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AN EVALUATION OF THE ETIOLOGY OF REDUCED CYP1A1 MESSENGER RNA EXPRESSION IN THE ATLANTIC TOMCOD FROM THE HUDSON RIVER, NEW YORK, USA, USING REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS

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Abstract—Adult Atlantic tomcod, *Microgadus tomcod,* from the Hudson River, New York State, USA, exhibit reduced inducibility of hepatic cytochrome P4501A1 (CYP1A1) mRNA compared with adult tomcod from the cleaner Miramichi River, New Brunswick, Canada, when treated with coplanar polychlorinated biphenyl (PCB) congeners or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In contrast, little difference in CYP1A1 inducibility is observed between tomcod from these two rivers when treated with polycyclic aromatic hydrocarbons (PAHs). We sought to determine if impaired hepatic CYP1A1 inducibility in Hudson River tomcod results from a multigenerational, genetic adaptation or a single generational, physiological acclimation. Embryos and larvae from controlled experimental crosses of Hudson River and Miramichi River parents were exposed for 24 h to water-borne PCB congener 77 (10 ppm), benzo[*a*]pyrene (B*a*P; 10 ppm), or dimethysulfoxide, and CYP1A1 expression was assessed in individual larva using competitive reverse transcriptase polymerase chain reaction (RT-PCR) analysis. The CYP1A1 mRNA was significantly induced in larvae from both populations by B*a*P (47- and 52-fold) and PCB 77 (9- and 22-fold), although levels of expression were higher in offspring of Miramichi matings. Most important, CYP1A1 mRNA was significantly induced by PCB 77 in larvae from Hudson River parents. Concentrations of dioxin, furan, and PCB congeners were measured in livers and eggs of female tomcod from these two locales to quantify the extent of maternal transfer of contaminants. For both rivers, wet-weight contaminant concentrations were significantly higher (4–7 times) in livers than in eggs of the same females, suggesting that a threshold level of contaminants may have to be reached before CYP1A1 transcription is impaired. We conclude that reduced inducibility of hepatic CYP1A1 mRNA in adult tomcod from the Hudson River is most consistent with single-generational acclimation.

Keywords—Cytochrome P4501A1 Gene expression Polychlorinated biphenyls Dioxins Furans

INTRODUCTION

Hepatic cytochrome P4501A1 (CYP1A1) gene expression in fishes is frequently used as a biomarker of exposure to aromatic hydrocarbons and early biological effect. The CYP1A1 transcription is dose-responsively induced in fishes by environmentally relevant doses of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), some coplanar polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) [1,2]. The prototypical CYP1A1 inducer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), may be recalcitrant to cytochrome P450-catalyzed metabolism, and as a result, its half-life in lipid-rich vertebrate tissue ranges up to at least 10 years [3]. The PCBs are believed metabolized by cytochrome P450s, albeit very slowly in fish hepatic tissues [4]. In contrast, PAHs are rarely quantifiable in fish livers because of their rapid metabolism by CYP1A1 catalyzed activities [5]. Reactive metabolites of bulky PAHs cause genetic lesions by adducting to DNA whereby they may initiate a carcinogenic process. Metabolically more recalcitrant PCDDs, PCDFs, and PCBs may also cause DNA damage by the generation of reactive oxygen species mediated by persistent induction of CYP1A1 activities [6].

The CYP1A1 transcription is mediated by activation of the aryl hydrocarbon receptor (AhR) pathway. Briefly, binding of ligand with AhR occurs in the cytoplasm and results in the release of two molecules of heat shock protein 90 and an additional 37 kDa AhR interacting protein from AhR [7]. Ligand-AhR complex translocates to the nucleus, where binding with ARNT (aryl receptor nuclear translocator) occurs. The activated AhR complex then binds with dioxin responsive elements (DREs) 5′ to proximal promoter elements of genes in the AhR battery such as CYP1A1. The AhR complex binding to DREs elicits DNA bending, chromatin and nucleosome disruption, increased access by constitutively expressed transcription factors to proximal promoter elements, and initiation of transcription. The AhR [8,9], ARNT [10], and DREs [11] have been identified and characterized in fishes, and their means of action apparently are similar to those in mammals. Studies with AhR-deficient mice have demonstrated that the AhR pathway function is needed for activation of CYP1A1 transcription [12], to mediate toxic responses to TCDD [13], and in the absence of xenobiotic exposure, AhR expression is required for normal in vitro hepatic cell cycling [14] and liver and immune system development [12,15].

Atlantic tomcod (*Microgadus tomcod)* is a very abundant, anadromous species found in Atlantic coast estuaries from Labrador, Canada, to the Hudson River, USA. Because of its bottom-dwelling existence, its dependence on benthic prey, its lipid-rich liver, and its year-round distribution within natal estuaries, tomcod from polluted locales often bioaccumulate high concentrations of halogenated aromatic hydrocarbon con-

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taminants [2,16]. Additionally, exposure of tomcod to elevated levels of PAHs can be inferred from the high levels of PAH metabolites spectrophotometrically detected in bile of tomcod from contaminated sites [17]. Biological activity of these contaminants may be inferred from the high levels of overall hepatic DNA damage as detected by ^{32}P postlabeling in tomcod from the Hudson and St. Lawrence rivers [17], the presence of an activated K-*ras* oncogene in liver tumor DNA in tomcod from the Hudson River [18], the elevated prevalence of hepatocellular carcinomas, and the truncated age structure of the Hudson River population [19].

Laboratory studies were conducted in which tomcod from the Hudson River (NY, USA) and the cleaner Miramichi River (NB, Canada) were ip injected with graded doses of TCDD; coplanar PCB congeners 77, 126, 169; B*a*P; beta naphthoflavone (β-NF); and 3-methylcholanthrene. CYP1A1 was not significantly inducible in tomcod from the Hudson River with either TCDD or the PCB congeners but was highly inducible with B*a*P, β-NF, and 3-methylcholanthrene. In contrast, CYP1A1 mRNA was highly inducible in tomcod from the Miramichi River with all seven chemicals [2,16; Wirgin et al., unpublished data]. This suggests that prior exposure of tomcod in the Hudson River to contaminants or genetic differences between populations may have diminished their CYP1A1 inducibility with halogenated aromatic hydrocarbons.

Down-regulation of CYP1A1 mRNA inducibility may occur through two mechanisms, i.e., genetic adaptation or physiological acclimation. Differentiating between these two alternatives is important in evaluating the persistence of this phenomenon in impacted populations. Genetic adaptation would indicate a multigenerational response that might persist despite the remediation of contaminated locales, whereas physiological acclimation would suggest a single generational response. Studies in higher vertebrates have demonstrated that mutations in functionally important AhR domains may significantly alter CYP1A1 inducibility and susceptibilities to other toxicological responses [20]. Additionally, physiological [21,22] and epigenetic [23] mechanisms have been demonstrated to modulate CYP1A1 transcription in rodent models acutely treated with halogenated aromatic hydrocarbon contaminants.

In this study, we attempted to distinguish between these explanations for reduced CYP1A1 inducibility in adult Hudson River tomcod by quantifying CYP1A1 mRNA expression in PCB congener 77- and B*a*P-exposed larval tomcod that were the offspring of a diallele cross between Hudson River and Miramichi River parents. The competitive reverse transcriptase polymerase chain reaction (RT-PCR) allowed us to quantify CYP1A1 mRNA expression in vehicle and contaminant-exposed individual tomcod larvae. The use of competitive RT-PCR as a dose-responsive measure of CYP1A1 expression has been validated in salmonid fishes [24].

METHODS

Broodstock collections and matings

Prespawning adult tomcod (20–25 cm total length; 2–3 years old) were collected from the commercial smelt fishery on the Miramichi River near Loggieville (NB, Canada) on January 7 and transported to the aquarium facility at the Gulf Fisheries Centre in Moncton, New Brunswick, Canada, where they were held in filtered 15-ppt seawater in a 1,000-L fiberglass tank at 4°C. Sperm was expressed from 21 males into individual plastic tubes on January 11 and divided into two aliquots. One aliquot of sperm from each of the 21 specimens was shipped chilled to New York, USA, to make M (Miramichi) × H (Hudson) crosses, and the second aliquot was used to fertilize eggs from 21 Miramichi River females to make M × M crosses.

Tomcod from the Hudson River were collected on January 12 with boxtraps set at Garrison (NY, USA) at river mile 50. Triplicate H × H crosses were made immediately at the collection site, each with eggs from two females and sperm from six males. Sperm from an additional 14 males was expressed on site into individual tubes and transported on ice to the airport. Six female Hudson River fish were transported alive to the airport, where three M × H crosses were made, each with eggs from two Hudson River females and incoming sperm from six Miramichi River males. Resulting H × H and M × H embryos were placed in plastic bottles filled with fresh water, were chilled on ice, and were sent back to Moncton along with the sperm from the 14 Hudson River males. In Moncton, aliquots of eggs expressed from the 21 Miramichi River females were fertilized with the incoming sperm from Hudson River males to make H × M crosses. For all crosses, fertilization was achieved by mixing sperm into pooled eggs, stirring for 5 min, then adding fresh water and letting stand for 5 min, after which sperm was rinsed away with three water changes. All embryos from matings within a cross were mixed to create one pool from each cross, and these were randomly assigned to treatments.

Egg incubations

Embryos from each of the four crosses were divided equally by volume among three Macdonald jars for incubation. The surface of each jar was covered by a screen of 500-μm Nytex mesh to prevent embryos from escaping. Dipnets were hung in the outfall of each jar to confirm that no embryos had escaped. Water flows were set at 3.5 to 4.0 L/min/jar to maintain embryos in suspension. Embryos were incubated at 3 to 5°C in dechlorinated city water.

All 12 jars were suspended within a single, darkened 1.5-m fiberglass tank. Water exiting the top of the jars spilled down into the tank, from which it was pumped up to a headtank with ports that redistributed the water back to the jars. Through this design, all groups of embryos shared the same water throughout their incubation. Water was aerated during its fall, and 60 L (approximately 6%) of water was replaced from the system approximately every 4 d to minimize accumulation of ammonia, which never exceeded 0.9 mg/L.

Approximately 100 embryos were removed from each jar 3 to 4 d after fertilization and examined under a dissecting microscope to assess their development. Between 12 and 31% of embryos in each jar were alive and developing normally, with an average survivorship of 20%.

Experimental exposures

For each cross, one group was exposed to 10 ppm PCB congener 77 (Ultra Scientific, Kingston, RI, USA), the second group to 10 ppm B*a*P (Sigma Aldrich, Oakville, ON, Canada), and the third group to vehicle (0.5% DMSO) used to dissolve congener 77 and B*a*P. Exposures were made in three-quarter filled, 1-L glass jars maintained at 3 to 4°C in a temperature bath and equipped with air stones. Embryos or larvae were exposed 37 to 40 d postfertilization (i.e., during the period of hatch) for 24 h, rinsed twice with clean water, and then transferred to clean water for an additional 24 h for B*a*P; 72, 168,

or 192 h for congener 77; and 48 h for DMSO. Sacrifice times and doses of congener 77 and B*a*P were selected to maximize CYP1A1 mRNA induction based on previous work with adult tomcod ip injected with these chemicals [2].

Individual yolk sac larvae were collected and placed in individual 1.5-ml microcentrifuge tubes with approximately 50 μl of H_2O, were snap frozen in liquid nitrogen, and were maintained at −70°C until processing. Larvae were not measured or weighed in this experiment, but in other studies, the mean length of individual Hudson River tomcod larvae at 7 d posthatch was 6.96 mm and mean wet weight was 0.214 mg. Dry weights of larvae were approximately 10% of wet weight values (C. Chambers, personal communication).

Isolation of total RNA from individual larvae

Frozen individual larvae were homogenized in 500 μl of Ultraspec reagent (Biotecx, Houston, TX, USA) directly in the 1.5-ml microcentrifuge tubes. The homogenates were incubated on ice for 10 min and 100 μl of chloroform was added. The homogenates were then vortexed and incubated on ice for an additional 10 min. Homogenates were centrifuged at 15,000 *g* for 15 min at 4°C, and the aqueous phase was saved and mixed with an equal volume of cold isopropanol and then incubated on ice for 45 min. Homogenates were centrifuged at 15,000 *g* for 15 min at 4°C to pellet total RNA. The RNA pellets were washed twice with cold 70% EtOH, air dried, resuspended in 50 μl of diethyl pyrocarbonate-treated water, and stored at −80°C.

Quantitation of total RNA concentrations in sample preparations

Initially, spectrophotometry was used to quantify total RNA concentrations and to determine purity of individual preparations; however, RNA concentrations were too low for reliable estimations. Instead, we used slot blot analysis for this task. Two- and 10-μl volumes of each larval RNA preparation were applied directly to Nytran Nylon Plus membranes (Schleicher and Schuell, Keene, NH, USA) with a Schleicher and Schuell Slot Blot Manifold along with four dilutions (0.5, 2.0, 5.0, and 12.5 ng) of total RNA previously isolated from a single adult tomcod liver and for which RNA concentrations were determined by spectrophotometric analysis. Membranes were hybridized to ^{32}P radiolabeled rat 18 S rRNA (pHRR118) probes [25] and washed as described in Wirgin et al. [17]. Hybridization signals for larval and liver RNAs were quantified with a Molecular Dynamics Phosphorimager (Sunnyvale, CA, USA). A standard curve of total liver RNA concentrations versus rRNA hybridization signal was generated from adult samples on each slot blot, and this was used to calculate total RNA concentrations for each larval RNA on that blot.

Northern blot analysis

Northern blot analysis was used to evaluate the integrity of all larval RNA preparations. Ten microliters of total RNA from each specimen were denatured and separated in 1.2% formaldehyde agarose gels [26], transferred to Schleicher and Schuell Nytran membranes by capillary action overnight, and fixed to the membranes by baking in a vacuum oven at 80°C for 2 h. Membranes were prehybridized and hybridized at 65°C to ^{32}P radiolabeled rat 18 S rRNA probes as described above, and rRNA bands were evaluated for integrity from autoradiographs.

Preparation of truncated control RNA standard

Control truncated CYP1A1 RNA standard was prepared as described by Celi et al. [27]. A truncated DNA fragment was amplified from tomcod CYP1A1 cDNA [28] using primers U1 (5′-GGGATCCACCAGGAGATCAAGGCCTGCCCTTCAC-CATCCC-3′) and D1 (5′-GGCTGCAGATATAGCAGACAG-3′). Primer U1 is 40 nucleotides (nt) in length; nt 7 through 22 are identical to tomcod CYP1A1 cDNA nt 1140 through 1155 (in exon 4) and U1 nt 23 through 40 are identical to tomcod CYP1A1 cDNA nt 1253 through 1270 (in exon 5). The D1 primer extends from tomcod CYP1A1 cDNA nt 1705 to 1685 (in exon 7). Use of U1 and D1 results in the amplification of a 469 bp product with a 97 bp deletion extending from CYP1A1 nt 1156 to nt 1252. These primers contained *Bam* HI (U1) and *Pst* I (D1) restriction sites, which were used to insert this CYP1A1 fragment into Bluescript(II) vector to generate the plasmid pZPY1. This recombinant was sequenced to confirm that it contained the truncated tomcod CYP1A1 fragment. An RNA Transcription Kit (Stratagene, La Jolla, CA, USA) with T3 RNA polymerase was used to generate sense strand RNA from pZPY1 as described by the manufacturer. The RNA preparations were subjected to RNAse-free DNAse digestion and purified by alcohol precipitations. Truncated CYP1A1 RNA was diluted to 50 pg/μl and stored at −70°C until use.

Competitive RT-PCR quantitation of CYP1A1 mRNA expression

For each larval sample, two to five different volumes of total RNA were each mixed with 0.2 pg of truncated CYP1A1 RNA standard in 10-μl volumes. Two μl of random hexamers (Life Technologies, Gaithersburg, MD, USA) (150 pmoles each) were added, incubated at 75°C for 5 min, and chilled on ice for 5 min. Eight microliters of reverse transcriptase mixture (1 X M-MLV reverse transcriptase buffer, 0.5 mM dNTPs, 4 units [U] of RNasin ribonuclease inhibitor, and 20 U of M-MLV reverse transcriptase [Promega, Madison, WI, USA]) was added to each reaction and incubated at 42°C for 1 h. Reaction products were denatured at 98°C for 5 min, chilled on ice for 5 min, and an aliquot was PCR amplified. The PCR conditions were initially optimized so that reactions were stopped in the logarithmic phase of amplification. The PCR reactions were in 25-μl volumes containing 1 X reaction buffer, 0.2 mM dNTPs, 1 μM of each of the primers U2 (5′-CAC-CAGGAGATCAAGG-3′) and D2 (5′-CTGCAGATATAGCA-GACAG-3′), and 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA). Cycling conditions were denaturation at 95°C for 5 min, 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min.

Reaction products were electrophoresed in 1.0% agarose gels and stained with either ethidium bromide and visualized on an ultraviolet transilluminator and photographed or with SYBR Green I (Amresco, Solon, OH, USA) and visualized by scanning with a Molecular Dynamics Phosphorimager.

The intensities of the truncated control and native CYP1A1 bands were determined from scans using the IQMac Version 1.2 (Molecular Dynamics) application. Initially, ethidium bromide-stained gels were also subjected to Southern hybridization with ^{32}P-radiolabeled tomcod CYP1A1 cDNA probes [28]. This was discontinued because sufficient product was obtained to visualize DNA fragments with either ethidium bromide or SYBR Green I staining (Molecular Probes, Eugene, OR, USA).

Each larval RNA was diluted two to five times, and these were analyzed in RT-PCR to determine the RNA concentration that would produce an amount of product that was between 75 and 125% of that obtained from the truncated control RNA standard. Calculations of CYP1A1 mRNA in larval samples were made from the relationship $(A/B)\cdot C$, where A and B were the intensities of the products from total RNA and control RNA, respectively, and C was the amount of the control RNA used in the reverse transcriptase reaction. Consideration was not given to the size of the CYP1A1 mRNA in calculating amount present.

Measurement of hepatic and gonadal levels of HAHs

Concentrations of PCBs, PCDDs, and PCDFs were measured in the pooled livers and eggs of 10 mature female tomcod (total length 18.4–25.5 cm) collected from the Hudson River at Garrison and five groups of four to five tomcod each (total length 21.5–31.5 cm) collected from the Miramichi River at Loggieville. Analyses were carried out at Fisheries and Oceans Canada's Institute of Ocean Sciences in Sidney, British Columbia. Dioxin/furan analyses were performed in accordance with the Environment Canada Reference Methods 1/RM/19 and 1/RM/23 [29,30]. During carbon fiber cleanup of the dioxin samples, all 209 PCB congeners were also collected from an automated high-performance liquid chromatography system. From this system, four fractions were collected, where fraction 1 contained the di-*ortho*-PCBs, fraction II contained the mono-*ortho*-PCBs, fraction III contained the non-*ortho*-PCBs, and fraction IV contained the PCDDs and PCDFs. Fractions were concentrated to less than 10 μl, spiked with corresponding ^{13}C-labeled method performance standards, and analyzed by high-resolution gas chromatography/high resolution mass spectrometry. Details on methods, surrogate internal standard solution composition, and the solvents and conditions used in all the clean-up and fractionation steps can be found in MacDonald et al. [31]. Tissue samples were analyzed in batches of 12, which included one procedural blank, one certified reference material, and nine real samples, of which one was analyzed in duplicate. The criteria for PCB identification and quantification and the quality assurance and quality control measures undertaken for the sample work-up and the high-resolution gas chromatography/high resolution mass spectrometry analysis were similar to those outlined in the Environment Canada protocols for PCDD/PCDF analyses [29,30]. Total PCDDs and PCDFs are expressed as toxic equivalent quotients using toxic equivalence factor values reported in [32].

Lipid determinations

Percentage moisture and lipid were determined for each sample to permit expression of data as dry weight or as lipid normalized. Approximately 5 g of homogenized sample was accurately weighed, dried with sodium sulfate, and extracted with 100 ml of 1:1 hexane:dichloromethane from a glass column by gravity flow. The extract was reduced to 1 ml using a Turbovap and was quantitatively transferred to a weigh boat using hexane/dichloromethane. The extract was placed in a 40°C oven overnight and subsequently placed in a dessicator to cool to room temperature. Then it was accurately weighed. The weights of the oven-dried extract and the original sample were used to calculate the percent lipid. Precision of lipid analysis was checked by reanalyzing four of the five Miramichi liver pools and the single Hudson liver pool. Mean deviation of the two analyses was 0.87% lipid, with a range of 0.47 to 1.65%.

Data analysis

The CYP1A1 mRNA. For all statistical comparisons, CYP1A1 mRNA data (CYP1A1 pg/μg of total RNA) were $\ln(X + 1)$ transformed to improve normality, determined by examination of probability plots. Data presented are back-transformed means and 95% confidence intervals. Differences in CYP1A1 mRNA levels over time (3, 7, 8 d) after exposure to PCB congener 77 were tested by one-way ANOVA to determine whether responses had peaked by day 3 and were therefore suitable for comparison with other treatments. Within each cross (e.g., H × H), CYP1A1 mRNA levels of fish exposed to congener 77 and B*a*P were compared with fish exposed only to the vehicle DMSO (i.e., negative control) by one-way ANOVA followed by Tukey's multiple range test to assess statistical significance of induction. The CYP1A1 mRNA levels in offspring of the four crosses to the three different treatments were compared by two-way ANOVA. Because this analysis revealed a significant difference among crosses in overall CYP1A1 mRNA levels, irrespective of treatment, the following analysis was conducted to determine whether there was a difference among crosses in response to treatments (i.e., CYP1A1 inducibility). The CYP1A1 mRNA levels in congener 77- and B*a*P-treated fish were reduced by the mean CYP1A1 mRNA level in the DMSO controls of their cross. The M × H cross was deleted from this analysis because only a single fish survived congener 77 exposure. The CYP1A1 mRNA levels were then compared among the three remaining crosses exposed to either congener 77 or B*a*P by two-way ANOVA.

Dioxin, furan, and PCB concentrations in liver and eggs. The relationship between contaminant burdens in eggs and livers of the same females ($n = 5$ pools for Miramichi and 1 pool for Hudson) was examined by Pearson correlation. Paired t tests were carried out to determine whether contaminant loads differed significantly between the two tissues. All analyses were carried out on log-transformed data and significance levels were not corrected for multiple comparisons. All statistical analyses were carried out in SYSTAT 7.0 (SPSS, Chicago, IL, USA). Tissue contaminant concentrations are presented both on wet weight and lipid-normalized bases. Lipid normalization permits comparison of our results to some previously published data sets; however, we feel that wet weight concentrations provide the most accurate measure of intracellular levels of contaminants available to activate the AhR pathway.

RESULTS

Determination and yield of total RNA concentrations in individual specimens

To express results as CYP1A1 mRNA pg/μg total RNA, it was necessary to quantitate concentrations of total RNA in each isolation. Because the size of the individual larva was small (mean wet wt = 0.214 mg) and yield of RNA was low, we used slot blot analysis to quantitate total RNA concentrations in each larval RNA isolation. Mean yield of total RNA from each larva was 1.01 μg (± 0.121 μg), or 0.005 μg/mg tissue. Total RNA isolated from individual larva was also subjected to northern blot analysis with the 18S rRNA probe to confirm the integrity of each RNA preparation (Fig. 1).

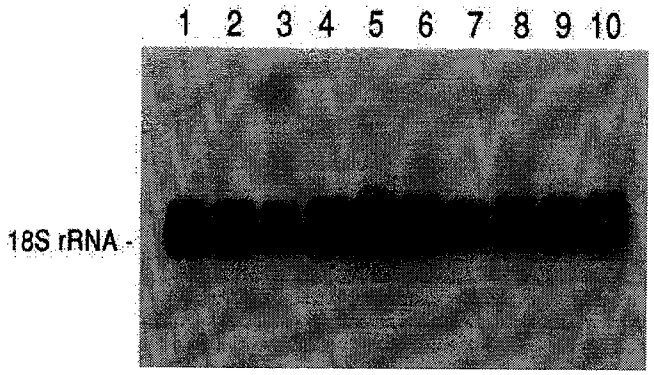

Fig. 1. An autoradiograph of a northern blot hybridization of 2.5 μl of total RNA from 10 individual tomcod larvae hybridized to a ^{32}P-radiolabeled rat 18 S rRNA probe (pHRR118). Hybridizations were done to evaluate the integrity of all RNA preparations.

Comparison of CYP1A1 mRNA levels among tomcod larvae

Use of RT-PCR permitted us to quantify basal levels of CYP1A1 mRNA expression in individual DMSO-exposed tomcod larva (Fig. 2, lanes 1–6) and levels of gene induction in B*a*P- (Fig. 2, lanes 7–12) and congener 77-exposed fish. Mean levels of CYP1A1 mRNA were approximately twofold higher in DMSO-exposed larvae from the Miramichi River (34.6 pg CYP1A1 mRNA/μg total RNA) than in DMSO-exposed larvae from the Hudson River (16.1 pg CYP1A1 mRNA/μg total RNA).

There were no significant differences among the CYP1A1 mRNA levels of H × H larvae exposed to congener 77 and sampled on days 3, 7, or 8 ($F(2, 28) = 0.909$, $p = 0.414$) (data not shown). Sample size for day 8 was only two, but comparing day 7 ($n = 10$) to day 3 ($n = 16$) also showed no difference in mean CYP1A1 mRNA levels ($F(1, 24) = 0.969$, $p = 0.335$). Similarly, in the only other cross for which sample sizes permitted testing of congener 77 effect over days 3 through 8, H × M, there was no significant difference among days in CYP1A1 mRNA levels ($F(2, 21) = 0.754$, $p = 0.483$). Therefore, CYP1A1 mRNA levels appeared to have stabilized

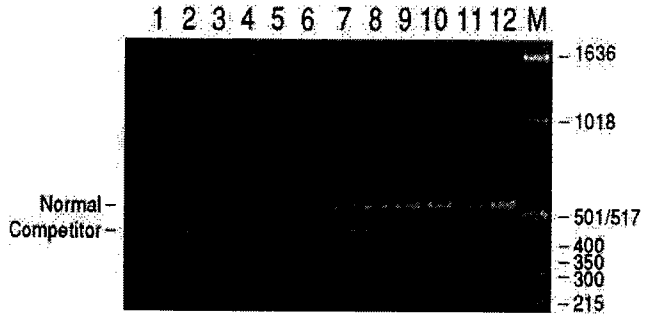

Fig. 2. Competitive RT-PCR analysis of CYP1A1 mRNA expression in individual tomcod larvae exposed to dimethysulfoxide (DMSO) or benzo[*a*]pyrene (B*a*P). Variable volumes of total larval RNA and a constant amount (0.2 pg) of competitor CYP1A1 mRNA truncated standard were used in each reaction. The molecular size of native CYP1A1 PCR products was 566 bp and the truncated CYP1A1 standard was 469 bp. Lanes 1 through 5 contain total RNA from a DMSO-exposed larva (0.5, 1.0, 2.0, 5.0, and 10 μl) and lanes 7 through 11 contain RNA from a B*a*P-exposed larva (same volumes). Lanes 6 and 12 contain larval RNA and no truncated standard. Lane M contains a 1-kB DNA ladder. The 1.0% agarose gel was stained with ethidium bromide and photographed under ultraviolet illumination.

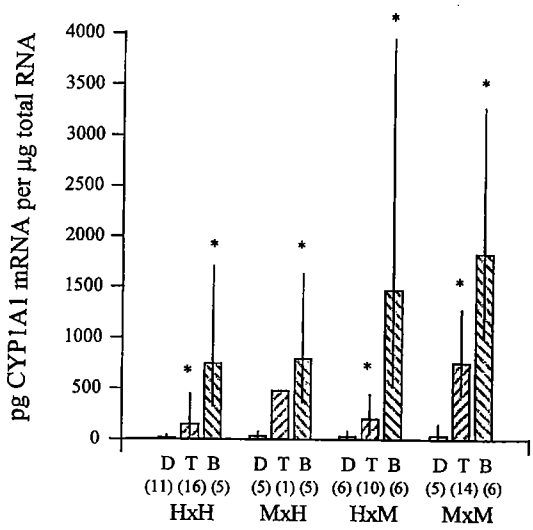

Fig. 3. The CYP1A1 mRNA levels in tomcod larvae exposed to benzo[*a*]pyrene (B), PCB 77 (T), or vehicle control (DMSO) (D). Data are back-transformed means and 95% confidence intervals from analysis of variance (ANOVA) with log-transformed data. Numbers below bars are sample sizes. Asterisks denote significant induction over respective DMSO control for each cross ($p < 0.01$, Tukey's test following one-way ANOVA for each cross). Crosses are male × female for Hudson River, New York State, USA (H), and Miramichi, New Brunswick, Canada (M), broodstock.

3 d after exposure to congener 77, and only day 3 data were used for subsequent comparison with other treatments.

The CYP1A1 mRNA was significantly induced over DMSO controls in offspring of all crosses exposed to either congener 77 or B*a*P ($p < 0.01$, Tukey's test following one-way ANOVA for each cross) (Fig. 3). Differences in CYP1A1 mRNA levels were examined among the four crosses and three treatments by a two-way analysis of variance. Treatment effect was highly significant ($F(2, 78) = 58.355$, $p < 0.001$), but interestingly, there was also a significant difference among crosses in CYP1A1 mRNA levels ($F(3, 78) = 3.236$, $p = 0.027$), which did not differ significantly across treatments (cross × treatment interaction term, $F(6, 78) = 0.618$, $p = 0.715$). Across all treatments, M × M offspring showed higher CYP1A1 mRNA levels than H × H offspring ($p = 0.004$; Tukey's test following one-way ANOVA, $F(3, 86) = 4.157$, $p = 0.008$), with hybrids intermediate and not significantly different from one another or from pure offspring from either parental stock.

The important question in this experiment, though, was whether the different crosses showed a similar degree of CYP1A1 induction after congener 77 or B*a*P exposures rather than similar absolute levels of CYP1A1 mRNA. This question was addressed in the following way. The CYP1A1 mRNA levels in congener 77- and B*a*P-treated fish were reduced by the mean CYP1A1 mRNA level shown by DMSO control of their cross. The M × H cross was deleted from this analysis because only a single fish survived congener 77 exposure. The CYP1A1 mRNA levels were then compared among the three remaining crosses exposed to either B*a*P or congener 77 by two-way ANOVA.

Induction levels were similar among crosses (cross, $F(2, 51) = 0.889$, $p = 0.417$; cross × treatment, $F(2, 51) = 0.688$, $p = 0.507$), ranging from 7- to 22-fold for congener 77 and 24- to 52-fold for B*a*P (fold inductions calculated on back-transformed data). Though high, CYP1A1 mRNA levels induced by 24-h exposure to 10 ppm congener 77 were signif-

Table 1. Concentrations of chlorinated aromatic hydrocarbons that induce CYP1A in fish found in livers and eggs of female Atlantic tomcod sampled January 27, 1999, from Loggieville, New Brunswick, Canada, in the Miramichi River estuary and on January 8, 1999, from Garrison, New York State, USA, river mile 50 on the Hudson River estuary; concentrations are expressed on a wet weight basis (wet) as well as lipid-normalized basis (lipid) (g lipid/g wet liver); dry weights may be calculated from percent moisture (water)

Sample (*n* in pool)	Water (% wet wt)	Lipid (% wet wt)	2,3,7,8-TCDD[a] (ng/kg)		Total dioxins and furans (2,3,7,8-TCDD TEQ[a])		PCB[a] congener 77 (ng/kg)		Total coplanar PCBs (ng/kg)[b]	
			Wet	Lipid	Wet	Lipid	Wet	Lipid	Wet	Lipid
Miramichi livers										
Pool 1 (5)	75	5	1	13	2	33	40	853	67	1,412
Pool 2 (4)	69	9	2	25	5	58	57	608	111	1,185
Pool 3 (4)	70	10	1	14	3	34	51	522	99	1,021
Pool 4 (5)	74	4	1	35	3	74	12	327	26	705
Pool 5 (5)	69	11	1	10	3	28	141	1,293	198	1,815
Mean	72	8	1	20	3	45	60	721	100	1,227
SD	3	3	1	11	1	20	48	372	64	417
n	5	5	5	5	5	5	5	5	5	5
Miramichi eggs										
Pool 1 (5)	73	2	0	8	0	19	13	564	19	835
Pool 2 (4)	73	2	0	11	1	22	21	873	27	1,121
Pool 3 (4)	74	2	0	9	0	18	11	458	15	648
Pool 4 (5)	72	2	1	29	1	56	12	498	18	733
Pool 5 (5)	72	2	0	5	0	12	16	666	20	820
Mean	73	2	0	12	1	25	15	612	20	831
SD	1	0	0	10	0	17	4	166	5	178
n	5	5	5	5	5	5	5	5	5	5
Hudson livers (10)	60	14	22	158	41	291	7,582	54,003	8,443	60,133
Hudson eggs (10)	74	2	6	275	8	376	1,342	60,467	1,457	65,641

[a] TCDD = tetrachlorodibenzo-*p*-dioxin; TEQ = toxic equivalent quotient; PCB = polychlorinated biphenyls.
[b] Total coplanar PCBs = sum of concentrations of 3,3′4,4′-tetrachlorobiphenyl (77), 3,3′4,4′,5-pentachlorobiphenyl (126), and 3,3′,4,4′,5,5′-hexachlorobiphenyl (169).

icantly lower than levels induced by a similar duration of exposure to 10-ppm B*a*P ($F(1, 51) = 14.454$, $p < 0.001$).

Wet-weight concentrations of dioxins, furans, and PCBs in liver and eggs

Contaminant burdens were much higher in the livers and eggs of tomcod from the Hudson River than from the Miramichi River (Table 1). Hepatic concentrations of TCDD, total PCDDs and PCDFs expressed in TCDD toxic equivalent quotients, congener 77, and total coplanar PCBs were 16-, 13-, 126-, and 84-fold higher, respectively, in tomcod from the Hudson River than from the Miramichi River. Corresponding levels of these contaminants in eggs were 21-, 14-, 92-, and 74-fold higher in Hudson River tomcod compared with fish from the Miramichi River. While a statistical comparison of tissues from the two rivers is not possible with only a single sample from the Hudson River, it is informative that all contaminant categories in both tissue levels in tomcod from the Hudson River far exceeded the 99% confidence interval for the five Miramichi River samples.

Wet-weight concentrations of TCDD, total PCDDs and PCDFs expressed in TCDD toxic equivalent quotients, congener 77, and total coplanar PCBs were significantly higher (4–7 times) in livers than eggs of fish from the Miramichi River (paired *t* tests, $t = 3.3$–7.1, $p = 0.002$–0.030, $n = 5$). Hudson River females showed the same pattern in the single pools of tissues with four- to sixfold higher contaminant concentrations in livers than in eggs. Levels of each contaminant measured were significantly correlated between livers and eggs of the same females ($r = 0.884$–0.950, $p < 0.02$, $n = 6$), relationships which were driven almost entirely by the single sample from the Hudson River (Miramichi River samples only, $r = 0.009$–0.448, $p > 0.4$ for each, $n = 5$).

Lipid-normalized concentrations of dioxins, furans, and PCBs in livers and eggs

Lipid levels (% wet weight) in eggs were almost identical between fish from the Hudson and Miramichi rivers (2%), whereas the hepatic lipid level was almost twice as high in the Hudson River sample (14%) compared with the Miramichi River samples (8%). Levels of all lipid-normalized hepatic and egg PCDD, PCDF, and PCB congeners were much higher in tomcod from the Hudson River than from the Miramichi River. For example, levels of congener 77 were 99- and 75-fold higher, respectively, in the eggs and liver of Hudson River tomcod compared with Miramichi River tomcod. Levels of TCDD were 22- and 8-fold higher in the eggs and liver, respectively, of Hudson River tomcod than of Miramichi River tomcod.

Lipid normalization did alter the relationship between liver and egg concentrations of contaminants in fish from both rivers. Contaminant concentrations were higher in eggs than livers of Hudson River females and were of comparable concentrations in livers and eggs of Miramichi River females for congener 77 and total coplanar PCBs ($p > 0.08$, paired *t* tests), though TCDD and total PCDDs and PCDFs were still higher in liver than eggs of Miramichi River fish (paired *t* tests, $t = 5.679$, $p = 0.007$; and $t = 5.460$, $p = 0.005$, respectively). Contaminant concentrations in livers and eggs were still highly correlated ($r = 0.974$–0.985, $p < 0.001$, $n = 6$), but unlike with wet weight data, correlations for TCDD and total PCDDs and PCDFs were also detected within Miramichi River samples ($r = 0.933$, $p = 0.021$, $n = 5$; and $r = 0.908$, $p = 0.033$, $n = 5$, respectively).

DISCUSSION

In previous studies, we reported that hepatic levels of aromatic hydrocarbon contaminants, including PCDDs, PCDFs,

and coplanar PCB congeners that induce CYP1A1, were much higher in male and female adult tomcod from two sites in the Hudson River estuary (Hackensack River, NJ, USA, and Garrison, NY, USA) compared with fish from the cleaner Miramichi and Margaree rivers, Canada [2,16]. For example, the level of TCDD was 554 ng/kg wet weight in pooled livers of male tomcod from the Hackensack River compared with 1 ng/kg wet weight and 48 ng/kg wet weight, respectively, in fish from the Margaree (females) and Miramichi (males) rivers. Similarly, hepatic levels of PCBs were much higher in tomcod from both sites in the Hudson River estuary than in fish from the cleaner reference rivers. For example, hepatic levels of total CYP1A1-active non-*ortho* PCB congeners were 62- and 200-fold higher in male tomcod from Garrison than in fish from the Miramichi and Margaree rivers, respectively [2]. At the same time, we observed that hepatic CYP1A1 mRNA inducibility with halogenated aromatic hydrocarbons such as congener 77 or TCDD was significantly less in ip-injected tomcod from the Hudson River than in similarly treated tomcod from the Miramichi River [2,16]. In several experiments with tomcod depurated for periods up to 305 d, little or no evidence of CYP1A1 mRNA induction was observed in fish treated with TCDD or three different coplanar PCB congeners known to induce CYP1A1 in fish, including congener 77.

Based on these results, we suggested that CYP1A1 inducibility may be decreased in Hudson River tomcod by prior exposure to persistent aromatic hydrocarbons or other contaminants [16] and that impaired inducibility may be due to down-regulation of AhR pathway function [2]. Because of the role of CYP1A1 in the activation of procarcinogens to reactive electrophiles [1] and in the generation of DNA-damaging reactive oxygen species [6], it is conceivable that down-regulation of CYP1A1 provides a selective advantage to populations residing in highly contaminated ecosystems. In this regard, genetic polymorphisms in AhR that impact susceptibility to TCDD-induced toxicity have been reported in rodent models [33]. We observed genetic polymorphisms in the 3′ end of tomcod AhR and differences in the frequencies of AhR alleles among tomcod populations [9]; however, the functional significance of variant alleles has yet to be evaluated. Experimental evidence in rodent models indicates that exposure to aromatic hydrocarbons or other contaminants can physiologically regulate AhR function and subsequent CYP1A1 expression [21] or epigenetically silence CYP1A1 transcription [23]. Thus, it is possible that genetic, physiological, epigenetic, or a combination of these mechanisms may be operative to down-regulate CYP1A1 inducibility in impacted populations.

In this current study, using RT-PCR analysis, we found that basal levels of CYP1A1 mRNA expression in DMSO-exposed larval tomcod were uniformly low but always detectable in larvae from both the Hudson River and Miramichi River populations (Figs. 2 and 3). This permitted us to quantify fold induction of CYP1A1 expression in chemically exposed individual larva. Larvae from both populations exhibited significant CYP1A1 mRNA induction for both chemicals. Larvae from the Hudson (47-fold) and Miramichi rivers (52-fold) exhibited very similar levels of gene induction when exposed to 10 ppm B*a*P. Significant induction of CYP1A1 in PAH-treated larvae from both populations is consistent with hepatic CYP1A1 expression in adult fish from these two populations, although levels of induction in larvae were not as high as those observed in adult livers (>180-fold induction) [2].

Most importantly, significant CYP1A1 mRNA induction was observed in Hudson River tomcod larvae that were exposed to congener 77. The CYP1A1 induction was lower, but not significantly, in Hudson River (9-fold) than Miramichi River (22-fold) larvae treated with congener 77 (10 ppm). Induction of CYP1A1 expression in congener 77-exposed Hudson River larvae is contradictory to what we observed in livers of adult tomcod from the Hudson River that were ip injected with 10 mg/kg body weight of congener 77 [2,16]. These results are most consistent with the hypothesis that reduced inducibility of hepatic CYP1A1 mRNA in adult tomcod from the Hudson River treated with halogenated aromatic hydrocarbons is due to physiological acclimation or to epigenetic effects in adult fish because of chronic exposure to high levels of contaminants. This may result from increasing tissue loads of contaminants during growth of environmentally exposed fish or to variation in ontogenetic factors that regulate CYP1A1 expression during development.

Significantly lower concentrations of aromatic hydrocarbons in eggs (and presumably larvae) than in adult livers would suggest that a threshold concentration of active contaminants needs to be reached before modulation of hepatic CYP1A1 inducibility occurs. However, are levels of contaminants that induce CYP1A1 lower in eggs than in livers of tomcod from the Hudson River population? Hepatic levels of all measured aromatic hydrocarbon contaminants in female tomcod collected from the Hudson River at Garrison in January 1999 were high (Table 1) and very similar to those observed in adult tomcod collected in December 1997 from the same site [2]. Hepatic levels of these contaminants were significantly lower in Miramichi River than Hudson River tomcod. Overall concentrations of these contaminants expressed on a wet-weight basis were far lower in eggs than in their matched livers. For example, levels of TCDD, total PCDDs and PCDFs, and coplanar PCB congeners that induce CYP1A1 in the eggs of Miramichi River fish were 26 to 40% of those in matching livers. Levels of these contaminants in the eggs of Hudson River fish ranged between 17 and 28% of hepatic levels. These results support the contention that threshold tissue concentrations of these or other Hudson River-borne contaminants exist that modulate hepatic CYP1A1 inducibility in adults.

Several examples of physiological alteration of CYP1A1 inducibility can be found in the mammalian literature. In vitro and in vivo studies in rodent models have demonstrated significant reduction in AhR levels by TCDD treatment. For example, hepatic expression of AhR was reduced by 88% in Sprague-Dawley rats 8 h after administration of a single oral dose of 10 μg TCDD/kg body weight [21]. Reductions in AhR expression were also observed in lung, spleen, and thymus of TCDD-treated animals [21]. Effects of reduced AhR levels on CYP1A1 expression were also tested by in vitro exposure of mouse hepatoma cells (Hepa-1c1c7) and rat smooth muscle cells (A-7) to 1 nM TCDD for 12 d. This exposure resulted in significantly reduced inducibility of a transfected TCDD-inducible reporter gene construct [21]. Thus, depletion of cellular pools of AhR provides a physiological mechanism whereby CYP1A1 inducibility could be compromised in populations that are chronically exposed to very high levels of persistent contaminants.

But how does the dose of TCDD used in these in vivo experiments with rodents (10 μg TCDD/kg body wt) relate to actual tissue burdens of TCDD? In other studies, treatment of Sprague-Dawley rats and B6C3F1 mice with single oral doses of 10 μg TCDD/kg body weight resulted in hepatic tissue

burdens of 85 and 68 ng TCDD/g tissue, respectively [34]. Clearly, hepatic TCDD toxic equivalent quotients in tomcod from the most contaminated reaches of the Hudson River estuary do not approach those used to deplete in vivo AhR levels in TCDD-treated rats and are in fact approximately three orders of magnitude lower. Experimental studies with much lower levels of TCDD or other AhR agonists are needed to evaluate the likelihood of AhR depletion in chronically exposed populations.

Recent isolation and characterization of the murine AhR repressor (AhRR) provides an alternative mechanism whereby AhR function could be physiologically down-regulated [22]. The AhRR inhibits AhR function by competing with AhR for ARNT and binding to DRE sequences, thereby occluding access to DREs by AhR-ligand complexes. Because AhRR is inducible by 3-methylcholanthrene, it can be envisioned that chronic exposure to aromatic hydrocarbons in polluted environments could result in chronic induction of AhRR expression and down-regulation of AhR levels and function. The presence of AhRR in fish and its role in the environmental regulation of AhR function have yet to be addressed.

Epigenetic down-regulation of CYP1A1 or AhR transcription might also be occurring in chronically exposed populations. Studies have demonstrated that metals commonly found in polluted systems such as the Hudson River, including Ni, Cr, and As, can alter methylation levels at CpG sites in promoter sequences and thereby silence expression of a variety of inducible genes [35]. Furthermore, Shen and Whitlock [23] demonstrated that methylation of CpG dinucleotides in CYP1A1 DREs diminishes protein–DNA interactions and inhibits the enhancer function of the DNA and CYP1A1 induction by TCDD. Studies are needed to address the potential of these or other xenobiotics to methylate and silence inducible genes, such as CYP1A1, in environmentally exposed populations.

It is also possible that levels of aromatic hydrocarbons needed to induce or to inhibit CYP1A1 mRNA induction differ between larvae and adult tomcod liver. Wang et al. [36] recently examined expression and inducibility of CYP1A1, AhR, and ARNT in oocytes and developing embryos of zebrafish (*Danio rerio*) that were treated with graded doses of TCDD. Using RT-PCR analysis, these workers found that expression of AhR and ARNT were detectable in unfertilized eggs and in embryos shortly after fertilization. Using northern blot analysis, CYP1A1 mRNA was detectable at the same periods of development and levels of CYP1A1 expression were comparable with those observed in the livers of adult zebrafish treated with the equivalent doses of TCDD. These results seem to contradict the suggestion that larvae and adults respond differently to a given exposure of these contaminants.

Noninducibility of CYP1A1 protein and EROD activity has also been observed in killifish *Fundulus heteroclitus* from several estuaries along the Atlantic coast of the United States highly contaminated with PCBs [37], TCDD [38], and creosote (PAHs) [39]. This suggests that impaired inducibility of CYP1A1 is a common phenomenon in fish and perhaps other vertebrate populations from aromatic hydrocarbon-contaminated locales. These same populations also exhibit resistance to aromatic hydrocarbon-induced overt lethality in both embryos [37,40] and adults [40], suggesting a possible common underlying mechanistic basis for impaired CYP1A1 inducibility and resistance to overt toxicity. Because of the demonstrated role of AhR in activating CYP1A1 transcription and in mediating many toxic responses to these xenobiotics [13], AhR down-regulation may provide the mechanistic bases for these phenomena. Studies of the heritability of resistance in killifish from New Bedford Harbor, Massachusetts, USA (PCBs) [37] and the Elizabeth River, Virginia, USA (creosote) [39] suggest that resistance is transmitted to the F1 generation; however, the mechanistic bases of these observations are only beginning to be explored.

In total, these studies suggest that impaired inducibility of CYP1A1 and perhaps resistance to overt aromatic hydrocarbon-induced toxicity is a common phenomenon in fish and perhaps other vertebrate populations from highly contaminated sites. Initial studies suggest that several different mechanisms, both genetic and nongenetic, may be operative to decrease responsiveness of CYP1A1 inducibility and incur resistance in impacted populations. If AhR is down-regulated in impacted populations by chronic exposure to persistent pollutants, the use of CYP1A1 expression as a biomarker of exposure is compromised. Given the demonstrated importance of AhR expression in normal liver and immune system development in the absence of contaminants in AhR-deficient mice, it is not difficult to envision that perturbations to AhR function from chronic exposure may result in persistent damage to organism and population performance both in contaminated and perhaps in remediated ecosystems.

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