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# CHARACTERIZATION OF THE ARYL HYDROCARBON RECEPTOR REPRESSOR AND A COMPARISON OF ITS EXPRESSION IN ATLANTIC TOMCOD FROM RESISTANT AND SENSITIVE POPULATIONS

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**Abstract**—Atlantic tomcod from the Hudson River, USA, are resistant to cytochrome P4501A1 (CYP1A1) mRNA induction and early life stage toxicities induced by coplanar polychlorinated biphenyls (PCBs) or tetrachlorodibenzo-*p*-dioxins but not polycyclic aromatic hydrocarbons. We sought to determine if basal expression or inducibility of aryl hydrocarbon receptor repressor (AHRR) mRNA is higher in tomcod from the resistant Hudson River population than in those from sensitive populations. Tomcod AHRR cDNA was characterized and its expression quantified in different tissues and life stages of tomcod from the Hudson River, Miramichi River, Canada (sensitive), and among environmentally exposed tomcod from these two sources and the St. Lawrence River, Canada. Phylogenetic analysis revealed that tomcod AHRR falls within the clade of other vertebrate aryl hydrocarbon receptors (AHRs) but is most closely related to the four previously identified AHRR genes. Induction of AHRR mRNA was observed in all tissues of PCB77-treated juvenile tomcod of Miramichi River descent, and expression differed among tissues and was significantly related to levels of CYP1A1 mRNA expression. Aryl hydrocarbon receptor repressor mRNA was similarly inducible in F2 embryos of Miramichi and Hudson River descent by benzo[*a*]pyrene but less by PCB77 in Hudson River offspring. A significant, positive correlation was observed between CYP1A1 mRNA and AHRR mRNA concentrations in environmentally exposed tomcod from the three rivers. We conclude that differences in basal expression or inducibility of AHRR mRNA are not the mechanistic basis of resistance but that levels of AHRR often mirror those of CYP1A1, suggesting that a common AHR pathway–related mechanism may modulate expression of both genes.

**Keywords**—Resistance Aryl hydrocarbon receptor pathway Gene expression Polychlorinated biphenyls Polycyclic aromatic hydrocarbons

## INTRODUCTION

Aromatic hydrocarbon (AH) compounds are known to elicit a variety of toxic effects in fishes including DNA damage, early life stage toxicities, and neoplasia. For example, early life stage toxicities elicited by 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) are the likely cause of chronic reproductive failure and eventual extirpation of the Lake Ontario, USA, and Canada lake trout *Salvelinus namaycush* population [1]. Epizootics of hepatic neoplasia in several North American fish populations probably resulted from exposure to polycyclic aromatic hydrocarbons (PAHs) and perhaps other AH compounds [2]. These toxic effects are often related to increased levels of DNA damage in fishes from these impacted populations. Damage from AHs to DNA may result from oxidation of PAHs to bulky reactive metabolites that adduct to DNA [3] or from the generation of reactive oxygen species that can modify DNA bases [4].

Sediments in the Hudson River Estuary (New York–New Jersey) are highly contaminated with AH compounds including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans, and PAHs. Two hundred miles of the Hudson River downstream of Ft. Edwards (NY, USA) is designated a U.S. federal Superfund site, the largest in the nation, because of PCB contamination. Similarly, the lower Passaic River (NJ, USA), a tributary of Newark Bay in the western Hudson River Estuary, is a federal Superfund site because of contamination with TCDD. Also, much of the lower Hudson River Estuary is highly polluted with PAHs [5].

Atlantic tomcod *Microgadus tomcod* is an abundant, anadromous, bottom-dwelling fish species in the Hudson River Estuary. Tomcod are resident year-round in their natal estuaries, but they undergo seasonal migrations in late autumn to spawning areas at or above the salt front [6]. Because tomcod have extremely lipid-rich livers [7] and consume a variety of benthic prey, they may serve as effective sentinels of sediment-borne, lipophilic contaminants such as AHs. Additionally, because tomcod are the only wintertime spawners in the Hudson River Estuary, their young life stages are important prey to resource species in freshwater, brackish, and marine sectors of the estuary [8]. It has been demonstrated that during the summer months, juvenile tomcod serve as the dominant prey of many predatory finfish species in the deepwater channels of the river.

Unfertilized eggs [9] and livers from juvenile (<1 year old) [10] and adult [11] tomcod from the Hudson River Estuary exhibit much higher burdens of halogenated aromatic hydrocarbons than conspecifics from cleaner Atlantic coast rivers. For example, juvenile tomcod from the Hackensack River (NJ,

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USA) and Newark Bay exhibited 930 times higher hepatic TCDD toxic equivalency quotients (a summary measure of the toxicity of environmental samples) than similarly aged tomcod from the Miramichi River. Levels of hepatic TCDD (the most toxic PCDD congener) in tomcod from the Hackensack River and Newark Bay were among the highest ever reported in a natural population of any species [10]. Because of their contamination and unique position in the food chain, tomcod probably are important vectors of contaminant exposure to top predators in the Hudson River food web [11].

In the early 1980s, tomcod from the Hudson River exhibited an extraordinarily high prevalence of hepatic tumors compared to conspecifics from cleaner North American estuaries [7,12,13]. Over 90% of two-year-old and 40% of one-year-old tomcod exhibited hepatic tumors [13]. Concurrently, the age structure of the Hudson River spawning aggregation was significantly truncated compared to those in other rivers [13]. Decreased longevity of the Hudson River population may have resulted from exposure to chemical pollutants, thermal stress during the warmer months, genetically programmed interpopulation differences in longevity, or a combination of these or other factors. Also, results from long-term monitoring programs over the past two decades suggest an overall decline in the abundance of spawning tomcod in the Hudson River (M. Mattson, Normandeau Associates, Bedford, NH, USA, personal communication).

Hepatic cytochrome P4501A1 (CYP1A1) expression is widely used as a biomarker to monitor exposure and early biological effect of AH contaminants in natural populations of fishes. Expression of CYP1A1 at the transcriptional and translational levels is a sensitive and dose-responsive measure of exposure of fishes to coplanar AH compounds [14,15]. Also, induction of hepatic CYP1A1 enzyme activity is mechanistically linked to perturbations at higher levels of biological organization, including DNA damage, early life stage toxicities, and neoplasia.

Transcription of CYP1A1 is activated by the aryl hydrocarbon receptor (AHR) pathway in all vertebrates, including fishes [16]. Briefly, diffusion of lipophilic AH compounds (ligand) into the cell results in their binding to AHR in the cytoplasm; release of two molecules of heat-shock protein 90 (Hsp90), ARA9 and p23, from AHR; and translocation of the AHR–ligand complex by an unknown mechanism into the nucleus. Within the nucleus, the AHR–ligand complex binds with a second transcription factor, the aryl hydrocarbon nuclear translocator (ARNT). The AHR–ligand–ARNT complex then binds to dioxin response enhancer elements (DREs) in the 5′ regulatory region of genes in the AHR battery, such as CYP1A1, and activate their transcription. Furthermore, it is believed that activation of the AHR pathway is needed for most, if not all, toxic responses to AH exposure and that CYP1A1 expression is predictive of most toxic responses to these chemicals [17]. The AHR in fishes, including tomcod [18], is structurally similar to that in mammals, although two functional forms of AHR—AHR1 and AHR2—are seen in many fish taxa [19].

Recently, an aryl hydrocarbon receptor repressor (AHRR) that inhibits AH activation of CYP1A1 transcription was identified in mouse [20], human [21], rat [22], and Atlantic killifish *Fundulus heteroclitus* [23]. Expression of AHRR provides negative feedback regulation of induction of CYP1A1 transcription, probably other inducible genes in the AHR battery, and perhaps AH-induced toxicities. The AHRR shares many, although not all, of the structurally and functionally conserved domains that are characteristic of other genes in the Per-AHR/ARNT-Sim (PAS) family, including basic–helix–loop–helix (bHLH) and PAS-A domains. However, truncation of PAS-B and an absence of Q-rich domains prevent AHRR from activating gene expression. Because its 5′ regulatory region contains multiple DREs [23,24], AHRR expression is inducible by AH compounds through AHR pathway activation as described previously for CYP1A1. Induced AHRR competes with AHR for binding to ARNT, and the AHRR–ARNT complex binds to DREs of genes in the AHR battery but does not activate their transcription. Occupancy of DREs by AHRR–ARNT occludes access to them by AHR–ARNT and thereby represses transcription of genes such as CYP1A1.

In earlier studies, hepatic CYP1A1 mRNA expression was found to be significantly higher in environmentally exposed adult tomcod from the Hudson River than in those from four cleaner Atlantic coast estuaries [25]. In controlled laboratory experiments, however, adult tomcod from the Hudson River were two orders of magnitude less sensitive to hepatic and extrahepatic CYP1A1 induction by halogenated aromatic hydrocarbons than those collected from cleaner locales [15,26]. This phenomenon was observed for three coplanar PCBs and TCDD [26]. Similarly, tomcod embryos and larvae of Hudson River descent were significantly less sensitive than those from reference sites to early life stage toxicities induced by exposure to TCDD or a mixture of coplanar PCBs [27]. However, inducibility of CYP1A1 mRNA was similar between adult or young life stages of tomcod of Hudson River and reference river descent when treated with either of two PAHs, benzo[*a*]pyrene (B*a*P), or beta-naphthoflavone (β-NF) [15,27]. These results indicate that resistance to halogenated aromatic hydrocarbons but not nonhalogenated PAH-induced gene expression and early life stage toxicities has developed in the Hudson River tomcod population.

It is important to know the mechanistic basis of resistance in the Hudson River tomcod population in order to predict its persistence in the population and ramifications at the ecosystem level. The phenomenon of resistance in tomcod also provides a unique model to explore gene–environment interactions. Because CYP1A1 transcription and early life stage toxicities are activated by the AHR pathway, we sought to determine if differences in AHRR expression might underlie the resistance phenotype. Our hypothesis was that basal or inducible expression of AHRR would be greater in tomcod of Hudson River ancestry than in tomcod from less contaminated rivers such as the Miramichi River and would therefore serve to down-regulate AHR pathway–mediated CYP1A1 inducibility and early life stage toxicities.

## MATERIALS AND METHODS

### *Cloning of tomcod AHRR*

Three previously published deduced AHRR amino acid sequences (mouse, human, and killifish) were aligned, and conserved areas were identified from which to design primers for polymerase chain reaction (PCR) amplification of AHRR in tomcod. Because AHR and AHRR peptides are similar at their amino terminus, sequences were excluded that are shared by the two genes. Three upstream (TRRU1, TRRU2, and TRRU3) and two downstream (TRRD1 and TRRD2) primers were designed (Table 1), and combinations of these were tested on tomcod cDNA in reverse transcriptase PCR (RT-PCR) reactions. The combination of TRRU1 and TRRD1 amplified a

Table 1. Sequences of oligonucleotide primers used in the polymerase chain reaction amplification of Atlantic tomcod aryl hydrocarbon receptor repressor cDNA and genomic DNA

| Primer name | Primer sequence |
|---|---|
| TRRU1 | TCAAAAGCTTCTTCCAAG |
| TRRU2 | CTTTGCCTTGGTGGTGAG |
| TRRU3 | GCCAGCTCCACTGGGCCA |
| TRRD1 | CATGGCCCAGTGGAGCTG |
| TRRD2 | GTCAAAAATCCAGAGGT |
| TRRS1 | GCTGGCCCTGTTCTGTGT |
| TRRP1 | CGAGGCGGACTTCTACGA |
| TRRP2 | CCAGCACGGTCACTACGA |
| TRRP3 | CAACCACAACAGCCACAA |
| TRRP4 | CTGGAATTAACTGATAATCACACCGTG |
| TRRP5 | AATACTGCAGCGACACCATTCCCTTCAC |
| TRRP6 | CCCTGAATGTCTCCTGAA |
| MBA03S | ATGGATGATGACATTGCCGC |
| MBA04AS | ACGCTCCGTCAGGATCTTCA |

311-bp product. This fragment was sequenced using TRRU1 and TRRD1, and the resulting sequence showed similarity to the published AHRR sequences.

This amplicon was then used to screen our tomcod β-NF–induced cDNA library developed from the liver of one Hudson River tomcod [28]. In primary screenings, 40 plaques were positive, of which nine were positive after secondary screenings. All had inserts of about 2.1 kb, which is smaller than expected when compared with the published AHRR sequences. Insert fragments from four phage clones were subcloned into sequencing vectors and sequenced with TRRU1, TRRD2, and TRRS1 using a Beckman Coulter (Fullerton, CA, USA) CEQ2000 automated sequencer. All four clones had identical sequences of 2,087 bp.

Comparison of this 2,087-bp sequence with the published AHRR sequences revealed that the 3′ end of the coding sequence was absent. To obtain the remainder of the gene, a tomcod liver genomic DNA library [29] was screened with the 2,087-bp tomcod cDNA fragment. Twenty-one plaques were positive in primary screening, 14 in secondary screening, and four were positive after tertiary screening, and these were isolated. The four plaques contained different-sized tomcod inserts. The DNAs from these genomic DNA clones were digested with *Bam* HI and *Pst* I and Southern blotted and hybridized with a 941-bp fragment from the 3′ end of the tomcod AHRR cDNA. The 4.0-kb *Bam* HI (New England Biolabs, Beverly, MA, USA), 2.3-kb *Pst* I, and 1.3-kb *Pst* I fragments hybridized to the AHRR cDNA probe and were isolated. These fragments were subcloned and sequenced using M13 forward, M13 reverse, TRRP1, TRRP2, TRRP3, TRRP4, TRRP5, and TRRP6 primers (Table 1). A combination of the 2,087 bp of cDNA and a1125 bp of genomic DNA fragment yielded a contiguous tomcod AHRR sequence of 3,212-bp fragment.

### *Husbandry of tomcod*

Adult tomcod were collected from the Miramichi River (Loggieville, NB, Canada) during early December 2001 and transported alive back to the Department of Fisheries and Oceans Canada facility in Moncton, New Brunswick, where they were maintained until transport to the National Oceanic and Atmospheric Administration (NOAA) Laboratory (Sandy Hook, NJ, USA), where they were reared until spawning. Adult tomcod were collected in December 2001 from the Hudson River at Garrison, New York (River Mile 51) and transported alive to the NOAA Laboratory. Fish from the two populations were kept separately, although under identical conditions, until spawning. At least 10 male and 10 female fish from each population were used as parents to create F1 and F2 offspring.

### *Treatment of embryos*

Fourteen-day-old F2 embryos were waterborne exposed to two doses of both 3,3′,4,4′-tetrachlorobiphenyl (PCB77) (1 ppm and 10 ppm) (Ultra Scientific, North Kingstown, RI, USA) and B*a*P (0.1 and 1 ppm) (Sigma, St. Louis, MO, USA) in 0.01% acetone in glass Petri dishes for 7 d (PCB77) or 2 d (B*a*P) at 6°C. The congener PCB77 was chosen because it is the most abundant coplanar PCB congener by wet weight in livers [10] and unfertilized eggs [9] of environmentally exposed tomcod from the Hudson River. Polychlorinated biphenyl 77 has a toxic equivalency factor (a measure of toxic potency of individual PCDD/Fs and PCB congeners compared to that of TCDD) of 0.0001 in fish [30]. Controls were exposed to 0.01% acetone vehicle alone. The PCB77 treatment solutions of embryos were renewed every 48 h. At the conclusion of treatments, embryos were extensively washed three times in 5-ppt seawater and frozen at −70°C in pools of three embryos in microcentrifuge tubes.

### *Treatment of juveniles*

Six-month-old laboratory-reared F1 juvenile tomcod of Miramichi River ancestry (gender unknown) were intraperitoneal (ip) injected with 10 ppm of PCB77 in corn oil and maintained for 7 d in clean 5-ppt seawater at 15°C until sacrifice. Negative control fish were ip injected with corn oil alone. Immediately after sacrifice, individual organs (brain, gill, gonad, heart, intestine, kidney, liver, and spleen) were harvested and stored individually at −70°C in microcentrifuge tubes.

### *Collections of environmentally exposed adult tomcod*

Environmentally exposed adult tomcod were collected off Manhattan Island, New York, on the Hudson River with bottom trawls in mid-March 1990 and 1993; off Sheldrake Island on the Miramichi River with bottom trawls in early June 1993; and at La Baleine (Ile-aux-Coudres, PQ, Canada) in the St. Lawrence Estuary with weirs installed at angles to the shoreline in May of each year from 1998 to 2001. Shortly after collections, livers were excised and frozen in liquid nitrogen or dry ice and stored at −70°C until use.

### *Isolation of RNAs*

The RNAs were isolated from pooled embryos ($n$ = 3/pool) or tissues from individual juvenile or adult tomcod as described [9,15]. Approximately 50 mg of tissues were used from juveniles and adults. Frozen embryos and tissues were homogenized in Ultraspec reagent (Biotecx, Houston, TX, USA), and RNA was isolated following the manufacturer's recommendations. The RNA pellets were resuspended in 50 μl (tissues) or 20 μl (embryos) of diethyl-pyrocarbonate–treated $H_2O$ and stored at −70°C until analysis. Total RNA concentrations were determined spectrophotometrically.

### *RT-PCR analysis of AHRR and actin*

First-strand cDNAs were generated from 10 and 100 ng of total RNAs in reverse transcription reactions. A 0.5-μg amount of random hexamers (Invitrogen, Carlsbad, CA, USA) was added to the RNAs in total volumes of 15 μl and incubated

at 75°C for 5 min. The mixture was chilled, and 10 μl of RT-mix were added so that the final reaction mix contained 1× Moloney Murine Leukemia Virus (MMLV) reaction buffer, 20 units of MMLV reverse transcriptase (Promega, Madison, WI, USA), 0.1 μg of RNasin RNase inhibitor (Promega), and 0.5 mM deoxynucleotide triphosphates (dNTPs) (Pharmacia, Kalamazoo, MI, USA). Reactions were incubated at 42°C for 1 h, and products were denatured at 98°C for 5 min and chilled on ice.

First-strand cDNA products were used as templates to PCR amplify tomcod AHRR (TRRP4 and TRRP6) and actin (MBA03S and MBA04AS) in two separate reactions using the primers listed in Table 1. The expected product size of AHRR was 1,032 bp, and that of actin was 588 bp. The PCR reactions were in 30-μl final volumes that contained 6 μl of the cDNA product, 1× PCR buffer (Roche Biochemicals, Indianapolis, IN, USA), 1 μM of each of the PCR primers, 0.2 mM of each of the dNTPs, and 1 unit of Taq DNA polymerase (Roche Biochemicals). Amplification parameters were 95°C for 5 min, 30 cycles (actin) or 35 (AHRR) cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 7 min.

Half the volume of PCR reactions were electrophoresed in 1.4% agarose gels and photographed with Kodak EDAS290 or AlphaImager 2200™ (Alpha Innotech Corp, San Leandro, CA, USA) gel documentation systems. One lane in each tier of each gel contained a 100-bp DNA ladder (New England Biolabs) that was used to quantitate DNA concentrations of AHRR and actin. Kodak 1D Image Analysis Software Version 3.5 or AlphaEase®FC Imaging Software was used to quantify the amount of PCR product in each lane.

### *CYP1A1 mRNA quantification*

The CYP1A1 mRNA levels were quantified using slot blot analysis. The quality of RNAs from each embryo pool or tissue was evaluated by denaturing agarose gel electrophoresis and ethidium bromide staining to evaluate the integrity of the 28S and 18S rRNA bands [15]. Those samples with degraded rRNAs were reisolated or omitted from analysis.

Two micrograms of total RNAs from pooled embryos or tissues were applied directly to Nytran Plus membranes (Schleicher and Schuell, Keene, NH, USA) using a 72-slot slot blot manifold (Schleicher and Schuell). Prehybridizations were for 2 h at 65°C, and hybridizations were overnight at 65°C as described in Courtenay et al. [15] using $^{32}$P-radiolabeled, β-NF–induced, full-length tomcod CYP1A1 cDNA probes [28]. Final wash conditions were 0.5× SSPE/0.1% sodium dodecyl sulfate at 65°C for 1 h. The CYP1A1 mRNA levels were quantified from phosphor imaging screens using a Storm 860 Scanner and Molecular Dynamics ImageQuant™ for Macintosh software Version 1.0 (Sunnyvale, CA, USA).

To ensure equal loading of RNAs, membranes were stripped of the CYP1A1 probes and rehybridized exactly as described previously to $^{32}$P-radiolabeled, rat 18S rRNA probes, pHRR118 [31]. The rRNA concentrations were quantified as described previously. The CYP1A1 mRNA levels are expressed as CYP1A1 OD units/rRNA OD units.

### *Data analyses*

*Comparison of DNA sequences.* Clustal W (ver 1.75) [32] was used to align AHRR, AHR, and ARNT amino acid sequences. The unweighted pair-group method using arithmetic averages (UPGMA) and maximum parsimony methods in MEGA2 [33] were used for phylogenetic analyses of tomcod AHRR and 20 vertebrate AHR, ARNT, and AHRR genes and *Caenorhabditis elegans* AHR. In each case, bootstrap values were obtained from 1,000 replications.

*Comparison of gene expression in embryos from two populations.* The CYP1A1 mRNA data were normalized to respective rRNA concentrations and natural log transformed to improve normality. The AHRR mRNA data were also log transformed but not normalized to respective actin concentrations. The CYP1A1 and AHRR mRNA concentrations in B*a*P- and PCB77-treated embryos were compared to respective controls for each population separately by analysis of variance (ANOVA) followed by the Tukey multiple range test. The correlation between concentrations of CYP1A1 mRNA and AHRR mRNA in all embryos was examined by Pearson correlation.

*Comparison of gene expression among tissues*. The CYP1A1 mRNA expression data in tissues were normalized to respective rRNA levels. Data were analyzed nonparametrically because AHRR data were nonnormally distributed even after log transformation. Comparisons of gene expression between PCB77 treated fish and corn oil controls were made with Mann–Whitney *U* test. Comparisons of CYP1A1 mRNA levels and AHRR mRNA levels across tissues were made for treated fish and control fish separately by Kruskal–Wallis test. Correlations of CYP1A1 and AHRR mRNA levels within each tissue (controls and PCB77-treated fish pooled) were made by Spearman rank correlation.

*Comparison of gene expression in environmentally exposed tomcod from three rivers.* The CYP1A1 mRNA expression data in environmentally exposed adult tomcod from three rivers were normalized to respective rRNA levels and log transformed to improve normality. The AHRR mRNA data were also log transformed but not normalized to respective actin concentrations. Comparisons among tomcod sampled from different rivers were assessed by ANOVA followed by the Tukey multiple range test. Sample means and 95% confidence intervals were back transformed to original units for presentation.

The AHRR mRNA expression in adult tomcod from the three rivers was quantified by RT-PCR on three separate occasions, and each data set was analyzed separately as described previously. The Pearson correlation was examined between AHRR expression and the CYP1A1 mRNA data that were quantified for the same fish. The CYP1A1 mRNA data were quantified only once, so the same data set was used for correlation with all three AHRR data sets, one of which included only nine fish that were also quantified for CYP1A1 mRNA. Differences in AHRR mRNA concentrations between male and female tomcod, recorded in a subsample of one of the three AHRR analyses, were compared by ANOVA. This subsample included tomcod from both the Hudson River and the Miramichi River (six to eight of each sex from each river). However, no significant differences were detected in AHRR mRNA levels between the rivers or in interaction between river and sex, so fish from the two rivers were pooled for comparison of sexes by one-way ANOVA.

## RESULTS

### *Cloning and characterization of tomcod AHRR*

The RT-PCR using primers (TRRU1 and TRRD1) based on conserved sequence among previously characterized AHRRs amplified a 311-bp product from tomcod cDNA that

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   1 ATGATTCCTCCGGGAGATTGCATGTACGCG GGACGCAAGAGGAGGAAACCCGTCCAAAAA CAGAAGCCTTCGTCGTCCAGCGAGAAGACT AACCCGTCGAAGCGGCACCGCGACCGTCTG
     M  I  P  P  G  D  C  M  Y  A   G  R  K  R  R  K  P  V  Q  K   Q  K  P  S  S  S  S  E  K  T   N  P  S  K  R  H  R  D  R  L    40
 121 AATGCCGAGCTGGACCGGCTGGCCAGCCTG CTGCCCTTCCCCCCCCGACGTCATCACAAAG CTGGACAAGCTCTCGGTGCTGCGCCTGGCC GTCTCCTACCTCCGCGTCAAGAGCTTCTTC
     N  A  E  L  D  R  L  A  S  L   L  P  F  P  P  D  V  I  T  K   L  D  K  L  S  V  L  R  L  A   V  S  Y  L  R  V  K  S  F  F    80
 241 CAAGCAAATCCAGAGAAACCGACCAGGAAG AACAGCATCAGCCCGGCCGCCTCCGTCCCT GAGCCCAGCAAAGGGCCCCTTCCCCTGGGC CCCTCCACCACCGAGAGCAGCCTCCTGCTG
     N  Q  A  P  E  K  P  T  R  K   N  S  I  S  P  A  A  S  V  P   E  P  S  K  G  P  L  P  L  G   P  S  T  T  E  S  S  L  L  L   120
 361 GATTCGCTGACAGGCTTTGCGTTGGTTGTC AGCAGTGATGGGATGGTGTTCTACGCCTCC TCCACCATCGTGGACTACCTGGGTTTCCAT CAGACGGACGTGATGCACCAGAATGTGTTT
     D  S  L  T  G  F  A  L  V  V   S  S  D  G  M  V  F  Y  A  S   S  T  I  V  D  Y  L  G  F  H   Q  T  D  V  M  H  Q  N  V  F   160
 481 GACTACATCCACATTGACGACCGCCAGGAG TTCAAACGCCAGCTACACTGGGCCATGTGT CCCCCACAGCACAAGGGGGCGCACCAGGAG CACCAGCTCCCACCGGGGACTGGTGAGGAG
     D  Y  I  H  I  D  D  R  Q  E   F  K  R  Q  L  H  W  A  M  C   P  P  Q  H  K  G  A  H  Q  E   H  Q  L  P  P  G  T  G  E  E   200
 601 TACATGGTGAGCAATCTCTTCCACTCCGCT GACGGAGGCAGTACCCCCCCGGAGCTCAAC TCCTTCCTCAGCCGTTGCTTCATTGCCCGG GTGCGCTGCCTGTTGGACAGCACCTCTGGC
     Y  M  V  S  N  L  F  H  S  A   D  G  G  S  T  P  P  E  L  N   S  F  L  S  R  C  F  I  A  R   V  R  C  L  L  D  S  T  S  G   240
 721 TTCCTGACTATGCAGTTCCAGGGACGACTG AAGTTCCTCGTGGGGCAGAAGAAGAAGTCT GGCTCGGGGGCCCTGCTGCCGCCCCAGCTG GCCCTGTTCTGTGTGGCCGTGCCCCTGCTG
     F  L  T  M  Q  F  Q  G  R  L   K  F  L  V  G  Q  K  K  K  S   G  S  G  A  L  L  P  P  Q  L   A  L  F  C  V  A  V  P  L  L   280
 841 ATGCCCTCCATCACAGAGATGAAGATGAAG AGCCTGCTGCTGAGGGGGAAGAGCAGAGGA GGCCCAGGGCTCCTCCCAGGCATGGATCCC AGCAGCGAGCGAGGGGAGACATTCAGGAGA
     M  P  S  I  T  E  M  K  M  K   S  L  L  L  R  G  K  S  R  G   G  P  G  L  L  P  G  M  D  P   S  S  E  R  G  E  T  F  R  R   320
 961 CATTCAGGGAACGGAGGGATGGGTGAGGCC TGTGACCCTCTCTTGCTCCCTTGCTCCACG CCGAATGGTGCCCAGCGGGGCCAGCACACC CCCTGGACCCCCCTCTCCAAGGACAACATC
     H  S  G  N  G  G  M  G  E  A   C  D  P  L  L  L  P  C  S  T   P  N  G  A  Q  R  G  Q  H  T   P  W  T  P  L  S  K  D  N  I   360
1081 AAGTACCGGCCGGAGGGCTTCTACAACCAG GACGAGCCCCTCAACTACTGTAAGACCTCA ATGGCTCCCCACAAAGGCCTCAGCCCAGGC CTGGGCGGTGGGTGGCCTCAGCGGCCCAAC
     K  Y  R  P  E  G  F  Y  N  Q   D  E  P  L  N  Y  C  K  T  S   M  A  P  H  K  G  L  S  P  G   L  G  G  G  W  P  Q  R  P  N   400
1201 TCCGGAGCCATCAGGGCGGGGCAGGGAGGT GGCTACCTCCCCTCTAACAGGATGAACAAG GCCAACCACTACGGGAAGCCGTACCGCCAG GCCCCCAGCTGCAACGGAGGCCGGGGTGGC
     S  G  A  I  R  A  G  Q  G  G   G  Y  L  P  S  N  R  M  N  K   A  N  H  Y  G  K  P  Y  R  Q   A  P  S  C  N  G  G  R  G  G   440
1321 GAGGTGTTTGTCTCTCGTCTCTATGGCAAC CTCCAGAGCCCAACCGAGCCGGACACGTAC TGCGTGGACCTGGTGAAGAGCGAGGGCGGC TACGGCGAGTGCTACAATGCACACCTGATG
     E  V  F  V  S  R  L  Y  G  N   L  Q  S  P  T  E  P  D  T  Y   C  V  D  L  V  K  S  E  G  G   Y  G  E  C  Y  N  A  H  L  M   480
1441 GCGGAGATGCCGCCCATCAAGGTGGAGCAC GACTCGGACTCGGAGAACGGCCTGGACCCG TACGGCCGGCCCTGGGCCTGCCGCGACCCG GGCCTCATGGAGCGTTGCTACGGCAACGCG
     A  E  M  P  P  I  K  V  E  H   D  S  D  S  E  N  G  L  D  P   Y  G  R  P  W  A  C  R  D  P   G  L  M  E  R  C  Y  G  N  A   520
1561 CTGTACGACCCCAACACCGGCGTCCAGCTC AAATCCGAGGCGGACTTCTACGACCAGCAG TACTCGCCGTGTCAGCGGGGGAAGGCCGCC ATCAGCCCGCCGTACGTCCAGCACGGTCAC
     L  Y  D  P  N  T  G  V  Q  L   K  S  E  A  D  F  Y  D  Q  Q   Y  S  P  C  Q  R  G  K  A  A   I  S  P  P  Y  V  Q  H  G  H   560
1681 TACGACAACTACGCCGCCGTCAACGGCGTC AACGCGCCCGGGCGGCTGCAGAAGTACAAC AAAGACCCCGGCCACATGCACAACAACAAC AACAACAACAACAACAACAACCACAACAGC
     Y  D  N  Y  A  A  V  N  G  V   N  A  P  G  R  L  Q  K  Y  N   K  D  P  G  H  M  H  N  N  N   N  N  N  N  N  N  N  H  N  S   600
1801 CACAACAGCCAGTTCAGCCCCCACCGGGGC CCGCACCCGGACCCCCTTTGTAGCAGCCAG ACGCTGAACCTCTTGGACGGCCAGGCCTAC CAGGGCTCCCCCATGGAGCCCCACAAGGCC
     H  N  S  Q  F  S  P  H  R  G   P  H  P  D  P  L  C  S  S  Q   T  L  N  L  L  D  G  Q  A  Y   Q  G  S  P  M  E  P  H  K  A   640
1921 TTCATGCAGCAGGACTATGGCAAGCACAAC AGCTACGAGTTCAAGGGTCACGGCATCATC CACTCCATCAAGCGGGAGCCCATGGACTCC CCGCCGTGGTCGGAGAACGGCCACGACATG
     F  M  Q  Q  D  Y  G  K  H  N   S  Y  E  F  K  G  H  G  I  I   H  S  I  K  R  E  P  M  D  S   P  P  W  S  E  N  G  H  D  M   680
2041 GGCCAGGCCTTGATGGGTGCCGCCCGGAAC GTTATGCCCTGCGCCATGAACGCAGGCCAC CTCAAAGCCAACCCGTACGTGTACATGCAG TGA
     G  Q  A  L  M  G  A  A  R  N   V  M  P  C  A  M  N  A  G  H   L  K  A  N  P  Y  V  Y  M  Q   *    711
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Fig. 1. Nucleotide and deduced amino acid sequences of Atlantic tomcod aryl hydrocarbon receptor repressor (AHRR) coding sequence. Nucleotides are numbered on the left and amino acids on the right. GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) accession numbers for the sequences are AY841897 and AY841898.

exhibited extensive sequence similarity with mouse, human, and killifish AHRRs. This putative tomcod AHRR amplicon was then used as a probe to screen a tomcod cDNA library, and numerous hybridizable plaques were identified from which a subset was isolated and characterized. A 2,087-bp cDNA fragment was isolated that, based on previously characterized AHRRs, terminated 221 bp upstream of the putative AHRR start codon and that contained the entire 5′ tomcod AHRR coding region but was missing part of its 3′ coding sequence. Characterization of other tomcod cDNA clones failed to identify one that contained the missing 3′ coding sequence (Fig. 1).

A tomcod liver genomic DNA library was then screened with the 2,087-bp tomcod AHRR cDNA fragment as probe; four plaques were positive after tertiary screening, and these were characterized. This allowed us to isolate a genomic DNA fragment that contained the final 267 nucleotides of the 2,133-bp AHRR coding sequence.

To determine whether the predicted contiguous cDNA sequence exists in vivo, we designed several upstream primers (TRRP1, TRRP2, and TRRP3) 5′ from the end of the contiguous AHRR cDNA sequence and TRRP5 3′ downstream of the predicted termination codon (Table 1). The expected fragment sizes from amplification with TRRP1-TRRP5, TRRP2-TRRP5, and TRRP3-TRRP5 were 583, 511, and 390 bp, respectively. Reverse transcriptase-PCR with these primer pairs was used to amplify total hepatic RNA obtained from a Miramichi River tomcod treated with 10 ppm B*a*P. Electrophoresis of the RT-PCR products showed that they matched the predicted size of these amplicons. This confirmed that the contiguous sequence obtained by combining the 2,087-bp cDNA with the sequence obtained from the genomic DNA clone can be considered as the putative mRNA sequence of tomcod AHRR. We sequenced 859 nucleotides past the predicted tomcod AHRR stop codon and did not find a typical AATAAA polyadenylation signal sequence; instead, an alternative AAGAAA polyadenylation signal was found 452 nucleotides downstream of the stop codon.

### *Comparison of tomcod AHRR with other PAS genes*

The putative coding sequence of tomcod AHRR is 2,133 bp, and the deduced peptide is 711 amino acid residues with a molecular weight of 78.3 kDa (Fig. 1). The complete tomcod AHRR coding nucleotide sequence shares 74% identity with that of killifish AHRR. Insufficient similarity exists at the 3′ end of AHRR to quantitatively compare the full-length tomcod and mammalian genes. When comparing its first 5′ 900 nucleotides, tomcod AHRR shared 78, 70, and 66% with AHRR of killifish, human, and mouse, respectively. The complete tomcod AHRR peptide shares 75% identity with that of killifish. The first 5′ 300 amino acid residues of tomcod AHRR share 83, 75, and 72% identity, respectively, with AHRR of killifish, human, and mouse. Putative bHLH, PAS-A, and PAS-B domains in the tomcod AHRR peptide were compared with previously identified AHRR and AHR sequences (Fig. 2). The bHLH domain of tomcod AHRR shares 94% identity with that in killifish and somewhat less with that in mouse AHRR (81%). The bHLH domains of tomcod AHRR and tomcod AHR share 77% identity (Fig. 3). Sequence of the PAS-A domain in tomcod and killifish AHRR are identical, with the exception of

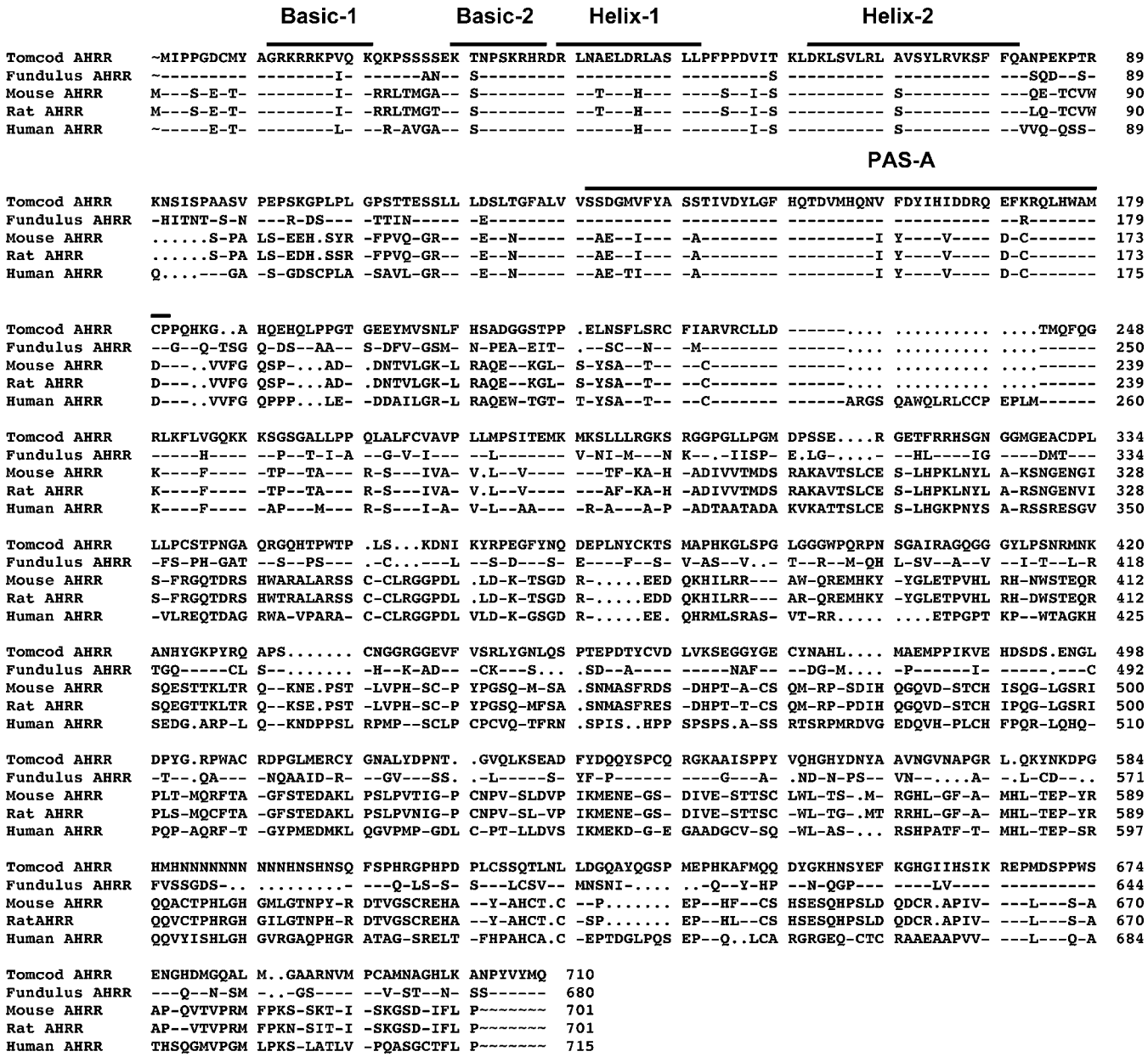


Fig. 2. An alignment of deduced Atlantic tomcod aryl hydrocarbon receptor repressor (AHRR) amino acid sequences with AHRR amino acid sequences previously identified in *Fundulus heteroclitus* (Atlantic killifish), mouse, rat, and human using the Pileup option in the GCG computer software package (Wisconsin Package Ver 10.3-UNIX, Accelrys, San Diego, CA, USA). Basic–helix–loop–helix and Per-AHR/ARNT-Sim (PAS)-A domains are highlighted with brackets. Dashes indicate identity, and dots identify deletions.

one residue at position 172 (Fig. 2). Overall, the PAS-A domain of tomcod AHRR has higher identity to mouse AHRR (80%) than to tomcod AHR (59%). The PAS-B domain of tomcod AHRR is less similar to that of killifish (49%) and mouse (9%) compared to that of the PAS-A domain. The PAS-B domain of tomcod AHRR has no similarity to that of the PAS-B domain of tomcod AHR (Fig. 3).

In phylogenetic analyses using both distance (UPGMA and neighbor joining) and maximum parsimony approaches, tomcod AHRR tightly clustered with the four previously identified AHRR genes, with 100% bootstrap support using either approach (Fig. 4). Not unexpectedly, the tomcod AHRR gene clustered most closely with the one previously identified fish AHRR (*F. heteroclitus*) compared to mammalian AHRRs. The five AHRR sequences were most closely aligned to that of AHRs rather than ARNT genes.

*Comparison among tissues of gene expression*

Induced and basal expression of CYP1A1 mRNA and AHRR mRNA were compared among eight tissues (brain, gill, gonad, heart, intestine, kidney, liver, and spleen) of PCB77-treated (single ip injection of 10 ppm PCB77) and corn oil–treated six-month-old F1 tomcod of Miramichi River descent. Juvenile tomcod that were ip injected with PCB77 and sacrificed 7 d later showed significantly higher CYP1A1 mRNA levels than corn oil–injected controls in all eight tissues sampled (Mann–Whitney $U$ tests, $p < 0.05$; Fig. 5A). Significant differences were observed in CYP1A1 expression among tissues of both PCB77-treated (Kruskal–Wallis test statistic = 48.870, $p < 0.001$) and control fish (Kruskal–Wallis = 39.303, $p < 0.001$), with levels being highest in liver for both groups. When comparing inducibility among tissues, liver showed the

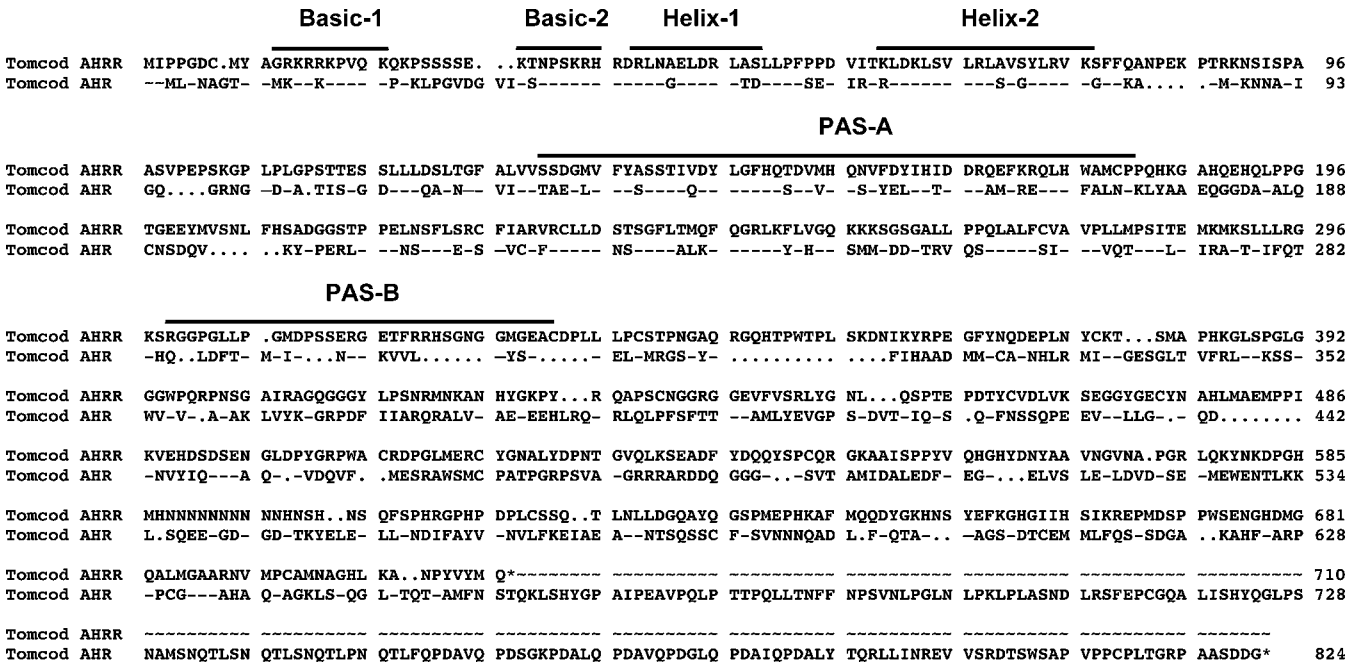


Fig. 3. An alignment of deduced Atlantic tomcod aryl hydrocarbon receptor repressor (AHRR) and Atlantic tomcod aryl hydrocarbon receptor (AHR) amino acid sequences using the Pileup option in the GCG computer software package (Wisconsin Package Ver 10.3-UNIX, Accelrys, San Diego, CA, USA). Basic–helix–loop–helix, Per-AHR/ARNT-Sim (PAS)-A, and PAS-B domains are highlighted with brackets. Dashes indicate identity, and dots are used to identify areas of deletions.

greatest CYP1A1 mRNA response to PCB77 in absolute terms (i.e., treated median CYP1A1 mRNA − control median CYP1A1 mRNA). However, expressed as a percent increase over basal levels of expression (i.e., [treated − control]/control), heart was most responsive (190-fold induction), followed by intestine (127), kidney (24), spleen (18), gill (11), liver (9), gonad (6), and brain (5). Brain was least responsive by both absolute and percentage increase in CYP1A1 mRNA expression.

The AHRR mRNA levels were also significantly higher in all tissues of tomcod treated with PCB77 than controls (Mann–Whitney $U$ tests, $p < 0.05$; Fig. 5B). Significant differences were observed in AHRR mRNA levels among tissues of both PCB77 injected (Kruskal–Wallis = 22.587, $p = 0.002$) and control (Kruskal–Wallis = 26.572, $p < 0.001$) fish. Basal levels of AHRR expression in untreated tomcod were observed in all tissues except liver and brain. Highest basal levels of AHRR expression were observed in gonad and intestine. Heart showed the highest absolute AHRR response to PCB77, followed by spleen and liver. Expressed as a percentage increase over control levels, spleen showed the greatest AHRR response, followed by liver and kidney (fold induction not reported because of zero responses recorded for some controls).

Expression of CYP1A1 mRNA and AHRR mRNA were significantly, positively correlated in all tissues except gonad and brain (Spearman rank correlations for each tissue separately = 0.664–0.810, $p < 0.05$ to $<0.005$, $n = 10$–12, respectively; controls and PCB77 treated fish pooled). This correlation was strongest in liver.

### *Gene expression in chemically treated F2 embryos from two populations*

It is possible that levels of gene expression are altered by the substantial maternal transfer of AHs to F1 early life stages of tomcod from the Hudson River [9,15]; therefore, gene expression was quantified in F2 embryos to further evaluate the heritability of CYP1A1 and AHRR expression phenotypes. In these experiments, gene expression was determined in embryos that were the offspring of Hudson River and Miramichi River parents that were exposed to two concentrations of waterborne B*a*P or PCB77 and acetone. As shown in Figure 6, no significant difference was observed between F2 embryos of Hudson and Miramichi origin in basal levels of CYP1A1 mRNA ($F_{1,13} = 1.706$, $p = 0.214$) or AHRR mRNA expression ($F_{1,13} = 0.224$, $p = 0.644$), data logged for each analysis). Both doses of B*a*P and both doses of PCB77 significantly induced CYP1A1 in embryos of Miramichi River origin (ANOVA $F_{4,26} = 42.279$, $p < 0.001$; Tukey test $p < 0.001$ for all four comparisons to controls). Exposure to B*a*P at concentrations of 0.1 and 1 ppm significantly induced CYP1A1 mRNA over vehicle-treated controls in F2 embryos of Hudson River origin (ANOVA $F_{4,33} = 328.726$, $p < 0.001$; Tukey test $p < 0.001$; Fig. 6A). In contrast, no induction was seen in F2 embryos of Hudson River descent exposed to waterborne PCB77 at a concentration of 1 ppm, and 10 ppm produced only marginally significant induction (Tukey test $p = 0.042$).

Both doses of B*a*P significantly elevated AHRR over controls in Hudson River embryos (ANOVA $F_{4,33} = 23.924$, $p < 0.001$; Tukey test $p < 0.001$), but neither dose of PCB77 did (Fig. 6B). By contrast, both doses of B*a*P and PCB77 significantly induced AHRR over controls in embryos of Miramichi River origin (ANOVA $F_{4,26} = 11.306$, $p < 0.001$; Tukey test $p = 0.015$ [0.1 ppm B*a*P], $p = 0.012$ [1 ppm B*a*P], $p = 0.003$ [1 ppm PCB77], and $p < 0.001$ [10 ppm PCB77]).

For F2 embryos from both populations pooled, a positive correlation was observed between embryonic levels of CYP1A1 mRNA and AHRR mRNA (Pearson correlation $R = 0.758$, $p < 0.001$, $n = 69$; Fig. 6C).

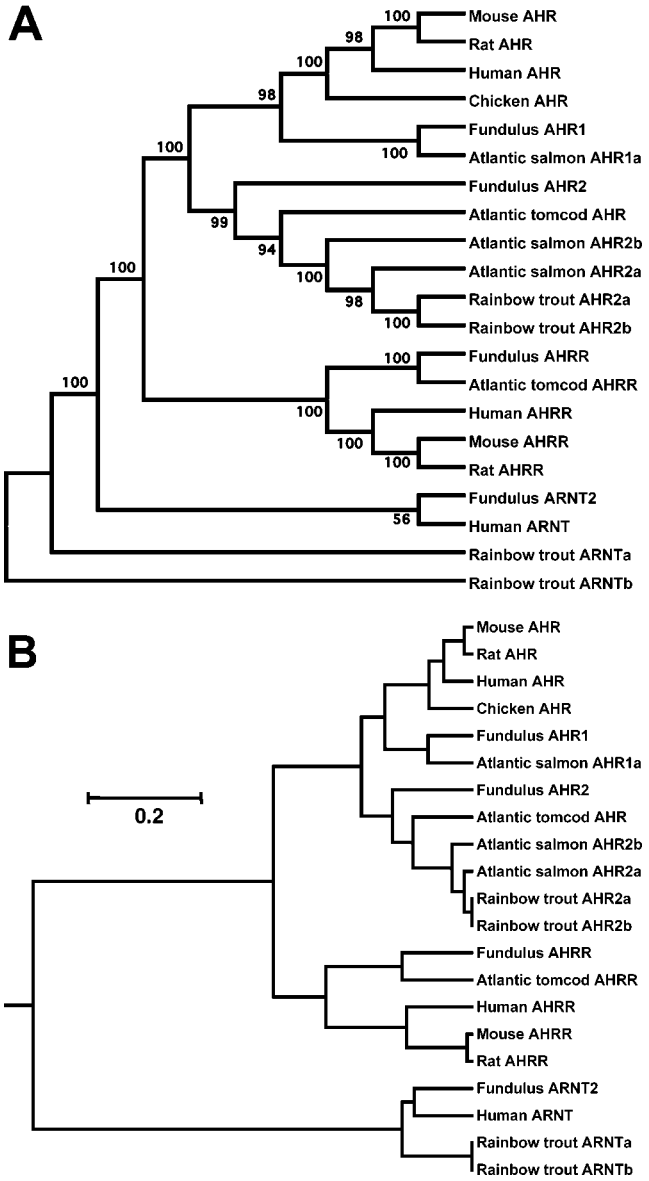


Fig. 4. Phylogenetic analyses of full-length peptide sequences of 21 genes in the vertebrate Per-AHR/ARNT-Sim family including aryl hydrocarbon receptor 1 (AHR1), AHR2, aryl hydrocarbon receptor repressor (AHRR), aryl nuclear translocator 1 (ARNT1), and ARNT2. (**A**) An unrooted tree generated by the unweighted pair-group method using arithmetic averages method (UPGMA) analysis in MEGA2 [33]; bootstrap values (1,000 replications) are indicated on the tree's nodes. (**B**) A rooted tree generated by the maximum parsimony method in Mega2. GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) accession numbers of the analyzed sequences are chicken AHR (AF260832), *Fundulus heteroclitus* AHR1 (AF024591), *F. heteroclitus* AHR2 (U29679), *F. heteroclitus* ARNT2 (AF402781), *F. heteroclitus* AHRR (AF443441), human AHR (L19872), human ARNT (M69238), human AHRR (AF293639), mouse AHR (D38417), mouse AHRR (AB015140), rainbow trout AHR2α (AF065137), rainbow trout AHR2β (AF065138), rainbow trout ARNTα (U73840), rainbow trout ARNTβ (U73841), rat AHR (U04860), rat AHRR (AY367561), Atlantic salmon AHR1α (AY456090), Atlantic salmon AHR2α (AJ608767), Atlantic salmon AHR2β (AJ608768), Atlantic tomcod AHR (AF050489), and Atlantic tomcod AHRR (AY841898).

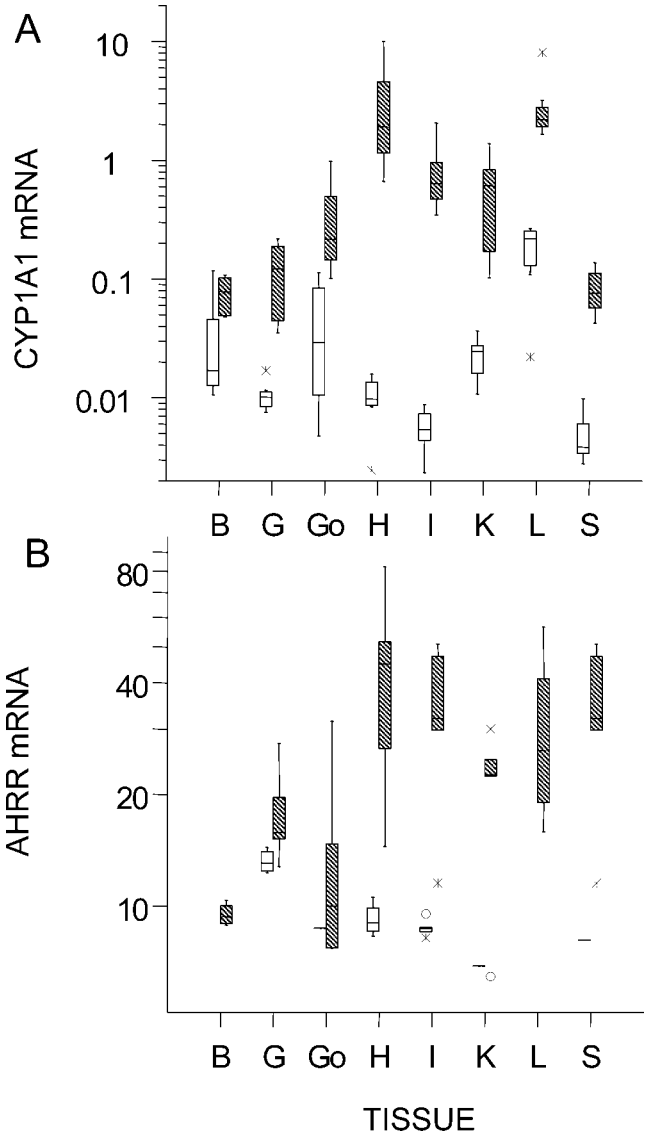


Fig. 5. Box plots comparing cytochrome P4501A1 (CYP1A1) mRNA (**A**) and aryl hydrocarbon receptor repressor (AHRR) mRNA (**B**) expression in eight tissues of laboratory-reared juvenile F1 tomcod of Miramichi River (Canada) ancestry that were intraperitoneally injected with 10 ppm of polychlorinated biphenyl 77 (hatched bars) or corn oil (open bars), transferred to clean water, and sacrificed after 7 d. $n$ = 7 or 8 per bar for CYP1A1 mRNA and 5 or 6 per bar for AHRR mRNA. Where bars are not apparent (e.g., AHRR data for B controls), all data are zero. B = brain; G = gill; Go = gonad; H = heart; I = intestine; K = kidney; L = liver; S = spleen. Note log $Y$ scale in both plots. The CYP1A1 mRNA expression was determined by slot blot analysis using $^{32}$P-radiolabeled tomcod CYP1A1 cDNA probes and is expressed as relative optical density (OD) units of CYP1A1 mRNA/relative OD units of rRNA. The AHRR mRNA expression was determined by semiquantitative reverse transcriptase-polymerase chain reaction and is expressed as relative OD units of AHRR mRNA.

### *Comparison of gene expression in environmentally exposed tomcod from three rivers*

Previous studies demonstrated significant variation among environmentally exposed adult tomcod from the Hudson River, St. Lawrence River, and Miramichi River in their hepatic burdens of AH contaminants [10,15] and hepatic CYP1A1 mRNA expression [25]. We hypothesized that environmentally exposed adult tomcod from the resistant Hudson River population would show higher levels of hepatic AHRR mRNA expression than fish from the two sensitive populations and that this might explain their reduced inducibility of CYP1A1. Twelve individual specimens from each river were sacrificed immediately

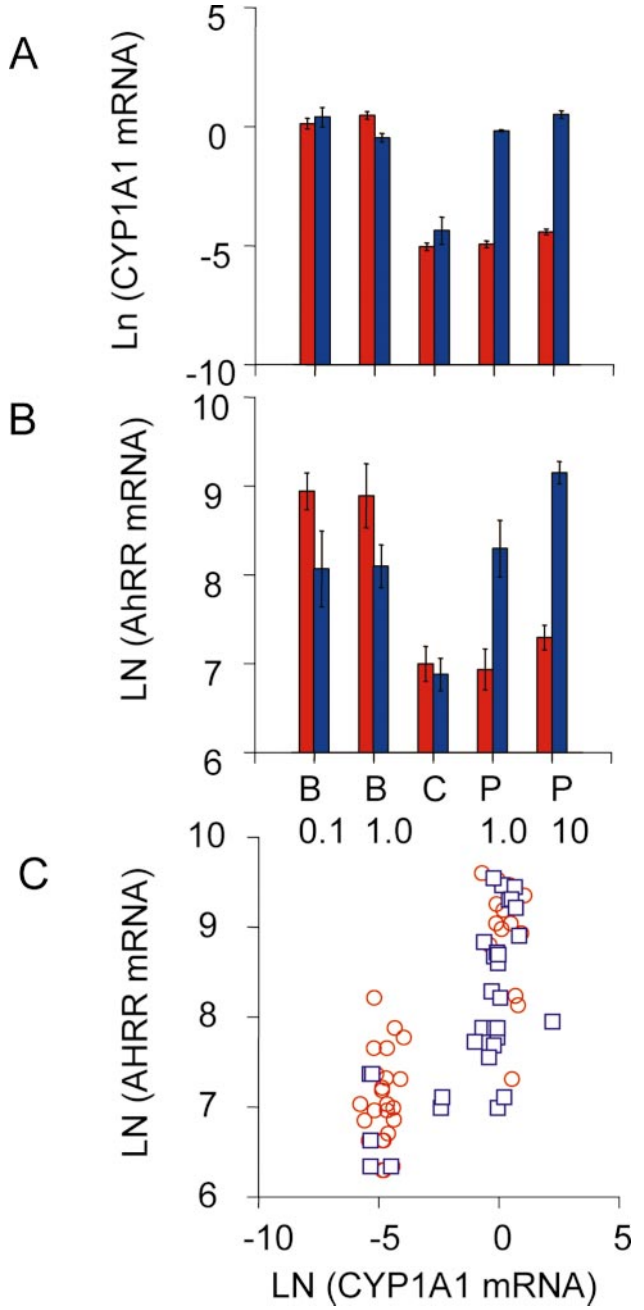


Fig. 6. (**A**) Comparison of cytochrome P4501A1 (CYP1A1) mRNA expression in F2 tomcod embryos of Hudson River (USA) origin (red bars) and Miramichi River (Canada) origin (blue bars). Each bar represents the mean (±1 standard error, data log transformed) of six or eight pools of three embryos each; LN refers to the natural log. Embryos were exposed at 14 d of age to waterborne concentrations of benzo[*a*]pyrene (B*a*P) (0.1 or 1.0 ppm) (B01 and B1) for 2 d, polychlorinated biphenyl (PCB) 77 (1 or 10 ppm) (P1 and P10) for 7 d, or acetone vehicle (C) (for 2 and 7 d). The CYP1A1 mRNA levels were determined by slot blot analysis and are expressed as CYP1A1 mRNA/rRNA mRNA. (**B**) Comparison of aryl hydrocarbon receptor repressor (AHRR) mRNA expression in F2 tomcod embryos of Hudson River origin (red bars) and Miramichi River origin (blue bars). Each bar represents the mean (±1 standard error, data log transformed) of six or eight pools of three embryos each. Embryos were exposed to waterborne concentrations of B*a*P (0.1 or 1.0 ppm) (B0.1 and B1) for 2 d, PCB77 (1 or 10 ppm) (P1 and P10) for 7 d, or acetone vehicle (C) (for 2 and 7 d). (**C**) Correlation of AHRR mRNA expression and CYP1A1 mRNA expression in F2 tomcod embryos of Hudson River (red circles) and Miramichi River (blue squares) origin (data log transformed). Pearson $R = 0.758$, $p < 0.001$, $n = 69$). Data set includes members of both populations exposed to waterborne B*a*P (0.1 or 1.0 ppm), PCB77 (1 or 10 ppm), or acetone vehicle.

after capture and were analyzed for hepatic CYP1A1 mRNA and a greater number for expression of AHRR mRNA.

Hepatic CYP1A1 mRNA levels were significantly higher in tomcod from the Hudson River than Miramichi River, and both were significantly higher than in tomcod from the St. Lawrence River ($F_{2,33} = 38.514$, $p < 0.001$; Tukey $p = 0.024$ [Hudson vs Miramchi], $p < 0.001$ [St. Lawrence vs both other rivers]; Fig. 7A). Expression of CYP1A1 was higher in tomcod from the Hudson River by 11.7- and 2.8-fold compared to those from the St. Lawrence River and the Miramichi River, respectively.

Reverse transcriptase-PCR was used to quantify AHRR mRNA levels in environmentally exposed tomcod from the three rivers. None of the three RT-PCR analyses conducted showed significant differences in hepatic AHRR mRNA levels among tomcod sampled from the three rivers. Furthermore, no significant difference was detected in AHRR mRNA levels between environmentally exposed male and female tomcod from these rivers. Interindividual variation in expression of AHRR mRNA was very high in fish from at least two of the three rivers. Expression of AHRR mRNA was not detectable in most fish from all three rivers. Nine of 33 tomcod from the Hudson River and 11 of 28 fish from the Miramichi River exhibited detectable levels of hepatic AHRR, compared to only 1 of 16 fish from the St. Lawrence River.

Expression of AHRR mRNA and CYP1A1 mRNA were directly related in one of the three analyses ($R = 0.394$, $p = 0.021$, $n = 34$; Fig. 7B). Similarly weak, positive correlations were observed in the other two data sets, though these were not statistically significant ($R = 0.311$, $p = 0.065$, $n = 36$; $R = 0.623$, $p = 0.073$, $n = 9$). When populations were examined separately, only the Hudson River population, in one of the three data sets, showed a significant correlation between hepatic concentrations of AHRR mRNA and CYP1A1 mRNA ($R = 0.591$, $p = 0.043$, $n = 12$; Fig. 7C).

## DISCUSSION

Sediment and biota of the Hudson River Estuary have been contaminated for at least five decades with PCBs and PCDD/Fs and probably longer than that with PAHs [34]. All life stages of tomcod from the Hudson River Estuary bioaccumulate high levels of PCBs and PCDD/Fs [10], including early life stages [9] that are highly sensitive to the toxicities of these contaminants in other species. Tomcod from the Hudson River are resistant to hepatic [15] and extrahepatic CYP1A1 mRNA induction [26] and early life stage toxicities induced by coplanar PCBs and TCDD but not PAHs [27]. We propose that resistance in Hudson River tomcod results from strong selective pressure for PCB/TCDD-resistant phenotypes in this population. Because resistance is observed at all life stages, in all tissues, and is largely heritable, we postulate that it is mechanistically based in alterations of the AHR pathway. That is because the AHR pathway is central to transcriptional activation of genes that metabolically detoxify and activate AH contaminants and in eliciting overt toxic responses [17].

Previously, we observed no significant difference between tomcod from the Hudson River and sensitive populations in basal or induced expression of AHR mRNA or ARNT mRNA [35]. A third component of this pathway is AHRR, which recently was characterized in three mammalian species and a single species of fish. It has been shown that transcription of AHRR is inducible by PAHs [20,36–38], TCDD [38], and PCBs [23] in vivo and in vitro in mammalian and fish models,

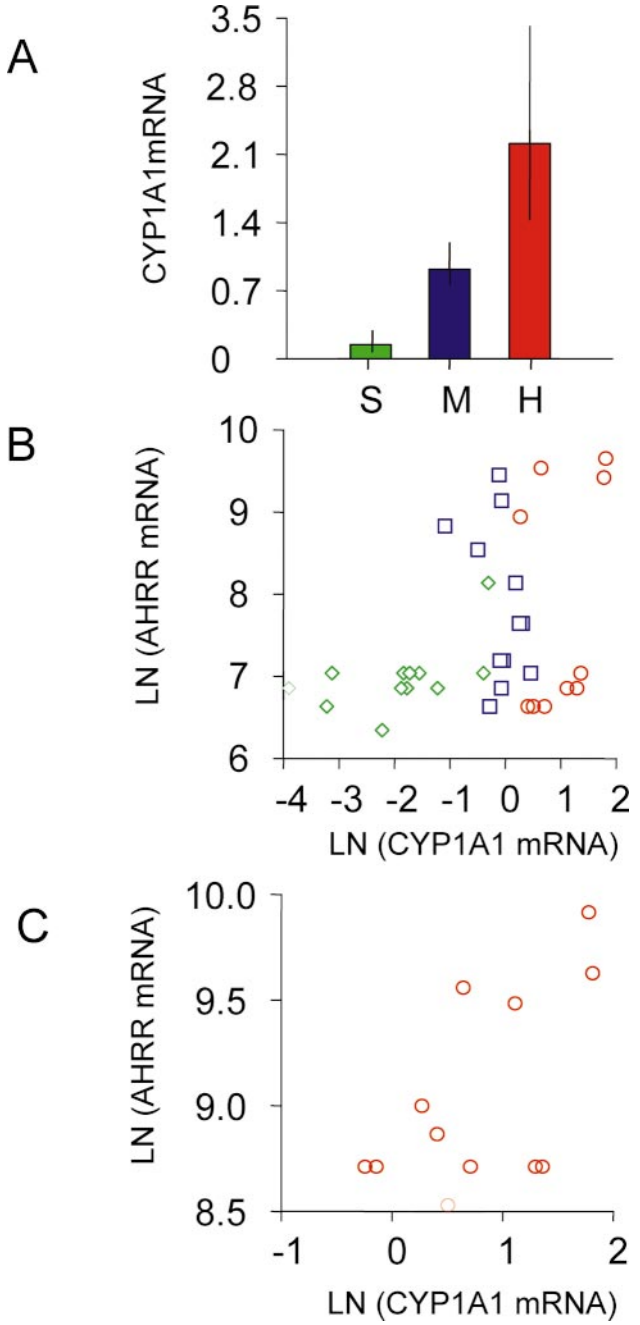


Fig. 7. (**A**) Comparison of hepatic cytochrome P4501A1 (CYP1A1) mRNA concentrations (mean and 95% confidence intervals, $n = 12$ per bar, optical density units) for adult tomcod collected from the St. Lawrence River (S; Canada), Miramichi River (M; Canada), and Hudson River (H; USA) and immediately sacrificed. The CYP1A1 mRNA expression levels were determined by slot blot analysis and expressed as CYP1A1 mRNA/rRNA mRNA. (**B**) Positive correlation of hepatic aryl hydrocarbon receptor repressor (AHRR) mRNA and CYP1A1 mRNA expression (both natural log transformed) in pooled tomcod from the St. Lawrence River (green diamonds), Miramichi River (blue squares), and Hudson River (red circles) ($R = 0.394$, $p = 0.021$, $n = 34$). (**C**) Positive correlation of hepatic AHRR mRNA and CYP1A1 mRNA concentrations (both natural log transformed) in Hudson River tomcod ($R = 0.591$, $p = 0.043$, $n = 12$); LN refers to the natural log.

is AHR mediated, and serves to repress AHR activated transcription of CYP1A1 and presumably overt toxicities. Our objective in this study was to compare AHRR expression in tomcod from resistant and sensitive populations, driven by the hypothesis that basal or induced expression of AHRR is higher in tomcod from the resistant Hudson River than sensitive populations.

### *Characterization and phylogenetic analysis of tomcod AHRR*

We first cloned and characterized full-length AHRR cDNA from tomcod cDNA and genomic DNA libraries. Tomcod AHRR shared some of the structural highlights of other PAS proteins, including very high sequence identity with killifish and mammals at bHLH and PAS-A domains. These domains allow AHRR to recognize cognitive DNA sequences, such as DREs, in promoters of inducible genes in the AHR battery and interact with its transcription partner, ARNT. Similar to other AHRRs, tomcod AHRR can be distinguished from AHR, ARNT, and other PAS proteins by severe truncation of its PAS-B domain that prevents AHRRs from binding ligands [23]. Also, AHRR can be distinguished from AHR by the absence of Q-rich domains that are critical to transactivation of proximal promoter elements in responsive genes in the AHR battery.

Phylogenetic comparison of putative tomcod AHRR to the four previously identified AHRRs, fish, and other vertebrate AHRs (AHR1s and AHR2s) and ARNTs (ARNT1 and ARNT2) revealed that the AHRRs, including that of tomcod, formed a single distinct clade in both neighbor joining and maximum parsimony trees with strong bootstrap support. Not unexpectedly, tomcod AHRR clustered most closely with killifish AHRR. Also, the AHRR clade was part of a larger AHR grouping that included both AHR1s and AHR2s. These results indicate that AHRR and AHRs are distinctly separate genes and are supported by studies in other taxa that demonstrate that AHRR and AHR1 and AHR2 map to separate linkage groups [23].

### *Comparison among tissues of basal and induced expression of AHRR*

We sought to confirm that AHRR is inducible by an AH compound of environmental relevance, PCB77, in juvenile tomcod from a sensitive population and to determine its profile of basal and induced expression in a panel of tomcod tissues. We hypothesized that AHRR expression varies significantly among tissues and that its expression across tissues is negatively correlated with CYP1A1 inducibility. This hypothesis is supported, for example, by results from Mimura et al. [20], who show that liver and thymus express relatively low levels of AHRR in TCDD-treated mice, a phenotype that was consistent with the sensitivity of these tissues to the toxic effects of TCDD.

In tomcod, AHRR mRNA was significantly inducible in all tissues tested, and, as expected, basal and induced levels of AHRR expression differed significantly among individual tissues. Surprisingly, basal and induced AHRR expression directly tracked that of CYP1A1 across tissues. Expression levels of the two genes were significantly positively correlated. Tissues that expressed the highest levels of CYP1A1 also expressed elevated levels of AHRR.

Basal expression of AHRR was observed in all tissues investigated, except liver and brain. This result was similar to that in adult and fetal tissues from humans, where basal expression of AHRR was detected in many but not all tissues [37] and differed from that in mouse, where basal AHRR expression in untreated animals was virtually nondetectable across all tissues [20]. In tomcod, inducibility of both genes was generally highest in liver, heart, and intestine and lowest

in brain. We found high AHRR expression in the heart, liver, and spleen of tomcod treated with PCB77, which is similar to results in killifish treated with a PCB mix [23]. Meyer et al. [36] also reported moderate AHRR induction in liver of killifish treated with β-NF. While induction of AHRR in liver by PCBs was high in both tomcod and killifish from reference populations, that was not the case for rat [22]. Interestingly, Karchner et al. [23] also observed differences in AHRR inducibility among tissues of killifish when treated with a PCB mixture compared to TCDD. In total, these results suggest that expression patterns of AHRR mRNA differ among tissues, species, and perhaps even AHR agonists.

*Comparison of AHRR expression between tomcod from two populations*

Adult and early life stages of tomcod from the Hudson River and cleaner locales exhibit dramatic interpopulation differences in susceptibility to a variety of toxicities elicited by coplanar PCBs and TCDD. It is possible that decreased susceptibility to toxicities from these compounds results from up-regulation of AHRR expression in resistant populations. We evaluated this possibility by comparing basal and induced AHRR expression in embryos of F2 tomcod from the resistant and sensitive population. Although AHRR was often detectable in early life stages of tomcod, we found no differences in expression between untreated tomcod embryos from the two populations. Furthermore, AHRR mRNA expression was equally inducible by B*a*P treatments in early life stage offspring of F2 tomcod from the two populations. However, PCB77 exposure significantly induced AHRR mRNA in tomcod offspring from the Miramichi River but less so in those from the Hudson River. Thus, neither basal nor induced levels of AHRR were higher in early life stages of tomcod of Hudson River ancestry but instead generally paralleled that of CYP1A1 mRNA. Thus, up-regulation of AHRR does not explain resistance of early life stages of Hudson River tomcod to coplanar PCBs and TCDD.

Atlantic killifish populations from three Atlantic coast estuaries that are highly contaminated with AHs are highly resistant to AH-induced toxicities and CYP1A induction. But unlike tomcod, killifish populations typically exhibit resistance to both halogenated AHs (PCBs, PCDDs, and polychlorinated dibenzofurans) and nonhalogenated PAHs (reviewed in Wirgin and Waldman [39]). Two studies compared basal and inducible AHRR expression in killifish from resistant and sensitive populations. Karchner et al. [23] found no evidence of up-regulation of AHRR expression in eight tissues of TCDD or PCB-treated adult killifish from the resistant New Bedford Harbor population compared to those from a sensitive population. Meyer et al. [36] compared basal and induced hepatic AHRR expression in killifish from the resistant Elizabeth River and a sensitive population. Basal AHRR expression was similar between fish from the two populations, but AHRR mRNA was significantly inducible three- to fourfold by β-NF treatment in killifish from the reference population but not in those from the resistant Elizabeth River population. Thus, results in resistant killifish from the Elizabeth River were similar to those we observed in tomcod; that is, AHRR was more inducible in fish from the sensitive compared to the resistant population. But tomcod were unique in that AHRR was significantly inducible by B*a*P but not PCB77 in tomcod from the resistant population.

In summary, gene expression patterns for AHRR in tomcod from sensitive and resistant populations usually mirror those for CYP1A1. The AHRR and CYP1A1 were inducible by PAHs but not PCB77 in tomcod from the Hudson River, whereas both genes were inducible by PAHs and PCB77 in tomcod from the Miramichi River. These results are inconsistent with the hypothesis that up-regulation of basal or inducibility of AHRR is the mechanistic basis of resistance in the Hudson River tomcod population. Rather than provide the mechanistic basis of resistance, AHRR inducibility exhibits the same profile as CYP1A1 expression and early life stage toxicities. This suggests that all three manifestations of resistance, CYP1A1 mRNA inducibility, AHRR inducibility, and early life stage toxicities, share the same but presently unknown mechanistic bases. Thus, rather than serve as the mechanistic basis of resistance, reduced inducibility of AHRR in the Hudson River population probably results from the same phenomenon as that for CYP1A1, that is, impairment of AHR pathway function for halogenated rather than nonhalogenated AHs in this population.

While altered expression of neither AHR2 nor ARNT2 was observed in tomcod from the Hudson River compared to that in tomcod from the Miramichi River, it is possible that genetic variants at these loci or novel proteins yet to be identified modulate activation of the AHR pathway in tomcod from the Hudson River. For example, Hansson [40] has proposed the existence of three AHR2 and two AHR1 genes in the typical fish genome, the functional attributes of which are unknown. It is possible that these multiple AHRs may interact to differentially impact activation of the AHR pathway in tomcod from resistant and sensitive populations. Furthermore, the differential expression patterns of AHRR and CYP1A1 for halogenated compared to nonhalogenated AHs in tomcod from the Hudson River populations provide further evidence for multiple pathways, AHR dependent and AHR independent, that modulate AH-induced toxicity in this population.

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