio, 2011). This difference between fishes and other vertebrates is further exasperated by multiple isoforms of the AHR in fishes, unique tissue-specific patterns of expression, and less conservation among species of fishes relative to conservation among species of birds (Doering et al., 2013).

1.6.1 Multiple aryl hydrocarbon receptor isoforms in fishes

Fishes possess multiple isoforms of the AHR that can be grouped into at least three distinct clades (AHR1, AHR2, AHR3) and with each clade possibly containing multiple isoforms (α, β, δ, γ) (Hahn, 2001; 2002). This is in contrast to other vertebrates where expression of a great diversity in AHRs is less pronounced (Hahn, 2001; 2002). It is hypothesized that vertebrates underwent an ancient genome duplication event which resulted in multiple clades of the AHR, with some fishes, such as salmonids, having undergone a second such duplication event which resulted in multiple isoforms of each AHR clade (Hahn, 2001; 2002). Complete redundancy of function after gene duplication is unstable and over time results in inactivation or functional divergence of duplicated genes (Abnet et al., 1999). This raises the question as to the function of the retained clades and isoforms of the AHR and whether they are significant in determining differential sensitivities among fishes. The fact that multiple clades of the AHR are

likely present in all fishes is significant. The AHR2 has been demonstrated through knockout studies to be the toxicologically active form in fishes studied to date (Clark et al., 2010; Incardona et al., 2006; Prasch et al., 2003; Scott et al., 2011; Teraoka et al., 2003; 2010; Van Tiem and Di Giulio., 2011), with the AHR1 not binding DLCs or binding with lesser affinity than the AHR2 (Andreasen et al., 2002; Fraccalvieri et al., 2013; Karchner et al., 1999; 2005). This is indicative of divergent toxicological roles of these two AHR clades within fishes (Hahn, 2001; 2002). No information is currently available regarding the functional significance of the AHR3 clade, which is known only in some cartilaginous fishes.

Multiple isoforms of the AHR have been isolated from some fishes, including Atlantic salmon (*Salmo salar*), fugu (*Takifugu rubripes*), and rainbow trout (*O. mykiss*) (Hahn et al., 2006). Rainbow trout are known to have at least two isoforms of the AHR2, designated AHR2α and AHR2β (Abnet et al., 1999). These isoforms are 95 % identical at the amino acid level and both are capable of high-affinity binding of TCDD (Abnet et al., 1999). Likewise, Atlantic salmon are known to have at least four isoforms of the AHR2, designated AHR2α, AHR2β, AHR2δ, and AHR2γ (Hansson and Hahn, 2008). Significant differences in sensitivity to activation by TCDD have been demonstrated between these isoforms of the AHR2 in both rainbow trout and Atlantic salmon (Abnet et al., 1999; Hansson and Hahn, 2008). In addition to isoform specific sensitivity to activation by TCDD, there are tissue-specific differences in levels of expression among isoforms in these species (Abnet et al., 1999; Hansson and Hahn, 2008). This could suggest that there are divergent functional roles among isoforms of the AHR in addition to divergent functional roles among AHR1s and AHR2s.

1.6.2 Tissue-specific expression of aryl hydrocarbon receptor isoforms

AHR clades and isoforms have tissue-specific patterns of expression in fishes. Among fishes studied to date, the AHR1 is primarily expressed in brain, heart, and gonad, while the AHR2 is uniformly expressed in all tissues (Abnet et al., 1999; Hansson and Hahn, 2008; Yamauchi et al., 2005). Different isoforms of the AHR2 are known to be differentially expressed in tissues of rainbow trout and Atlantic salmon, and likely in other fishes (Abnet et al., 1999; Hansson and Hahn, 2008). AHR2β of rainbow trout has greater expression than AHR2α; with 10-, 4-, and 4-fold greater expression in the heart, liver, and brain, respectively (Abnet et al., 1999). Greater expression of AHR2β of rainbow trout has also been detected in kidney, blood, spleen, intestine, and ovary (Abnet et al., 1999). Greater expression of AHR2β in liver and heart are of interest because these are major target organs for adverse effects of exposure to DLCs (Antkiewicz et al., 2005; Yamauchi et al., 2006).

In addition to tissue-specific expression of different AHR clades and isoforms; differences in autoregulation of expression of the AHR and stability of AHR protein could also result in differences in sensitivity to DLCs among fishes. Up-regulation in abundance of transcript of AHR2 has been demonstrated in some fishes following exposure to agonists of the AHR and the degree of this up-regulation could be a factor in sensitivity of fishes (Tanguay et al., 1999; Yamauchi et al., 2006). However, it is currently unclear whether an up-regulation in abundance of transcripts of AHR2 results in differences in responsiveness to agonists of the AHR among species or among tissues. Further, some authors have hypothesized that differences in protein confirmation of the AHR could be a factor in differences in sensitivity to DLCs among populations of the Atlantic tomcod (*Microgadus tomcod*) (Wirgin et al., 2011). Furthermore,

differences in expression, response, and function of the AHRR, ARNT, and cofactors of the AHR in determining differences in sensitivity to DLCs among fishes are not known. Finally, other unknown differences in dynamics of the AHR-pathway could also exist between birds and fishes.

1.6.3 Constraints intrinsic of an application to fishes

A linkage between sensitivity to DLCs and structural or functional properties of the AHR have not yet been identified for fishes. This is largely due to a number of current limitations to investigation into the AHRs of fishes and their role in sensitivity to DLCs. There is less conservation among available sequences of amino acids of the AHR of fishes relative to other vertebrates due to the great diversity in species. Greater than 97 % similarity in the identity of amino acids of the LBD of the AHR1 was found among fourteen species of birds (Head et al., 2008), while similiarity in the identity of amino acids in the LBD of the AHR2 of fishes can be less than 70 % based on publicly available sequences in GenBank. Additionally, it has been hypothesized that the great sensitivity of salmonids and some other fishes to adverse effects of exposure to DLCs could be due, at least in part, to their expressing multiple, functional AHR genes (Hansson and Hahn, 2008). Furthermore, implications of expression of AHR3 and its role in the sensitivity to DLCs of cartilaginous fishes is completely unknown (Hahn, 2002). Upon initiation of investigation into the specific mechanisms that result in differences in sensitivity to DLCs among fishes in May of 2010, full-length sequences of AHR2s were only available for six species of fishes, with partial sequences from an additional fifteen species (National Center for Biotechnology Information). And additional isoforms could be yet undiscovered in these species.

Finally, the sensitivity to DLCs is only known for four species of fishes with available sequence information for the AHR1 and AHR2 (Buckler et al., 2015; Elonen et al., 1998; Guiney et al., 2000; Henry et al., 1997; Park et al., 2014; Tillitt et al., 2016; Toomey et al., 2001; Walker et al., 1991; 1992; 1994; Yamauchi et al., 2006). Because of these significant differences in the AHR-pathway of fishes relative to birds, elucidating the mechanisms that result in differences in sensitivity to DLCs among species of fish is more complex and potentially different than in birds. However, identifying these mechanisms and developing a robust predictive relationship between the AHR and sensitivity among fishes would significantly improve the risk assessment of DLCs to fishes, particularly species of special concern such as endangered species.

1.6.4 Future perspectives and research needs:

In order to develope a robust predictive relationship that could be used to determine the sensitivity of any species of fish to any DLC, four main steps would be required:

1) The molecular sequencing and characterization of AHR1s, AHR2s, and AHR3s in a range of fishes would be necessary. Determining the amino acid sequence of the LBD of the AHR would be essential for the characterization of structure-activity relationships and the tissue-specific patterns of expression for each AHR would facilitate proper assessment. Ideal candidates for sequencing and characterization of AHRs would include fishes whose sensitivity has been established in embryos (Figure 1.3).

2) Knowledge of the ligand binding affinity and sensitivity to activation of AHRs in numerous fishes of known sensitivity would be necessary in order to determine whether sensitive fishes have AHRs with greater sensitivity and insensitive species have AHRs with lesser sensitivity. This would ascertain that differences in sensitivity among fishes were driven, at least in part, by differences in AHRs and not other factors.

3) Chimeric AHRs substituting hypothesized critical amino acid sequences in the AHR of fishes could be used in an attempt to alter the ligand binding affinity of the AHR2 in a sensitive species into that of an insensitive species. Additionally, chimeric AHRs could be used in an attempt to turn responsive AHR2s into unresponsive AHR1s. Alternatively, chimeric AHRs can be investigated *in silico* by use of homology models. If successful, this approach would validate which amino acid differences in the LBD were critical to affinity of binding and could be employed as a genetic screen for predicting the sensitivity to DLCs among fishes.

4) Differences in affinity of binding or sensitivity to activation of the AHR might not be the driving factor for differences in sensitivity to DLCs among fishes. Additionally, some fishes, such as salmonids, have complex dynamics of the AHR, which involve multiple isoforms from each clade. Mechanisms that result in differences in sensitivity among these species might be too complex for genetic screening techniques based on the AHR. In this case, a means of integrating potential confounding factors would be required. One option would be to elucidate a relationship between *in vivo* sensitivity and *in vitro* response of whole cells to exposure to DLCs, such as induction of ethoxyresorufin *O*-deethlase (EROD) or up-regulation in abundance of transcript of cytochrome P450 1A (CYP1A). This alternate method could predict an ecologically relevant

endpoint, such as embryo-lethality, by use of an *in vitro* approach. Such a relationship has been developed for birds (Head and Kennedy, 2010). Since whole cells are employed, this approach would integrate numerous confounding factors, including interactions among multiple AHRs, differences in levels of expression among AHRs, and interplay between AHRs and AHRR, ARNT, and other cofactors.

1.7 Candidate for application: Sturgeon

Sturgeon would be an ideal candidate for the development and application of such a predictive relationship for sensitivity to DLCs among fishes. A total of twenty-four extant species of sturgeon are known and all of them are likely endangered with pollution being one likely culprit (LeBreton et al., 2004). Sturgeon are uniquely susceptible to the bioaccumulation of lipophilic pollutants, such as DLCs. They are long-lived, require numerous years before they begin reproducing, and then spawn only intermittently. Sturgeon live in close association with sediments where DLCs tend to accumulate and are most persistent. Sturgeon feed largely on benthic organisms which might accumulate sediment-borne DLCs. Sturgeon have greater lipid content than some other fishes which could facilitate bioaccumulation (LeBreton et al., 2004). Due to these risk factors, elevated concentrations of DLCs have been detected in tissues and eggs of sturgeons (Foster et al, 2001; Foster et al, 1999; Kruse and Webb, 2006; Kruse and Scarnecchia, 2002; MacDonald, 1997; U.S. EPA, 2002). However, little is currently known regarding the sensitivity of sturgeon to DLCs. Toxicity studies conducted with sturgeon have found them to be among the most sensitive fishes to the adverse effects of other environmental pollutants, such as endocrine disruptors, methylmercury, and metal ions, which suggests

sturgeon might also be sensitive to other anthropogenic pollutants, including DLCs (Dwyer et al., 2005; Lee et al., 2012; Vardy et al., 2011; 2013). This evidence justifies the hypothesis that sturgeon could be sensitive to DLCs and considering known elevated concentrations in tissues and eggs, some populations could be at risk. However, due to their endangered status and a general lack of toxicity testing protocols for sturgeons, it is difficult to perform robust toxicity studies with these fishes. A predictive relationship developed for fishes by use of structural or functional properties of the AHR could allow prediction of the sensitivity of any species of sturgeon to DLCs and other agonists of the AHR. Acquiring a single tissue sample from each species of sturgeon and knowledge of concentrations of DLCs in tissues of wild individuals could allow for the accurate assessment of risk to exposure to DLCs for sturgeons. This information could be essential in aiding the world-wide conservation of these, and other, endangered fishes.

1.8 Objectives

Limited information is available regarding the sensitivity of sturgeons, or other endangered species of fish, to exposure to DLCs. Nothing is known regarding the mechanism(s) that result in differences in sensitivity to DLCs among fishes that would enable the development of a mechanism-based biological model for predicting the sensitivity to DLCs of any species of fish, including sturgeons. This lack of knowledge on the mechanism(s) that determine sensitivity among fishes is largely a result of the current lack of key information regarding sequences of amino acids, structural and functional properties, and patterns of expression of AHRs among phylogenetically diverse species of fish. Therefore, the overall objective of the research

conducted under this Ph.D. thesis was to develop a mechanism-based biological model that uses *in vitro* data for the assessment of species-specific sensitivity of sturgeons in particular, but fishes in general, to DLCs and enable the accurate risk assessment of exposed populations. For the purposes of this research, white sturgeon (*Acipenser transmontanus*) and lake sturgeon (*Acipenser fulvescens*) were used as model sturgeons. The specific research objectives, hypotheses, and experimental approaches employed are outlined below:

Objective 1. Characterize tissue specificity of aryl hydrocarbon receptor (AHR) mediated responses and relative sensitivity of white sturgeon (*Acipenser transmontanus*) to an AHR agonist (Chapter 2).

Little research has been conducted to date that characterizes the responses of sturgeon to activation of the AHR by exposure to DLCs. Since sturgeon are ancient species of fish, their responses might differ from those of more advanced vertebrates, including teleost fishes. Therefore, this study investigated tissue-specific molecular and biochemical responses of white sturgeon (*A. transmontanus*) *in vivo* to a model agonist of the AHR, β-naphthoflavone (βNF). These responses were compared to those of the well-characterized model species, rainbow trout (*O. mykiss*) as well as other species of fishes from the literature. Therefore, the specific objective and associated null hypothesis was:

1) To determine whether white sturgeon respond to activation of the AHR by βNF through up-regulation of the abundance of transcripts (CYP1A, AHR) and induction in enzyme activity (CYP1A1, CYP1A2, CYP1B1) of genes of the AHR gene battery among different tissues in accordance with responses previously reported for teleost fishes.

H_0: Exposure to βNF does not significantly change abundance of transcript of CYP1A or AHR and enzyme activity of CYP1A1, CYP1A2, or CYP1B1 in liver, gill, or intestine relative to control in white sturgeon.

Objective 2. Identify aryl hydrocarbon receptors (AHR1 and AHR2) in white sturgeon (*Acipenser transmontanus*) and investigate levels of expression in an evolutionary context regarding sensitivity of white sturgeon to dioxin-like compounds (Chapter 3).

White sturgeons were found to be among the more responsive species of fish to exposure to the model agonist of the AHR, βNF (Chapter 2). These responses were mediated primarily through activation of the AHR. However, no full-length sequences of amino acids of AHRs are currently available for sturgeons. Further, no information is currently available regarding tissue-specific patterns of expression of AHRs or autoregulation of expression following exposure to an agonist of the AHR in sturgeons. Therefore, in order to better characterize AHR mediated responses and relative sensitivity of sturgeons to exposure to DLCs, this study determined the primary amino acid structure of AHR1 and AHR2 of white sturgeon and characterized patterns of expression and response to exposure to an agonist of the AHR. Therefore, the specific objectives and associated null hypotheses were:

1) To determine whether AHR1 or AHR2 have tissue-specific levels of expression in white sturgeon.

   H_0: Abundance of transcripts of AHR1 or AHR2 is not significantly different among liver, brain, gill, heart, spleen, stomach, intestine, head kidney, or muscle of white sturgeon.

2) To determine whether AHR1 and AHR2 have different levels of basal expression in white sturgeon.

   H_0: Basal abudance of transcripts of AHR1 is not significantly different from basal abundance of transcripts of AHR2 in white sturgeon.

3) To determine whether expression of AHR1 or AHR2 is differentially regulated by exposure to an agonist of the AHR in white sturgeon.

   H_0: Exposure to βNF does not significantly change abundance of transcripts of AHR1 or AHR2 in liver, gill, or intestine relative to controls in white sturgeon.

Objective 3. Characterize functionality of aryl hydrocarbon receptors (AHR1 and AHR2) of white sturgeon (*Acipenser transmontanus*) and the implications for the risk assessment of dioxin-like compounds (Chapter 4).

AHRs identified in white sturgeon were found to have primary amino acid structures and patterns of expression unique from those of other studied fishes, which might alter responses of sturgeons to DLCs relative to other fishes (Chapter 3). Therefore, this study characterized functionality of the AHR1 and AHR2 of white sturgeon by investigating relative potencies to activation of each AHR by six selected DLCs of environmental relevance to populations of white sturgeon in Canada. Specifically, sensitivities to activation of AHRs of white sturgeon were determined by use of an *in vitro* luciferase reporter gene (LRG) assay by use of COS-7 cells transfected with AHR1 or AHR2 of white sturgeon. Currently, risk assessment of DLCs in fishes uses TEFs developed for the World Health Organization (WHO) that are based on studies of embryo-lethality with salmonids. However, it is uncertain whether TEFs developed by use of salmonids are protective of sturgeons. TEQs based on TEFs were compared to TCDD

equivalents determined from relative potencies of AHRs of white sturgeon for environmental concentrations of these six selected DLCs in tissues of endangered populations of white sturgeon from the upper Columbia River and Fraser River. This methodology was proposed as an *in vitro* means of predicting the relative sensitivity of white sturgeon to selected DLCs and improving the risk assessment of DLCs to endangered populations of white sturgeon. Therefore, the specific objectives and associated null hypotheses were:

1) To determine whether AHR1 or AHR2 of white sturgeon were activated by exposure to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachloro-dibenzofuran (TCDF), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), or 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105).

   H_0: Relative luciferase units are not significantly different in COS-7 cells transfected with AHR1 or AHR2 of white sturgeon that were exposed to TCDD, PeCDF, TCDF, PCB 126, PCB 77, or PCB 105 relative to controls.

2) To Determine whether TEQs based on TEFs developed by the WHO for fishes are comparable to TCDD equivalents based on relative potencies of AHRs of white sturgeon by use of measured concentrations of TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 in tissues from white sturgeon collected from the upper Columbia and Fraser Rivers.

   H_0: TEQs are not different from TCDD equivalents for measured concentrations of TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 in tissues from white sturgeon collected from the upper Columbia and Fraser Rivers.

Objective 4. Elucidate whether differences in activation of aryl hydrocarbon receptors of white sturgeon relative to lake sturgeon are predicted by identities of key amino acids in the ligand binding domain (Chapter 5).

White sturgeons were predicted to be among the most sensitive species of fish to exposure to DLCs because AHR1 and AHR2 of white sturgeon were found to be activated by concentrations of DLCs less than those required to activate AHRs of other vertebrates tested to date (Chapter 4). However, it is unknown whether all species of sturgeon are equally sensitive to DLCs or whether there are great differences in sensitivity among species. Therefore, sensitivities to activation of AHRs of a second species of sturgeon, the lake sturgeon, were determined by use of COS-7 cells transfected with AHR1 or AHR2 of lake sturgeon that were exposed to serial concentrations of TCDD, PeCDF, TCDF, PCB 126, PCB 77, or PCB 105 as conducted for AHRs of white sturgeon (Chapter 4). These sensitivities to activation by DLCs of AHRs of lake sturgeon were compared to previously generated sensitivities to activation by DLCs of AHRs of white sturgeon. Since AHR2 is believed to be the primary driver of adervse effects of exposure to DLCs in fishes, differences in sensitivity to activation by DLCs of AHR2 *in vitro* between white sturgeon and lake sturgeon were hypothesized to be suggestive of differences in sensitivity to exposure to DLCs *in vivo* between white sturgeon and lake sturgeon. Homology modeling and *in silico* mutagenesis were used to identify critical amino acid residues in the LBD of the AHRs of white sturgeon and lake sturgeon that could be used as a genetic screen that is predictive of differences in both *in vitro* activations by DLCs and *in vivo* sensitivity to DLCs in these, and potentially other fishes. Therefore, the specific objectives and associated null hypotheses were:

1) To determine whether AHR1 or AHR2 of lake sturgeon were activated by exposure to TCDD, PeCDF, TCDF, PCB 126, PCB 77, PCB 105.

H_0: Relative luciferase units are not significantly different in COS-7 cells transfected with AHR1 or AHR2 of lake sturgeon that were exposed to TCDD, PeCDF, TCDF, PCB 126, PCB 77, or PCB 105 relative to controls.

2) To Determine whether there are differences in sensitivity to TCDD, PeCDF, TCDF, PCB 126, PCB 77, or PCB 105 between white sturgeon and lake sturgeon.

   H_0: Sensitivities of AHR1 or AHR2 to TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 are not different between white sturgeon and lake sturgeon.

3) To Determine whether differences in sensitivity to TCDD, PeCDF, TCDF, PCB 126, PCB 77, or PCB 105 between white sturgeon and lake sturgeon can be linked to identities of amino acids in the ligand binding domain of the AHR.

   H_0: Differences in sensitivities to TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 between AHR1 or AHR2 of white sturgeon and lake sturgeon cannot be explained by identities of amino acids in the ligand binding domain of the AHR.

Objective 5. Characterize conservation in transcriptomic and proteomic response of white sturgeon to equipotent concentrations of 2,3,7,8-TCDD, PCB 77, and benzo[a]pyrene (Chapter 6).

Relative potencies and relative sensitivities for TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 determined for AHR2 of white sturgeon (Chapter 4) and lake sturgeon (Chapter 5) in the LRG assay were indistinguishinable from relative potencies and relative sensitivities determined for these species in liver explants (Eisner et al., 2016). However, little is known about the links between activation of the AHR by DLCs, the resulting cascade of molecular, biochemical, and histological events, and the eventual manifestation of apical adverse effects.

Therefore, this study investigated linkages across levels of biological organization by use of high-throughput, next-generation whole transcriptome and whole proteome analysis in livers of juvenile white sturgeon exposed to equipotent concentrations of three different agonists of the AHR, namely TCDD, PCB 77, and benzo[a]pyrene (BaP). Responses of the transcriptome and proteome were then compared for conservation in response between chemicals at equipotent concentrations and between levels of biological conservation. Therefore, the specific objectives and associated null hypotheses were:

1) To determine whether exposure to TCDD, PCB 77, and BaP results in altered expression of genes at either the level of the transcriptome or the proteome.

   H_0: Abundance of transcripts or proteins are not statistically different following exposure to TCDD, PCB 77, or BaP relative to controls in liver of white sturgeon

2) To determine whether exposure to TCDD, PCB 77, and BaP results in different identities of responses at either the level of the transcriptome or the proteome.

   H_0: Identities of up- or down-regulated transcripts or proteins by exposure to TCDD, PCB 77, or BaP are not different in liver of white sturgeon

3) To determine whether exposure to TCDD, PCB 77, and BaP results in different magnitude of responses at either the level of the transcriptome or the proteome.

   H_0: Magnitude of up- or down-regulation of transcripts or proteins by exposure to TCDD, PCB 77, or BaP are not different in livers of white sturgeon.

4) To determine whether exposure to TCDD, PCB 77, and BaP results in different responses at the level of the transcriptome relative to the level of the proteome.

   H_0: Transcriptomes and proteomes following exposure to TCDD, PCB 77, and BaP are not statistically different in livers of white sturgeon.

5) To determine whether exposure to TCDD, PCB 77, and BaP results in alteration in different physiological processes at either the level of the transcriptome or the proteome.

H_0: Physiological processes altered by exposure to TCDD, PCB 77, or BaP are not different in livers of white sturgeon.

Objective 6. Identify whether *in vitro* activation of AHR2 or AHR1 are predictive of *in vivo* sensitivity to 2,3,7,8-TCDD across phylogenetically diverse species of fish (Chapter 7).

Equal activation of the AHR2 of white sturgeon by three different agonists was demonstrated to result in similar global responses and magnitude of responses across levels of biological organization and would be predicted to result in similar adverse effects and severity of adverse effects at the level of the whole organism (Chapter 7). This is suggestive of response-response relationships between activation of the AHR in the LRG assay and apical level adverse effects, such as mortality of embryos. Therefore, this study developed a mechanism-based biological model to predict the sensitivity of any species of fish, including endangered species such as sturgeons, to DLCs. Specifically, this study 1) investigated sensitivities to activation by TCDD in the LRG assay of a total of five AHR1s and ten AHR2s across seven species of fish that are known to differ in sensitivity of embryos by almost 40-fold, and 2) characterized the relationship between sensitivity to activation of AHR1s and AHR2s by TCDD and sensitivity of embryos to TCDD. This mechanism-based biological model has the potential to guide more objective ecological risk assessment of DLCs for species of fish that are not easily studied, including threatened or endangered species such as sturgeons. Therefore, the specific objectives and associated null hypotheses were:

1) To determine whether AHR1s or AHR2s of lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), white sucker (*Catostomus commersonii*), or northern pike (*Esox lucius*) were activated by exposure to TCDD.

    H_0: Relative luciferase units are not statistically different in COS-7 cells transfected with AHR1 or AHR2 of lake trout, brook trout, fathead minnow, Japanese medaka, white sucker, or northern pike and exposed to TCDD relative to controls.

2) To determine whether there is a linear relationship between sensitivity to activation of AHR1 by TCDD and sensitivity of embryos to TCDD among fathead minnow, lake sturgeon, Japanese medaka, and northern pike.

    H_0: There is no statistically significant linear relationship between sensitivity to activation of AHR1 by TCDD and sensitivity of embryos to TCDD among fishes.

3) To determine whether there is a linear relationship between sensitivity to activation of AHR2 by TCDD and sensitivity of embryos to TCDD among lake trout, brook trout, fathead minnow, lake sturgeon, Japanese medaka, white sucker, and northern pike.

    H_0: There is no statistically significant linear relationship between sensitivity to activation of AHR2 by TCDD and sensitivity of embryos to TCDD among fishes.

CHAPTER 2

2 TISSUE SPECIFICITY OF ARYL HYDROCARBON RECEPTOR (AHR) MEDIATED RESPONSES AND RELATIVE SENSITIVITY OF WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) TO AN AHR AGONIST

PREFACE

As an early step towards predicting the sensitivity of sturgeons to exposure to dioxin-like compounds (DLCs), the aim of Chapter 2 was a general *in vivo* characterization of molecular and biochemical responses to activation of the aryl hydrocarbon receptor (AHR) in a representative of the Acipenseridae, the white sturgeon (*Acipenser transmontanus*). Since sturgeons are an ancient family of fishes, they might not respond to activation of the AHR in a manner that is consistent with responses of more modern teleost fishes whose responses are well characterized. Pattern and magnitude of responses were compared to the model teleost, rainbow trout (*Oncorhynchus mykiss*), and other fishes from the literature. Demonstrating that sturgeons respond to exposure to DLCs in a manner consistent with teleost fishes was necessary in order to support development of a mechanism-based biological model for predicting the sensitivity of any species of sturgeon based on data derived partially, or completely, from teleosts.

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Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Steve Wiseman (University of Saskatchewan) provided inspiration, scientific input, guidance, and training, commented on and edited the manuscript.

Shawn C. Beitel and Brett J. Tendler (both University of Saskatchewan) provided laboratory assistance with the *in vivo* exposure and time-sensitive biochemical analyses.

Drs. John P. Giesy and Markus Hecker (both University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1 Abstract

Sturgeons are endangered in some parts of the world. Due to their benthic nature and longevity sturgeon are at greater risk of exposure to bioaccumulative contaminants such as dioxin-like compounds that are associated with sediments. Despite their endangered status, little research has been conducted to characterize the relative responsiveness of sturgeon to dioxin-like compounds. In an attempt to study the biological effects and possible associated risks of exposure to dioxin-like compounds in sturgeon, the molecular and biochemical responses of white sturgeon (*Acipenser transmontanus*) to a model aryl hydrocarbon receptor (AHR) agonist, β-naphthoflavone (βNF) were investigated. White sturgeons were injected intraperitoneally with one of three doses of βNF (0, 50, or 500 mg/kg, bw). Rainbow trout (*Oncorhynchus mykiss*) were used as a reference species since their responses have been well characterized in the past. Three days following injection with βNF, fish were euthanized and livers, gills, and intestines collected for biochemical and molecular analyses. White sturgeon exposed to βNF had significantly greater ethoxyresorufin *O*-deethylase (EROD) activity in liver (up to 37-fold), gill (up to 41-fold), and intestine (up to 36-fold) than did unexposed controls. Rainbow trout injected with βNF exhibited EROD activity that was significantly greater in liver (88-fold), than that of controls, but was undetectable in gills or intestine. Abundance of CYP1A transcript displayed a comparable pattern of tissue-specific induction with intestine (up to 189-fold), gills (up to 53-fold), and liver (up to 21-fold). Methoxyresorufin *O*-deethylase (MROD) and pentoxyresorufin *O*-deethylase (PROD) activities were undetectable in unexposed white sturgeon tissues while exposed tissues displayed MROD activity that was only moderately greater than the activity that could be detected. Differential inducibility among liver, gill, and intestine following exposure to

an AHR agonist is likely associated with tissue-specific regulation of the AHR signalling pathway. Liver and gill of white sturgeon had significantly greater AHR transcript abundance than did the intestine, however following exposure to βNF, significantly greater induction in AHR transcript abundance was detected in intestine (up to 35-fold) compared to liver (up to 5-fold) or gills (up to 11-fold). It was shown that white sturgeons are responsive to AHR agonists in the liver, gill, and intestine and could be among the more sensitive fish species with regard to inducibility of CYP1A.

2.2 Introduction

World-wide populations of certain species of sturgeon (Acipenseridae) have been decreasing with some species nearing extinction. This has resulted in interest for conservation of sturgeon. In the northwestern USA and British Columbia, Canada there is particular concern about populations of the white sturgeon (*Acipenser transmontanus*), which is the largest freshwater fish in North America. Decreases in the sizes of populations have been attributed to several human activities, including overfishing, construction of dams, alteration of habitat, competition from introduced species, and pollution (Birstein, 1993; Coutant, 2004; Gisbert and Williot, 2002; Irvine et al., 2007; Luk'yanenko et al., 1999; Paragamian and Hansen, 2008; Scott and Crossman, 1973). In contrast to the literature describing the impact of overfishing, impoundment of rivers, and alteration of habitats on populations of the white sturgeon, little is currently known about the potential effects of pollutants on long-term survival of this species (LeBreton et al., 2004). Some persistent pollutants including metals, bioaccumulative organochlorine pesticides, and halogenated aromatic hydrocarbons (HAHs) have been detected in sturgeon at concentrations sufficient to warrant concern (Foster et al., 1999, 2001; Kruse and Scarnecchia, 2002; Kruse and Webb, 2006; MacDonald et al., 1997; U.S. EPA, 2002; Mierzykowski, 2010). In fact, sturgeon might be particularly susceptible to the effects of bioaccumulation of lipophilic pollutants. During a survey of the Columbia River, USA, out of twelve fish species examined, it was found that sturgeon contained the greatest concentrations of contaminants in their tissues (U.S. EPA, 2002). Sturgeons are long-lived, and require up to thirty years until they begin reproducing, and then spawn only intermittently. Sturgeon live in close association with the sediment and feed primarily on benthic organisms. Once fertilized sturgeon

eggs adhere to the substratum by means of a sticky glycoprotein layer which could expose developing embryos to chemicals present in the sediment (Hochleithner and Gessner, 2001). Sturgeon have a greater lipid content than some other fishes (LeBreton et al., 2004). These attributes give sturgeon a greater potential of exposure to contaminants associated with sediments and increases the likelihood of bioaccumulation of these contaminants into fatty tissues.

HAHs, which include polychlorinated biphenyl congeners (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) are known contaminants in sediments of some rivers, such as the Columbia River, which are inhabited by white sturgeon and have been detected at elevated concentrations in white sturgeon tissues and eggs (Foster et al., 1999, 2001; Kruse and Scarnecchia, 2002; Kruse and Webb, 2006; MacDonald et al., 1997; U.S. EPA, 2002). Of the HAHs, those that can bind to the aryl hydrocarbon receptor (AHR) are known as dioxin-like compounds (Okey, 2007). Effects mediated by activation of the AHR by dioxin-like compounds are pleiotrophic and can include hepatotoxicity, immune suppression, reproductive toxicity, teratogenicity, carcinogenicity, endocrine dysfunction, and anorexia (Kawajiri and Fujii-Kuriyama, 2007). Little is currently known about the sensitivity of sturgeon to dioxin-like compounds. However, induction of phase I, mixed function monoxygenase enzymes and deformities have been observed in white sturgeon collected from the Columbia River, British Columbia, Canada (Foster et al., 2001; Kruse and Webb, 2006). Damage to the liver, thought to result from exposure to dioxin-like compounds has been observed in lake sturgeon (*Acipenser fluvescens*) collected from the St. Lawrence River, Canada (Doyon et al., 1999).

The objective of this study, was to characterize tissue-specific responsiveness of white sturgeon to the model AHR agonist, β-naphthoflavone (βNF) and compare this responsiveness to that of other previously characterized fishes. Specifically, ethoxyresorufin O-deethylase (EROD), methoxyresorufin O-deethylase (MROD), and pentoxyresorufin O-deethylase (PROD) activity as well as expression of the CYP1A gene in liver, intestine, and gills of exposed and unexposed, juvenile white sturgeon was compared to that of rainbow trout (*Oncorhynchus mykiss*). To further elucidate the mechanisms of tissue-specific responsiveness of white sturgeon to AHR agonists responsiveness of these endpoints was compared to tissue-specific gene expression of the AHR. Comparison of EROD activity with other fishes suggests that responsiveness of the AHR pathway in sturgeon might be different from that of other fishes. Thus, a phylogenetic tree based on the amino acid sequence of CYP1A and AHR was developed. To our knowledge, this is the first report of expression of the gene coding for AHR and CYP1A in white sturgeon.

2.2 Materials and methods

2.2.1 Fish

White sturgeon (*A. transmontanus*), ranging in mass from 12 to 27 g were randomly selected from in-house stock reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Rainbow trout (*O. mykiss*) ranging from 9 to 24 g were randomly selected from in-house stock reared from eggs acquired from a commercial supplier (Troutlodge, Sumner, WA, USA). White sturgeon and rainbow trout were maintained in separate 712 L tanks under

flow-through conditions at approximately 12 °C and fed approximately 2 % of their body weight daily. White sturgeons were fed frozen bloodworms (Hagen, Montreal, QC, Canada) and rainbow trout were fed commercial trout feed (Martin Classic Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada).

2.2.2 Exposure protocol

Individual white strugeon and rainbow trout were randomly assigned to one of five 400 L tanks that were maintained at approximately 12 °C under flow-through conditions. Tanks were designated as white sturgeon 0 mg/kg, white sturgeon 50 mg/kg, white sturgeon 500 mg/kg, rainbow trout 0 mg/kg, and rainbow trout 50 mg/kg. Seven individuals of each species were maintained in each tank. Following a four-week acclimation period, each fish was injected intraperitoneally (i.p.) with one of three doses of βNF (purity > 98 %; Sigma-Aldrich, Oakville, ON, Canada) dissolved in corn oil. An injection volume of approximately 4 ml/kg was used. Three days following injection, all fish were euthanized by overdose of tricaine mechanesulfonate (MS-222, Sigma-Aldrich). Livers and intestines were collected and immediately snap-frozen in liquid nitrogen. Gill arches were excised and stored in ice-cold HEPESCortland (HC) buffer (0.38 g KCl, 7.74 g NaCl, 0.23 g MgSO4–7H2O, 0.23 g CaCl2-H2O, 0.41 g NaH2PO4-H2O, 1.43 g HEPES, and 1 g glucose per 1 L of H2O, pH 7.7) and analyzed for EROD activity within 72 h by use of the methods described by Jonsson et al. (2002).

2.2.3 Ethoxy-, methoxy-, pentoxyresorufin *O*-deethylase assay

Microsomes were prepared for EROD, MROD, and PROD assays by use of the methods described by Kennedy and Jones (1994). Briefly, approximately 300 mg of liver or intestinal tissue was minced into small pieces with cold razor blades and quantitatively transferred into a 4 ml ultracentrifuge tube containing ice-cold Tris buffer (0.05 M Tris, 1.15 % potassium chloride, pH 7.5). Tissue was then homogenized with approximately 5 strokes using a Powergen 125 (FTH-115) blade-type homogenizer (Thermo Fisher Scientific, Nepean, ON, Canada). The homogenate was centrifuged at 10,000 xg in a Sorvall WX Ultraspeed Centrifuge (Thermo Fisher Scientific) for 10 min at 4 °C. The supernatant of each sample was transferred into separate ultracentrifuge tubes and centrifuged again at 100,000 xg for 30 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 1 ml of ice-cold microsome stabilizing buffer (20 % glycerol, 0.1 M KH2PO4, 1 mM EDTA, 1 mM dithiothreitol) per gram of tissue originally used. Aliquots were stored at -80 °C until analyzed.

Preparation of gill tissue for EROD assays was performed according to the methods described by Jonsson et al. (2002) with a few modifications. Briefly, primary gill filaments were cut immediately above the septum with a cold razor blade into pieces of about 2 mm by 2 mm. Gill filament pieces were stored in ice-cold HC buffer until all gill samples had been prepared and then immediately used for EROD analysis.

Activities of EROD, MROD, and PROD in each preparation of microsomes and EROD activity in gill filaments were assayed in 96-well plates. Dilutions of resorufin (Sigma-Aldrich) were used to establish standard curves. Each preparation of microsomes or gill tissue was analyzed in triplicate. All EROD reaction wells contained 16 µl of microsomes, 30 µl of 7-

ethoxyresorufin (7-ER, Sigma-Aldrich) working solution (2.5 μM final well concentration), and 95 μl HEPES buffer (0.05 M HEPES, pH 7.8). All MROD and PROD reaction wells contained 30 μl of 7-methoxyresorufin (7-MR, Sigma-Aldrich) or 7-pentoxyresorufin (7-PR, Sigma-Aldrich) working solution (5 μM final well concentration) in place of 7-ER solution. Samples of gill were analyzed by carefully placing each piece of tissue into individual wells of a 96-well plate which contained reaction solution. Following a 5 min incubation at room temperature, enzymatic reactions were initiated by addition of 30 μl nicotinamide adenine dinucleotide phosphate (NADPH, Sigma-Aldrich) to make a final well concentration of 0.3 mM. Plates were immediately placed in a fluorescence plate reader (POLARstar OPTIMA, BMG LAB-TECH, Cary, NC, USA) according to the methods described by Kennedy et al. (1995) and read on a time-course every 5 min for a total reaction time of 60 min. Resorufin was quantified at 530 nm excitation and 590 nm emission wave-lengths, respectively. Concentrations of protein in each well were determined by use of the bicinchoninic acid (BCA; Sigma-Aldrich) method with dilutions of bovine serum albumin (BSA; Sigma) used to produce standard curves.

2.2.4 Amplification and sequencing of white sturgeon CYP1A cDNA

Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) and synthesized by Invitrogen (Burlington, ON, Canada). Because a CYP1A sequence from white sturgeon was not available in Genbank, degenerate primers (Table 2.1) were designed by aligning conserved regions of CYP1A1 sequences from red seabream (*Pagrus major*; accession # 159895623), zebrafish (*Danio rerio*; accession # 39653366), rainbow trout (*O. mykiss*;

Table 2.1. Sequences, annealing temperatures, primer efficiency, and corresponding target gene Genbank accession number of oligonucleotide primers used in cloning of white sturgeon CYP1A and quantitative real-time PCR.

Target Gene	Accession #	Primer Sequence (5'-3')	Efficiency (%)	Annealing Temp (°C)
CYP1A (degenerate)	NA	Forward: TGGTCWGTGATGTACTTGGTGRC Reverse: CGTTTGTGCTTCATTGTGAGA	NA	60
β-actin	FJ205611	Forward: CCGAGCACAATGAAAATCAA Reverse: ACATCTGCTGGAAGGTGGAC	96	60
CYP1A	JQ660369	Forward: GATCCCTCCACCTTCTCTCC Reverse: GCCGATAGACTCACCAATGC	99	60
AHR	AY880254	Forward: TGGAGATCAGGACCAAGACC Reverse: GTGTAACCCAGCACCACCTT	90	60

accession # 1778054), terapon (*Terapon jarbua*; accession # 167599358), flathead mullet (*Mugil cephalus*; accession # 167599362), and tilapia (*Oreochromis niloticus*; accession # 224042458) by use of CLUSTALW multiple sequence aligner available through the SDSC molecular biology workbench (Subramaniam, 1998). The polymerase chain reaction (PCR) was performed and the PCR product was purified by use of the QIAQuick PCR purification system (Qiagen, Mississauga, ON, CA) according to the manufacturer's protocol. Purified PCR products were cloned into the pGEM-T easy vector using a DNA ligation kit (Invitrogen) and transformed into competent JM109 *Escherichia coli* cells (Promega, Madison, WI, USA). Plasmids were isolated with a Qiagen plasmid purification kit and the products were sequenced at the National Research Council of Canada's Plant Biotechnology Institute (University of Saskatchewan). Gene-specific primers for quantitative PCR (Table 2.1) were designed based on the partial cDNA sequences determined for white sturgeon CYP1A. Gene-specific qPCR primers for β-actin and AHR were designed from publicly available sequences by use of Primer3 software (Table 2.1) (Rozen and Skaletsky, 2000) and synthesized by Invitrogen.

2.2.5 Quantitative real-time PCR

Total RNA was extracted from approximately 30 mg of intestine or gill tissue by use of the RNeasy Plus Mini Kit (Qiagen) or 30 mg of liver tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen), both according to the manufacturer's protocol. Purified RNA was quantified by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). Purified RNA samples were stored at -80 °C until analyzed. First-strand cDNA synthesis was performed by use of the QuantiTect Reverse Transcription Kit (Qiagen) with 1 μg of total RNA

according to the manufacturer's protocol. The cDNA samples were stored at -20 °C until analyzed.

Real-time PCR (qPCR) was performed in 96-well plates using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 70 µl reaction mixture of 2x concentrated Power SYBR Green master mix (Applied Biosystems), an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. qPCR primers for CYP1A, β-actin, and AHR were designed as described above. Reactions were conducted in triplicate with 20 µl reaction volumes per well. The PCR reaction mixture was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile consisted of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Target gene transcript abundance was quantified by normalizing to β-actin according to the methods described by Simon (2003).

2.2.6 Phylogenetic tree

Phylogenetic trees were generated for fish CYP1A and AHR using the CLC Genomics Workbench v.4.7.2 (Katrinebjerg, Aarhus, Denmark). Relatedness of complete and partial amino acid sequences among various fish species was determined for partial amino acid sequences of white sturgeon CYP1A and AHR.

2.2.7 Statistical analysis

Statistical analyses were conducted by use of SPSS 19 software (SPSS, Chicago, IL, USA). A logarithmic transformation was used whenever necessary to ensure homogeneity of variance. However, non-transformed values are presented in the figures. Normality of each dataset was determined using the Kolmogorov-Smirnov test and homogeneity of variance was determined by use of Levene's test. Data were analyzed using either analysis of variance (ANOVA) or Kruskal-Wallis test, followed by Tukey's test or Mann-Whitney U test, respectively. Bonferroni Correction was applied following multiple comparisons using the Mann-Whitney U test. A probability level of $p \leq 0.05$ was considered significant. All data are shown as mean ± standard error of mean (S.E.M.). Data points of greater or less than ± 2 standard deviations (S.D.) from the mean were removed as outliers.

2.4 Results

2.4.1 Phylogeny

The cloned nucleotide sequence of white sturgeon CYP1A was uploaded to GenBank (Table 2.1). The CYP1A cDNA sequence cloned from the white sturgeon clustered closely with the Siberian sturgeon (*Acipenser baerii*). However, it was more distant from CYP1A sequences of Atlantic sturgeon (*Acipenser oxyrhynchus*), shortnose sturgeon (*Acipensesr brevirostrum*), and the sterlet (*Acipenser ruthenus*) (Figure 2.1). CYP1A of white sturgeon was more distant from CYP1A sequences of rainbow trout and other salmonids.

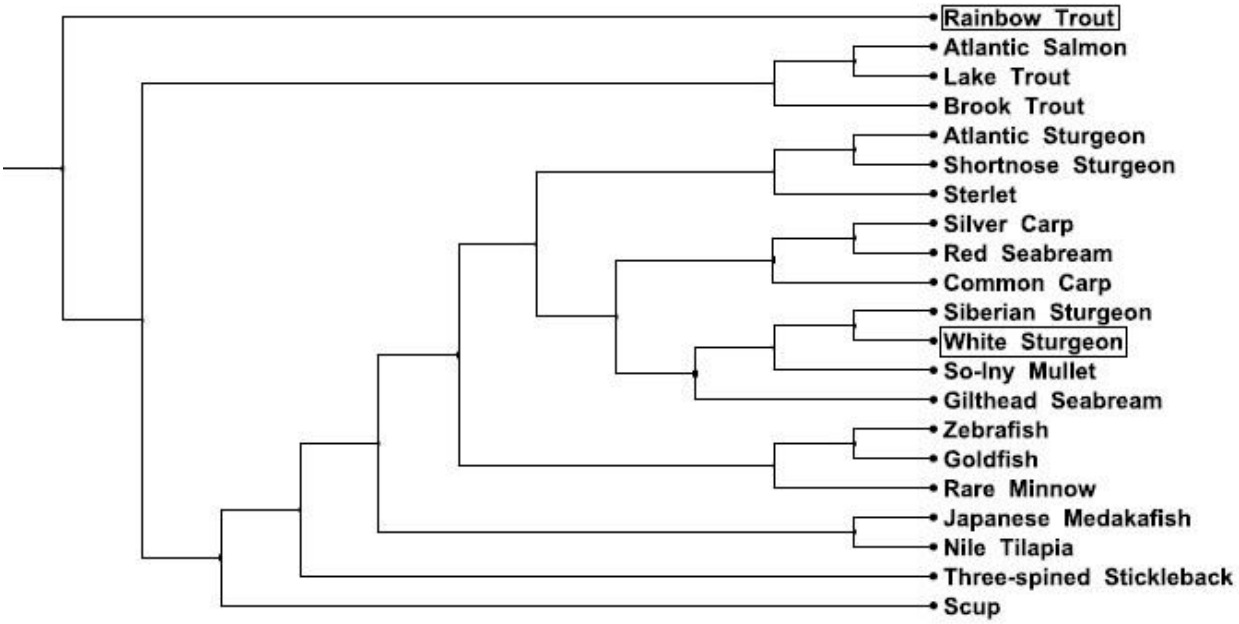


Figure 2.1. Phylogenetic tree for relatedness of CYP1A amino acid sequences among various fish species. White sturgeon and rainbow trout are highlighted. Branch lengths represent bootstrap values based on 1,000 samplings. Accession numbers used were rainbow trout (AAB69383.1), Atlantic salmon (*Salmo salar*; AAK52513.3), lake trout (*Salvelinus namaycush*; AAQ10900.1), brook trout (*Salvelinus fontinalis*; AAQ10899.1), Atlantic sturgeon (*Acipenser oxyrinchus*; ADX94782.1), shortnose sturgeon (*Acipenser brevirostrum*; ADX94783.1), sterlet (*Acipenser ruthenus*; AEN19340.2), silver carp (*Hypophthalmichthys molitrix*; ACH53596.1), red seabream (*Pagrus major*; ABV24471.1), common carp (*Cyprinus carpio*; BAB39379.1), Siberian sturgeon (*Acipenser baerii*; ADM47436.1), so-iny mullet (*Liza haematocheila*; ACO55176.1), gilthead seabream (*Sparus aurata*; AAB62887.1), zebrafish (*Danio rerio*; BAB90841.1), goldfish (*Carassius auratus*; ABF60890.1), rare minnow (*Gobiocypris rarus*; ABV01348.1), Japanese medakafish (*Oryzias latipes*; NP_001098557.1), nile tilapia (*Oreochromis niloticus*; ACJ60906.2), three-spined stickleback (*Gasterosteus aculeatus*; ADO15701.1), and scup (*Stenotomus chrysops*; AAA74969.1).

The sequence of AHR from white sturgeon clustered closely with sequences of AHR from other cartilaginous fishes such as the smooth dogfish (*Mustelus canis*) and little skate (*Leucoraja erinacea*). However, it was more distant from AHR sequences of most teleost fishes such as the rainbow trout and other salmonids (Figure 2.2). AHR sequences from other sturgeons are currently unavailable.

2.4.2 Enzyme activities and CYP1A transcript abundance in white sturgeon

Basal EROD activity was detected in livers of white sturgeon and rainbow trout, however it was undetectable in intestines or gills from either species (Table 2.2). Basal EROD activity in livers of white sturgeon was not significantly ($p \leq 0.05$) different than the basal EROD activity in livers of rainbow trout. Basal MROD and PROD activities were undetectable in livers and intestines of white sturgeon (Table 2.2). Average basal MROD activity in livers of rainbow trout was less than average basal EROD activity (Table 2.2). Basal MROD activity was not detected in intestines of rainbow trout. Due to a lack of tissue, MROD and PROD activity could not be determined in gills of white sturgeon or rainbow trout.

Transcripts of CYP1A were detectable in livers, gills, and intestines of white sturgeon not exposed to βNF. Under basal conditions, the abundance of transcripts of CYP1A was significantly ($p \leq 0.05$) greater in livers when compared to gill and intestine (Figure 2.3A). The abundance of transcripts of CYP1A was significantly ($p \leq 0.05$) less in intestine compared to that in gill (Figure 2.3A).

Exposure to βNF significantly induced ($p \leq 0.05$) EROD activity in liver, gill, and intestine of white stugeon (Figure 2.4). EROD activity was significantly less in liver of white

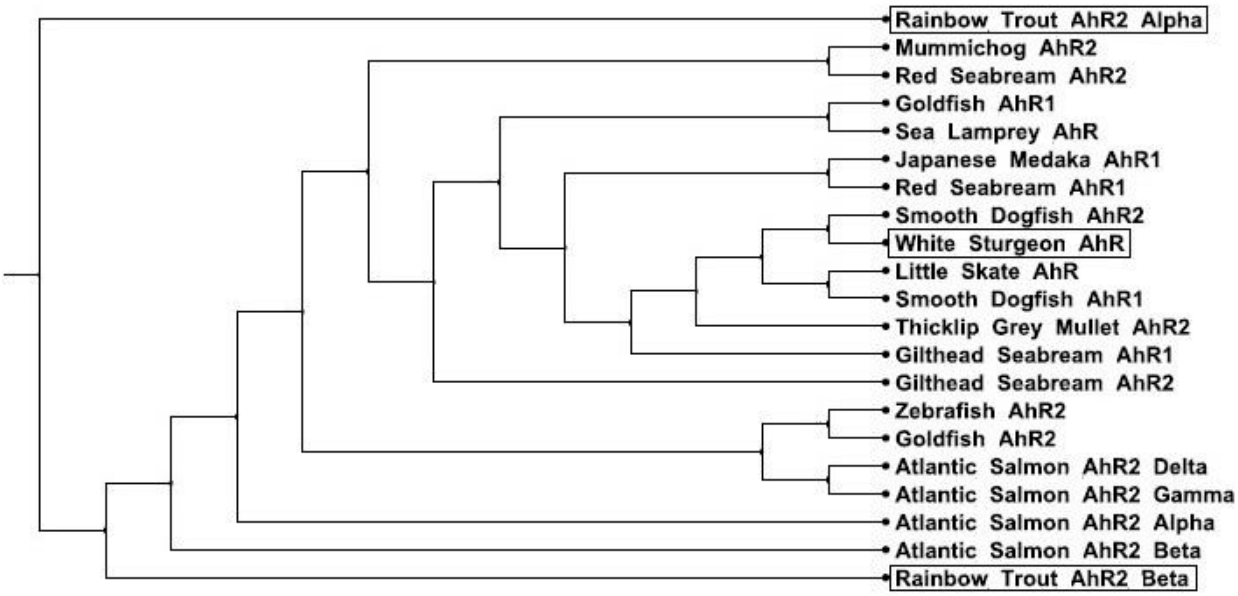
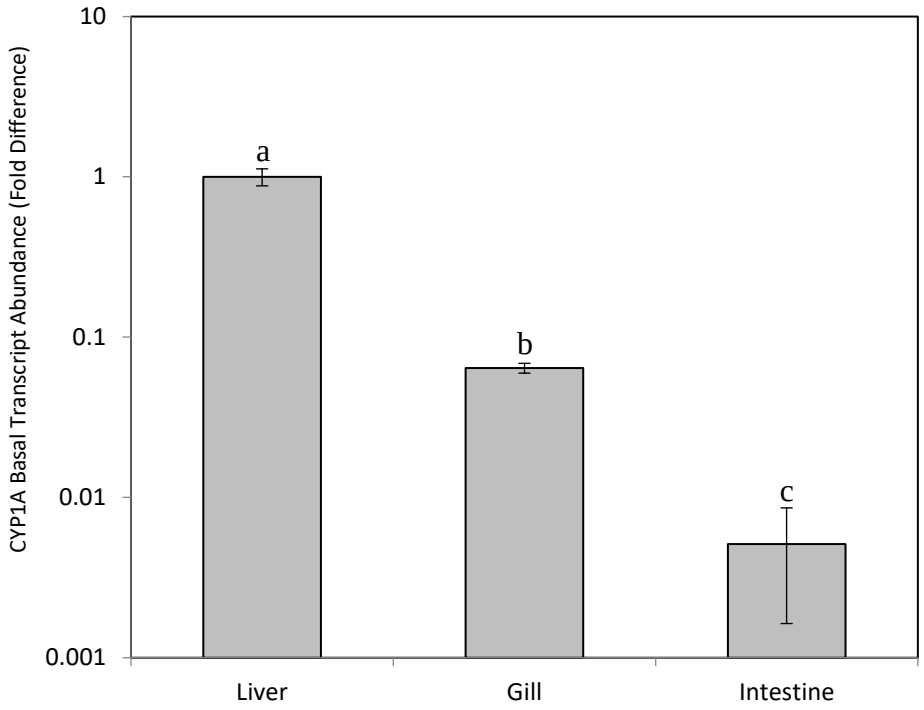


Figure 2.2. Phylogenetic tree for relatedness of AHR amino acid sequences among various fish species. White sturgeon and rainbow trout are highlighted. Branch lengths represent bootstrap values based on 1,000 samplings. Accession numbers used were rainbow trout AHR2α (NP_001117723.1), mummichog AHR2 (*Fundulus heteroclitus*; AAC59696.3), red seabream AHR2 (*Pagrus major*; BAE02825.1), goldfish AHR1 (*Carassius auratus*; ACT79400.1), sea lamprey AHR (*Petromyzon marinus*; AAC60338.2), Japanese medakafish AHR1 (*Oryzias latipes*; NP_001098148.1), red seabream AHR1 (*P. major*; BAE02824.1), smooth dogfish AHR2 (*Mustelus canis*; AAC60336.1), white sturgeon (AAX18240.1), little skate AHR (*Leucoraja erinacea*; AAC60337.1), smooth dogfish AHR1 (*M. canis*; AAC60335.1), thicklip grey mullet AHR2 (*Chelon labrosus*; AEI165611.1), gilthead seabream AHR1 (*Sparus aurata*; ABY82367.1), gilthead seabream AHR2 (*Sparus aurata*; AAN05089.1), zebrafish AHR2 (*Danio rerio*; NP_571339.1), goldfish AHR2 (*Carassius auratus*; ACT79401.1), Atlantic salmon AHR2δ (*Salmo salar*; NP_001117015.1), Atlantic salmon AHR2γ (*S. salar*; NP_001117037.1), Atlantic salmon AHR2α (*S. salar*; NP_001117156.1), Atlantic salmon AHR2β (*S. salar*; NP_001117028.1), and rainbow trout AHR2β (NP_001117724.1).

Table 2.2. Average EROD, MROD, and PROD activity in white sturgeon (WS) and rainbow trout (RT) i.p. injected with βNF at 0 mg/kg, 50 mg/kg, or 500 mg/kg. Livers and intestines were measured in pmol/min/mg protein, however gills were measured in pmol/min/mg tissue. Standard error of the mean (S.E.M.) shown in brackets. < D represents undetectable activity in all individuals.

			0 mg/kg	50 mg/kg	500 mg/kg
EROD	Liver	WS	14 (2.0)	510 (83)	241 (69)
		RT	9.6 (2.4)	850 (65)	-
	Intestine	WS	< D	4.8 (1.8)	9.0 (1.9)
		RT	< D	< D	-
	Gill	WS	< D	0.032 (0.0078)	0.068 (0.017)
		RT	< D	< D	-
MROD	Liver	WS	<D	1.3 (0.44)	1.2 (0.27)
		RT	3.8 (0.51)	280 (19)	-
	Intestine	WS	< D	0.84 (0.19)	2.7 (0.36)
		RT	< D	< D	-
PROD	Liver	WS	< D	< D	< D
		RT	< D	< D	-
	Intestine	WS	< D	< D	< D
		RT	< D	< D	-


10
1
0.1
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CYP1A Basal Transcript Abundance (Fold Difference)
a
b
c
Liver
Gill
Intestine

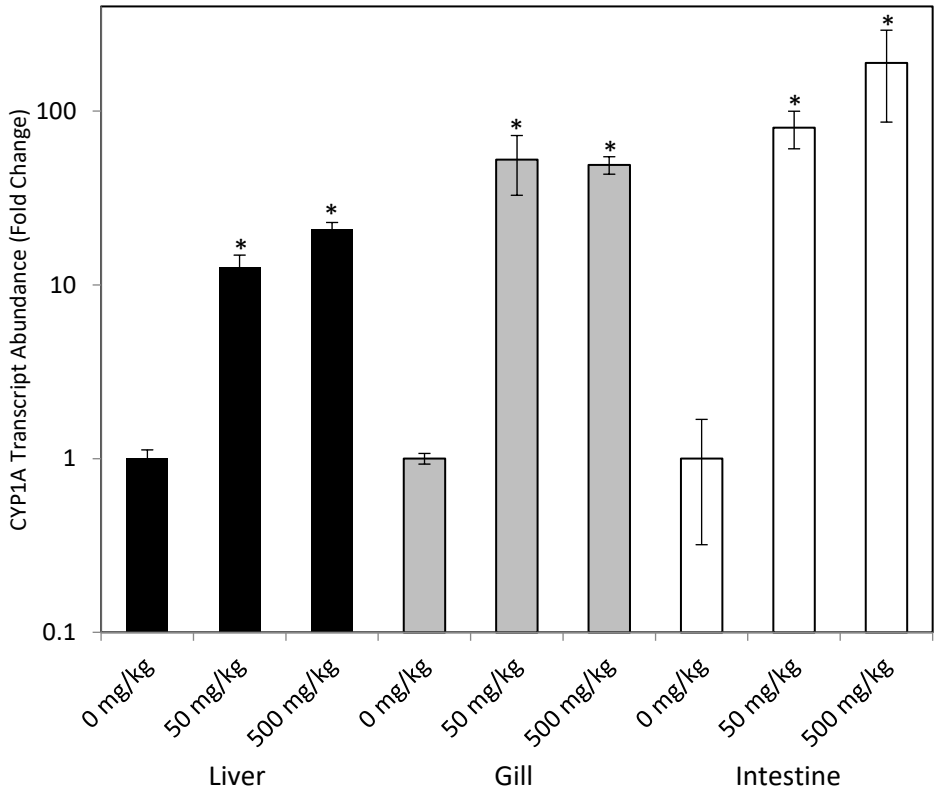

100
10
1
0.1
CYP1A Transcript Abundance (Fold Change)
*
0 mg/kg
50 mg/kg
500 mg/kg
Liver
Gill
Intestine

Figure 2.3. Comparison of basal CYP1A transcript abundance among liver, gill, and intestinal tissues in white sturgeon (A). Gill and intestine transcript abundance shown as fold difference from liver transcript abundance. Different letters indicate significant difference (Kruskal Wallis test; $p \leq 0.05$). Fold up-regulation in CYP1A transcript abundance determined by quantitative real-time PCR in white sturgeon liver, gill, and intestinal tissues 3 days following injection of either 50 or 500 mg βNF/kg, bw (B). Data represents mean ± S.E.M. (n = 5 fish) *Significantly different than liver, gill, or intestinal tissue at 0 mg βNF/kg, bw (one-way ANOVA; $p \leq 0.05$).

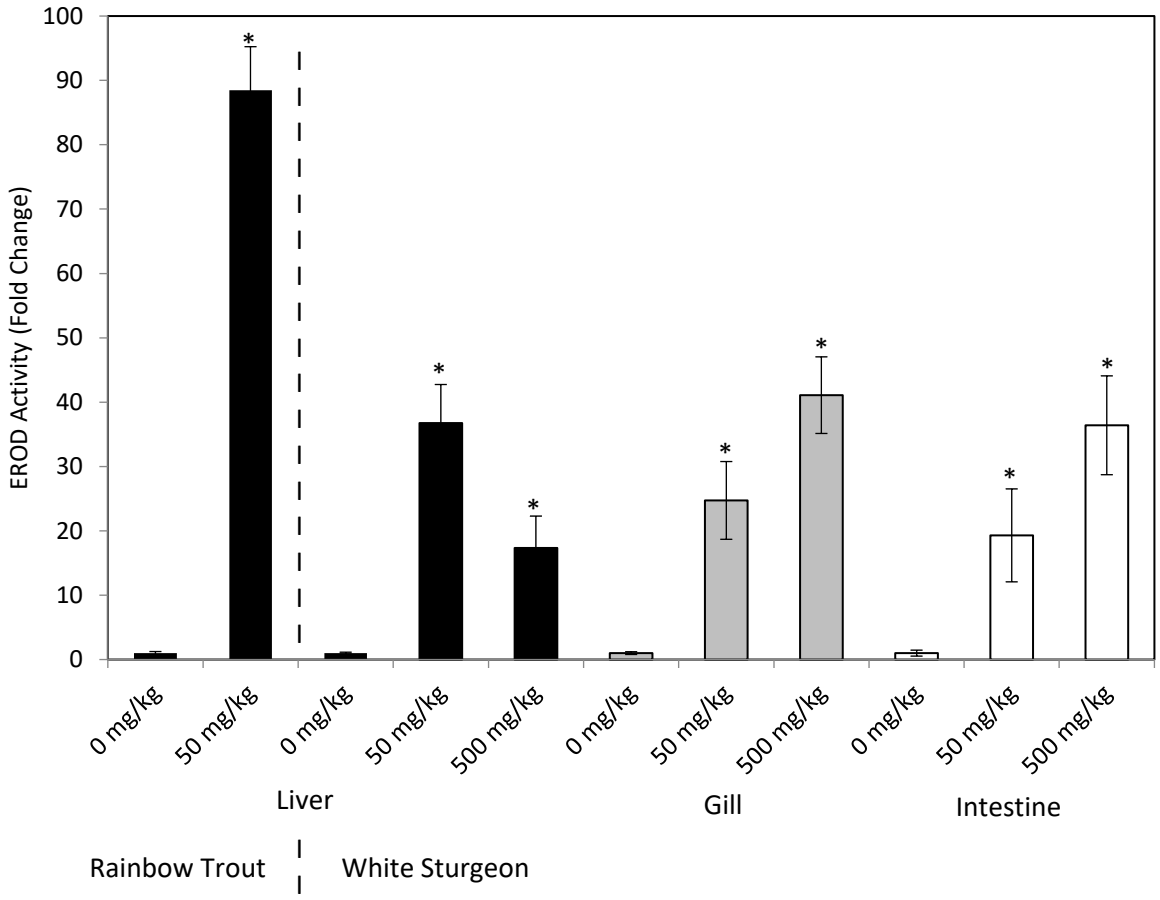


Figure 2.4. EROD activity shown as fold change over basal activity in liver, gill, and intestine of white sturgeon following injected with 50 or 500 mg βNF/kg, bw. Rainbow trout had undetectable activity in exposed and unexposed gill and intestine (data not shown). Induction of gill and intestine of white sturgeon is based upon ½ the detection limit due to undetectable basal activity. * indicates significant (Kruskal Wallis test; $p \leq 0.05$) difference over basal activity.

sturgeon exposed to 500 mg βNF/kg, bw (Figure 2.4). The greatest fold-induction in EROD activity was observed in gill of white sturgeon exposed to 500 mg βNF/kg, bw (Figure 2.4). The greatest absolute activity was detected in liver of white sturgeon exposed to 50 mg βNF/kg, bw (Table 2.2). EROD activity was detectable only in liver of rainbow trout exposed to βNF (Table 2.2). Significant induction in EROD activity was detected in liver of both rainbow trout and white sturgeon following exposure to 50 mg βNF/kg, bw; however, induction was significantly ($p \leq 0.05$) greater in rainbow trout (Figure 2.3).

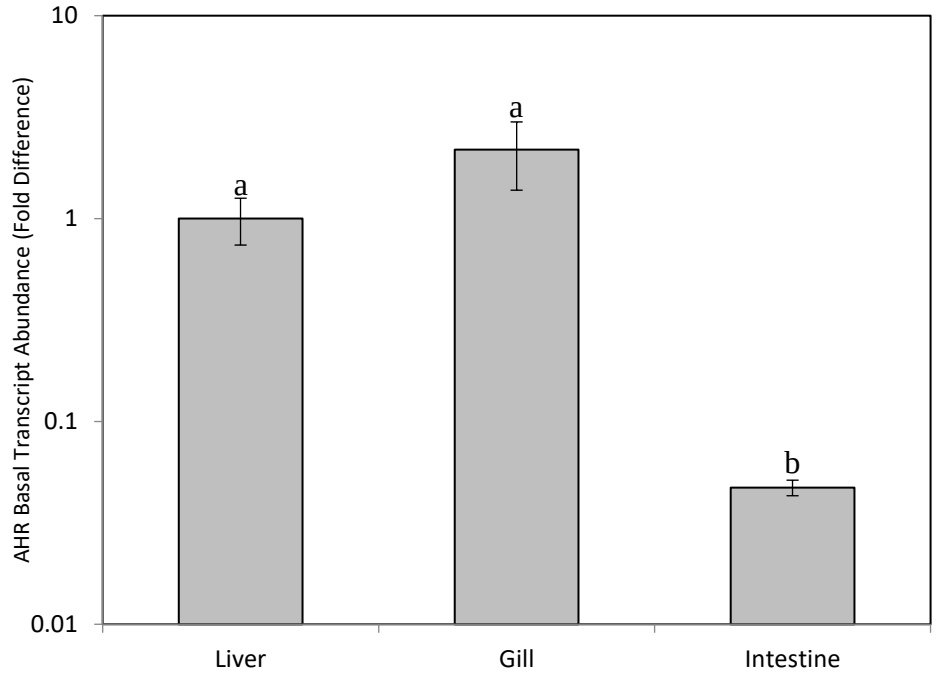
Exposure to βNF resulted in significantly ($p \leq 0.05$) greater MROD activity in liver and intestine of white sturgeon (Table 2.2). MROD activity detected in liver of white sturgeon exposed to 500 mg βNF/kg, bw and individuals exposed to 50 mg βNF/kg, bw were comparable (Table 2.2). The greatest MROD activity was observed in intestine of white sturgeon exposed to 500 mg βNF/kg, bw (Table 2.2). MROD activity was detectable in liver, but not in intestine of rainbow trout exposed to βNF (Table 2.2). Although MROD activity was detected in liver of rainbow trout and white sturgeon following exposure to 50 mg βNF/kg, bw, activity was greater in rainbow trout than in white sturgeon (Table 2.2). PROD activity was not detected in liver or intestine of white sturgeon or rainbow trout that had been exposed to βNF (Table 2.2). Transcript abundance of CYP1A was significantly ($p \leq 0.05$) greater in liver, gill, and intestine of white sturgeon exposed to either 50 or 500 mg βNF/kg, bw compared to controls (Figure 2.3B). The greatest observed up-regulation of CYP1A transcript abundance was in intestine (up to 189-fold), followed by gill (up to 53-fold), and then liver (up to 21-fold).

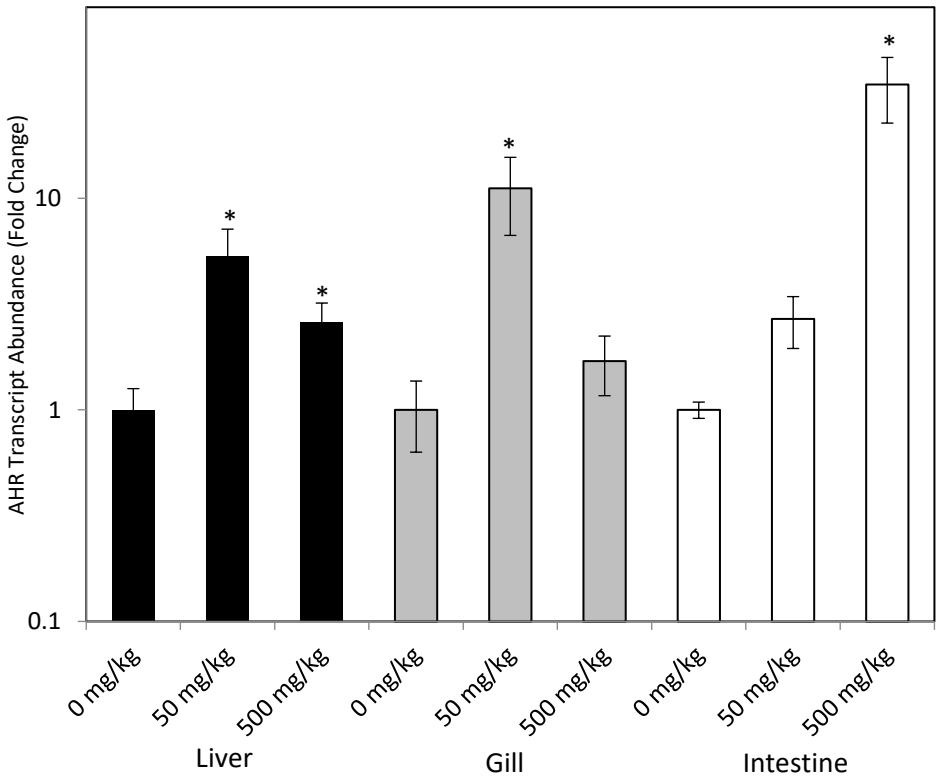
2.4.3 AHR transcript abundance in white sturgeon

Transcript abundance of AHR was significantly ($p \leq 0.05$) greater in gill and liver compared to intestine of white sturgeon not exposed to βNF (Figure 2.5A). No significant ($p \leq 0.05$) difference was observed between abundance of transcripts of the AHR in liver or gill of white sturgeon not exposed to βNF (Figure 2.5A). Significant ($p \leq 0.05$) up-regulation of AHR transcript abundance was observed in liver of white sturgeon exposed to 50 or 500 mg βNF/kg, bw (Figure 2.5B). Significant ($p \leq 0.05$) up-regulation of AHR transcript abundance was only observed in gill following exposure to 50 mg βNF/kg, bw while significant ($p \leq 0.05$) up-regulation of AHR transcript abundance was only observed in intestine following exposure to 500 mg βNF/kg, bw (Figure 2.5B). The greatest observed up-regulation was in intestine (up to 35-fold), followed by gill (up to 11-fold), and then liver (up to 5-fold).

2.5 Discussion

The biology and life-history of sturgeons might make them particularly susceptible to bioaccumulation of lipophilic pollutants. Therefore, better knowledge of how these ancient species respond to dioxin-like compounds has been the objective of this and previous investigations. In the present study, βNF was chosen as a model compound to study the biological effects following exposure to an AHR agonist and to further investigate the AHR signalling pathways of white sturgeon.


10
1
0.1
0.01
AHR Basal Transcript Abundance (Fold Difference)
a
a
b
Liver
Gill
Intestine


10
1
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AHR Transcript Abundance (Fold Change)
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0 mg/kg
50 mg/kg
500 mg/kg
0 mg/kg
50 mg/kg
500 mg/kg
0 mg/kg
50 mg/kg
500 mg/kg
Liver
Gill
Intestine

Figure 2.5. Comparison of basal AHR transcript abundance among liver, gill, and intestinal tissues in white sturgeon (A). Gill and intestine transcript abundance shown as fold difference from liver transcript abundance. Different letters indicate significant difference (Kruskal Wallis test; $p \leq 0.05$). Fold up-regulation of AHR transcript abundance determined by quantitative real-time PCR in white sturgeon liver, gill, and intestinal tissues 3 days following injection of either 50 or 500 mg βNF/kg, bw (B). Data represents mean ± S.E.M. (n = 5 fish) *Significantly different than liver, gill, or intestinal tissue at 0 mg βNF/kg, bw (Kruskal Wallis test; $p \leq 0.05$).

2.5.1 Relative responsiveness of white sturgeon

Basal EROD activities detected in liver of white sturgeon and rainbow trout were consistent with basal EROD activities previously published for these species (Table 2.3) (Foster et al., 2001; Zhang et al., 1990). Basal hepatic EROD activity detected in white sturgeon was comparable to activity published for Adriatic sturgeon (*Acipenser naccarii*), however basal hepatic EROD activity published for lake sturgeon (*A. fluvescens*) was less than that observed in this study for white sturgeon (Table 2.3) (Agradi et al., 1999; Rousseaux et al., 1995). Hepatic EROD activity for the similarly ancient species, the little skate (*L. erinacea*) was greater than that observed for the white sturgeon during the study reported upon here (Table 2.3) (Hahn et al., 1998). Differences in basal EROD activity are not uncommon between or among fishes and several reasons related to the biology of the fishes and physical environments can result in these differences (Whyte et al., 2000). Differences in basal EROD activity within a species are not uncommon, however basal hepatic EROD activity in immature white sturgeon of very different size classes did not differ significantly (Table 2.3) (Foster et al., 2001; Whyte et al., 2000). This is unlike rainbow trout where differences in basal hepatic EROD activity were observed among individuals of different sizes (Table 2.3) (Zhang et al., 1990). In addition, differences in sex, strain, nutritional status, and environmental parameters could also impact EROD activities between individuals (Whyte et al., 2000). Based on studies conducted with sturgeon, it appears that basal hepatic EROD activity varies little both among and within species of sturgeon; however, EROD activity in sturgeons and other cartilaginous fish species has not been well characterized.

Table 2.3 Average hepatic EROD activity in fish species i.p. injected with βNF (pmol/min/mg protein). Species sorted from least to greatest evolutionary position.

Species		0 mg/kg	50 mg/kg	Fold Induction	Source
Little Skate	*Raja erinacea*	109	852	8x	Hahn et al., 1998
Adriatic Sturgeon	*Acipenser naccarii*	15	-	-	Agradi et al., 1990
Lake Sturgeon	*Acipenser fluvescens*	3.4	-	-	Rousseaux et al., 1995
White Sturgeon[a]	*A.transmontaus*	14	511	37x	This study.
White Sturgeon[b]	*A.transmontanus*	11	-	-	Foster et al., 2001
Mudfish	*Clarias anguillaris*	64	337	5x	Gadagbui et al., 1996
Mummichog	*Fundulus heteroclitus*	244	1386	6x	Kloepper-Sams and Stegeman, 1989
European Eel	*Anguilla anguilla*	24.7	1843	75x	Fenet et al., 1998
Northern Pike	*Esox lucius*	480	4660	10x	Forlin and Celander., 1993
Atlantic Salmon	*Salmo salar*	50	1600	32x	Grosvik et al., 1997
Brook Trout	*Salvelinus fontinalis*	25	1960	78x	Elskus and Stegeman., 1989
Brown Trout	*Salmo trutta*	44	12480	284x	Forlin and Celander, 1993
Rainbow Trout[c]	*O.mykiss*	9.6	849	88x	This study.
Rainbow Trout[d]	*O.mykiss*	80	7000	88x	Zhang et al., 1990
Largemouth Bass	*Micropterus salmoides*	739	2700	6x	Zhang et al., 1991
Nile Tilapia	*Oreochromis niloticus*	80	485	6x	Gadagbui et al., 1996
Dab	*Limanda limanda*	530	7070	13x	Forlin and Celander, 1993
European Perch	*Perca fluviatilis*	440	2560	6x	Forlin and Celander, 1993

[a] White sturgeon weighted 12 to 27 g.

[b] White sturgeon weighted greater than 2 kg.

[c] Rainbow trout weighed 9 to 24 g.

[d]Rainbow Trout weighed 100 to 200 g.

White sturgeons were less responsive to βNF than rainbow trout, which is known to be one of the most responsive fishes (Table 2.3) (Zhang et al., 1990). In fact, white sturgeons were more responsive than most fishes, other than salmonids, that have been studied to date (Table 2.3). Although there is uncertainty regarding the cause and effect relationship between EROD responsiveness and population-level endpoints, this observation suggests that white sturgeon could be among the more sensitive species to the effects of dioxin-like compounds (Whyte et al., 2000). Since few studies have characterized responsiveness of EROD in other sturgeons, it cannot be determined whether different species of sturgeon have comparable responsiveness to AHR agonists. However, one study with the Adriatic sturgeon (*A. naccarii*) i.p. injected with 80 mg βNF/kg, bw found an 11-fold induction in EROD activity, a value that is less than that observed in this study of white sturgeon exposed to either 50 or 500 mg βNF/kg, bw (Agradi et al., 1999). This suggests that some variability in responsiveness among sturgeons exists.

White sturgeon exhibited relatively little basal MROD activity and responsiveness compared to rainbow trout (Table 2.2). Lesser MROD activity which is associated with CYP1A2 indicates lesser expression of this CYP isoform in white sturgeon compared to rainbow trout and could be a characteristic of ancient fish species. No published work is currently available characterizing MROD activity or reports on the presence of the CYP1A2 isoform in sturgeons or other cartilaginous fish species. Likewise, PROD activity which is associated with CYP1B1 was undetectable in white sturgeon and has not been characterized in sturgeons or cartilaginous fishes, which suggests the same scenario as CYP1A2 could exist. The functional significance, if any, of the lesser MROD and PROD activity in white sturgeon is unknown.

2.5.2 Tissue-specific responsiveness

The greater induction of EROD activity and CYP1A transcript abundance observed in gill and intestine of white sturgeon was unexpected since this is the first report of equal or greater relative induction of EROD activity and transcripts of CYP1A in gill and intestine than in liver following i.p. injection with an AHR agonist. EROD activity was not detected in gill of sea bass (*Dicentrarchus labrax*) i.p. injected with 80 mg βNF/kg, bw (Novi et al., 1998). However significant induction was observed in liver (Novi et al., 1998). Similarly, the rock cod (*Trematomus bernacchii*) showed less than a 2-fold induction of EROD activity in gill and intestine following i.p. injection with 80 mg βNF/kg, bw (Di Bello et al., 2007). In the shortnose sturgeon (*A. brevirostrum*) i.p. injection with PCB 126 resulted in a comparable induction in CYP1A transcript abundance in liver and intestine as in white sturgeon, but no statistically significant ($p \leq 0.05$) induction was observed in gill (Roy et al., 2011).

Several metabolic responses to xenobiotics are known to be inducible in intestine of fishes under certain conditions (Hanninen et al., 1987). An average 33-fold induction in intestinal EROD activity was observed in spot (*Leiostomus xanthurus*) collected from one site in Chesapeake Bay, MD, USA heavily contaminated by polycyclic aromatic hydrocarbons (PAHs) (Van Veld et al., 1988). Although induction of CYP1A transcript abundance and EROD activity was greatest in intestine of white sturgeon, absolute activities were still less than in liver. These results suggest that the liver is the primary tissue responsible for metabolism of xenobiotics in white sturgeon, but other tissues such as gill and intestine have the capacity to respond to exposure to AHR agonists. Since no detectable induction of EROD activity was observed in gill or intestine of rainbow trout following i.p. injection with βNF, greater xenobiotic metabolism

capacity of these tissues could either be specific to sturgeon or a trait of ancient fishes. Regardless, it is currently unclear as to the functional significance of this capacity of metabolism in gill and intestine of white sturgeon. These tissues could be important in the excretion of readily metabolized compounds such as PAHs that are likely to first come into contact with the gill and intestine of a benthic species such as the sturgeon. Differential inducibility of different tissues following exposure to AHR agonists is likely linked to tissue-specific regulation of the AHR signalling pathway.

2.5.3 Effects on AHR transcript abundance

Previous studies have demonstrated induction of AHR expression in fishes following exposure to an AHR agonist (Andreasen et al., 2002; Tanguay et al., 1999). Up-regulation of AHR2 transcript abundance and subsequent expression of the protein catalyst has been observed in primary hepatocytes of rainbow trout exposed to βNF (Wiseman and Vijayan, 2007). However, induction of AHR expression is not necessarily the rule in vertebrates. Unlike in rainbow trout, exposure of clawed frogs (*Xenopus tropicalis*) to PCB 126 resulted in no significant difference in AHR transcript abundance (Jonsson et al., 2011). In contrast to AHR, significantly greater CYP1A transcript abundance and EROD activity were detected in clawed frogs following exposure to PCB 126 (Jonsson et al., 2011). Therefore, AHR transcript abundance observed in white sturgeon is consistent with previous results observed for fishes, but differs from that of some other vertebrates.

Tissue-specific up-regulation of expression of AHR transcript following exposure to an AHR agonist is currently unknown. Under basal conditions white sturgeon showed uniform

expression of AHR transcript in liver and gill, but significantly less in intestine, while in the mummichog (*Fundulus heteroclitus*) uniform expression of AHR transcript under basal conditions was observed in heart, liver, ovary, testis, brain, kidney, and gill, but intestine was not investigated (Karchner et al., 1999). By contrast, in salmonids, tissue-specific expression of AHR transcript varied among tissues by orders of magnitude, with greatest expression in the liver and heart (Abnet et al., 1999; Hansson and Hahn, 2008). The results of this study on white sturgeon suggests that the up-regulation observed in CYP1A transcript is accompanied by up-regulation of AHR transcript and presumably greater transcription to protein for increased ligand binding. At this time no reliable antibodies exist to measure AHR protein content of white sturgeon, but future studies should investigate the relationship between AHR transcript abundance and actual translation to protein. Previous work with primary hepatocytes of rainbow trout found lesser up-regulation in AHR transcript than that observed in white sturgeon (Aluru et al., 2005). It is possible that white sturgeon exhibit greater up-regulation of transcription of the AHR due to either: (1) lesser mRNA-protein translation than other fishes, (2) unstable transcripts, or (3) lesser basal levels of either transcript or protein.

Gills of white sturgeon exhibited lesser responsiveness of AHR transcript upregulation following exposure to 500 compared to 50 mg βNF/kg, bw. No studies could be located presenting comparable negative feedback of AHR transcript abundance following exposure to greater doses of an AHR agonist. Alternatively, the system could have been overwhelmed and reduced AHR transcript abundance is a first sign of overt toxicity. At lesser doses of an AHR agonist the gill appears to be more responsive with up-regulation in AHR and CYP1A transcript abundance. However, following a greater exposure the gill showed little responsiveness of AHR transcript abundance. It is speculated that this could be a protective mechanism against oxidative

damage at the delicate gill surface. A negative feedback system for AHR transcript abundance was not observed in the liver or intestine of white sturgeon.

Relative to other cartilaginous fishes, sturgeons are believed to possess AHR genes comprising three clades: AHR1, AHR2, and AHR3 (Hahn et al., 2006). Although the role of each isoform is not yet known, in zebrafish (*D. rerio*) AHR2 is the active form while AHR1 has been found to be inactive (Andreasen et al., 2002). Little information is available regarding the AHR3 clade known only in some cartilaginous fishes (Hahn et al., 2006). It is not definitively known which AHR gene was sequenced during this study. Considering AHR2 is the known active isoform in fishes, it is likely that the AHR2 was characterized in white sturgeon. This AHR gene had clear tissue-specific expression and induction following exposure to an AHR agonist. Nevertheless, the possibility exists for other AHR genes to follow comparable induction patterns, particularly the relatively unknown AHR3 clade. It has been hypothesized that the responsiveness of salmonids to the effects of AHR agonists could be due in part to their having multiple, functional AHR genes (Hansson and Hahn, 2008). Expression, binding affinity, and implications to dioxin-like compound sensitivity of sturgeon resulting from expression of AHR2 and AHR3 genes are unknown. Characterizing the isoform specific expression of AHR genes in white sturgeon would require isoform specific primers and was beyond the scope of this study.

2.6 CONCLUSION

This study demonstrated that white sturgeons are responsive to AHR agonists and could be among the most responsive of fishes with regard to inducibility of CYP1A. Although the liver is the main location of these responses, other organs such as the gill and intestine have the

capacity to respond as evidenced by both the level of induction in EROD activity as well as induction of both CYP1A and AHR transcript abundance. Upon further study of AHR transcript abundance in white sturgeon, it appears that AHR dynamics could be both species and tissue-specific in fishes. It is currently unclear whether induction of AHR transcript abundance has an impact on species or tissue sensitivity to dioxin-like compounds. For this reason, white sturgeon could be a candidate for researching AHR function and differential species sensitivity to dioxin-like compounds in ancient and cartilaginous fish species.

CHAPTER 3

3 IDENTIFICATION AND EXPRESSION OF ARYL HYDROCARBON RECEPTORS (AHR1 AND AHR2) PROVIDE INSIGHT IN AN EVOLUTIONARY CONTEXT REGARDING SENSITIVITY OF WHITE STURGEON (*ACIPENSER TRANSMONTANUS*) TO DIOXIN-LIKE COMPOUNDS

PREFACE

Chapter 2 demonstrated that white sturgeon (*Acipenser transmontanus*) respond to activation of the AHR in a manner that is generally consistent with that of modern teleost fishes, namely through induction of phase I biotransformation enzymes. However, nothing was known regarding the aryl hydrocarbon receptors (AHRs) of sturgeons. Since the AHR drives most, if not all, adverse effects of exposure to dioxin-like compounds (DLCs) in vertebrates, the aim of Chapter 3 was to identify AHRs expressed in white sturgeon and characterize their tissue-specific patterns of expression and autoregulation of expression following exposure to an agonist of the AHR. Better characterization of the AHR pathway of sturgeons is critical in order to develop a mechanism-based biological model that allows prediction of the sensitivity of any species of sturgeon to exposure to DLCs.

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Author contributions:

Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Steve Wiseman (University of Saskatchewan) provided inspiration, scientific input, guidance, and training, commented on and edited the manuscript.

Shawn C. Beitel (University of Saskatchewan) provided laboratory assistance with the *in vivo* exposure, sample collection, and polymerase chain reaction.

Drs. John P. Giesy and Markus Hecker (both University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1 Abstract

Sturgeons are ancient fishes, which are endangered in many parts of the world. Due to their benthic nature and longevity, sturgeons are at great risk of exposure to bioaccumulative contaminants such as dioxin-like compounds (DLCs). Despite their endangered status, little research has been conducted to characterize the relative sensitivity of sturgeons to DLCs. Proper assessment of risk of DLCs posed to these fishes therefore, requires a better understanding of this sensitivity and the factors that are driving it. Adverse effects associated with exposure to DLCs are mediated by the aryl hydrocarbon receptor (AHR). This study identified and characterized two distinct AHRs, AHR1 and AHR2, in white sturgeon (*Acipenser transmontanus*) for the first time as a first step in studying the relative sensitivities of sturgeons to DLCs. Furthermore, tissue-specific expression of both AHRs under basal conditions and in response to exposure to the model DLC, β-naphthoflavone (βNF), was determined. The sequence of amino acids of AHR1 of white sturgeon had greater similarity to AHRs of tetrapods, including amphibians, birds, and mammals, than to AHR1s of other fishes. The sequence of amino acids in the ligand binding domain of the AHR1had greater than 80 % similarity to AHRs known to bind DLCs and was less similar to AHRs not known to bind DLCs. AHR2 of white sturgeon had greatest similarity to AHR2 of other fishes. Profiles of expression of AHR1 and AHR2 in white sturgeon were distinct from those known in other fishes and appear more similar to profiles observed in birds. Expressions of both AHR1 and AHR2 of white sturgeon were greatest in liver and heart, which are target organs for DLCs. Furthermore, abundances of transcripts of AHR1 andAHR2 in all tissues from white sturgeon were greater than controls (up to 35-fold) following exposure to βNF. Based upon both AHRs having similar abundances of transcript in target organs of DLC

toxicity, both AHRs being up-regulated following exposure to βNF, and both AHRs having greatest similarity to AHRs known to bind DLCs, it is hypothesized that both AHR1 and AHR2 of white sturgeon might mediate effects of DLCs in this species. Since current risk assessments are based on data derived largely from highly divergent fishes within the Salmonidae, presence of two functional AHRs in white sturgeon, one of which has greatest similarity to AHRs of birds, might have significant implications for the sensitivity of sturgeons to DLCs compared to other fishes.

3.2 Introduction

Sturgeons (Acipenseridae) are ancient fishes with recognizable fossils of modern species dating back at least 65 million years (Wilimovsky, 1956). Today, sturgeons are endangered over much of their range, which has rendered them of great interest in context with ecological risk assessment. In the northwestern USA and British Columbia, Canada there is particular concern about declines of some populations of white sturgeon (*Acipenser transmontanus*), the largest freshwater species of fish in North America. These decreases in populations of white sturgeon have been attributed to several human activities, including pollution (Birstein, 1993; Coutant, 2004; Gisbert and Williot, 2002; Irvineet al., 2007; Luk'yanenko et al., 1999; Paragamian and Hansen, 2008; Scott and Crossman, 1973). Sturgeons are long-lived, sexual maturity is attained slowly, they spawn only intermittently, live in close association with sediments, and have a greater lipid content than numerous other fishes which increases the likelihood of bioaccumulation of lipophilic pollutants (Birstein, 1993). Dioxin-like compounds (DLCs), which include polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyls (PCBs), and certain polycyclic aromatic hydrocarbons (PAHs) are contaminants of concern because of their ability to bioaccumulate and because they can be persistent under certain conditions, such as in sediments (Birnbaum and DeVito,1995). Some DLCs have been detected in white sturgeon at concentrations sufficient to warrant concern (Foster et al., 1999, 2001; Kruse and Scarnecchia, 2002; Kruse and Webb, 2006; MacDonald et al., 1997) given chronic effect levels in some species of fishes (Giesy et al., 2002; Rigaud et al., 2013). Due to their specific life history, white sturgeon could be particularly susceptible to the

adverse effects of bioaccumulation of DLCs. However, little is currently known regarding the sensitivity of sturgeons or other ancient fishes to these contaminants.

PCDDs, PCDFs, PCBs, and other DLCs share structural similarities and bind with relatively high affinity to the aryl hydrocarbon receptor (AHR) (Giesy et al., 1994). Following ligand binding, the AHR heterodimerizes with the aryl hydrocarbon nuclear translocator(ARNT) allowing binding to consensus dioxin responsive elements on DNA, resulting in pleiotropic expression of a suite of biotransformation enzymes and regulating all known effects of exposure to DLCs (Okey, 2007). Activation of AHR-mediated pathways causes a range of adverse effects in vertebrates, including hepatotoxicity, immune suppression, reproductive and endocrine impairment, teratogenicity, carcinogenicity, and loss of weight (Kawajiri and Fujii-Kuriyama, 2007). Some fishes, such as salmonids, are among the vertebrates of greatest sensitivity to adverse effects from exposure to DLCs (Johnson et al., 1998; Walker et al., 1991). Despite the potential for adverse effects of DLCs to sturgeons, little is known about the sensitivity of these and other ancient fishes to these chemicals. Sturgeons and some other ancient fishes, including sharks, rays, and skates share similar molecular responses to DLCs with regards to cytochrome P450 enzymes that are consistent with the more modern teleost fishes (Agradi et al., 1999; Doering et al., 2012; Hahn et al., 1998; Roy et al., 2011). Toxicity studies conducted with sturgeons have found them to be among the most sensitive fishes to adverse effects of other environmental pollutants, such as endocrine disrupting chemicals and metal ions (Dwyer et al., 2005; Vardy et al., 2011, 2012). This evidence justifies the hypothesis that sturgeons could be sensitive to DLCs, which together with their great risk of exposure, warrants further investigations into the AHR signalling pathway of sturgeons.

Knowledge of the specific structure of the AHRs of sturgeons is important because it has been shown that in birds, sensitivity to DLCs is related to the sequence of amino acids of the ligand binding domain of the AHR (Karchner et al., 2006; Farmahin et al., 2012, 2013; 2012, 2013; Head et al., 2008). However, a similar relationship between species sensitivity to DLCs and the sequence of amino acids of the AHR has not yet been established for fishes (Doering et al., 2013). To establish such relationships, a better understanding of the AHR indifferent species of fishes would be required, with the ultimate goal of allowing the prediction of the sensitivity of species of concern, such as some species of sturgeons, to DLCs. Therefore, objectives of this study were to identify full-length amino acid sequences of AHRs expressed in different tissues of white sturgeon and to determine their tissue-specific expression under basal conditions and in response to exposure to a model DLC. This work will supplement current knowledge on evolutionary aspects of the AHR pathway among ancient fishes and allow for a better understanding of the mechanisms by which sturgeons respond and their sensitivity to exposure to DLCs.

3.3 Materials and methods

3.3.1 Fish

Juvenile white sturgeon (*A. transmontanus*), ranging in mass from 12 to 27 g (approximately 1.5 years of age) were randomly selected from an in-house stock reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). White sturgeons were maintained in separate 712 L tanks under flow-through conditions at approximately 12 °C

and fed frozen bloodworms (Hagen, Montreal, QC, Canada) at approximately 2 % of their body weight daily.

3.3.2 Exposure protocol

The protocol for exposing juvenile white sturgeon has been described previously (Doering et al., 2012). Briefly, twenty-one individuals were injected intraperitoneally (i.p.) with one of three doses (n = 7) of beta-naphthoflavone (βNF purity > 98 %; Sigma-Aldrich, Oakville, ON, Canada) dissolved in corn oil. Doses used were 0 mg βNF/kg-bw (0 mM), 50 mg βNF/kg-bw (46 mM), and 500 mg βNF/kg-bw (460 mM). Three days following injection, all fish were euthanized by overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich). Livers, gills, and intestines were collected from fish exposed to βNF and immediately snap-frozen in liquid nitrogen. Brains, hearts, livers, gills, stomachs, intestines, spleens, head kidney, and muscle were collected from control fish for basal expression studies and immediately snap-frozen in liquid nitrogen.

3.3.3 Identification and sequencing of AHRs in white sturgeon

Full-length AHR1 and AHR2 genes had not yet been identified for sturgeons. However, a fragment of 609 nucleotides from an AHR-like gene of white sturgeon was available online (Accession#: AY880254.1). Additional nucleotide fragments of AHR-like genes were identified in a library of the transcriptome of liver from white sturgeon that was generated by paired-end sequencing by use of the *Illumina* Hi-Seq 2000 platform (Illumina, San Diego, CA, USA).

Methods used for *Illumina* paired-end transcriptome sequencing have been described previously (Tompsett et al., 2013; Wiseman et al., 2013). Full-length cDNA sequences for each AHR were acquired by use of rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). cDNA was synthesized by use of the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and RACE-PCR was performed by use of the Advantage 2 PCR Kit (Clontech), both according to the protocol provided by the manufacturer. Gene-specific RACE-PCR primers for white sturgeon AHR1 and AHR2 were designed by use of Primer3 software (Table 3.1) (Rozen and Skaletsky, 2000) according to the protocol provided by the manufacturer and synthesized by Invitrogen (Burlington, ON, Canada). Purified PCR products were cloned into pGEM-T easy vectors using a DNA ligation kit (Invitrogen) and transformed into competent JM109 *Escherichia coli* cells (Promega, Madison, WI, USA). Plasmids were isolated by use of a plasmid kit (Qiagen; Toronto, ON, Canada) and the products were sequenced by the University of Calgary's University Core DNA Services (Calgary, AB, Canada). Primers for amplification of full-length cDNAs of AHR1 and AHR2 were designed as described above (Table 3.1) and products were amplified by use of a LongRange PCR Kit (Qiagen). Full-length cDNAs were cloned into vectors and sequenced as described above. Consensus nucleotide sequences for AHR1 and AHR2 were determined by aligning of three or more replicated sequences by use of the Biology Workbench v.3.2 (Subramaniam, 1998).

3.3.4 Sequence alignment and phylogeny

Amino acid sequence alignments and phylogenetic tree were generated for vertebrate AHRs using the CLC Genomics Workbench v.4.7.2 (Katrinebjerg, Aarhus, Denmark). Accession

Table 3.1 Sequences, efficiency, and corresponding target gene Genbank accession number of white sturgeon oligonucleotide primers used in rapid amplification of cDNA ends PCR, in sequencing of full-length cDNA, and in quantitative real-time PCR. Annealing temperatures were 70, 67, and 60 °C for RACE, full, and qPCR, respectively.

Assay	Target Gene	Accession #	Primer Sequence (5'-3')	Efficiency (%)
RACE	AHR1	NA	Forward: AAAAGACCTGCTGGAAATGGCCTCC	NA
RACE	AHR2	AY880254	Forward: AATCTGAGCAGGGCACGGAACTCAT Reverse: CAAACAGAGCCAGCTGAGAGGGGAC	NA
Full	AHR1	NA	Forward: ATGTATGCAAGCCGCAAAAGGC Reverse: TGGAAAGCCACTGGATGTGG	NA
Full	AHR2	NA	Forward: AAGGTTTCTTTGGGCTTCGGSTSTT Reverse: TGGCGGTCTAAAATACAGGATACTCATC	NA
qPCR	β-actin	FJ205611	Forward: CCGAGCACAATGAAAATCAA Reverse: ACATCTGCTGGAAGGTGGAC	96
qPCR	AHR1	KJ420394	Forward: GAATTGCGCCTTTTATCGAG Reverse: TTTGCACTTTTCTGCACTGG	94
qPCR	AHR2	KJ420395	Forward: TGGAGATCAGGACCAAGACC Reverse: GTGTAACCCAGCACCACCTT	90

numbers used were: Goldfish AHR1 (ACT79400.1); Zebrafish AHR1a (AAM08127.1); Spiny Dogfish Shark AHR3 (AFR24094.1); Sea Lamprey AHR (AAC60338.2); Japanese Medakafish AHR1b (BAB62011.1); Japanese Medakafish AHR1a (BAB62012.1); Red Seabream AHR1 (BAE02824.1); Zebrafish AHR1b (AAI63508.1); Mummichog AHR1 (AAR19364.1); Spiny Dogfish Shark AHR1 (AFR24092.1); Goldfish AHR2 (ACT79401.1); Zebrafish AHR2 (AAI63711.1); Mummichog AHR2 (AAC59696.3); Red Seabream AHR2 (BAE02825.1); Rainbow Trout AHR2b (NP 001117724.1); Rainbow Trout AHR2a (NP 001117723.1); Spiny Dogfish Shark AHR2a (AFR24093.1); Albatross AHR2 (BAC87796.1); Cormorant AHR2 (BAF64245.1); Hamster AHR (NP 001268587.1); Mouse AHR (NP 038492.1); Guinea Pig AHR (NP 001166525.1); Albatross AHR1 (BAC87795.1); Cormorant AHR1 (BAD01477.1); Quail AHR1 (ADI24459.2); Chicken AHR1 (NP 989449.1); *Xenopus* AHR (JC7993).

3.3.5 Quantitative real-time PCR

Total RNA was extracted from approximately 30 mg each of brain, heart, muscle, liver, gill, stomach, intestine, spleen, head kidney, or muscle from each individual fish by use of the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the protocol provided by the manufacturer. Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples of RNA were stored at -80 °C until analyzed. First-strand cDNA synthesis was performed by use of the QuantiTect Reverse Transcription Kit (Qiagen) with 1 μg of total RNA according to the protocol provided by the manufacturer and the samples of cDNA were stored at -20 °C until analyzed.

Real-time PCR (qPCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 70 µl reaction mixture of 2x concentrated Power SYBR Green master mix (Qiagen), 3.5 µl cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water was prepared for each cDNA sample and primer combination. qPCR primers for β-actin of white sturgeon were acquired from Doering et al., 2012 (Table 3.1). qPCR primers for AHR1 and AHR2 of white sturgeon were designed from consensus nucleotide sequences as described in Section 2.3 (Table 3.1). Reactions were conducted in triplicate with 20 µl reaction volumes per well. The reaction mixture for PCR was denatured at 95 °C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Target gene transcript abundance was quantified by normalizing to β-actin according to methods described previously (Simon, 2003).

3.3.6 Statistical analysis

Statistical analyses were conducted using SPSS 19 software (SPSS, Chicago, IL, USA). A logarithmic transformation was used whenever necessary to ensure homogeneity of variance, however, non-transformed values are presented in the figures. Normality of each dataset was tested by use of the Kolmogorov-Smirnov test and homogeneity of variance was determined using Levene's test. Data were analyzed using t-test, analysis of variance (ANOVA), or Kruskal-Wallis test, as appropriate. Tests were followed by either Tukey's test or Mann-Whitney U test where applicable. Bonferroni Correction was applied where applicable. All data are shown as mean ± standard error of mean (SE) with n = 5.

3.4 Results

3.4.1 Identification of AHRs of white sturgeon

Full-length sequences of two distinct AHRs were identified in livers from white sturgeon. One receptor was homologous to the AHR1 and had a length of 834 amino acids, while the other was homologous to the AHR2 and had a length of 1,100 amino acids. These two receptors are 39 % similar at the amino acid level. Full-length nucleotide and amino acid sequences for AHR1 (Accession#: KJ420394) and AHR2 (Accession #: KJ420395) of white sturgeon have been made available online.

3.4.2 Phylogeny of AHRs of white sturgeon

The putative full-length sequence of amino acids of AHR1 of white sturgeon clustered closely with AHRs of tetrapods, including amphibians, birds, and mammals, and was more divergent fromAHR1s of other fishes (Figure 3.1). The putative sequence of amino acids in the ligand binding domain of the AHR1 of white sturgeon had greater than 80 % similarity with avian AHR1s and amphibian AHRs, as well as AHRs from some other ancient species of fishes (Table 3.2).

The putative full-length amino acid sequence of AHR2 of white sturgeon clustered closely with AHR2s of other fishes (Figure 3.1). The putative sequence of amino acids in the ligand binding domain of the AHR2 of white sturgeon was greater than 80 % similar to the ligand binding domain of AHR2s of some fishes, including the shark, as well as avian AHR2

sequences (Table 3.2). An alignment of the putative amino acid sequence of the ligand binding domain of AHR1and AHR2 of white sturgeon with the ligand binding domains of AHRs of other vertebrates is illustrated (Figure 3.2).

3.4.3 Tissue distribution and basal expression of AHRs in white sturgeon

Transcripts of AHR1 and AHR2 were amplified in livers, brains, gills, hearts, spleens, stomachs, intestines, head kidney, and muscle from white sturgeon not exposed to βNF (Figure 3.3A and B). Abundance of transcripts of AHR1 was significantly ($p \leq 0.01$) greater in livers and hearts relative to other tissues of white sturgeon not exposed to βNF (Figure 3.3B). There was no significant difference ($p > 0.01$) in abundance of transcripts among brain, gill, spleen, stomach, intestine, head kidney, or muscle of white sturgeon not exposed to βNF (Figure 3.3B). Abundance of transcripts of AHR2 was significantly ($p \leq 0.01$) greater in gill relative to other tissues of white sturgeon not exposed to βNF (Figure 3.3B). There was no significant difference ($p > 0.01$) in abundance of transcripts in liver, brain, spleen, stomach, intestine, or head kidney of white sturgeon not exposed to βNF (Figure 3.3B). Muscle had significantly ($p \leq 0.01$) lesser abundance of transcripts of AHR2 than all other tissues of white sturgeon not exposed to βNF (Figure 3.3B). Abundance of transcripts of AHR1 and AHR2 were not significantly different ($p > 0.01$) in livers from white sturgeon not exposed to βNF (Figure 3.3A).

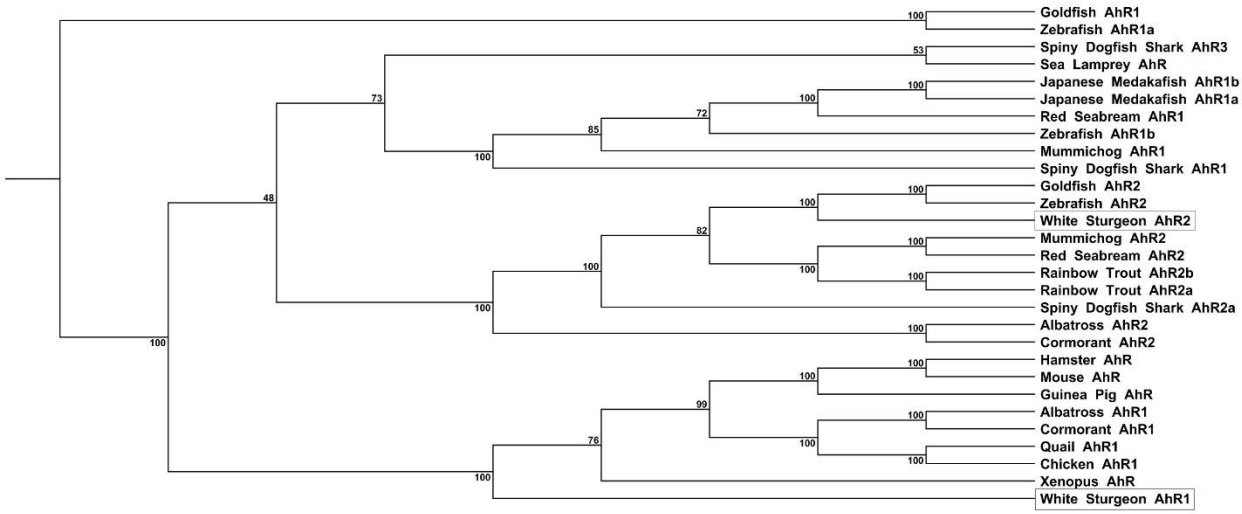

Figure 3.1. Phylogenetic tree for relatedness of AHR amino acid sequences among various vertebrates. AHR1 and AHR2 of white sturgeon are highlighted. Branch lengths represent bootstrap values based on 1,000 samplings.

Table 3.2. Percent similarity of the putative amino acid sequence of the ligand binding domain of the AHR1 and AHR2 of white sturgeon compared to the ligand binding domain of select AHRs of other vertebrates.

	Species	Percent Similarity
White Sturgeon AHR1		
	Pheasant AHR1	86 %
	Spiny Dogfish Shark AHR1	85 %
	Xenopus AHR	82 %
	Red Seabream AHR1	82 %
	Zebrafish AHR1b	81 %
	Lamprey AHR	80 %
	Guinea Pig AHR	79 %
	Goldfish AHR1	75 %
	Spiny Dogfish Shark AHR3	71 %
	Zebrafish AHR1a	69 %
White Sturgeon AHR2		
	Zebrafish AHR2	86 %
	Goldfish AHR2	85 %
	Spiny Dogfish Shark AHR2a	82 %
	Cormorant AHR2	81 %
	Lamprey AHR	76 %
	Rainbow Trout AHR2a	76 %
	Xenopus AHR	74 %
	Red Seabream AHR2	72 %
	Guinea Pig AHR	70 %
	Spiny Dogfish Shark AHR3	69 %

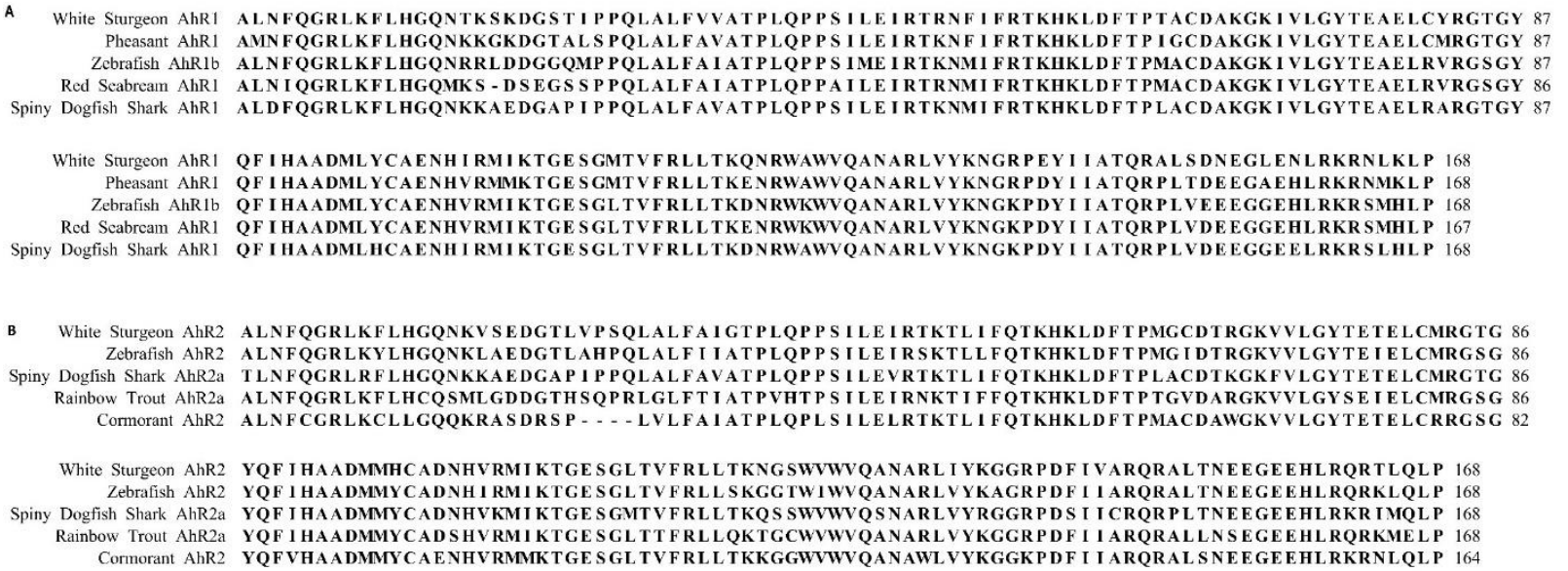

Figure 3.2. Alignment of the putative amino acid sequence of the ligand binding domain of the AHR1 (A) and AHR2 (B) of white sturgeon with selected AHRs of other vertebrates.

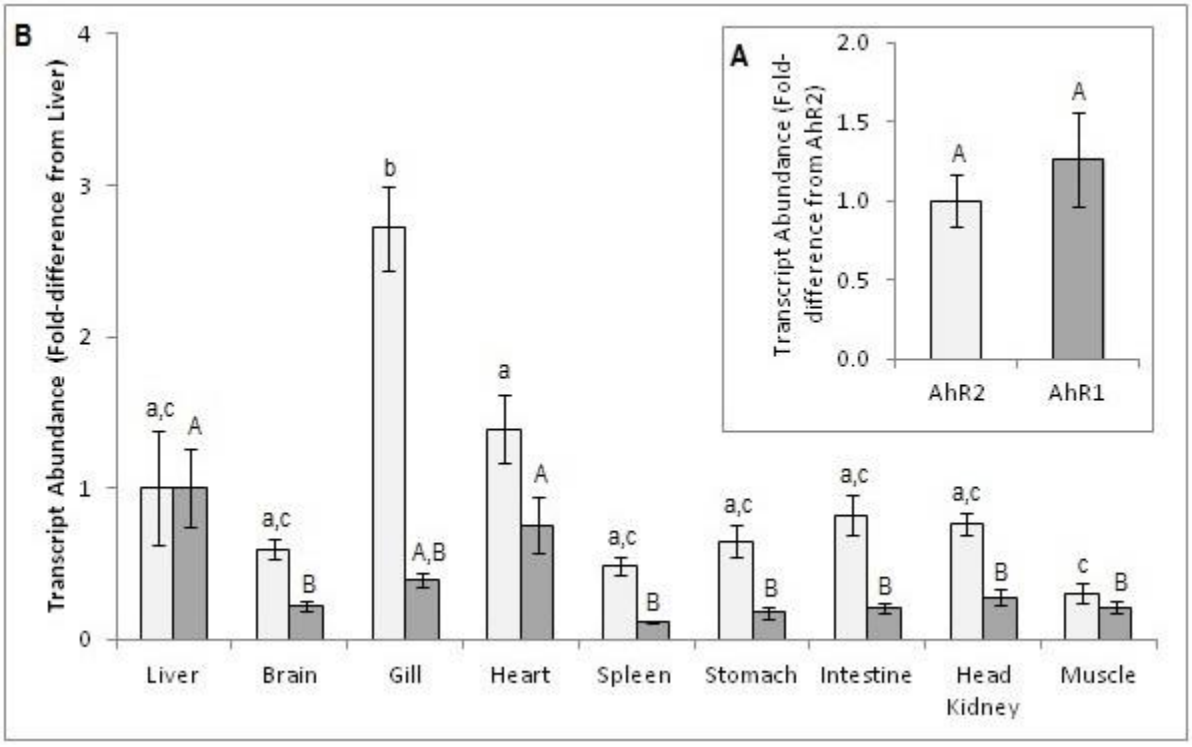

Figure 3.3. A: Comparison of basal abundances of transcripts of AHR1 and AHR2 in livers from white sturgeon (n = 5 fish). Different letters indicate significant difference (t-test; $p \leq 0.01$). B: Comparison of basal AHR1 and AHR2 transcript abundance among tissues from white sturgeon. Data are shown as fold-difference from transcript abundance in liver and data represents mean ± S.E. (n = 5 fish). Different upper case letters indicate significant differences in abundance of transcript of AHR1 and different lower case letters indicate significant differences in abundance of AHR2 (one-way ANOVA; $p \leq 0.01$).

3.4.4 Transcript abundance of AHRs in white sturgeon following exposure to βNF

Significantly ($p \leq 0.05$) greater abundance of transcripts of AHR1was observed in liver and gill from white sturgeon exposed to 50 mg βNF/kg-bw relative to 0 mg βNF/kg-bw (Figure 3.4). Significantly greater abundance of transcripts of AHR1 was observed in intestine of white sturgeon exposed to either 50 or 500 mg βNF/kg-bw relative to 0 mg βNF/kg-bw (Figure 3.4), but the magnitude of effect (up to 2-fold) was lesser than in liver or gill (both up to 5-fold) (Figure 3.4). Abundance of transcripts of AHR2 in white sturgeon exposed to βNF was adapted from Doering et al. (2012). Briefly, significantly ($p \leq 0.05$) greater abundance of transcripts of AHR2 was observed in livers (up to 5-fold), gills (up to 11-fold), and intestines (up to 35-fold) of white sturgeon exposed to βNF relative to 0 mg βNF/kg-bw (Figure 3.4).

3.5 Discussion

Results presented here demonstrate for the first time, that white sturgeons express at least two distinct forms of the AHR (AHR1 and AHR2). Expression of these two forms of AHR was unique both under basal conditions and in response to a model DLC. AHR1 and AHR2 of white sturgeon had amino acid compositions unique from other studied fishes (Figure 3.1). These unique amino acid compositions and expression might provide some insight into the mechanistic basis of altered sensitivity of sturgeons to DLCs compared to some other species of fishes.

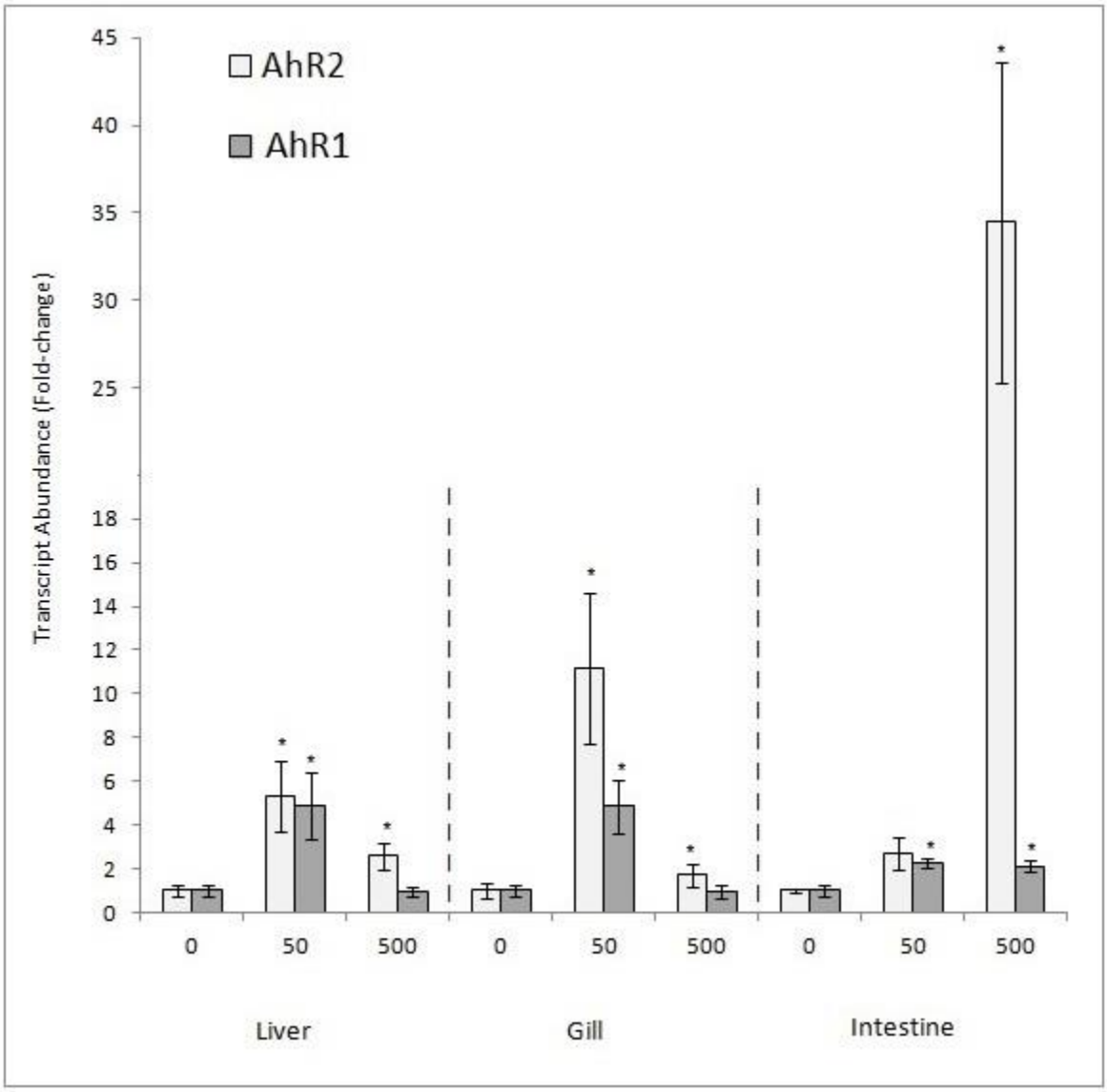

Figure 3.4. Abundances of transcripts of AHR1 and AHR2 in livers, gills, and intestines from white sturgeon 3 days following ip injection with either 0, 50, or 500 mg βNF/kg-bw. AHR2 transcript abundance adapted from Doering et al (2012). Data represents mean ± S.E. (n = 5 fish). An asterisk (*) represents significant change in abundance of transcripts in tissues from white sturgeon exposed to 50 or 500 mg βNF/kg-bw compared to the abundance in tissues of white sturgeon exposed to 0 mg βNF/kg-bw (Kruskal Wallis test; $p \leq 0.05$).

3.5.1 Evolutionary perspectives

Sturgeons (Chondrostei) are estimated to have diverged from the lineage leading to teleosts approximately 300 million years ago following divergence of the Actinopterygii (which includes both sturgeons and teleost fishes) from the Sarcopterygii (which includes the tetrapods) approximately 450 million years ago (Kumar and Hedges, 1998; Blair and Hedges, 2005). Although an in depth essay on evolution of the AHR in ancient fishes is beyond the scope of this paper, the AHR1 of white sturgeon had greatest similarity to AHRs of tetrapods, while the AHR2 of white sturgeon has greatest similarity to the AHR2 of other fishes. Another ancient species of fish, the smooth dogfish shark (*Mustelus canis*), has an AHR1 that also has greater similarity to the AHRs of tetrapods than do the AHR1 of other fishes (Hahn et al., 1997). However, another ancient fish, the little skate (*Leucoraja erinacea*) has an AHR which is greatly diverged from AHRs of other vertebrates (Hahn et al., 1997). The AHR is a member of the Per-Arnt-Sim (PAS) family of proteins and shares some structural similarities with other PAS proteins, including ARNT, aryl hydrocarbon receptor repressor (AHRR), and hypoxia inducible factor 1α (HIF1α). Although little is known about the AHR, ARNT, or AHRR of sturgeons or other ancient fishes, HIF1 α has been studied in Russian sturgeon (*Acipenser gueldenstaedtii*). Alignment of the sequence of HIF1α of Russian sturgeon revealed that this protein resembles an intermediate between a teleost and a tetrapod (Rytkonen et al., 2007). The fact that AHR, HIF1α, and likely other proteins in sturgeons most closely resemble those of tetrapods is not necessarily surprising. Beyond the relatively close evolutionary relationship between the first sturgeons and the first tetrapods, sturgeons have an unusually low degree of protein evolution, with genetic divergence between subpopulations of some species of teleost fishes being greater than is

observed between different species of sturgeons (Birstein et al., 1997). This relatively slow evolution might indicate that structure and function of proteins within the Acipenseridae has greater similarity to ancestral forms of proteins than do proteins within the more advanced and highly divergent teleost fishes. Furthermore, the structure and function of ancestral AHRs might have significant implications for sturgeons in context with the ecotoxicology of DLCs, especially considering that current ecological risk assessments for fishes are based upon data derived largely from highly divergent fishes within the Salmonidae that might not be representative of sturgeons or other ancient fishes (Van den Berg et al., 1998).

3.5.2 Possible involvement of AHR1and AHR2 in dioxin-like responses of white sturgeon

Knock-down studies in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*) have shown that effects of DLCs are mediated by the AHR2 in these species (Clark et al., 2010; Prasch et al., 2003; Van Tiem and Di Giulio, 2011). Similarly, the AHR2 has been hypothesized to mediate effects of DLCs in some other fishes (Bak et al., 2013; Hanno et al., 2010). However, there remains uncertainty regarding roles of AHR1 and AHR2 in mediating dioxin-like effects among fishes. Species such as the red seabream (*Pagrus major*) and mummichog have been shown to have two AHRs (AHR1 and AHR2) that are activated by DLCs (Bak et al., 2013; Karchner et al., 1999). In contrast, the zebrafish expresses three AHRs (AHR1a, AHR1b, AHR2) with the AHR2 and AHR1b exhibiting activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), while the AHR1a did not bind TCDD (Karchner et al., 2005). The red seabream and mummichog are among the most sensitive species of fishes to DLCs with regards to embryo-lethality, and both species are known to express AHR1 and AHR2 proteins that bind and are

activated by DLCs (Bak et al., 2013; Karchner et al., 1999; Toomey et al., 2001; Yamauchi et al., 2006). Furthermore, the great sensitivity of salmonids to DLCs is hypothesized to be caused, at least in part, by expression of multiple AHR2 proteins that bind and are activated by DLCs (Hansson and Hahn, 2008). Thus, the presence of multiple AHRs in sturgeon that are responsive to exposure to DLCs might be indicative of a species that has greater sensitivity to DLCs than species of fishes that do not have multiple, responsive AHRs.

Mammals express a single AHR that mediates effects of DLCs in this class of animals (Fernandez-Salguero et al., 1996; Shimizuet al., 2000). In contrast, birds express two forms of the AHR (AHR1 and AHR2), both of which bind TCDD and interact with dioxin-responsive elements in upstream regulatory regions of tar-get genes (Yasui et al., 2007). However, avian AHR2 has both lesser affinity for TCDD and lesser hepatic transcript abundance than avian AHR1 (Yasui et al., 2007). In the common cormorant (*Phalacrocorax carbo*), the toxicologically functional AHR1 is expressed relatively uniformly among liver, kidney, heart, spleen, muscle, pancreas, lung, intestine, brain, and gonad tissues, while xpression of AHR2, which is of lesser toxicological functionality, is localized in the liver (Yasui et al., 2007). In fishes, AHR1 is expressed primarily in brains, hearts, and gonads with relatively lesser expression in liver, while expression of AHR2 is relatively uniform in all tissues of all fishes examined to date (Abnet et al., 1999; Hansson and Hahn, 2008; Karchner et al., 1999; Yamauchi et al., 2006). Therefore, it is possible that among different species of vertebrates, profiles of tissue expression could indicate differences in physiological function between AHR1 and AHR2.

Profiles of expression of AHR1 and AHR2 in white sturgeon were distinct from those known in other fishes and appear more similar to profiles observed in birds. The AHR2 of white sturgeon has relatively uniform expression across tissues similar to that of the toxicologically

functional AHR1 of birds. Expression of AHR1 of white sturgeon was centralized in the liver which is similar to the less toxicologically functional AHR2 of birds. However, in livers of birds, the abundance of transcripts of AHR2 is lesser than the abundance of AHR1, while the abundances of transcripts of AHR1 and AHR2 in livers of white sturgeon were very similar. In liver of red seabream and mummichog the abundance of transcripts of AHR1 is lesser than the abundances of AHR2, which indicates the possibility of lesser toxicological significance of AHR1 compared to AHR2 in these two species (Bak et al., 2013; Karchner et al., 1999). The liver, along with the heart, are major target organs of DLC toxicity in fishes (Wisk and Cooper, 1990). Because embryos of fishes have great sensitivity to cardiovascular toxicity following exposure to DLCs, the relatively great expression of AHR1 and AHR2 in hearts of white sturgeon could indicate that embryos of this species are at increased risk of DLC induced cardiovascular toxicity. However, this hypothesis has to be confirmed in future studies.

Changes in abundances of transcripts of AHR following exposure to an AHR agonist varies among vertebrates. Greater abundance of transcripts of AHR2 following exposure to DLCs has been observed in tissues from several species of fishes (Andreasen et al., 2002; Doering et al., 2012; Hanno et al., 2010; Tanguay et al., 1999; Wiseman and Vijayan, 2007). In contrast, AHR in species of birds, mammals, and amphibians studied to date are not known to be up-regulated following exposure to DLCs (Iwata et al., 2010; Jonsson et al., 2011; Nault et al., 2013). However, these vertebrates have toxicologically, functional receptors. Because abundances of transcripts of AHR1 and AHR2 in all studied tissues from white sturgeon were greater following exposure to a model DLC, there is reason to hypothesize that both AHRs might have a role in responses to DLCs in white sturgeon.

3.5.2 AHR structure and future directions

A molecular basis for sensitivity of different species of birds to toxic effects of DLCs has been demonstrated. Specifically, differences in sensitivity to DLCs are related to affinity of the AHR1 for DLCs, where species with AHR1s that have greater affinity for DLCs are more sensitive than species with AHR1s of lesser affinity for DLCs (Farmahin et al., 2012). These differences in affinity are due to subtle differences in the sequence of amino acids in the ligand binding domain of the AHR1 (Karchner et al., 2006; Head et al., 2008). However, the relationship between sensitivity to DLCs and the structure of the AHR is not well understood among different species of fishes (Doering et al., 2013; Fraccalvieri et al., 2013). When the putative sequence of amino acids of the ligand binding domain of the AHR1 of white sturgeon was compared to that of other vertebrates, the sequence had greatest similarity to AHRs that are known to bind DLCs and had less similarity to AHRs that are known not to bind DLCs. The sequence had 82 and 81 % similarity to red seabream AHR1 and zebrafish AHR1b, respectively, which are both known to bind DLCs (Bak et al., 2013; Karchner et al., 2005). However, the sequence had 69 % similarity to zebrafish AHR1a which is known not to bind DLCs (Karchner et al., 2005). Furthermore, based upon critical amino acids identified in the ligand binding domain of the AHR1 of birds, the AHR1 of white sturgeon would be classified as a pheasant-type or moderately sensitive species (Head et al., 2008). Currently, the physiological or toxicological role of AHR1 in white sturgeon is not known. The largely restricted expression of AHR1 to heart and liver tissues might suggest a specialized role of this AHR, possibly related to cardiovascular or hepatic function or development. It is possible that the functionality of the AHR1 was lost in some species of teleost fishes, but was retained in avian and mammalian

AHRs and is present in AHRs in ancient sturgeons and some other fishes. Although only two AHRs were identified in this study, sturgeons have up to at least a hexadecaploid (16 n) genome, and therefore, it is likely that they express additional forms or isoforms of AHRs (Birstein et al., 1997). Another polypoid fish, the Atlantic salmon (*Salmo salar*), is known to express two isoforms of the AHR1 (α, β) and four isoforms of the AHR2 (α, β, γ, δ) (Hansson and Hahn,2008). Although the distinct role of each isoform of the AHR and their involvement in mediating effects of DLCs is unclear, future studies should investigate the possibility of additional AHRs in sturgeons and whether they have a role in the sensitivity of sturgeons to DLCs.

Since sturgeons are endangered and are at increased risk to elevated exposure to DLCs, future research should investigate whether this distinct AHR1 identified in the current study is capable of binding and mediating effects of DLCs, which might alter sensitivity of white sturgeon to some DLCs. For example, compared to birds and mammals, fishes are relatively insensitive to mono-ortho PCBs (Van den Berg et al., 1998). Additionally, 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF) is the most potent known DLC to some birds (Cohen-Barnhouse et al., 2011). Elevated sensitivity to PeCDF relative to TCDD has not yet been adequately confirmed in fishes. Since AHR1 of white sturgeon has greater similarity to the AHR of birds and mammals than to the AHR1 of other fishes it is hypothesized that white sturgeon might be substantially more sensitive to mono-ortho PCBs and some furans than other fishes. If AHR1 is capable of eliciting a response to DLCs, sturgeon specific toxic equivalence factors (TEFs) might be required to ensure proper risk assessment of DLCs to endangered sturgeons.

3.6 Conclusion

Taken together, it appears that both AHR1 and AHR2 of white sturgeon might contribute to dioxin-like effects in this species based upon both AHRs having similar abundances of transcript in target organs of DLC toxicity, both AHRs being up-regulated following exposure to a model DLC, and both AHRs having greatest similarity to AHRs known to bind DLCs. The presence of two functional AHRs in white sturgeon might have implications for the sensitivity of sturgeons to DLCs compared to other fishes. Proper risk assessment of these endangered fishes to exposure to DLCs, therefore, requires a better understanding of this sensitivity and the factors that are driving it.

CHAPTER 4

4 FUNCTIONALITY OF ARYL HYDROCARBON RECEPTORS (AHR1 AND AHR2) OF WHITE STURGEON (ACIPENSER TRANSMONTANUS) AND IMPLICATIONS FOR THE RISK ASSESSMENT OF DIOXIN-LIKE COMPOUNDS

PREFACE

Chapter 3 identified two aryl hydrocarbon receptors (AHRs) in white sturgeon, one AHR1 and one AHR2. In studied teleosts, AHR2 is known to drive adverse effects of exposure to dioxin-like compounds (DLCs) due to its greater levels of expression in target organs and greater affinity for DLCs, while AHR1 is known to have little or no role in toxicity due to its lesser levels of expression in target organs and little or no affinity for DLCs. In contrast, Chapter 3 demonstrated that AHR1 and AHR2 of white sturgeon have comparable levels of expression in target tissues and have primary amino acid structures more similar to those of AHR1s and AHR2s known to bind DLCs with high affinity. This is suggestive that AHR1 and AHR2 of sturgeons might both mediate adverse effects of exposure to DLCs in these species in contrast to other fishes and complicate development of a mechanism-based biological model for predicting the sensitivity of any species of sturgeon to DLCs. Therefore, the aim of Chapter 4 was to determine whether both AHR1 and AHR2 of white sturgeon are activated by DLCs and characterize their relative sensitivities to six selected DLCs of environmental relevance. Support for, or against, the hypothesis that both AHR1 and AHR2 of sturgeon drive toxicity of DLCs is critical in order to develop a mechanism-based biological model that allows the prediction of the sensitivity of any species of sturgeon to exposure to DLCs.

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dioxin-like compounds" 48 (14), 8219-8226. Copyright (2014), with permission from American Chemical Society.

Author contributions:

Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Reza Farmahin (Environment Canada and University of Ottawa) provided scientific input, guidance, and training, commented on and edited the manuscript.

Dr. Steve Wiseman (University of Saskatchewan) provided inspiration, scientific input, guidance, and training, commented on and edited the manuscript.

Dr. Sean W. Kennedy (Environment Canada and University of Ottawa) provided scientific input and guidance, commented on and edited the manuscript, and provided funding for the research conducted at the the National Wildlife Research Centre, Environment Canada.

Dr. John P. Giesy (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript.

Dr. Markus Hecker (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

4.1 Abstract

Worldwide, populations of sturgeons are endangered, and it is hypothesized that anthropogenic chemicals, including dioxin-like compounds (DLCs), might be contributing to the observed declines in populations. DLCs elicit their toxic action through activation of the aryl hydrocarbon receptor (AHR), which is believed to regulate most, if not all, adverse effects associated with exposure to these chemicals. Currently, risk assessment of DLCs in fishes uses toxic equivalency factors (TEFs) developed for the World Health Organization (WHO) that are based on studies of embryo-lethality with salmonids. However, there is a lack of knowledge of the sensitivity of sturgeons to DLCs, and it is uncertain whether TEFs developed by the WHO are protective of these fishes. Sturgeons are evolutionarily distinct from salmonids, and the AHRs of sturgeons differ from those of salmonids. Therefore, this study investigated the sensitivity of white sturgeon (*Acipenser transmontanus*) to DLCs in vitro via the use of luciferase reporter gene assays using COS-7 cells transfected with AHR1 or AHR2 of white sturgeon. Specifically, activation and relative potencies (RePs) of 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachloro-dibenzofuran (TCDF), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), and 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105) were determined for each AHR. It was demonstrated that white sturgeon expresses AHR1s and AHR2s that are both activated by DLCs with EC_{50} values for 2,3,7,8-TCDD that are lower than those of any other AHR of vertebrates tested to date. Both AHRs of white sturgeon had RePs for polychlorinated dibenzofurans more similar to TEFs for birds, while RePs for polychlorinated biphenyls were most similar to TEFs for fishes. Measured concentrations of select DLCs in tissues of white sturgeon from British Columbia,

Canada, were used to calculate toxic equivalents (TEQs) by use of TEFs for fishes used by the WHO and TCDD equivalents (TCDD-EQs) via the use of RePs for AHR2 of white sturgeon as determined by transfected COS-7 cells. TCDD-EQs calculated for endangered populations of white sturgeon were approximately 10-fold greater than TEQs and were within ranges known to cause adverse effects in other fishes, including other species of sturgeons. Therefore, TEFs used by the WHO might not adequately protect white sturgeon, illuminating the need for additional investigation into the sensitivity of these fish to DLCs.

4.2 Introduction

Most species of sturgeon (Acipenseridae) are endangered worldwide (U.S. Fish and Wildlife Service), which has rendered these fishes of great interest in context with their susceptibility to anthropogenic stressors. There is particular concern about declines of populations of white sturgeon (*Acipenser transmontanus*) in the northwestern United States and British Columbia, Canada (Species at Risk Public Registry). These declines have been attributed to several activities of humans, including overharvesting, alteration of habitats, and pollution (Hildebrand et al., 2013; Irvine et al., 2007). Sturgeons are long-lived, and their sexual maturity is attained slowly (Birstein, 1993). They spawn intermittently, live in close association with sediments, and have a lipid content greater than that of numerous other fishes, which increases the likelihood of bioaccumulation of lipophilic pollutants (Birstein, 1993). Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs), are contaminants of particular concern with regard to sturgeons because of the ability of DLCs to bioaccumulate and because they can be persistent under certain conditions, such as those found in sediments. Some DLCs have been detected in white sturgeon at concentrations sufficient to warrant concern (Foster et al., 1999; Kruse and Scarnecchia, 2002; Kruse and Webb, 2006; MacDonald et al 1997). However, currently little is known about the sensitivity of sturgeons to these contaminants.

Dioxin-like compounds share structural similarities and bind with relatively great affinity to the aryl hydrocarbon receptor (AHR) (Denison and Heath-Pagliuso, 1998). The AHR is a ligand-activated transcription factor in the Per-Arnt-Sim (PAS) family of proteins, which

mediates the pleiotropic expression of a suite of genes and is believed to regulate most, if not all, adverse effects associated with exposure to DLCs (Okey, 2007). In vertebrates, such effects can include hepatotoxicity, immune suppression, reproductive and endocrine impairment, teratogenicity, carcinogenicity, and loss of body mass (Kawajiri and Fujii-Kuriyama, 2007). It has been hypothesized that vertebrates underwent an ancient genome duplication event, which resulted in AHR1 and AHR2 clades (Hahn, 2001; 2002). Some fishes, such as salmonids, then underwent a second duplication event, which gave rise to multiple isoforms of AHR1 and AHR2 (Hahn, 2001; 2002). The effects of DLCs have been shown to be mediated through AHR2, not AHR1, in all fishes studied to date, (Clark et al., 2010; Hanno et al., 2010; Prasch et al., 2003; Van Tiem and Di Giulio., 2011) while AHR1, not AHR2, drives effects of DLCs in birds (Karchner et al., 2006). White sturgeons express at least two forms of the AHR, AHR1 and AHR2, with AHR1 being most identical to the AHRs of tetrapods, such as birds, mammals, and amphibians, while AHR2 is most identical to AHR2s of other fishes (Doering et al., 2014b). Both AHR1 and AHR2 have similar levels of expression in target tissues of toxicity of DLCs and are upregulated following exposure to DLCs (Doering et al., 2014b; 2012). This has raised questions about the function of these AHRs and whether both have roles in mediating the toxicity of DLCs to sturgeons.

Currently, the assessment of risks posed by DLCs to fishes uses toxic equivalency factors (TEFs) developed by the World Health Organization (WHO) that are based largely on embryolethality studies with salmonids (Van den Berg et al., 1998). Sturgeons and some other ancient fishes, including sharks, rays, and skates, respond to exposure to DLCs through induction of cytochrome P4501A (CYP1A), which is consistent with responses of salmonids. (Agradi et al., 1999; Doering et al., 2012; Hahn et al., 1998; Roy et al., 2011). However, sturgeons and

other ancient fishes are evolutionarily distinct from more modern fishes, such as salmonids, and because the sequence of amino acids of AHR1 and AHR2 of white sturgeon differ from those of salmonids, it is hypothesized that AHRs of white sturgeon might function differently (Doering et al., 2014b). This raises the question of whether TEFs currently suggested by the WHO for fishes ($TEF_{WHO\text{-}Fish}$) are adequately protective of white sturgeon.

The objective of this study was to investigate whether AHR1 and AHR2 of white sturgeon are activated by exposure to a suite of PCDDs, PCDFs, and coplanar PCBs. To determine this, a luciferase reporter gene (LRG) assay with COS-7 cells transfected with AHR1 or AHR2 of white sturgeon was used. Relative potencies (RePs) of selected DLCs were determined for each AHR of white sturgeon. RePs developed for white sturgeon in this study were compared against $TEF_{WHO\text{-}Fish}$ by use of measured concentrations of select DLCs in tissues from endangered populations of white sturgeon. This work supplements our current knowledge of evolutionary aspects of the AHR pathway among ancient fishes and allows for a better understanding of mechanisms by which sturgeons respond to DLCs, as well as a better understanding of the sensitivity of sturgeons to exposure to DLCs. This information could be essential in guiding more objective risk assessment of sturgeons to DLCs as part of ongoing conservation efforts worldwide.

4.3 Materials and methods

4.3.1 Chemicals

Stock solutions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF), and 2,3,7,8-tetrachloro-dibenzofuran (TCDF) were prepared in dimethyl sulfoxide (DMSO) from > 98 % pure standards (Wellington Laboratories, Guelph, ON). Stock solutions of 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), and 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105) were prepared in DMSO from 100 % pure standards (Chromographic Specialties, Brockville, ON). Nominal concentrations and purities of each stock solution were confirmed by use of high-resolution gas chromatography and mass spectrometry (GC-MS) according to U.S. EPA Method 1668 (2014). Serial dilutions for each compound were made in DMSO on the basis of measured concentrations of stock solutions. Doses of 0.003 to 100 nM PCDDs and PCDFs and 0.01 to 9,000 nM PCBs were used.

4.3.2 Development of Expression Constructs for AHR1, AHR2, and ARNT2 of White Sturgeon

Full-length sequences of AHR1 and AHR2 of white sturgeon have been published previously (Doering et al., 2014b). ARNT2 of white sturgeon was acquired according to the methods described for the AHR by Doering et al. (2014b). In short, the full-length sequence was generated by paired end transcriptome sequencing by use of the *Illumina* HiSeq 2000 platform (Illumina, San Diego, CA) and cloned into vectors. A consensus nucleotide sequence was

determined by aligning three or more replicated sequences. Expression constructs for AHR1, AHR2, and ARNT2 of white sturgeon were generated by use of the pENTR Directional TOPO entry vector kit (Invitrogen, Burlington, ON) and the pcDNA 3.2/V5-DEST gateway vector kit (Invitrogen) according to the protocol provided by the manufacturer. Primers used to amplify full-length AHR1, AHR2, and ARNT2 of white sturgeon for ligation into expression vectors were designed according to the protocol provided by the manufacturer (Invitrogen) and included a CACC 5′-overhang and the Kozak consensus sequence (CACCATGA) in the forward primer, with the stop codon being removed from the reverse primer (Table C4.S1). Expression constructs for AHR1, AHR2, and ARNT2 of white sturgeon were sequenced by the University of Calgary's University Core DNA Services (Calgary, AB), and products of expression constructs were synthesized by use of the TnT Quick-Coupled Reticulocyte Lysate System kit and FluoroTect GreenLys (Promega, Madison, WI) according to the protocol provided by the manufacturer. Bands were visualized by use of a Typhoon Trio imager (Molecular Dynamics, Sunnyvale, CA) to confirm the proper product size of each protein.

4.3.3 Transfection of COS-7 Cells, the Luciferase Reporter Gene (LRG) Assay, and AHR/ARNT Protein Expression

Culture of COS-7 cells, transfection of constructs, and the LRG assay were performed in 96-well plates according to methods described by Farmahin et al. (2012) with minor modifications. Optimized amounts of expression vectors transfected into cells were 8 ng of white sturgeon AHR1 or AHR2, 1.5 ng of white sturgeon ARNT2, 20 ng of rat CYP1A1 reporter construct (donated by M. Denison, University of California, Davis, CA), and 0.75 ng of *Renilla*

luciferase vector (Promega). The total amount of DNA that was transfected into cells was kept constant at 50 ng by addition of salmon sperm DNA (Invitrogen). Western blot analysis was performed according to the methods described by Farmahin et al. (2012). In brief, AHR1, AHR2, ARNT2, and β-actin protein concentrations in COS-7 cells were determined by use of the Bradford assay (Farmahin et al., 2012). The anti-V5-HRP antibody (Invitrogen) was used for detecting V5-AHR1/AHR2 and V5-ARNT2, and anti-β-actin peroxidase (Sigma-Aldrich, Oakville, ON) was used as a loading control, both according to methods described by Farmahin et al. (2012). Blots were visualized by enhanced chemiluminescence by use of a Typhoon Trio imager (Molecular Dynamics) to confirm expression of proteins in COS-7 cells.

4.3.4 Concentration-Response Curves and Statistical Analysis

Three concentration-response curves, each with four technical replicates per concentration of chemical, were obtained from three independent experiments for each combination of AHR and DLC. Response curves and effect concentrations (ECs) were developed by use of GraphPad Prism version 5.0 (San Diego, CA). Data were fit to a four-parameter logistic model. Lowest observed effect concentrations (LOECs) were defined as the first treatment dose that was statistically significant ($p \leq 0.05$) from the DMSO control treatment by use of analysis of variance (ANOVA) followed by Dunnett's test. The homogeneity of variance of each data set was determined by use of Levene's test. A logarithmic transformation was used whenever necessary to ensure homogeneity of variance. All data are shown as mean ± the standard error of the mean (SE).

4.3.5 Calculation of ReS and ReP Values

The relative sensitivity (ReS) and relative potency (ReP) were calculated by use of three points on the concentration-response curve according to methods described below, unless otherwise stated. The ReS between AHR1 and AHR2 of white sturgeon was calculated by use of the formula (Equation 4.1).

$$\mathrm{ReS} = \frac{\mathrm{ECxx\ of\ AhR1\ or\ AhR2}}{\mathrm{ECxx\ of\ AhR2}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.1)$$

where EC_{XX} of AHR1 or AHR2 is the average of the concentration to elicit a 20 % (EC_{20}), 50 % (EC_{50}), or 80 % (EC_{80}) response in COS-7 cells transfected with AHR1 or AHR2 for selected DLCs. ReP values were calculated by use of the formula (Equation 4.2).

$$\mathrm{ReP} = \frac{\mathrm{ECxx\ TCDD}}{\mathrm{ECxx\ DLC}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4.2)$$

where EC_{XX} is the average of the concentration to elicit EC_{20}, EC_{50}, and EC_{80} in COS-7 cells exposed to TCDD or the selected DLC.

4.3.6 Calculation of TEQ and TCDD-EQ

Published concentrations of TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 in liver, muscle, and eggs of white sturgeon from the Fraser River (n = 6) or upper Columbia River (n = 1) in British Columbia, Canada (Kruse and Webb, 2006; MacDonald et al., 1997) were used

to calculate toxic equivalents (TEQs) and TCDD equivalents (TCDD-EQs) expressed as picograms of TCDD per gram of tissue by use of $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998) and RePs developed by use of responses in COS-7 cells transfected with AHR2 of white sturgeon, respectively. AHR2 was selected as TEQs and TCDD-EQs calculated by using AHR2 represented a more sensitive estimate of toxicity relative to those calculated using AHR1. Where concentrations from multiple individuals were available, the greatest concentration of each DLC was selected because of the limited number of individuals sampled and to be the most conservative by representing a worst-case scenario.

4.4 Results

4.4.1 Concentration-Dependent Effects of TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105

4.4.1.1 Relative Sensitivity

AHR1 and AHR2 of white sturgeon were activated in a concentration-dependent manner by exposure to TCDD, PeCDF, TCDF, PCB 126, and PCB 77 (Figure 4.1 and Table 4.1). However, concentrations of PCB 105 as great as 9,000 nM did not activate either AHR (Figure 4.1 and Table 4.1). The sensitivity of AHR1 and AHR2 of white sturgeon to DLCs was approximately equal (Table 4.2).

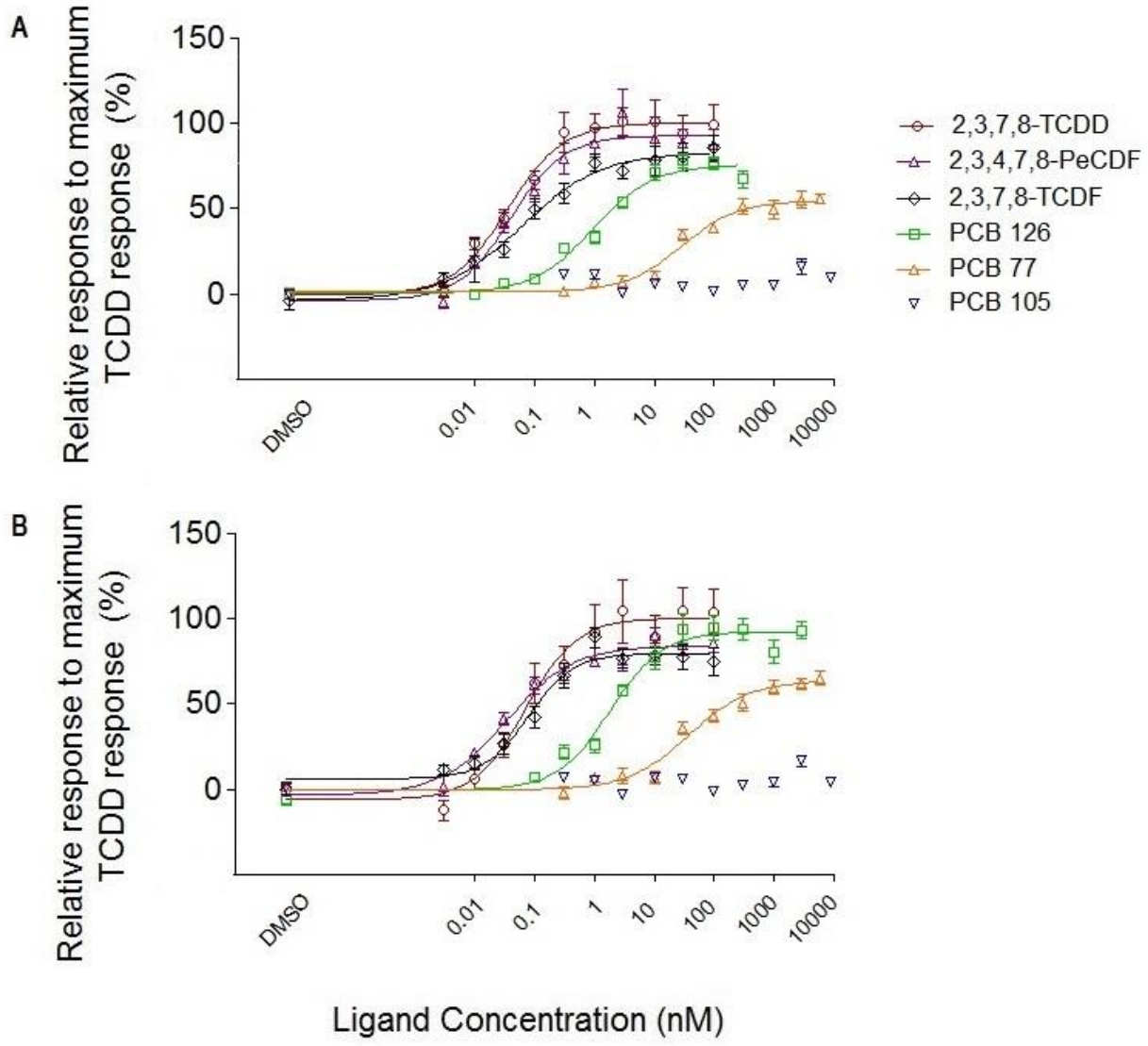

Figure 4.1. Responses of COS-7 cells transfected with AHR1 (A) or AHR2 (B) of white sturgeon following exposure to six dioxin-like compounds (DLCs). Dose-response curves of each DLC are presented as a percentage relative to the maximal response of TCDD. Data are presented as means ± SE based on three replicate assays conducted in quadruplicate.

Table 4.1. Calculated LOECs (nM), ECs (nM), and maximum responses relative to the maximum response of TCDD (%) for AHR1 and AHR2 of white sturgeon. Values that could not be calculated are indicated with "-". Standard error of the mean (S.E.) is presented in brackets.

	White sturgeon AHR1					White sturgeon AHR2				
	LOEC	EC_{20}	EC_{50}	EC_{80}	Max. Response	LOEC	EC_{20}	EC_{50}	EC_{80}	Max. Response
TCDD	0.01	0.0081 (± 0.001)	0.036 (± 0.008)	0.16 (± 0.05)	100	0.03	0.018 (± 0.005)	0.070 (± 0.02)	0.28 (± 0.09)	100
PeCDF	0.01	0.0097 (± 0.002)	0.040 (± 0.01)	0.16 (± 0.04)	93	0.01	0.058 (± 0.002)	0.034 (± 0.01)	0.20 (± 0.08)	84
TCDF	0.01	0.0073 (± 0.007)	0.060 (± 0.02)	0.49 (± 1)	83	0.003	0.024 (± 0.02)	0.079 (± 0.03)	0.26 (± 0.1)	79
PCB 126	0.1	0.19 (± 0.08)	0.94 (± 0.1)	4.7 (± 0.8)	76	0.1	0.45 (± 0.2)	1.8 (± 0.4)	7.4 (± 1)	92
PCB 77	1	6.5 (± 3)	28 (± 2)	124 (± 56)	54	1	7.5 (± 3)	38 (± 10)	193 (± 67)	64
PCB 105	-	-	-	-	< 20	-	-	-	-	< 20

Table 4.2. Relative sensitivity (ReS) of AHR1 relative to AHR2 of white sturgeon to selected dioxin-like compounds. Values that could not be calculated are indicated with "-".

	TCDD	**PeCDF**	**TCDF**	**PCB 126**	**PCB 77**	**PCB 105**
White sturgeon AHR1	1.8	1.4	0.7	1.1	1.2	-
White sturgeon AHR2	1.0	1.0	1.0	1.0	1.0	-

4.4.1.2 Relative Potency

DLCs had chemical and receptor specific potencies in transfected COS-7 cells. TCDD and PeCDF were both the most potent DLCs with respect to AHR1 (Table 4.3). However, PeCDF was the most potent DLC with respect to AHR2 (Table 4.3). The order of potency for AHR1 based on ReP was TCDD = PeCDF > TCDF > PCB 126 > PCB 77 > PCB 105 (Table 4.1). The order of potency for AHR2 based on ReP was PeCDF > TCDD = TCDF > PCB 126 > PCB 77 > PCB 105 (Table 4.1). TCDD elicited the greatest maximal response to both AHR1 and AHR2 (Figure 4.1; Table 4.1).

RePs developed for white sturgeon based on responses in COS-7 cells transfected with AHR1 or AHR2 were distinct from those based on responses in COS-7 cells transfected with AHRs of other vertebrates or $TEF_{WHO\text{-}Fish}$, which is based on mortalities of embryos of salmonids (Table 4.3). Both AHR1 and AHR2 of white sturgeon had RePs for PeCDF and TCDF more similar to $TEF_{WHO\text{-}Bird}$, while AHR1 and AHR2 of white sturgeon had RePs for PCBs most similar to $TEF_{WHO\text{-}Fish}$ (Table 4.3).

4.4.2 Comparison of TEQs in White Sturgeon

A comparison of TCDD-EQ developed from RePs derived by use of COS-7 cells transfected with AHR2 of white sturgeon (this study) and TEQs developed from $TEF_{WHO\text{-}Fish}$ to exposure data previously reported for white sturgeon (Kruse and Webb, 2006; MacDonald et al., 1997) showed that the greatest contributions to TCDD-EQs and TEQs were observed for TCDD and TCDF in liver, muscle, and eggs of white sturgeon collected from the Fraser River and the

upper Columbia River (Table 4.4). On the basis of RePs for AHR2 of white sturgeon derived from the study presented here, TCDD-EQs for the six DLCs were approximately 10-fold greater in liver, muscle, and eggs from white sturgeon from the Fraser River and upper Columbia River relative to TEQs developed by use of $TEF_{WHO\text{-}Fish}$ (Table 4.4; 4.5).

4.5 Discussion

4.5.1 Relative Sensitivity of White Sturgeon AHR1 and AHR2 Compared to the Sensitivity of Those of Other Species

As a first step in characterizing the sensitivity of white sturgeon to DLCs, this study investigated the *in vitro* sensitivity of AHR1 and AHR2 of white sturgeon to several prototypic DLCs. Both AHR1 and AHR2 of white sturgeon were activated by exposure to DLCs and had EC_{50}s for TCDD less than those of any other AHR tested to date with values of 0.036 and 0.070 nM, respectively. By comparison, EC_{50}s of other fishes derived for TCDD with AHR1- and AHR2-transfected COS-7 cells ranged from 0.073 to 5.9 nM and from 0.1 to 1.9 nM, respectively (Andrease et al., 2002; Abnet et al., 1999; Bak et al., 2013; Evans et al., 2005; Hansson and Hahn, 2008; Karchner et al., 1999; Tanguay et al., 1999; Wirgin et al., 2011). It needs to be acknowledged, however, that there are some uncertainties with regard to the comparability of ECs derived by different studies with fish using COS-7 cells transfected with AHR1 or AHR2 and, thus, whether they allow for accurate prediction of relative sensitivity among species. This is mainly due to differences in the methods that were applied by these studies and which have been shown to affect ECs by > 10-fold (Farmahin et al., 2012). Although

Table 4.3. Relative potency (ReP) of selected dioxin-like compounds (DLCs) to AHRs of white sturgeon compared to AHRs of other vertebrates. Values that could not be calculated are indicated with "-". Compounds that were not analyzed in the referenced study are indicated with "NA".

	TCDD	**PeCDF**	**TCDF**	**PCB 126**	**PCB 77**	**PCB 105**
White sturgeon AHR1	1.0	1.0	0.4	0.04	0.001	-
Red Seabream AHR1[a]	1.0	1.5	2.5	-	NA	NA
Chicken AHR1[b]	1.0	1.0	1.0	0.07	0.002	0.000,03
Human AHR[c]	1.0	0.01	0.04	0.01	0.0001	-
White sturgeon AHR2	1.0	1.3	1.0	0.04	0.002	-
Rainbow Trout AHR2α[c]	1.0	0.2	0.5	0.2	0.01	-
Zebrafish AHR2[c]	1.0	0.4	1.6	-	-	-
Red Seabream AHR2[a]	1.0	0.9	1.5	0.009	NA	NA
Embryos of Pallid Sturgeon[d]	1.0	NA	NA	0.08	NA	NA
Embryos of Shovelnose Sturgeon[d]	1.0	NA	NA	0.07	NA	NA
Embryos of Rainbow Trout[e]	1.0	0.3	0.03	0.005	0.0002	-
$TEF_{WHO\text{-}Fish}$[f]	1.0	0.5	0.05	0.005	0.0001	< 0.000,005
$TEF_{WHO\text{-}Bird}$[f]	1.0	1.0	1.0	0.1	0.05	0.0001
$TEF_{WHO\text{-}Mammal}$[g]	1.0	0.3	0.1	0.1	0.0001	0.000 03

[a] RePs were calculated based on an average of the minimum and maximum ReP of each DLC by use of luciferase reporter gene assays using COS-7 cells transfected with the respective AHR (Bak et al., 2013).

[b] RePs were calculated according to methods described in Villeneuve et al. (2000) by use of luciferase reporter gene assays using COS-7 cells transfected with AHR1 (Farmahin et al., 2012; Manning et al., 2012).

[c] RePs were calculated based on EC_{50} values by use of luciferase reporter gene assays using COS-7 cells transfected with the respective AHR (Abnet et al., 1999).

[d] RePs were calculated based on LD_{50} values in embryos of pallid sturgeon and shovelnose sturgeon (Buckler, 2011).

[e] RePs were calculated based on LD_{50} values in embryos of rainbow trout (Zabel et al., 1995).

[f] TEF values developed by the WHO (Van den Berg et al., 1998).

[g] TEF value developed by the WHO (Van den Berg et al., 2006).

Table 4.4. Maximum TEQs and TCDD-EQs of Fraser River white sturgeon liver, muscle, and eggs for select DLCs based on maximum measured concentrations (Kruse and Webb, 2006; MacDonald et al., 1997). TEQs calculated by use of $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998) and TCDD-EQs calculated by use of RePs developed by use of COS-7 cells transfected with AHR2 of white sturgeon (this study). ReP for PCB 105 was set at 0.000,01 based on no response at up to 9,000 nM. All concentrations are expressed in pg/g-ww.

Fraser River White Sturgeon									
	Liver			**Muscle**			**Eggs**		
	Measured Conc.	TCDD-EQ	TEQ	Measured Conc.	TCDD-EQ	TEQ	Measured Conc.	TCDD-EQ	TEQ
TCDD	20.0	20.0	20.0	34.8	34.8	35.0	4.20	4.20	4.20
PeCDF	3.80	4.94	1.90	7.60	9.88	3.80	0.80	1.04	0.40
TCDF	390	390	19.5	520	520	26.0	42.6	42.6	2.10
PCB 126	7.80	0.312	0.390	10.7	0.428	0.054	1.80	0.072	0.009
PCB 77	59.0	0.118	0.006	62.9	0.126	0.006	7.0	0.014	0.001
PCB 105	9 795	0.098	0.049	21 337	0.213	0.110	2 707	0.027	0.014
Total		415	41.8		565	65.0		48.0	6.72

Table 4.5. Maximum TEQs and TCDD-EQs of upper Columbia River white sturgeon liver, muscle, and eggs for select DLCs based on maximum measured concentrations (Kruse and Webb, 2006; MacDonald et al., 1997). TEQs calculated by use of $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998) and TCDD-EQs calculated by use of RePs developed by use of COS-7 cells transfected with AHR2 of white sturgeon (this study). ReP for PCB 105 was set at 0.000,01 based on no response at up to 9,000 nM. All concentrations are expressed in pg/g-ww.

Upper Columbia River White Sturgeon									
	Liver			**Muscle**			**Eggs**		
	Measured Conc.	TCDD-EQ	TEQ	Measured Conc.	TCDD-EQ	TEQ	Measured Conc.	TCDD-EQ	TEQ
TCDD	0.146	0.146	0.150	0.172	0.172	0.170	2.37	2.37	2.40
PeCDF	0.276	0.359	0.140	0.166	0.216	0.083	0.550	0.719	0.280
TCDF	20.6	20.6	1.0	13.3	13.3	0.670	67.2	67.2	3.40
PCB 126	16.1	0.644	0.081	5.15	0.206	0.026	15.9	0.636	0.080
PCB 77	33.5	0.067	0.003	24.5	0.049	0.003	27.7	0.055	0.003
PCB 105	4 680	0.047	0.023	3 740	0.037	0.019	3 750	0.038	0.019
Total		21.9	1.40		14.0	0.971		71.0	6.18

caution must be used when comparing EC_{50}s derived for other fishes, the EC_{50}s of chicken (*Gallus gallus*) described by Farmahin et al. (2012) and Manning et al. (2012) were derived by use of the same methods used in the study presented here and therefore can be compared directly. Chicken is the species of bird known to be most sensitive to TCDD with an LD_{50} to embryos of 210 pg/g of egg and an EC_{50} for AHR1 in transfected COS-7 cells of 0.22 nM (Cohen-Barnhouse et al., 2011; Farmahin et al., 2012). On the basis of EC_{50}s, AHR1 and AHR2 of white sturgeon were more sensitive to TCDD, PeCDF, TCDF, PCB 126, and PCB 77 than AHR1 of chicken (Farmahin et al., 2012; Manning et al., 2012). However, AHR1 of chicken was more sensitive to PCB 105, which did not show any significant response in white sturgeon (Manning et al., 2012). The fact that white sturgeons have two AHRs with great sensitivity to PCDDs, PCDFs, and non-ortho PCBs might indicate that white sturgeon also have in vivo sensitivity to these compounds greater than those of most other vertebrates.

The sensitivity of AHRs *in vitro* might be an indicator of the sensitivity of fishes *in vivo* and could represent one method of predicting the relative sensitivity of endangered fishes, such as sturgeons, to DLCs by use of an *in vitro* approach (Doering et al., 2013). EC_{50}s for TCDD in COS-7 cells transfected with AHR1 (0.073 nM) or AHR2 (0.51 nM) of red seabream (*Pagrus major*) were compared to EC_{50}s for upregulation of CYP1A in whole embryos exposed to TCDD (0.30 to 0.91 nM), with EC_{50}s for upregulation of CYP1A and activation of AHR2 being most similar, indicating that AHR2 activation might be predictive of responses in embryos (Bak et al., 2013). However, little is known about the sensitivity of embryos of sturgeons to DLCs, and substantial variability exists in the available data. Embryo-lethality studies of pallid sturgeon (*Scaphirhynchus albus*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) determined these sturgeons to be the least sensitive fishes with LD_{50}s of 12,000 and 13,000 pg of TCDD/g of

egg, respectively (Buckler, 2011). In contrast, an elevated incidence of malformations of embryos of shortnose sturgeon (*Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrinchus*) has been observed at concentrations as low as 50 pg of TCDD/g of egg (Chambers et al., 2012). However, LD_{50}s were not achieved at concentrations as great as 600 and 1450 pg of TCDD/g of egg for shortnose and Atlantic sturgeon, respectively (Chambers et al., 2012). In contrast, LD_{50}s of teleost fishes ranged from 65 to 2610 pg of TCDD/g of egg (Doering et al., 2013). Evidence collected to date supports the hypothesis that there might be significant diversity in sensitivity to DLCs among sturgeons, with some species exhibiting great sensitivity to DLCs. On the basis of the greater sensitivities of both AHR1 and AHR2 *in vitro*, and the great inducibility of CYP1A *in vivo* (Doering et al., 2012), it is hypothesized that the white sturgeon is sensitive to DLCs.

4.5.2 Relative Potency of Select Dioxin-like Compounds to AHR1 and AHR2 of White Sturgeon

On the basis of the results derived from COS-7 cells transfected with AHR1 or AHR2 of white sturgeon, it was found that PCDFs and non-ortho PCBs had greater potency relative to TCDD compared to the current $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998). RePs determined for AHR2 of white sturgeon following exposure to PeCDF, TCDF, PCB 126, and PCB 77 were 3-, 20-, 8-, and 20-fold greater, respectively, than $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998). However, neither AHR1 nor AHR2 exhibited a measurable response to mono-ortho PCB 105, which is consistent with what has been shown in all other fishes studied to date (Abnet et al., 1999; Van den Berg et al., 1998). RePs derived for both AHR1 and AHR2 of white sturgeon were more

similar to $TEF_{WHO\text{-}Bird}$ than to $TEF_{WHO\text{-}Fish}$ (Van den Berg et al., 1998). However, there is uncertainty about how accurately *in vitro* activation in COS-7 cells transfected with AHR mirrors environmentally relevant end points *in vivo*. In birds, it has been demonstrated that activation of AHR1 in transfected COS-7 cells is predictive of effects of DLCs *in vivo* in a range of model and wild species (Farmahin et al., 2012; 2013; Manning et al., 2012). Although fishes have not been studied to the same level of detail as birds, there is considerable similarity between RePs for PCDDs, PCDFs, and PCBs derived from activation of AHR2α of rainbow trout (*Oncorhynchus mykiss*) in transfected COS-7 cells and RePs derived from embryos of rainbow trout (Abnet et al., 1999). In embryos of pallid and shovelnose sturgeons exposed to serial doses of either TCDD or PCB 126, it was found that PCB 126 had RePs of 0.08 and 0.07, respectively, relative to TCDD compared to the RePs of 0.04 derived for both AHR1 and AHR2 of white sturgeon (Buckler, 2011); a difference of 2-fold compared to an 8-fold difference from the $TEF_{WHO\text{-}Fish}$ for PCB 126 of 0.005 (Table 4.3). Although *in vitro* systems do not consider differences in metabolism between congeners, fishes have been shown to metabolize DLCs more slowly than other vertebrates (Van den Berg et al., 2006), and therefore, on the basis of the similarity between in vitro and *in vivo* RePs in fishes, it appears that RePs that are derived from COS-7 cells transfected with AHR1 or AHR2 might be representative of environmentally relevant effects on embryos of sturgeons.

4.5.3 Application to Risk Assessment

Previous studies have shown that COS-7 cells transfected with AHR1s are predictive of in vivo sensitivity of birds to DLCs (Farmahin et al., 2012). Assuming that the greater sensitivity

of white sturgeon to some PCDFs relative to TCDD as determined by COS-7 cells transfected with AHR1 or AHR2 is similarly predictive of *in vivo* sensitivity, this would have significant implications for the assessment of risk to populations of this species. Several DLCs were detected in tissues and eggs of adult white sturgeon from the Fraser River and upper Columbia River in British Columbia, Canada, with PCDFs having among the greatest concentrations (Kruse and Webb, 2006; MacDonald et al., 1997). On the basis of concentrations of TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 in individuals collected from the Fraser River, TEQs calculated by use of $TEF_{WHO\text{-}Fish}$ were 65.0 and 41.8 pg of TCDD/g of wet weight in muscle and liver, respectively, whereas TEQs of the fishes collected in the upper Columbia River were 0.971 and 1.40 pg of TCDD/g of wet weight in muscle and liver, respectively (Table4. 4). TEQs calculated for eggs of white sturgeon were 6.18 and 6.70 pg of TCDD/g of egg wet weight for fishes from the Fraser River and upper Columbia River, respectively. Although there is no consensus about whether TEQs within these ranges represent a significant concern (Abalos et al., 2008; Elonen et al., 1998; Johnson et al., 1998; Toomey et al., 2001; Walker et al., 1991; Yamauchi et al., 2006), adverse effects have been observed in some fishes at concentrations that are significantly less (Giesy et al., 2002). In adult rainbow trout exposed to environmentally relevant concentrations of TCDD via the diet for 300 days, the most sensitive end points measured were survival of adult females and effects on behavior, both of which occurred at LOECs of 0.22 pg of TCDD/g of wet weight in liver and 0.21 pg of TCDD/g of wet weight in muscle (Giesy et al., 2002). However, early life stages of fishes, such as embryos, are known to be among the most sensitive to DLCs (Elonen et al., 1998). TEQs for eggs of white sturgeon exceed the LOEC of 0.3 pg of TCDD/g of egg wet weight that was observed in one study following maternal transfer of TCDD to embryos of rainbow trout (Giesy et al., 2002). However,

other studies had LOECs for embryos of salmonids ranging from 15 to 34 pg of TCDD/g of egg wet weight (Johnson et al., 1998; Walker et al., 1991).

TCDD-EQs derived from responses in COS-7 cells transfected with AHR2 of white sturgeon were approximately 10-fold greater than TEQs derived from $TEF_{WHO\text{-}Fish}$ in liver, muscle, and eggs from fishes from the Fraser River and upper Columbia River (Table 4.4; 4.5). On the basis of RePs, concentrations of TCDD-EQs in muscle and liver of white sturgeon from the Fraser River were 565 and 415 pg of TCDD/g of wet weight, respectively, whereas TCDD-EQs in muscle and liver of the white sturgeon collected from the upper Columbia River were 14.0 and 21.9 pg of TCDD/g of wet weight, respectively (Table 4.4; 4.5). These concentrations of TCDD-EQs significantly exceed concentrations shown in several studies to cause adverse effects in fishes (Giesy et al., 2002; Fisk et al., 1997; Walter et al., 2000) and are likely to have some chronic impacts on white sturgeon from these rivers. TCDD-EQs in eggs were 48.0 and 71.0 pg of TCDD/g of egg wet weight in white sturgeon collected in the Fraser River and upper Columbia River, respectively (Table 4.4; 4.5). These concentrations significantly exceed effect concentrations for several fishes (Giesy et al., 2002; Toomey et al., 2001; Walker et al., 1991; Yamauchi et al., 2006), including shortnose, Atlantic, pallid, and shovelnose sturgeons (Buckler, 2011; Chambers et al., 2012).

In conclusion, this study demonstrates that white sturgeon express two distinct AHR proteins, AHR1 and AHR2 that are responsive to exposure to DLCs. More importantly, the EC_{50}s derived in this study for 2,3,7,8-TCDD were less than those previously reported for any other AHR of vertebrates tested to date. These unique and sensitive patterns of response mediated by AHRs of white sturgeon might be indicative of greater sensitivity of white sturgeon to some DLCs relative to other fishes, in particular PCDFs. On the basis of RePs developed for

TCDD, PeCDF, TCDF, PCB 126, PCB 77, and PCB 105 by use of COS-7 cells transfected with AHR2 of white sturgeon, it appears $TEF_{WHO\text{-}Fish}$ might not accurately predict the risk of DLCs to endangered populations of white sturgeon. Because numerous species of sturgeons are endangered and can have elevated levels of exposure to mixtures of DLCs, future research should investigate whether RePs derived using COS-7 cells transfected with AHR1 or AHR2 accurately represent RePs derived by use of *in vivo* end points of biological relevance such as embryo-lethality or other environmentally relevant end points and establish the relative sensitivity of white sturgeons to DLCs compared to salmonids. The development of sturgeon specific RePs could be essential for objective risk assessments of endangered sturgeons worldwide.

CHAPTER 5

5 DIFFERENCES IN ACTIVATION OF ARYL HYDROCARBON RECEPTORS OF WHITE STURGEON RELTIVE TO LAKE STURGEON ARE PREDICTED BY IDENTITIES OF KEY AMINO ACIDS IN THE LIGAND BINDING DOMAIN

PREFACE

Chapter 4 demonstrated that both aryl hydrocarbon receptors (AHR1 and AHR2) of white sturgeon are activated by exposure to dioxin-like compounds (DLCs) with sensitivity to activation greater than that of any other AHR of vertebrates tested to date. This is suggestive that both AHR1 and AHR2 might drive toxicity of DLCs in sturgeons and that white sturgeon might be among the most sensitive species of fish to exposure to DLCs. However, nothing is known regarding AHRs of other species of sturgeon. Therefore, the aim of Chapter 5 was to identify AHRs of another species of sturgeon, the lake sturgeon (*Acipenser fulvescens*) and determine whether both AHR1 and AHR2 are activated by DLCs and compare their sensitivities to activation to those of AHR1 and AHR2 of white sturgeon. Better characterizing diversity in AHR structure and function among members of the Acipenseridae is critical in order to develop a mechanism-based biological model that is capable of predicting the sensitivity of any species of sturgeon to DLCs.

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Author contributions:

Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Dr. Reza Farmahin (Environment Canada and University of Ottawa) provided inspiration, scientific input, guidance, and training, commented on and edited the manuscript.

Dr. Steve Wiseman (University of Saskatchewan) provided inspiration, scientific input, guidance, and training, commented on and edited the manuscript.

Shawn C. Beitel (University of Saskatchewan) provided laboratory assistance with computational analyses and sample collection, commented on and edited the manuscript.

Dr. Sean W. Kennedy (Environment Canada and University of Ottawa) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research conducted at the the National Wildlife Research Centre, Environment Canada.

Dr. John P. Giesy (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript.

Dr. Markus Hecker (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

5.1 Abstract

Dioxin-like compounds (DLCs) are pollutants of global environmental concern. DLCs elicit their adverse outcomes through activation of the aryl hydrocarbon receptor (AHR). However, there is limited understanding of the mechanisms that result in differences in sensitivity to DLCs among different species of fishes. Understanding these mechanisms is critical for protection of the diversity of fishes exposed to DLCs, including endangered species. This study investigated specific mechanisms that drive responses of two endangered fishes, white sturgeon (*Acipenser transmontanus*) and lake sturgeon (*Acipenser fulvescens*) to DLCs. It determined whether differences in sensitivity to activation of AHRs (AHR1 and AHR2) can be predicted based on identities of key amino acids in the ligand binding domain (LBD). White sturgeons were 3- to 30-fold more sensitive than lake sturgeon to exposure to five different DLCs based on activation of AHR2. There were no differences in sensitivity between white sturgeon and lake sturgeon based on activation of AHR1. Adverse outcomes as a result of exposure to DLCs have been shown to be mediated through activation of AHR2, but not AHR1, in all fishes studied to date. This indicates that white sturgeons are likely to have greater sensitivity *in vivo* relative to lake sturgeon. Homology modeling and in silico mutagenesis suggests that differences in sensitivity to activation of AHR2 result from differences in key amino acids at position 388 in the LBD of AHR2 of white sturgeon (Ala-388) and lake sturgeon (Thr-388). This indicates that identities of key amino acids in the LBD of AHR2 could be predictive of both *in vitro* activations by DLCs and *in vivo* sensitivity to DLCs in these, and potentially other, fishes.

5.2 Introduction

Some dioxin-like compounds (DLCs), including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs), are ubiquitous, persistent, and bioaccumulative pollutants of environmental concern globally. DLCs share similarities in structure and bind to the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor in the Per-Arnt-Sim (PAS) family of proteins that mediates the pleiotropic expression of a suite of genes and is believed to regulate most, if not all, adverse outcomes associated with exposure to DLCs (Okey, 2007). In vertebrates, these adverse outcomes can include hepatotoxicity, immune suppression, impairment of reproductive and endocrine processes, teratogenicity, and carcinogenicity (Kawajiri and Jujii-Kuriyama, 2007). Although fishes are among the most sensitive vertebrates to exposure to DLCs, there are great differences in sensitivity among species. For example, differences in concentrations of DLCs that cause embryo-lethality can be as great as 200-fold (Buckler et al., 2015; Elonen et al., 1998; Johnson et al., 1998; Toomey et al., 2001; Walker et al., 1991; Yamauchi et al., 2006). These differences in sensitivity combined with the more than 25,000 known species of fish hamper objective and efficient assessments of risks of exposure to DLCs for this class of vertebrates.

The process of assessing risks of exposure to pollutants is currently undergoing significant changes. Traditional toxicity testing is conducted by use of live animals and requires large numbers of individuals. Considering the number of species to be protected, such *in vivo* approaches are not feasible from an economic, temporal, ethical, or cultural perspective. As a consequence, there has been increasing focus on the development of alternative approaches that

alleviate these limitations while allowing for reliable and objective assessments of risks to all species associated with exposure to pollutants. One approach that has been proposed is that of the adverse outcome pathway (AOP). An AOP is a conceptual framework that organizes knowledge concerning biologically plausible and empirically supported links between molecular-level alteration of a biological system and an adverse outcome at a level of biological organization of regulatory relevance, such as survival, growth, or reproduction (Ankley et al., 2010). Establishing linkages between molecular initiating events (MIEs; such as binding of DLCs to the AHR) and adverse outcomes is critical in order to move away from a dependence on in vivo toxicity testing of large numbers of individuals of multiple species and to improve predictive in vitro approaches necessary to advance risk assessment (Ankley et al., 2010). In particular, knowledge of the sensitivity of endangered species and whether they are being adversely affected by pollution is of great interest to ongoing conservation efforts worldwide (Doering et al., 2013). Since it is difficult and unethical to acquire endangered species in numbers necessary for in vivo toxicity testing, the development of alternative testing approaches that can reliably predict adverse outcomes to such species is of even greater importance in this context.

Of the 149 species of fishes listed as endangered or threatened in the USA (U.S. Fish and Wildlife Service) and numerous others in countries worldwide, one group of particular interest are sturgeons (Acipenseridae). Most of the twenty-four species of sturgeons found worldwide are endangered (U.S. Fish and Wildlife Services). Pollution has been implicated as a probable factor contributing to some of the observed decreases in sizes of populations of several species of sturgeons throughout North America, Europe, and Asia (Bergman et al., 2008; Dadswell, 2006; Hildebrand and Parsley, 2013; Hensel and Holcik, 1997; Hu et al., 2009; Khodorevskaya et al.,

1997; Lenhardt et al., 2006). Although the influence of pollution on declines in populations of sturgeons is not well understood, they are at particular risk of exposure to DLCs and other bioaccumulative pollutants because they are long-lived, attain sexual maturity slowly, spawn only intermittently, live in close association with sediments, and have greater lipid content than some other fishes. Based upon previous investigation into potencies of selected DLCs *in vitro* to white sturgeon (*Acipenser transmontanus*), concentrations of DLCs detected in endangered populations of white sturgeon (Kruse and Webb, 2006; MacDonald et al., 1997) exceed concentrations shown in several studies to cause chronic impacts in other fishes (> 71 pg of TCDD equivalents/g of egg), (Doering et al., 2014a) which warrants concern. Despite being at a great risk of exposure to DLCs due to their life history traits, little is known regarding the sensitivity of sturgeons, or other endangered fishes, to DLCs.

Evidence collected to date supports the hypothesis that there might be significant diversity in sensitivity to DLCs among members of the family Aciperseridae (Buckler et al., 2015; Chambers et al., 2012; Doering et al., 2014a), with the potential for some species to have great sensitivity (Chambers et al., 2012; Doering et al., 2014a). Therefore, in order to enable objective and efficient assessments of risks posed by DLCs to sturgeons, and other fishes, methods are needed that enable accurate prediction of their relative sensitivity. Results of previous studies have shown that the MIE that is likely to determine *in vivo* sensitivity of certain other vertebrates to DLCs is sensitivity to activation of the AHR and the identity of key amino acids in the ligand binding domain (LBD) that determine affinity of binding. Key amino acid residues have been shown to be responsible for differences in sensitivity to DLCs *in vivo* and with regard to activation of AHRs *in vitro* among strains of mice (*Mus musculus*) (Pandini et al., 2007) and among species of birds (Farmahin et al., 2013; Head et al., 2008; Karchner et al.,

2006; Manning et al., 2012). Key amino acids in the LBD have also been demonstrated to drive differences in sensitivity to activation between AHR1α and AHR1β of *Xenopus laevis* (Odio et al., 2013) and between AHR1a and AHR2 of zebrafish (*Danio rerio*) (Fraccalvieri et al., 2013). However, specific molecular determinants of differences in sensitivity to DLCs in vivo and with regard to activation of AHRs in vitro among different species of fishes are currently unknown (Doering et al., 2013).

In order to identify specific mechanisms that determine differences in sensitivity to DLCs among species of fishes, the objectives of this study were to investigate the AHR1s and AHR2s of two species of sturgeons which are listed as endangered in the United States (U.S. Fish and Wildlife Sercices) and Canada (Species at Risk Public Registry), white sturgeon (*A. transmontanus*) and lake sturgeon (*Acipenser fulvescens*), to determine whether there are differences in sensitivity to activation of AHRs by PCDDs, PCDFs, or coplanar PCBs, and to determine whether differences can be linked to identities of key amino acids in the LBD. Support for the hypothesis that sensitivity of fishes *in vivo* and with regard to activation of AHRs *in vitro* is determined by key amino acids in the LBD of AHR would serve as an early step in developing a mechanism-based biological model to predict *in vivo* sensitivity to DLCs among fishes which would enable incorporation of the relative sensitivity among species into the AOP framework and guide more objective risk assessments of sturgeons, and other fishes, to DLCs.

5.3 Materials and methods

5.3.1 Identification, Sequencing, and Phylogeny of AHRs of Lake Sturgeon

Sequences of transcripts of AHR1 and AHR2 had not yet been identified for lake sturgeon. Full-length cDNAs of AHR1 and AHR2 of lake sturgeon were amplified by use of a LongRange PCR Kit (Qiagen; Toronto, ON) by use of gene-specific primers for AHR1 and AHR2 of white sturgeon described previously (Table C5.S1) (Doering et al., 2014a). Polymerase chain reaction (PCR) products were purified by use of a QIAquick PCR Purification Kit (Qiagen) and cloned into pGEM-T easy vectors by use of a DNA ligation kit (Invitrogen; Burlington, ON) and transformed into competent JM109 *E. coli* cells (Promega, Madison, WI). Plasmids were isolated by use of a Plasmid Mini Kit (Qiagen), and products were sequenced at the University of Calgary's University Core DNA Services (Calgary, AB). Consensus nucleotide sequences for AHR1 and AHR2 were determined by aligning sequences of three or more PCR products. The evolutionary relationship of AHR1 and AHR2 proteins from lake sturgeon to AHR proteins from other vertebrates was constructed by use of CLC Genomics Workbench v.4.7.2 (Katrinebjerg, Aarhus).

5.3.2 Development of Expression Constructs for AHR1 and AHR2 of Lake Sturgeon

Expression constructs for AHR1 and AHR2 of lake sturgeon were generated by use of methods described previously (Doering et al., 2014a). Primers used to amplify full-length AHR1 and AHR2 of lake sturgeon for ligation into expression vectors were described previously for

white sturgeon (Table C5.S1) (Doering et al., 2014a). Expression constructs for AHR1 and AHR2 of lake sturgeon were sequenced by the University of Calgary's University Core DNA Services (Calgary, AB) and products of expression constructs were synthesized by use of the TnT Quick-Coupled Reticulocyte Lysate System kit and FluoroTect GreenLys (Promega).

5.3.3 Dosing Solutions

2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF), 2,3,7,8-tetrachloro-dibenzofuran (TCDF), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), and 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105) were acquired from commercial suppliers, and stock solutions were prepared in dimethyl sulfoxide (DMSO) as described previously (Doering et al., 2014a). Concentrations of stock solutions were confirmed as described previously (Doering et al., 2014a).

5.3.4 Transfection of COS-7 Cells, Luciferase Reporter Gene (LRG) Assay, and AHR/ARNT Protein Expression

Culture of COS-7 cells, transfection of constructs, and the LRG assay were performed in 96-well plates according to methods described previously (Doering et al., 2014a). In brief, optimized amounts of expression vectors transfected into cells were 8 ng of lake sturgeon AHR1 or AHR2, 1.5 ng of white sturgeon ARNT2 (Doering et al., 2014a), 20 ng of rat CYP1A1 reporter construct (donated by M. Denison - University of California, Davis, CA) (Han et al., 2004; Rushing et al., 2002), and 0.75 ng of *Renilla* luciferase vector (Promega). The total amount

of DNA that was transfected into cells was kept constant at 50 ng by addition of salmon sperm DNA (Invitrogen). Expression of AHR1, AHR2, and ARNT2 proteins in COS-7 cells was assessed as described previously (Doering et al., 2014a).

5.3.5 Concentration−Response Curves

All concentration-response curves were obtained from three independent experiments, each with four technical replicates per concentration of chemical for each combination of AHR and DLC. Response curves and effect concentrations (ECs) were developed by use of methods described previously (Doering et al., 2014a). Lowest observed effect concentrations (LOECs) were defined as the least dose of DLC that caused an effect that was statistically significant ($p \leq 0.05$) from response of the DMSO control. All data are shown as mean ± standard error of the mean (SE).

5.3.6 Calculation of ReS and ReP Values

Relative sensitivity (ReS) to activation of AHR1 and AHR2 and relative potency (ReP) of each DLC were calculated by use of three points on the concentration-response curve according methods described previously (Doering et al., 2014a). ReS of each DLC were also calculated by use of the LOEC. ReS between AHR1 and AHR2 of lake sturgeon were calculated

by use of the formula (Equation 5.1).

$$ReS = \frac{ECxx\ AhR2}{ECxx\ AhR1\ or\ AhR2} \quad \text{..... (5.1)}$$

where EC_{XX} of AHR1 or AHR2 is the mean of the concentration to elicit a 20 (EC_{20}), 50 (EC_{50}), and 80 % (EC_{80}) response or LOEC in COS-7 cells transfected with AHR1 or AHR2 exposed to each DLC.

Differences in ReS between AHRs of white sturgeon (WS) and lake sturgeon (LS) were calculated by use of the formula (Equation 5.2).

$$ReS = \frac{ECxx\ WS}{ECxx\ LS} \quad \text{..... (5.2)}$$

where EC_{XX} of LS or WS is the mean of the concentration to elicit an EC_{20}, EC_{50}, and EC_{80} in COS-7 cells transfected with AHR1 or AHR2 of lake sturgeon for selected DLC relative to ECxx of white sturgeon.

ReP values were calculated by use of the formula (Equation 5.3).

$$ReP = \frac{ECxx\ TCDD}{ECxx\ DLC} \quad \text{..... (5.3)}$$

where EC_{XX} is the mean of the concentration to elicit an EC_{20}, EC_{50}, and EC_{80} in COS-7 cells exposed to TCDD or the selected DLC.

5.3.7 Homology Modeling

Homology modeling of the LBD of AHR1 and AHR2 of white sturgeon and lake sturgeon was conducted by use of a modification of methods described previously (Farmahin et al., 2013). In brief, sequences that produced the most significant alignment with ligand binding domains (LBDs) of AHRs of white sturgeon and lake sturgeon were identified by use of PSI-Blast against the Protein Data Bank (PDB) (Bergman et al., 2000). Models for LBD of AHRs of white sturgeon and lake sturgeon were generated by use of Modeler 9.13 (University of California, San Francisco, CA) run through EasyModeller 4.0 by use of a docked complex template containing HIF-2α and ARNT of *Homo sapiens* (PBD ID: 4H6J-A).35 Amino acids 273 to 380 and 274 to 381 for AHR1 of white sturgeon and lake sturgeon, respectively, were modeled, while amino acids 290 to 397 for AHR2s of white sturgeon and lake sturgeon were modeled. Accuracy of the models, indicated as a *z*-score measuring the deviation of total energy of the model relative to random confirmations, was assessed by use of ProSA (Wiederstein and Sippl, 2007). Stereochemical quality, measured as percent of amino acid residues that reside in the most favored areas of a Ramachandran plot, was assessed by use of PROCHECK (Laskowski et al., 1993). The Computed Atlas of Surface Topography of Proteins (CASTp) server was used to predict which amino acid residues in the LBD contribute to the internal cavity and the volume of the cavity in the Connolly's molecular surface (Dundas et al., 2006). In silico mutagenesis was conducted by use of PyMOL 1.3 (DeLano, 2002). Mutant models were energetically refined by

use of Swiss-PbdViewer 4.1 (Guex and Peitsch, 1996). The CLC Drug Discovery Workbench 1.0.2 (Qiagen) was used for three-dimensional (3-D) visualization and imaging of protein structures.

5.4 Results

5.4.1 Identification and Phylogeny of AHR1 and AHR2 of Lake Sturgeon

The putative, full-length sequences of AHR1 (AIV00618.1) and AHR2 (AIW39681.1) of lake sturgeon were 868 and 1,101 amino acids, respectively. The AHR1 of lake sturgeon clustered closely with AHR1 of white sturgeon being 94 % similar at the amino acid level (Figure C5.S1). The AHR2 of lake sturgeon clustered closely with AHR2 of white sturgeon being 94 % similar at the amino acid level (Figure C5.S1).

5.4.2 Relative Sensitivity of AHR1 and AHR2 of White Sturgeon and Lake Sturgeon in Vitro

AHR1 and AHR2 of white sturgeon and lake sturgeon were activated in a concentration-dependent manner by TCDD, PeCDF, TCDF, PCB 126, and PCB 77 (Figure 5.1). Concentrations of PCB 105 as great as 9,000 nM did not activate either AHR of either sturgeon (Figure 5.1; Table 5.1). In general, the sensitivity to activation of AHR1 of lake sturgeon was greater than the sensitivity to activation of AHR2 of lake sturgeon (Tables 5.1; 5.2).

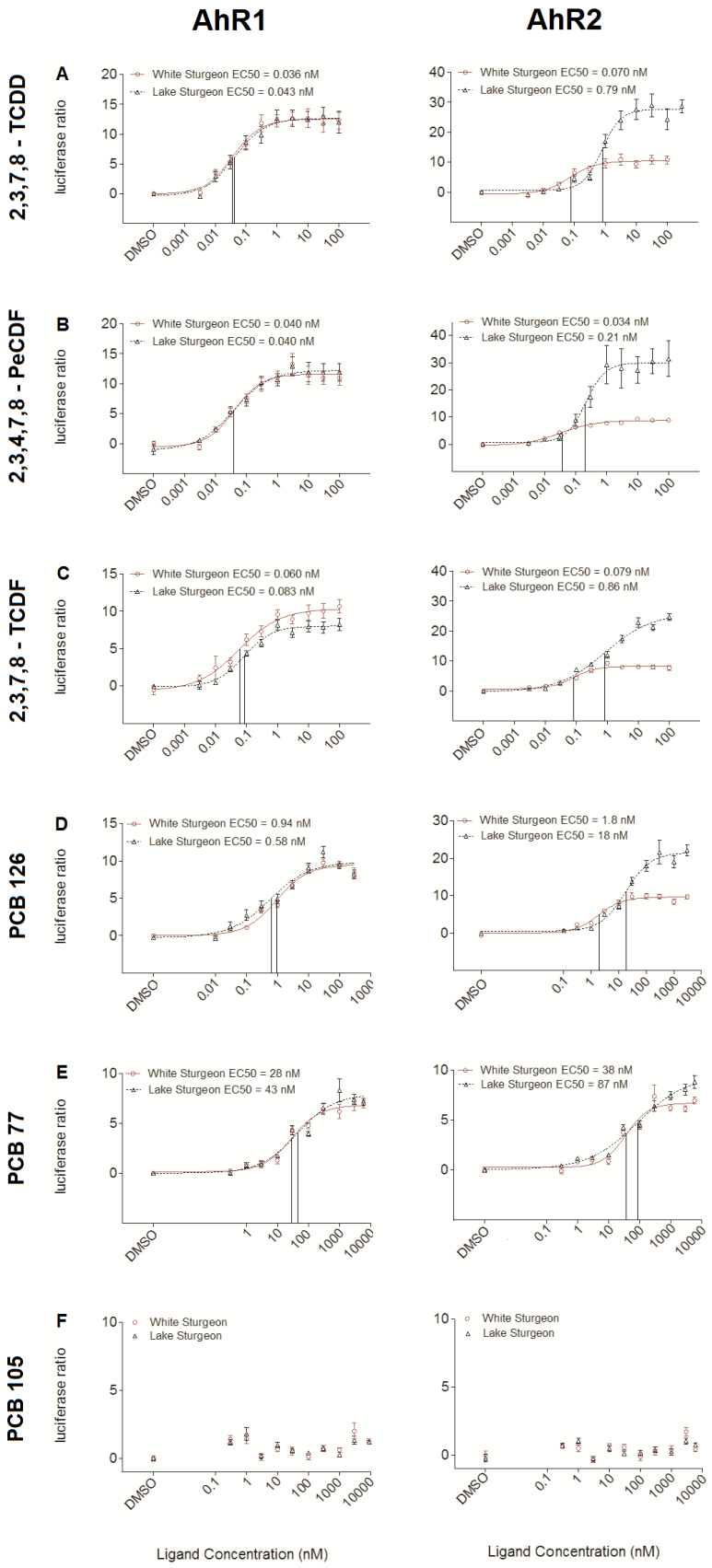

AhR1
AhR2
A
2,3,7,8 - TCDD
White Sturgeon EC50 = 0.036 nM
Lake Sturgeon EC50 = 0.043 nM
White Sturgeon EC50 = 0.070 nM
Lake Sturgeon EC50 = 0.79 nM
B
2,3,4,7,8 - PeCDF
White Sturgeon EC50 = 0.040 nM
Lake Sturgeon EC50 = 0.040 nM
White Sturgeon EC50 = 0.034 nM
Lake Sturgeon EC50 = 0.21 nM
C
2,3,7,8 - TCDF
White Sturgeon EC50 = 0.060 nM
Lake Sturgeon EC50 = 0.083 nM
White Sturgeon EC50 = 0.079 nM
Lake Sturgeon EC50 = 0.86 nM
D
PCB 126
White Sturgeon EC50 = 0.94 nM
Lake Sturgeon EC50 = 0.58 nM
White Sturgeon EC50 = 1.8 nM
Lake Sturgeon EC50 = 18 nM
E
PCB 77
White Sturgeon EC50 = 28 nM
Lake Sturgeon EC50 = 43 nM
White Sturgeon EC50 = 38 nM
Lake Sturgeon EC50 = 87 nM
F
PCB 105
White Sturgeon
Lake Sturgeon
luciferase ratio
DMSO
0.001
0.01
0.1
1
10
100
1000
10000
Ligand Concentration (nM)
Ligand Concentration (nM)

Figure 5.1. Dose response curves of COS-7 cells transfected with AHR1 or AHR2 of white sturgeon (red) and lake sturgeon (black) following exposure to TCDD (A), PeCDF (B), TCDF (C), PCB 126 (D), PCB 77 (E), and PCB 105 (F). Data are presented as mean ± S.E. based on three replicated assays each conducted in quadruplicate. EC_{50}s (nM) for white sturgeon and lake sturgeon are indicated as vertical lines. Dose response curves of white sturgeon are based on findings described previously (Doering et al., 2014a).

Table 5.1. Calculated LOECs (nM), ECs (nM), and maximum responses relative to the maximum response of TCDD (%) for AHR1 and AHR2 of lake sturgeon.

	Lake sturgeon AHR1					Lake sturgeon AHR2				
	LOEC	EC_{20}	EC_{50}	EC_{80}	Max. Response	LOEC	EC_{20}	EC_{50}	EC_{80}	Max. Response
TCDD	0.01	0.0090 (± 0.005)	0.043 (± 0.01)	0.21 (± 0.04)	100	0.1	0.30 (± 0.05)	0.79 (± 0.04)	2.1 (± 0.5)	100
PeCDF	0.01	0.0065 (± 0.001)	0.040 (± 0.003)	0.25 (± 0.07)	96	0.03	0.077 (± 0.02)	0.21 (± 0.01)	0.58 (± 0.3)	108
TCDF	0.03	0.019 (± 0.006)	0.083 (± 0.02)	0.35 (± 0.09)	63	0.03	0.078 (± 0.04)	0.86 (± 0.3)	9.6 (± 3.6)	94
PCB 126	0.1	0.18 (± 0.08)	0.58 (± 0.2)	4.4 (± 1.7)	78	0.3	4.1 (± 1.4)	18 (± 1.6)	135 (± 69)	78
PCB 77	1	6.4 (± 0.6)	43 (± 11)	294 (± 141)	63	0.3	20 (± 1.4)	87 (± 37)	2075 (± 908)	34
PCB 105	-	-	-	-	< 20	-	-	-	-	< 20

Values that could not be calculated are indicated with '-'. Standard error of the mean (S.E.) is presented in brackets.

Table 5.2. Relative sensitivity (ReS) of AHR1s and AHR2s of sturgeons to selected dioxin-like compounds based on the average of EC_{20}, EC_{50}, and EC_{80}.

	TCDD	**PeCDF**	**TCDF**	**PCB 126**	**PCB 77**	**PCB 105**
Lake Sturgeon AHR1[a]	12	2.9	23	30	6.4	-
Lake Sturgeon AHR2[a]	1.0	1.0	1.0	1.0	1.0	-
White Sturgeon AHR1[b]	1.8	1.4	0.7	1.1	1.2	-
White Sturgeon AHR2[b]	1.0	1.0	1.0	1.0	1.0	-
Lake Sturgeon AHR1[c]	0.8	0.7	1.2	1.1	0.5	-
White Sturgeon AHR1[c]	1.0	1.0	1.0	1.0	1.0	-
Lake Sturgeon AHR2[c]	0.1	0.3	0.03	0.06	0.1	-
White Sturgeon AHR2[c]	1.0	1.0	1.0	1.0	1.0	-

ReS of white sturgeon are based on findings described previously (Doering et al., 2014a). Values that could not be calculated are indicated with '-'.

[a] Calculated by use of Equation 5.1.

[b] Adapted from previously published results (Doering et al., 2014a)

[c] Calculated by use of Equation 5.2.

Based on the mean of concentrations to elicit EC_{20}, EC_{50}, and EC_{80}, sensitivity to activation of AHR1 of lake sturgeon to TCDD, PeCDF, TCDF, PCB 126, and PCB 77 was approximately equal to that of AHR1 of white sturgeon (Table 5.2). In contrast, AHR2 of lake sturgeon was 3- to 30-fold less sensitive to activation to TCDD, PeCDF, TCDF, PCB 126, and PCB 77 than AHR2 of white sturgeon (Table 5.2). LOECs were also used to make comparisons of the sensitivity to activation of AHRs of white sturgeon and lake sturgeon. AHR1 of white sturgeon and lake sturgeon had the same LOEC-based sensitivities (Table C5.S2). In contrast, AHR2 of lake sturgeon was 3- to 10-fold less sensitive to activation to TCDD, PeCDF, TCDF, and PCB 126 than AHR2 of white sturgeon (Table C5.S2).

5.4.3 Relative Potency of Select DLCs to AHR1 and AHR2 of Lake Sturgeon

Each DLC had chemical- and receptor-specific potencies in COS-7 cells transfected with AHR1 or AHR2 of lake sturgeon (Figure 5.1). TCDD was the most potent DLC to AHR1 (Table C5.S3). PeCDF was the most potent DLC to AHR2 (Table C5.S3). Based on responses in COS-7 cells, RePs for AHR1 of lake sturgeon were similar to RePs for AHR1 of white sturgeon, but RePs for AHR2 of lake sturgeon were more distinct from RePs for AHR2 of white sturgeon (Table C5.S3).

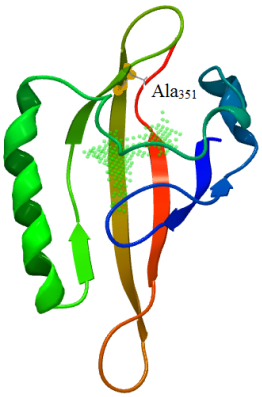
5.4.4 Homology Modeling of AHR1 and AHR2 of White Sturgeon and Lake Sturgeon

Models had ProSA z-scores between -3.51 and -4.54, which are within the range of values for native protein structures of similar size and are comparable to *z*-scores for models of

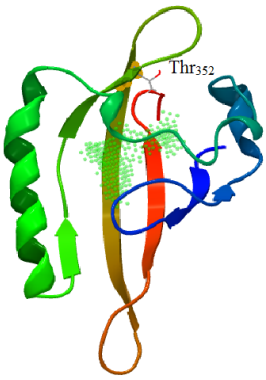
AHR reported by other authors (Figure C5.S2) (Farmahin et al., 2013; Fraccalvieri et al., 2013; Pandini et al., 2007). Greater than 85 % of amino acid residues resided in the most favored areas of the Ramachandran plot indicating that the dihedral angles are favorable (Figure C5.S3). The LBD of the AHR1 of both white sturgeon and lake sturgeon has a single amino acid difference at position 351 in the AHR1 of white sturgeon and position 352 in the AHR1 of lake sturgeon (Figure 5.2B). However, the position of this amino acid is not predicted to contribute to the structure of the internal cavity (Figure 5.2A, 5.2B). The LBD of the AHR2 of white sturgeon and lake sturgeon has four differences in amino acids at positions 305, 313, 321, and 388 (Figure 5.2D). However, only a single amino acid difference in white sturgeon (Ala-388) and lake sturgeon (Thr-388) are predicted to contribute to the structure of the internal cavity (Figure 5.2C, 5.2D). Volumes of the main cavity of the LBD of AHR1s of white sturgeon and lake sturgeon were similar at 295 and 299 $Å^3$, respectively (Figure 5.2A). However, the volume of the main cavity of the LBD of AHR2 of white sturgeon (292 $Å^3$) was greater than the volume of the main cavity of the LBD of the AHR2 of lake sturgeon (273 $Å^3$) (Figure 5.2C). *In silico* mutagenesis indicated that the volume of the main cavity of the LBD of a T388A mutant of AHR2 of lake sturgeon has a restored volume of the main cavity 289 $Å^3$ (Figure 5.2E) while the volume of the main cavity of the LBD of a T305M, A313V, or E321G mutant were unchanged (274 Å3) (Figure C5.S4). When LBDs of the AHR2 of all thirteen fishes with available AHR sequence data were aligned, no other species had Thr at the position equivalent to 388 in AHR2 of sturgeons (Figure C5.S5).

A

White Sturgeon AhR1
cavity volume = 295 Å^3



Lake Sturgeon AhR1
cavity volume = 299 Å^3

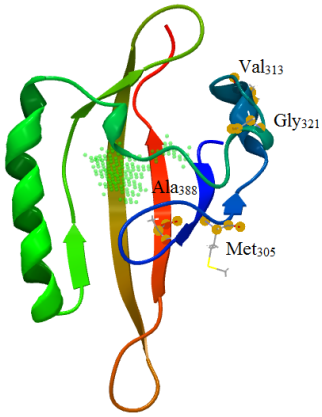


B

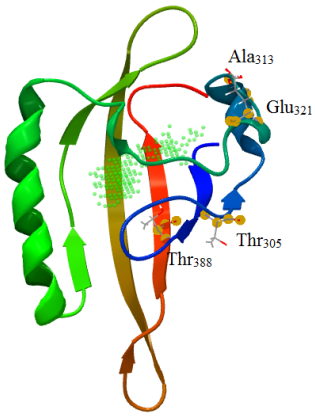
```
White Sturgeon AhR1  NFIFRTKHKL  DFTPTACDAK  GKIVLGYTEA  ELCYRGTGYQ  FIHAADMLYC  AENH 327
Lake Sturgeon AhR1   ..........  ..........  ..........  ..........  ..........  .... 328

                                                  351
White Sturgeon AhR1  IRMIKTGESG  MTVFRLLTKQ  NRWAWVQANA  RLVYKNGRPD  YIIATQRALS  DNE 380
Lake Sturgeon AhR1   ..........  ..........  ...T......  ..........  ..........  ... 381
                                                  352
```

C

White Sturgeon AhR2
cavity volume = 292 Å^3



Lake Sturgeon AhR2
cavity volume = 273 Å^3



D

```
                                  305          313          321
White Sturgeon AhR2  TLIFQTKHKL  DFTPMGCDTR  GKVVLGYTDT  GLCMRGTGYQ  FIHAADMMHC  ADNH 344
Lake Sturgeon AhR2   ..........  ....T.....  ..A.......  E.........  ..........  .... 344

                                                                     388
White Sturgeon AhR2  VRMIKTGESG  LTVFRLLTKN  GSWVWVQANA  RLIYKGGRPD  FIVARQRALT  NEE 397
Lake Sturgeon AhR2   ..........  ..........  ..........  ..........  ...T......  ... 397
```

E

Lake Sturgeon AhR2 T388A Mutant
cavity volume = 289 Å^3

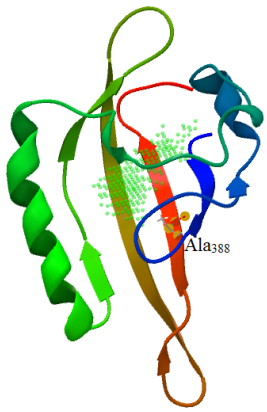


Figure 5.2. 3-D model of the ligand binding domains (LBDs) of the AHR1 (A) and AHR2 (C) of white sturgeon and lake sturgeon is shown. Predicted binding pocket is indicated as a dotted region and the cavity volume is indicated ($Å^3$). Amino acid differences between AHR1 (A) of white sturgeon and lake sturgeon and between AHR2 (C) of white sturgeon and lake sturgeon are labelled and shown as 'stick structures'. Alignment of the amino acid sequences of the LBDs of AHR1 (B) and AHR2 (D) of white sturgeon and lake sturgeon. Amino acids which contribute to the internal cavities are highlighted in boxes. Amino acid residues are indicated at position 351 or 352 for AHR1s and 305, 313, 321, and 388 for AHR2s. 3-D model of the LBD of the *in silico* T388A mutant of AHR2 of lake sturgeon (E) is shown. Amino acid mutation is labelled and shown as a 'stick structure' (E).

5.5 Discussion

As an initial step toward identifying specific mechanisms that determine differences in sensitivity to DLCs among species of fishes, this study compared AHR1 and AHR2 from white sturgeon and lake sturgeon with regard to *in vitro* activation by DLCs and differences in the primary and tertiary structure of the LBD of each AHR. Studies have shown that differences in activation of AHR1 transfected into COS-7 cells were predictive of *in vivo* sensitivity of birds to DLCs (Farmahin et al., 2012). However, the role of AHR1, if any, in sensitivity to DLCs *in vivo* among fishes is not completely understood (Bak et al., 2013; Clark et al., 2010; Doering et al., 2013; Hanno et al., 2010; Karchner et al., 1999; Prasch et al., 2003; Van Tiem and Di Giulio, 2011). AHR1s from white sturgeon and lake sturgeon were not different in their sensitivity to activation by any of the six DLCs tested in this study when transfected into COS-7 cells. This suggests that differences in activation of AHR1 would not result in differences in sensitivity between white sturgeon and lake sturgeon *in vivo* despite both AHR1s being activated by exposure to DLCs. Adverse outcomes as a result of exposure to DLCs have been shown to be mediated through activation of AHR2 in all fishes studied to date (Bak et al., 2013; Clark et al., 2010; Doering et al., 2013; Hanno et al., 2010; Karchner et al., 1999; Prasch et al., 2003; Van Tiem and Di Giulio, 2011). Sensitivity to activation of AHR2 from white sturgeon was 3- to 30-fold greater for TCDD, PeCDF, TCDF, PCB 126, and PCB 77 than AHR2 of lake sturgeon, which suggests that white sturgeons are likely to have greater sensitivity to DLCs in vivo compared to lake sturgeon. Although embryo-lethality assays have not been conducted to date on white sturgeon, it is hypothesized that white sturgeon have relatively great sensitivity to DLCs *in vivo* as white sturgeon have been shown to express two AHRs with EC_{50}s for TCDD less than

any other tested vertebrate (Doering et al., 2014a; 2014b) and have been shown to be among the most responsive of fishes with regard to induction of CYP1A *in vivo* (Doering et al., 2012). No previous studies have investigated the sensitivity of lake sturgeon to DLCs *in vivo* or *in vitro*.

The Per-Arnt-Sim B (PAS B) domain or LBD of the AHR is involved in binding of DLCs and has been the focus of intensive study in context with furthering our understanding of the MIE of adverse outcomes in vertebrates as a result of exposure to DLCs. However, investigation into the LBD of the AHR among fishes is complicated by a lack of conservation with shared amino acid identity being < 70 % among diverse fishes (Doering et al., 2013) relative to > 96 % among diverse birds (Farmahin et al., 2013). This makes investigation into white sturgeon and lake sturgeon a useful starting point as great differences in sensitivity among sturgeons has been evidenced (Bucker et al., 2015; Chambers et al., 2012; Doering et al., 2014a), yet conservation in amino acid identity is > 96 % between the LBD of AHR1s and AHR2s of white sturgeon and lake sturgeon. Within the LBD of the AHR1 of white sturgeon and lake sturgeon, there is a single amino acid difference at positions 351 and 352, respectively. However, this difference is not located within the binding cavity and, therefore, is predicted to have no significant effect on binding of DLCs and thus sensitivity to activation by DLCs. This prediction is in agreement with in vitro data where no difference in sensitivity to activation was observed between AHR1s of white sturgeon and lake sturgeon. Within the LBD of the AHR2 of white sturgeon and lake sturgeon, there were four differences in amino acids at positions 305, 313, 321, and 388. Amino acids at positions 305, 313, and 321 do not contribute to the binding cavity and are predicted to have no significant effect on binding of DLCs. However, the amino acid at position 388 is located at a critical position within the binding cavity. The difference at position 388 of AHR2 of white sturgeon (Ala-388) and lake sturgeon (Thr-388) is in the equivalent

position to amino acid 380 in the AHR1 of birds, 375 in the AHR of mice, and 386 in the AHR1a and AHR2 of zebrafish. This amino acid has been identified as one of seven residues required for high affinity binding of DLCs to the AHR (Pandini et al., 2009). In birds, amino acid identities within the LBD of AHR1 at positions 324 and 380 explain differences in in vivo sensitivity to DLCs and in vitro sensitivity to activation of AHR1 by DLCs (Farmahin et al., 2013; Head et al., 2008). Similarly, in mammals, amino acid identities at position 375 within the LBD of AHR have been shown to result in differences in sensitivity to TCDD between strains of mice (Beischlag et al., 2008; Bisson et al., 2009; Hankinson, 1995; Nguyen and Bradford, 2008). The C57BL/6J strain with Ala-375 has 10-fold greater sensitivity to DLCs *in vivo* relative to the DBA/2 strain with Val-375 (Bisson et al., 2009; Ema et al., 1993). Mutation of Ala-375 to Val-375 by site-directed mutagenesis resulted in a reduction of approximately 10-fold in binding affinity of AHR for TCDD (Pandini et al., 2007). In fish, the lack of binding of DLCs by AHR1a of zebrafish has been shown to result from the presence of Tyr-296 and Thr-386 residues compared to His-296 and Ala-386 residues in the AHR2, which binds DLCs (Fraccalvieri et al., 2013).

The amino acids Ala-388 in the LBD of AHR2 of white sturgeon and Thr-388 in the LBD of AHR2 of lake sturgeon have side chains that are oriented toward the binding pocket, which indicates that they are directly involved in binding of DLCs (Bisson et al., 2009), while amino acids at positions 305, 313, and 321 have side chains oriented away from the binding pocket, and therefore, are not predicted to affect binding of DLCs (Pandini et al., 2007; 2009). It has been proposed that replacing Ala (CH3) at this critical position in the LBD of the AHR with a Val (CH(CH3)2) or Thr (CHCH3OH) residue, the R-groups of which are greater in size than the R-group of Ala, would partially impair proper binding of ligands by altering the volume of

the binding pocket (Bisson et al., 2009). Replacing Ala with Leu (CH2CH(CH3)2), the R-group of which is even greater in size than the R-group of Val or Thr, completely blocks binding of ligands (Bisson et al., 2009). Because AHR2 of white sturgeon has an Ala-388 residue but AHR2 of lake sturgeon has a Thr-388 residue, the reduced volume of the binding cavity for AHR2 of lake sturgeon compared to the binding cavity for AHR2 of white sturgeon would be suggestive of a lesser sensitivity to activation of AHR2 of lake sturgeon as was observed *in vitro*. This prediction was confirmed since the cavity volume for AHR2 of white sturgeon (292 $Å^3$) was 7 % greater than the cavity volume for AHR2 of lake sturgeon (273 $Å^3$). This is similar to the 5 % reduction in cavity volume observed in mice AHR1 mutants with A375V (Bisson et al., 2009). In silico mutation of Thr-388 to Ala-388 in AHR2 of lake sturgeon restored the cavity volume to within 1 % of that of AHR2 of white sturgeon (289 $Å^3$), while mutation of amino acids at position 305, 313, or 321 did not alter the cavity volume (274 $Å^3$). This provides further evidence of the importance of this key amino acid in the cavity volume of the LBD of the AHR2 of lake sturgeon. However, there is uncertainly whether the cavity volume of the LBD alone or in combination with knowledge of the R-group of amino acids lining the cavity are predictive of sensitivity to activation of AHRs by DLCs. Further, when all thirteen species of fishes with known sequences of the LBD of the AHR2 were aligned, none had a Thr residue in the equivalent of position 388 of AHR2 of lake sturgeon, which indicates that other as yet unidentified amino acids are also likely to be involved with determining differences in sensitivity among diverse fishes. However, thirteen species are an insignificant percentage of the greater than 25,000 species of fishes known to exist, and therefore sequence information on numerous other species would be required to further confirm this hypothesis.

Although this study provided evidence for the hypothesis that differences in sensitivity among fishes to DLCs *in vivo* and with regard to activation of AHRs *in vitro* is determined by identities of key amino acids in the LBD of AHR2, it needs to be acknowledged that further studies are necessary to test this hypothesis. In order to provide additional evidence for the hypothesis that differences in sensitivity to activation of AHR2 of white sturgeon and lake sturgeon is driven by Ala-388 and Thr-388, site-directed mutagenesis studies should be the focus of ongoing research, as has previously been used to provide evidence for similar hypotheses in birds (Farmahin et al., 2012; 2013; Karchner et al., 2006) and mammals (Pandini et al., 2007). In context with advancing AOPs, the pleiotropic alteration of the expression of a suite of genes might be the basis of adverse outcomes as a result of activation of AHR by DLCs (Carney et al., 2004; Finne et al., 2007; Nault et al., 2013; Alexeyenko et al., 2010). Because the *in vitro* assays used in this study are based on expression of cytochrome P4501A (CYP1A), they might not be representative of adverse outcomes of regulatory relevance to embryos or adult animals or give indications on whether there are differences in responsiveness of other genes in the AHR gene battery of white sturgeon and lake sturgeon. Whole transcriptome sequencing technologies, such as RNA-seq, would provide valuable insight into whether differences in sensitivities to activation of AHR2s of white sturgeon and lake sturgeon by DLCs have implications for responses of the whole AHR gene battery in these, or potentially other, fishes. Further, embryo-lethality assays with white sturgeon and lake sturgeon would enable linking of differences in sensitivity to activation of AHR2s to differences in sensitivity to an *in vivo* adverse outcome of regulatory relevance.

In summary, this study demonstrated that white sturgeons are 3- to 30-fold more sensitive than lake sturgeon to exposure to DLCs with regard to activation of AHR2, but not AHR1, in

COS-7 cells transfected with AHRs from these species. This difference in relative sensitivity to activation of AHR2 is suggested to result from key amino acid identities at position 388 of the LBD of the AHR2 of white sturgeon (Ala-388) and lake sturgeon (Thr-388) based on homology modeling and *in silico* mutagenesis. This is a first report linking specific differences in the structure of the AHR2 protein to differences in activation of the AHR2 *in vitro* between two different species of fishes. It marks an initial step in providing a molecular understanding of differences in species sensitivity of fishes to DLCs with the ultimate goal to integrate this information into 21st century risk assessment approaches, such as the AOP concept. Further, this study provides evidence that ongoing investigations into the LBD of AHR2 might identify the specific molecular mechanisms responsible for differences in sensitivity among the largest and most diverse group of vertebrates, the fishes, to exposure to DLCs.

CHAPTER 6

6 HIGH CONSERVATION IN TRANSCRIPTOMIC AND PROTEOMIC RESPONSE OF WHITE STURGEON TO EQUIPOTENT CONCENTRATIONS OF 2,3,7,8-TCDD, PCB 77, AND BENZO[A]PYRENE

PREFACE

Chapter 5 demonstrated that both aryl hydrocarbon receptors (AHR1 and AHR2) of lake sturgeon (*Acipenser fulvescens*) are activated by dioxin-like compounds (DLCs) as was demonstrated in Chapter 4 for AHR1 and AHR2 of white sturgeon (*Acipenser transmontanus*). However, little is known about the links between activation of the AHR by a DLC, and the resulting cascade of molecular, biochemical, and histological events that eventually manifest as an apical adverse effect. Therefore, the aim of Chapter 6 was to investigate these linkages across levels of biological organization in white sturgeon by use of high-throughput, next-generation whole transcriptome and whole proteome analyses. Demonstrating high conservation in global response across levels of biological organization given equal activation of the AHR by agonists would provide support for predictive links between sensitivity to activation of the AHR and apical adverse effects of regulatory relevance, such as mortality of embryos. Demonstrating such links is critical in order to develop a mechanism-based biological model that enables the prediction of the sensitivity of any species of sturgeon to exposure to DLCs.

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Author contributions:

Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, conducted the transcriptome analyses, polymerase chain reaction, and *in vivo* exposure, prepared most figures, and drafted the manuscript.

Drs. Song Tang and Hui Peng (both University of Saskatchewan) provided scientific input and guidance, conducted bioinformatics analyses, prepared some figures, commented on and edited the manuscript.

Bryanna K. Eisner (University of Saskatchewan) provided laboratory assistance with the transcriptomics and *in vivo* exposure.

Dr. Jianxian Sun (University of Saskatchewan) provided laboratory assistance with proteomics.

Dr. John P. Giesy (University of Sasaktchewan) provided scientific input and guidance, commented on and edited the manuscript.

Dr. Steve Wiseman (University of Saskatchewan) provided scientific input, guidance, and training, assisted with bioinformatics, commented on and edited the manuscript.

Dr. Markus Hecker (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

6.1 Abstract

Adverse effects associated with exposure to dioxin-like compounds (DLCs) are mediated primarily through activation of the aryl hydrocarbon receptor (AHR). However, little is known about the cascades of events that link activation of the AHR to apical adverse effects. Therefore, this study used high-throughput, next-generation molecular tools to investigate similarities and differences in whole transcriptome and whole proteome responses to equipotent concentrations of three agonists of the AHR, 2,3,7,8-TCDD, PCB 77, and benzo[a]pyrene, in livers of a non-model fish, the white sturgeon (*Acipenser transmontanus*). A total of 926 and 658 unique transcripts were up- and down-regulated, respectively, by one or more of the three chemicals. Of the transcripts shared by responses to all three chemicals, 85 % of up-regulated transcripts and 75 % of down-regulated transcripts had the same magnitude of response. A total of 290 and 110 unique proteins were up- and down-regulated, respectively, by one or more of the three chemicals. Of the proteins shared by responses to all three chemicals, 70 % of up-regulated proteins and 48 % of down-regulated proteins had the same magnitude of response. Among treatments there was 68 % similarity between the global transcriptome and global proteome. Pathway analysis revealed that perturbed physiological processes were indistinguishable between equipotent concentrations of the three chemicals. The results of this study contribute toward more completely describing adverse outcome pathways associated with activation of the AHR.

6.2 Introduction

Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs), are ubiquitous, persistent, and bioaccumulative pollutants of environmental concern globally. Exposure to DLCs can lead to a variety of adverse effects in vertebrates, with fishes being among the most sensitive. Fishes are particularly sensitive to DLCs during early life stages (Buckler et al., 2015; Doering et al., 2013; Elonen et al., 1998; Johnson et al., 1998; Park et al., 2014; Toomey et al., 2001; Walker et al., 1991; Yamauchi et al., 2006; Zabel et al., 1995). Adverse effects associated with exposure of early life stages of fishes include craniofacial and cardiovascular malformation, pericardial and yolk sac edema, and posthatch mortality (King-Heiden et al., 2012; Okey, 2007). Adverse effects associated with exposure of less sensitive juvenile or adult life stages of fishes primarily include wasting syndrome, fin necrosis, and hepatotoxicity (Kleeman et al., 1988; Spitsbergen et al., 1986; Walker et al., 2000) but can also include reduced hemopoiesis, hyperplasia of gill filaments, histological lesions, immune suppression, impaired reproductive and endocrine processes, carcinogenesis, and mortality (Giesy et al., 2002; Spitsbergen et al., 1988a; 1988b; Okey, 2007).

DLCs share similarities in structure and a total of seven PCDDs, ten PCDFs, and twelve PCBs are considered dioxin-like because they bind with relatively great affinity to the aryl hydrocarbon receptor (AHR) (Denison and Heath-Pagliuso 1998; Van den Berg et al., 1998). The AHR is a ligand-activated transcription factor in the basic/helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of proteins (Okey, 2007). Most, if not all, critical adverse effects associated with exposure to DLCs are mediated through activation of the AHR and dysregulation of AHR

responsive genes (Okey, 2007). Exposure to other anthropogenic contaminants that bind to the AHR, including some polycyclic aromatic hydrocarbons (PAHs), can also result in dioxin-like adverse effects in fishes (Billiard et al., 1999; 2002; Jayasundara et al., 2015; Van Tiem and Di Giulio, 2011). Since all DLCs exert toxicity via a single, specific mechanism, toxicity of a mixture of DLCs follows an approximately additive toxicity model (Van den Berg et al., 1998). Manifested adverse effects, such as pericardial and yolk sac edema, as well as mortality were indistinguishable between embryos of rainbow trout (*Oncorhynchus mykiss*) exposed to mixtures of DLCs or single DLCs at concentrations that result in equal activation of the AHR (Walker et al., 1996; Zabel et al., 1995). Because equipotent concentrations of DLCs result in indistinguishable apical effects in fishes, it would be hypothesized that equal activation of the AHR by any DLC or mixture of DLCs results in indistinguishable response across levels of biological organization (i.e., transcript, protein, tissue).

Despite decades of research into the molecular initiating event (i.e., binding of DLCs to the AHR) and apical adverse effects of DLCs, little is known about the cascade of key events that link activation of the AHR with apical adverse effects, particularly in non-mammalian and non-model species. Therefore, in order to investigate these key events and improve linkages between molecular data sets and apical adverse effects of regulatory relevance, this study used high-throughput, next-generation molecular tools as an initial 'proof of concept' for characterizing the similarities (or lack thereof) in acute responses between the transcriptome and proteome to equipotent concentrations of three different agonists of the AHR in livers of juvenile white sturgeon (*Acipenser transmontanus*). White sturgeons are a non-model, ecologically relevant, endangered species of fish. White sturgeons have been the focus of a series of recent investigations into responses to DLCs as part of ongoing assessments of risk to white sturgeon in

particular and sturgeons in general (Doering et al., 2012; 2014a; 2014b; 2015b; Eisner et al., 2016). Specifically, juvenile white sturgeons were exposed to equipotent concentrations of the PCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), the PCB, 3,3',4,4'-tetrachlorobiphenyl (PCB 77), and the PAH, benzo[a]pyrene (BaP). TCDD is the prototypical agonist of the AHR, while PCB 77 is as much as 5000-fold less potent than TCDD (Van den Berg et al., 1998). BaP is among the strongest agonists of the AHR among the PAHs but can also elicit several non-AHR-mediated adverse effects, including carcinogenic, mutagenic, and immunotoxic effects (Neilson, 1998). Unlike DLCs, BaP can be rapidly metabolized and bioactivated (Neilson, 1998). Therefore, the specific objectives of this study were to 1) determine global responses of the whole transcriptome and proteome; 2) compare responses of the transcriptome to responses of the proteome; and 3) compare perturbation of physiological processes and predict similarities and differences in key events that could result in apical adverse effects on whole organisms.

6.3 Materials and methods

6.3.1 Animals

Juvenile white sturgeon of approximately three years of age and ranging in body mass (bm) from 460 to 1512 g were reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC). The studies reported here were approved by the Animal Research Ethics Board at the University of Saskatchewan (Protocol #20110082). Use of endangered white sturgeon was permitted by Environment Canada (Permit #SARA305).

6.3.2 Intraperitoneal Exposure

The protocol used for exposing white sturgeon has been described previously (Doering et al., 2012). In brief, sixteen white sturgeons (n = 4 per treatment) were randomly assigned to one of four 400 L tanks that were maintained at approximately 12 °C under flow-through conditions. Each individual received one intraperitoneal injection containing either 5 μg of TCDD/kg-bm (purity > 98 %; Wellington Laboratories, Guelph, ON), 5 mg of PCB 77/kg-bm (purity 100 %; Chromographic Specialties, Brockville, ON), or 30 mg of BaP/kg-bm (purity ≥ 96 %; Sigma-Aldrich, Oakville, ON) in corn oil. Control individuals received corn oil alone. Doses were chosen to represent equally potent concentrations of each chemical based on maximal activation of the AHR as determined during previous *in vitro* and *in vivo* investigations of sensitivity of white sturgeon to agonists of the AHR (Doering et al., 2012; 2014a; 2014b; 2015b; Eisner et al., 2016) and represent the lowest observed effect concentration (LOEC) in rainbow trout 4 weeks after treatment (Spitsbergen et al., 1988b). Three days following injection, livers from all individuals were excised and frozen in liquid nitrogen.

6.3.3 Transcriptomics

Total RNA was extracted from approximately 30mg of liver from each individual by use of the RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON). Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE). Quality of RNA was determined through RNA Integrity Number (RIN) by use of a 2100 Bioanalyzer (Agilent, Clara, CA). Only samples with an RIN ≥ 8 were used in

subsequent analysis (Control n = 3; TCDD, PCB 77, and BaP n = 4). Equal amounts of RNA from each individual were pooled to create one sample of 2 µg RNA per treatment. One RNA-Seq library per treatment was prepared by use of the Tru-Seq RNA Sample Prep Kit (*Illumina*, San Diego, CA). Quality of libraries was confirmed by use of a 2100 Bioanalyzer (Agilent). Each library was loaded onto a separate Mi-Seq v3 150 cycle cartridge (*Illumina*) and run as 75 base-pair (bp) paired-end reads on a Mi-Seq sequencer (*Illumina*) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK). Raw sequences have been made available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (Accession #GSE79624).

No public databases for either the genome or transcriptome of white sturgeon were available. Therefore, a comprehensive reference transcriptome was constructed by use of *de novo* assembly from reads for liver of white sturgeon described here and in earlier studies described elsewhere (Doering et al., 2014b). The reference transcriptome has been made available in the NCBI GEO (Accession #GSE79624). Specifically, a total of six Mi-Seq (*Illumina*) runs at 75 bp paired-end reads and four Hi-Seq (*Illumina*) runs on individual lanes at 100 bp paired-end reads were used. Prior to assembly, the quality of raw reads was assessed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the terminal 5' and 3' nucleotides were trimmed from all sequences to ensure high qualities (Phred quality score $\geq$ 30). All subsequent analyses were based on these cleaned reads. A merging step was then carried out on the paired reads from each of these four samples so that any overlapping reads were merged into a single read. Contigs (continuous, overlapping sequences assembled from individual sequencing reads) for the reference transcriptome were *de novo* assembled from the merged

reads and unmerged paired-end reads from individual sequencing reads by use of CLC genomics workbench v.5.0 (CLC Bio, Aarhus, Denmark) with default parameters. The minimum contig length was set to 200 bp and this assembly process generated 69,312 unique contigs. The mean contig size was 1,060 bp. Contigs comprising the reference transcriptome were annotated by use of BlastX searches in Blast2GO v.2.5.0 software (Conesa et al 2005) with a minimum *E*-value of $<10^{-6}$ against sequences in the NCBI non-redundant protein database for zebrafish.

Both merged and unmerged reads comprising each treatment were aligned to the reference transcriptome. The total numbers of paired-end reads were 21,113,596; 20,913,356; 21,935,312; and 19,015,255 for the control, TCDD, PCB 77, and BaP treatments, respectively. Mapped reads were filtered based on a count per million (CPM) of > 5 in at least one of the four treatments and normalized by use of the 'edgeR' package in R software v.3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Fold-changes for each contig for each treatment were calculated based on CPM relative to CPM in the control. Value of variation of biological variation (BCV) was set to 0.2 and significance of differentially expressed contigs was scored by a cutoff false discovery rate (FDR) of 0.05. Since statistical analysis cannot be conducted on the pooled libraries, significance of differentially expressed transcripts was scored by a change in abundance greater than or equal to a threshold of ± 2-fold relative to controls. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to confirm results of transcriptome analysis and assess variance among pooled individuals for a subset of six genes as described previously (Doering et al., 2012; 2014a; 2015a; Eisner et al., 2016). qRT-PCR primers for amplification of genes of interest were designed by use of Primer3 software (Rozen and Skaletsky et al., 2000) based on sequences from the white sturgeon liver transcriptome described here or were based on

primers of white sturgeon published previously (Table C6.S1) (Doering et al., 2012). Abundance of transcripts was quantified by normalizing to β-actin according to methods described previously (Simon, 2003).

6.3.4 Proteomics

Livers from individuals of each treatment group were pooled at equal mass to create one sample per treatment (Control n = 3; TCDD, PCB 77, and BaP n = 4). Pooled livers were homogenized on ice in a lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM KCl, 420 mM NaCl, 20 % Glycerol, and protease inhibitor cocktail, pH 7.4) by use of a model 100 Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA). Liver lysates were centrifuged at 15,000 xg for 15 min at 4 °C and soluble supernatant was transferred to a new tube and centrifuged again as a final clearing step. Total concentrations of protein in the supernatant were determined by use of the Bradford assay (Bradford, 1976), with a bovine serum albumin (BSA) standard. Filter-aided sample preparation was used for digestion of proteins by use of spin ultrafiltration filters with a molecular weight cut off of 30,000 daltons (30kDa). Aliquots of the lysates containing approximately 100 µg of protein were transferred to YM-30 microcon filter units (Millipore, Burlington, ON) and centrifuged at 14,000 xg for 30 min. Salt and other interferences in the buffer were removed from samples by washing three times with 0.1 M Tris-HCl. Samples were reduced for 60 min at 37 ºC with 50 µL of 5 mM DTT, and then carboxymethylated in the dark at room temperature for 30 min with 15 mM iodoacetamide. Samples were further digested overnight with 5 µg trypsin at 20:1 protein to trypsin and with gentle shaking. Digestion was terminated by adding formic acid to 1 % (v/v). The final sample

was collected by centrifugation at 14,000 xg for 30 min. Samples were stored at -80 °C until analyzed.

Each sample was loaded in duplicate onto a 75 mm inner diameter fused silica microcapillary column (Polymicron Technologies, Phoenix, AZ) packed with 10 cm of Luna 3-mC18 100 Å reversed phase particles (Phenomenex, Torrance, CA) and placed in-line with a nanoLC-electrospray ion source (Proxeon, Mississauga, ON) interfaced to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific) at the University of Toronto (Toronto, ON). The organic gradient was driven by the EASY-nLC system at 300 nL/min. The mobile phase consisted of 95 % acetonitrile with 0.1 % formic acid (A) and 5% acetonitrile (B). B at 2 % was increased to 6 % after 2 min, 62 mins later B was increased to 24 %, 26 min later B was increased to 90 % and held static for 5 min, and then decreased to initial conditions of 2 % of B and held for 8 min for equilibration. Positive precursor ions (400 to 2,000 m/z) were subjected to data-dependent collision-induced dissociation as the instrument cycled through one full mass scan at 60,000 full-width at half maximum followed by 17 successive MS/MS scans targeting the most intense precursors with dynamic exclusion and +2/+3 charge state selection enabled. Raw MS/MS sequences have been deposited to the ProteomeXchange Consortium via the Proteomics Identifications (PRIDE) partner repository (Accession #PXD003840) (Vizcaino et al., 2014).

No public databases for proteome sequences of white sturgeon were available. Therefore, a comprehensive, genome-free, artifact-free reference proteome was constructed based on the liver transcriptome of white sturgeon through the online pipeline at

http://kirschner.med.harvard.edu/tools/mz_ref_db.html (Wuhr et al., 2014). The reference proteome has been made available in the PRIDE partner repository (Accession #PXD003840). Raw MS/MS files were analyzed by use of MaxQuant v.1.5.1.2 (Cox et al., 2008). MS/MS spectra were searched against the reference proteome containing forward and reversed (decoy) sequences, allowing for variable modifications of methionine oxidation and N-terminal acetylation and fixed cysteine carbamidomethylation. Parent mass and fragment ions were matched by use of a maximal initial mass deviation of 7 p.p.m and 0.5 Th, respectively. Protein FDR was set to 0.01. Spectral count was used for label-free quantification of the proteins, and only proteins with spectral counts greater than four were used in further analyses. Sequences comprising each treatment were aligned to the reference proteome. Since statistical analysis cannot be conducted on the pooled samples, significance of differentially expressed proteins was scored by a change in abundance greater than or equal to a threshold of ± 2-fold relative to controls.

6.3.5 Data Analysis

Associations between multivariate descriptors of expression of transcriptome (69,312 contigs) and proteome (520 proteins) were inferred by use of coinertia analysis (CIA). A Monte Carlo test with 5,000 permutations was used for validating results of CIA. Venn plots were produced by use of the VennDiagram package with R software (Chen et al., 2011). Density ternary plots were produced by use of the ggtern package (ggtern.com) with R software. Only transcripts and proteins that had changes in abundance greater than or equal to a threshold of ± 2-fold were used in venn plots and ternary plots. Coinertia analysis (CIA) was performed by use of

the ade4 and ggplot2 (http://ggplot2.org) packages with R software (Dray et al., 2007). Accession numbers for each altered protein were converted to mRNA accession numbers from zebrafish prior to pathway analysis by use of the database to database (db2db) tool in bioDBnet (http://biodbnet.abcc.ncifcrf.gov/db/db2db.php). Only transcripts and proteins that had changes in abundance greater than or equal to a threshold of ± 2-fold were used in pathway analysis. Linear regression was performed by use of GraphPad Prism version 6.0 (San Diego, CA).

6.3.6 Pathway Analysis

Pathway analyses (functional grouped Gene Ontology (GO) terms analysis) were visualized by use of ClueGO v.2.1.637 run through Cytoscape v.3.2.138 by use of ontologies based on zebrafish in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the GO Consortium Biological Processes.

6.4 Results and discussion

6.4.1 Equipotent Concentrations of TCDD, PCB 77, and BaP

The AHR is conserved among vertebrate taxa and is best known for its ability to regulate expression of cytochrome P450 1A (CYP1A) as part of an adaptive response to exposure to certain xenobiotics (Hahn, 2002). Equipotent concentrations of TCDD, PCB 77, and BaP increased abundance of transcripts of CYP1A relative to controls ($p \leq 0.05$), but there were no statistical differences among these treatments ($p > 0.05$) (Figure C6.S2). This indicated

approximately equal activation of the AHR and suggests that approximately equipotent concentrations were administered, although a full dose-response would be required to confirm this hypothesis. Further, prior studies on white sturgeon have shown maximal abundance of transcripts of CYP1A to range from approximately 10- to 20-fold greater than controls following exposure to DLCs, with abundance of transcripts of CYP1A in the study presented here being 25-, 15-, and 24-fold greater than controls for TCDD, PCB 77, and BaP, respectively (Figure C5.S2) (Doering et al., 2012; Eisner et al., 2016). This indicates that approximately maximal response of activation of the AHR was achieved by exposure to each of the three chemicals.

6.4.2 Comparison among Responses to TCDD, PCB 77, and BaP

Abundances of 674, 818, and 923 transcripts were altered by ≥ 2-fold relative to controls in livers of white sturgeon exposed to TCDD, PCB 77, and BaP, respectively (Table C5.S4). Of the altered transcripts, 378, 493, and 529 were increased while 296, 325, and 394 were decreased by TCDD, PCB 77, and BaP, respectively (Table C5.S4). Few studies to date have investigated responses to activation of the AHR across the whole transcriptome, particularly in nonmodel and nonmammalian species; and no studies have investigated whole proteome responses. One commonality among the few studies conducted to date is the great number of genes and physiological processes that were altered, either directly or indirectly, as a result of activation of the AHR. Abundances of 2,000 altered transcripts were detected by use of microarray in embryos of zebrafish exposed to TCDD (Alexeyenko et al., 2010), and 1,058 altered transcripts were detected by use of serial analysis of gene expression (SAGE) sequencing in livers of adult zebrafish exposed to TCDD (Li et al., 2013). In studies with embryos of killifish (*Fundulus*

heteroclitus), 1,167 altered transcripts were detected by use of microarray after exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) (Whitehead et al., 2010), and 1,392 altered transcripts were detected by use of Mi-Seq sequencing in livers of juvenile roach (*Rutilus rutilus*) exposed to TCDD (Brinkmann et al., 2016). Less is known regarding responses of the transcriptome or proteome of fishes following exposure to PAHs. However, abundances of 241 altered transcripts were detected by use of microarray in one study on embryos of zebrafish exposed to BaP (Huang et al., 2014).

Because the equipotent concentrations that were used caused an approximately equal activation of the AHR, it was hypothesized that global responses of the transcriptome would be similar among chemicals. These responses would then be predicted to result in similar responses at the level of the proteome and then eventually result in similar apical adverse effects and severity of adverse effects at the level of the whole organism. At the level of the transcriptome, expression of a total of 926 unique transcripts was up-regulated by ≥ 2-fold relative to controls by TCDD, PCB 77, or BaP; but only 14 % (129) of these transcripts were common to responses to all three chemicals, and 37 % (344) of these transcripts were common to responses to two or more chemicals (Figure 6.1A). A total of 658 unique transcripts were down-regulated by ≥ 2-fold relative to controls by TCDD, PCB 77, or BaP, with 9 % (57) of these transcripts being common to responses to all three chemicals and 38 % (248) of these transcripts being common to responses to two or more chemicals (Figure 6.1B). Variability at the level of the transcriptome might be expected, and it was hypothesized that numerous changes in abundances of transcripts were likely due to effects not mediated by the AHR and could represent compensatory responses; however, some evidence supports agonist-dependent responses despite the same mode of action (Brinkmann et al., 2016; Boutros et al., 2008; Kopec et al., 2010). Transcripts for which

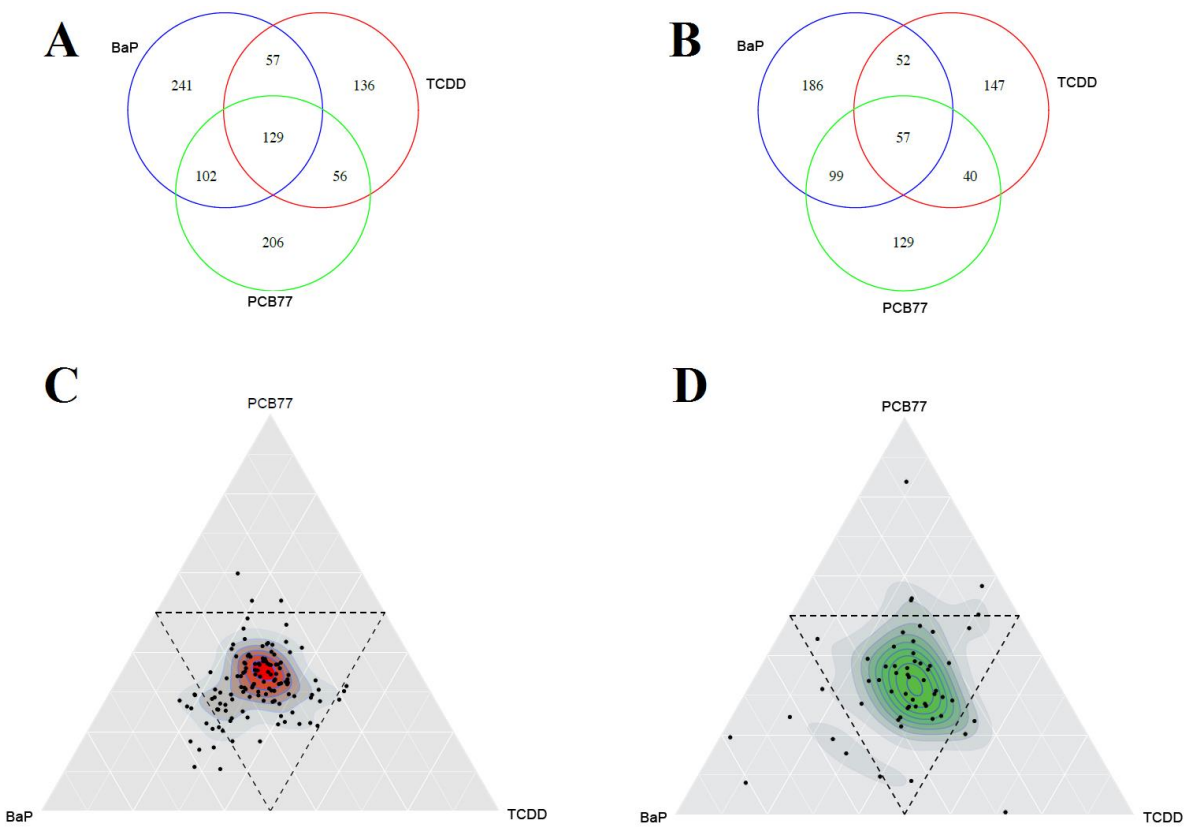


Figure 6.1. Venn diagrams representing number of transcripts with increased abundance (A) and number of transcripts with decreased abundance (B) based on transcriptome responses in livers of white sturgeon exposed to TCDD (red), PCB 77 (green), and BaP (blue). Density ternary plots representing transcripts with increased abundance (C) and transcripts with decreased abundance (D) shared by TCDD, PCB 77, and BaP. Each dot (black) represents a transcript with 129 transcripts represented in (C) and 57 transcripts represented in (D). The position of a transcript is specified as the center of mass (barycenter) of masses placed at the vertices of an equilateral triangle, and the proportions of the three treatments sum to 100 %. Each point represents a different composition of the three treatments, with the maximum proportion (100 %) of each treatment in each corner of the triangle, and the minimum proportion (0 %) at the opposite line. Color gradient represents kernel density estimation for up-regulated (red) and down-regulated (green) transcripts. Dashed lines border the barycenter which contains transcripts with equal fold-change following exposure to TCDD, PCB 77, or BaP. Transcripts outside of the dashed lines had greater fold-change following exposure to the chemical in the corresponding corner relative to the other chemicals.

expression was altered by all three chemicals might represent core genes of the AHR-gene battery, and, therefore, these transcripts were plotted in a density ternary plot incorporating fold-change in order to determine whether shared transcripts had similar magnitudes of response to equipotent concentrations as would be expected for core genes in the AHR-gene battery (Figure 6.1C; 6.1D). Of the 129 and 57 shared up- and downregulated transcripts, 85 % (110) and 75 % (43), respectively, clustered in the middle of the ternary plot indicating that equipotent concentrations of each chemical caused comparable fold-changes in expression at the level of the transcriptome. Equal magnitudes of responses at equipotent concentrations of each chemical were confirmed by qRT-PCR for six selected genes. No statistically significant differences ($p > 0.05$) were detected between response to TCDD, PCB 77, and BaP for CYP1A (Figure C6.S2), aryl hydrocarbon receptor repressor (AHRR) (Figure C6.S3), fructose-1,6-bisphosphatase 1a (FBP1A) (Figure C6.S4), superoxide dismutase (SOD) (Figure C6.S5), sex determining region Y-box 9 (SOX9) (Figure C6.S6), or cholesterol 7-alpha-monooxygenase 1a (CYP7A1A) (Figure C6.S7). Similar magnitudes of responses for the majority of shared transcripts due to exposure to equipotent concentrations of the three chemicals support the hypothesis that these transcripts might represent core genes of the AHR-gene battery.

Compared to changes across the transcriptome, fewer proteins were altered in livers of white sturgeon across the proteome. A total of 282, 359, and 307 proteins were altered by ≥ 2-fold relative to controls by TCDD, PCB 77, and BaP, respectively (Table C6.S4). A greater number of proteins were up-regulated by TCDD, PCB 77, and BaP (180, 270, and 218, respectively) than were down-regulated (102, 89, and 89, respectively) (Table C6.S4). A total of 290 unique proteins were up-regulated by ≥ 2-fold relative to controls by TCDD, PCB 77, or BaP, with 36 % (103) and 76 % (219) of these proteins being common to all three or to two or

more chemicals, respectively (Figure 6.2A). A total of 110 unique proteins were down-regulated by ≥ 2-fold relative to controls by TCDD, PCB 77, or BaP, with 26 % (29) and 76 % (87) of these proteins being common to all three or to two or more chemicals, respectively (Figure 6.2B). Of the 103 shared proteins that were up-regulated by each of the three chemicals, 70 % (72) were clustered in the middle of the ternary plot (Figure 6.2C); and of the 29 proteins that were down-regulated by each of the three chemicals, 48 % (14) were clustered in the middle of the ternary plot (Figure 6.2D) indicating comparable fold-changes in abundances of proteins in response to equipotent concentrations of the three chemicals. As with the transcriptome, the similar magnitude of response for the majority of shared proteins by equipotent concentrations of the three chemicals supports the hypothesis that these proteins might represent core genes of the AHR-gene battery.

6.4.3 Comparison between the Global Transcriptome and Global Proteome

Although transcripts are a prerequisite for translation of genes to proteins, there is uncertainty regarding whether altered abundance of transcripts has functional consequence at the level of the cell, tissue, or whole organism. Therefore, changes in abundances of transcripts were compared to changes in abundances of proteins. Across treatments there was 68 % similarity between the global transcriptome and global proteome (Figure 6.3). This is in contrast to some studies comparing global transcriptomes with global proteomes that have reported weak correlations as a result of post-translational effects (Juschke et al., 2013). Investigations at the protein level are considered more representative of dysregulation of physiology of organisms relative to investigations at the transcript level (Tomanek et al., 2011). Due to covariation

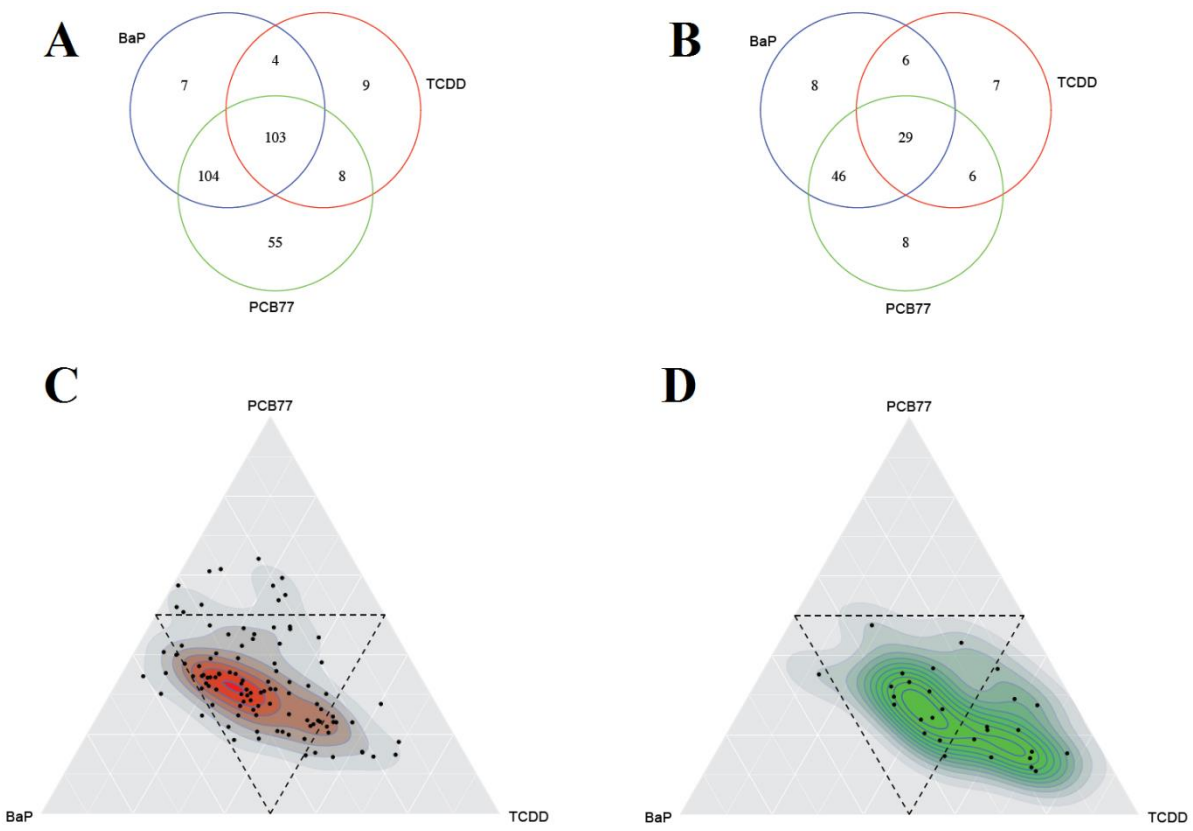


Figure 6.2. Venn diagrams representing number of proteins with increased abundance (A) and number of proteins with decreased abundance (B) based on proteome responses in livers of white sturgeon exposed to TCDD (red), PCB 77 (green), and BaP (blue). Density ternary plots representing proteins with increased abundance (C) and proteins with decreased abundance (D) shared by TCDD, PCB 77, and BaP. Each dot (black) represents a protein with 103 proteins represented in (C) and 29 proteins represented in (D). The position of a protein is specified as the center of mass (barycenter) of masses placed at the vertices of an equilateral triangle, and the proportions of the three treatments sum to 100 %. Each point represents a different composition of the three treatments, with the maximum proportion (100 %) of each treatment in each corner of the triangle, and the minimum proportion (0 %) at the opposite line. Color gradient represents kernel density estimation for up-regulated (red) and down-regulated (green) proteins. Dashed lines border the barycenter which contains proteins with equal fold-change following exposure to TCDD, PCB 77, or BaP. Proteins outside of the dashed lines had greater fold-change following exposure to the chemical in the corresponding corner relative to the other chemicals.

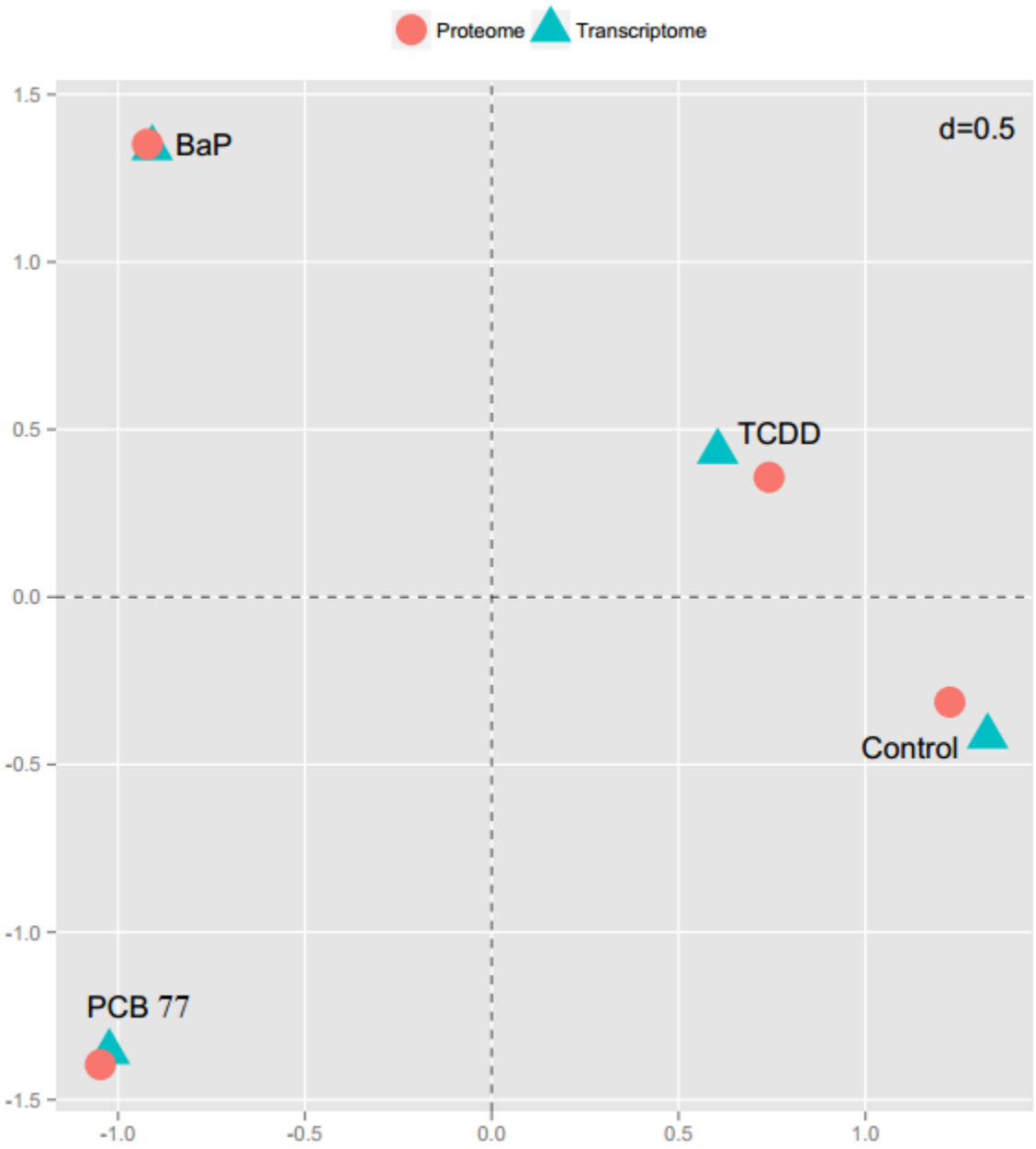


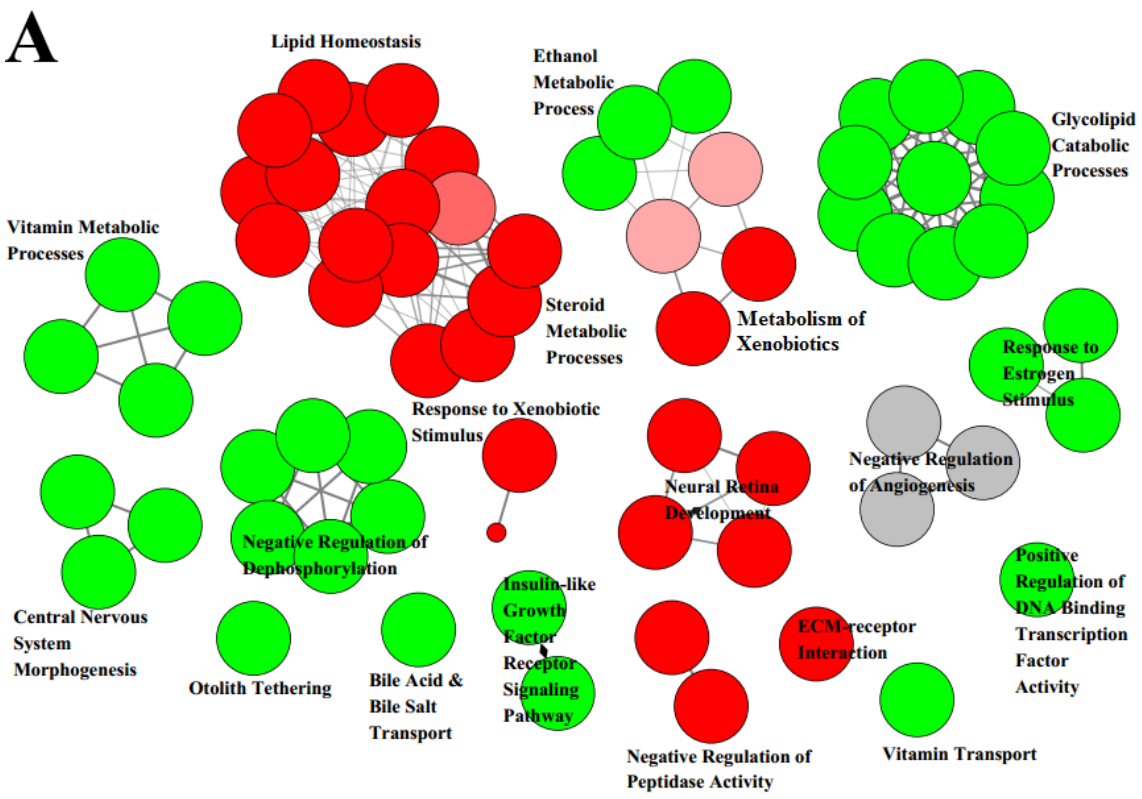
Figure 6.3. Association between transcriptome and proteome by use of coinertia analysis (CIA) of transcriptome (red circle) and proteome (green triangle) responses in liver of white sturgeon following exposure to TCDD, PCB 77, or BaP. CIA is a multivariate method that identifies trends or co-relationships in multiple data sets. Global similarity (coinertia) between transcriptomic and proteomic datasets is 68 % (p = 0.1639).

between the transcriptome and proteome, this indicates that responses to activation of the AHR at the level of the transcriptome are largely representative of responses of the proteome. It is hypothesized that this agreement between changes that translate across levels of molecular organization (i.e., transcriptome to proteome) could be indicative of further downstream physiological alterations representing apical adverse effects at the level of the whole organism.

6.4.4 Perturbations Among Physiological Pathways

The AHR-gene battery up-regulates genes in response pathways involved in response to xenobiotics, including CYP1A, aldehyde dehydrogenases (ADH), glutathione S-transferases (GST), NAD(P)H quinone oxidoreductases (NQO), and UDPglucuronosyltransferases (UGT), as well as the AHRR (Garner et al., 2012; Timme-Laragy et al., 2007; 2009). Responses of both the transcriptome and proteome in livers of white sturgeon exposed to TCDD, PCB 77, or BaP included greater abundances of transcripts and proteins in these response pathways relative to controls, including genes such as CYP1, CYP2, CYP3, UGT, GST, sulfotransferases (SULT), and AHRR (Figures 6.4; C6.S2; C6.S3). Due to the great increase in expression of Phase I enzymes in response to exposure to DLCs, oxidative stress as a result of chronic, increased expression of CYPs has been proposed as a mode of action for toxicity of DLCs (Dalton et al., 2002). However, antisense repression of CYP1s fails to prevent developmental toxicity of TCDD, which indicates that toxicity of AHR-agonists is predominantly due to CYP-independent mechanisms (Carney et al., 2004) and therefore necessitates further investigation into the modes of action for toxicity by DLCs.

Despite an important role in responses to exposure to xenobiotics, it has been proposed that ancestral functions of the AHR were regulation of developmental and regenerative processes


A
Lipid Homeostasis
Ethanol Metabolic Process
Glycolipid Catabolic Processes
Vitamin Metabolic Processes
Steroid Metabolic Processes
Metabolism of Xenobiotics
Response to Estrogen Stimulus
Response to Xenobiotic Stimulus
Negative Regulation of Angiogenesis
Neural Retina Development
Negative Regulation of Dephosphorylation
Positive Regulation of DNA Binding Transcription Factor Activity
Insulin-like Growth Factor Receptor Signaling Pathway
Central Nervous System Morphogenesis
Otolith Tethering
Bile Acid & Bile Salt Transport
ECM-receptor Interaction
Negative Regulation of Peptidase Activity
Vitamin Transport

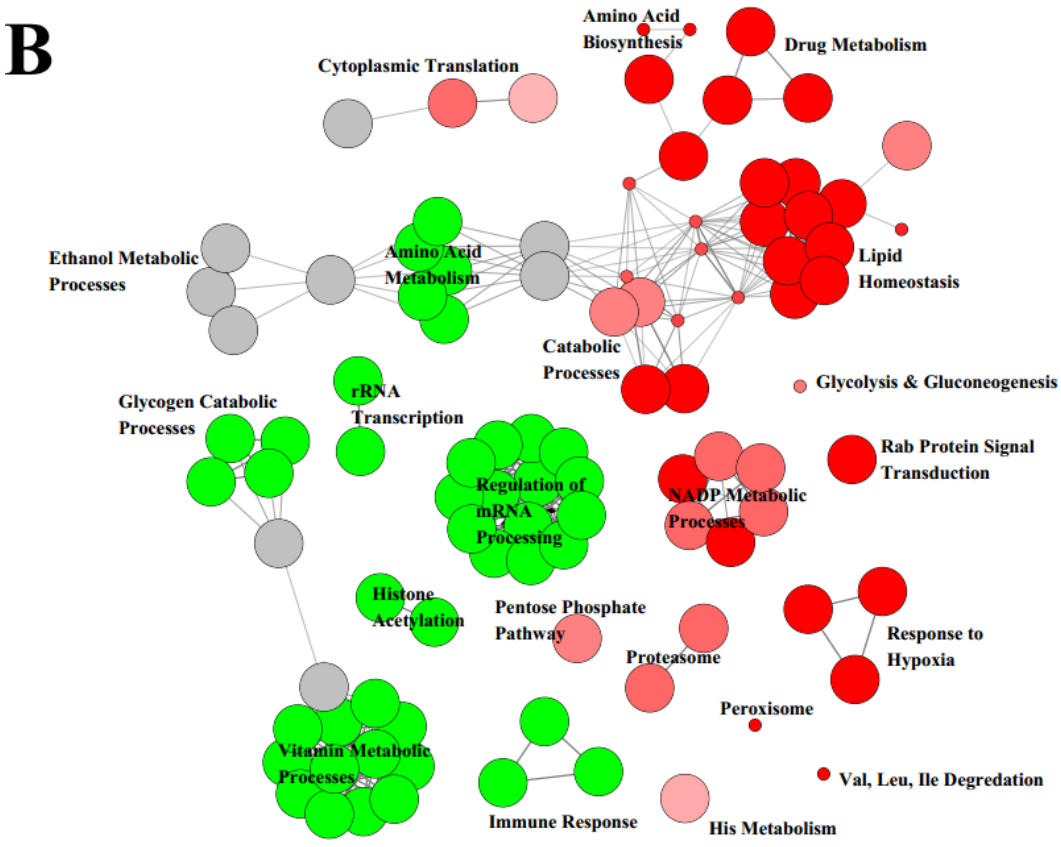

B
Amino Acid Biosynthesis
Drug Metabolism
Cytoplasmic Translation
Ethanol Metabolic Processes
Amino Acid Metabolism
Lipid Homeostasis
Catabolic Processes
Glycolysis & Gluconeogenesis
Glycogen Catabolic Processes
rRNA Transcription
Rab Protein Signal Transduction
Regulation of mRNA Processing
NADP Metabolic Processes
Histone Acetylation
Pentose Phosphate Pathway
Response to Hypoxia
Proteasome
Peroxisome
Vitamin Metabolic Processes
Val, Leu, Ile Degredation
Immune Response
His Metabolism

Figure 6.4. Cytoscape visualization of ClueGO clustering results of shared physiological processes altered by TCDD, PCB 77, and BaP at the level of the transcriptome (A) and proteome (B) in liver of white sturgeon. Clusters with a greater proportion of up-regulated processes are shown in (red) while clusters with a greater proportion of down-regulated processes are shown in (green). Degree of red or green shows relative abundance of up-regulated vs down-regulated processes in each cluster. Grey clusters consist of 50 % up-regulated processes and 50 % down-regulated processes. Size of cluster represents the relative number of processes in the cluster. Interconnection between pathways is represented by grey interconnecting lines indicating that these categories share transcript(s) or protein(s). The statistical test was set to a right-sided hypergeometrical test with a Bonferroni (step down) *p-v*alue correction and a *kappa* score of 0.4. Response pathways for shared genes are representative of complete response pathways for individual chemicals, namely TCDD (Figure C6.S8), PCB 77 (Figure C6.S9), and BaP (Fig C6.S10).

of liver, immune system, gonad, heart, and sensory systems (Hahn, 2002). Therefore, potent activation of the AHR by agonists could result in dysregulation of a range of critical developmental and homeostatic processes in embryos, juveniles, and adults. One example is the wingless-type MMTV integrated site family (WNT) signaling pathway, which has been proposed as a critical target of the AHR (Lammi et al., 2004; Logan et al., 2004). WNTs are a large family of proteins that are highly conserved across taxa and play important roles in development and maintaining normal homeostasis by regulating cell proliferation through control of cell cycle regulators (Logan et al., 2004). Dysregulation of WNT signaling, and the interrelated Hedgehog (HH) signaling pathway, lead to a variety of adverse effects, including carcinogenesis and developmental deformities, making negative or positive feedback control of WNT signaling critical for proper functioning of an organism (Lammi et al., 2004; Logan et al., 2004). Expression of several transcripts in the WNT signaling pathway, including WNT5B and Indian hedgehog (IHH) A, was up-regulated at the level of the transcriptome by TCDD, PCB 77, and BaP (Figures 6.4; C6.S8; C6.S9; C6.S10). Pathway analysis indicated that dysregulation of the WNT signaling pathway might have affected several developmental processes, including those involved with the sensory and lateral line systems, neuromast development, tissue and fin regeneration and wound healing, development of the endocrine pancreas, and endocrine and cardiovascular systems, angiogenesis and development of blood vessels, somite development, and axis elongation, among others (Figures 6.4; C6.S8; C6.S9; C6.S10). Several other signaling pathways have also been suggested to be regulated by the AHR or altered through cross-talk, including the pregnane X receptor (PXR), endocrine, hypoxia, and peroxisome proliferation-activated receptor (PPAR) signaling pathways (King-Heiden et al., 2012; Mathew et al., 2009; Teraoka et al., 2006). Responses of the transcriptome in liver of white sturgeon exposed to

TCDD, PCB 77, and BaP included up-regulation of key nuclear receptors representative of several of these signaling pathways, including, AHR1 and AHR2, PPAR, androgen receptor (AR), and hypoxia inducible factor-1α (HIF-1α). However, nuclear receptors were not detected in the proteome, mainly due to their low abundance which could not be detected by the present proteomic analytical method. Taken together, this indicates possible dysregulation of several critical developmental and homeostatic processes that could lead to a range of adverse effects, including deformities, endocrine disruption, carcinogenesis, and impaired growth and reproduction.

Widespread changes in expressions of genes have been suggested to be a likely reason for pleiotropic apical adverse effects associated with activation of the AHR (Alexeyenko et al., 2010; Boutros et al., 2008; Li et al., 2013). However, it is also thought that most AHR-responsive genes play no direct role in mediating apical adverse effects and that only a core of species- and chemical-independent genes, which share common functions, is responsible for observed toxicities (Boutros et al., 2008). In the study presented here, 129 up-regulated and 57 down-regulated genes were observed at the level of the transcriptome (Figure 6.1) and 103 up-regulated and 29 down-regulated proteins were observed at the level of the proteome (Figure 6.2) that were common to all three chemicals. These transcripts and proteins were used to identify physiological processes regulated directly by activation of the AHR (Figure 6.4). Pathway analysis of these altered transcripts and proteins indicated that similar physiological processes were perturbed at both the level of the transcriptome (Figure 6.4A) and proteome (Figure 6.4B), including processes that were identified in other studies by use of transcriptome analysis (Alexeyenko et al., 2010; Brinkmann et al., 2016; Li et al., 2013). These processes included responses to xenobiotics, lipid and carbohydrate homeostasis, developmental and regenerative

processes, and processes involved with transcription and translation of genes. Further, the major physiological processes that were perturbed by all three chemicals at the level of both the transcriptome and proteome were similar to the processes that were perturbed when all transcripts or proteins altered by TCDD, PCB 77, or BaP at the level of the transcriptome or proteome were analyzed individually (Figures 6.4; C6.S8; C6.S9; C6.S10). These results support the hypothesis that the AHR mediates widespread perturbation through direct or indirect control over a large number of genes.

The zebrafish has been used as a model fish to successfully elucidate specific mechanisms for several of the classical adverse effects associated with exposure of early life stages of fishes to DLCs. Specifically, craniofacial malformations of zebrafish have been shown to be caused through dysregulation of genes involved in development of skeletal elements, namely downregulation of sonic hedgehog (SS) A and B and SOX9B and upregulation of forkhead box (FOX) Q1A (Andreasen et al., 2007; Planchart et al., 2010; Teraoka et al., 2006). Also, cardiovascular malformations in zebrafish have been shown to be caused through dysregulation of genes involved in development of the heart and vasculature, namely up-regulation of cytochrome c oxidase (COX) 2 and bone morphogenetic protein (BMP) 4 and down-regulation of the cell cycle gene cluster (Carney et al., 2006; Mehta et al., 2008; Teraoka et al., 2009). Less is known regarding specific mechanisms of adverse effects of DLCs on juvenile or adult fishes. However, hepatic steatosis is believed to result from AHR mediated up-regulation of fatty acid translocase (FAT) and PPARα (Mellor et al., 2016). SS was not identified in the transcriptome or proteome of white sturgeon, while COX and BMP had several isoforms that were unaltered by any of the three chemicals. However, in agreement with results of previous studies of zebrafish, SOX9 was down-regulated and FOX was up-regulated at the level

of the transcriptome by all three chemicals in livers of white sturgeon. Although PPARα was not altered, PPARδ was up-regulated at the level of the transcriptome by all three chemicals which is in agreement with previously demonstrated modes of toxic action for hepatic steatosis (Mellor et al., 2016). FAT was not identified in the transcriptome or proteome of white sturgeon; however, physiological processes involved in lipid homeostasis that are likely to be under control of PPAR and interrelated with FAT were altered at both the level of the transcriptome and the proteome. However, there is uncertainty in the accuracy of genome-free transcriptome or proteome sequencing to identify transcripts or proteins to the isoform level. Therefore, IHH, FOX, SOX, COX, BMP, and PPAR transcripts might be identified as the wrong isoform, which could alter interpretation of predicated perturbation to physiological processes or magnitude of response to exposure to chemicals. These uncertainties at the level of the exact transcript or protein highlight the advantage of investigating entire physiological pathways by use of whole transcriptome or proteome analysis as opposed to investigating individual genes. Also, individual genes affected by each DLC were not always identical; however, the same major processes were perturbed by equipotent concentrations of all three chemicals when entire physiological pathways were considered, which provides confidence in the whole pathway analysis approach.

BaP can also exert effects that are independent of the AHR (Neilson, 1998). Therefore, it was hypothesized that some responses to BaP at the level of the transcriptome and proteome would be unique from responses to TCDD and PCB 77. All, or most, of these non-AHR-mediated effects are believed to be caused by metabolites of BaP that are formed by reactions catalyzed by CYP enzymes (Shou et al., 1996). Most effects of BaP have been tested only in species such as mice (*Mus musculus*) and rainbow trout, which have greater rates of CYP-mediated biotransformation relative to other species (Liu et al., 2012; Nisbet and LaGoy, 1992).

However, the rate of CYP-mediated biotransformation in liver of white sturgeon has been shown to be slower than in other fishes. Previously, PAHs have been classified based on their potency for causing carcinogenesis, which is believed to be mediated largely through non-AHR-mediated pathways (Nisbet and LaGoy, 1992); however, carcinogenesis is not an end point of regulatory relevance in context with the risk assessment of fishes. In the current study, physiological processes that were perturbed by BaP did not differ from those of individuals exposed to TCDD or PCB 77, which suggests that activation of the AHR is likely to be the predominant driver of acute responses to exposure to BaP in white sturgeon. However, responses of the transcriptome and proteome might become less similar over time as reactive metabolites of BaP are formed and result in a greater non-AHR-mediated response. If BaP does elicit predominantly AHR-mediated effects on some fishes, it would support the development of toxic equivalency factors (TEFs) for certain PAHs, similar to those currently used for DLCs (Van den Berg et al., 1998). However, the weighted importance of AHR-mediated effects relative to non- AHR-mediated effects in eliciting acute or chronic apical adverse effects associated with exposure to PAHs remains unclear, especially among fishes (Billiard et al., 2002).

6.4.5 Toward Predicting Apical Adverse Effects in Fishes

Recently, there has been an increasing desire to make better use of growing quantities of mechanistic toxicology data (including both transcriptomics and proteomics) in support of chemical risk assessment and regulatory decision making (Villeneuve et al., 2014). One approach that has been proposed in this context and that is increasingly gaining acceptance across the scientific and regulatory communities is that of the adverse outcome pathway (AOP).

An AOP is a conceptual framework that establishes biologically plausible links between molecular-level perturbation of a biological system and an adverse outcome of regulatory relevance, such as survival, growth, or reproduction (Ankley et al., 2010). However, due to the complexity of AHR-mediated responses and how they are related to or are linked with adverse outcomes, no complete AOPs are currently available for activation of the AHR (https://aopkb.org/aopwiki/index.php/AOP_List). Toward the objective of describing more complete AOPs associated with activation of the AHR, the study presented here demonstrated three main contributions: 1) AOPs by definition are not chemical specific, and any chemical that triggers the molecular initiating event (i.e., activation of the AHR) should elicit the same chain of downstream key events given equal potency at the molecular initiating event and similar adsorption, distribution, metabolism, and elimination (ADME) (Villeneuve et al., 2014). It could be demonstrated that equal activation of the AHR by three different agonists (Figure C6.S2) resulted in similar global responses and magnitude of responses at both the level of the transcriptome (Figure 6.1) and the proteome (Figure 6.2) and would be predicted to result in similar adverse outcomes and severity of adverse outcomes (Figure 6.4). It was also demonstrated that where differences existed with regard to the exact transcripts or proteins that were altered, the actual physiological processes that were perturbed were indistinguishable (Figures 6.4; C6.S8; C6.S9; C6.S10). Therefore, future AOPs developed for activation of the AHR will likely be common to all DLCs and other agonists of the AHR at different levels of biological organization, given equal activation of the AHR and comparable ADME. 2) Key events and the linking key event relationships are not unique to a single AOP or to a single chemical or chemical class (Villeneuve et al., 2014). Therefore, common key events are reusable and do not need to be generated independently for each new AOP (Villeneuve et al., 2014).

Results of the study presented here demonstrated perturbed physiological processes and their associated pathways common to all three chemicals which are likely to represent core perturbations for activation of the AHR (Figure 6.4). Some of these pathways are well understood, including the WNT, PPAR, HH, ER, AR, and hypoxia pathways, supporting the development of putative AOPs associated with perturbation of these critical pathways as a result of activation of the AHR. However, the link between activation of the AHR and perturbation of these pathways remains an ongoing question. 3) AOPs are not static, and there is no benchmark for when an AOP is complete (Villeneuve et al., 2014). AOPs and the associated key events and key event relationships are able to grow over time as new information is generated. Therefore, in order for results of the study presented here to inform future investigation into AOPs associated with activation of the AHR, all data has been made available online for investigation into all transcripts and proteins altered by each of the three chemicals but also the associated physiological processes for each transcript or protein and the fold-change relative to abundances in the control. As such, this comprehensive transcriptomic and proteomic data set will be able to support as yet unrevealed or unexpected biological connections between molecular level perturbations in livers of fishes by DLCs and certain PAHs as a result of activation of the AHR.

CHAPTER 7

7 *IN VITRO* ACTIVATION OF AHR2, BUT NOT AHR1, IS PREDICTIVE OF *IN VIVO* SENSITIVITY TO 2,3,7,8-TCDD ACROSS PHYLOGENETICALLY DIVERSE SPECIES OF FISH

PREFACE

Chapter 6 demonstrated that equal activation of the AHR by agonists resulted in similar global responses and magnitude of responses at both the level of the transcriptome and level of the proteome and would be predicted to result in similar adverse effects and severity of adverse effects at the level of the whole organism. This supports the hypothesis that activation of the AHR is predictive of apical level adverse effects of regulatory relevance, such as mortality of embryos. Therefore, the aim of Chapter 7 was to develop a mechanism-based biological model that is capable of predicting the sensitivity of embryos of any species of sturgeon, or other species of fish, based on sensitivity to activation of the AHR in *in vitro* assays. Development of this predictive model has the potential to guide more objective ecological risk assessment of DLCs and potentially other agonists of the AHR for species of fish that are not easily studied but which represent major receptors for exposure at contaminated sites.

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Author contributions:

Jon A. Doering (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed all data, prepared figures, and drafted the manuscript.

Drs. Steve Wiseman and John P. Giesy (both University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript.

Dr. Markus Hecker (University of Saskatchewan) provided inspiration, scientific input, and guidance, commented on and edited the manuscript, and provided funding for the research.

7.1 Abstract

Adverse effects of exposure to dioxin-like compounds (DLCs) in vertebrates are primarily driven by activation of the aryl hydrocarbon receptor (AHR). However, mechanisms for the great differences in sensitivity to these effects among species of fish were unknown. Therefore, this study 1) investigated sensitivities to activation by the model DLC, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), of AHR1s and AHR2s among seven species of fish known to differ in sensitivity to TCDD by almost 40-fold, and 2) characterized the relationship between *in vitro* sensitivity to activation of AHRs to TCDD and *in vivo* sensitivity of embryos to TCDD. All AHR1s and AHR2s were activated in a concentration-dependent manner by exposure to TCDD. There was no significant linear relationship ($R^2 = 0.24$) between EC_{50} of AHR1 and LD_{50} of embryos. However, a highly significant positive linear relationship ($R^2 = 0.96$) was observed between EC_{50}s of AHR2s and LD_{50}s of embryos. The slope and *y*-intercept for this linear relationship for AHR2 of fishes is not statistically different from the slope and *y*-intercept for the previously determined significant linear relationship among EC_{50} of AHR1 and LD_{50} of embryos of birds to TCDD. Results of this study suggest that sensitivity to activation of AHR2, but not AHR1, mediates adverse effects of and sensitivity to TCDD among phylogenetically diverse species of fish with a comparable relationship as previously demonstrated for AHR1 of birds. This co-relationship resulted in a single equation for predicting sensitivity to TCDD across distantly related species of oviparous vertebrates from EC_{50}s of AHRs. This mechanism-based biological model has the potential to guide more objective ecological risk assessment of DLCs for species of fish that are not easily studied, including threatened or endangered species.

7.2 Introduction

Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and co-planar polychlorinated biphenyls (PCBs), are ubiquitous, persistent, and bioaccumulative pollutants of environmental concern globally. DLCs share similarities in structure and a total of seven PCDDs, ten PCDFs, and twelve PCBs are considered dioxin-like because they bind with relatively great affinity to the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor in the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of proteins and is believed to mediate most, if not all, critical adverse effects of exposure to DLCs in vertebrates (Okey, 2007). These adverse effects can include hepatotoxicity, suppression of immune, reproductive, and endocrine processes, teratogenicity, and carcinogenicity (Kawajiri and Fujii-Kuriyama, 2007). Exposure to other anthropogenic contaminants that bind to the AHR, including some other halogenated aromatic hydrocarbons (HAHs) (Van den Berg et al., 2006) and certain polycyclic aromatic hydrocarbons (PAHs), can also result in dioxin-like adverse effects in vertebrates (Billiard et al., 1999; 2002; Jayasundara et al., 2015). Although all DLCs exert toxicity via a single, specific, and highly conserved mechanism, differences in relative sensitivity greater than 1000-fold to the most studied DLC, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), exist both among and within vertebrate taxa (Cohen-Barnhouse et al., 2011; Doering et al., 2013; Hengstler et al., 1999; Korkalainen et al., 2001).

Differences in binding affinity and transactivation of the AHR have been implicated as a key mechanism contributing to differences in sensitivity to DLCs among vertebrates. However, linking differences in functionality of the AHR to differences in sensitivity of species to DLCs is

complicated by significant diversity in numbers and types of AHRs, both among and within vertebrate taxa that have occurred during the evolution of vertebrates as a result of whole-genome duplications and lineage-specific duplications and losses of genes (Hahn, 2002; Hahn et al., 2006). Birds and fishes express two forms of the AHR, AHR1 and AHR2, while the single AHR expressed by mammals is orthologous to the AHR1 (Hahn et al., 2006). Little is known regarding AHRs of amphibians or reptiles (Hahn, 2002). Despite this diversity in AHRs among vertebrates, differences in binding affinity of the AHR1 have been demonstrated to explain the more than 40-fold differences in sensitivity to TCDD and to other DLCs among birds (Farmahin et al., 2012; 2013; Karchner et al., 2006; Manning et al., 2012). Further, differences in binding affinities and transactivation of the AHR have been suggested to explain the more than 1000-fold difference in sensitivity among mammals (Ema et al., 1993; Wang et al., 2013), while low binding affinity of the AHR1 has been suggested as a mechanism for the tolerance of some amphibians to DLCs (Lavine et al., 2005; Shoots et al., 2015). However, the role that differences in binding affinity and transactivation of the AHR play in explaining the almost 200-fold difference in sensitivity to TCDD among species of fishes is currently unknown (Doering et al., 2013; Buckler et al., 2015).

Fishes represent a paraphyletic assemblage of species that comprises more than 50 % of all living vertebrates with more than 25,000 extant species (Nelson, 1994). Fishes represent among the most sensitive vertebrates to DLCs, with the lake trout (*Salvelinus namaycush*) being the most sensitive known fish with lethal doses that cause 50 % mortality (LD_{50}) to embryos of between 47 and 90 pg TCDD/g-egg (Guiney et al., 2000; Walker et al., 1991; 1992; 1994). Extirpation of lake trout from Lake Ontario in the 1960's has been largely attributed to mortality of sac fry as a result of exposure to mixtures of DLCs (Cook et al., 2003). However, elucidating

the specific mechanisms that explain differences in sensitivity among fishes has presented several challenges. There is less conservation in identities of amino acids in AHRs among fishes relative to AHRs among other vertebrates as a result of the paraphyletic classification, massive diversity of species, and nearly 500 million years of fish evolution (Doering et al., 2013; Gagnier et al., 1989). Further, salmonids and some other fishes have undergone subsequent genome duplication events, which have resulted in multiple isoforms of the AHR1 and AHR2, with each isoform displaying distinct species- and tissue-specific profiles of expression (Hahn, 2001; 2002; Le Comber et al., 2004). Up to six distinct AHRs have been identified in genomes of some fishes, presenting the challenge of identifying the relative roles of each AHR in contributing to dioxin-like adverse effects (Hansson et al., 2004). As a result of these challenges that are specific to fishes, much less is known about the AHRs of fishes relative to what is known about AHRs of birds and mammals. This is especially true with regard to differences in AHRs among species that might mediate the observed 200-fold difference in sensitivity to TCDD.

Therefore, the aim of this study was to begin to elucidate the role of the AHR in the observed differences in sensitivity among fishes through investigation into AHR1s and AHR2s among phylogenetically diverse species. Specifically, this study investigated sensitivity to activation by TCDD of a total of five AHR1s and ten AHR2s among seven species of fish of known sensitivity of embryos to TCDD. These seven species span an almost 40-fold difference in LD_{50} for TCDD, comprise five orders and six families, and include both teleost and non-teleost species (Table C7.S1). The species chosen for the current study were lake trout and brook trout (*Salvelinus fontinalis*) that are defined as being the most sensitive fishes to exposure to TCDD, fathead minnow (*Pimephales promelas*) and lake sturgeon (*Acipenser fulvescens*) that are defined as being moderately sensitive to exposure to TCDD, and Japanese medaka (*Oryzias*

latipes), white sucker (*Catostomus commersonii*), and northern pike (*Esox lucius*) that are defined as being the least sensitive to exposure to TCDD (Table C7.S1). The ultimate goal of this research was to provide evidence for the role of AHR1s and AHR2s in driving the observed differences in sensitivities to DLCs among fishes and to develop a mechanism-based biological model that enables the prediction of the sensitivity to TCDD of any species of fish. This model would guide more objective ecological risk assessment of native fishes to exposure to DLCs and other agonists of the AHR.

7.3 Materials and methods

7.3.1 Identification, sequencing, phylogeny, and expression of AHRs

Sequences of AHRs had not yet been identified for lake trout, brook trout, fathead minnow, white sucker, or northern pike. Sequences of AHRs already were known for lake sturgeon and Japanese medaka. AHRs were identified and isolated from samples of liver obtained from lake trout from Lac la Plonge (Saskatchewan, Canada); brook trout from Southwest River (Prince Edward Island, Canada); fathead minnow from a laboratory culture obtained from the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK); and white sucker and northern pike from Lake Diefenbaker (Saskatchewan, Canada). Known AHRs were isolated from samples of liver obtained from Japanese medaka from a laboratory culture obtained from the Aquatic Toxicology Research Facility at the University of Saskatchewan (Saskatoon, SK). Known AHRs had previously been isolated from samples of liver obtained from lake sturgeon from a laboratory culture raised from larvae from

Wild Rose State Fish Hatchery (Wild Rose, WI). Total RNA was extracted from approximately 30 mg of liver by use of the RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON). First-strand cDNA was synthesized from 1 μg total RNA by use of the QuantiTect Reverse Transcription Kit (Qiagen). Full-length cDNAs of AHRs from each species were amplified by use of primers designed from sequences obtained from a combination of degenerate primers, rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR), transcriptome sequencing (Beitel et al., 2015; Wiseman et al., 2013), and published sequences from the National Center for Biotechnology Information (NCBI) database. Gene-specific full-length primers were designed by use of Primer3 software (Table C7.S2) (Rozen and Skaletsky, 2000). Full-length products were amplified by use of the LongRange PCR Kit (Qiagen). Purified full-length products were cloned into pGEM-T easy vectors by use of a DNA ligation kit (Invitrogen) and transformed into competent JM109 *Escherichia coli* cells (Promega, Madison, WI). Plasmids were isolated by use of the QIAprep Spin Miniprep Kit (Qiagen) and sequenced by the University of Calgary's University Core DNA Services (Calgary, AB). A consensus nucleotide sequence was determined by aligning three or more replicated sequences and has been made publicly available in the NCBI database.

Basal abundances of transcripts of AHR1s and AHR2s of each species were quantified in livers from a total of six adult individuals (3 males and 3 females) for lake trout, brook trout, fathead minnow, Japanese medaka, and northern pike, or six juvenile individuals (sex unknown) for lake sturgeon. Abundances of transcripts were determined by use of quantitative real-time polymerase chain reaction (qRT-PCR) according to standard methods that have been described previously (Doering et al., 2014b; 2015a; 2016). Gene-specific qRT-PCR primers were designed by use of Primer3 software (Rozen and Skaletsky, 2000) or were published previously (Table

C7.S3) (Beitel et al., 2015; Doering et al., 2012; 2014b; 2015a; Wiseman et al., 2011). Abundances of transcripts were normalized to β-actin according to methods described previously (Simon, 2003).

7.3.2 Development of expression constructs for AHRs

Expression constructs for AHR1s and AHR2s were generated by use of the pENTR Directional TOPO entry vector kit (Invitrogen, Burlington, ON) and pcDNA 3.2/V5-DEST gateway vector kit (Invitrogen) according to methods described previously (Doering et al., 2014a; Farmahin et al., 2012). Gene-specific primers used to amplify full-length AHRs for ligation into expression vectors were designed according to the protocol provided by the manufacturer (Invitrogen) (Table C7.S4). Expression constructs for AHRs of lake sturgeon had previously been generated by use of the same methods (Doering et al., 2015b).

7.3.3 Luciferase reporter gene (LRG) assay

Culture of COS-7 cells, transfection of constructs, and the luciferase reporter gene (LRG) assay were performed in 96-well plates according to methods described previously (Farmahin et al., 2012), with minor modifications (Doering et al., 2014a). In order to strengthen direct comparison, each AHR was assessed by use of the same methods, equipment, dosing solutions, and personnel. Amounts of expression constructs that had previously been optimized for transfection of AHR1 and AHR2 of lake sturgeon also were used for transfection of AHRs of lake trout, brook trout, fathead minnow, Japanese medaka, white sucker, and northern pike in

order to strengthen direct comparison between studies (Doering et al., 2015b). Amounts of expression constructs transfected into cells were 8ng of AHR, 1.5 ng of white sturgeon ARNT2 (Doering et al., 2014a), 20 ng of rat CYP1A1 reporter construct (pGudLuc 6.1) (Han et al., 2004; Rushing and Denison, 2002), and 0.75 ng of *Renilla* luciferase vector (Promega). The total amount of DNA that was transfected into cells was kept constant at 50 ng by addition of salmon sperm DNA (Invitrogen). Transfected COS-7 cells were dosed with serial concentrations of TCDD ranging from 0.003 to 300 nM. TCDD (purity > 98 %) was acquired from Wellington Laboratories (Guelph, ON) and stock solutions were prepared in dimethyl sulfoxide (DMSO). Concentrations of TCDD were confirmed by high-resolution gas chromatography and mass spectrometry (GC-MS) according to Agilent methods 7890A and 5975C, respectively. Luciferase was measured by use of a POLARstar OPTIMA microplate reader (BMG Labtech, New Orleans, LA).

7.3.4 Concentration-response curves and statistical analysis

All concentration-response curves for each AHR were obtained from three independent experiments, each with a different passage of cells, and each with four technical replicates per concentration of TCDD. Response curves, effect concentrations (ECs), and linear regressions were developed by use of GraphPad Prism 6 software (San Diego, CA). Response curves were fit by use of a four-parameter logistic model. Due to differences in values among the three independent experiments of each AHR, the maximum response of each independent experiment was standardized to 100 for each dose-response curve. Comparison of slopes and *y*-intercepts for linear regression were performed by use of analysis of covariance (ANCOVA). Statistical

analyses were performed by use of IBM SPSS Statistics 20 software (Armonk, NY) or GraphPad Prism 6 software.

7.4 Results and discussion

7.4.1 Identifying AHRs of fishes

Neither nucleotide nor amino acid sequences of AHR1s and AHR2s were known for most species of fish of known sensitivity of embryos to TCDD. Therefore, AHRs were identified in lake trout, brook trout, fathead minnow, white sucker, and northern pike. A single AHR1 was identified in liver of fathead minnow and two AHR1s (AHR1a and AHR1b) were identified in liver of northern pike (Table C7.S1). A fragment of AHR1 was known from transcriptome sequencing of liver and gonad from white sucker; however, AHR1 was not found to be expressed in livers of the white sucker investigated here (Beitel et al., 2015). No AHR1s could be identified in livers of lake trout or brook trout. Two AHR1s (AHR1a and AHR1b) are known from the genome of Japanese medaka; however, only AHR1b was found to be expressed in livers of the Japanese medaka investigated here (Table C7.S1). A single AHR1 of lake sturgeon had been identified previously (Doering et al., 2015b). Putative amino acid sequences of AHR1s for fathead minnow, Japanese medaka, and northern pike clustered closely with sequences of AHR1s of other species of fish (Figure C7.S1). However, the putative amino acid sequence for AHR1 of lake sturgeon clustered closest with sequences of AHR1s of tetrapods (Figure C7.S1). Genome sequencing of these species might identify additional AHR1s that were not identified here.

Two AHR2s (AHR2a and AHR2b) each were isolated from livers of lake trout, brook trout, and northern pike, while a single AHR2 was isolated each from liver of fathead minnow and white sucker (Table C7.S1). Two AHR2s (AHR2a and AHR2b) are known from the genome of Japanese medaka; however, only AHR2b was found to be expressed in livers of the Japanese medaka investigated here (Table C7.S1). A single AHR2 in lake sturgeon had been identified previously (Doering et al., 2015b). Putative amino acid sequences for AHR2s of lake trout, brook trout, fathead minnow, lake sturgeon, Japanese medaka, white sucker, and northern pike clustered closely with sequences of AHR2s of other species of fish (Figure C7.S1). Putative amino acid sequences for AHR2a of lake trout, brook trout, and northern pike clustered most closely with sequences for AHR2α and AHR2β of salmonids, while sequences for AHR2b of lake trout, brook trout, and northern pike clustered most closely with sequences for AHR2δ and AHR2γ of salmonids (Figure C7.S1). However, genome sequencing of these species might identify additional AHR2s that were not identified here.

7.4.2 Roles of AHR1 and AHR2 in eliciting dioxin-like effects across fishes

An almost 200-fold difference in sensitivities to TCDD with regard to LD_{50} of embryos has been observed among the seventeen species of fish investigated to date. However, the mechanism(s) for these differences remained unknown. Previous authors have suggested that differences in sensitivities among embryos of different species of fish might be due to differences in levels of expression of AHRs (Karchner et al., 2000) or differences in developmental times of embryos (Elonen et al., 1998). There are limited numbers of antibodies against different AHRs that are available for different species of fish making assessments of

basal levels of protein abundance for AHRs among phylogenetically diverse fishes impractical. Further, no significant relationship ($R^2 = 0.17$; $p = 0.11$) was observed between developmental times measured as degree days to hatch and sensitivities of embryos to TCDD across the seventeen fishes of known sensitivity (Figure C7.S8). Therefore, the study presented here investigated whether differences in sensitivity to TCDD among fishes is primarily mediated by differences in activation of the AHR, as has previously been demonstrated for birds (Farmahin et al., 2012; 2013; Karchner et al., 2006; Manning et al., 2012) and suggested for mammals and amphibians (Ema et al., 1993; Hengstler et al., 1999; Korkalainen et al., 2001; Lavine et al., 2005; Shoots et al., 2015; Wang et al., 2013) as opposed to being mediated by differences in levels of expression of AHRs or development times of embryos.

In contrast to tetrapods, in fishes the AHR2 is believed to be the primary mediator of dioxin-like adverse effects, while AHR1 is believed to have little or no role in mediating toxicity (Doering et al., 2013). This hypothesis is largely based on results of studies investigating AHRs of two model fishes, zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*). These and other studies identified several characteristic differences between AHR1 and AHR2 of fishes: 1) In all fishes studied to date, expression of AHR2 is ubiquitous among tissues, while expression of AHR1 is primarily localized in brain, liver, heart, and gonad (Andreasen et al., 2002; Doering et al., 2014b; Karchner et al., 1999; Yamauchi et al., 2005). This suggests a more specialized role of AHR1 relative to AHR2. 2) Expression of AHR2 is up-regulated by exposure to TCDD, while expression of AHR1 is unchanged or up-regulated by a smaller magnitude (Andreasen et al., 2002; Doering et al., 2012; 2014b; Karchner et al., 2005; Tanguay et al., 1999; Yamauchi et al., 2006). 3) Binding affinity for DLCs and sensitivity to activation are greater for AHR2 relative to AHR1 in both zebrafish and mummichog, with AHR1a of zebrafish not binding TCDD

(Andreasen et al., 2002; Fraccalvieri et al., 2013; Karchner et al., 1999; 2005). 4) Knockdown of expression of AHR2 prevents toxicity of DLCs and dioxin-like PAHs in zebrafish and mummichog, while knockdown of expression of AHR1 does not alter toxicity (Clark et al., 2010; Incardona et al., 2006; Prasch et al., 2003; Scott et al., 2011; Teraoka et al., 2003; 2010; Van Tiem et al., 2011). Despite this strong evidence for AHR2 driving dioxin-like adverse effects in zebrafish and mummichog, greater sensitivity to activation by DLCs of AHR1 relative to AHR2 in red seabream (*Pagrus major*) and sturgeons (*Acipenser* spp.) suggest a possible role of AHR1 in mediating adverse effects of DLCs in some species of fish (Bak et al., 2013; Doering et al., 2014a; 2015b). This raises the question as to whether studies on a limited number of model species (i.e. zebrafish and mummichog) are representative of the phylogenetic diversity of fishes with regard to functionality of AHR1s and AHR2s and their respective roles in mediating dioxin-like adverse effects. Results of the study presented here support the hypothesis that AHR2 mediates dioxin-like adverse effects among fishes, while AHR1 has little or no role in mediating adverse effects. Basal expression of AHR2 is 6- to 9-fold greater relative to AHR1 in livers of adult fathead minnow, Japanese medaka, and juvenile lake sturgeon, but not in livers of northern pike where basal expression of AHR1a is 20- and 25-fold greater relative to AHR2a and AHR2b, respectively (Figure C7.S2 to C7.S5). However, caution must be exercised when interpreting these results because abundances of transcripts of AHRs might not accurately reflect abundances of proteins of AHRs due to post-translational effects and relative abundances of transcripts in livers of adults or juveniles might not reflect relative abundances of transcripts in embryos (Juschke et al., 2013).

All five AHR1s that were investigated here were activated in a concentration-dependent manner by exposure to TCDD (Figure 7.1). In contrast to AHR1s of zebrafish and mummichog,

AHR1s of fathead minnow, lake sturgeon, Japanese medaka, and northern pike all had greater sensitivities to TCDD relative to AHR2s (Figure 7.1; Figure 7.2; Table C7.S5). This might suggest that a toxicological role of AHR1 of fishes is more widespread than previously recognized. However, there was no linear relationship ($R^2 = 0.24$; $p = 0.41$) between EC_{50} of AHR1 and LD_{50} of embryos among these fishes (Figure 7.3B). It has been suggested that the pleiotropic functions of the AHR in mammals have been partitioned between the AHR1 and AHR2 in fishes or that the multiple AHRs in fishes might have evolved novel physiological functions not present in mammals (Karchner et al., 2005). Therefore, activation of AHR1 by DLCs might result in certain subtle, sub-lethal, or tissue-specific adverse effects in embryos of fishes that do not correlate with LD_{50}s. Alternatively, AHR1s might function with great sensitivity *in vitro* but their response *in vivo* is too little to result in adverse effects. In common with AHR1s, all ten AHR2s that were investigated here were activated in a concentration-dependent manner by exposure to TCDD (Figure 7.2). However, in contrast to AHR1s, a highly significant, positive linear relationship ($R^2 = 0.96$; $p = < 0.0001$) was observed between EC_{50}s of AHR2s and LD_{50}s of embryos among the seven species (Figure 7.3C). This suggests a prominent role of AHR2 in mediating both adverse effects of and sensitivities to TCDD among phylogenetically diverse species of fish.

Despite the strong relationship between EC_{50}s of AHR2s and LD_{50}s of embryos among the fishes investigated here, several uncertainties remain, particularly regarding expression and identification of multiple isoforms of the AHR. It has been proposed that the great sensitivity of salmonids to DLCs might be related to the fact that they express up to four AHR2s, each of which is activated by DLCs (Hansson and Hahn, 2008). Results of studies with rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) suggest AHR2α is the primary

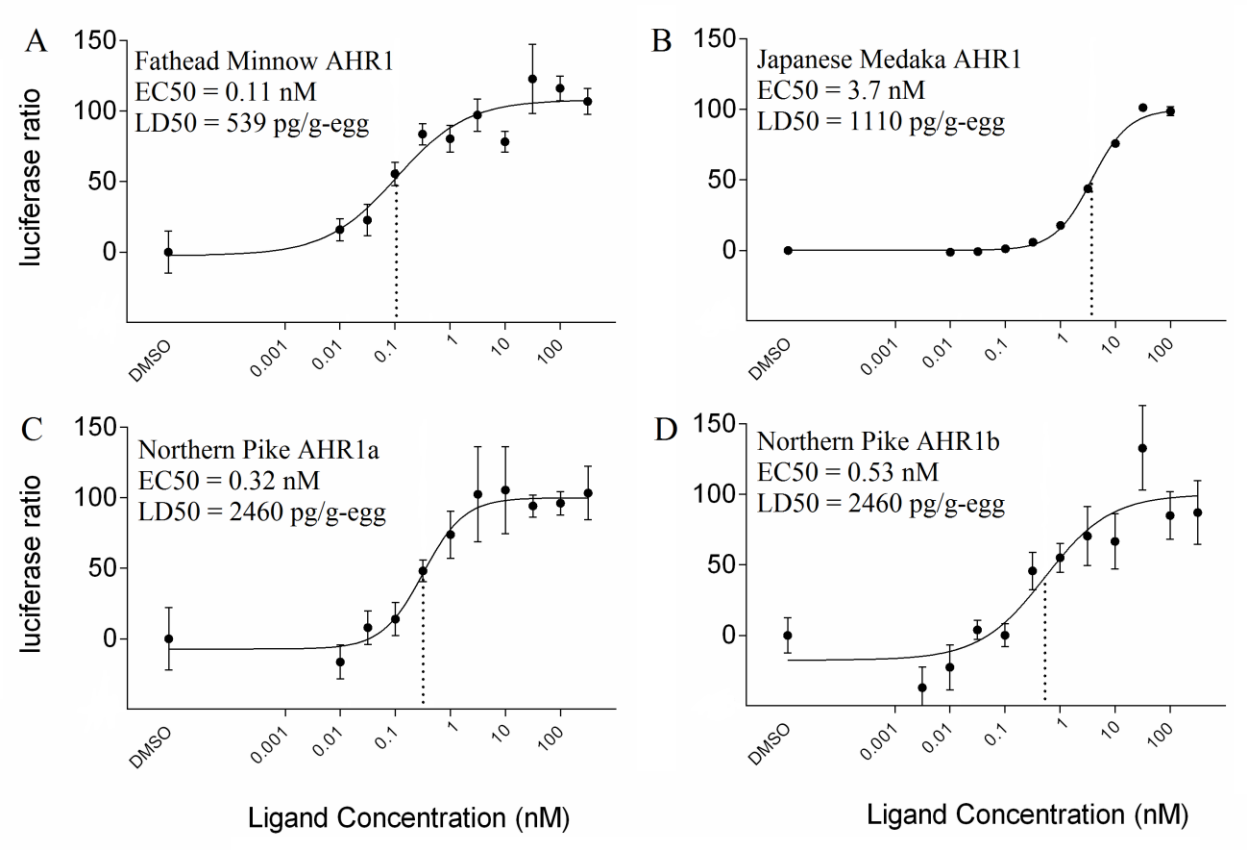

Figure 7.1. Dose-response curves for activation of AHR1 of fathead minnow (A) and Japanese medaka (B), and AHR1a (C) and AHR1b (D) of Northern pike by TCDD. The EC_{50} is represented by a dotted line. DMSO control is standardized to 0 and maximum response is standardized to 100. The dose-response curve for activation of AHR1 of lake sturgeon by TCDD has been published previously with an EC_{50} of 0.043 nM and is not shown again here (Doering et al., 2015b).

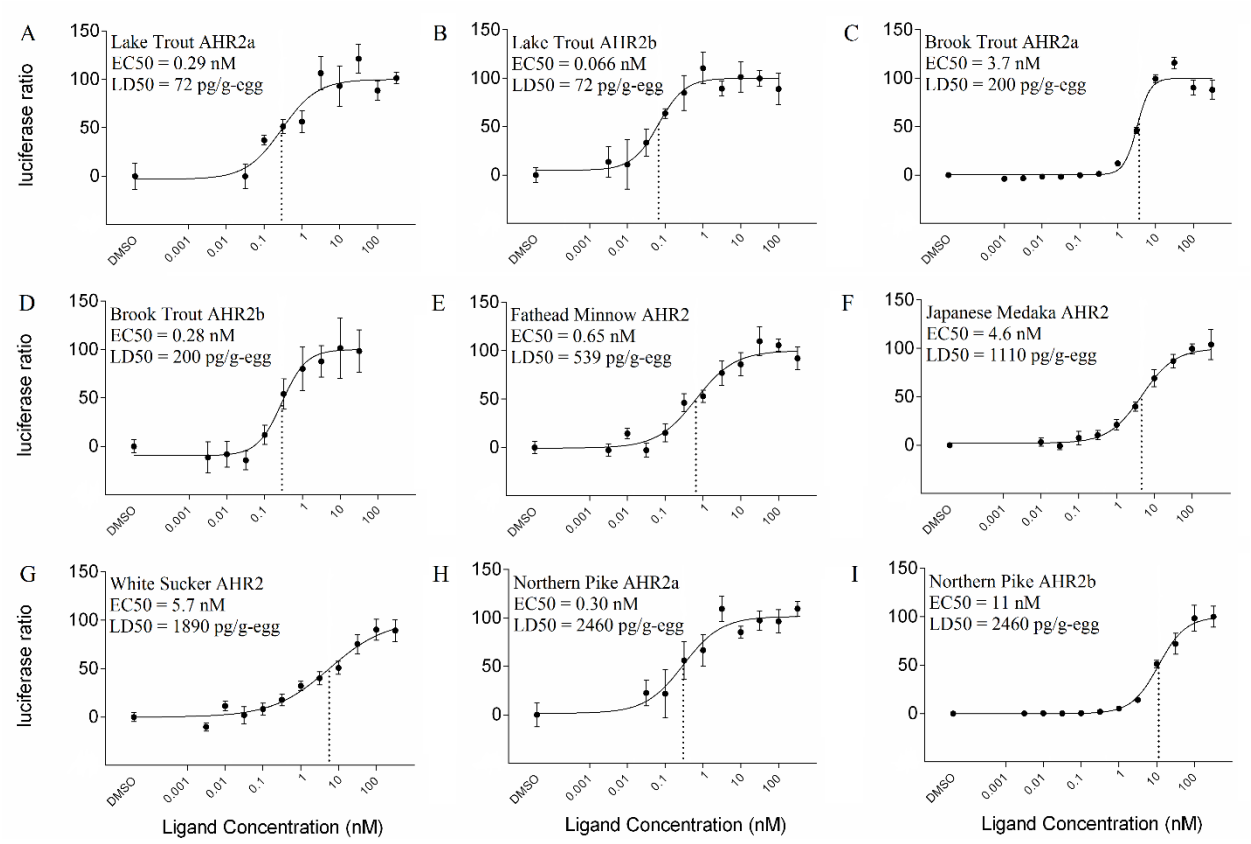

Figure 7.2. Dose-response curves for activation of lake trout AHR2a (A) and AHR2b (B), brook trout AHR2a (C) and AHR2b (D), fathead minnow AHR2 (E), Japanese medaka AHR2 (F), white sucker AHR2 (G), and northern pike AHR2a (H) and AHR2b (I) by TCDD. The EC_{50} is represented by a dotted line. Maximum response is standardized to 100. The dose-response curve for activation of AHR2 of lake sturgeon by TCDD has been published previously with an EC_{50} of 0.79 nM and is not shown again here (Doering et al., 2015b).

mediator of sensitivity to DLCs in these species based on its greater basal expression and greater sensitivity to activation by DLCs (Abnet et al., 1999; Hansson and Hahn, 2008). AHR2αs were not identified in lake trout, brook trout, or northern pike because AHR2a has the greatest similarity to AHR2β, while AHR2b has the greatest similarity to AHR2δ (Table C7.S6), while relative expression between isoforms is inconsistent among these species (Figure C7.S5 to Figure C7.S7). The number of AHRs expressed by lake trout and brook trout is not known, while genome (Assembly Accession #GCF_000721915) and transcriptome sequencing (Beitel et al., 2015) of northern pike has only identified the four AHRs investigated here. Despite this uncertainty, the EC_{50} of AHR2a of lake trout, brook trout, and northern pike did not fit the relationship between EC_{50} and LD_{50} (Figure 7.3C), while the EC_{50} of AHR2b of these species fit the relationship with AHR2s of the other four species. This is suggestive that AHR2b (greatest similarity to AHR2δ) mediates sensitivity to TCDD in these species or that multiple isoforms of the AHR2 of some salmonids have EC_{50}s that fit the relationship between EC_{50} and LD_{50} that might together mediate sensitivity to TCDD. However, identification of additional AHRs through genome sequencing of lake trout and brook trout in particular, and knockdown studies of AHRs in more species of fish is necessary in order to fully elucidate whether AHRs of fishes contribute simultaneously, additively, or individually to adverse effects of and sensitivity to TCDD.

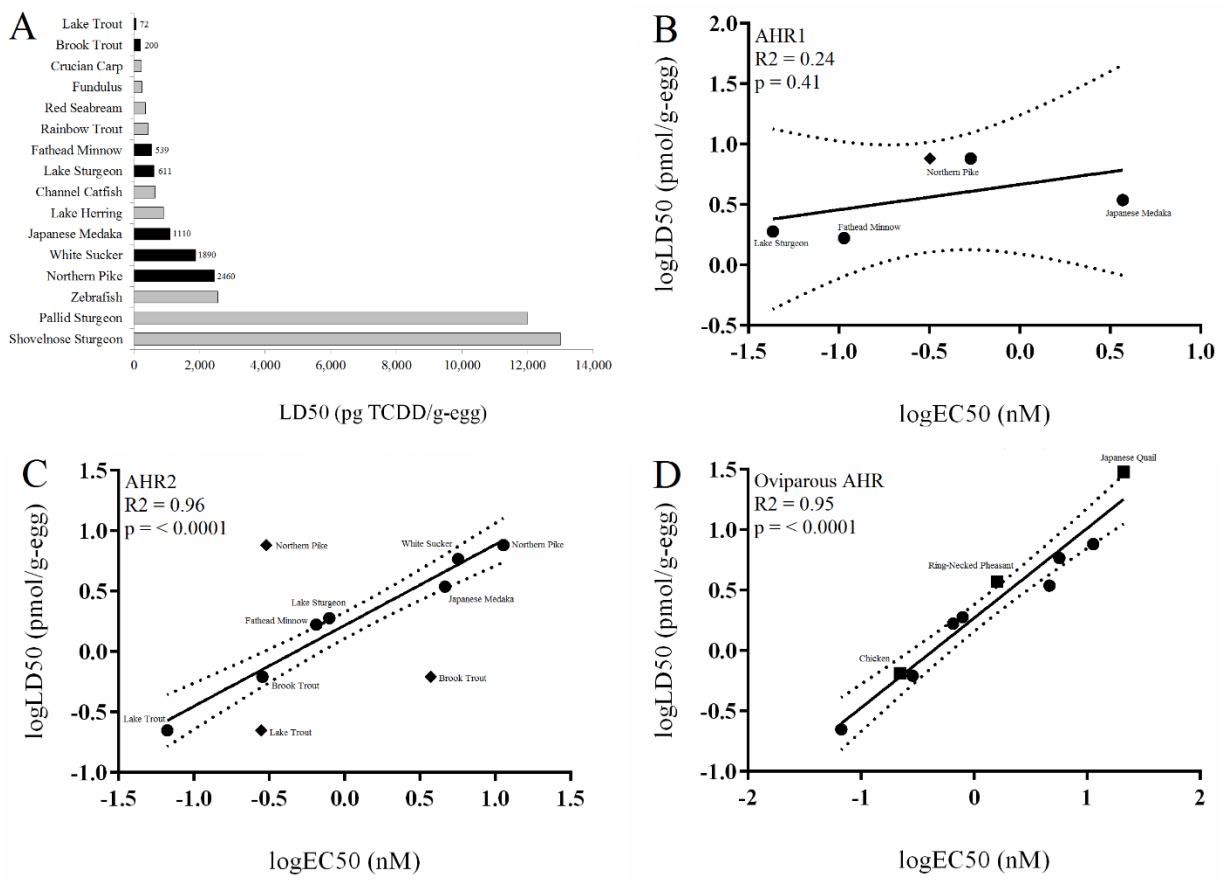

Figure 7.3. Distribution of sensitivities of embryos of different species of fish to TCDD based on lethal dose to cause 50% mortality (LD_{50}) (A) (Buckler et al., 2015; Elonen et al., 1998; Guiney et al., 2000; Henry et al., 1997; Park et al., 2014; Tillitt et al., 2016; Toomey et al., 2001; Walker et al., 1991; 1992; 1994; 1994; Yamauchi et al., 2006). The seven species used in the study presented here are highlighted in black and list the LD_{50} (pg TCDD/g-egg). Linear regressions for sensitivity to activation (EC_{50}) of AHR1s (B) and AHR2s (C) against sensitivity to TCDD of embryos (LD_{50}) across fishes. AHR1a and AHR2a (diamond) are differentiated from AHR1b, AHR2b, and AHR2 (circle). AHR2a are not included in the regression. The equation of the line for AHR1 is Y = 0.2094*X+0.6676 (Equation 7.1) with a slope of 0.21 ± 0.2 and *y*-intercept of 0.67 ± 0.2. The equation for the line for AHR2 is Y = 0.6693*X+0.2185 (Equation 7.2) with a slope of 0.67 ± 0.06 and *y*-intercept of 0.22 ± 0.04. Linear regression across oviparous vertebrates for sensitivity to activation (EC_{50}) of AHR2s of fishes (circles) and AHR1s of birds (squares) against sensitivity to TCDD of embryos (LD_{50}) (D). The equation of the line for AHRs of oviparous vertebrates is Y = 0.7429*X+0.2715 (Equation 7.3) with a slope of 0.74 ± 0.06 and *y*-intercept of 0.27 ± 0.05. Best fit lines for regressions are indicated and 95 % confidence intervals are represented as dotted lines.

7.4.3 Predicting sensitivities of fishes and other oviparous vertebrates to dioxins

Accurate ecological risk assessment is presented with several challenges, including the need to assess potential adverse effects on a wide range of species that exhibit different sensitivities to pollutants. One approach that builds on conservation of physiological responses to exposure to contaminants between species and is quickly gaining acceptance across the scientific and regulatory communities is that of the adverse outcome pathway (AOP). AOPs organize the growing quantity of data on mechanisms of toxic action into a conceptual framework that establishes biologically plausible links between perturbation at the molecular-level of a biological system and an adverse outcome of regulatory relevance, such as survival, growth, or reproduction (Ankley et al., 2010). Currently, no complete AOPs are available for activation of the AHR due to the complex pleiotropic responses mediated by activation of the AHR and the wide range of resulting adverse effects (Doering et al., 2016). In the study presented here, the complex series of molecular, biochemical, and histological events following activation of the AHR were bypassed by linking the molecular initiating event (i.e. activation of AHR2) to an adverse outcome (i.e. survival). The strong relationship ($R = 0.96$; $p = < 0.0001$) between sensitivity to activation of AHR2 (EC_{50}) and sensitivity of embryos (LD_{50}) produces a quantitative, putative AOP linking differences in the molecular initiating event among phylogenetically diverse species of fish to differences in sensitivity for an endpoint of regulatory relevance (Figure 7.3C). Therefore, this relationship has been developed into an equation (Equation 1) and can theoretically be used to predict the LD_{50} of embryos to TCDD for any species of fish based on *in vitro* derived EC_{50}s. EC_{50}s for activation of AHR2 by TCDD and LD_{50}s of embryos to TCDD are known in the literature for three species of fishes: red seabream,

rainbow trout, and zebrafish (Abnet et al., 1999; Bak et al., 2013; Doering et al., 2013). The relationship developed here for EC_{50} of AHR2 was able to predict LD_{50}s of these three species to within 2-fold of the measured LD_{50} (Table 7.1) with much of this error likely being a result of differences in methods that were applied by these studies and which have been shown to result in inter-laboratory differences in ECs for activation of the AHR (Farmahin et al., 2012). Despite the possible uncertainty in comparability of ECs across studies, the strong predictive capability of this relationship could still significantly improve ecological risk assessment of DLCs and other agonists of the AHR by allowing for effective site-specific and species-specific assessments, while reducing need for costly and often challenging *in vivo* toxicity testing approaches.

The above discussed predictive relationship could be applied to threatened or endangered species which cannot be subjected to traditional *in vivo* toxicity testing approaches for ethical or practical reasons, yet for which information on sensitivities to pollutants is of great interest. This is important since 137 and 29 species of fish in the United States and Canada, respectively, are currently listed as endangered or threatened (Species at Risk Public Registry; U.S. Fish and Wildlife Service). Two such species are the Atlantic salmon and white sturgeon, both of which have endangered populations in the United States and Canada (Species at Risk Public Registry; U.S. Fish and Wildlife Service). Lethality data is unavailable regarding the sensitivity of embryos of Atlantic salmon or white sturgeon (*Acipenser transmontanus*) to DLCs. However, the EC_{50}s for activation of AHR2 by TCDD have previously been determined for both species (Doering et al., 2014a; Hansson and Hahn, 2008). Therefore, the linear relationship among fishes (Equation 7.2) was used to predict the sensitivities of Atlantic salmon and white sturgeon to TCDD. EC_{50}s of AHR2α and AHR2δ of Atlantic salmon predicts LD_{50}s of 116 and 208 pg TCDD/g-egg, respectively (Table 7.1). It is predicted that Atlantic salmon, and potentially other

Salmo spp. of salmonids, are among the most sensitive species of fishes to TCDD with regard to LD_{50} of embryos, with sensitivity comparable to *Salvelinus* spp. such as lake trout and brook trout (Figure 7.3A). The EC_{50} of AHR2 of white sturgeon predicts an LD_{50} of 91 pg TCDD/g-egg (Table 1). It is predicted that white sturgeons are among the most sensitive species of fishes to TCDD with regard to LD_{50} of embryos and suggests significant diversity in sensitivity to TCDD among the Acipenseridae (Figure 7.3A) (Buckler et al., 2015; Doering et al., 2015b; Eisner et al., 2016; Tillitt et al., 2016). Predicted sensitivities to TCDD of these and other endangered species of fish based on *in vitro* data could guide more objective risk assessments of these important species and represents an important application of this mechanism-based biological model.

Comparable relationships between *in vitro* and *in vivo* endpoints that allow prediction of sensitivities to DLCs (LD_{50}) of any species based on sensitivity to activation of AHR1 (EC_{50}) already exist for birds (Farmahin et al., 2012; 2013; Karchner et al., 2006; Manning et al., 2012). However, no relationship was available for other oviparous vertebrates, such as fishes, amphibians, or reptiles. The slope and *y*-intercept for the linear relationship for AHR2 of fishes developed here (Figure 7.3C; slope = 0.67; *y*-intercept = 0.22) are not statistically different (p = 0.11 and p = 0.052, respectively) from the slope and *y*-intercept for the linear relationship between EC_{50} of AHR1 and LD_{50} of embryos to TCDD among species of birds (Figure C7.S9; slope = 0.84; *y*-intercept = 0.38). The well-studied relationship between sensitivity to activation of AHR1 and embryo-lethality of birds being mirrored so closely by the same relationship for AHR2 of fishes provides strong support for the hypothesis that AHR2 mediates adverse effects of and sensitivities to TCDD among fishes in a fashion comparable to that of AHR1 mediating adverse effects of and sensitivities to TCDD among species of birds. Further, it supports the

hypothesis for the plausibility of a single predictive relationship between EC_{50} of the AHR and LD_{50} of embryos across oviparous vertebrates and spanning distantly related fishes, amphibians, reptiles, and avian taxa. Therefore, AHR1s of birds were combined with AHR2s of fishes to produce a single relationship for AHR across oviparous vertebrates (Figure 7.3D). This resulted in a strong relationship (R^2= 0.95; p = < 0.0001) across these two distantly related vertebrate taxa (Figure 3D) and was developed into an equation (Equation 2). No LD_{50}s in units of TCDD/g-egg of either amphibians or reptiles are available; however, amphibians studied to date are known to be very tolerant to exposure with DLCs (Beatty et al., 1976; Jung and Walker, 1997). Although AHRs of amphibians and reptiles are poorly understood, AHR1 is believed to be the primary mediator of dioxin-like adverse effects in these taxa (Shoots et al., 2015; Oka et al., 2016). EC_{50}s for TCDD of AHR1 of two species of amphibians, the African clawed frog (*Xenopus laevis*) and Mexican axolotl (*Ambystoma mexicanum*), are comparable to those of AHR1 of the insensitive Japanese quail (*Coturnix japonica*) (Farmahin et al., 2012; Shoots et al., 2015). This suggests that sensitivity of amphibians might be predicted by sensitivity to activation of AHR1. Therefore, the linear relationship for AHRs among oviparous vertebrates (Equation 7.3) was used with the EC_{50}s for AHR1s of African clawed frog and Mexican axolotl to predict LD_{50}s of 7 070 and 6 276 pg TCDD/g-egg, respectively (Table 7.1). This suggests that among classes of oviparous vertebrates that have been studied to date, the African clawed frog and Mexican axolotl are among the least sensitive to TCDD with regard to LD_{50} of embryos.

Validating this predictive relationship across several species of amphibians, reptiles, and a greater phylogenetic diversity of fishes could yield a strong mechanism-based biological model for predicting sensitivities to TCDD of any species of oviparous vertebrate. This relationship likely could also prove to be valid across DLCs, including PCDDs, PCDFs, and PCBs, which has

Table 7.1. Predicted versus measured LD_{50}s of TCDD to embryos of fishes (A) determined by use of Equation 7.2 (Figure 7.3C) or amphibians (B) determined by use of Equation 7.3 (Figure 7.3D) based on EC_{50} for activation of AHR2 or AHR1, respectively.

A Fishes	Species Name	Isoform	EC_{50} (nM)	LD_{50} (pg/g-egg)	Predicted LD_{50} (pg/g-egg)	Accuracy (Fold-difference)
Atlantic Salmon	*Salmo salar*	AHR2α	0.10[a]	NA[c]	116	NA[c]
Atlantic Salmon	*Salmo salar*	AHR2δ	0.24[a]	NA[c]	208	NA[c]
White Sturgeon	*Acipenser transmontanus*	AHR2	0.070[b]	NA[c]	91	NA[c]
Red Seabream	*Pagrus major*	AHR2	0.51[d]	360[e]	345	1.04
Rainbow Trout	*Oncorhynchus mykiss*	AHR2α	2.0[f]	439[g]	860	1.96
Zebrafish	*Danio rerio*	AHR2	4.9[f]	2555[h,i]	1,567	1.63
B Oviparous Vertebrates		Isoform	EC_{50} (nM)	LD_{50} (pg/g-egg)	Predicted LD_{50} (pg/g-egg)	Accuracy (Fold-difference)
African Clawed Frog	*Xenopus Laevis*	AHR1	27[j]	NA[c]	7,070	NA[c]
Mexican Axolotl	*Ambystoma mexicanum*	AHR1	23[j]	NA[c]	6,276	NA[c]

[a] Adapted from previously published results (Hansson and Hahn, 2008)

[b] Adapted from previously published results (Doering et al., 2014a).

[c] Not available.

[d] Adapted from previously published results (Bak et al., 2013).

[e] Adapted from previously published results (Yamauchi et al., 2006).

[f] Adapted from previously published results (Abnet et al., 1999).

[g] Adapted from previously published results (Walker et al., 1991)

[h] Adapted from previously published results (Elonen et al., 1998).

[i] Adapted from previously published results (Henry et al., 1997).

[j] Adapted from previously published results (Shoots et al., 2015).

already been demonstrated among birds (Farmahin et al., 2013; Manning et al., 2012). This could lead to relationships for predicting sensitivities across oviparous vertebrates for other agonists of the AHR, including dioxin-like PAHs and certain emerging contaminants that act as AHR agonists, both of which are of ongoing concern to risk assessors. Further, substitutions of key amino acid residues in the ligand binding domain (LBD) of the AHR have been demonstrated to explain differences in sensitivity to activation by TCDD among AHRs of birds and some mammals, amphibians, and fishes (Andreasen et al., 2002; Bisson et al., 2009; Doering et al., 2015b; Farmahin et al., 2012; 2013; Fraccalvieri et al., 2013; Head et al., 2008; Karchner et al., 2006; Manning et al., 2012; Shoots et al., 2015). However, structural elements of the AHR2 that confer differences in sensitivity to activation by TCDD among phylogenetically diverse species of fish are unknown. Future identification of key structural elements within the AHR1 or AHR2 that determine sensitivities to activation and likely also determine sensitivities *in vivo* across phylogenetically diverse species of fish or other oviparous vertebrates would revolutionize how DLCs and other agonists of the AHR are assessed and would result in a powerful model that is accurate across laboratories, methodologies, and vertebrate taxa.

CHAPTER 8

8 GENERAL DISCUSSION

8.1 Introduction

Anthropogenic pollutants, including dioxin-like compounds (DLCs), have been implicated as one probable factor contributing to declines in populations of several species of sturgeons throughout North America, Europe, and Asia (Bergman et al., 2008; Dadswell, 2006; Hildebrand and Parsley, 2015; Hensel and Holcik, 1997; Hu et al., 2009; Khodorevskaya et al., 1997; Lenhardt et al., 2006; MacDonald et al., 1997). However, despite growing interest in the conservation of sturgeons (Birstein, 1993), few toxicity studies have been conducted with sturgeons as a result of practical and ethical concerns regarding research with endangered species that are not easy to acquire or maintain in the laboratory. Therefore, the specific goal of this thesis was to develop a mechanism-based biological model that allows the prediction of the relative sensitivity of any species of sturgeon to adverse effects of exposure to one class of anthropogenic pollutants, DLCs, through *in vitro* approaches. Further, development of a mechanism-based biological model that can be conducted by use of non-lethal samples would allow investigation into sensitivities to DLCs of even critically endangered species of sturgeon.

Sturgeons are long-lived, spawn intermittently, live in close association with sediments, and have greater lipid content than some other fishes (Hochleithner and Gessner, 2001). These attributes could give sturgeons a greater potential of exposure to and bioaccumulation of DLCs and other lipophilic chemicals. This exposure has been confirmed by detection of significant concentrations of DLCs and other chemicals in tissues of sturgeons in several studies (Kruse and Webb, 2006; MacDonal et al., 1997; Mierzykowski, 2010). Despite the possible risks to sturgeons as a result of exposure to DLCs, at the initiation of this research in May of 2010, only a few studies were published regarding responses of sturgeons following exposure to DLCs or

other agonists of the aryl hydrocarbon receptor (AHR) (Agradi et al., 1999; Foster et al., 2001; Palace et al., 1996; Palumbo et al., 2009; Rousseaux et al., 1995), while no studies were published regarding sensitivity of sturgeons to DLCs or other agonists of the AHR. Since 2010, several studies have been published that have investigated sturgeons with regards to responses and sensitivity to exposure to DLCs or other agonists of the AHR, studies included within this thesis (Doering et al., 2012; 2014a; 2014b; 2015b; 2016) and those from other researchers (Buckler et al., 2015; Chambers et al., 2012; Eisner et al., 2016; Roy et al., 2011; Tillitt et al., 2016). Published studies involved several species of sturgeons from North America, namely shortnose sturgeon (*Acipenser brevirostrum*), lake sturgeon (*Acipenser fulvescens*), Atlantic sturgeon (*Acipenser oxyrinchus*), white sturgeon (*Acipenser transmontanus*), pallid sturgeon (*Scaphirhynchus albus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*). These studies have all contributed toward the overall goal of developing a mechanism-based biological model that allows the prediction of the sensitivity of any species of sturgeon to DLCs. These studies demonstrate that sturgeons, despite being ancient species of fish, respond to exposure to activation of the AHR in a manner that is generally consistent with that of more modern and better characterized teleost fishes. Specifically, sturgeons share responses of teleost fishes, such as induction in Phase I biotransformation enzymes (Doering et al., 2012; Eisner et al., 2016; Roy et al., 2011) and other known AHR-mediated genes (Doering et al., 2016) and manifest adverse effects observed in teleost fishes, including pericardial and yolk sac edema, deformities of the head and eyes, reduced growth, and embryo-mortality (Buckler et al., 2013; Carney et al 2006; Chambers et al., 2012; Tillitt et al., 2016). This conservation in general response and effect to exposure to DLCs also appears to span sharks and rays (Hahn et al., 1998), amphibians (Shoots et al., 2015), and birds (Cohen-Barnhouse et al., 2011; Karchner et al., 2006). This provides

support for the use of teleost fishes in developing a mechanism-based biological model for predicting the relative sensitivity of all sturgeons, and possibly all oviparous vertebrates, to DLCs.

8.2 Predicting the relative sensitivity of sturgeons to aryl hydrocarbon receptor agonists

Results of Chapter 7 demonstrated that sensitivity (dose to cause 50 % mortality; LD_{50}) of embryos of fishes to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could be predicted to within 2-fold by use of the generated linear relationship (Figure 7.3D; Table 7.1) from the sensitivity to activation (concentration to cause 50 % effect; EC_{50}) of the AHR2. Sensitivity to activation of AHR1s and AHR2s of two sturgeons were investigated here, white sturgeon in Chapter 4 (Doering et al., 2014a) and lake sturgeon in Chapter 5 (Doering et al., 2015b). It was demonstrated that AHR2 of white sturgeon is approximately 10-fold more sensitive to activation by TCDD, and other selected DLCs, relative to AHR2 of lake sturgeon (Doering et al., 2015b). This resulted in the hypothesis that embryos of white sturgeon are 10-fold more sensitive to TCDD, and other DLCs, relative to embryos of lake sturgeon (Doering et al., 2015b). In another study, cytochrome P450 1A (CYP1A) expression was investigated in liver explants of white sturgeon and lake sturgeon (Eisner et al., 2016). In this study, juvenile white sturgeon had comparable sensitivity as juvenile lake sturgeon based on the average of the EC_{20}, EC_{50}, and EC_{80}, but juvenile white sturgeons were 10-fold more sensitive than juvenile lake sturgeon based on lowest observed effect concentration (LOEC) (Eisner et al., 2016). However, relative sensitivity of up-regulation of CYP1A in liver explants of juvenile sturgeons might not accurately reflect relative sensitivity of embryos or apical endpoints, such as mortality. Since

conducting these *in vitro* investigations of AHRs of white sturgeon and lake sturgeon, information has become available regarding the LD_{50} of lake sturgeon to TCDD (Tillitt et al., 2016), but the LD_{50} of white sturgeon to TCDD remains unknown. Based upon the linear relationship for AHR2 developed in Chapter 7 (Equation 7.2), embryos of white sturgeon are predicted to have an LD_{50} of 91 pg TCDD/g-egg relative to 611 pg TCDD/g-egg measured for embryos of lake sturgeon (Tillitt et al., 2016). Therefore, embryos of white sturgeon are predicted to be approximately 7-fold more sensitive to TCDD with regard to median lethality relative to lake sturgeon (Table 8.1). Studies with two other *Acipenser* spp., the shortnose sturgeon and Atlantic sturgeon, observed elevated mortality of embryos at concentrations as little as 50 pg TCDD/g-egg however, LD_{50}s were not achieved at the greatest investigated concentrations of 600 and 1,450 pg TCDD/g-egg, respectively (Chambers et al., 2011). Therefore, embryos of white sturgeon are predicted to be greater than 7-fold and 16-fold more sensitive to TCDD relative to shortnose sturgeon and Atlantic sturgeon, respectively. Embryos of *Scaphirhynchus* spp. of sturgeon that have been studied were the least sensitive known species of fish to TCDD, with LD_{50}s of 12,000 and 13,000 pg TCDD/g-egg for pallid sturgeon and shovelnose sturgeon, respectively (Buckler et al., 2015). Based upon embryo-lethality studies of sturgeons conducted to date, sensitivity to TCDD ranges by approximately 20-fold between the most sensitive studied sturgeon, the lake sturgeon and the least sensitive studied sturgeon, the shovelnose sturgeon (Buckler et al., 2015; Chambers et al., 2011; Tillitt et al., 2016) (Table 8.1). Based upon the predicted sensitivity of white sturgeon, sensitivity of embryos to TCDD could range by more than 140-fold among sturgeons (Table 8.1). This is significant because teleost fishes studied to date range in sensitivity of embryos by approximately 35-fold between the most sensitive studied teleost, the lake trout (*Salvelinus namaycush*) and the least sensitive studied

Table 8.1 Predicted and measured LD_{50}s to TCDD across investigated species of sturgeon.

Common Name	Species Name	LD_{50} (pg TCDD/g-egg)	Relative Sensitivity
White Sturgeon	*Acipenser transmontanus*	91[a]	1.0
Lake Sturgeon	*Acipenser fulvescens*	611[b]	0.2
Shortnose Sturgeon	*Acipenser brevirostrum*	> 600[c]	> 0.2
Atlantic Sturgeon	*Acipenser oxyrinchus*	> 1,450[c]	> 0.06
Pallid Sturgeon	*Scaphirhynchus albus*	12,000[d]	0.008
Shovelnose Sturgeon	*Scaphirhynchus platorynchus*	13,000[d]	0.007

[a] Adapted from previously published results (Chapter 7).

[b] Adapted from previously published results (Tillitt et al., 2016).

[c] Adapted from previously published results (Chambers et al., 2012).

[d] Adapted from previously published results (Buckler et al., 2015).

teleost, the zebrafish (*Danio rerio*) (Doering et al., 2013).

Results of Chapter 7 demonstrate that the linear relationship for fishes between sensitivity to activation of AHR2 by TCDD and sensitivity of embryos to TCDD is not statistically different from the same relationship for AHR1 of birds. This suggests that the relationship between sensitivity to activation of the AHR and sensitivity to apical adverse effects on embryos is indistinguishable across these distantly related oviparous vertebrates. The relationship between sensitivity to activation of the AHR1 and sensitivity to embryo-lethality in birds is much better understood than this relationship between AHR2 and embryo-lethality in fishes (Doering et al., 2013). In birds, studies have demonstrated that sensitivity to activation of AHR1 is highly predictive of sensitivity of embryos among birds for polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) (Farmahin et al., 2012; Manning et al., 2012). However, robust information on both sensitivities to activation of AHR2 and sensitivities of embryos across PCDDs, PCDFs, and PCBs is completely lacking for fishes, with the exception of 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) for lake sturgeon (Doering et al., 2015b; Tillitt et al., 2016). When available information on EC_{50}s and LD_{50}s across PCDDs, PCDFs, and PCBs for birds and fishes is combined, the linear relationship remains highly significant ($R^2 = 0.87$; $p < 0.0001$) (Figure 8.1). The relationship among PCDDs, PCDFs, and PCBs has greater variability than the relationship among species for TCDD alone (Figure 8.1). This variability has been hypothesized to result from differences in rates of adsorption, distribution, metabolism, and excretion (ADME) among PCDD, PCDF, and PCB congeners (Farmahin et al., 2012). However, the difference between predicted and measured LD_{50}s is still less than 4-fold (Table 8.2). This single, highly predictive, linear

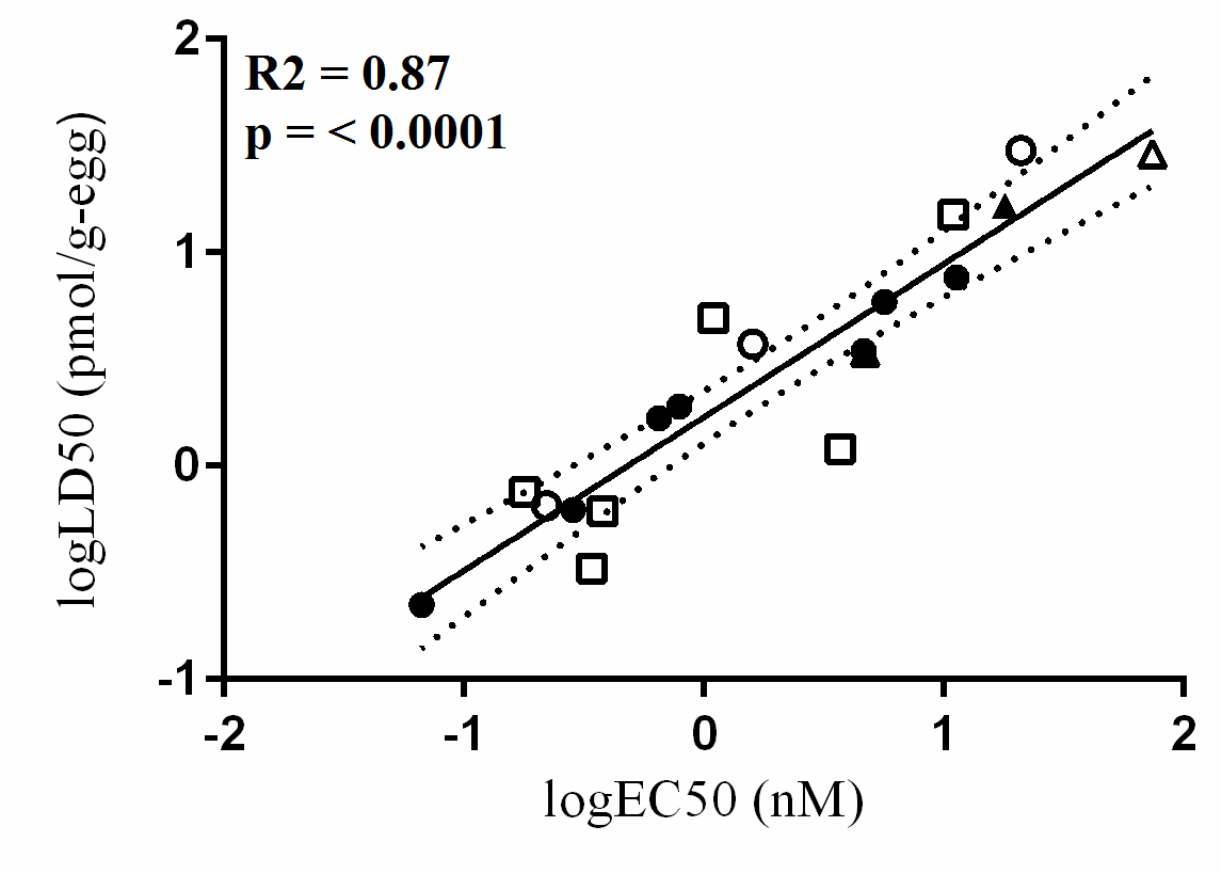

Figure 8.1. Linear regression across oviparous vertebrates for sensitivity to activation (EC_{50}) of AHR2s of fishes (solid) and AHR1s of birds (open) against sensitivity of embryos (LD_{50}) for polychlorinated dibenzo-*p*-dioxins (circle), polychlorinated dibenzofurans (square), and polychlorinated biphenyls (triangle). The equation of the line for AHRs of oviparous vertebrates across DLCs is Y = 0.7183*X+0.2287 (Equation 8.1) with a slope of 0.72 ± 0.07 and *y*-intercept of 0.23 ± 0.06. Best fit line for regression is indicated and 95 % confidence intervals are represented as dotted lines. EC_{50} and LD_{50} data was generated previously (Brunstrom and Anderson, 1988; Chapter 7; Doering et al., 2015b; Farmahin et al., 2012; Head et al., 2008; Manning et al., 2012; Tillitt et al., 2016).

Table 8.2 Predicted versus measured LD_{50}s of polychlorinated dibenzofurans (A) and polychlorinated biphenyls (B) to embryos of birds and fishes determined by use of Equation 8.1 (Figure 8.1).

A PCDFs	Congener	EC_{50} (nM)	LD_{50} (pg/g-egg)	Predicted LD_{50} (pg/g-egg)	Accuracy (Fold-difference)
Chicken	PeCDF	0.18[a]	260[d]	168	1.6
	TCDF	0.34[a]	100[d]	236	2.4
Pheasant	PeCDF	0.38[a]	210[d]	285	1.4
	TCDF	3.65[a]	370[d]	1,313	3.6
Quail	PeCDF	1.1[a]	1,700[d]	596	2.9
	TCDF	11[a]	4,600[d]	2,900	1.6
B PCBs	Isoform	EC_{50} (nM)	LD_{50} (pg/g-egg)	Predicted LD_{50} (pg/g-egg)	Accuracy (Fold-difference)
Chicken	PCB 126	4.7[b]	1,100[e]	1,678	1.5
	PCB 77	74[b]	8,600[f]	10,883	1.3
Lake Sturgeon	PCB 126	18[c]	5,400[g]	4,401	1.2

[a] Adapted from previously published results (Farmahin et al., 2012).

[b] Adapted from previously published results (Manning et al., 2012).

[c] Adapted from previously published results (Doering et al., 2015b).

[d] Adapted from previously published results (Cohen-Barnhouse et al., 2011).

[e] Adapted from previously published results (Head et al., 2008).

[f] Adapted from previously published results (Brunstrom and Anderson, 1988).

[g] Adapted from previously published results (Tillitt et al., 2016).

relationship that spans dioxin-like PCDDs, PCDFs, and PCBs among birds and fishes (Figure 8.1) is suggestive that this linear relationship is also predictive of sensitivities to PCDDs, PCDFs, and PCBs among other classes of oviparous vertebrates, such as amphibians and reptiles, as well as among a greater phylogenetic diversity of fishes.

Chapter 4 investigated potencies for activation of AHR2 by six selected DLCs of environmental relevance to white sturgeon and investigated mixtures of these six DLCs measured in eggs of white sturgeon from the Fraser River and upper Columbia River in B.C., Canada (Doering et al., 2014a). It was hypothesized that relative potencies among DLCs calculated from the average of EC_{20}, EC_{50}, and EC_{80} of AHR2 could be utilized in estimating the potency of mixtures of DLCs to white sturgeon in TCDD equivalents (Doering et al., 2014a). The linear relationship developed here (Figure 8.1) supports the hypothesis that sensitivity to activation of AHRs (EC_{50}s) across DLCs is predictive of potencies to embryos (LD_{50}s). Based on relative potencies derived from sensitivities to activation of AHR2 for TCDD, 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF), 2,3,7,8-tetrachloro-dibenzofuran (TCDF), PCB 126, 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), and 2,3,3′,4,4′-pentachlorobiphenyl (PCB 105) and measured concentrations of the same six DLCs in eggs of white sturgeon, there was up to 44 pg TCDD equivalents/g in eggs from the Fraser River and up to 65 pg TCDD equivalents/g in eggs from the upper Columbia River (Doering et al., 2014a; Kruse and Webb, 2006; Macdonald et al., 1997). Based upon the predicted potencies of mixtures of DLCs in eggs of white sturgeon and the predicted LD_{50} of white sturgeon of 82 to 91 pg TCDD/g-egg (Table 8.1; Table 8.3) these maximum measured environmental concentrations approach or exceed concentrations that would be expected to cause elevated mortality and other adverse effects (Chambers et al., 2011; Tillitt

Table 8.3 Predicted LD_{50}s of white sturgeon (A) and lake sturgeon (B) to embryos determined by use of Equation 8.1 (Figure 8.1).

A White Sturgeon	EC_{50} (nM)	Predicted LD_{50} (pg/g-egg)
TCDD	0.070[a]	82
PeCDF	0.034[a]	51
TCDF	0.079[a]	84
PCB 126	1.8[a]	842
PCB 77	38[a]	6,743
B Lake Sturgeon	**EC_{50} (nM)**	**Predicted LD50 (pg/g-egg)**
TCDD	0.79[b]	611 (measured)[c]
PeCDF	0.21[b]	188
TCDF	0.86[b]	465
PCB 126	18[b]	5,400 (measured)[c]
PCB 77	87[b]	12,225

[a] Adapted from previously published results (Doering et al., 2014a).

[b] Adapted from previously published results (Doering et al., 2015b).

[c] Adapted from previously published results (Tillitt et al., 2016).

et al., 2016). However, studies confirming the sensitivity of embryos of white sturgeon and further developing the predictive linear relationship among PCDDs, PCDFs, and PCBs for fishes are necessary in order to support these hypotheses.

8.3 Considering structural differences in AHRs across fishes and other oviparous vertebrates

Studies across birds and some mammals, amphibians, and fishes have identified key amino acid substitutions in the ligand binding domain (LBD) of the AHR that confer differences in binding affinity for DLCs, and therefore determine sensitivities to activation among species (Andreasen et al., 2002; Bisson et al., 2009; Doering et al., 2015b; Ema et al., 1993; Farmahin et al., 2012; 2013; Fraccalvieri et al., 2013; Head et al., 2008; Karchner et al., 2006; Odio et al., 2013; Shoots et al., 2015; Manning et al., 2012). These studies of birds have led to AHR genotyping and identification of key amino acid residues in the LBD as a quick, inexpensive, accurate, and non-lethal means of predicting the sensitivity of any species of bird to DLCs (Farmahin et al., 2013; Manning et al., 2013). Substitutions of key amino acid residues have also been demonstrated to explain differences in sensitivities to activation by TCDD among AHR1a, AHR1b, and AHR2 of zebrafish and between AHR2α and AHR2β of rainbow trout (Andreasen et al., 2002; Fraccalvieri et al., 2013). However, structural elements of the AHR2 that confer differences in sensitivity to activation by TCDD among phylogenetically diverse species of fish are unknown (Figure 8.2). Alignment of the LBD of AHR2s from lake trout, brook trout

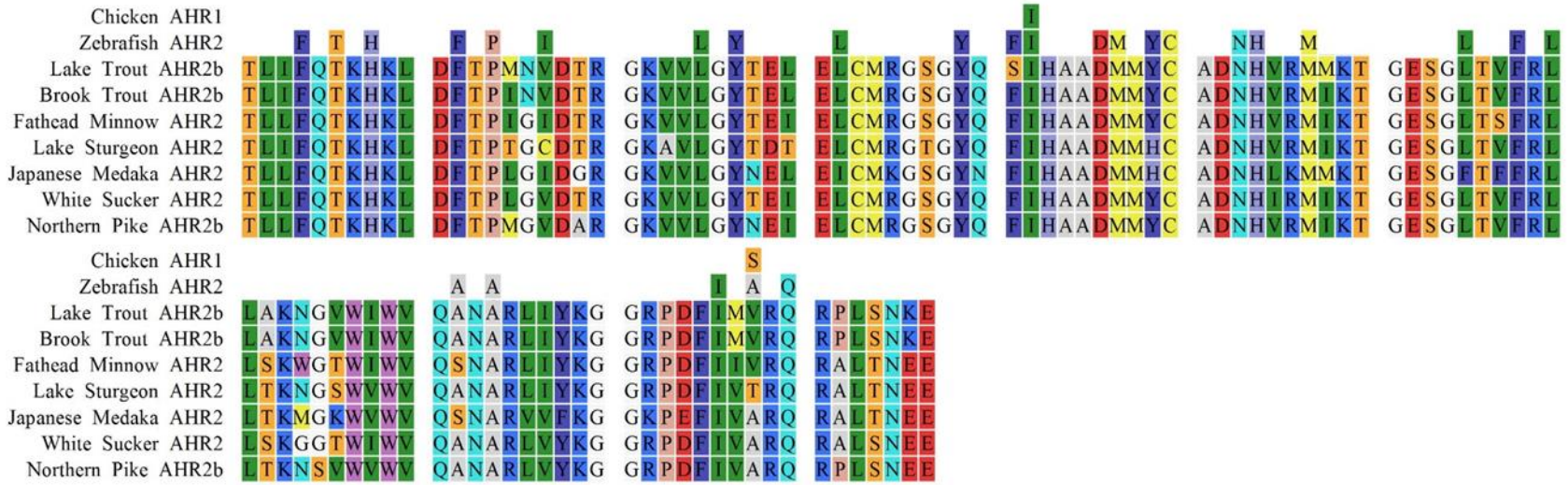

Figure 8.2. Alignment of amino acid sequences of the ligand binding domains (LBD) of AHR2s of fishes used to develop a predictive linear regression (Chapter 7; Equation 7.2). Key amino acids at position 324 and 380 of the LBD of AHR1 of chicken (*Gallus gallus*) are shown (Head et al., 2008). Amino acids of the AHR2 of zebrafish (accession # AF063446.1) that were previously identified by use of the CASTp server (Dundas et al., 2006) as being internal to the binding cavity are shown (Fraccalvieri et al., 2013). Coloring based on the Rasmol color scheme.

(*Salvelinus fontinalis*), fathead minnow (*Pimephales promelas*), lake sturgeon, Japanese medaka *(Oryzias latipes*), white sucker (*Catostomus commersonii*), and northern pike (*Esox lucius*) that were used in the linear regression (Chapter 7; Equation 7.2) demonstrate that 31 % of amino acid positions (33 of 107) are different across the length of the LBD of these AHR2s (Figure 8.2) and 26 % of critical amino acid positions (7 of 27), previously identified as being internal to the binding cavity of AHR2 of zebrafish, are different among these AHR2s (Figure 8.2) (Fraccalvieri et al., 2013). No pattern of key amino acid substitutions is present that previously had been demonstrated to confer differences in sensitivities among AHR1s of birds (Figure 8.2) and no clear patterns are present among critical amino acids identified as being internal to the binding cavity of AHR2 of zebrafish (Figure 8.2). This suggests that the specific mechanisms that mediate differences in sensitivities to activation of AHR2s across the phylogenetic diversity of fishes are different, and are potentially more complex, than those for AHR1s of birds. It also might suggest that structural characteristics that determine sensitivity to activation for AHR2s of phylogenetically diverse fishes are outside of the LBD and might involve critical amino acid substitutions in other domains which have been found to be important for activation of the AHR, such as the basic/helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) A, or transactivation domains (Ko et al., 1997; Pongratz et al., 1998; Whitelaw et al., 1994; Wu et al., 2013).

8.4 Future Research

The research contained within this thesis led to the development of a mechanism-based biological model that is capable of predicting the sensitivity to TCDD, and other DLCs, of any species of sturgeon based on *in vitro* data (Figure 7.3; 8.1). However, this research provides a

foundation for several areas of further study: 1) Additional information is necessary in order to strengthen the hypothesis that sensitivity to activation of the AHR2 is predictive of sensitivity of embryos among dioxin-like PCDDs, PCDFs, and PCBs. The linear relationship illustrated in Figure 8.1 includes significant data among fishes and birds for TCDD. However, the majority of the data for PCDFs and PCBs is for birds, apart from data for PCB 126 in lake sturgeon (Figure 8.1). Future studies should strengthen this linear relationship and hypothesis through acquiring EC_{50}s for AHR2 and LD_{50}s of embryos for a greater number of DLCs, particularly PCDFs and PCBs, among a greater phylogenetic diversity of fishes. 2) Additional information is necessary in order to strengthen the hypothesis that the developed linear relationship (Figure 8.1) is predictive of sensitivities to DLCs among all oviparous vertebrates, including amphibians and reptiles. Future studies should strengthen this linear relationship and hypothesis through acquiring EC_{50}s for AHR1 and LD_{50}s of embryos for amphibians and potentially reptiles. 3) Structural characteristics of the AHR2 that determine differences in sensitivity to activation by TCDD were not successfully elucidated within this thesis. Future studies should investigate ligand binding affinities for TCDD among AHR2s of different sensitivities to activation in order to elucidate whether these differences are determined by structural characteristics within the LBD or whether they are determined by structural characteristics of other functional domains. 4) This thesis developed a linear relationship capable of predicting an endpoint of regulatory relevance, mortality of embryos. However, exposure to DLCs can result in numerous adverse effects on embryos, juveniles, and adults (Carney et al 2006; Doering et al 2016). Chapter 6 demonstrated that equal activation of the AHR by agonists results in similar global responses and magnitude of responses across levels of biological organization in white sturgeon (Doering et al., 2016). Future studies should determine whether equal activation of the AHR results in similar responses and

magnitude of responses among a greater phylogenetic diversity of fishes. This could suggest that predictive relationships can be developed for other endpoints of regulatory relevance. 5) The overall purpose of the research contained within this thesis was to predict the sensitivity of sturgeons to DLCs to improve the ecological risk assessment of these endangered species. However, AHR information is only publicly available for two species, white sturgeon and lake sturgeon (Doering et al., 2015b), and sensitivity of embryos is only known for lake sturgeon, pallid sturgeon, and shovelnose sturgeon (Buckler et al., 2015; Tillitt et al., 2016). It is predicted that sensitivity to TCDD could range by more than 140-fold among sturgeons (Table 8.1). Therefore, future studies should acquire AHR activation and embryo-lethality data for additional species of sturgeons. Particularly, embryo-lethality data for white sturgeon to confirm the predictions presented within this thesis and strengthen the confidence in future predictions of sturgeons that are critically endangered and cannot be investigated *in vivo*.

8.5 Conclusion

The purpose of the line of research contained within this thesis was to develop a method to predict the relative sensitivity of any species of sturgeon to adverse effects of exposure to any agonist of the AHR. Sturgeons were found to respond to activation of the AHR in a manner that was generally consistent with that of better studied teleost fishes and predictive linear relationships were successfully developed between sensitivity to activation of the AHR2 by TCDD and sensitivity of embryos to TCDD among seven fishes that span an almost 40-fold difference in LD_{50}. The relationship between sensitivity of the AHR and sensitivity of embryos was found to hold true across distantly related fishes and birds, and across available information

for PCDDs, PCDFs, and PCBs with predicted LD_{50}s demonstrated to be within 4-fold of measured LD_{50}s. Based upon available information on sturgeons as well as predictions, sensitivity to TCDD could vary by more than 140-fold among the most sensitive species of sturgeon and the least sensitive species of sturgeon, with environmental concentrations in the Fraser and upper Columbia Rivers exceeding concentrations that are likely to cause elevated mortality of embryos of the most sensitive species. Despite uncertainties in structural elements that determine sensitivity to activation of AHRs of fishes, it was demonstrated that sensitivity to activation of the AHR2, but not the AHR1, is likely to mediate adverse effects of, and sensitivity to, DLCs among fishes in a manner that is consistent with other oviparous vertebrates, namely birds. Future identification of key structural elements within the AHR that determine sensitivity to activation and likely also determine sensitivity *in vivo* across phylogenetically diverse species of fish, as well as other oviparous vertebrates, would result in a powerful and inexpensive model that is accurate among laboratories, methodologies, and vertebrate taxa. Despite the use of a genetic screen of sensitivity based solely on amino acid sequence being unsuccessful to date, the successfully developed linear predictive model could potentially be applied to even critically endangered sturgeons, and other fishes, as expression constructs for AHR2 could likely be developed based on sequences acquired from non-lethal samples, such as scales, blood, or from biopsied tissues (Jarque et al 2010; Quiros et al 2007). Although only PCDDs, PCDFs, and PCBs were investigated here, similar linear predictive relationships could exist between activation of the AHR and sensitivity of embryos to other agonists of the AHR, such as polycyclic aromatic hydrocarbons (PAHs) or other planar halogenated aromatic hydrocarbons (PHAHs). Regardless, the research demonstrated within this thesis adds significantly to the ecological risk assessment of DLCs to sturgeons and other fishes in particular, and oviparous vertebrates in general.

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APPENDIX[1]

[1] Supplementary data are included in this chapter. The figure or table number is presented as Cx.Sy format, where ‘Cx’ indicates chapter number; ‘Sy’ indicates figure or table number.

Table C4.S1. Accession #s of white sturgeon genes used to design oligonucleotide primers used in producing expression constructs.

Target Gene	Accession #	Primer Sequence (5'-3')
AHR1	KJ420394	Forward: CACCATGTATGCAAGCCGCAAAAG Reverse: TGGAAAGCCACTGGATGTGG
AHR2	KJ420395	Forward: CACCATGTTGGCCACCGGA Reverse: GTAATCACAGCAGTTGGCT
ARNT2	KJ959625	Forward: CACCATGGCAACTCCCGCAG Reverse: CTCGGAAAAAGGTGGAAACATGCC

.

Table C5.S1. Accession #s of lake sturgeon genes used to design oligonucleotide primers. Annealing temperatures were 67 and 70 °C for full-length and construct PCRs, respectively.

Assay	Target Gene	Accession #	Primer Sequence (5'-3')
Full	AHR1[a]	KJ420394	Forward: ATGTATGCAAGCCGCAAAAGGC Reverse: TGGAAAGCCACTGGATGTGG
Full	AHR2[a]	KJ420395	Forward: AAGGTTTCTTTGGGCTTCGGSTSTT Reverse: TGGCGGTCTAAAATACAGGATACTCATC
Construct	AHR1[b]	NA	Forward: CACCATGTATGCAAGCCGCAAAAG Reverse: TGGAAAGCCACTGGATGTGG
Construct	AHR2[b]	NA	Forward: CACCATGTTGGCCACCGGA Reverse: GTAATCACAGCAGTTGGCT

[a]Adapted from previously published results (Doering et al., 2014b).

[b]Adapted from previously published results (Doering et al., 2014a).

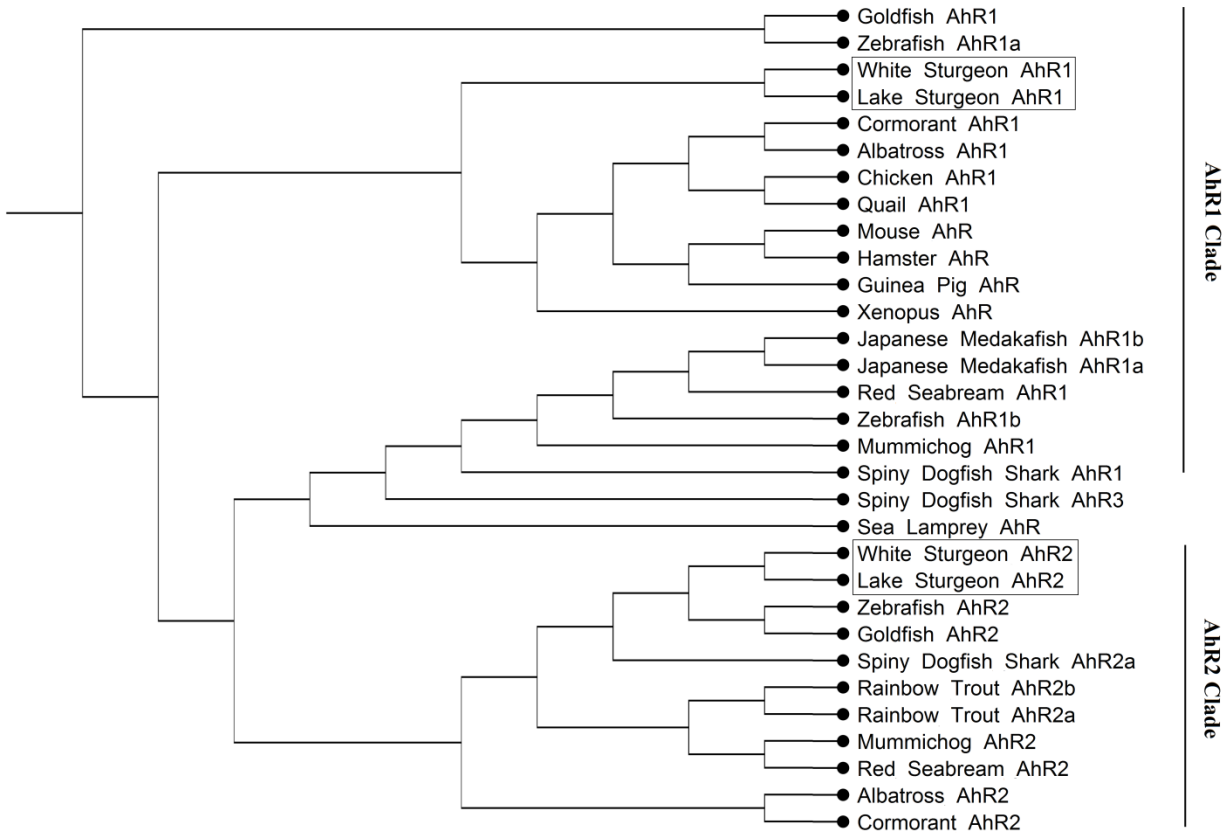


Figure C5.S1. Phylogenetic tree for relatedness of full-length amino acid sequences of AHRs among vertebrates. AHR1 of white and lake sturgeons and AHR2 of white sturgeon and lake sturgeon are highlighted. Branch lengths represent bootstrap values based on 1,000 samplings. The AHR1 and AHR2 clades are indicated. Accession numbers used were: Goldfish AHR1 (*Carassius* auratus; ACT79400.1); Zebrafish AHR1a (*Danio rerio*; AAM08127.1); White Sturgeon AHR1 (*Acipenser transmontanus*; AHX35737.1); Cormorant AHR1 (*Phalacrocorax carbo*; BAD01477.1); Albatross AHR1 (*Phoebastria nigripes*; BAC87795.1); Chicken AHR1 (*Gallus gallus*; NP_989449.1); Quail AHR1 (*Coturnix japonica*; ADI24459.2); Mouse AHR (*Mus musculus*; NP_038492.1); Hamster AHR (*Mesocricetus auratus*; NP_001268587.1); Guinea Pig AHR (*Cavia porcellus*; NP_001166525.1); Xenopus AHR (*Xenopus laevis*; JC7993); Japanese Medakafish AHR1b (*Oryzias latipes*; BAB62011.1); Japanese Medakafish AHR1a (*O. latipes*; BAB62012.1); Red Seabream AHR1 (*Pagrus major*; BAE02824.1); Zebrafish AHR1b (*D. rerio*; AAI63508.1); Mummichog AHR1 (*Fundulus heteroclitus*; AAR19364.1); Spiny Dogfish Shark AHR1 (AFR24092.1); Spiny Dogfish Shark AHR3 (*Squalus acanthias*; AFR24094.1); Sea Lamprey AHR (*Petromyzon marinus*; AAC60338.2); White Sturgeon AHR2 (*A. transmontanus*; KJ420395.1); Zebrafish AHR2 (*D. rerio*; AAI63711.1); Goldfish AHR2 (*C.* auratus; ACT79401.1); Spiny Dogfish Shark AHR2a (*S. acanthias*; AFR24093.1); Rainbow Trout AHR2b (*Oncorhynchus mykiss*; NP_001117724.1); Rainbow Trout AHR2a (*O. mykiss*;

NP_001117723.1); Mummichog AHR2 (*F. heteroclitus*; AAC59696.3); Red Seabream AHR2 (*P. major*; BAE02825.1); Albatross AHR2 (*P. nigripes*; BAC87796.1); Cormorant AHR2 (*P. carbo*; BAF64245.1).

Table C5.S2. Relative sensitivity (ReS) to selected dioxin-like compounds of AHR1 and AHR2 of lake sturgeon compared to AHR1 and AHR2 of white sturgeon based on the LOEC.

	TCDD	**PeCDF**	**TCDF**	**PCB 126**	**PCB 77**	**PCB 105**
Lake Sturgeon AHR1[a]	10.0	3.0	1.0	3.0	0.3	-
Lake Sturgeon AHR2[a]	1.0	1.0	1.0	1.0	1.0	-
White Sturgeon AHR1[b]	3.0	1.0	0.3	1.0	1.0	-
White Sturgeon AHR2[b]	1.0	1.0	1.0	1.0	1.0	-
Lake Sturgeon AHR1[c]	1.0	1.0	0.3	1.0	1.0	-
White Sturgeon AHR1[c]	1.0	1.0	1.0	1.0	1.0	-
Lake Sturgeon AHR2[c]	0.3	0.3	0.1	0.3	3.3	-
White Sturgeon AHR2[c]	1.0	1.0	1.0	1.0	1.0	-

ReS of white sturgeon are based on findings described previously (Doering et al., 2014a). Values that could not be calculated are indicated with '-'.

[a] Calculated by use of Equation 5.1.

[b] Adapted from previously published results (Doering et al., 2014a).

[c] Calculated by use of Equation 5.2.

Table C5.S3. Relative potency (ReP) of selected dioxin-like compounds to AHR1 and AHR2 of lake sturgeon compared to AHRs of other vertebrates.

	TCDD	**PeCDF**	**TCDF**	**PCB 126**	**PCB 77**	**PCB 105**
Lake Sturgeon AHR1[a]	1.0	0.9	0.6	0.05	0.0008	-
White Sturgeon AHR1[b]	1.0	1.0	0.4	0.04	0.001	-
Lake Sturgeon AHR2[a]	1.0	3.7	0.3	0.02	0.001	-
White Sturgeon AHR2[b]	1.0	1.3	1.0	0.04	0.002	-
$TEF_{WHO\text{-}Fish}$[c]	1.0	0.5	0.05	0.005	0.0001	< 0.000005
$TEF_{WHO\text{-}Bird}$[c]	1.0	1.0	1.0	0.1	0.05	0.0001
$TEF_{WHO\text{-}Mammal}$[d]	1.0	0.3	0.1	0.1	0.0001	0.00003

Values that could not be calculated are indicated with '-'. Compounds that were not analyzed in the referenced study are indicated with 'NA'.

[a] Calculated by use of Equation 5.3.

[b] RePs were derived by use of luciferase reporter gene assays using COS-7 cells transfected with the respective AHR (Doering et al., 2014a).

[c] Toxic equivalency factor (TEF) developed by the World Health Organization (WHO) (Van den Berg et al., 1998).

[d] TEF developed by the WHO (Van den Berg et al., 2006).

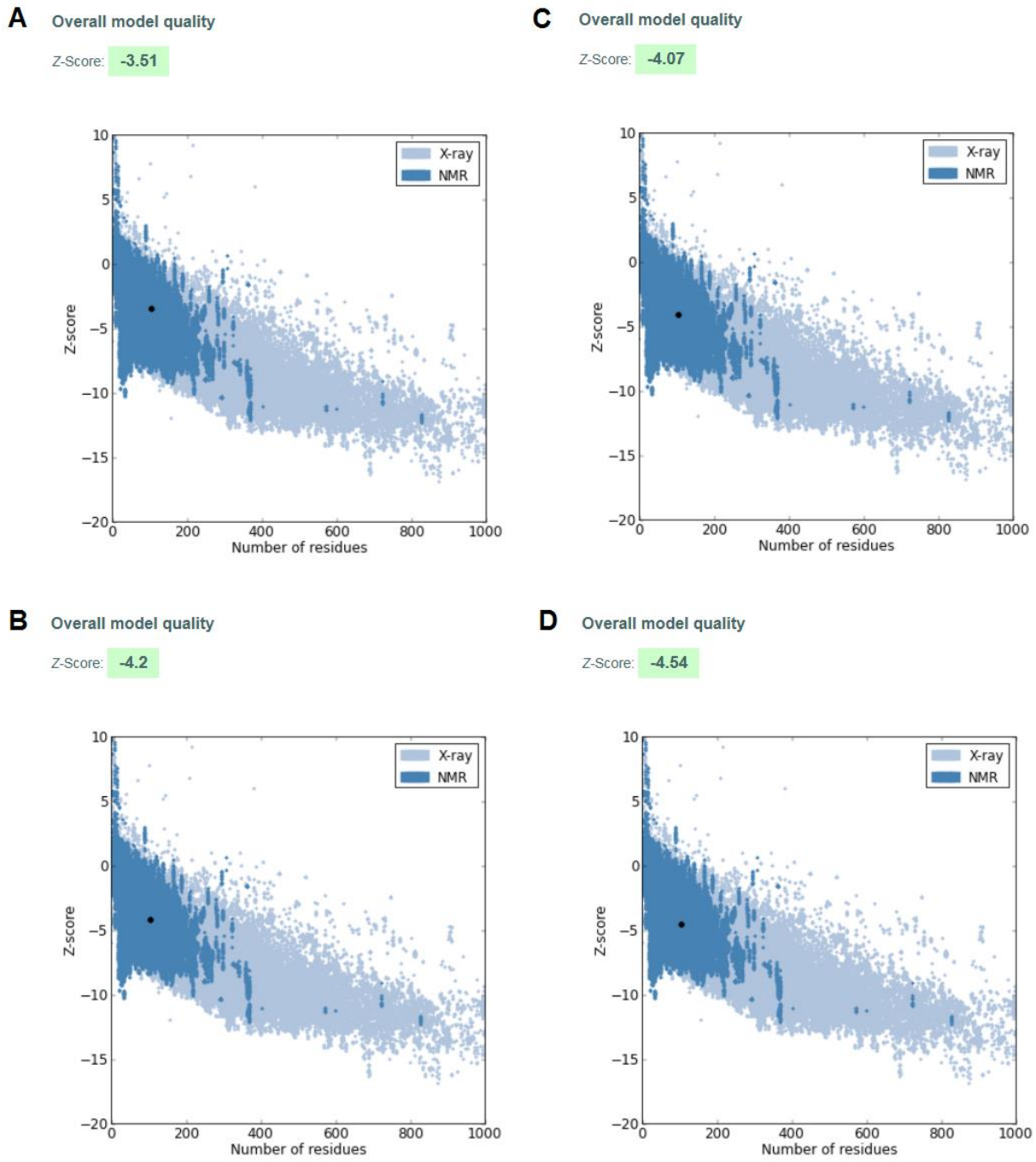


Figure C5.S2. Output from ProSA (Wiederstein et al., 2007) showing *z*-scores for models of AHR1 of white sturgeon (A), AHR2 of white sturgeon (B), AHR1 of lake sturgeon (C), and AHR2 of lake sturgeon (D). *Z*-scores are shown to be within the range of values for native protein structures of similar size.

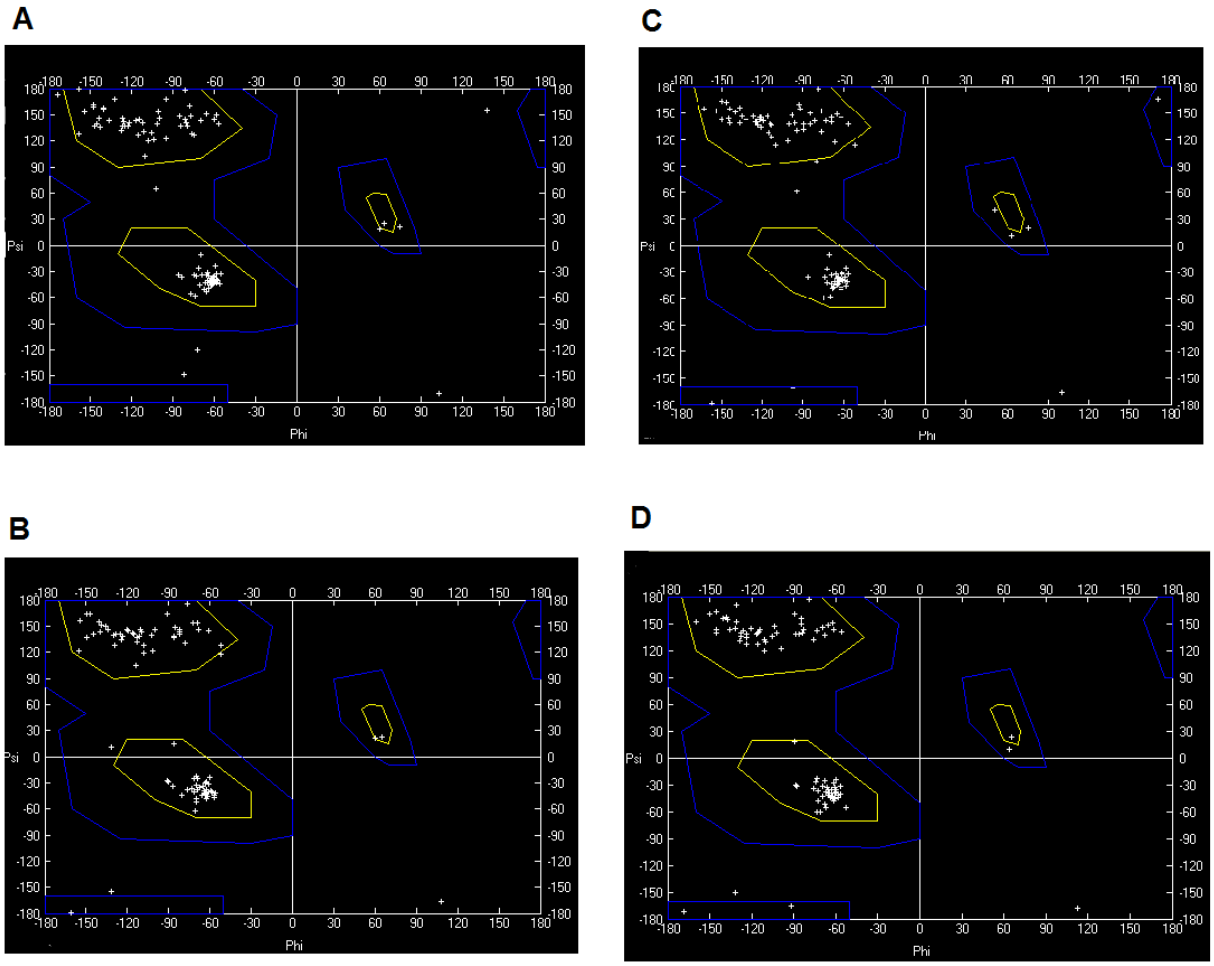


Figure C5.S3. Ramachandran plots for models of AHR1 of white sturgeon (A), AHR2 of white sturgeon (B), AHR1 of lake sturgeon (C), and AHR2 of lake sturgeon (D). Psi and phi dihedral angles for each amino acid residue are plotted. Ramachandran plots indicate that 97 %, 99 %, 98 %, and 98 % of amino acid residues are in the most favored areas of the Ramachandran plot for AHR1 of white sturgeon, AHR2 white sturgeon, AHR1 lake sturgeon, and AHR2 of lake sturgeon, respectively. Images were produced by use of Swiss-PbdViewer 4.1 (Guex et al., 1996).

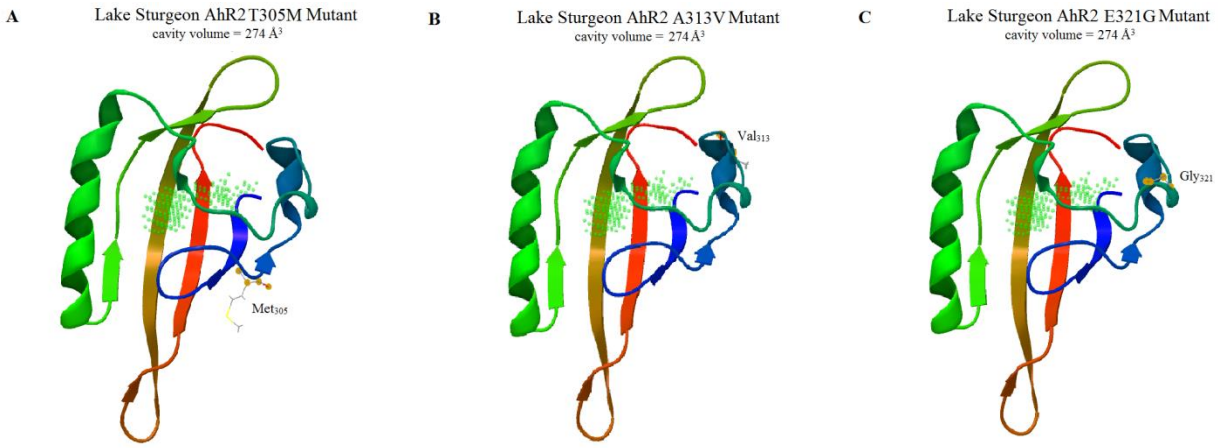


Figure C5.S4. 3-D models of the ligand binding domains (LBDs) of *in silico* T305M (A), A313V (B), and E321G (C) mutants of AHR2 of lake sturgeon is shown. Predicted binding pocket is indicated as a dotted region and the cavity volume is indicated ($Å^3$). Amino acid mutation is labelled and shown as a 'stick structure'.

Figure C5.S5. Aligment of the ligand binding domain (LBD) of the AHR2 of all fishes available in Genebank. Amino acid at the equivalent of position 388 in the AHR2 of white sturgeon and lake sturgeon is indicated by a black box. Accession numbers were the same as Figure S1 unless stated. Accession numbers used were: zebrafish (*D. rerio*; NP_571339.1); gilthead seabream (*Sparus aurata*; AAN05089.1); elephant shark (*Callorhinchus milii*; AFO95208.1); thicklip grey mullet (*Chelon labrosus*; AEI16511.1); Atlantic salmon (*Salmo salar*; NP_001117156.1, NP_001117028.1, NP_001117015.1, NP_001117037.1); fugu (*Takifugu rubripes*; NP_001033049.1, NP_001033052.1, NP_001033047.1); channel catfish (*Ictalurus punctatus*; AHH42811.1); Atlantic tomcod (*Microgadus tomcod*; AAC05158.2).

Table C6.S1. Sequences, annealing temperatures, primer efficiencies, and corresponding gene Genbank accession numbers of oligonucleotide primers used in quantitative real-time polymerase chain reaction (qRT-PCR).

Target Gene	Accession #	Primer Sequence (5'-3')	Efficiency (%)	Annealing Temp (°C)
β-actin[a]	FJ205611	Forward: CCGAGCACAATGAAAATCAA Reverse: ACATCTGCTGGAAGGTGGAC	96	60
CYP1A[a]	JQ660369	Forward: GATCCCTCCACCTTCTCTCC Reverse: GCCGATAGACTCACCAATGC	99	60
AHRR	NA	Forward: GATGCACCAGAATGTGTTCG Reverse: ATGGACCAGTGGAGCTGTGT	107	60
SOD	NA	Forward: GCAGGTCCGTGGTGATTCAT Reverse: TTCCGATGACACAGCAAGCT	87	60
FBP1A	NA	Forward: CAATGGTGGCTGATGTTCAC Reverse: GTGGATGCACTCTGGCTGTA	102	60
SOX9	NA	Forward: AAGGGCTATGACTGGACCCT Reverse: GTGAAGATGCGGGTACTGGT	100	60
CYP7A1A	NA	Forward: GCCATTGAAACCTCAAGGAA Reverse: AGTCCTTCTGTGGTCCATGC	103	60

[a]Adapted from previously published results (Doering et al., 2012).

Table C6.S2. Comparison of fold-changes for abundance of transcripts by use of transcriptome sequencing (A) and by use of quantitative real-time polymerase chain reaction (qRT-PCR) (B) in livers of white sturgeon three days following intraperitoneal injection with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm.[a]

	A Transcriptomics			B qRT-PCR		
Gene	TCDD	PCB 77	BaP	TCDD	PCB 77	BaP
CYP1A	33-fold	42-fold	50-fold	25-fold ± 4	15-fold ± 3	24-fold ± 8
AHRR	78-fold	105-fold	110-fold	196-fold ± 40	103-fold ± 22	210-fold ± 44
SOD	1.3-fold	1.1-fold	1.5-fold	1.1-fold ± 0.1	0.89-fold ± 0.3	1.2-fold ± 0.5
FBP1A	1.1-fold	1.2-fold	0.83-fold	0.52-fold ± 0.1	0.64-fold ± 0.4	0.48-fold ± 0.2
SOX9	0.21-fold	0.13-fold	0.39-fold	0.28-fold ± 0.04	0.22-fold ± 0.05	0.59-fold ± 0.08
CYP7A1A	0.12-fold	0.44-fold	0.85-fold	0.34-fold ± 0.1	0.28-fold ± 0.04	0.43-fold ± 0.09

[a]Transcriptomics data represented as the mean fold-change where multiple contigs had the same sequence description. qRT-PCR data represented as mean ± standard error of the mean (S.E.M.).

Table C6.S3. Comparison of the standard deviation (SD) in abundance of transcripts in livers from the control treatment (n = 3) assessed by use of quantitative real-time polymerase chain reaction (qRT-PCR).[a]

Gene	SD
CYP1A	± 0.33
AHRR	± 0.36
SOD	± 0.41
FBP1A	± 0.77
SOX9	± 0.25
CYP7A1A	± 0.36
Average	± 0.41

[a]SDs are based on a normalized control value of 1.

Table C6.S4. Number of genes up- or down-regulated by ≥ 2-fold in the transcriptome and proteome in livers of white sturgeon following exposure to TCDD, PCB 77, or BaP.

Transcriptome		Up-regulated	Down-regulated
	TCDD	378	296
	PCB 77	493	325
	BaP	529	394
Proteome		**Up-regulated**	**Down-regulated**
	TCDD	180	102
	PCB 77	270	89
	BaP	218	89

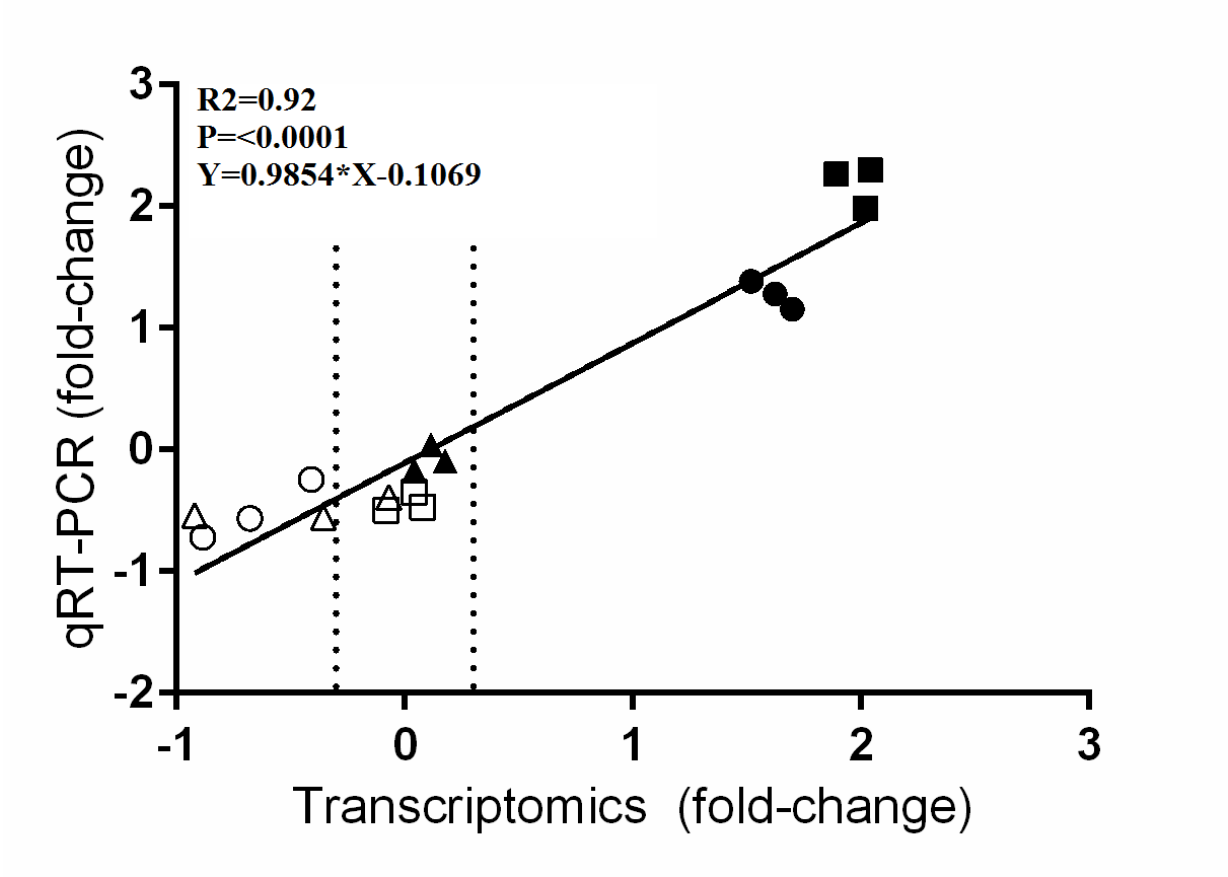


Figure C6.S1. Linear regression of fold-change in abundance of transcripts in livers of white sturgeon exposed to TCDD, PCB 77, or BaP relative to abundance in controls assessed by either quantitative real-time polymerase chain reaction (qRT-PCR) or transcriptome analysis by use of *Illumina* Mi-Seq. Plotted data represents the mean (n = 4) for CYP1A (solid circle), AHRR (solid square), SOD (solid triangle), FBP1A (open square), SOX9 (open circle), CYP7A1A (open triangle). Plotted data has been $\log_{10}$ transformed. Plotted data is presented elsewhere (Table C6.S2). Dotted lines indicate the threshold of 2-fold down-regulated and 2-fold up-regulated.

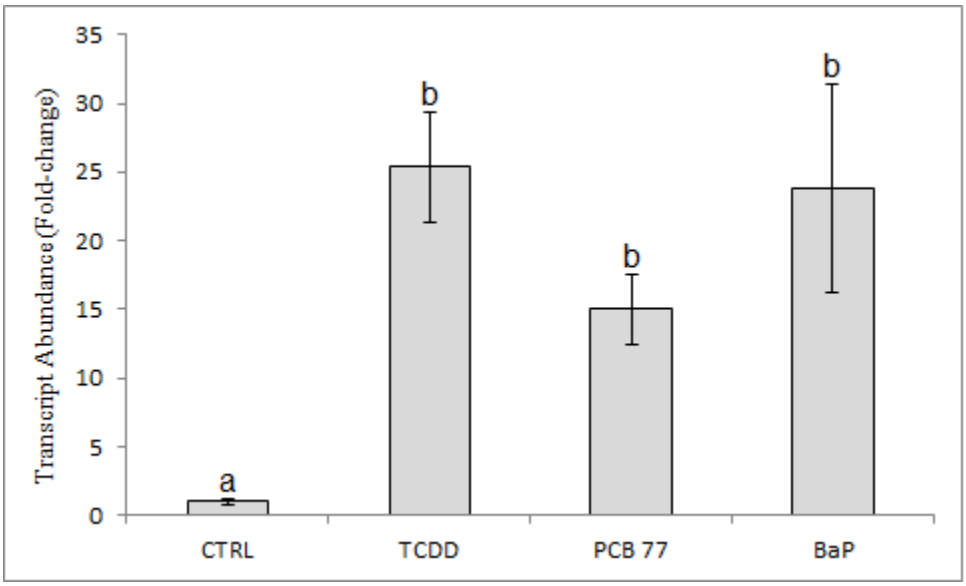


Figure C6.S2. Abundance of transcripts of cytochrome P450 1A (CYP1A) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean±standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

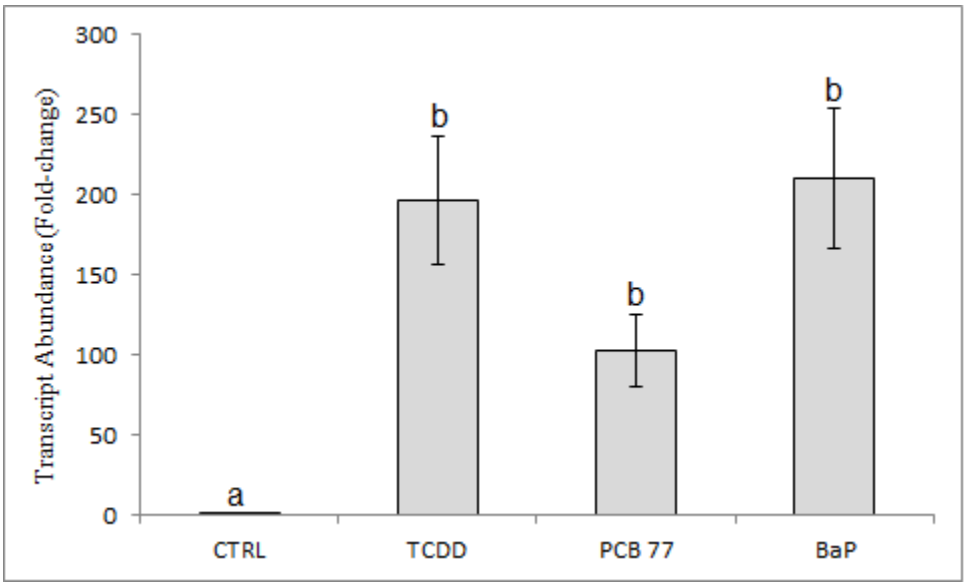


Figure C6.S3. Abundance of transcripts of aryl hydrocarbon receptor repressor (AHRR) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean ± standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

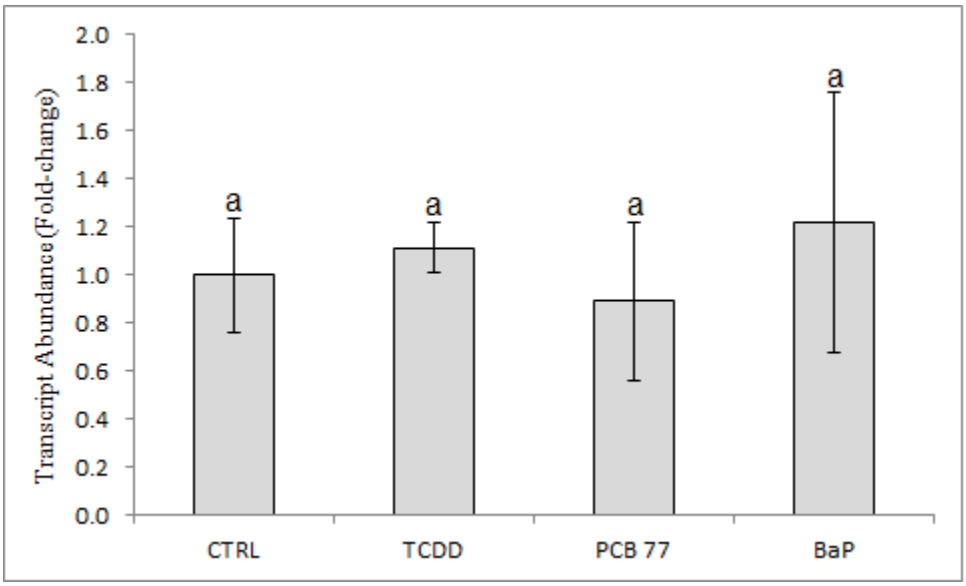


Figure C6.S4. Abundance of transcripts of superoxide dismutase (SOD) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean ± standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

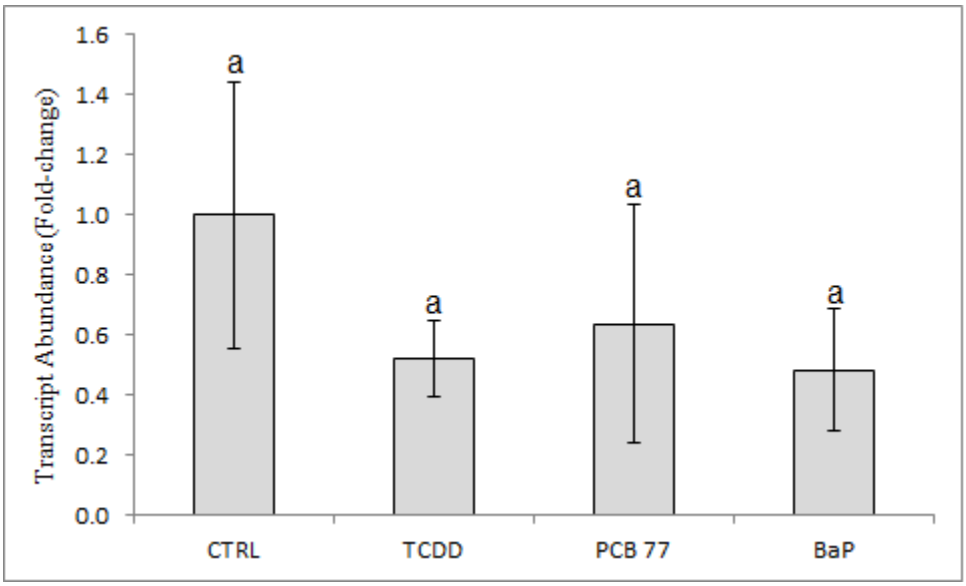


Figure C6.S5. Abundance of transcripts of fructose-1,6-bisphosphatase 1a (FBP1A) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean ± standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

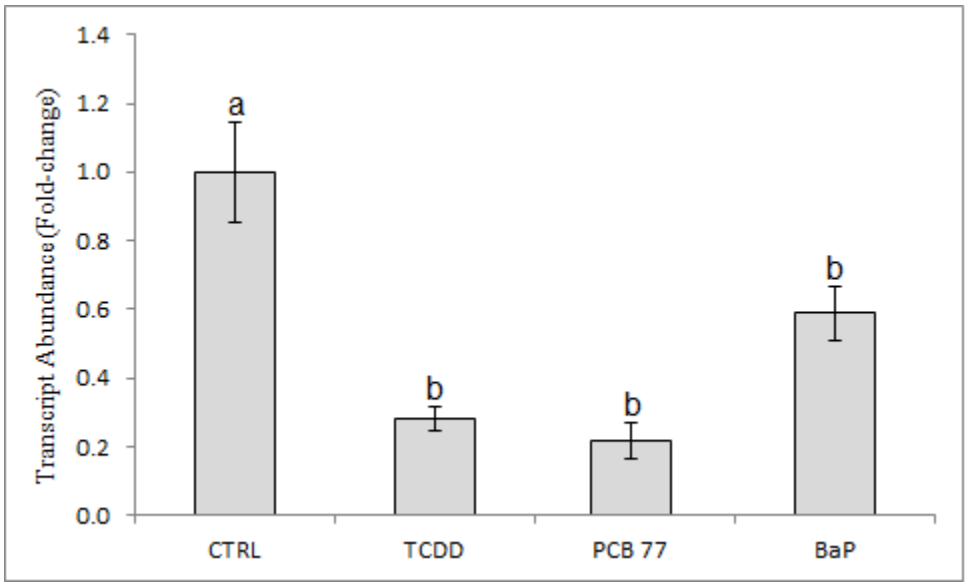


Figure C6.S6. Abundance of transcripts of sex determining region Y-box 9 (SOX9) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean ± standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

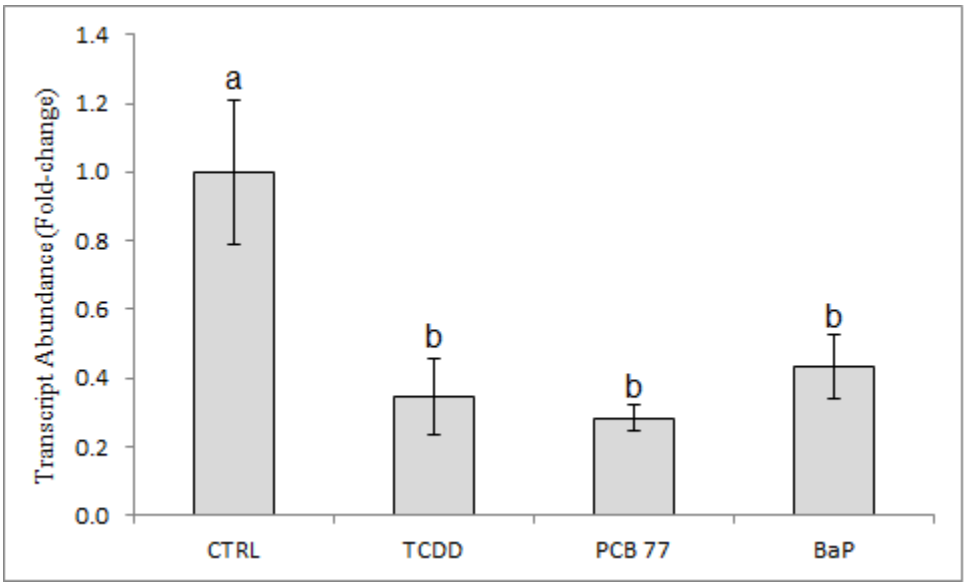


Figure C6.S7. Abundance of transcripts of cholesterol 7-alpha-monooxygenase 1a (CYP7A1A) in livers of white sturgeon three days following intraperitoneal injection with either corn oil alone, or corn oil with 5 µg TCDD/kg-bm, 5 mg PCB 77/kg-bm, or 30 mg BaP/kg-bm. Data represent mean ± standard error of the mean (S.E.M.). Different letters represent statistical difference by use of analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p \leq 0.05$).

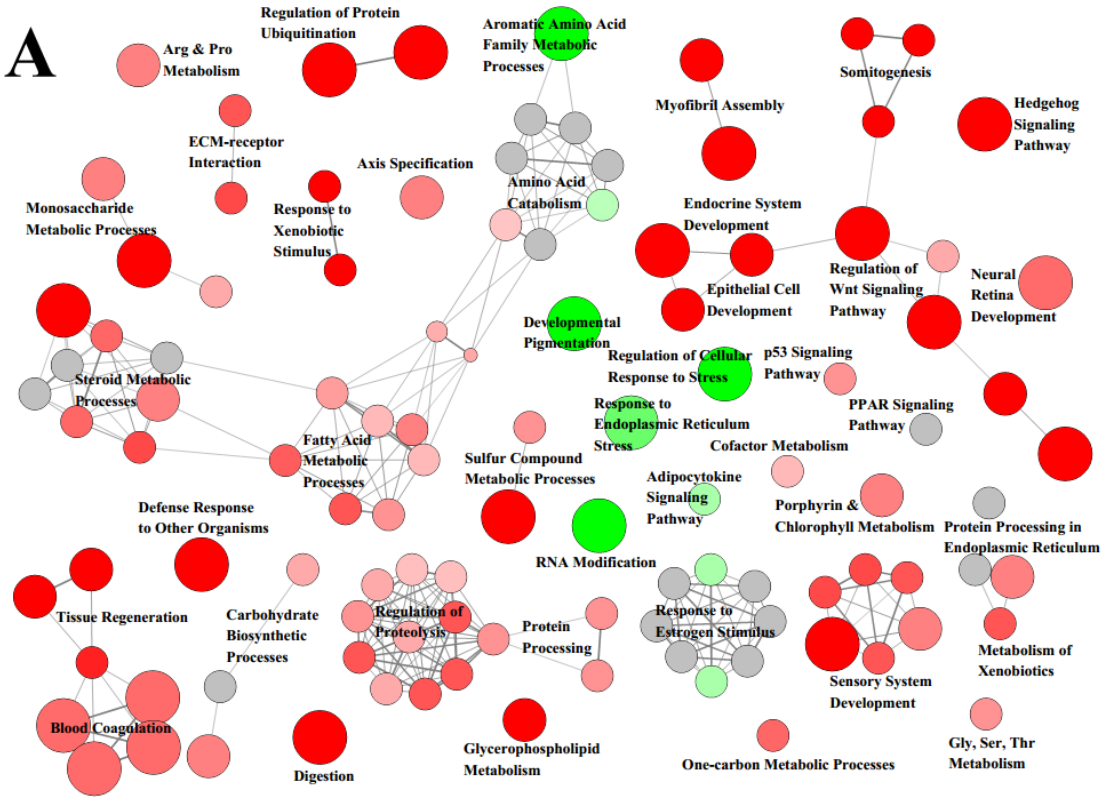

A
Regulation of Protein Ubiquitination
Arg & Pro Metabolism
Aromatic Amino Acid Family Metabolic Processes
Somitogenesis
Myofibril Assembly
Hedgehog Signaling Pathway
ECM-receptor Interaction
Axis Specification
Amino Acid Catabolism
Monosaccharide Metabolic Processes
Response to Xenobiotic Stimulus
Endocrine System Development
Regulation of Wnt Signaling Pathway
Neural Retina Development
Epithelial Cell Development
Developmental Pigmentation
Regulation of Cellular Response to Stress
p53 Signaling Pathway
Steroid Metabolic Processes
Response to Endoplasmic Reticulum Stress
PPAR Signaling Pathway
Cofactor Metabolism
Fatty Acid Metabolic Processes
Sulfur Compound Metabolic Processes
Adipocytokine Signaling Pathway
Porphyrin & Chlorophyll Metabolism
Protein Processing in Endoplasmic Reticulum
Defense Response to Other Organisms
RNA Modification
Tissue Regeneration
Carbohydrate Biosynthetic Processes
Regulation of Proteolysis
Protein Processing
Response to Estrogen Stimulus
Sensory System Development
Metabolism of Xenobiotics
Blood Coagulation
Digestion
Glycerophospholipid Metabolism
One-carbon Metabolic Processes
Gly, Ser, Thr Metabolism
B
Val, Leu, Ile Degradation
Lipid Catabolic Processes
Catabolic Processes
Peroxisome
Pyruvate Metabolism
PPAR Signaling Pathway
Tyr Metabolism
Pentose & Glucuronate Interconversion
Proteasome
Carbohydrate Catabolic Processes
His Metabolism
Amino Acid Metabolism & Biosynthesis
Pentose Phosphate Pathway
Ascorbate & Aldarate Metabolism
Metabolism of Xenobiotics
Glycolysis & Gluconeogenesis
Trp Metabolism
Protein Folding
Response to Hypoxia
Gly, Ser, Thr Metabolism
Rab Protein Signal Transduction
Ribosome Assembly
Nuclear Transport
Translation
Nucleoside Metabolic Processes
NADP Metabolic Processes
Ribosome
ATP Metabolic Processes

Figure C6.S8. Cytoscape visualization of ClueGO clustering results of physiological processes altered by TCDD at the level of the transcriptome (A) and proteome (B) in liver of white sturgeon. Clusters with a greater proportion of up-regulated processes are shown in (red) while clusters with a greater proportion of down-regulated processes are shown in (green). Degree of red or green shows relative abundance of up-regulated vs down-regulated processes in each cluster. Grey clusters consist of 50 % up-regulated processes and 50 % down-regulated processes. Size of cluster represents the relative number of processes in the cluster. Interconnection between pathways is represented by grey interconnecting lines indicating that these categories share transcript(s) or protein(s).

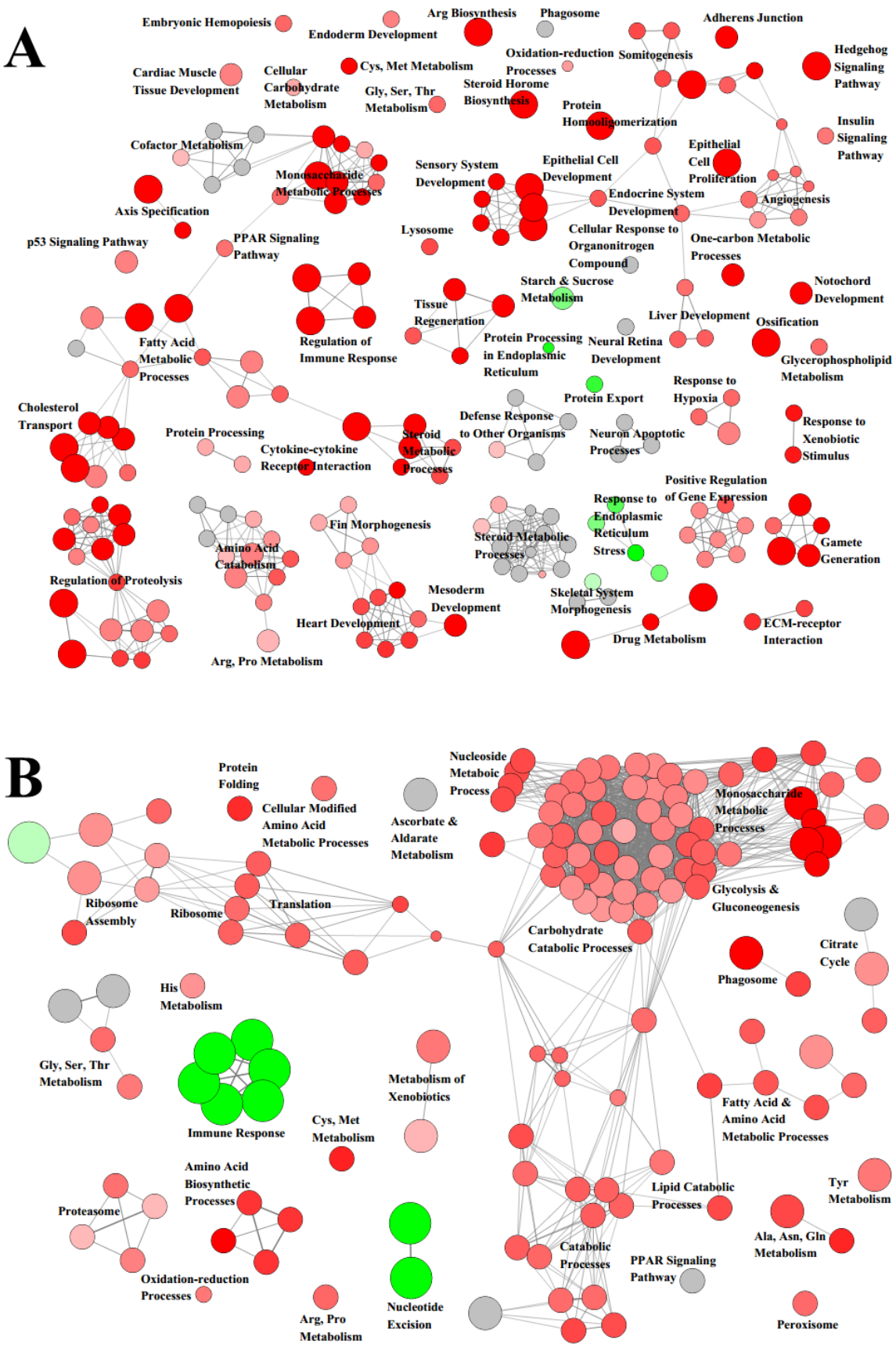

A
Embryonic Hemopoiesis
Endoderm Development
Arg Biosynthesis
Phagosome
Adherens Junction
Cardiac Muscle Tissue Development
Cellular Carbohydrate Metabolism
Cys, Met Metabolism
Gly, Ser, Thr Metabolism
Oxidation-reduction Processes
Steroid Horome Biosynthesis
Somitogenesis
Hedgehog Signaling Pathway
Protein Homooligomerization
Insulin Signaling Pathway
Cofactor Metabolism
Monosaccharide Metabolic Processes
Sensory System Development
Epithelial Cell Development
Epithelial Cell Proliferation
Axis Specification
Endocrine System Development
Angiogenesis
Lysosome
Cellular Response to Organonitrogen Compound
One-carbon Metabolic Processes
p53 Signaling Pathway
PPAR Signaling Pathway
Starch & Sucrose Metabolism
Notochord Development
Tissue Regeneration
Liver Development
Ossification
Fatty Acid Metabolic Processes
Regulation of Immune Response
Protein Processing in Endoplasmic Reticulum
Neural Retina Development
Glycerophospholipid Metabolism
Protein Export
Response to Hypoxia
Cholesterol Transport
Defense Response to Other Organisms
Response to Xenobiotic Stimulus
Protein Processing
Cytokine-cytokine Receptor Interaction
Steroid Metabolic Processes
Neuron Apoptotic Processes
Positive Regulation of Gene Expression
Response to Endoplasmic Reticulum Stress
Fin Morphogenesis
Amino Acid Catabolism
Steroid Metabolic Processes
Gamete Generation
Regulation of Proteolysis
Mesoderm Development
Skeletal System Morphogenesis
Heart Development
Drug Metabolism
ECM-receptor Interaction
Arg, Pro Metabolism
B
Protein Folding
Cellular Modified Amino Acid Metabolic Processes
Ascorbate & Aldarate Metabolism
Nucleoside Metaboic Process
Monosaccharide Metabolic Processes
Ribosome Assembly
Ribosome
Translation
Glycolysis & Gluconeogenesis
Carbohydrate Catabolic Processes
Citrate Cycle
Phagosome
His Metabolism
Gly, Ser, Thr Metabolism
Metabolism of Xenobiotics
Fatty Acid & Amino Acid Metabolic Processes
Immune Response
Cys, Met Metabolism
Amino Acid Biosynthetic Processes
Lipid Catabolic Processes
Tyr Metabolism
Proteasome
Ala, Asn, Gln Metabolism
Catabolic Processes
PPAR Signaling Pathway
Oxidation-reduction Processes
Nucleotide Excision
Arg, Pro Metabolism
Peroxisome

Figure C6.S9. Cytoscape visualization of ClueGO clustering results of physiological processes altered by PCB 77 at the level of the transcriptome (A) and proteome (B) in liver of white sturgeon. Clusters with a greater proportion of up-regulated processes are shown in (red) while clusters with a greater proportion of down-regulated processes are shown in (green). Degree of red or green shows relative abundance of up-regulated vs down-regulated processes in each cluster. Grey clusters consist of 50 % up-regulated processes and 50 % down-regulated processes. Size of cluster represents the relative number of processes in the cluster. Interconnection between pathways is represented by grey interconnecting lines indicating that these categories share transcript(s) or protein(s).

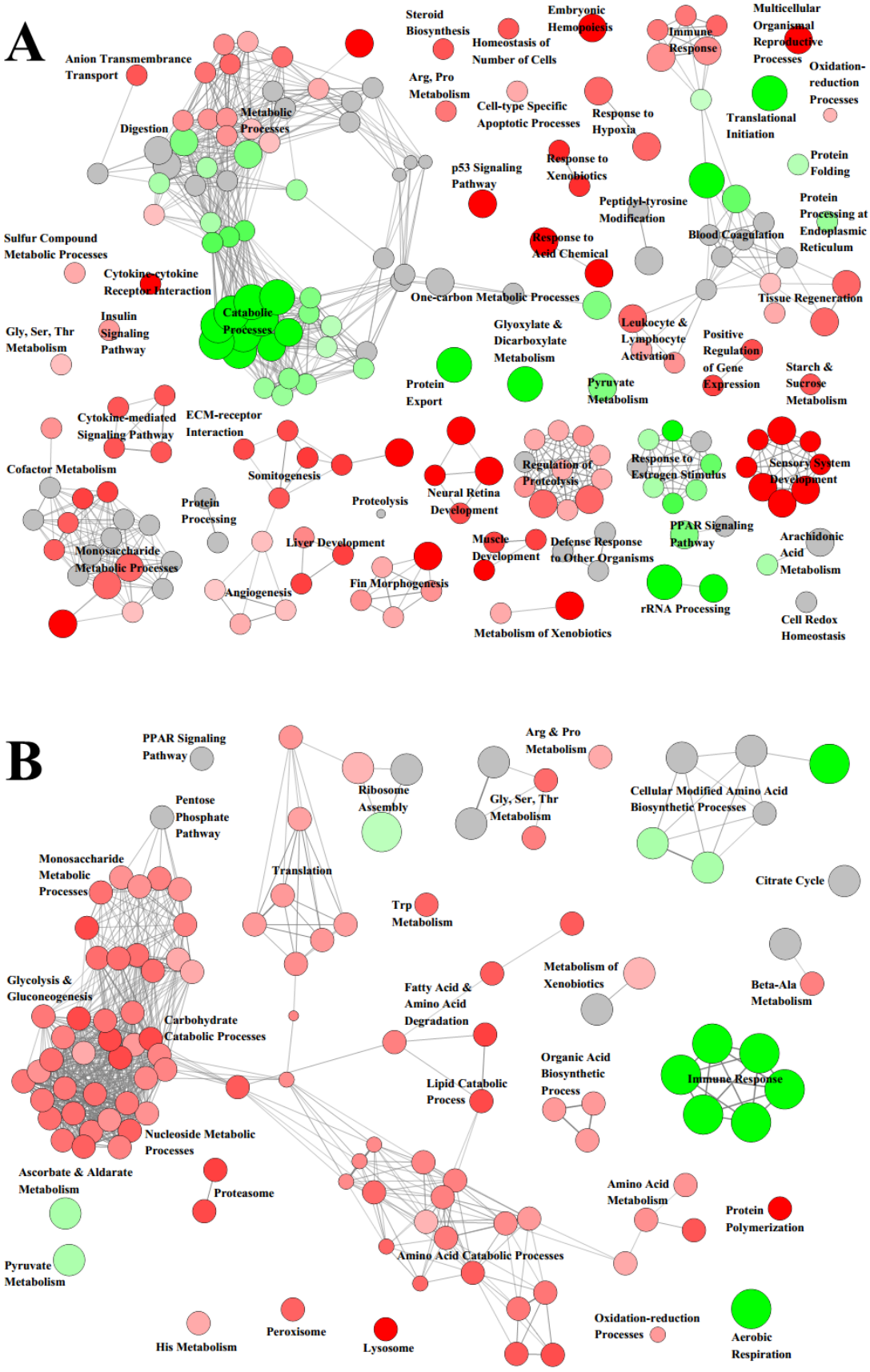

A
Anion Transmembrance Transport
Digestion
Metabolic Processes
Steroid Biosynthesis
Homeostasis of Number of Cells
Embryonic Hemopoiesis
Immune Response
Multicellular Organismal Reproductive Processes
Oxidation-reduction Processes
Arg, Pro Metabolism
Cell-type Specific Apoptotic Processes
Response to Hypoxia
Translational Initiation
p53 Signaling Pathway
Response to Xenobiotics
Protein Folding
Peptidyl-tyrosine Modification
Protein Processing at Endoplasmic Reticulum
Blood Coagulation
Sulfur Compound Metabolic Processes
Response to Acid Chemical
Cytokine-cytokine Receptor Interaction
Catabolic Processes
One-carbon Metabolic Processes
Tissue Regeneration
Insulin Signaling Pathway
Gly, Ser, Thr Metabolism
Glyoxylate & Dicarboxylate Metabolism
Leukocyte & Lymphocyte Activation
Positive Regulation of Gene Expression
Protein Export
Pyruvate Metabolism
Starch & Sucrose Metabolism
Cytokine-mediated Signaling Pathway
ECM-receptor Interaction
Cofactor Metabolism
Somitogenesis
Regulation of Proteolysis
Response to Estrogen Stimulus
Sensory System Development
Protein Processing
Proteolysis
Neural Retina Development
PPAR Signaling Pathway
Arachidonic Acid Metabolism
Monosaccharide Metabolic Processes
Liver Development
Muscle Development
Defense Response to Other Organisms
Angiogenesis
Fin Morphogenesis
rRNA Processing
Cell Redox Homeostasis
Metabolism of Xenobiotics
B
PPAR Signaling Pathway
Ribosome Assembly
Arg & Pro Metabolism
Gly, Ser, Thr Metabolism
Cellular Modified Amino Acid Biosynthetic Processes
Pentose Phosphate Pathway
Monosaccharide Metabolic Processes
Translation
Citrate Cycle
Trp Metabolism
Metabolism of Xenobiotics
Glycolysis & Gluconeogenesis
Fatty Acid & Amino Acid Degradation
Beta-Ala Metabolism
Carbohydrate Catabolic Processes
Organic Acid Biosynthetic Process
Immune Response
Lipid Catabolic Process
Nucleoside Metabolic Processes
Ascorbate & Aldarate Metabolism
Proteasome
Amino Acid Metabolism
Protein Polymerization
Amino Acid Catabolic Processes
Pyruvate Metabolism
Oxidation-reduction Processes
Aerobic Respiration
His Metabolism
Peroxisome
Lysosome

Figure C6.S10. Cytoscape visualization of ClueGO clustering results of physiological processes altered by BaP at the level of the transcriptome (A) and proteome (B) in liver of white sturgeon. Clusters with a greater proportion of up-regulated processes are shown in (red) while clusters with a greater proportion of down-regulated processes are shown in (green). Degree of red or green shows relative abundance of up-regulated vs down-regulated processes in each cluster. Grey clusters consist of 50 % up-regulated processes and 50 % down-regulated processes. Size of cluster represents the relative number of processes in the cluster. Interconnection between pathways is represented by grey interconnecting lines indicating that these categories share transcript(s) or protein(s).

Table C7.S1. Phylogenetic classification of species of fish of known sensitivity to TCDD of embryos based on LD_{50} (pg TCDD/g-egg).

Common Name	LD_{50}	Infraclass	Superorder	Order	Family	Genus	Species
Lake Trout	72[a]	Teleostei	Protacanthopterygii	Salmoniformes	Salmonidae	Salvelinus	namaycush
Brook Trout	200[b]	Teleostei	Protacanthopterygii	Salmoniformes	Salmonidae	Salvelinus	fontinalis
Crucian Carp	240[c]	Teleostei	Ostariophysi	Cypriniformes	Cyprinidae	Carassius	carassius
Mummichog	250[d]	Teleostei	Acanthopterygii	Cyprinodontiformes	Fundulidae	Fundulus	heteroclitus
Red Seabream	360[e]	Teleostei	Acanthopterygii	Perciformes	Sparidae	Pagrus	major
Rainbow Trout	439[f]	Teleostei	Protacanthopterygii	Salmoniformes	Salmonidae	Oncorhynchus	mykiss
Fathead Minnow	539[g]	Teleostei	Ostariophysi	Cypriniformes	Cyprinidae	Pimephales	promelas
Lake Sturgeon	611[h]	Chondrostei	NA[i]	Acipenseriformes	Acipenseridae	Acipenser	fulvescens
Channel Catfish	644[g]	Teleostei	Ostariophysi	Siluriformes	Ictaluridae	Ictalurus	punctatus
Lake Herring	902[g]	Teleostei	Protacanthopterygii	Salmoniformes	Salmonidae	Coregonus	artedi
Japanese Medakafish	1110[g]	Teleostei	Acanthopterygii	Beloniformes	Adrianichthyidae	Oryzias	latipes
White Sucker	1890[g]	Teleostei	Ostariophysi	Cypriniformes	Catostomidae	Catostomus	commersonii
Northern Pike	2460[g]	Teleostei	Protacanthopterygii	Esociformes	Esocidae	Esox	lucius
Zebrafish	2555[j]	Teleostei	Ostariophysi	Cypriniformes	Cyprinidae	Danio	rerio
Pallid Sturgeon	12 000[k]	Chondrostei	NA[i]	Acipenseriformes	Acipenseridae	Scaphirhynchus	albus
Shovelnose Sturgeon	13 000[k]	Chondrostei	NA[i]	Acipenseriformes	Acipenseridae	Scaphirhynchus	platorynchus

[a]Average LD_{50} from previously published results (Guiney et al., 2000; Walker et al., 1991; Walker et al., 1992; Walker et al., 1994).
[b]LD_{50} from previously published results (Walker et al., 1994).
[c]LD_{50} from previously published results (Park et al., 2014).
[d]LD_{50} from previously published results (Toomey et al., 2001).
[e]LD_{50} from previously published results (Yamauchi et al., 2006).
[f]LD_{50} from previously published results (Walker et al., 1992).
[g]LD_{50} from previously published results (Elonen et al., 1998).
[h]LD_{50} from previously published results (Tillitt et al., 2016).
[i]No classification is denoted by 'NA'.
[j]Average LD_{50} from previously published results (Elonen et al., 1998; Henry et al., 1997).
[k]LD_{50} from previously published results (Buckler et al., 2015).

Table C7.S2. Sequences and annealing temperatures of oligonucleotide primers used to sequence full-length AHRs.

Species	Target Gene	Primer Sequence (5'-3')	Annealing Temp (°C)
Lake Trout	AHR2a	Forward: ATGTTGAGTAACGCTGGAGTCTATGCTG	60
		Reverse: TTAGAAGTTGCAATAGTTGGTTTGGTTGTGCTCTG	
	AHR2b	Forward: ATGTTGGGGAGTACGGCG	50
		Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	
Brook Trout	AHR2a	Forward: ATGTTGAGTAACAACGCTGGAGTCTATGC	48
		Reverse: TTAGAAGTTGCAATAGTTGATTTGGTTGTGC	
	AHR2b	Forward: ATGTTGGGGAGTACGGCG	60
		Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	
Fathead Minnow	AHR1	Forward: ATGTACGCGGGACGAAAAAGAAGAA	48
		Reverse: TCAGAGGTATAAACCAGTAGTCTGAGGGTCTG	
	AHR2	Forward: ATGTCGAGGAGTATCGGTATCTATGCG	50
		Reverse: CTAATAGTCACAGCACTTGCTTTGGTTGTTCTC	
Japanese Medaka	AHR1	Forward: ATGTACGCCGGGCGCAAACG	60
		Reverse: AGTTTCTAAACAGGTGGCAGGGTTC	
	AHR2	Forward: ATGCTGTCCGGCACCGCCATGTA	60
		Reverse: CTTGTTCTCGGTAAAGCAGGTGTTCC	
White Sucker	AHR2	Forward: ATGTCGAGGAGTATCGGTATATATGCGGTC	48
		Reverse: CTAATAGTCACAGCAACTGCTTTGGTTGTTCTC	
Northern Pike	AHR1a	Forward: ATGAGCAGCAGCACATATGCCAGT	60
		Reverse: TTACAGCTGCCCTGCGGCGT	
	AHR1b	Forward: ATGTATGCTGGACGTAAAAGGAGAAAACC	60
		Reverse: CTAGTTCCACACCCCACTGGACTGGA	
	AHR2a	Forward: ATGCTAAGTAATGCTAGAGTATATGCTGTCAAGAA	60
		Reverse: TTAGAAGTCGCAATAGTTGGTTTGGTTG	
	AHR2b	Forward: ATGTTGGGGAGTACGGCG	60
		Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	

Table C7.S3. Sequences, annealing temperatures, and efficiencies of oligonucleotide primers used for quantitative real-time polymerase chain reaction (qRT-PCR).

Species	Target Gene	Primer Sequence (5'-3')	Efficiency (%)	Annealing Temp (°C)
Lake Trout	β-actin[a]	Forward: AGAGCTACGAGCTGCCTGAC	94	60
		Reverse: GCAAGACTCCATACCGAGGA		
	AHR2a	Forward: CCACAGAACCCCAACAGACT	106	60
		Reverse: ACGTGGCTGTTTCCATTAGG		
	AHR2b	Forward: ACAGTGAGTAACGTTCGGGG	101	60
		Reverse: GTCAGGGAAGGGTTAAAGGC		
Brook Trout	β-actin[a]	Forward: AGAGCTACGAGCTGCCTGAC	101	60
		Reverse: GCAAGACTCCATACCGAGGA		
	AHR2a	Forward: CCACAGAACCCCAACAGACT	97	60
		Reverse: ACGTGGCTGTTTCCATTAGG		
	AHR2b	Forward: ACAGTGAGTAACGTTCGGGG	108	60
		Reverse: GTCAGGGAAGGGTTAAAGGC		
Fathead Minnow	β-actin	Forward: GTGCCCATCTACGAGGGTTA	90	60
		Reverse: TCTCAGCTGTGGTGGTGAAG		
	AHR1	Forward: CAGCGTATGCAGCAACATCT	105	60
		Reverse: ACTGGAATGGGACAAGCAAC		
	AHR2	Forward: ACCTGGTGACACTTTCGTCC	98	60
		Reverse: TCCATTCTCTTGCACAGCAC		
Lake Sturgeon	β-actin[b]	Forward: CCGAGCACAATGAAAATCAA	90	60
		Reverse: ACATCTGCTGGAAGGTGGAC		
	AHR1[c]	Forward: GAATTGCGCCTTTTATCGAG	94	60
		Reverse: TTTGCACTTTTCTGCACTGG		
	AHR2[d]	Forward: TGGAGATCAGGACCAAGACC	90	60
		Reverse: GTGTAACCCAGCACCACCTT		
Japanese Medaka	β-actin	Forward: GAGACTTTCAACAGCCCTGC	97	60
		Reverse: GAGCGTAGCCCTCGTAGATG		
	AHR1	Forward: GCGAGCTATTTAGTGCCCTG	109	60
		Reverse: CTGGCCAGAGTTCAGGAGTC		
	AHR2	Forward: TCTCCCTCAGCCTTTTCTCA	100	60

		Reverse: TCCGTTACTGAAAACCGGAC		
Northern Pike	β-actin[e]	Forward: AGAGCTACGAGCTGCCTGAC	99	60
		Reverse: GCAAGACTCCATACCGAGGA		
	AHR1a	Forward: AACTCCCACAGGAGGATGTG	84	60
		Reverse: CTGACACCCAGGTTCCTCAT		
	AHR1b	Forward: CACAACAAAAGCACCCATTG	105	60
		Reverse: GAATGGACAATGGACATCCC		
	AHR2a	Forward: CGGCTTTCCAGCTATGTCTC	101	60
		Reverse: CAAACCTTTCGCTGGTGATT		
	AHR2b	Forward: CTCACAACCCCAGATCCACT	90	60
		Reverse: GTCCTTGTAGGCAGCTCGAC		

[a]Adapted from previously published results (Wiseman et al., 2011).

[b]Adapted from previously published results (Doering et al., 2015b).

[c]Adapted from previously published results (Doering et al., 2014a).

[d]Adapted from previously published results (Doering et al., 2012).

[e]Adapted from previously published results (Beitel et al., 2015).

Table C7.S4. Sequences and annealing temperatures of oligonucleotide primers used to produce expression constructs.

Species	Target Gene	Primer Sequence (5'-3')	Annealing Temp (°C)
Lake Trout	AHR2a	Forward: CACCATGTTGAGTAACGCTGGAGTCTATGCTG	60
		Reverse: GAAGTTGCAATAGTTGGTTTGGTTGTG	
	AHR2b	Forward: CACCATGTTGGGGAGTACGGCG	60
		Reverse: GAAGTTGCAACAGTTGGTTTGATTG	
Brook Trout	AHR2a	Forward: CACCATGTTGAGTAACGCTGGAGTCTATGCTG	60
		Reverse: GAAGTTGCAATAGTTGGTTTGGTTGTG	
	AHR2b	Forward: CACCATGTTGGGGAGTACGGCG	60
		Reverse: GAAGTTGCAACAGTTGGTTTGATTG	
Fathead Minnow	AHR1	Forward: CACCATGTACGCGGGACGAAAAAGAAGAA	60
		Reverse: GAGGTATAAACCAGTAGTCTGAGGGTCTGGG	
	AHR2	Forward: CACCATGTCGAGGAGTATCGGTATCTATGCG	60
		Reverse: ATAGTCACAGCACTTGCTTTGGTTGTTC	
Japanese Medaka	AHR1	Forward: CACCATGTACGCCGGGCGCAAACG	60
		Reverse: AGTTTCTAAACAGGTGGCAGGGTTC	
	AHR2	Forward: CACCATGCTGTCCGGCACCGCCATGTA	60
		Reverse: CTTGTTCTCGGTAAAGCAGGTGTTCC	
White Sucker	AHR2	Forward: CACCATGTCGAGGAGTATCGGTATCTATGCG	60
		Reverse: ATAGTCACAGCACTTGCTTTGGTTGTTC	
Northern Pike	AHR1a	Forward: CACCATGAGCAGCAGCACATATGCCAGT	60
		Reverse: CAGCTGCCCTGCGGCGTG	
	AHR1b	Forward: CACCATGTATGCTGGACGTAAAAGGAGAAAACC	60
		Reverse: GTTCCACACCCCACTGGACTGGAC	
	AHR2a	Forward: CACCATGCTAAGTAATGCTAGAGTATATGCTGTCAAGAA	60
		Reverse: GAAGTCGCAATAGTTGGTTTGGTTG	
	AHR2b	Forward: CACCATGTTGGGGAGTACGGCG	60
		Reverse: GAAGTCGCAACAGTTGGTTTGATTG	

Table C7.S5. Calculated effect concentrations (ECs) (nM) for sensitivity to activation by TCDD of AHR1s and AHR2s of fishes. Standard error of the mean (S.E.M.) is presented in brackets.

Species	Isoform	EC_{20}	EC_{50}	EC_{80}
Lake Trout	AHR2a	0.080 (± 0.04)	0.29 (± 0.07)	1.3 (± 0.07)
	AHR2b	0.021 (± 0.01)	0.066 (± 0.01)	0.27 (± 0.09)
Brook Trout	AHR2a	1.9 (± 0.3)	3.3 (± 0.4)	5.7 (± 0.6)
	AHR2b	0.10 (± 0.008)	0.28 (± 0.1)	1.1 (± 0.6)
Fathead Minnow	AHR1	0.033 (± 0.01)	0.11 (± 0.06)	0.78 (± 0.5)
	AHR2	0.12 (± 0.02)	0.65 (± 0.1)	3.8 (± 2)
Lake Sturgeon	AHR1[a]	0.0090 (± 0.005)	0.043 (± 0.01)	0.21 (± 0.04)
	AHR2[a]	0.30 (± 0.05)	0.79 (± 0.04)	2.1 (± 0.5)
Japanese Medaka	AHR1	1.3 (± 0.2)	3.7 (± 0.4)	11 (± 0.7)
	AHR2	1.2 (± 0.2)	4.6 (± 0.3)	19 (± 4)
White Sucker	AHR2	0.58 (± 0.3)	5.7 (± 0.7)	96 (± 40)
Northern Pike	AHR1a	0.17 (± 0.1)	0.32 (± 0.2)	1.7 (± 0.7)
	AHR1b	0.083 (± 0.009)	0.53 (± 0.2)	4.3 (± 2)
	AHR2a	0.11 (± 0.09)	0.30 (± 0.2)	1.1 (± 0.5)
	AHR2b	4.2 (± 0.7)	11 (± 2)	39 (± 14)

[a]Adapted from previously published results (Doering et al., 2015b).

Table C7.S6. Percent similarity of the putative, full-length amino acid sequence of AHR2a and AHR2b of lake trout based on the top hits from an NCBI Blast against the non-redundant protein sequence database.

	Species	Percent Similarity
Lake Trout AHR2a		
	Brook Trout AHR2a	98 %
	Atlantic Salmon AHR2β	96 %
	Rainbow Trout AHR2β	95 %
	Rainbow Trout AHR2α	93 %
	Atlantic Salmon AHR2α	80 %
	Northern Pike AHR2a	78 %
Lake Trout AHR2b		
	Brook Trout AHR2b	98 %
	Atlantic Salmon AHR2δ	95 %
	Atlantic Salmon AHR2γ	92 %
	Northern Pike AHR2b	72 %

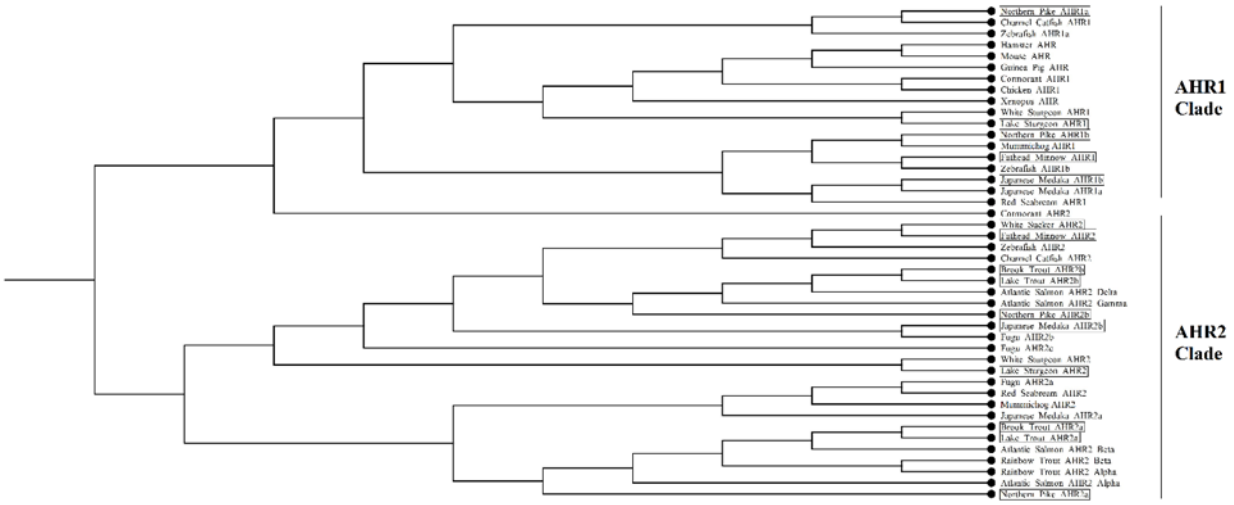


Figure C7.S1. Phylogenetic tree for relatedness of full-length amino acid sequences of AHRs across vertebrates. AHRs of fishes that were included in the linear regressions (Figure 3B and Figure 3C) are highlighted. Branch lengths represent bootstrap values based on 1000 samplings. Accession numbers used are: channel catfish AHR1 (*Ictalurus punctatus*; AHH42151.1); zebrafish AHR1a (*Danio rerio*; AAM08127.1); hamster AHR (*Mesocricetus auratus*; NP_001268587.1); mouse AHR (*Mus musculus*; NP_038492.1); guinea pig AHR (*Cavia porcellus*; NP_001166525.1); cormorant AHR1 (*Phalacrocorax carbo*; BAD01477.1); chicken AHR1 (*Gallus gallus*; NP_989449.1); Xenopus AHR (*Xenopus laevis*; JC7993); white sturgeon AHR1 (*Acipenser transmontanus*; AHX35737.1); lake sturgeon AHR1 (*Acipenser fulvescens*; AIV00618.1); mummichog AHR1 (*Funduus heteroclitus*; AAR19364.1); zebrafish AHR1b (*D. rerio*; AAI63508.1); Japanese medakafish AHR1a (*Oryzias latipes*; BAB62012.1); red seabream AHR1 (*Pagrus major*; BAE02824.1); cormorant AHR2 (*P. carbo*; BAF64245.1); zebrafish AHR2 (*D. rerio*; AAI63711.1); channel catfish AHR2 (*I. punctatus*; AHH42811.1); Atlantic salmon AHR2 delta (*Salmo salar*; NP_001117015.1); Atlantic salmon AHR2 gamma (*S. salar*; NP_001117037.1); fugu AHR2b (*Takifugu rubripes*; NP_001033052.1); fugu AHR2c (*T. rubripes* NP_001033047.1); white sturgeon AHR2 (*A. transmontanus*; KJ420395.1); lake sturgeon AHR2 (*A. fluvescens*; AIW39681.1); fugu AHR2a (*T. rubripes*; NP_001033049.1); red seabream AHR2 (*P. major*; BAE02825.1); mummichog AHR2 (*F. heteroclitus*; AAC59696.3); Japanese medaka AHR2a (*O. latipes*; XP_011488315.1); Atlantic salmon AHR2 beta (*S. salar*; NP_001117028.1); rainbow trout AHR2 beta (*Oncorhynchus mykiss*; NP_001117724.1); rainbow trout AHR2 alpha (*O. mykiss*; NP_001117723.1); Atlantic salmon AHR2 alpha (*S. salar*; NP_001117156.1). Phylogenetic tree was constructed by use of CLC Genomics Workbench v.7.0.4 (Katrinebjerg, Aarhus).

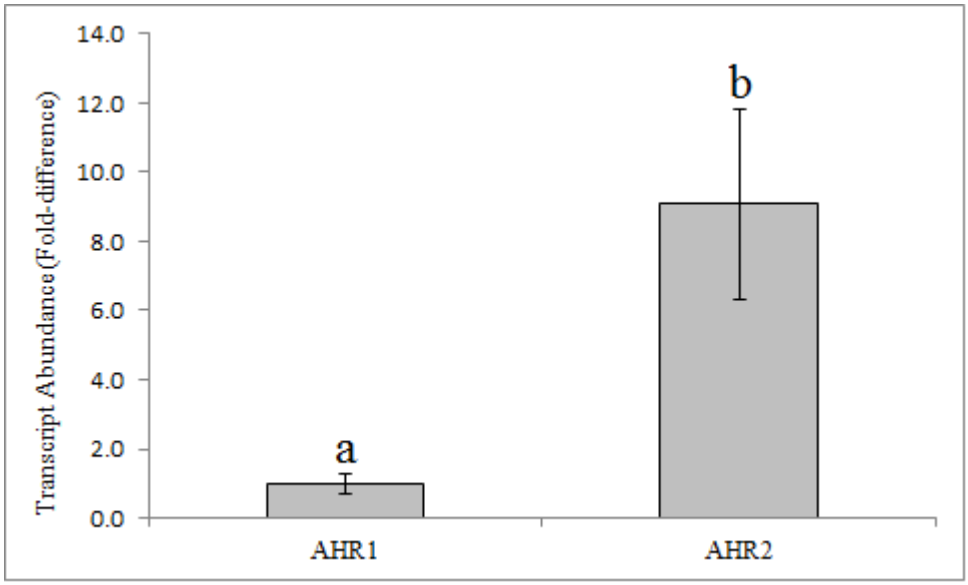


Figure C7.S2. Comparison of basal abundance of transcripts of AHR1 and AHR2 in livers of adult fathead minnow. Data represents mean ± standard error of the mean (S.E.M.) of six individuals (n = 3 males and n = 3 females). Different letters represent statistical difference determined by use of t-test ($p < 0.05$).

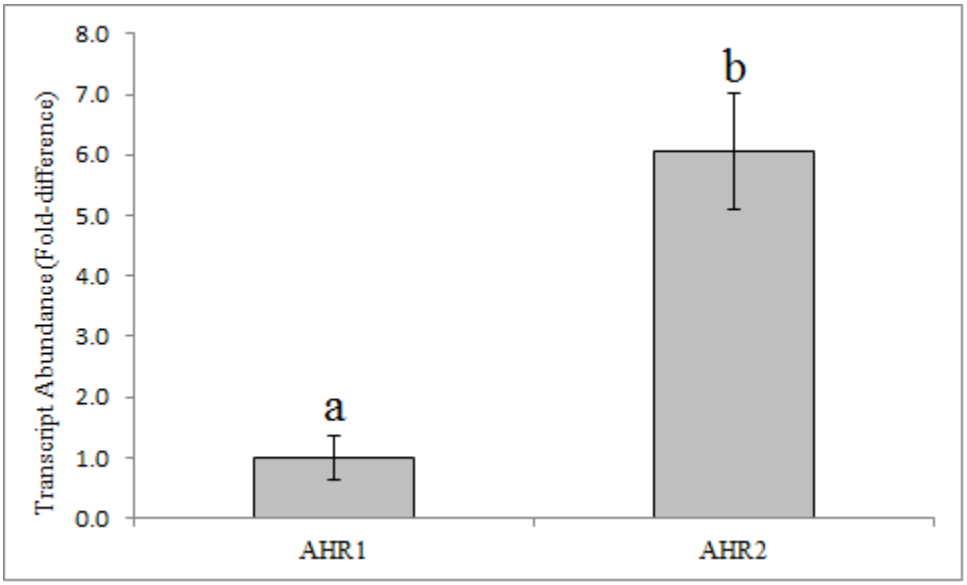


Figure C7.S3. Comparison of basal abundance of transcripts of AHR1 and AHR2 in livers of juvenile lake sturgeon. Data represents mean ± standard error of the mean (S.E.M.) of six individuals of unknown sex. Different letters represent statistical difference determined by use of t-test ($p < 0.05$).

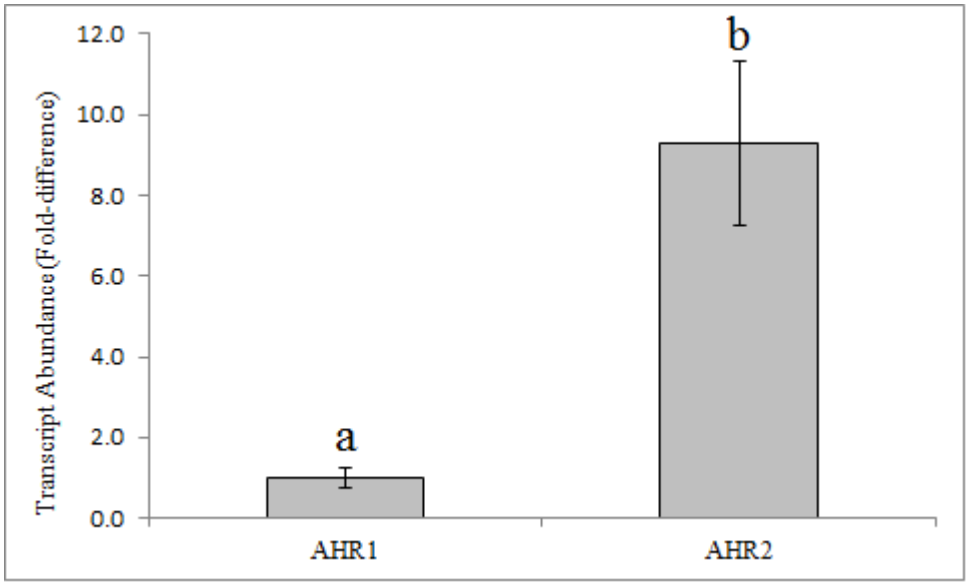


Figure C7.S4. Comparison of basal abundance of transcripts of AHR1 and AHR2 in livers of adult Japanese medaka. Data represents mean ± standard error of the mean (S.E.M.) on six individuals (n = 3 males and n = 3 females). Different letters represent statistical difference determined by use of t-test ($p < 0.05$).

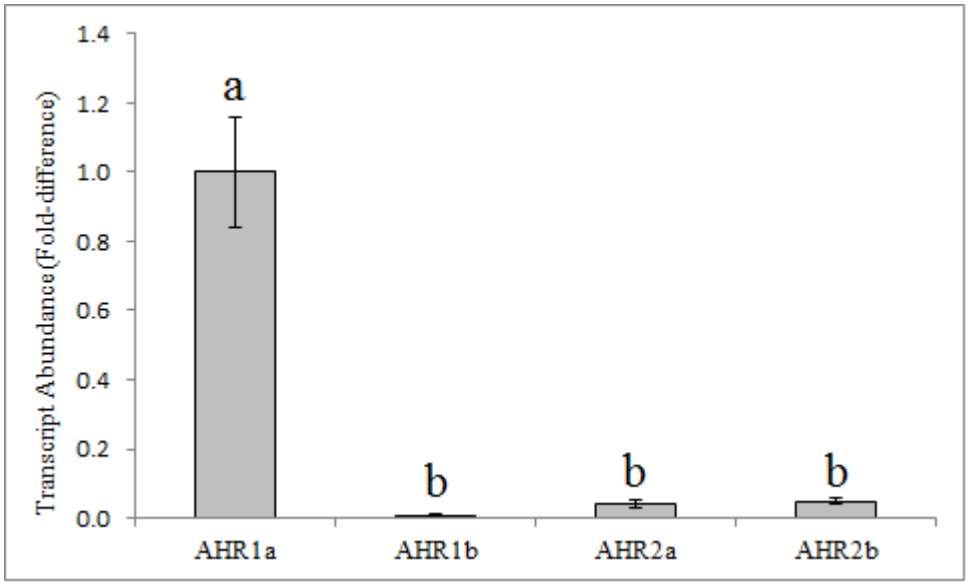


Figure C7.S5. Comparison of basal abundance of transcripts of AHR1a, AHR1b, AHR2a, and AHR2b in livers of adult northern pike. Data represents mean ± standard error of the mean (S.E.M.) of six individuals (n = 3 males and n = 3 females). Different letters represent statistical difference determined by use of analysis of variance (ANOVA) followed by Tukey's post-doc test ($p < 0.05$).

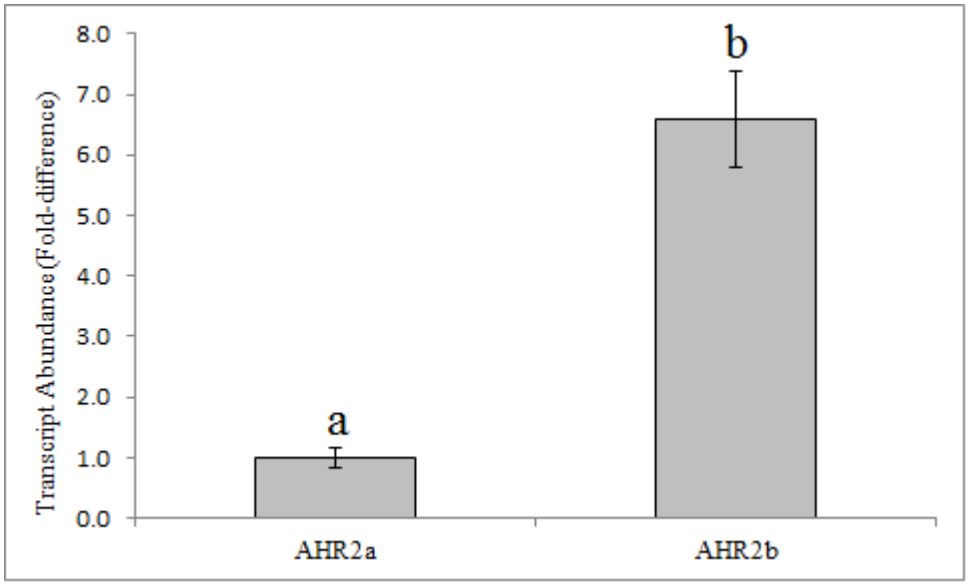


Figure C7.S6. Comparison of basal abundance of transcripts of AHR2a and AHR2b in livers of adult lake trout. Data represents mean ± standard error of the mean (S.E.M.) of six individuals (n = 3 males and n = 3 females). Different letters represent statistical difference determined by use of t-test ($p < 0.05$).

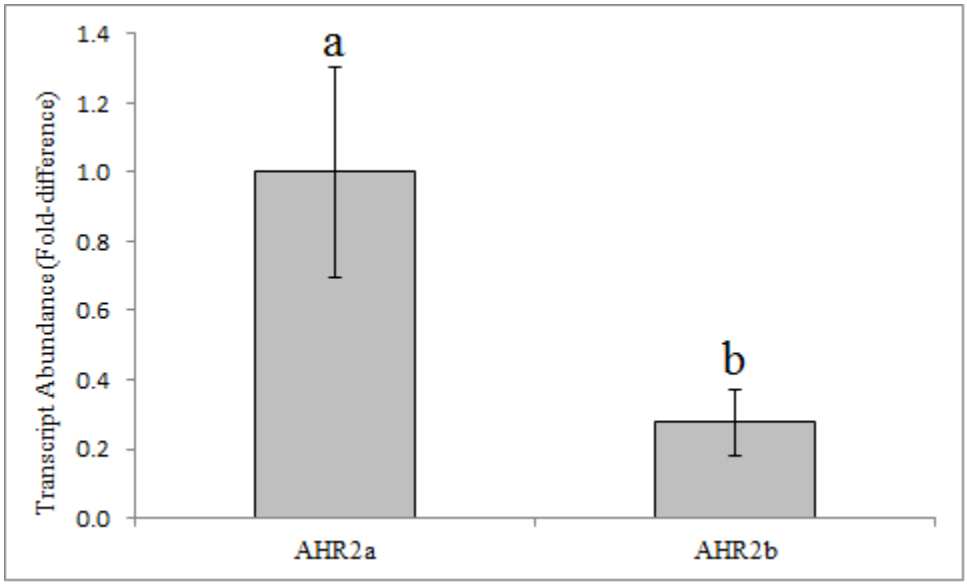
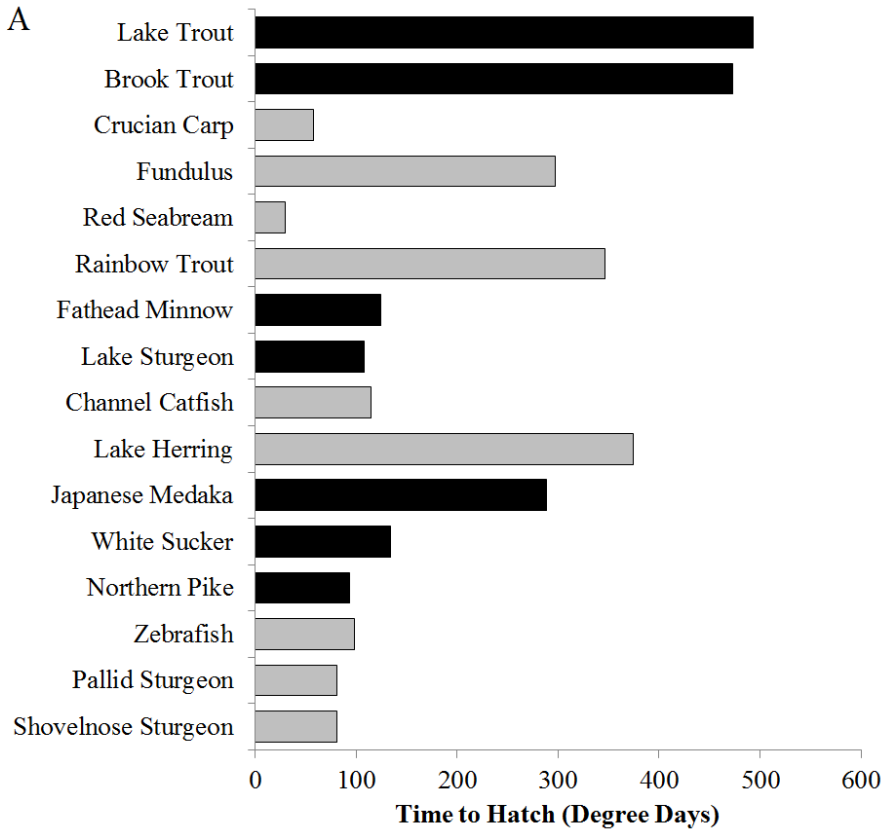


Figure C7.S7. Comparison of basal abundance of transcripts of AHR2a and AHR2b in livers of adult brook trout. Data represents mean ± standard error of the mean (S.E.M.) of six individuals (n = 3 males and n = 3 females). Different letters represent statistical difference determined by use of t-test ($p < 0.05$).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Fundulus
Red Seabream
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Japanese Medaka
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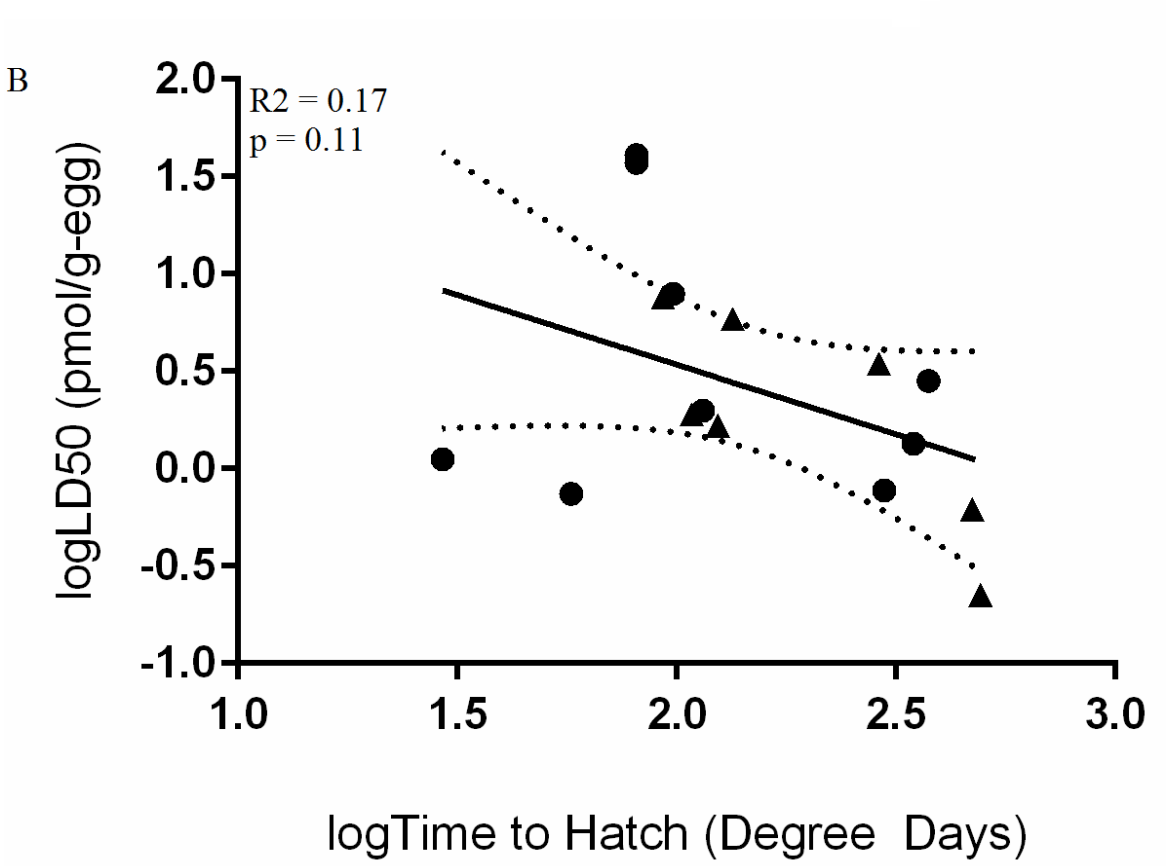
500
600
Time to Hatch (Degree Days)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p = 0.11
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1.5
1.0
0.5
0.0
-0.5
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logLD50 (pmol/g-egg)
1.0
1.5
2.0
2.5
3.0
logTime to Hatch (Degree Days)

Figure C7.S8. Average time to hatch in degree days for embryos of sixteen species of fish of known sensitivity to TCDD in order from most sensitive (top) to least sensitive (bottom) (A). Times to hatch and temperatures are based on descriptions given in studies of embryo toxicity or are based on the best estimates of the authors in cases that studies do not present time to hatch information. Species investigated in the study presented here are highlighted in black. Linear regression of sensitivity (LD_{50}) against average time to hatch (degree days) (B). Species investigated in the study presented here are indicated by a triangle. The equation of the line is Y=0.7153*X+1.965 with a slope of -0.72 ± 0.4 and *y*-intercept of 2.0 ± 0.9. The best fit line for the regression is indicated and 95 % confidence intervals are represented as dotted lines.

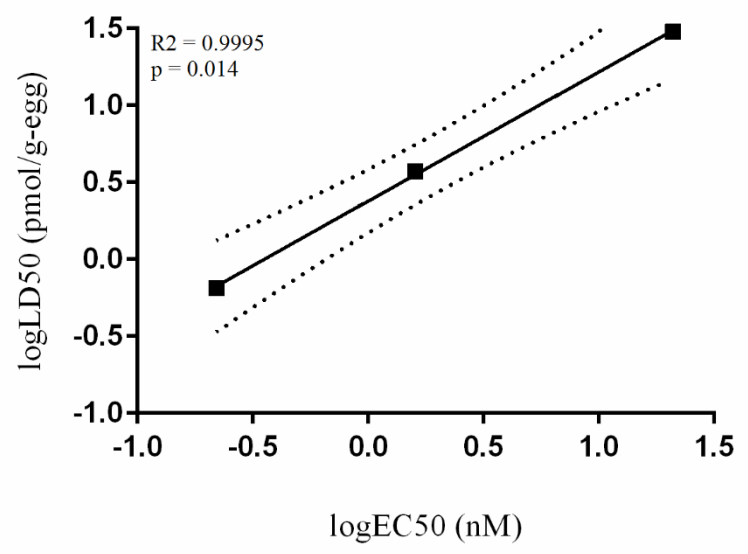

Figure C7.S9 Linear regression across chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), and Japanese quail (*Coturnix japonica*) for sensitivity to activation (EC_{50}) of AHR1 against sensitivity to TCDD of embryos (LD_{50}) (Cohen-Barnhouse et al., 2011; Farmahin et al., 2012). The equation of the line is Y=0.8394*X+0.3785 with a slope of 0.84 ± 0.02 and *y*-intercept of 0.38 ± 0.01. Best fit line for regression is indicated and 95 % confidence intervals are represented as dotted lines.