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Characterization and Expression of Cytochrome P4501A in Atlantic Sturgeon and Shortnose Sturgeon Experimentally Exposed to Coplanar PCB 126 and TCDD

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Abstract

The AHR pathway activates transcription of CYP1A and mediates most toxic responses from exposure to halogenated aromatic hydrocarbon contaminants such as PCBs and PCDD/Fs. Therefore, expression of CYP1A is predictive of most higher-level toxic responses from these chemicals. To date, no study had developed an assay to quantify CYP1A expression in any sturgeon species. We addressed this deficiency by partially characterizing CYP1A in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) and shortnose sturgeon (*Acipenser brevirostrum*) and then used derived sturgeon sequences to develop reverse transcriptase (RT)-PCR assays to quantify CYP1A mRNA expression in TCDD and PCB126 treated early life-stages of both species. Phylogenetic analysis of CYP1A, CYP1B, CYP1C and CYP3A deduced amino acid sequences from other fishes and sturgeons revealed that our putative Atlantic sturgeon and shortnose sturgeon CYP1A sequences most closely clustered with previously derived CYP1A sequences. We then used semi-quantitative and real-time RT-PCR to measure CYP1A mRNA levels in newly hatched Atlantic sturgeon and shortnose sturgeon larvae that were exposed to graded doses of waterborne PCB126 (0.01–1000 parts per billion (ppb)) and TCDD (0.001–10 ppb). We initially observed significant induction of CYP1A mRNA compared to vehicle control at the lowest doses of PCB126 and TCDD used, 0.01 ppb and 0.001 ppb, respectively. Significant induction was observed at all doses of both chemicals although lower expression was seen at the highest doses. We also compared CYP1A expression among tissues of i.p. injected shortnose sturgeon and found significant inducibility in heart, intestine, and liver, but not in blood, gill, or pectoral fin clips. For the first time, our results indicate that young life-stages of sturgeons are sensitive to AHR ligands at environmentally relevant concentrations, however, it is yet to be determined if induction of CYP1A can be used as a biomarker in environmental biomonitoring.

Keywords

gene expression; PCBs; PCDD/Fs; contaminant effects

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Introduction

There are 26 species of sturgeon worldwide and populations of most are severely reduced or threatened with extirpation (Bemis and Kynard, 1997). All sturgeons mature at advanced ages, do not spawn annually, exhibit only moderate fecundity for their large size, and are long-lived. These life history parameters are thought to render sturgeons highly vulnerable to the anthropogenic effects of overharvest, habitat alteration, and chemical toxicity (Birstein et al., 1997a). While the effects of overharvest and habitat modification have on occasion been empirically evaluated, the effects of toxic chemicals have rarely been experimentally tested in any sturgeon species. Early and adult life-stages of all sturgeons are bottom-dwelling and adults are generalist omnivores; making them vulnerable to benthic-borne lipophilic halogenated aromatic hydrocarbons (HAHs) such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins and furans (PCDD/Fs).

Within North America, there are eight species of sturgeons and three are currently listed as endangered while a fourth is being considered for that status under the U.S. federal Endangered Species Act (ESA). Two species, Atlantic sturgeon *Acipenser oxyrinchus oxyrinchus* and shortnose sturgeon *A. brevirostrum* co-occur along the Atlantic Coast of North America from the Canadian Maritime provinces to Georgia and northern Florida. Shortnose sturgeon has been listed as endangered under the U.S federal ESA since its inception in 1973 and Atlantic sturgeon has recently been recommended as endangered in parts of its geographic range including the New York Bight. Both species are long-lived with Atlantic sturgeon and shortnose sturgeon achieving estimated maximum ages of 60 and 67 years, respectively. Atlantic sturgeon reside within their natal estuary for the first 2 to 6 years of life and then undergo extensive coastal migrations not returning until they sexually mature at 5 to 34 years of age depending on their gender and river of origin (Smith and Clugston, 1997). Shortnose sturgeon is amphidromous and were believed restricted to their natal estuary for their entire life history (Kynard, 1997). However, recent evidence suggests that they may foray into coastal waters more frequently and for greater distances than previously thought (Fernandes et al., 2010; Wirgin et al., 2010). Thus, young life-stages of both species are likely to be highly exposed to contaminants within some natal estuaries and are potentially susceptible to their toxic effects. Both species are polyploid with Atlantic sturgeon thought to be tetraploid and shortnose sturgeon believed to be dodecaploid (Birstein et al., 1997b).

PCDD/Fs and coplanar PCBs induce many shared toxicities because of their common mode of action that is mediated through the ligand-activated aryl hydrocarbon receptor (AHR) pathway. AHR structure and function are conserved across all vertebrate taxa including fishes (Hahn, 1998). However, unlike mammalian taxa, fishes have two forms of AHR (Hahn et al., 1997) of which one (AHR2) is usually more functionally active than the other (AHR1) (Clark et al., 2010; Pandini et al., 2007). While AHR1 and AHR2 have been characterized in several fish taxa, none is as primitive as the sturgeons.

Early life-stages of fishes are sensitive to PCDD/Fs and coplanar PCBs induced toxicities. These toxicities include pericardial and yolk-sac edema, craniofacial malformations, and abnormal spinal curvature. It is believed that these toxicities from exposure to AHR ligands emanate from structural and functional damage to the developing heart (Incardona et al., 2004). Furthermore, studies have demonstrated that these toxicities from HAHs are mediated by the AHR pathway. Although, all fishes tested exhibit vulnerability to these toxicities, interspecific variation in sensitivity among fishes is substantial (Elonen et al., 1998) perhaps due to variation in the abilities of ligands to bind their AHRs as demonstrated among bird species (Head et al., 2008) and among populations of fishes (Wirgin et al., 2011).

CYP1A transcriptional activation is mediated by the AHR pathway in vertebrate taxa (Ma, 2001). In many fishes, CYP1A mRNA, CYP1A protein, and CYP1A encoded EROD enzyme activity are highly sensitive and dose responsive to induction by exposure to PCDD/Fs, coplanar PCBs, and some PAHs. Because CYP1A transcription is mediated by the AHR pathway, CYP1A expression is often used as a biomarker of exposure and early biological effect in fishes (Bucheli and Fent, 1995). Furthermore, induced expression of CYP1A is often correlated with higher level toxic effects in fishes including elevated DNA damage, early life-stage toxicities, and several cancers (Wirgin and Theodorakis, 2002a).

Because Atlantic and shortnose sturgeon spawn in estuaries, often in those that are urbanized and industrialized, early life-stages of their offspring have the potential to be highly exposed to damaging levels of contaminants. It has been hypothesized, although never empirically evaluated, that toxic chemicals have contributed to the failure of impacted shortnose sturgeon and Atlantic sturgeon populations to rebuild to historical levels despite the imposition of moratoriums on their harvest and attempts to restore degraded habitats. For example, PCBs and PCDD/Fs levels are highly elevated in sediments of both the Hudson River (Bopp et al., 1991; Farley and Thomann, 1998) and Delaware River (McCoy et al., 2002). Furthermore, shortnose sturgeon from both rivers have been shown to bioaccumulate these chemicals (Environmental et al., 2002; NOAA, 2010). However, no study has determined if actual toxic effects of these compounds have impacted these populations.

As a first step in evaluating potential toxicities to these species, we have partially characterized CYP1A cDNA in both Atlantic coast sturgeons and from these data developed semi-quantitative and real-time RT-PCR assays to quantify CYP1A mRNA expression. We then empirically evaluated these assays in larval offspring of both species that were experimentally exposed under controlled laboratory conditions to environmentally relevant graded exposure concentrations of coplanar PCB126 and TCDD. We also compared CYP1A expression among tissues, including blood and fin clips, of shortnose sturgeon that were i.p. injected with graded concentrations of PCB126 to initially evaluate the feasibility of using CYP1A inducibility as a biomarker of exposure and early effect of PCBs and PCDD/Fs in sturgeons.

Materials and Methods

Sources of embryos

Sturgeon embryos were obtained from ongoing research and aquaculture programs that culture both species. Fertilized shortnose sturgeon eggs of Connecticut River origin were obtained from the Conte Anadromous Fish Lab, USGS, Turners Falls, Massachusetts, USA. Fertilized Atlantic sturgeon and additional shortnose sturgeon eggs both of Saint John River, New Brunswick (NB), Canada, ancestry were obtained from Cornel Ceapa, Acadian Sturgeon and Caviar Inc., Saint John, NB. In both cases, broodstock was collected from natural populations. Neither river is thought to have major inputs of HAH contamination near or upstream of where sturgeon broodstock were collected, however, tissue burdens of these pollutants have yet to be empirically quantified in sturgeons from either river.

Rearing of embryos

Embryos of both species were transported to the Howard Marine Science Laboratory of NOAA Fisheries, Sandy Hook, New Jersey. Embryos were maintained initially in modified Macdonald's jars until they were counted into groups of 30 that were allocated to glass dishes filled to 150 mL with 0.01 ppt salt water. Embryos were checked for mortalities during twice-daily water changes. Hatched larvae were transferred to 250-mL glass beakers filled to 150 mL with 0.01 ppt salt water. All sturgeon were maintained in environmental

control chambers with an air temperature of 15 °C and 14 hr light:10 hr dark light regime. Survival to hatching, size and morphometrics at hatching, and yolk-sac absorption rates were recorded and will be reported elsewhere.

Chemical treatments

Twenty-five Atlantic sturgeon larvae 48-h posthatch were exposed for 26 to 27 h at 14° C in triplicate beakers to graded exposure concentrations of TCDD (nominal doses of 0.001 ppb, 0.01 ppb, 0.1 ppb, 1.0 ppb, and 10 ppb), (AccuStandard, New Haven, CT) (99.1% purity), in acetone vehicle (0.05–0.1% acetone) or PCB126 (nominal doses of 0.01 ppb, 0.1 ppb, 1.0 ppb, 10 ppb, 100 ppb, 1000 ppb), (AccuStandard) (99.7% purity) in water alone, and acetone vehicle alone. All exposures were in 100 ml glass beakers in 50 ml of 0.1 ppt artificial seawater. Immediately after exposure, larvae were harvested and snap frozen in pools of 5 larvae in 1.5 ml plastic microcentrifuge tubes and maintained at –80°C until processing.

Thirty shortnose sturgeon 5-d old embryos were exposed for 26–27 hr to the same graded exposure concentrations of TCDD and PCB126 and replication level as described above. After exposure, embryos were transferred to clean water and reared to hatch (7–10 d) and snap frozen in plastic vials as individuals 2 d after hatch.

CYP1A expression in tissues of juvenile shortnose sturgeon treated with PCB126

Hatchery-reared juvenile shortnose sturgeon of Connecticut River ancestry were treated with graded concentrations of PCB126 (0.01 ppb, 0.1 ppb, 1 ppb, 10 ppb, and 50 ppb) to compare tissue specific expression of CYP1A mRNA expression. Treatments occurred at the Conte Lab of the USGS. Five fish per treatment group were i.p. injected with the graded concentrations of PCB126 in 25 µl of corn oil vehicle or 25 µl of corn oil alone. Each treatment group was marked with a different color latex dye and maintained in a single tank of ambient Connecticut River water with the exception of the corn oil alone-treated group that were unmarked, but kept in a separate tank. Eight days after treatment, fish were sacrificed, tissues harvested (blood, gill, heart, intestine, liver, pectoral fin clip), snap frozen and maintained at –80° until processing.

Quantification of uptake of PCB126 in sturgeon embryos

Atlantic sturgeon embryos were exposed to radiolabeled PCB126 so that whole body burdens of PCB126 could be correlated with levels of CYP1A mRNA in gene expression assays. ³HPCB126 purchased from American Radiolabeled Chemicals (5 Ci/mmol, 99% purity in hexane) was diluted in acetone and cold PCB126 to working stocks of 20 ng PCB126/µl, 2 ng PCB126/µl and 0.2 ng PCB126/µl and 25 µl of each stock was used in exposure solutions. Exposures were for 2, 8, and 24 hr in triplicate to 10 ppb, 1 ppb, and 0.1 ppb PCB126 (final nominal concentration) in 50 ml of 0.1% artificial sea water in 100 ml glass beakers maintained at 14° C. Each beaker contained ten 5 day-old Atlantic sturgeon embryos. After exposures, embryos were washed three times with distilled water, weighed, solubilized overnight at 50° C in 2 ml of Soluene-350 (PerkinElmer Life and Analytical Science, Boston, MA), cooled the next morning to room temperature, 10 ml of Hionic Fluor (Packard Bioscience Co., Meriden, CT) was added, and counted for 10 min on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard Bioscience).

Isolation and characterization of Atlantic sturgeon CYP1A

Perusal of the GenBank database indicated the absence of CYP1A sequence data for any sturgeon species. However, we found 21 partial or complete sequences for CYP1A cDNA from other fishes but none as primitive as sturgeons. We selected three of these fish CYP1A

sequences (lake trout *Salvelinus namaycush*, rainbow trout *Oncorhynchus mykiss*, and Japanese eel *Anguilla japonica*,) from which we identified highly conserved sequences. Fifteen different primers derived from these three sequences were empirically tested in various combinations for their abilities to amplify Atlantic sturgeon cDNA that had been isolated from pooled untreated Atlantic sturgeon larvae. Of these 15 primers, six provided reliable amplification and sequences of Atlantic sturgeon cDNA (Table 1; Sturcyp255F, Sturcyp691F, Sturcyp922F, Sturcyp1589R, Sturcyp707R, and Sturcyp941R). Three cDNA fragments of 668 bp, 453 bp, and 251 bp were reproducibly amplified with different combinations of these primers pairs. Atlantic sturgeon derived sequences were then used to construct five new derived primers (Table 1; Sturcyp538F, Sturcyp864F, Sturcyp880R, Sturcyp1240R, and Sturcyp1305R) to generate a contiguous 1335 bp Atlantic sturgeon cDNA sequence (GenBank Accession Number HQ439359). This cDNA sequence was then translated into a peptide sequence of 444 amino acids.

Development of β -actin primers

To normalize CYP1A expression in both semi- and quantitative RT-PCR analyses, we developed primers to amplify Atlantic sturgeon β -actin cDNA. Partial β -actin sequence was available in the literature for two sturgeon species; white sturgeon *Acipenser transmontanus* (Genbank Accession Numbers FJ205611 and AY878120) and Chinese sturgeon *Acipenser sinensis* (Genbank Accession Number AJ745100). Two upstream (Table 1; Sturact168F and Sturact193F) and two downstream (Table 1; Sturact380R and Sturact393R) β -actin primers were designed based on conservation between these sequences. All combinations of these upstream and downstream primers successfully amplified Atlantic sturgeon cDNA and the size of the amplicons closely approximated that expected based on characterization of β actin in the two sturgeon species (188 bp, 201 bp, 212 bp, and 225 bp). These four DNA fragments were sequenced to develop Atlantic sturgeon and shortnose sturgeon specific β -actin primers (Atlantic sturgeon and shortnose sturgeon GenBank Accession Numbers, HQ439361 and HQ439362, respectively).

CYP1A inducibility in Atlantic sturgeon larvae treated with TCDD and PCB126 RNA isolation

Total RNA was isolated from 3 pools of 5 Atlantic sturgeon larvae each, individual shortnose sturgeon larvae, or tissues from individual juvenile shortnose sturgeon using Ultraspec reagent (Biotexc, Houston, TX) as per the manufacturer's recommendations and modified in (Yuan et al., 2006). RNA concentrations and purities were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technol. Inc., Wilmington, DE).

Reverse transcription

First strand cDNAs were generated from 100 ng of total RNA preparations. Five hundred ng of random hexamers (Integrated DNA Technol. Inc., Coralville, IA) were added to the RNAs in total volumes of 15 μ l and incubated at 75° C for 5 min. The mixture was chilled on ice, and 10 μ l of RT-mix was added, so that the final reaction mix contained 1 X MMLV reaction buffer, 20 U MMLV reverse transcriptase (Promega Life Science, Madison, WI), 10 U RNasin RNase inhibitor (Promega), and 0.5 mM dNTPs (GE Healthcare Bio-Sciences Corp. Piscataway, NJ). Reactions were incubated at 42° C for 1 hr, products were denatured at 98° C for 5 min, and chilled until PCR.

Semi quantitative RT-PCR of Atlantic sturgeon CYP1A mRNA

PCR primers were designed based on our derived Atlantic sturgeon sequence to amplify CYP1A initially in a semi-quantitative RT-PCR assay. Primers Sturcyp864F and Sturcyp1240R (Table 1) amplified a 366 bp DNA fragment. Four different numbers of PCR

cycles; 23, 27, 32, and 37 cycles, were empirically tested for their abilities to distinguish among treatment groups and 23 cycles was selected for gene expression experiments. Amplicons were visualized in 1.4% agarose gels stained with ethidium bromide. NIH ImageJ 1.42 q software was used to quantify the relative amounts of the amplicons.

Real-time RT-PCR of Atlantic sturgeon CYP1A

To more quantitatively compare CYP1A expression among exposure groups, a real-time RT-PCR assay was also developed. Adoption of this approach required the development of new primers for CYP1A and β -actin that would amplify shorter amplicons (<200 bp) than used for semi-quantitative RT-PCR (Table 1; CYP1A-StycypRT231F and StycypRT287R; β -actin-Sturact60F and Sturact125R). Reactions contained 3 μ l of RT product, 2 μ l of primer mix (final conc. 100 pM of each primer) and 5 μ l of Power Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA). Quantitative RT-PCR amplifications and analyses were performed on an ABI 7900 Real-Time PCR instrument.

Characterization of shortnose sturgeon CYP1A cDNA

We used the derived Atlantic sturgeon sequence to partially characterize shortnose sturgeon CYP1A cDNA. Initially, Atlantic sturgeon primers Sturcyp864F and Sturcyp1240R (Table 1) were used to amplify a 366 bp product from shortnose sturgeon cDNA. We then tested several pairs of CYP1A primers conserved among fish species or specific to Atlantic sturgeon to amplify additional portions of shortnose sturgeon CYP1A including Sturcyp255F and Sturcyp707R, Sturcyp 538F and Sturcyp 880R, and Sturcyp864F and Sturcyp1589R. These three pairs provided PCR products of 453 bp, 346 bp, and 726 bp, respectively. These three amplicons were characterized and their sequences were aligned to produce a contiguous shortnose sturgeon CYP1A sequence of 1335 bp (GenBank Accession number HQ439360). This shortnose sturgeon cDNA sequence was then translated into a peptide sequence of 444 amino acids.

CYP1A inducibility in shortnose sturgeon larvae

Characterization of shortnose sturgeon CYP1A cDNA sequence allowed us to develop species-specific primers for real-time RT-PCR analysis of CYP1A expression in TCDD and PCB126 exposed shortnose sturgeon larvae. Embryos were exposed at 5 d post fertilization to graded exposure concentrations of TCDD and PCB126 for 24 hr. Embryos were then transferred to clean water where they remained until hatching. Larvae at 2 d posthatch (mean hatch time was 10–11 d) were sacrificed, snap frozen as individuals, and maintained at -80° C until processing. RNA was isolated from individual larvae and cDNAs generated as described above. Real-time RT-PCR was used as described above to compare CYP1A mRNA expression among treatment groups for each chemical and compared to controls. Primers used for CYP1A were SNScypRT158F and SNScypRT230R and for β -actin Sturact60F and Sturact125R.

Statistical Analysis

The Unweighted Pair Group Method with Arithmetic Means (UPGMA) implemented in MEGA version 4 was used to construct a dendrogram of teleost cytochrome P450 1A, 1B, 1C, 1D and 3A sequences.

The relative comparison $\Delta\Delta$ Ct method for real-time RT-PCR, as described in Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen, 2001) was used to calculate β -actin-normalized fold induction of CYP1A1 mRNA in real-time RT-PCR assays for the TCDD and PCB126-treated groups compared expression in the acetone control group.

Analysis of variance (ANOVA) initially was used to determine whether significant differences ($p < 0.05$) existed in gene expression among all groups in semi-quantitative or real time RT-PCR assays; those individual groups that significantly differed in expression were identified by Tukey's multiple range test.

Results

Characterization of Atlantic sturgeon CYP1A cDNA

The 1335 bp Atlantic sturgeon putative CYP1A cDNA sequence exhibited mean 71.6% similarity with CYP1A nucleotide sequences from 21 other fishes in the literature. The deduced 444 amino acid Atlantic sturgeon putative CYP1A sequence exhibited mean 80% sequence similarity with CYP1A peptide sequences from these other fishes. UPGMA analysis was done to determine the amino acid sequence relatedness of the Atlantic sturgeon peptide with other fish cytochrome P450s amino acid sequences including CYP1A1, CYP1A2, CYP1A3, CYP1B1, CYP1B2, CYP1C1, CYP1C2, and CYP3A (Fig 1). The Atlantic sturgeon sequence clustered with 100% bootstrap support with the other fish CYP1A sequences. This confirmed that we had isolated and partially characterized CYP1A from Atlantic sturgeon. To our knowledge, this was the first characterization of a CYP1A sequence from any sturgeon species.

CYP1A inducibility in Atlantic sturgeon larvae

Agarose gel electrophoresis of the amplicons of semi-quantitative RT-PCR of Atlantic sturgeon CYP1A cDNA revealed only a single band despite Atlantic sturgeon being a tetraploid species (Fig 2A). Also, comparison of the sequences of many CYP1A RT-PCR products did not show any polymorphisms over the stretch of 1335 bp that we report here. Our results are most consistent with CYP1A mRNA being transcribed from only a single locus in Atlantic sturgeon and that duplicated forms that most likely existed following whole genome duplication in the sturgeons were lost. CYP1A mRNA expression detected by semi-quantitative RT-PCR was low in the water and even lower in the acetone control group (Fig. 2B). ANOVA revealed significant differences in CYP1A expression among the control and TCDD exposure groups. Use of Tukey's HSD test further showed that expression in all TCDD treated groups was significantly induced compared to that in control groups. The lowest TCDD concentration, 0.001 ppb, induced significant 18-fold CYP1A expression in Atlantic sturgeon larvae compared to the acetone control group. Maximum induction to TCDD was in larvae exposed to 0.1 ppb TCDD (42 fold). Expression was slightly lower in larvae exposed to 1.0 and 10 ppb TCDD (40 fold).

CYP1A mRNA expression was also responsive to exposure concentration in Atlantic sturgeon larvae treated with PCB126, although not to the same extent as with TCDD (Fig. 3). ANOVA revealed significant differences in CYP1A expression among the PCB126 and control exposure groups. Tukey's HSD test revealed that all exposure concentrations of PCB126 induced significant CYP1A expression compared to the acetone control group. Initial 2.4-fold induction with PCB126 occurred at the lowest exposure concentration used, 0.01 ppb. Maximum induction of 10.8-fold occurred at the highest exposure concentration used, 1000 ppb.

Results with real-time RT-PCR analysis of CYP1A expression in Atlantic sturgeon larvae were similar to those with semi-quantitative RT-PCR, but the magnitude of induction detected was greater using the real-time approach. For TCDD, we observed significant 22-fold induction of CYP1A at the lowest exposure concentration used, 0.001 ppb. Maximum induction of 89-fold was observed at the second highest exposure concentration used, 1 ppb (Fig 4A).

For PCB126, significant 6.5-fold induction of CYP1A was observed at the lowest exposure concentration used, 0.01 ppb. At 0.1 ppb PCB126, induction increased dramatically to 82-fold and maximum induction of 120-fold was observed at 100 ppb PCB126. Expression decreased slightly to 118-fold at the highest exposure concentration of PCB126 used, 1000 ppb (Fig 4B).

Uptake of PCB126 by Atlantic sturgeon embryos

We also estimated whole body burdens of PCB126 in these gene expression experiments by exposing Atlantic sturgeon embryos to three concentrations of ^3H labeled PCB126 (0.1 ppb, 1.0 ppb, and 10 ppb) in acetone vehicle for 2 hr, 8 hr, and 24 hr after which they were immediately sacrificed and uptake quantified. We found that, as expected, the greatest uptake of PCB126 was at the highest exposure concentrations, but also very little difference between exposure durations of 8 hr or 24 hr (Table 2). The highest body burdens in embryos exposed to 10 ppb PCB126 were between 27 and 29 ng/g embryos.

Characterization of shortnose sturgeon CYP1A cDNA

The 1335 bp shortnose sturgeon putative CYP1A cDNA (GenBank Accession Number HQ439360) and deduced amino acid sequences shared 98.3% and 98.5% identity, respectively, with those of Atlantic sturgeon. The shortnose sturgeon sequence exhibited 79.5% sequence similarity with CYP1A cDNAs and 71.8% amino acid sequence similarity with the aforementioned 21 fish CYP1A sequences. The shortnose sturgeon CYP1A peptide sequence was then compared in UPGMA analysis to CYP1A1, CYP1A2, CYP1A3, CYP1B1, CYP1B2, CYP1C1, CYP1C2, and CYP3A sequences from other fishes and it clustered most closely with that of Atlantic sturgeon CYP1A. The shortnose sturgeon putative CYP1A sequence clustered with 100% support within the clade containing other fish CYP1A sequences and was distant from fish CYP1B1, CYP1B2, CYP1C1, CYP1C2, CYP1D1, and CYP3A sequences (Fig. 1).

CYP1A inducibility in shortnose sturgeon larvae

We used the derived shortnose sturgeon CYP1A sequence to develop a real-time RT-PCR assay to evaluate CYP1A mRNA inducibility in shortnose sturgeon larvae that were exposed as embryos for 24 h to graded exposure concentrations of TCDD and PCB126. Unlike treatment of Atlantic sturgeon larvae, shortnose sturgeon were not sacrificed immediately after exposure, but instead were transferred to clean water for 7–10 d and maintained until hatching and then sacrificed 2 d later. This protocol allowed us to evaluate the persistence of the induction response, but did not support a comparison of gene inducibility between the two sturgeon species.

ANOVA revealed significant heterogeneity of CYP1A mRNA expression among both the TCDD, PCB126, and control treatment groups. Initial significant 7.7-fold induction of CYP1A in TCDD-treated shortnose sturgeon occurred at the lowest exposure concentration used, 0.001 ppb (Fig. 5A). Levels of gene expression increased at 1 ppb TCDD to 142-fold and maximum 987-fold induction was observed at 10 ppb. Slightly lower 800-fold induction was seen at the highest exposure concentration used, 100 ppb. For PCB126, initial significant induction of CYP1A (3-fold) occurred at the lowest exposure concentration used, 0.01 ppb, and then increased to 83-fold at 1 ppb, 245-fold at 100 ppb and 424-fold induction at the highest exposure concentration, 1000 ppb PCB126 (Fig. 5B).

CYP1A inducibility among tissues of juvenile shortnose sturgeon

Hatchery-reared juvenile shortnose sturgeon were i.p. injected with graded doses of PCB126 to compare levels of gene expression among different tissues and to evaluate the feasibility

of detecting induced expression in non-invasively secured tissues such as blood or fin clips. Significant CYP1A mRNA induction was detected in intestine, liver, and heart, but not in blood, fin clips, or gills. Significant induction in intestine, liver, and heart was not observed until the second highest 10 ppb (intestine and liver) or highest dose used 50 ppb (heart).

Discussion

This is the first demonstration that sturgeon species are sensitive to an AHR-mediated biological effect at environmentally relevant concentrations of two potent AHR ligands; TCDD and PCB126. Significant induction of CYP1A mRNA occurred in larvae of both species, in response to both compounds at the lowest doses used and at concentrations consistent with likely tissue burdens of these contaminants (analyzed as TCDD TEQs) in sturgeons from impacted estuaries such as the Hudson River and Delaware River. CYP1A expression levels were dose responsive in both species at the lower doses of both chemicals and plateaued or even decreased at higher exposure levels.

Reports have chronicled elevated tissue levels of total PCBs and total PCDD/Fs in Atlantic sturgeon and shortnose sturgeon from the Hudson River (NOAA, 2010) and Delaware River (Environmental et al., 2002) compared to conspecifics from cleaner estuaries or benchmark concentrations known to induce toxicities in other fishes. But, to our knowledge, no study has quantified actual burdens of total PCDD/Fs and PCBs and combined on a congener specific basis in tissues of either species from chemically impacted natural populations. Therefore it is problematic to compare actual tissues burdens of PCBs or PCDD/Fs on a TCDD toxicity equivalency quotients (TCDD TEQs) basis in environmentally exposed sturgeons to exposure concentrations of the two congeners used in our study.

For comparative purposes, we consider hepatic burdens of both coplanar PCBs and PCDD/Fs expressed as TCDD TEQs from a short-lived benthic species, Atlantic tomcod (*Microgadus tomcod*), largely sympatric to young life-stages of both sturgeons in the tidal Hudson River estuary. Mean levels (n=10) of hepatic TCDD TEQs exclusively from PCDD/Fs in adult male and female tomcod collected from the main stem Hudson River at river kilometer (RK) 80 were 99 pg/g wet weight (ww) and 31 pg/g ww, respectively. Mean levels (n=10) of total TCDD TEQs from both PCDD/Fs and coplanar PCBs in young-of-the-year tomcod from the main stem Hudson River ranged from 79 pg/g ww at RK 27 to 5 pg/g ww at RK 132 with an overall mean of 34 pg/g ww across nine Hudson River sites from RK 1 to RM 132 (Fernandez et al., 2004). We observed initial significant induction of CYP1A mRNA in both assays in Atlantic sturgeon and shortnose sturgeon at nominal exposure concentrations of 0.001 and 0.01 ppb, for TCDD and PCB126, respectively. Thus, hepatic TCDD TEQs (ww) in tomcod from the Hudson River always exceeded those that significantly induced CYP1A in larval Atlantic and shortnose sturgeon under controlled laboratory conditions.

Because we estimated whole body burdens of PCB126 in Atlantic sturgeon embryos using radiolabeled PCB126, we are able to identify the concentrations of PCB126 associated with significant induction of CYP1A in our gene expression experiments. We found that burdens of PCB126 in Atlantic sturgeon embryos exposed to 0.1 ppb PCB126 (the lowest concentration used in uptake experiments) for 8 hr and 24 hr were 1.04 and 0.634 ng/g ww, respectively. We also found that waterborne exposure of Atlantic sturgeon larvae to nominal 0.1 ppb PCB126 for 26–27 hr resulted in significant 80-fold induction of CYP1A. Therefore, we can conclude that a minimum whole body burden of about 1 ng/g ww PCB126 is sufficient to highly induce CYP1A mRNA in young life stages of Atlantic sturgeon. However, we do caution that uptake was estimated in embryos and gene

expression was measured in larvae and it is likely that uptake of PCB will differ between these two early life stages.

Two questions remain to be addressed. First, CYP1A expression is often used as an easily quantifiable and dose-responsive biomarker of exposure and early effect in environmentally exposed fishes. We have demonstrated that gene induction in sturgeon larvae is sensitive to environmentally relevant concentrations of AHR ligands. However, early life-stages of sturgeons are rarely collected from natural populations. Because both species are listed or being considered for federal listing as endangered, it is not permissible to invasively harvest tissue such as liver which are traditionally analyzed in gene expression studies. It is likely that only fin clips or perhaps blood will be made available for biomarker studies in these species. To empirically address the feasibility of using CYP1A mRNA expression in fin clips and blood as a biomarker in environmentally exposed sturgeons, we evaluated CYP1A mRNA inducibility in multiple tissues of shortnose sturgeon that were i.p. injected with environmentally relevant concentrations of PCB126. CYP1A was significantly inducible in heart, intestine, and liver but not in blood, fin clips or gill suggesting that these latter three tissues may not be for analysis in biomonitoring studies. But a caveat to this conclusion should be considered. Our treatment of juvenile shortnose sturgeon was via an i.p. route, however, in natural environments the primary route of exposure of bottom-dwelling fishes to benthic-borne PCBs and PCDD/Fs is through diet and secondarily through the waterborne route. It is possible that we failed to detect induced expression in fin clips and blood because PCB126 burdens in these tissues were insufficient to induce expression whereas in environmentally exposed natural populations that may not be true. It would have been informative to measure actual concentrations of PCB126 in tissues of our treated fish and compare them to concentrations in environmentally exposed sturgeons from populations in chemically impacted populations. That would have allowed us to determine if our failure to detect induced expression in our study was due to insufficient levels of PCB126 in blood and fin clips or reduced sensitivity of CYP1A inducibility in these tissues.

Second, the relationship between elevated levels of CYP1A in larvae and higher-level early life-stage toxicities has yet to be determined in sturgeons. In many studies, induction of CYP1A and teratogenicity co-occur in TCDD, PCB, or PAH-treated fishes because of their common mode of action through activation of the AHR pathway (Wirgin and Theodorakis, 2002b). Many studies have demonstrated that elevated expression of CYP1A precedes and is often correlated with development of differing endpoints of toxicity in fishes including reduced survivorship, altered hatching rate, and teratogenicity in early life-stages exposed to AHR ligands. It has been demonstrated that CYP1A activity is required for circulation failure, edema (Teraoka et al., 2003), and apoptotic cell death in the vasculature induced by TCDD (Cantrell et al., 1998), but not other toxicities in embryonic fishes (Carney et al., 2004). Although, we report here only on levels of gene expression in sturgeons exposed to TCDD and PCB126, in parallel studies we have observed sublethal toxic responses at the organism level (e.g., morphometrics, developmental rates) and in increased mortality at environmentally relevant levels of these contaminants (Chambers and Wirgin, In preparation).

In conclusion, for the first time we have partially characterized CYP1A in a sturgeon species and demonstrated that its expression is significantly inducible in young life-stages of both Atlantic sturgeon and shortnose sturgeon at environmentally relevant concentrations of TCDD and a coplanar PCB. We have also determined CYP1A is inducible in tissues that are likely targets of chemical induced toxicity, heart and liver. Based on these results, and the known role of AHR activation in mediating overt toxicities, it is likely that sturgeons are sensitive to PCDD/Fs and PCBs induced toxicities at environmentally relevant

concentrations. However, it remains to be determined if CYP1A inducibility can be used as a biomarker of exposure in environmentally exposed sturgeons.

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Fig. 1. UPGMA dendrogram of relatedness of CYP1A, 1B, 1C, and 3A amino acid sequences among various teleost fishes and the sturgeons reported here. Genetic distance is indicated at the bottom.

Fig. 2.

Semi-quantitative RT-PCR analysis of CYP1A mRNA expression in Atlantic sturgeon larvae exposed to graded exposure concentrations of waterborne TCDD (0.001 to 10 ppb) in acetone vehicle for 26–27 h and immediately sacrificed. A. Agarose gel (1.4%) with semi-quantitative RT-PCR amplification of CYP1A cDNA (top panel) and β -actin cDNA (bottom panel). B. Mean $\pm$ CYP1A/ β -actin relative expression in TCDD exposed Atlantic sturgeon larvae as determined by semi-quantitative RT-PCR. N= 3 pools of 5 individuals per treatment group. * Significantly different than acetone controls at $P < 0.05$.

Fig. 3.

Semi-quantitative RT-PCR analysis of CYP1A mRNA expression in Atlantic sturgeon larvae exposed to graded exposure concentrations of waterborne PCB126 (0.01 to 1000 ppb) in acetone vehicle for 26–27 h and immediately sacrificed. A. Agarose gel (1.4%) with semi-quantitative RT-PCR amplification of CYP1A cDNA (top panel) and β -actin cDNA (bottom panel). B. Mean $\pm$ CYP1A/ β -actin relative expression in PCB126 exposed Atlantic sturgeon larvae as determined by semi-quantitative RT-PCR. N= 3 pools of 5 individuals per treatment group. * Significantly different than acetone controls at $P < 0.05$.

Fig. 4.

Fold induction of CYP1A expression determined by quantitative real-time RT-PCR in whole Atlantic sturgeon larvae that were waterborne exposed for 26–27 h to graded exposure concentrations of TCDD (0.001 to 10 ppb) (Panel A) and graded exposure concentrations of PCB126 (0.01 to 1000 ppb) (Panel B) in acetone vehicle and immediately sacrificed after treatment. Fold induction in chemically treated larvae is compared to acetone vehicle controls. N= 6 pools of 5 individuals per treatment group. * Significantly different than acetone controls at $P < 0.05$.

Fig. 5.

Fold induction of CYP1A expression determined by quantitative real-time RT-PCR in whole shortnose sturgeon larvae that were waterborne exposed as embryos to graded exposure concentrations of waterborne TCDD (Panel A) and PCB126 (Panel B) in acetone vehicle for 24 h and transferred to clean H₂O for 7 d to 10 d until hatching. Fold induction in chemically treated larvae is compared to acetone vehicle exposed controls. N= 5–6 individual larvae per treatment group. * Significantly different than acetone controls at $P < 0.05$.

Fig. 6. Fold induction of CYP1A expression determined by quantitative real-time RT-PCR in six tissues of juvenile shortnose sturgeon that were i.p. injected with five graded exposure concentrations of PCB126 in corn oil vehicle for 8 d. Fold induction in PCB126 fish is compared to corn oil vehicle exposed controls. * Significantly different than acetone controls at $P < 0.05$.

Table 1

Primers based on conserved CYP1A sequences among lake trout, rainbow trout, and Japanese eels that were successfully used to amplify Atlantic sturgeon CYP1A cDNA fragments			
Sturcyp255F		CCCTCACCTCAGCCTGAC	
Sturcyp691F		GGCCGGCGCTACAGCCA	
Sturcyp922F		ATCACTGACTCCCTCATTG	
Sturcyp1589R		CGCTTGTGCTTCATGGTGA	
Sturcyp707R		TGGCTGTAGCGCCGGCC	
Sturcyp941R		TCAATGAGGGAGTCAGTG	
Primers based on derived Atlantic sturgeon sequences that were used to amplify and partially characterize CYP1A in Atlantic sturgeon			
Sturcyp538F		CCAGCAGTACTCCTGC	
Sturcyp864F		CGTCCAGAACATTGTGAC	
Sturcyp880R		GTCACAATGTTCTGGACG	
Sturcyp1240R		GGGAGGAAGGAGGAGTG	
Sturcyp1305R		AAGATACACGTGTCC	
Primers based on conserved sequences between white sturgeon and Chinese sturgeon that were used to amplify and partially characterize β -actin in Atlantic sturgeon and shortnose sturgeon			
Sturact168F		GGTGATGAGGCTCAGAGC	
Sturact193F		GGTATCCTGACCCTGAAG	
Sturact380R		TCAAACATGATCTGGGTC	
Sturact393R		GGTGTGAAGGTTTCAAAC	
Primers used in semi-quantitative RT-PCR analysis of CYP1A and β -actin mRNA expression in TCDD and PCB126 treated Atlantic sturgeon larvae			
<u>Primer Name</u>	<u>Species</u>	<u>Gene</u>	<u>Primer Sequence</u>
Sturcyp864F	AS	CYP1A	CGTCCAGAACATTGTGAC
Sturcyp1240R	AS	CYP1A	GGGAGGAAGGAGGAGTG
Sturact168F	AS	β -actin	GGTGATGAGGCTCAGAGC
Sturact393R	AS	β -actin	GGTGTGAAGGTTTCAAAC
Primers used in real-time RT-PCR analysis of CYP1A and β -actin mRNA expression in TCDD and PCB126 treated Atlantic sturgeon (AS) and shortnose sturgeon (SS) larvae			
<u>Primer Name</u>	<u>Species</u>	<u>Gene</u>	<u>Primer Sequence</u>
StycypRT231F	AS	CYP1A	GCAAGCTGGCACAGAATGC
StycypRT287R	AS	CYP1A	TGCTGGTCGGGGTTTCAAC
SNScypRT158F	SS	CYP1A	CCTTTTGGAGCTGGCTTTG
SNScypRT230C	SS	CYP1A	AGGGTGGGACACCAAGTACATT
Sturact60F	AS & SS	β -actin	CATTGTCACCACTGGGATGAC
Sturact125R	AS & SS	β -actin	ACACGCAGCTCATTGTAGAAGGT

Table 2

Uptake of ^3H radiolabeled PCB126 in acetone vehicle by Atlantic sturgeon embryos exposed for 2 hr, 8 hr, and 24 hr

Dose (ppb)	Time of Exposure	Mean Uptake PCB126 (ng/g embryos)
0.1	2 hr	0.000
1.0	2 hr	0.800
10	2 hr	9.847
0.1	8 hr	1.041
1.0	8 hr	5.409
10	8 hr	28.89
0.1	24 hr	0.634
1.0	24 hr	3.927
10	24 hr	27.02