

# A Cross-species Quantitative Adverse Outcome Pathway for Activation of the Aryl Hydrocarbon Receptor Leading to Early Life Stage Mortality in Birds and Fishes

Jon A. Doering,<sup>\*,†</sup> Steve Wiseman,<sup>†,‡</sup> John P. Giesy,<sup>†,§</sup> and Markus Hecker<sup>†,||</sup>

<sup>†</sup>Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B3, Canada

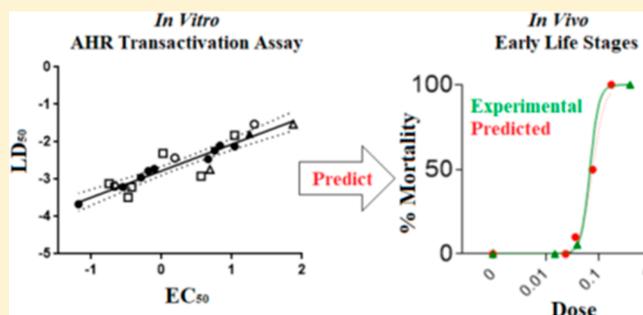
<sup>‡</sup>Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada

<sup>§</sup>Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

<sup>||</sup>School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C8, Canada

## Supporting Information

**ABSTRACT:** Dioxin-like compounds (DLCs) elicit adverse effects through activation of the aryl hydrocarbon receptor (AHR). Prior investigations demonstrated that sensitivity to activation of AHR1 in an in vitro AHR transactivation assay is predictive of early life stage mortality among birds. The present study investigated the link between sensitivity to activation of AHR1s and AHR2s and early life stage mortality among fishes. A significant, linear relationship was demonstrated between sensitivity to activation of AHR2 and early life stage mortality among nine fishes, while no relationship was found for AHR1. The slope and *y*-intercept for the linear relationship between sensitivity to activation of AHR1 and early life stage mortality in birds was not statistically different from the same relationship for AHR2 in fishes. Data for fishes and birds across DLCs were expanded into four significant, linear regression models describing the relationship between sensitivity to activation of AHR and the dose to cause early life stage mortality of 0%, 10%, 50%, or 100%. These four relationships were combined to form a quantitative adverse outcome pathway which can predict dose–response curves of early life stage mortality for DLCs to any bird or fish from species- and chemical-specific responses in an in vitro AHR transactivation assay.



## INTRODUCTION

Along with habitat loss and overexploitation, pollution is a primary cause for decreases in populations of wildlife. Currently, ecological risk assessments are hampered by dramatic differences in sensitivity to chemicals among a vast diversity of different species and the reality that all species of regulatory concern cannot be pragmatically evaluated. This is particularly true for ecological risk assessments of legacy contamination by a class of industrial chemicals known as dioxin-like compounds (DLCs). 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. DLCs share similarities in structure and bind with relatively great affinity to the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor that is thought to mediate most, if not all, adverse effects associated with exposure to DLCs in vertebrates through direct or indirect pleiotropic dysregulation of genes.<sup>1</sup> The most severe adverse effects occur in embryos of oviparous vertebrates and include craniofacial and cardiovascular malformations, pericardial and yolk sac edema, and early life stage mortality.<sup>2</sup> Despite all DLCs exerting toxicity via a single, specific, and highly

conserved mechanism, differences in relative sensitivity of embryos as great as 200-fold to the most studied DLC, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), exist both among and within vertebrate taxa.<sup>3–5</sup>

In 2007, the National Research Council (NRC) outlined a vision and strategy for toxicity testing in the 21<sup>st</sup> century.<sup>6</sup> The NRC envisioned a shift from whole-animal toxicity testing of apical effects to a focus on interactions of chemicals with specific molecular targets leading to perturbation of a physiological pathway.<sup>6</sup> This vision of predictive toxicology led to the development of a conceptual framework known as the adverse outcome pathway (AOP).<sup>7</sup> AOPs describe existing knowledge and weight of evidence supporting a defensible link between adverse apical effects of regulatory relevance, termed the adverse outcome, and the interaction of a chemical with a specific molecular target, termed the molecular initiating event.<sup>7</sup> The most advanced developments in AOPs, known as quantitative AOPs (qAOPs), have potential utility to quantitative

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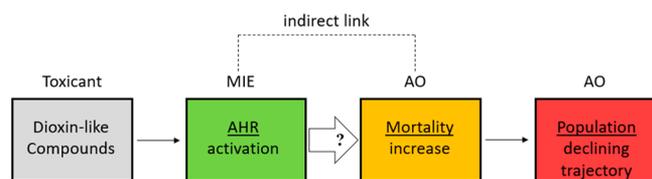
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ecological risk assessments.<sup>8–10</sup> A qAOP describes quantitative response–response relationships linking the molecular initiating event and adverse outcome to enable quantitative prediction of the probability of occurrence or severity of an adverse outcome for a given magnitude of chemical interaction with a molecular initiating event.<sup>8–10</sup> Depending upon the extent of mechanistic understanding and the needs in terms of regulatory application, a qAOP could be as simple as a linear regression that quantitatively links the molecular initiating event to the adverse outcome, or as complex as a consecutive series of nonlinear models which describe responses at several levels of biological organization and simulate associated internal and external modifying factors.<sup>8,9</sup> However, because of the time and resources required to describe even the simplest qAOPs, few have been developed and none have been validated beyond a small subset of laboratory model species, which might not be representative of the diversity of native species of regulatory concern.<sup>8,10–12</sup>

Activation of the AHR is believed to cause early life stage mortality in birds and fishes through circulatory failure as a result of cardiovascular teratogenesis.<sup>13,14</sup> Several plausible mechanisms by which activation of the AHR might cause cardiovascular teratogenesis have been investigated, including through dysregulation of vascular endothelial growth factor (VEGF), members of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily, cyclooxygenase-2 (COX-2), or through production of AHR-dependent reactive oxygen species (ROS).<sup>13,15,16</sup> However, the precise series of events which link activation of the AHR to early life stage mortality are currently unknown because the AHR directly or indirectly regulates thousands of genes in numerous physiological pathways (Figure 1).<sup>17–19</sup>



**Figure 1.** Adverse outcome pathway (AOP) for activation of the aryl hydrocarbon receptor (AHR) by dioxin-like compounds (DLCs) as the molecular initiating event (MIE) leading to an increase in early life stage mortality and declining population trajectory as adverse outcomes (AOs). The precise series of events which link the MIE and AO is not known, as indicated. However, an indirect, quantitative link between the MIE and AO was proposed in the study presented here in order to address this uncertainty and enable development of a quantitative AOP (qAOP).

This uncertainty in key events and the fact that comparable quantitative data are unavailable for most end points makes it impossible to describe a complete qAOP for activation of the AHR leading to early life stage mortality at present. However, results of prior investigations suggest an indirect, quantitative link between sensitivity to activation of the AHR and sensitivity to early life stage mortality among birds (Figure 1).<sup>20–23</sup> Specifically, *in vitro* AHR transactivation assays using COS-7 cells transfected with the AHR1 isoform of different species of birds has been used to explain the more than 40-fold difference in sensitivity to TCDD, and other DLCs, observed within this taxonomic group.<sup>20–23</sup> However, whether differences in transactivation of AHR isoforms (AHR1 or AHR2) explain differences in sensitivity to DLCs among other vertebrates was unknown.<sup>5</sup> Therefore, this study identified the specific

mechanism for the almost 200-fold difference in sensitivity observed within the largest group of vertebrates, the fishes.<sup>3,5</sup> Specifically, this study investigated sensitivity to activation by TCDD in an *in vitro* AHR transactivation assay for seven AHR1s and 12 AHR2s among nine species of fish for which sensitivities of embryos to TCDD were known. The nine species were lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*), red seabream (*Pagrus major*), fathead minnow (*Pimephales promelas*), lake sturgeon (*Acipenser fulvescens*), Japanese medaka (*Oryzias latipes*), white sucker (*Catostomus commersonii*), northern pike (*Esox lucius*), and zebrafish (*Danio rerio*). These nine species span an almost 40-fold difference in sensitivity to TCDD (Figure S1 of the Supporting Information, SI), comprise six orders and seven families, and include both freshwater and marine and both teleost and nonteleost species. Mechanistic understanding among chicken (*Gallus gallus*), pheasant (*Phasianus colchicus*), and quail (*Coturnix japonica*) described by previous studies<sup>21,23</sup> and the nine fishes described here was then used to construct a qAOP describing activation of the AHR indirectly leading to early life stage mortality among birds and fishes (Figure 1). This qAOP can be used to guide more objective ecological risk assessments of native species to exposure to DLCs and demonstrates the potential of 21<sup>st</sup> century approaches in predictive toxicology proposed by the NRC.

## MATERIALS AND METHODS

### Identification, Sequencing, and Phylogeny of AHRs.

Sequences of AHRs had not yet been identified for lake trout, brook trout, fathead minnow, white sucker, or northern pike. AHRs were isolated from samples obtained from each species (Table S1). Total RNA was extracted from approximately 30 mg of liver by use of the RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON). First-strand cDNA was synthesized from 1  $\mu$ g total RNA by use of the QuantiTect Reverse Transcription Kit (Qiagen). Full-length products were amplified by use of the LongRange PCR Kit (Qiagen) with gene-specific primers (Table S2) that were designed from partial sequences obtained from a combination of degenerate primers, rapid amplification of cDNA ends (RACE) PCR, transcriptome sequencing,<sup>24,25</sup> and published sequences in the National Center for Biotechnology Information (NCBI) database. Purified full-length products were cloned into pGEM-T easy vectors by use of a DNA ligation kit (Invitrogen, Burlington, ON) 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 National Center for Biotechnology Information (NCBI) database (Table S2).

### 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) and pcDNA 3.2/V5-DEST gateway vector kit (Invitrogen) according to methods described previously<sup>21,26</sup> using gene-specific primers (Table S3).

**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,<sup>21</sup> with minor modifications.<sup>26</sup> Amounts of expression constructs transfected into cells were

8 ng of AHR, 1.5 ng of white sturgeon ARNT<sub>2</sub>,<sup>26</sup> 20 ng of rat CYP1A1 reporter construct (pGudLuc 6.1),<sup>27,28</sup> 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). The LRG assay had previously been performed for AHRs from birds,<sup>21–23</sup> red seabream,<sup>29</sup> lake sturgeon,<sup>30</sup> and zebrafish.<sup>31,32</sup>

**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. Lowest observed effect concentrations (LOECs) were defined as the least dose of TCDD that caused an effect that was statistically significant ( $p \leq 0.05$ ) from response of the DMSO control. Comparison of slopes and  $y$ -intercepts for linear regressions were performed by use of analysis of covariance (ANCOVA).

**Quantitative Adverse Outcome Pathway Development.** Relationships between the dose to cause mortality of 0% (LD<sub>0</sub>), 10% (LD<sub>10</sub>), 50% (LD<sub>50</sub>), or 100% (LD<sub>100</sub>) and EC<sub>50</sub> were developed based on data from birds and fishes for one PCDD, two PCDFs, and two coplanar PCBs from the published literature.<sup>4,33–38</sup> All values used in development of the qAOP are provided (Tables S4 and S5). The LD<sub>0</sub>, LD<sub>10</sub>, LD<sub>50</sub>, and LD<sub>100</sub> were chosen because sufficient information for these points could be extracted from the published literature enabling robust linear regressions. Data were only used if the study reported exposure measurements and % mortality, performed statistical analysis, and had matching LRG data not involving mutant constructs. Data for LD<sub>0</sub>, LD<sub>10</sub>, and LD<sub>100</sub> were only used for studies with greater than five doses. LD<sub>0</sub> was defined as the greatest concentration where mortality is not statistically different from controls. LD<sub>10</sub> was used if reported, otherwise was defined as the least dose where mortality was statistically different from controls. LD<sub>50</sub> was only used if reported. LD<sub>100</sub> was defined as the least dose where mortality was not statistically different from 100%. Predicted dose–response curves for early life stage mortality were validated against experimental dose–response curves from the published literature that reported exposure concentrations and % mortality.<sup>4,33–35,37,39–44</sup> Control mortality in the experimental dose–response curves were normalized to 0% by use of GraphPad Prism 6 software. Predictions were evaluated against experimental results based on fold-difference (FD), mean absolute error (MAE), root-mean-square error (RMSE), and mean absolute percent error (MAPE).

## RESULTS

### Identification of AHR1s and AHR2s of Fishes.

Sequences of AHRs had not yet been identified for most fishes of known sensitivity of embryos to TCDD. A single AHR1 was

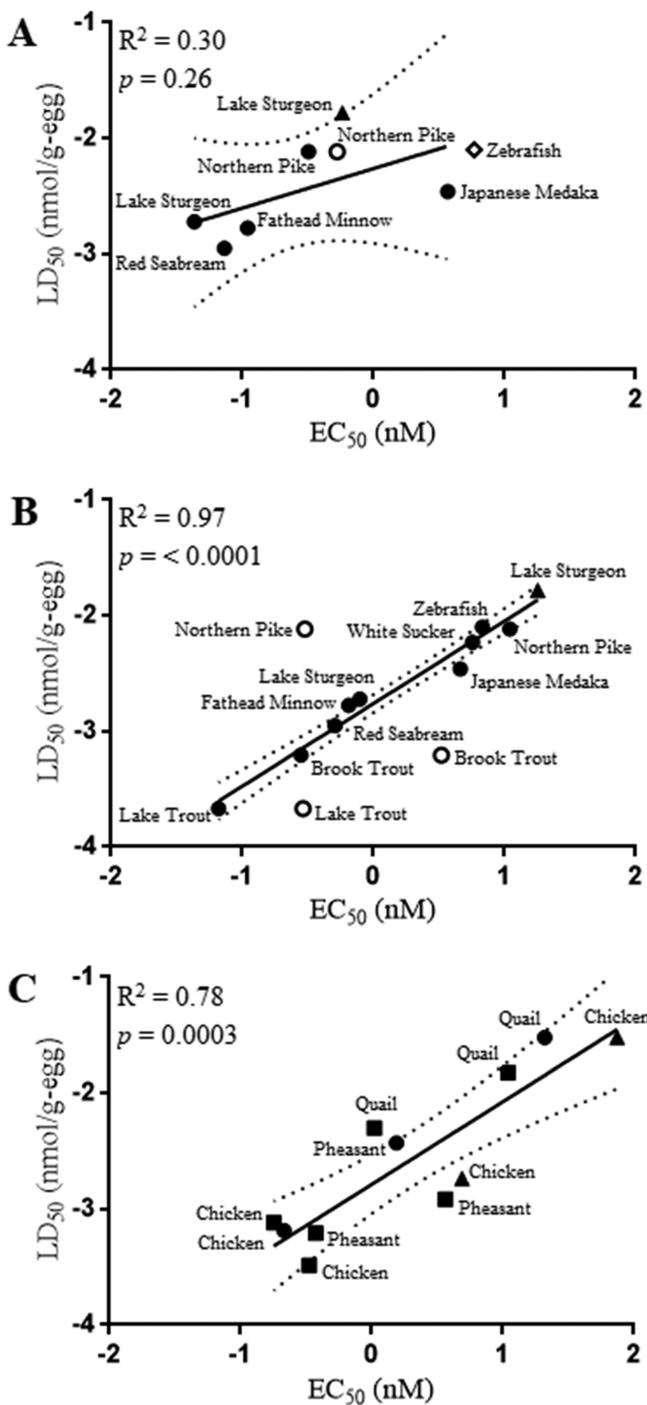
identified in fathead minnow and two AHR1s (AHR1a and AHR1b) were identified in northern pike. Two AHR2s (AHR2a and AHR2b) were identified in lake trout, brook trout, and northern pike, while a single AHR2 was identified in fathead minnow and white sucker. Putative amino acid sequences for AHR2a of lake trout, brook trout, and northern pike clustered most closely with sequences for AHR2 $\alpha$  and AHR2 $\beta$  of previously investigated salmonids, while sequences for AHR2b of lake trout, brook trout, and northern pike clustered most closely with sequences for AHR2 $\delta$  and AHR2 $\gamma$  (Figure S2 and Table S6).

**Activation of AHR1 and AHR2 of Fishes In Vitro.** All AHR1s and AHR2s that were investigated here were activated in a concentration-dependent manner by exposure to TCDD (Figures S3, S4, and Table S7). There was no significant, linear relationship ( $R^2 = 0.30$ ;  $p = 0.26$ ) between EC<sub>50</sub>s for activation of AHR1 and LD<sub>50</sub>s in early life stages of fishes (Figure 2A). A significant, linear relationship ( $R^2 = 0.97$ ;  $p \leq 0.0001$ ) was observed between EC<sub>50</sub>s for activation of AHR2s and LD<sub>50</sub>s in fishes (Figure 2B). AHR2b of lake trout, brook trout, and northern pike fit the linear relationship, while AHR2a did not (Figure 2B).

**Development and Validation of a Quantitative Adverse Outcome Pathway.** The slope and  $y$ -intercept for the linear relationship for AHR2s of fishes (Figure 2B; slope = 0.72;  $y$ -intercept =  $-2.8$ ) is not statistically different ( $p = 0.99$  and  $p = 0.82$ , respectively) from the slope and  $y$ -intercept for the linear relationship for AHR1s of birds (Figure 2C; slope = 0.71;  $y$ -intercept =  $-2.8$ ). Since the slopes and  $y$ -intercepts were not different, cross-chemical, -species, and -taxa data were combined for four points on the dose–response curve. Statistically significant, linear relationships were observed between EC<sub>50</sub>s for activation of AHR1 of birds or AHR2 of fishes and LD<sub>0</sub> ( $R^2 = 0.85$ ;  $p \leq 0.0001$ ), LD<sub>10</sub> ( $R^2 = 0.91$ ;  $p \leq 0.0001$ ), LD<sub>50</sub> ( $R^2 = 0.85$ ;  $p \leq 0.0001$ ), and LD<sub>100</sub> ( $R^2 = 0.86$ ;  $p \leq 0.0001$ ) (Figure 3). These four linear relationships were combined into a qAOP capable of predicting dose–response curves for early life stage mortality for DLCs using only the molar mass of the DLC and the chemical- and species-specific EC<sub>50</sub> for AHR1 for birds or AHR2 for fishes in an in vitro AHR transactivation assay as input variables. Predicted dose–response curves were compared to normalized experimental dose–response curves from the published literature for twenty-seven scenarios representing nine species of birds, eight species of fish, and up to five different DLCs (Figures 4 and S5). The qAOP produced predictions which are in good agreement with experimental dose–response curves across the 27 scenarios with an average difference of 2.2-fold between predicted and experimental results (Table S8). Calculated MAE, RMSE, and MAPE values also suggest a good fit model (Table S8). The final qAOP model is provided (Data Set S1).

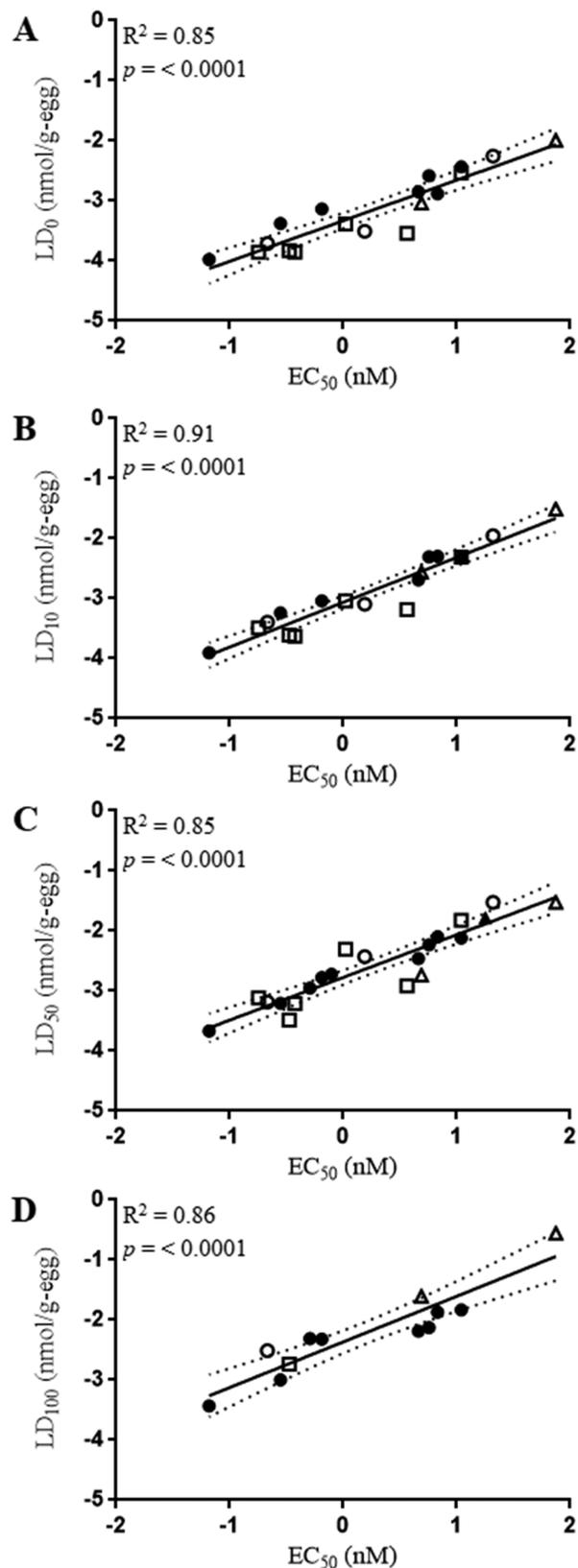
## DISCUSSION

Accurate ecological risk assessments are limited by several challenges, including the need to assess potential adverse effects to a wide range of species that can exhibit vastly different sensitivities to pollutants. To address the need for methods to assess sensitivities to DLCs in native species of regulatory concern, this study built on the vision of 21<sup>st</sup> century predictive toxicology outlined by the NRC by demonstrating an indirect, quantitative relationship between sensitivity to interaction of chemicals with the AHR and sensitivity to early life stage mortality among birds and fishes. This mechanistic understanding among species enabled development of a cross-species qAOP capable of accurately

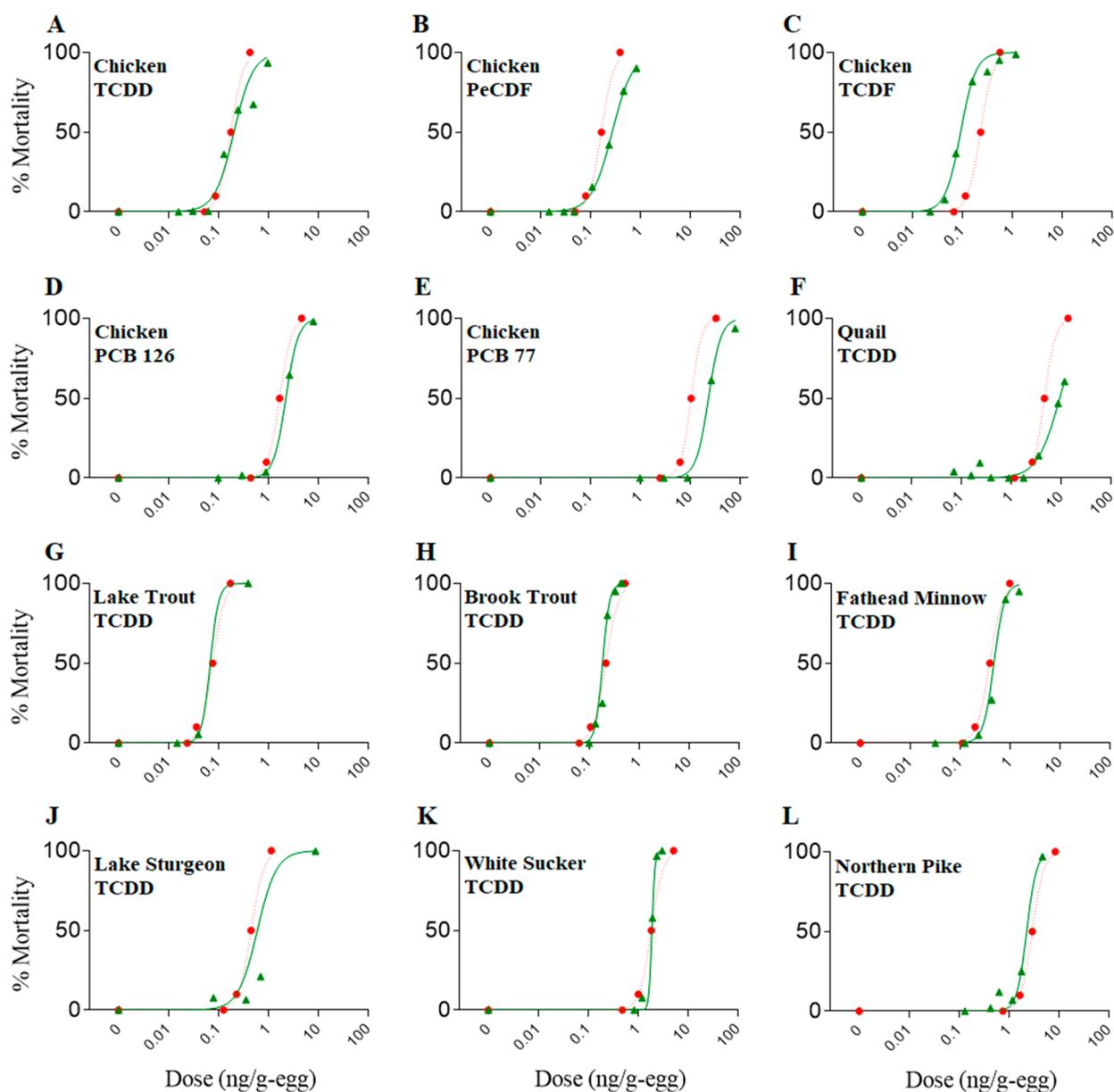


**Figure 2.** Linear regressions for early life stage mortality (LD<sub>50</sub>) against sensitivity to activation (EC<sub>50</sub>) of AHR1 of fishes (A), AHR2 of fishes (B), and AHR1 of birds (C) by PCDDs (circle), PCDFs (square), and coplanar PCBs (triangle). Isoform a (open) and isoform b (closed) are shown for species with multiple isoforms. Isoform a is not included in the regressions. AHR1b of zebrafish (open diamond) is shown (A) but is not included in the regression because it does not mediate early life stage mortality.<sup>52</sup> Best fit line and 95% confidence intervals are shown. Equations for the lines are  $y = 0.3416 \times x - 2.262$  (A),  $y = 0.714 \times x - 2.761$  (B), and  $y = 0.7126 \times x - 2.787$  (C).

predicting dose–response curves of early life stage mortality for species of birds and fishes to DLCs from the chemical molar mass and the chemical- and species-specific responses in an in vitro AHR transactivation assay as input variables.



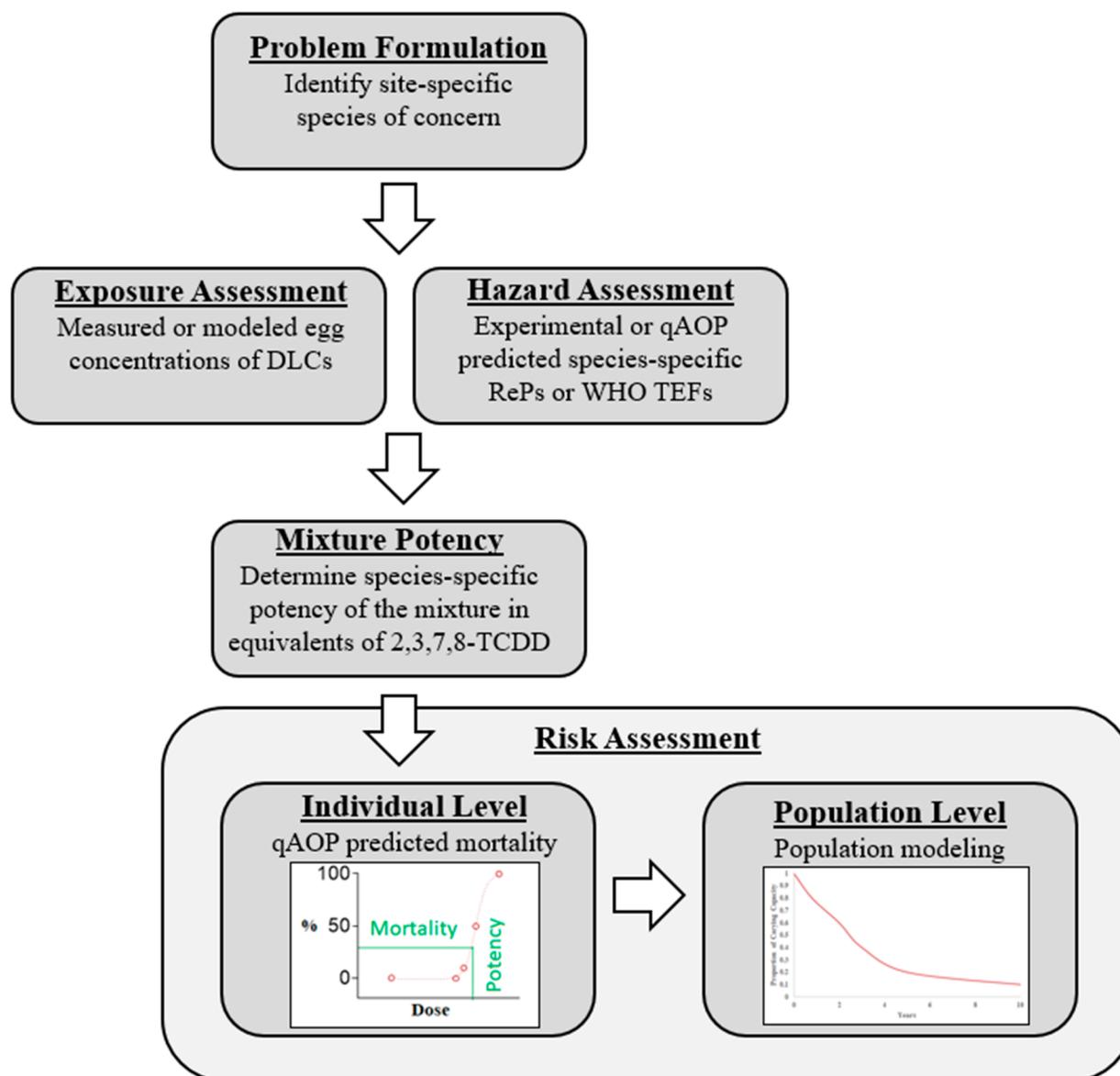
**Figure 3.** Linear regressions for early life stage mortality as LD<sub>0</sub> (A), LD<sub>10</sub> (B), LD<sub>50</sub> (C), and LD<sub>100</sub> (D) against sensitivity to activation (EC<sub>50</sub>) of AHR1s of birds (open) and AHR2s of fishes (solid) by PCDDs (circle), PCDFs (square), and coplanar PCBs (triangle). Best fit line and 95% confidence intervals shown. Equations for the lines are  $y = 0.6764 \times x - 3.33$  (A),  $y = 0.7471 \times x - 3.063$  (B),  $y = 0.7123 \times x - 2.775$  (C), and  $y = 0.7599 \times x - 2.365$  (D).



**Figure 4.** Dose–response curves for normalized experimental early life stage mortality data from the published literature (green triangle) and predicted using the qAOP (red circle) for birds (A–F) and fishes (G–L) exposed to 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), or 3,3',4,4'-tetrachlorobiphenyl (PCB 77). Best fit lines shown for both experimental and predicted dose–response curves. An additional 15 dose–response curve comparisons are presented elsewhere (Figure S5).

**Role of AHR Isoforms in Eliciting Early Life Stage Mortality.** An almost 200-fold difference in sensitivity to TCDD has been observed among the 17 species of fish investigated to date (Figure S1). However, the mechanism for these differences was unknown. In birds, the AHR1 isoform mediates early life stage mortality, while the AHR2 is thought to have little or no role in mediating critical toxicities.<sup>21–23</sup> In contrast to birds, the AHR2 isoform is thought to be the primary mediator of early life stage mortality in fishes, while the AHR1 isoform is thought to have little or no role in mediating critical toxicities. 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 isoforms in fishes. (1) In all fishes studied to date,

expression of AHR2 is ubiquitous among tissues, while expression of AHR1 is localized in brain, liver, heart, and gonad.<sup>45–48</sup> This suggests a more specialized role of AHR1 relative to AHR2. (2) Binding affinity for DLCs or sensitivity to activation in transactivation assays are greater for AHR2 than for AHR1 in both zebrafish and mummichog.<sup>31,45,47,49</sup> (3) Knockdown of expression of AHR2 prevents critical toxicities of DLCs and other AHR agonists in zebrafish and mummichog,<sup>50–55</sup> while knockdown of expression of AHR1 does not alter critical toxicities.<sup>50</sup> Despite this strong evidence for AHR2 driving critical toxicities of DLCs in zebrafish and mummichog, greater sensitivity to activation of AHR1 relative to AHR2 in red seabream and sturgeons (*Acipenser* spp.) suggested a possible role of AHR1 in mediating critical toxicities of DLCs in at least some fishes.<sup>26,29,30</sup>



**Figure 5.** Flowchart demonstrating a framework for application of the qAOP for activation of the AHR leading to early life stage mortality to ecological risk assessment of birds and fishes to exposure to complex mixtures of PCDDs, PCDFs, and coplanar PCBs.

In contrast to AHR1 of zebrafish and mummichog, AHR1 of all fishes investigated in the present study had greater sensitivity to activation by TCDD relative to AHR2 (Table S7). However, there is no significant, linear relationship between sensitivity to activation of AHR1 (as  $EC_{50}$ ) and early life stage mortality (as  $LD_{50}$ ) among these fishes (Figure 2A). But, it cannot be excluded that in some species of fish, activation of AHR1 by DLCs might result in certain subtle, sublethal, or tissue-specific adverse effects that do not correlate with early life stage mortality. In contrast to AHR1s, a statistically significant, linear relationship was observed between sensitivity to activation of AHR2 (as  $EC_{50}$ ) and early life stage mortality (as  $LD_{50}$ ) among these fishes (Figure 2B). Further, in the three investigated species with multiple AHR2 isoforms, namely lake trout, brook trout, and northern pike, the sensitivity to activation of the AHR2a isoform (most similar to AHR2 $\beta$ ) did not fit the linear relationship of AHR2s of the other fishes, while sensitivity of the AHR2b isoform (most similar to AHR2 $\delta$ ) did (Figure 2B). Results of previous studies have demonstrated comparable

sensitivities among AHR2 $\alpha$ , AHR2 $\delta$ , and AHR2 $\gamma$  isoforms in *in vitro* AHR transactivation assays, while sensitivity of AHR2 $\beta$  is different.<sup>56–58</sup> The study presented here could not distinguish whether the AHR2 $\alpha$ , AHR2 $\delta$ , or AHR2 $\gamma$  isoform, or a combination of isoforms, mediates early life stage mortality in these fishes as only AHR2 $\beta$  and AHR2 $\delta$  isoforms could be isolated here. However, if AHR2 $\alpha$ , AHR2 $\delta$ , and AHR2 $\gamma$  isoforms share comparable sensitivities to activation by DLCs, then knowledge of the specific mediator might not be important to predicting early life stage mortality. Overall, these results strongly support the hypothesis that AHR2 mediates early life stage mortality among phylogenetically diverse fishes, while AHR1 appears to have little or no role in mediating early life stage mortality.

**Defining the Domain of Applicability.** The study presented here demonstrates a mechanistic understanding between sensitivity to activation of AHR by PCDDs, PCDFs, and coplanar PCBs and sensitivity to early life stage mortality (Figure 2) which was used to develop a cross-species qAOP with demonstrated taxonomic applicability among phylogenetically diverse species

of birds and fishes (Figures 3 and 4). There are at least 10 000 species of birds and 34 000 fishes meaning this qAOP has probable applicability to more than 44 000 different species. Reptiles and amphibians bridge the evolutionary gap between birds and fishes. Studies suggest that the insensitivity to activation of AHR1 by DLCs might be predictive of the tolerance to early life stage mortality among amphibians, but insufficient information is currently available.<sup>59–63</sup> No information is currently available for sensitivity of AHRs or sensitivity to early life stage mortality of reptiles to DLCs. However, reptiles appear to express both AHR1 and AHR2 in common with birds and fishes,<sup>64,65</sup> while there is currently no evidence for an AHR2 in amphibians.<sup>66,67</sup> Regardless, considering the same quantitative relationship is present between activation of the AHR and early life stage mortality among evolutionarily divergent birds and fishes, it can be hypothesized that the same quantitative relationship also holds true among reptiles and amphibians. Therefore, this qAOP could have taxonomic applicability to more than 61 000 different species, if applicable to the 10 000 species of reptiles and 7000 amphibians. No information is currently available to support or refute whether this cross-species qAOP is applicable to agonists of the AHR other than PCDDs, PCDFs, and coplanar PCBs, but it is likely to have diverse chemical applicability.

**Utility of the Quantitative Adverse Outcome Pathway to Ecological Risk Assessment.** Ecological risk assessments and policy decisions are currently based on toxicological responses measured in a small number of model test species and extrapolation of those responses to native species of regulatory concern, including threatened or endangered species. The qAOP developed here can predict full dose–response curves of early life stage mortality for DLCs to any species of bird or fish from species- and chemical-specific responses in an *in vitro* AHR transactivation assay. The *in vitro* AHR transactivation assays are quick and inexpensive to perform relative to standard toxicity assays with embryos and can predict dose–response curves of early life stage mortality to within 2.2-fold of experimental results on average. This accuracy is comparable to the difference of up to 1.9-fold among four replicated dose–response curves of early life stage mortality from TCDD performed with lake trout.<sup>82,68–70</sup> Also, species-specific AHR constructs used in the *in vitro* AHR transactivation assay can be generated from nonlethal samples, such as blood, scales, or feathers, and biopsied or cold stored archival samples, and from as few as a single individual.<sup>71,72</sup> This not only provides an animal alternative approach to ecological risk assessments of DLCs for birds and fishes, but also enables the possibility of predicting dose–response curves of early life stage mortality for threatened or endangered species which often cannot be subjected to standard toxicity testing for ethical or practical reasons, yet information on sensitivities of threatened or endangered species is of interest to regulators.

Legacy contamination by DLCs consists of complex mixtures of PCDD, PCDF, and coplanar PCB congeners of varying potencies. To address this complexity, the toxic equivalency factor (TEF) approach was developed for the World Health Organization (WHO) based on the assumption of additive toxicity of DLCs due to a shared mechanism of toxicity.<sup>73</sup> However, TEFs are order of magnitude consensus values for the potency of a DLC relative to TCDD and are most useful for risk characterization and prioritization of areas of concern because differences in relative potencies among species can range by more than an order of magnitude, which introduces

significant uncertainty.<sup>4,74</sup> In contrast, the qAOP developed here can be used to accurately predict species-specific sensitivities to DLCs and therefore offers the possibility of a pragmatic species-specific framework for ecological risk assessments of mixtures of DLCs to birds and fishes (Figure 5). Concentrations of PCDD, PCDF, and coplanar PCB congeners in embryos can be acquired for site-specific species of concern through chemical quantification or can be predicted through chemical modeling or use of surrogate species (Figure 5). Species-specific relative potencies for identified congeners or a subset of congeners of greatest concern can then be generated using *in vitro* AHR transactivation assays and the qAOP (Figure 5). Using concentrations of congeners and predicted species-specific relative potencies for those congeners, toxic equivalents (TEQs) as TCDD equivalents can be generated and input into the qAOP dose–response output for TCDD to predict percent mortality expected due to the site-specific exposure to the species of concern (Figure 5). This individual level mortality information can then be used to inform the ecological risk assessment or further input into suitable population models (Figure 5). This framework demonstrates the immediate utility of this qAOP in guiding more objective ecological risk assessments of native species to legacy contamination by DLCs and demonstrates the potential of predictive toxicology and how these approaches can revolutionize ecological risk assessments in the 21<sup>st</sup> century.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b01438.

The final qAOP model (XLSX)

Additional information on construction of the qAOP, including sequencing of AHRs, dose–response curves for activation of fish AHRs, raw data used in development of the qAOP, and detailed model evaluation metrics (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: jad929@mail.usask.ca (J.A.D.).

### ORCID

Jon A. Doering: 0000-0001-6866-6456

### Notes

The authors declare no competing financial interest.

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## Supporting Information

### **A Cross-Species Quantitative Adverse Outcome Pathway for Activation of the Aryl Hydrocarbon Receptor Leading to Early Life Stage Mortality in Birds and Fishes**

Jon A. Doering,<sup>\*,†</sup> Steve Wiseman,<sup>†,‡</sup> John P. Giesy,<sup>†,§</sup> Markus Hecker<sup>†,||</sup>

<sup>†</sup> Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B3, Canada

<sup>‡</sup> Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, T1K 3M4, Canada

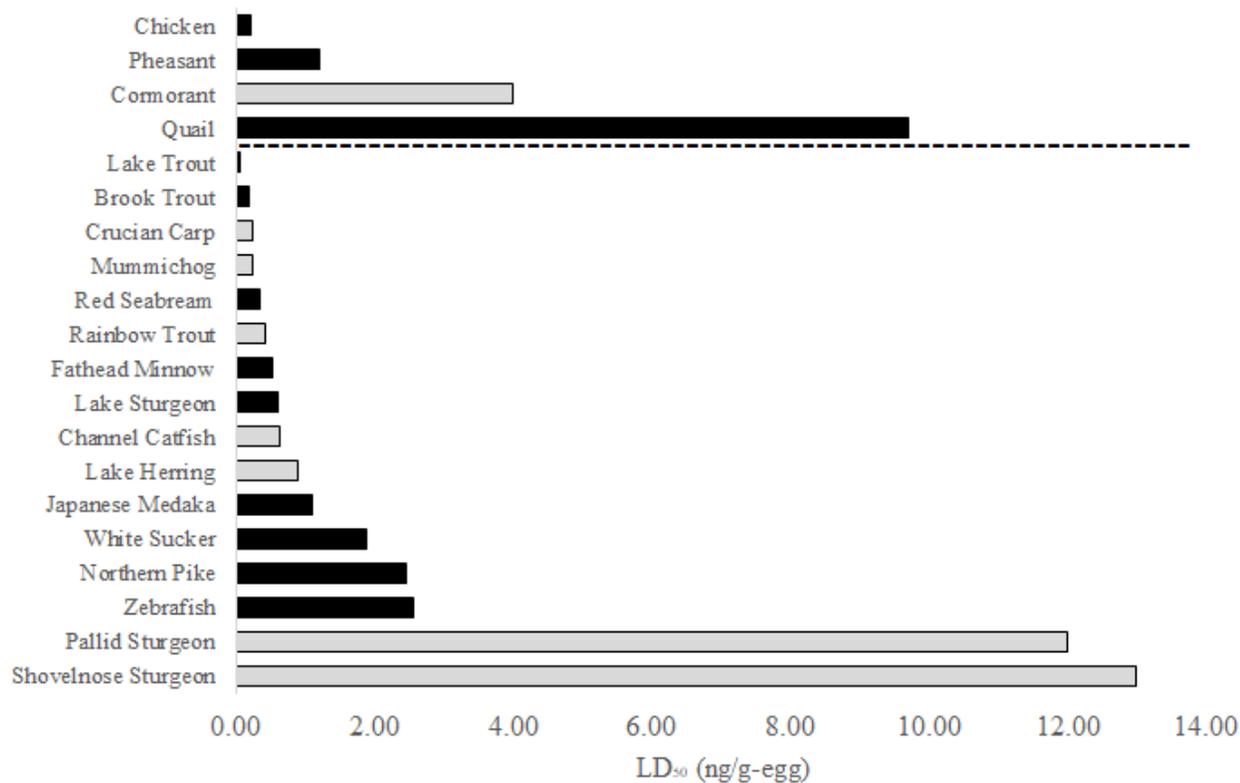
<sup>§</sup> Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B4, Canada

<sup>||</sup> School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5C8, Canada

Corresponding Author

\* E-mail: jad929@mail.usask.ca.

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**Figure S1.** Distribution of species-specific sensitivities to early life stage mortality (as LD<sub>50</sub>) following exposure to TCDD in birds (top) and fishes (bottom).<sup>1-13</sup> Species used in development of the qAOP are highlighted black.

**Table S1.** Information on fish used for sequencing of AHRs.

Common Name	Species	Age Class	Collection Source
Lake Trout	<i>Salvelinus namaycush</i>	Adult	Lac la Plonge, Saskatchewan, Canada
Brook Trout	<i>Salvelinus fontinalis</i>	Adult	Southwest River, Prince Edward Island, Canada
Fathead Minnow	<i>Pimephales promelas</i>	Adult	Laboratory Culture, University of Saskatchewan
Japanese Medaka	<i>Oryzias latipes</i>	Adult	Laboratory Culture, University of Saskatchewan
White Sucker	<i>Catostomus commersonii</i>	Adult	Lake Diefenbaker, Saskatchewan, Canada
Northern Pike	<i>Esox lucius</i>	Adult	Lake Diefenbaker, Saskatchewan, Canada

**Table S2.** Sequences, annealing temperatures, and corresponding target gene GenBank accession number of oligonucleotide primers used to sequence full-length AHRs.

Species	Target Gene	Accession #	Primer Sequence (5'-3')	Annealing Temp (°C)
Lake Trout	AHR2a	KX912256	Forward: ATGTTGAGTAACGCTGGAGTCTATGCTG Reverse: TTAGAAGTTGCAATAGTTGGTTTGGTTGTGCTCTG	60
	AHR2b	KX912257	Forward: ATGTTGGGGAGTACGGCG Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	50
Brook Trout	AHR2a	KX912258	Forward: ATGTTGAGTAACAACGCTGGAGTCTATGC Reverse: TTAGAAGTTGCAATAGTTGATTTGGTTGTGC	48
	AHR2b	KX912259	Forward: ATGTTGGGGAGTACGGCG Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	60
Fathead Minnow	AHR1	KX912260	Forward: ATGTACGCGGGACGAAAAGAAGAA Reverse: TCAGAGGTATAAACAGTAGTCTGAGGGTCTG	48
	AHR2	KX912261	Forward: ATGTCGAGGAGTATCGGTATCTATGCG Reverse: CTAATAGTCACAGCACTTGCTTTGGTTGTTCTC	50
Japanese Medaka	AHR1	KX912262	Forward: ATGTACGCCGGGCGCAAACG Reverse: AGTTTCTAAACAGGTGGCAGGGTTC	60
	AHR2	KX912263	Forward: ATGCTGTCCGGCACCGCCATGTA Reverse: CTTGTTCTCGGTAAAGCAGGTGTTCC	60
White Sucker	AHR2	KX912264	Forward: ATGTCGAGGAGTATCGGTATATATGCGGTC Reverse: CTAATAGTCACAGCAACTGCTTTGGTTGTTCTC	48
Northern Pike	AHR1a	KX912265	Forward: ATGAGCAGCAGCACATATGCCAGT Reverse: TTACAGCTGCCCTGCGGCGT	60
	AHR1b	KX912266	Forward: ATGTATGCTGGACGTAAAAGGAGAAAACC Reverse: CTAGTTCCACACCCCACTGGACTGGA	60
	AHR2a	KX912267	Forward: ATGCTAAGTAATGCTAGAGTATATGCTGTCAAGAA Reverse: TTAGAAGTCGCAATAGTTGGTTTGGTTG	60
	AHR2b	KX912268	Forward: ATGTTGGGGAGTACGGCG Reverse: CTAGAAGTTGCAACAGTTGGTTTGATTG	60

**Table S3.** Sequences, annealing temperatures, and corresponding target gene GenBank accession number of oligonucleotide primers used to produce expression constructs.

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

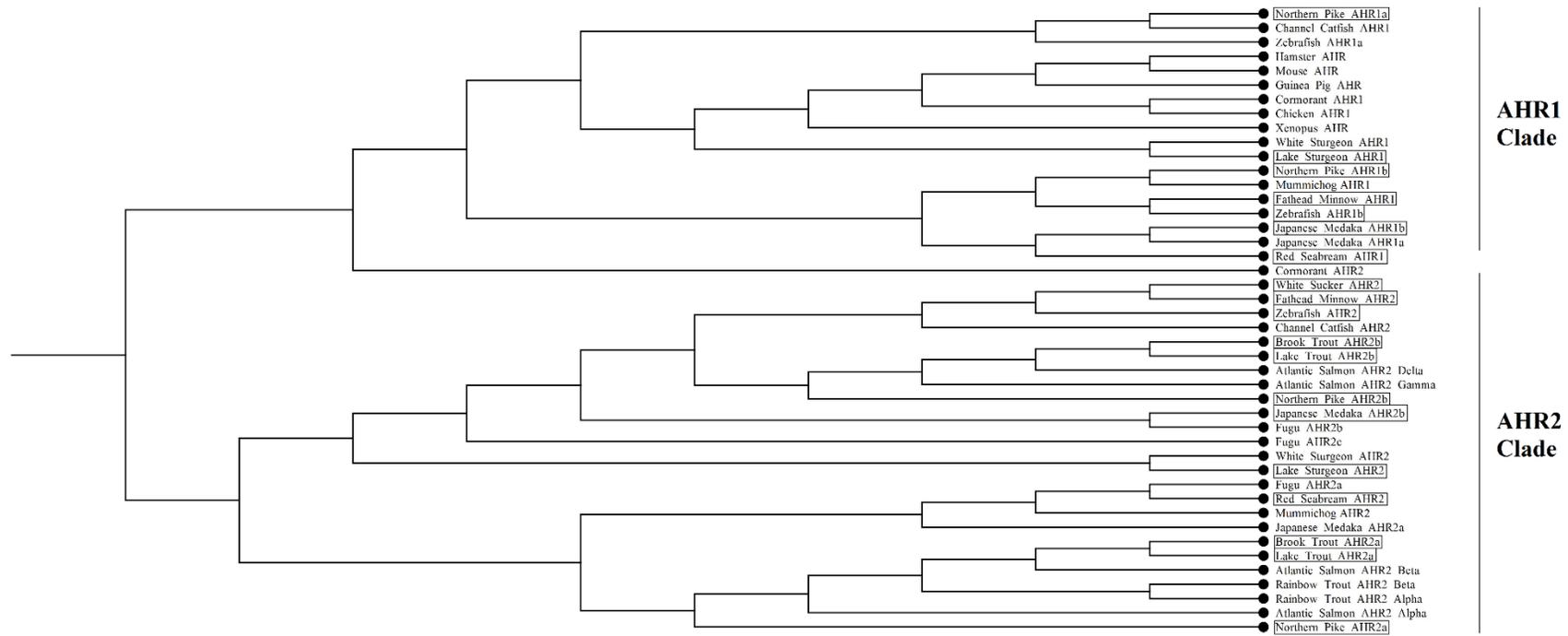
**Table S4.** Data from the *in vitro* AHR transactivation assay that were used in the construction of the qAOP.

Species	DLC	EC <sub>50</sub> nM	Reference
Zebrafish	TCDD	6.8	14
Northern Pike	TCDD	11	This study
White Sucker	TCDD	5.7	This study
Japanese Medaka	TCDD	4.6	This study
Lake Sturgeon	TCDD	0.79	15
Lake Sturgeon	PCB 126	18	15
Fathead Minnow	TCDD	0.65	This study
Red Seabream	TCDD	0.51	16
Brook Trout	TCDD	0.28	This study
Lake Trout	TCDD	0.066	This study
Quail	TCDD	21	17
Quail	PeCDF	1.1	17
Quail	TCDF	11	17
Pheasant	TCDD	1.6	17
Pheasant	PeCDF	0.37	17
Pheasant	TCDF	3.7	17
Chicken	TCDD	0.22	17
Chicken	PeCDF	0.18	17
Chicken	TCDF	0.34	17
Chicken	PCB 126	4.9	18
Chicken	PCB 77	75	18



**Table S5.** Early life stage mortality data used in the construction of the qAOP.

Species	DLC	LD <sub>0</sub>		LD <sub>10</sub>		LD <sub>50</sub>		LD <sub>100</sub>		Reference
		nM	ng/g-egg	nM	ng/g-egg	nM	ng/g-egg	nM	ng/g-egg	
Zebrafish	TCDD	0.0013	0.42	0.0050	1.6	0.0079	2.6	0.014	4.3	3
Northern Pike	TCDD	0.0037	1.2	0.0048	1.5	0.0076	2.5	0.015	4.8	3
White Sucker	TCDD	0.0026	0.85	0.0049	1.6	0.0059	1.9	0.0075	2.4	3
Japanese Medaka	TCDD	0.0014	0.46	0.0020	0.66	0.0035	1.1	0.0066	2.1	3
Lake Sturgeon	TCDD	-	-	-	-	0.0020	0.61	-	-	7
Lake Sturgeon	PCB 126	-	-	-	-	0.017	5.4	-	-	7
Fathead Minnow	TCDD	0.00073	0.24	0.00091	0.29	0.0017	0.54	0.0048	1.5	3
Red Seabream	TCDD	-	-	-	-	0.0011	0.36	0.0049	1.6	13
Brook Trout	TCDD	0.00042	0.14	0.00058	0.19	0.00062	0.20	0.0010	0.32	11
Lake Trout	TCDD	0.00011	0.034	0.00012	0.040	0.00021	0.069	0.00037	0.12	12
Quail	TCDD	0.0056	1.8	0.011	3.6	0.030	9.7	-	-	2
Quail	PeCDF	0.00041	0.14	0.00091	0.31	0.0050	1.7	-	-	2
Quail	TCDF	0.0029	0.89	0.0049	1.5	0.015	4.6	-	-	2
Pheasant	TCDD	0.00031	0.10	0.00081	0.26	0.0037	1.2	-	-	2
Pheasant	PeCDF	0.00014	0.048	0.00024	0.080	0.00062	0.21	-	-	2
Pheasant	TCDF	0.00029	0.088	0.00065	0.20	0.0012	0.37	-	-	2
Chicken	TCDD	0.00020	0.063	0.00040	0.13	0.00065	0.21	0.0031	0.99	2
Chicken	PeCDF	0.00014	0.048	0.00032	0.11	0.00076	0.26	-	-	2
Chicken	TCDF	0.00015	0.045	0.00025	0.075	0.00033	0.10	0.0018	0.56	2
Chicken	PCB 126	0.00092	0.30	0.0028	0.90	0.0018	0.60	0.025	8.1	19
Chicken	PCB 77	0.01027	3.0	0.031	9.0	0.030	8.8	0.28	81	19

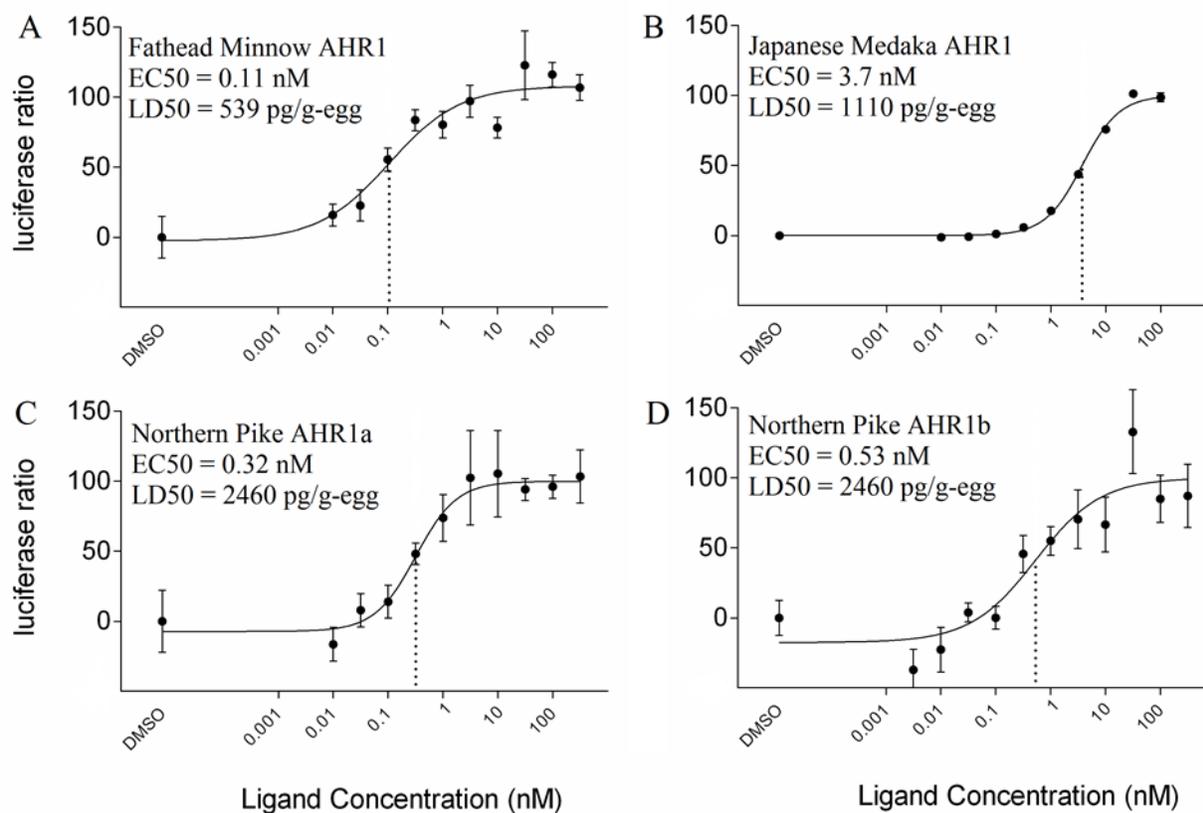


**Figure S2.** Phylogenetic tree for relatedness of full-length amino acid sequences of AHRs across vertebrates. AHRs of fishes that were included in the linear regressions (Figure 3) are highlighted. Branch lengths represent bootstrap values based on 1000 samplings. Accession numbers used are: channel 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 (*Fundulus 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. fulvescens*; AIW39681.1); fugu AHR2a (*T. rubripes*;

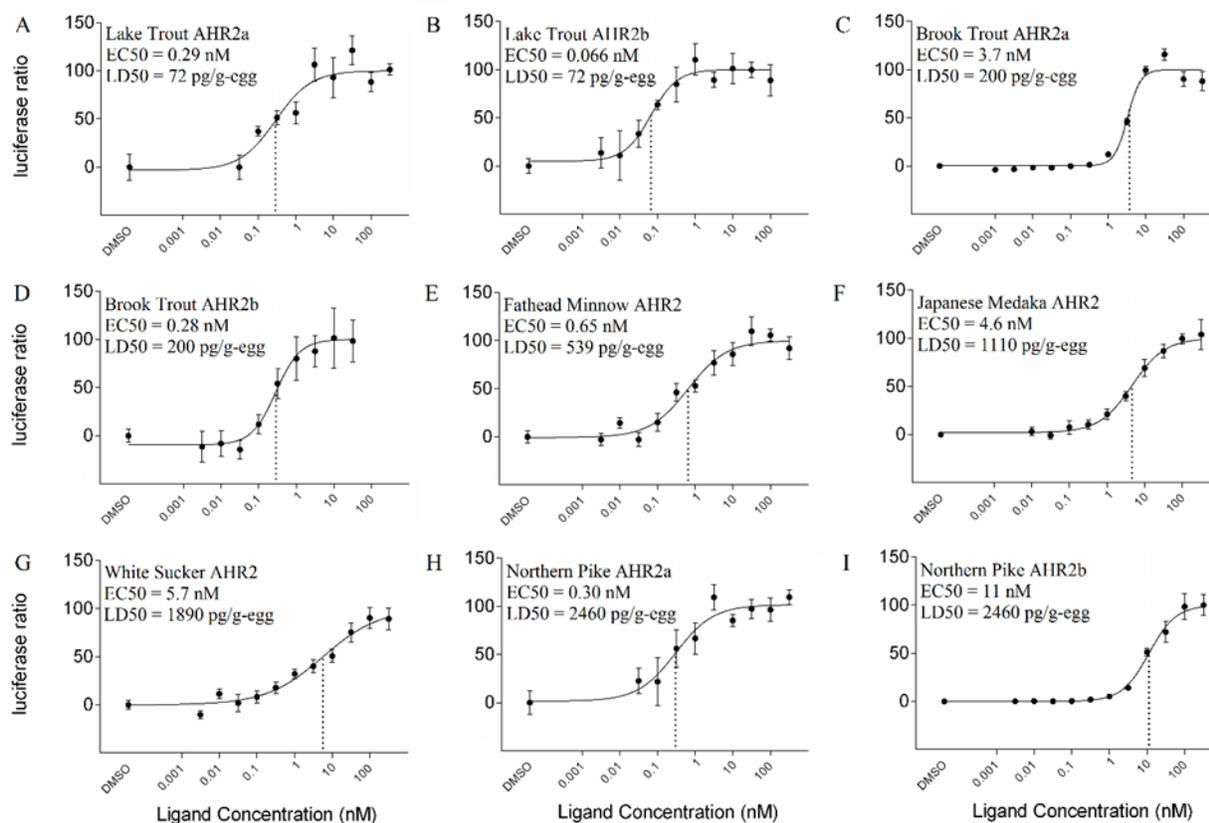
NP\_001033049.1); red seabream AhR2 (*P. major*; BAE02825.1); mummichog AHR2 (*F. heteroclitus*; AAC59696.3); Japanese medaka AHR2a (*O. latipes*; XP\_011488315.1); Atlantic salmon AHR2 beta (*S. salar*; NP\_001117028.1); rainbow trout AHR2 beta (*Oncorhynchus mykiss*; NP\_001117724.1); rainbow trout AHR2 alpha (*O. mykiss*; NP\_001117723.1); Atlantic salmon AHR2 alpha (*S. salar*; NP\_001117156.1). Phylogenetic tree for AHRs was constructed by use of CLC Genomics Workbench v.7.0.4 (Katrinebjerg, Aarhus).

**Table 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 AHR2 $\beta$	96%
	Rainbow Trout AHR2 $\beta$	95%
	Rainbow Trout AHR2 $\alpha$	93%
	Atlantic Salmon AHR2 $\alpha$	80%
	Northern Pike AHR2a	78%
Lake Trout AHR2b		
	Brook Trout AHR2b	98%
	Atlantic Salmon AHR2 $\delta$	95%
	Atlantic Salmon AHR2 $\gamma$	92%
	Northern Pike AHR2b	72%



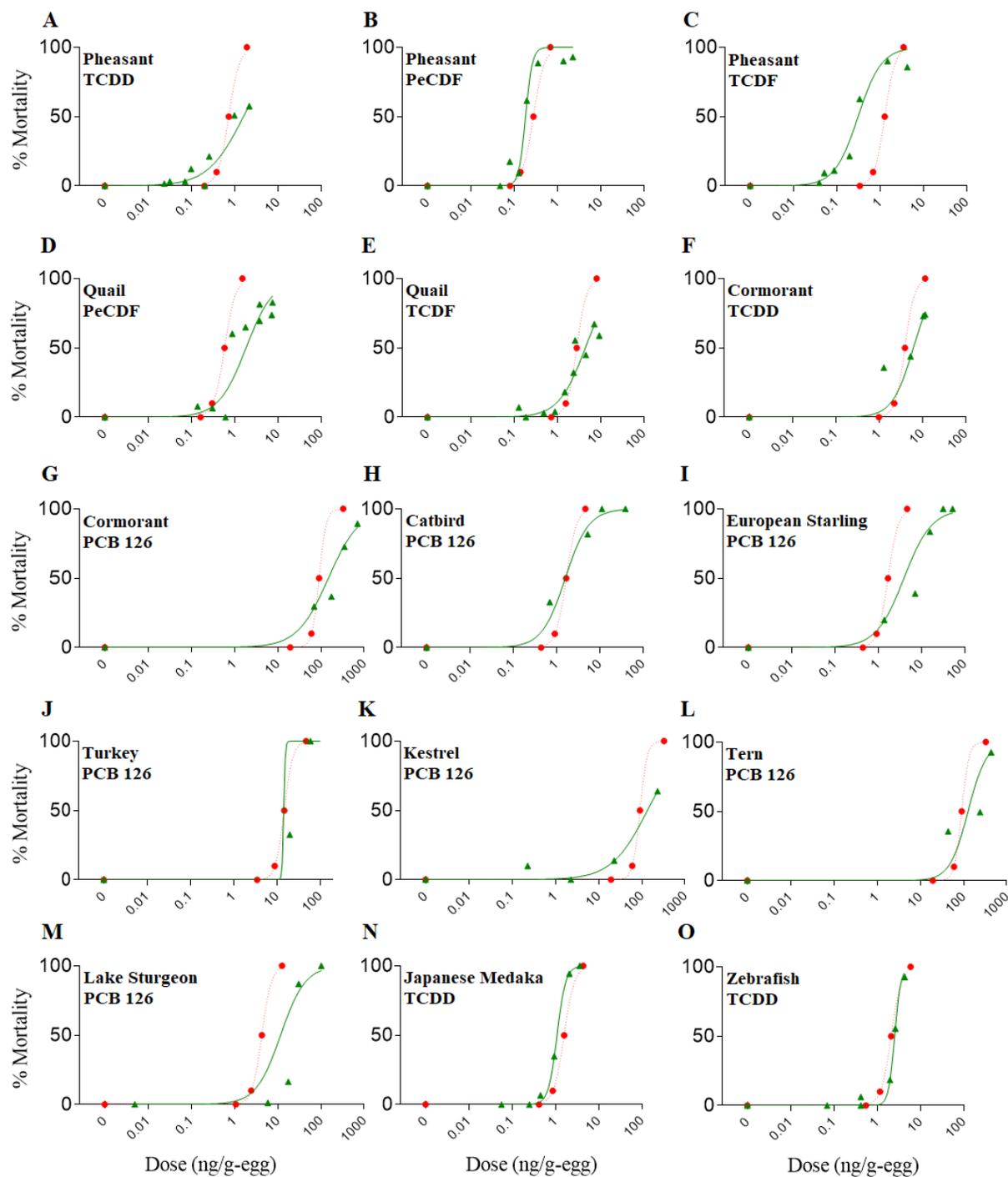
**Figure S3.** Dose-response curves for activation of AHR1 of fathead minnow (A) and Japanese medaka (B), and AHR1a (C) and AHR1b (D) of Northern pike by TCDD. The EC<sub>50</sub> is represented by a dotted line. Data are presented as the mean  $\pm$  standard error of the mean based on three replicate assays conducted in quadruplicate. DMSO control is standardized to 0 and maximum response is standardized to 100. Calculated EC<sub>50</sub> and LD<sub>50</sub> from the published literature are presented.<sup>3</sup>



**Figure S4.** 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<sub>50</sub> is represented by a dotted line. Data are presented as the mean  $\pm$  standard error of the mean based on three replicate assays conducted in quadruplicate. DMSO control is standardized to 0 and maximum response is standardized to 100. Calculated EC<sub>50</sub> and LD<sub>50</sub> from the published literature are presented.<sup>3,4,9,11,12</sup>

**Table S7.** Calculated effect concentrations (ECs) (nM) for sensitivity to activation by TCDD of AHR1s and AHR2s of fishes. Standard error of the mean is presented in brackets.

Species	Isoform	LOEC	EC <sub>20</sub>	EC <sub>50</sub>	EC <sub>80</sub>
Lake Trout	AHR2a	0.1	0.080 ( $\pm 0.04$ )	0.29 ( $\pm 0.07$ )	1.3 ( $\pm 0.07$ )
	AHR2b	0.03	0.021 ( $\pm 0.01$ )	0.066 ( $\pm 0.01$ )	0.27 ( $\pm 0.09$ )
Brook Trout	AHR2a	1.0	1.9 ( $\pm 0.3$ )	3.3 ( $\pm 0.4$ )	5.7 ( $\pm 0.6$ )
	AHR2b	0.1	0.10 ( $\pm 0.008$ )	0.28 ( $\pm 0.1$ )	1.1 ( $\pm 0.6$ )
Fathead Minnow	AHR1	0.1	0.033 ( $\pm 0.01$ )	0.11 ( $\pm 0.06$ )	0.78 ( $\pm 0.5$ )
	AHR2	0.3	0.12 ( $\pm 0.02$ )	0.65 ( $\pm 0.1$ )	3.8 ( $\pm 2$ )
Japanese Medaka	AHR1	0.3	1.3 ( $\pm 0.2$ )	3.7 ( $\pm 0.4$ )	11 ( $\pm 0.7$ )
	AHR2	0.3	1.2 ( $\pm 0.2$ )	4.6 ( $\pm 0.3$ )	19 ( $\pm 4$ )
White Sucker	AHR2	1.0	0.58 ( $\pm 0.3$ )	5.7 ( $\pm 0.7$ )	96 ( $\pm 40$ )
Northern Pike	AHR1a	0.3	0.17 ( $\pm 0.1$ )	0.32 ( $\pm 0.2$ )	1.7 ( $\pm 0.7$ )
	AHR1b	0.3	0.083 ( $\pm 0.009$ )	0.53 ( $\pm 0.2$ )	4.3 ( $\pm 2$ )
	AHR2a	0.3	0.11 ( $\pm 0.09$ )	0.30 ( $\pm 0.2$ )	1.1 ( $\pm 0.5$ )
	AHR2b	1.0	4.2 ( $\pm 0.7$ )	11 ( $\pm 2$ )	39 ( $\pm 14$ )



**Figure S5.** Dose-response curves for normalized experimental early life stage mortality data from the published literature (green triangle) and predicted using the qAOP (red circle) for birds (A-L) and fishes (M-O) exposed to 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachloro-dibenzofuran (PeCDF), 2,3,7,8-tetrachloro-dibenzofuran (TCDF), or 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Best fit lines shown for both experimental and predicted dose-response curves. An additional 12 dose-response curve comparisons are presented elsewhere (Figure 4).



**Table S8.** Evaluation statistics for predicted versus normalized experimental dose-response curves for early life stage mortality in birds and fishes.

	<b>FD<sup>a</sup></b>	<b>MAE<sup>b</sup></b>	<b>RMSE<sup>c</sup></b>	<b>MAPE<sup>d</sup></b>
LD <sub>Avg</sub> (all birds and fishes)	2.2	6.0	14.9	76
LD <sub>Avg</sub> (birds only)	2.4	7.7	15.9	99
LD <sub>Avg</sub> (fishes only)	1.7	2.6	6.7	31
LD <sub>Avg</sub> (species in model only)	2.0	2.5	6.5	69
LD <sub>Avg</sub> (species not in model only)	2.7	16.9	24.2	95
LD <sub>0</sub> (all birds and fishes)	2.3	4.5	12.2	117
LD <sub>0</sub> (birds only)	2.8	6.6	15.0	162
LD <sub>0</sub> (fishes only)	1.3	0.2	0.3	25
LD <sub>0</sub> (species in model only)	2.1	0.4	1.1	94
LD <sub>0</sub> (species not in model only)	2.9	16.0	23.9	183
LD <sub>10</sub> (all birds and fishes)	1.9	4.2	11.4	79
LD <sub>10</sub> (birds only)	2.2	6.1	14.0	108
LD <sub>10</sub> (fishes only)	1.3	0.2	0.3	21
LD <sub>10</sub> (species in model only)	1.9	0.5	1.4	74
LD <sub>10</sub> (species not in model only)	2.0	14.5	22.3	93
LD <sub>50</sub> (all birds and fishes)	1.7	6.5	16.0	46
LD <sub>50</sub> (birds only)	1.8	9.3	19.6	56
LD <sub>50</sub> (fishes only)	1.4	1.0	2.5	26
LD <sub>50</sub> (species in model only)	1.7	1.6	3.6	52
LD <sub>50</sub> (species not in model only)	1.5	20.6	30.9	29
LD <sub>100</sub> (all birds and fishes)	2.9	8.9	19.7	62
LD <sub>100</sub> (birds only)	2.8	8.8	15.3	70
LD <sub>100</sub> (fishes only)	2.9	9.0	23.8	52
LD <sub>100</sub> (species in model only)	2.4	7.5	19.8	59
LD <sub>100</sub> (species not in model only)	4.5	16.7	19.6	77

<sup>a</sup> Fold-difference<sup>b</sup> Mean Absolute Error<sup>c</sup> Root Mean Square Error<sup>d</sup> Mean Absolute Percent Error

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