Aryl hydrocarbon receptor nuclear translocators (ARNT1, ARNT2, and ARNT3) of white sturgeon (Acipenser transmontanus): Sequences, tissue-specific expressions, and response to β-naphthoflavone

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\textbf{ABSTRACT}

Sturgeons (Acipenseridae) are ancient fishes that have tissue-specific profiles of transcriptional responses to dioxin-like compounds (DLCs) that are unique from those generally measured in teleost fishes. Because DLCs exert their critical toxicities through activation of the aryl hydrocarbon receptor (AHR), this transcription factor has been the subject of intensive study. However, less attention has focused on the aryl hydrocarbon receptor nuclear translocator (ARNT), which is the dimerization partner of the AHR and required for AHR-mediated transcription. The present study sequenced ARNT1, ARNT2, and ARNT3 in a representative species of sturgeon, the white sturgeon (Acipenser transmontanus), and quantified tissue-specific basal transcript abundance for each ARNT and the response following exposure to the model agonist of the AHR, β-naphthoflavone. In common with other proteins in sturgeons, the amino acid sequences of ARNTs are more similar to those of tetrapods than are ARNTs of other fishes. Transcripts of ARNT1, ARNT2, and ARNT3 were detected in all tissues investigated. Expression of ARNTs are tightly regulated in vertebrates, but β-naphthoflavone caused down-regulation in gill, while an upward trend was measured in intestine. ARNTs are dimeric partners for multiple proteins, including the hypoxia inducible factor 1α (HIF1α), which mediates response to hypoxia. A downward trend in abundance of HIF1α transcript was measured in liver of white sturgeon exposed to β-naphthoflavone. Altered expression of ARNTs and HIF1α caused by activation of the AHR might affect the ability of certain tissues in sturgeons to respond to hypoxia when co-exposed to DLCs or other agonists.

1. Introduction

Dioxin-like compounds (DLCs), including polychlorinated dibenzo-\textregistered-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and co-planar polychlorinated biphenyls (PCBs), are a class of industrial pollutants of significant environmental concern. Exposure to DLCs can cause a range of adverse effects in fishes, including wasting syndrome, hepatotoxicity, decreased hemopoiesis, hyperplasia of gill filaments, fin necrosis, histological lesions, immune suppression, impaired reproduction, carcinogenesis, malformations, and mortality (Elonen et al., 1998; Giesy et al., 2002; Kleeman et al., 1988; Spitsbergen et al., 1986, 1988a, 1988b; Walter et al., 2000; Zabel et al., 1995). DLCs are agonists of the aryl hydrocarbon receptor (AHR) and most, if not all, critical adverse effects associated with exposure to DLCs are mediated through unrestricted activation of the AHR signalling pathway (Okey, 2007). Inactive AHRs are located in the cytosol in complex with a suite of interacting proteins (Kazlauskas et al., 1999, 2000). Upon binding of a ligand to the AHR, the interacting proteins dissociate, and the AHR-ligand complex translocates into the nucleus where it forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Nie et al., 2001; Reyes et al., 1992; Whitelaw et al., 1993). The AHR-ARNT heterodimer is able to interact with dioxin-responsive elements (DREs) to regulate transcription of target genes, including Phase I biotransformation enzymes such as cytochrome P450 1A (CYP1A) (Mimura and Fujii-Kuriyama, 2003; Whitlock Jr, 1999). In order to better understand the toxicities of DLCs, the protein structure, tissue-
specific expression, and molecular function of the AHR has been intensively investigated in fishes and other vertebrates, but significantly less is known about its dimerization partner, ARNT.

Several variants of ARNT have been identified in vertebrates, including fishes (Powell and Hahn, 2000). These ARNT variants are divided into three clades, namely ARNT1, ARNT2, and ARNT3, with each clade possibly containing multiple isoforms and splice variants (Hill et al., 2009; Lee et al., 2007, 2011; Powell and Hahn, 2000; Tanguay et al., 2000). ARNT1s function predominantly through heterodimerization with the AHR in response to exposure to various planar aromatic hydrocarbons and through heterodimerization with the hypoxia inducible factor 1α (HIF1α) in response to hypoxia (Nie et al., 2001; Prasch et al., 2004, 2006; Wang et al., 1995). Evidence suggests that ARNT2s function predominantly through heterodimerization with the single minded (SIM) transcription factor and serve roles in neurogenesis (Hirose et al., 1996). ARNT3s, which are also known as ARNT-like (ARNTL), brain and muscle ARNT-like-1 (BMAL1), or morphine preference 3 (MOP3), are believed to function predominantly through heterodimerization with the circadian locomotor output cycles kaput (CLOCK) transcription factor and serve roles in circadian rhythms (Gekakis et al., 1998; Onoue et al., 2019). However, functional redundancies in heterodimerization partners of ARNT1, ARNT2, and ARNT3 have been demonstrated in vitro (Doering et al., 2014b, 2015, 2018, Hirose et al., 1996, Hogenesch et al., 1997, Jain et al., 1998, Lee et al., 2007, 2011, Oka et al., 2013, Prasch et al., 2004, 2006, Zhang et al., 2019). ARNTs exist as shared dimeric partners for proteins in several physiological pathways so there is potential for cross-talk between these pathways through the altered abundance of ARNTs.

Currently, the limited research on ARNTs in fishes has primarily focused on more modern teleost fishes, with less known about ARNTs in more ancient species. Sturgeons (Acipenseridae) are an ancient family of fishes that emerged at least 65 million years ago (Willimovsky, 1956). Investigations to date suggest that sturgeons could be sensitive to a range of anthropogenic pollutants, including DLCs (Chambers et al., 2019). ARNT variants are di- stinct from those generally measured in teleosts (Doering et al., 2014b, 2015, 2018, Hirose et al., 1996, Hogenesch et al., 1997, Jain et al., 1998, Lee et al., 2007, 2011, Oka et al., 2013, Prasch et al., 2004, 2006, Zhang et al., 2019). ARNTs exist as shared dimeric partners for proteins in several physiological pathways so there is potential for cross-talk between these pathways through the altered abundance of ARNTs.

The protocol for exposing juvenile white sturgeon to βNF has been described previously (Doering et al., 2012). Briefly, seven white sturgeon were randomly assigned to each of three 400 L tanks that were maintained at approximately 12 °C under flow-through conditions. Following a four-week acclimation period, each fish was intraperitoneally injected with one of three doses (0 mg/kg-bm, 50 mg/kg-bm, or 500 mg/kg-bm) of βNF. Livers, gills, and intestines were collected for analysis and immediately snap-frozen in liquid nitrogen. Brains, hearts, stomachs, spleens, head kidney, and muscle were collected only from fish in the 0 mg/kg-bm treatment group. The tissue samples collected from this exposure study had previously been used for measurement of monooxygenase activity (Doering et al., 2012), abundance of CYP1A transcript (Doering et al., 2012), abundance of AHR1 and AHR2 transcripts (Doering et al., 2012, 2014a), and for transcriptomics (Doering et al., 2014a). As a result of these previous analyses, sufficient sample from this exposure was available only from four white sturgeon from each treatment group (n = 4) for use in the current study.

Transcriptomes for white sturgeon had previously been generated by use of Illumina paired-end transcriptome sequencing of RNA isolated from livers (Doering et al., 2014a; Doering et al., 2016). A full-length cDNA sequence of ARNT2 of white sturgeon had previously been identified from these transcriptomes and the consensus nucleotide sequence was obtained as described elsewhere (Doering et al., 2014b). However, sequences of other ARNTs were not known for any species of sturgeon. Partial nucleotide sequences of ARNT1 and ARNT3 were acquired by use of rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). Total RNA was extracted from approximately 30 mg of liver of white sturgeon by use of the RNaseasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) and RACE-cDNA was synthesized by use of the SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). RACE-PCR was performed by use of the Advantage 2 PCR Kit (Clontech). Gene-specific RACE-PCR and full-length primers for ARNT1 and ARNT3 of white sturgeon were designed by use of Primer3 software (Table 1, Rozen and Skaletsky, 2000) and synthesized by Invitrogen (Burlington, ON, Canada). Full-length PCR products for ARNT1 and ARNT3 were gel purified by use of the

2. Materials and methods

2.1. Fish

Juvenile white sturgeon of approximately 1.5 years of age and ranging in body mass (bm) from 12 to 27 g were randomly selected from an in-house stock maintained at the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan (Saskatoon, SK, Canada). White sturgeon were reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). The studies reported here were approved by the Animal Research Ethics Board at the University of Saskatchewan (Protocol #2007/0049).
**Table 1**

Sequences, efficiencies, annealing temperatures, and corresponding target gene Genbank accession numbers of white sturgeon oligonucleotide primers used to acquire full-length sequences and used in quantitative real-time PCR (qRT-PCR). Not available information is indicated (—).

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Accession #</th>
<th>PCR</th>
<th>Primer Sequence (5′-3′)</th>
<th>Efficiency (%)</th>
<th>Annealing Temp (°C)</th>
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<td>ARNT1</td>
<td>KX903304</td>
<td>RACE</td>
<td>3′: AAGTTATACAAAGGACCACTGGG</td>
<td>—</td>
<td>66</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Forward: ATGGAGGCGGGGTTAACC</td>
<td>—</td>
<td>67</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: TACCTGAGCTGGCGTTGGG</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<tr>
<td></td>
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<td></td>
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<td>60</td>
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<td></td>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td>Reverse: CCAGGACAAATGGAAAAATCAA</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: ACGGGTGAGCTGTGGTAATC</td>
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</table>

QIAquick Gel Extraction Kit (Qiagen) and then 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, USA). Plasmids were isolated by use of a QIAprep Spin Miniprep Kit (Qiagen) and the products were sequenced by the University of Calgary’s University Core DNA Services (Calgary, AB, Canada). Consensus nucleotide sequences for ARNT1 and ARNT3 were determined by aligning three or more replicated sequences by use of the CLUSTALW function in the Biology Workbench v.3.2 (Subramaniam, 1998).

2.4. Quantitative real-time PCR

Total RNA was extracted from approximately 30 mg of brain, heart, muscle, liver, gill, stomach, intestine, spleen, head kidney, or muscle of white sturgeon (n = 4) by use of the RNeasy Lipid Tissue Mini Kit (Qiagen). First-strand cDNA synthesis was performed by use of the QuantiTect Reverse Transcription Kit (Qiagen) with 1 μg of total RNA. The cDNA samples were stored at −20 °C until analyzed. Real-time PCR (qRT-PCR) was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50 μl reaction mixture of Quantifast SYBR Green master mix (Qiagen), 2.5 μl of cDNA, 10 pmol of gene-specific qRT-PCR primers, and nuclease free water was prepared for each cDNA sample and primer combination and reactions were conducted in duplicate with 20 μl reaction volumes per well. Gene-specific primers for quantification of ARNT1, ARNT2, and ARNT3 expressions were designed from full-length consensus sequences and gene-specific qRT-PCR primers for HIF1α were designed from conserved regions of the publicly available nucleotide sequence of HIF1α of Chinese sturgeon (*Acipenser sinensis*) (Table 1). Primers for qRT-PCR were designed by use of Primer3 software (Rozen and Skaletsky, 2000) and synthesized by Invitrogen. Each primer pair was validated through investigation of melt curves and gel electrophoresis to ensure amplification of a single amplicon. Reaction efficiency was determined by use of serial dilutions of a pool of cDNA, and all efficiencies were in the range of 80 to 110%. The qRT-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 expression was quantified by normalizing to β-actin according to methods described previously (Simon, 2003). Gene-specific qRT-PCR primers for β-actin of white sturgeon (Table 1) were developed and validated as described previously (Doering et al., 2012).

2.5. Phylogenetic tree

A phylogenetic tree was generated based on putative amino acid sequences of ARNTs of vertebrates by use of the CLC Genomics Workbench v.4.7.2 (Kattebierjeby, Aarhus, Denmark). Amino acid sequences that were predicted from genomic data were not included in the phylogenetic tree. Accession numbers used were: Atlantic croaker (*Micropogonias undulatus*) ARNT1 (ABD32161.1), Atlantic croaker ARNT2 (ABD32161.1), Atlantic tomcod (*Microgadus tomcod*) ARNT1 (ACX53266.1), chicken (*Gallus gallus*) ARNT1 (NP_989531.1), chicken ARNT2 (XP_413854.2), chicken ARNT3 (NP_001082130.1), clawed frog (*Xenopus laevis*) ARNT1 (NP_001082130.1), clawed frog (*Xenopus laevis*) ARNT2 (AA91608.1), clawed frog (*Xenopus laevis*) ARNT3 (AA91608.1), clawed frog (*Xenopus laevis*) ARNT2 (AA91608.1), clawed frog (*Xenopus laevis*) ARNT3 (AA91608.1), grass carp (*Ctenopharyngodon idella*) ARNT2b (AAP70730.1), grass carp ARNT2c (AAP70730.1), rainbow trout ( Oncorhynchus mykiss) ARNTa (AAG60051.1), rainbow trout ARNTb (AAG60052.1), zebrafish (*Danio
rerio) ARNT1a (NP_001007790.1), zebrafish ARNT1b (NP_001011712.1), zebrafish ARNT1c (NP_001038736.1), zebrafish ARNT2α (AAG25919.1), zebrafish ARNT2β (AAG25920.1), zebrafish ARNT2c (AAG25921.1), zebrafish ARNT3α (NP_571652.1), zebrafish ARNT3b (AAI34896.1).

2.6. Statistical analysis

Statistical analyses were conducted by use of IBM SPSS 20 software (Armonk, NY, USA). Normality of each dataset was determined by use of the Shapiro Wilk’s test and homogeneity of variance was determined by use of Levene’s test. A logarithmic transformation was used whenever necessary to ensure homogeneity of variance. Data was analyzed by use of either analysis of variance (one-way ANOVA) followed by either Dunnett’s test or Tukey’s test or by use of a Kruskal Wallis test followed by Mann Whitney U test. Bonferroni Correction was applied where applicable. All data are shown as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Identification and phylogeny of ARNTs

Full-length nucleotide sequences of ARNT1 and ARNT3 of white sturgeon were identified. The full-length nucleotide sequence of ARNT2 of white sturgeon had previously been identified (Doering et al., 2014b). ARNT1, ARNT2, and ARNT3 of white sturgeon clustered closely with ARNT1s, ARNT2s, and ARNT3s of other vertebrates, respectively (Fig. 1). However, ARNTs of white sturgeon clustered more closely with ARNTs of tetrapods than did ARNTs of other fishes (Fig. 1). ARNT1 and ARNT3 of white sturgeon share greatest amino acid similarity with ARNT1 or ARNT3 of chicken, respectively, while ARNT2 shares greatest amino acid similarity with ARNT2s of grass carp and zebrafish (Table 2). Full-length nucleotide sequences of ARNT1 (KX903304.1), ARNT2 (KJ959625.1), and ARNT3 (KX903304.1) of white sturgeon are publicly available in the NCBI database.

3.2. Basal expression of ARNTs

Transcripts of ARNT1, ARNT2, and ARNT3 were detected in liver, brain, gill, heart, spleen, stomach, intestine, head kidney, and muscle of juvenile white sturgeon (Fig. 2A). Basal expression in liver of juvenile white sturgeon was not statistically different (p > .05) among ARNT1, ARNT2, and ARNT3 (Fig. 2B). ARNT1 had greatest expression in liver, gill, heart, spleen, and head kidney of juvenile white sturgeon (Fig. 2A). ARNT2 had greatest expression in brain, gill, and heart (Fig. 2A). ARNT3 had greatest expression in liver and heart (Fig. 2A).

3.3. Expression of ARNTs following exposure to βNF

Intraperitoneal exposure of juvenile white sturgeon to βNF resulted in a statistically significant change (p < .05) in expression of ARNT1 in liver and gill and ARNT2 and ARNT3 in liver (Fig. 3). Expression of ARNT1 in liver was 0.24-fold lesser following exposure to 500 mg βNF/kg-bw, but expression was not statistically different (p > .05) between

<table>
<thead>
<tr>
<th>Species</th>
<th>Percent similarity (%)</th>
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<tbody>
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<td>White Sturgeon</td>
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</tr>
<tr>
<td></td>
<td>Mouse ARNT1</td>
</tr>
<tr>
<td></td>
<td>Zebrafish ARNT1c</td>
</tr>
<tr>
<td></td>
<td>Rainbow Trout ARNT1a</td>
</tr>
<tr>
<td></td>
<td>Rainbow Trout ARNT1b</td>
</tr>
<tr>
<td></td>
<td>Atlantic Tomcod ARNT1</td>
</tr>
<tr>
<td>White Sturgeon</td>
<td>Grass Carp ARNT2b</td>
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<tr>
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<td>Grass Carp ARNT2c</td>
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<tr>
<td>White Sturgeon</td>
<td>Zebrafish ARNT2a</td>
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<tr>
<td></td>
<td>Mouse ARNT3</td>
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<td>Zebrafish ARNT3b</td>
</tr>
<tr>
<td></td>
<td>Clowed Frog ARNT3</td>
</tr>
</tbody>
</table>

Table 2 Percent similarity of the putative, full-length amino acid sequence of ARNT1, ARNT2, and ARNT3 of white sturgeon compared to ARNTs of other vertebrates.

Fig. 1. Phylogenetic tree for relatedness of amino acid sequences of ARNTs among select species of vertebrates. ARNT1, ARNT2, and ARNT3 of white sturgeon are highlighted. Branch lengths represent bootstrap values based on 1000 samplings.
the 0 and 50 mg βNF/kg-bm treatments (Fig. 3). Expression of ARNT2 in liver was 0.21- and 0.10-fold lesser following exposure to 50 and 500 mg βNF/kg-bm, respectively (Fig. 3). Expression of ARNT3 in liver was 0.42-fold lesser following exposure to 500 mg βNF/kg-bm, but was not statistically different (p > .05) between the 0 and 50 mg βNF/kg-bm treatments (Fig. 3). Expression of ARNT1 in gills was 3.2-fold greater following exposure to 50 mg βNF/kg-bm, but expression was not statistically different (p > .05) between the 0 and 500 mg βNF/kg-bm treatments (Fig. 3). Expression of ARNT2 and ARNT3 in gills was not statistically different (p > .05) among any of the treatments (Fig. 3). Expression of ARNT1, ARNT2, and ARNT3 in intestine was not statistically different (p > .05) among any of the treatments (Fig. 3).

3.4. Expression of AHRs and HIF1α

Abundance of AHR1 and AHR2 transcripts had previously been determined in the same cDNA samples of juvenile white sturgeon as investigated here for ARNTs and HIF1α (Doering et al., 2014a). Therefore, expression of these genes were directly compared. Basal expressions of ARNTs in liver were 30-fold greater (p ≤ .05) relative to AHR1, AHR2, and HIF1α (Fig. 4). Basal expressions in liver were not different (p > .05) among AHR1, AHR2, and HIF1α (Fig. 4). Expression of HIF1α in liver was not statistically different (p > .05) among any of the treatments (Fig. 5), but there was a downward trend in the 50 and 500 mg βNF/kg-bm treatments (Fig. 5). Expression of HIF1α was not measured in gill or intestine because there were limited changes in expression measured for ARNT1, ARNT2, and ARNT3 in these tissues (Fig. 5).
and hematopoietic development (Abbott and Buckalew, 2000; Kozak et al., 1997; Maltepe et al., 1997), ARNT2 functions in development of the hypothalamus and nervous system (Hosoya et al., 2001; Keith et al., 2001), and ARNT3 functions in regulation of biological rhythms (Gekakis et al., 1998). In zebrafish, ARNTs have roles in developmental processes and responses to environmental cues in the liver, brain, heart, immune system, and possibly the peripheral nervous system (Alexeyenko et al., 2010; Cermakian et al., 2000; Hill et al., 2009). However, specific roles of ARNTs in other fishes have not yet been investigated. As in other fishes, ARNT1 and ARNT2 of white sturgeon were expressed in all examined tissues (Fig. 2A), which is unlike in tetrapods where ARNT1 is widely expressed while ARNT2 is restricted to endothelial tissues (Drutel et al., 1996; Hirose et al., 1996; Hoffman et al., 1991; Lee et al., 2007, 2011; Powell and Hahn, 2002). White sturgeon had comparable hepatic expression of ARNT1 and ARNT2 (Fig. 2B), which is also the case in the freshwater climbing perch (Anabas testudineus) (Zhang et al., 2019). In contrast, expression of ARNT2 is greater than ARNT1 in liver of zebrafish and mummichog (Powell and Hahn, 2002; Prasch et al., 2006; Tanguay et al., 2000) and expression of ARNT1 is greater than ARNT2 in liver of Atlantic cod (Gadus morhua) (Aranguren-Abadia et al., 2019). White sturgeon had greatest expression of ARNT3 in the liver, which has also been shown in mammals (Fang et al., 2014; Krishnaiah et al., 2017; Tong and Yin, 2013; Zhang et al., 2014), while in zebrafish the expression of ARNT3 is greater in brain, eye, heart, kidney, spleen, and gonad than in liver (Cermakian et al., 2000). However, there can be weak correlations between abundance of transcripts and abundance of proteins as a result of post-translational effects (Juschke et al., 2013). It is currently unknown whether these observed differences in levels of tissue-specific expression of ARNTs among species have any biological relevance due to uncertainty with post-translational effects and because only a few studies have investigated tissue-specific expression of ARNTs and only in a few species and life-stages.

Expression of ARNTs is tightly regulated in vertebrates because of their critical functions (Mandl and Depping, 2014). Lethal developmental abnormalities occur in mice that are mutated to be null for ARNT1 or ARNT2 and in zebrafish that are mutated to be either null or to over-express ARNT2 (Hill et al., 2009; Keith et al., 2001). Therefore, dysregulation of expressions of ARNTs by exposure to chemicals could result in adverse effects in fishes, particularly during early developmental stages. Alteration in expressions of ARNTs by exposure to DLCs, or other agonists of the AHR, have not been adequately investigated in fishes. Most studies on fishes measure no statistically significant change in expression of ARNTs following exposure to an agonist of the AHR (Andreasen et al., 2002; Brinkmann et al., 2016; Doering et al., 2016; Rahman and Thomas, 2019). Exposure to agonists of the AHR increased expression of ARNT1 and decreased expression of ARNT2 in liver of freshwater climbing perch (Zhang et al., 2019) and increased expression of ARNT1 and ARNT2 in liver of Atlantic croaker (Micropterus undulatus) (Rahman and Thomas, 2019). Effects of activation of the AHR on expression of ARNT3 is poorly understood with the exception of decreased hepatic expression of ARNT3 in mice (Fader et al., 2019). In the current study, expression of ARNT1, ARNT2, and ARNT3 was decreased in livers and expression of ARNT1 was increased in gills following exposure to βNF (Fig. 3). Further, an upward trend in expression of ARNT2 and ARNT3 was measured in gills and of ARNT1 and ARNT3 in intestines (Fig. 3). These differences in regulation of expression of ARNTs among species and tissues could be hypothesized to occur due to a variety of factors, including differences in durations of exposure, dose and potency of the agonist, and in adsorption, distribution, metabolism, and excretion (ADME). The liver typically elicits the greatest AHR-mediated response in fishes, but previous studies have shown that tissue-specific profiles of responses in white sturgeon are unique from those that are typically measured in teleosts (Doering et al., 2012). Exposure of white sturgeon to βNF caused an increase in abundance of CYP1A transcript in liver, gill, and intestine which was

**Fig. 5.** Expression of HIF1α in livers of juvenile white sturgeon 3 days following intraperitoneal injection with either 0, 50, or 500 mg βNF/kg-bm. Data represent mean ± S.E.M. (n = 4 fish). Expression in each treatment is shown relative to 0 mg βNF/kg-bm, which has been standardized to 1. An asterisk (*) indicates significant difference from livers of fish exposed to 0 mg βNF/kg-bm (ANOVA followed by Dunnett’s, p ≤ .05).

4. Discussion

Compared to the AHR, relatively less is known about differences in amino acid sequences, tissue-specific expressions, or functions of ARNTs among fishes. Results of the present study demonstrate that white sturgeon express three distinct clades of ARNTs, namely ARNT1, ARNT2, and ARNT3 (Fig. 1, Table 2). This is consistent with most other vertebrates that have been investigated (Powell et al., 1999). Although some differences in clades do exist among species; for example, an ARNT2 is not known in rainbow trout or scup (Stenotomus chrysops), and only divergent forms of ARNT1 are known (Pollenz et al., 1996; Powell and Hahn, 2000). Almost nothing is known about ARNTs in more ancient fishes, with sturgeons (Chondrostei) being among the earliest group of fishes with sequences of ARNTs currently in the NCBI database. Putative amino acid sequences of the three ARNTs identified in white sturgeon resemble an intermediate between sequences of other fishes and sequences of tetrapods (Fig. 1). Similar findings have been reported in sturgeons for other Per-Arnt-Sim (PAS) proteins, including the AHR (Doering et al., 2014a; Roy et al., 2018) and HIF1α (Rytikonen et al., 2007). This has been suggested to occur as a result of sturgeons being an ancient lineage that are less divergent from tetrapods relative to the more modern teleosts (Doering et al., 2014a). Further, sturgeons have a reduced degree of protein evolution, possibly as a result of a large genome that can be up to hexadecaploid (16 n), which might also add to their proteins being less divergent from those of ancestral species (Birstein, 1993; Doering et al., 2014a). As is the case with the AHR and HIF1α, it is unknown whether there is any functional significance of sturgeons expressing ARNTs that share greater identity with ARNTs of tetrapods relative to those of most other fishes. However, in vitro studies with several species of sturgeon indicate that both ARNT1 and ARNT2 can heterodimerize with both AHR1 and AHR2 and regulate gene expression through interaction with DREs (Doering et al., 2014b, 2015; Roy et al., 2018), which has also been demonstrated in numerous other species of fish and in reptiles and birds (Abnet et al., 1999a, 1999b, Bak et al., 2013, Doering et al., 2018, Hansson and Hahn, 2008, Hirose et al., 1996, Karchner et al., 1999, Lee et al., 2007, 2011, Oka et al., 2013, Virgin et al., 2011). Heterodimerization between ARNT3 and AHRs or between ARNTs and other partner proteins, such as HIF1α, has not yet been investigated in sturgeons and in general is less understood in fishes relative to heterodimerization between ARNT1 or ARNT2 and AHRs.

Expression of ARNTs is ubiquitous, constitutive, in abundance, and tightly regulated in vertebrates because of the various roles of ARNTs in developmental processes and responses to environmental cues (Mandl and Depping, 2014). In mammals, ARNT1 functions in normal vascular
accompanied by a relatively comparable increase in abundance of AHR1 and AHR2 transcripts in two previous investigations using the same samples as the current investigation into ARNT (Doering et al., 2012, 2014a). This increase in expression of AHR1 and AHR2 was hypothesized to be one mechanism for the greater responsiveness of gill, intestine, and to a lesser degree, liver, in white sturgeon relative to teleosts (Doering et al., 2012, 2014a). The present study adds to this hypothesis by showing that the direction of response of ARNTs to JNF are negatively correlated relative to responses of CYP1A and AHRs in liver but are comparable to responses of CYP1A and AHRs in gill and intestine (Fig. 3). The reason for this tissue-specific pattern of responses is currently unknown. It could be hypothesized that ARNTs are down-regulated in livers to repress uncontrolled activation of the AHR signalling pathway in this highly responsive tissue, while ARNTs are upregulated in gills and potentially in intestines in order to increase the capacity of these less-responsive tissues to activate the AHR signalling pathway in response to the exposure. The absence of any increase in expression of ARNTs in gills of white sturgeon exposed to 500 mg JNF/kg-bm (Fig. 3), which was also shown for AHR1 and AHR2 in the previous investigations of the same samples (Doering et al., 2012, 2014a), could be a compensatory mechanism to prevent over-activation of the AHR signalling pathway at the greatest exposure dose which could lead to tissue damage. However, ARNTs are expressed in great abundance in white sturgeon with approximately 30-fold greater basal expression relative to AHRs in liver (Fig. 4) so the measured changes in expression of ARNTs might have limited biological relevance in context with the capacity for activation of the AHR signalling pathway if the great abundance of transcript is representative of a great abundance of protein.

ARNTs are common dimeric partners for multiple proteins and as a result there is the possibility for cross-talk between signalling pathways in competition for ARNT. Exposure to DLCs causes activation of the AHR which might adversely affect the activation of one or more signalling pathways which share ARNT, for example the HIF1α signalling pathway that is critical for the response to hypoxia. In the case of cross-talk between the AHR and HIF1α, it has been proposed that the amount of ARNT is great enough to not represent a limiting factor in the co-activation of both signalling pathways (Park, 1999). It has been demonstrated in embryos of zebrafish that potent activation of the AHR did not affect responses to moderate hypoxia and in fact, the potency of the DLC was decreased, suggesting that HIF1α can outcompete AHR for available ARNTs (Prasch et al., 2004). However, zebrafish are a tolerant species to both hypoxia and DLCs, with embryos being capable of enduring anoxia for extended periods and doses of DLCs up to 40-fold greater than the most sensitive known fishes (Doering et al., 2013; Nikinmaa and Rees, 2005). Therefore, these findings in embryos of zebrafish might not hold true for exposure to severe hypoxia, in juveniles or adults, or in other species (Prasch et al., 2004). In particular, it is unclear whether HIF1α could compete with AHR for available ARNT during co-exposure to DLCs and hypoxia in species that are sensitive to DLCs and tolerant to hypoxia, tolerant to DLCs and sensitive to hypoxia, or sensitive to both DLCs and hypoxia. Sturgeons studied to date are known to be tolerant of hypoxia, possibly due to their characteristically lesser metabolic rate but also likely due to a relatively great capacity for activation of the HIF1α signalling pathway (Kieffer et al., 2001, 2011; Maxime et al., 1995; Randall et al., 1992). In contrast to zebrafish, some species of sturgeons, including white sturgeon, are likely to be among the most sensitive fishes to exposure to DLCs (Chambers et al., 2012, Doering et al., 2012, 2014b, Eisner et al., 2016, Tillitt et al., 2017). Further, exposure to JNF resulted in a downward trend in expression of HIF1α, a decrease in expressions of ARNT1, ARNT2, and ARNT3, and an increase in expressions of AHR1 and AHR2 in livers of white sturgeon (Doering et al., 2012, 2014a). This raises the question as to whether a decrease in expression of HIF1α and ARNTs accompanied by an increase in expression of AHRs would increase competition for available ARNTs and impair the ability of liver, or potentially other tissues with similar patterns of response in sturgeons, to respond to hypoxia when co-exposed to DLCs and hypoxia or anoxia. Depletion of dissolved oxygen in aquatic ecosystems is a growing concern, especially around areas of anthropogenic activity where exposure to pollutants, such as DLCs, is likely to occur. Therefore, further research should investigate whether the transcriptional responses of ARNTs following exposure of sturgeons to agonism of the AHR could increase their susceptibility to hypoxia or disrupt functioning of other signalling pathways that share ARNTs, such as circadian regulation.

5. Conclusion

This study identified ARNTs from three clades, namely ARNT1, ARNT2, and ARNT3, and characterized tissue-specific expression in a representative member of the Acipenseridae, the white sturgeon. The genetic diversity among fishes is greater than that of any other vertebrate group and there appears to be AHR signalling pathway diversity among fishes, with sturgeons possibly having tissue-specific transcriptional responses of ARNTs that are unique from those of some other fishes. Expression of ARNTs is tightly regulated in vertebrates as they play critical roles in numerous physiological and developmental processes, including responses to exposure to DLCs and hypoxia. Decreased expressions of ARNTs and HIF1α accompanied by increased expressions of AHRs following exposure to DLCs might increase the susceptibility of certain tissues in sturgeons to adverse effects resulting from co-exposure to DLCs and hypoxia or anoxia. Further information for sturgeons is necessary regarding interactions between ARNTs, AHRs, HIF1α, and other proteins that heterodimerize with ARNTs in the liver, gill, intestine, and other tissues and the possible implications for regulation of genes, developmental processes, environmental homeostasis, and toxicities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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