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Co-exposure to metals modulates CYP1A mRNA inducibility in Atlantic tomcod *Microgadus tomcod* from two populations

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Abstract

Populations from urbanized and industrialized sites are often exposed to mixtures of chemical contaminants including aromatic hydrocarbons (AHs) and heavy metals. The effects of mixtures of these contaminants on these populations are largely unknown. The Hudson River Estuary is highly contaminated with a variety of AHs including, PCBs and PAHs, and metals, and its population of Atlantic tomcod *Microgadus tomcod* bioaccumulates those which are persistent. The Hudson River's tomcod population exhibits resistance to persistent AHs as exemplified by significantly decreased inducibility of hepatic cytochrome P4501A (CYP1A) mRNA. We used hepatic CYP1A mRNA inducibility in tomcod from the Hudson River and a sensitive population to investigate the effects of acute co-exposure to metals on aryl hydrocarbon receptor (AHR)-mediated gene expression. Adult tomcod from the Hudson River and the cleaner Miramichi River were i.p. injected with one dose of benzo[*a*]pyrene (B[*a*]P) or coplanar PCB77 and graded doses of four metals, As, Cd, Cr, and Ni, and levels of hepatic CYP1A mRNA and protein were assayed. We observed no effects of metals treatment on basal levels of hepatic CYP1A mRNA expression, but all four metals significantly reduced CYP1A mRNA inducibility in tomcod from one or both populations. The magnitude of the inhibition of CYP1A mRNA inducibility differed among the metals and fish from the two populations. Also, the profile of the metals modulation of induced CYP1A mRNA showed differences that depended on the time after treatment of sacrifice. Our results demonstrate that co-exposure to several metals can impact inducible, but not basal levels of CYP1A expression and perhaps other toxicities mediated by the AHR.

Keywords: Co-exposure; CYPIA mRNA; Aromatic hydrocarbons; Metals

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1. Introduction

Fish populations inhabiting urban and industrialized estuaries are often exposed to high levels of complex mixtures of anthropogenic contaminants from early stages of development through adulthood. Sometimes tissue burdens of contaminants exceed those known to exert toxicities in naïve animals. Nonetheless, even in highly polluted locales, natural populations frequently thrive under conditions that would be harmful to conspecifics from more pristine ecosystems (Prince and Cooper, 1995a). Fish from wild populations chronically exposed to mixtures of pollutants sometimes develop resistance to the adverse effects of exposure to such xenobiotics (Wirgin and Waldman, 2004). Various mechanisms are proposed through which resistance to chemical pollutants can occur. Understanding these mechanisms provides insights in determining the likelihood of the persistence of resistant phenotypes after site remediation and about the potential biological cost to affected populations and perhaps communities and ecosytems.

Resistance may arise through genetic adaptations due to strong natural selection for phenotypes that are less sensitive to contaminant exposure. In this case, resistance will be transferred between generations and will usually, although not always (Levinton et al., 2003), persist even after site remediation. Alternatively, resistance may result from physiological acclimations and in this instance will cease after exposure. Adaptation may be viewed as a population level process—acclimation occurs at the individual level.

The Hudson River is among the most polluted estuaries in North America. A telling sign of its contamination is the existence of several U.S. federally designated Superfund sites including the Hudson River PCBs site which extends for almost 200 miles of the main stem river, the Marathon Battery Corp. site in Foundry Cove—Cold Spring, NY (deleted after remediation from the National Priority List (NPL)) for Cd and Ni, the Diamond Alkali site on the Passaic River, in Newark, NJ, for dioxin (TCDD), and several more for metals and other contaminants. In addition to these Superfund sites, the Hudson River ecosystem is also affected by the presence of other major polluters that discharge Mn, Ni, Cu, and Cr compounds into its waters (2001 Toxic Release Inventory).

Two populations of chemically resistant fishes have recently been described in the Hudson River Estuary, Atlantic killifish *Fundulus heteroclitus* (Prince and Cooper, 1995a, 1995b; Elskus et al., 1999) and Atlantic tomcod *Microgadus tomcod* (Wirgin et al., 1992; Courtenay et al., 1999). Fish from both populations exhibit significantly reduced inducibility of the cytochrome P4501A (CYP1A) gene and resistance to early life-stage toxicities by TCDD and TCDD-like PCBs. However, unlike resistant killifish from other locales, tomcod are only resistant to TCDD and coplanar PCBs, not polycyclic aromatic hydrocarbons (PAHs) despite the relatedness of these compounds and their common toxic activation through the aromatic hydrocarbon receptor (AHR) pathway.

The Atlantic tomcod population in the Hudson River has exhibited a checkered history of toxicant related perturbations. Over the past several decades, the overall abundance of spawning tomcod in the Hudson River has declined compared to historical levels (M. Mattson, Normandeau Associates, pers. commun.). In the early 1980s, hepatocellular carcinomas were commonly observed in tomcod from the Hudson River. Their prevalence was possibly among the highest ever observed in a wild population (Dey et al., 1993). Recently, a sharp decrease in the prevalence of gross hepatic lesions was observed in 2-year-old Hudson River tomcod (78% in 1983–1984 to 32% in 1995–1996, J. Young, Con Edison, pers. commun.). At the same time, the age structure of the Hudson River population was truncated compared to those from elsewhere. Its spawning aggregation was composed primarily of 1-year-old fish (97%; Dey et al., 1993), whereas in cleaner rivers spawning aggregations consisted of primarily 3- and 4-year-olds, and fish up to 7 years of age were observed. Recent years have witnessed a higher percentage of 2- and 3-year-old fish in the population (J. Young, Con Edison, pers. commun.).

Several mechanisms may be hypothesized to explain the lack of significant hepatic CYP1A mRNA inducibility in Hudson River tomcod exposed to halogenated aromatic hydrocarbons (HAHs). For example, genetic alteration(s) may have occurred in one or more key molecules in the AHR pathway or elsewhere that impact the structure or expression of key gene products that metabolize these compounds or transduce their signals through cellular networks. Indeed, genetic

polymorphisms have been observed in AHR in tomcod from the Hudson River that are not observed in tomcod from more sensitive populations (Roy and Wirgin, 1997; Yuan, 2003). Another possibility is that Hudson River tomcod may exhibit physiological acclimations impacting the bioavailability of these contaminants such as higher liver lipid content than tomcod from elsewhere (Cormier et al., 1989). Another hypothesis is that tomcod from the Hudson River are co-exposed to other contaminant(s) which counteract the effects of AHs. In this regard, metals are prime candidates for investigation for two reasons: (1) metals do co-occur with AHs in Hudson River biota, including tomcod, often at very high levels; and (2) several metals inhibit CYP1A expression and enzymatic activity, or some AH-related biological activities in other models.

In fish, Cd is the only metal shown to inhibit in vivo induction of CYP1A protein and enzymatic activity by AHs (Sandvik et al., 1997). Recently, several in vitro studies in fish (Bruschweiler et al., 1996; Risso-de Favernay et al., 2000a, 2000b; Ghosh et al., 2001) and mammalian cells (Tully et al., 2000; Maier et al., 2000) demonstrated that other metals (e.g., Cr, Pb, Cu, Zn, and As) modulate CYP1A expression. In a human lung adenocarcinoma cell line, co-exposure to arsenite attenuated B[*a*]P-induced cytotoxicity (Ho and Lee, 2002). Furthermore, clinically relevant concentrations of As_2O_3 inhibited 3-methylcholanthrene (3-MC)-induced CYP1A mRNA expression in primary human hepatocytes and in two human hepatoma cell lines. In contrast with the above studies, As was reported to increase the frequency of mutations induced by B[*a*]P at the HPRT locus (Maier et al., 2002), and in rats to increase pulmonary CYP1A mRNA, protein and activity (Seubert et al., 2002).

The overall goal of this study was to provide new information on the interactions between the biological effects of exposure to two classes of contaminants commonly present in industrial, urban, and natural environments: AHs and metals, and their consequences in exposed natural populations. The hypothesis proposed was that co-exposure to metals can modulate the biological effects of exposure to AHs and ultimately alter their toxicity. In addition, if this hypothesis is true, the co-occurrence of metals in the environment may contribute to the resistance to some of the effects of exposure to HAHs observed in the Atlantic tomcod population in the Hudson River.

2. Materials and methods

2.1. Collection and maintenance of fish

Unbaited boxtraps were used to collect tomcod from the Hudson River at Garrison, New York (NY) (River Kilometer 80) from mid-January to late February in 2001 and 2002. Smelt bag nets were used to collect adult tomcod from the Miramichi River, New Brunswick (NB), near Loggiville between December and February, 2001–2002. Hudson River fish were transferred within 1 h of collection to the NYU facility in Tuxedo, NY. Tomcod were housed at 4–6 °C (8:16 L:D photoperiod) in clean water at a salinity of 5 ppt. One-third of the volume of water was replaced weekly. Fish were maintained under the above conditions for 6–8 weeks for acclimation and depuration before treatment. After collection, Miramichi River tomcod were held temporarily at the aquarium facility of the Gulf Fisheries Centre, Moncton, NB, in 15 ppt seawater at 4 °C. After several weeks, fish were transported to the NYU facility in NY. Over a 10-day period, the salinity in the tank was gradually lowered to 5 ppt. All other holding conditions were identical to those for Hudson River fish.

2.2. Treatments

To determine if co-exposure to metals modulates the expression of genes induced by AHs, adult tomcod were i.p. injected with one AHR agonist (B[*a*]P or PCB77) and one metal salt ($CdCl_2$, $NiCl_2$, K_2CrO_4, and $NaAsO_2$), alone or in combination, as detailed below. AHR agonists were chosen to represent two different classes of AHs: B[*a*]P a prototypical PAH, and PCB77 a dioxin-like HAH.

Due to the resistance of Hudson River tomcod to PCBs, but not PAHs-induced CYP1A mRNA expression (Courtenay et al., 1999; Wirgin and Waldman, 2004), fish from this population were treated with B[*a*]P, whereas non-resistant fish from the Miramichi River were treated with PCB77. Groups of 4–9 adult Hudson River tomcod were i.p. injected in rapid sequence: (a) with B[*a*]P resuspended in corn oil (10 mg/kg) alone or with graded doses of; (b) $CdCl_2$ (2.5, 5, and 10 mg/kg), $NiCl_2$ (6.25, 12.5, 25, and 50 mg/kg), K_2CrO_4 (6.25, 12.5, and 25 mg/kg), or $NaAsO_2$ (1.25, 2.5, and 5 mg/kg) in $_{dd}H_2O$. The doses of metals tested were selected based on actual levels

measured in tissues of fish from the Hudson River (Wall et al., 1998). Negative (corn oil) and positive (B[*a*]P alone) control fish received an equal volume of $_{dd}H_2O$ as used for solvent in the metals exposed fish. In addition, groups of fish injected with corn oil and (a) $CdCl_2$ 0.5 and 10 mg/kg, (b) $NiCl_2$ 5 and 50 mg/kg, (c) K_2CrO_4 12.5 mg/kg, (d) $NaAsO_2$ 0.5 and 5 mg/kg were also included as negative controls. Fish from different treatment groups were housed in different tanks. All fish injected with B[*a*]P and all controls included in this set of experiments were sacrificed 2 days after treatment. CYP1A mRNA induction in B[*a*]P injected tomcod was previously determined to be maximum after this duration of exposure (Courtenay et al., 1999).

A similar experimental design tested the effects of co-exposure to these metals on PCB77 induced expression in tomcod from the Miramichi River. One notable exception was that PCB77 treated fish and related controls were sacrificed 7 days after injection because of the different kinetics of hepatic CYP1A mRNA induction between tomcod i.p. injected with B[*a*]P and PCB77 (Courtenay et al., 1999).

The effects of $NaAsO_2$ on the kinetics of CYP1A mRNA inducibility were investigated by i.p. injecting Hudson River tomcod with B[*a*]P (10 mg/kg) in combination with $NaAsO_2$ (2.5 mg/kg b.w.) and measuring hepatic CYP1A mRNA levels in groups of 4 fish each at different time-points (8, 48, and 120 h) after treatment. The effect of K_2CrO_4 on the kinetics of CYP1A mRNA induction by B[*a*]P (10 mg/kg) was determined in groups of 3–4 fish sacrificed 1, 2, 4, and 8 days after injections. In addition, one group of fish co-injected with B[*a*]P and K_2CrO_4, and one group injected with $NaAsO_2$ and corn oil received an additional injection of K_2CrO_4 (10 mg/kg) 4 days after the initial treatment and were sacrificed 1 week after the initial treatment. These exposure groups were included for the purpose of determining if a possible time-related decline in the metal-induced modulation of gene expression was due to a decreased bioavailability of metals.

Livers from all fish were excised immediately after sacrifice, flash-frozen, and stored at −70 °C until further use.

2.3. RNA extractions

Frozen sections from tomcod livers (approximately 10 mg) were homogenized in 500 μl of Ultraspec reagent (BIOTECX Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations with minor modifications (Yuan et al., 2001). RNA pellets were resuspended in 50 μl of DEPC-treated water and stored at −70 °C until further use.

2.4. Determination of RNA concentration and purity

The concentration and purity of total RNAs were determined by measuring absorbance at 260 and 280 nm with a Beckman DU650 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Integrity of all RNAs was verified by electrophoresis in northern gels. A 1 μg aliquot of total RNA from each sample was denatured and electrophoresed in 1% MOPS agarose gels (Fourney et al., 1988), stained with ethidium bromide (EtBr), and evaluated based on the integrity of the 18S and the 28S rRNA bands. Imaging of gels was performed using either a DS 34 Polaroid direct screen instant camera (Polaroid Corp., Waltham, MA) or a Kodak EDAS 290 (Kodak Scientific Imaging Systems, New Haven, CT). In the event that a RNA sample was degraded, it was re-isolated as described above.

2.5. Quantification of CYP1A mRNA expression

CYP1A mRNA expression levels were measured by slot-blot hybridizations. One microgram of denatured total RNA was applied directly to Nytran N nylon transfer membranes (Schlicher & Schuell, Keene, NH) using a 72-well Minifold II slot-blot manifold (Schlicher & Schuell) as described in Courtenay et al. (1999). Membranes were baked and were either hybridized immediately or stored in a vacuum dessicator until use.

Membranes were first washed in 1× SSC/0.1% SDS for 30 min at 65 °C, then pre-hybridized at 65 °C for 3 h, followed by overnight hybridization at 65 °C (Kreamer et al., 1991) using ^{32}P radiolabeled, beta naphthoflavone (β-NF) induced, full length, tomcod CYP1A cDNA probes (Roy et al., 1995). After hybridizations, membranes were washed two times in 6× SSC/0.1% SDS at room temperature followed by two 30 min washes each in 0.5× SSC/0.1% SDS. Membranes were partially dried and autoradiographed or exposed to phosphorimaging screens (Molecular

Dynamics, Sunnyvale, CA). Phosphorimaging screens were scanned using a Storm 860 Scanner (Molecular Dynamics, Sunnyvale, CA). Quantification of the optical density of the RNA bands was performed using ImageQuant™ for Macintosh ver. 1.2 build 039 (Molecular Dynamics). To ensure equal loading of samples, membranes were then stripped of the CYP1A probe by washing three times in boiling 0.1× SSPE/0.5% SDS. All membranes were then prehybridized, hybridized with 18S rat rRNA probes, pHRR118 (Chan et al., 1984), and quantified as described above for CYP1A.

2.6. Preparation of microsomes

Microsomes were prepared from approximately 100 mg of frozen livers from the same specimens from which CYP1A mRNA expression was measured. Liver samples were homogenized on ice in 2 ml of homogenization buffer (0.1 M sodium phosphate, 0.15 M KCl, 1 mM EDTA, 1 mM DTT, pH 7.4) containing 3% BSA and 1 mM phenylmethylsulfonyl fluoride (PMSF), centrifuged for 10 min at 13,800 × g at 4 °C, supernatants were retained and centrifuged at 28,000 × g for 2 h at 4 °C. Pellets were resuspended in homogenization buffer supplemented with 0.1 mM PMSF, centrifuged at 28,000 × g at 4 °C for 90 min. Pellets were resuspended in 200 μl of homogenization buffer. Protein concentration of each sample was estimated using Bio-Rad Protein Assay kits (BioRad Laboratories, Richmond, CA) according to the manufacturer's instructions.

2.7. Western blot analysis

Protein samples were resolved by electrophoresis in 10% SDS-polyacrylamide gels. An aliquot of 3× sample buffer (TRIS 625 mM, glycerol 50% (v/v), SDS 10%, BME 1% (v/v), and BPB 0.1% (w/v)) was added to each sample to a final dilution of 1×, samples were denatured for 5 min in boiling water, and then electrophoretically transferred to NitroBind 0.22 micron nitrocellulose membranes (Osmonics, Inc., Minnetonka, MN) overnight at 6 °C.

Membranes were stained with Ponceau dye to evaluate transfer and uniformity of loading. Membranes were rinsed 3× with 15 ml of $_{dd}H_2O$, washed 3× with 10 ml of TBST (TRIS 50 mM, NaCl 150 mM, and Tween 20 0.05%, v/v), and blocked with TBST containing BSA 3% (w/v) for 2 h. Membranes were incubated overnight at 6 °C in 7 ml of TBST supplemented with BSA 3% (w/v) and containing mouse anti-Atlantic cod (*Gadus morhua*) CYP1A monoclonal antibody NP-7 (final dilution 1:1000; Biosense Laboratories AS, Bergen, Norway). Membranes were washed with 10 ml of TBST 4× for 10 min each. Secondary antibody (anti-mouse IgG (H&L) AP conjugate (Promega Corp.)) was added at a final dilution of 1:3000 in 7 ml of TBST and incubated for 3 h. Secondary antibody was removed, membranes were washed 4× for 10 min each in TBST, and then 3× for 10 min each with TBS (TRIS 50 mM, NaCl 150 mM). Membranes were stained with 10 ml of Western Blue™ Stabilized Substrate for alkaline phosphatase (Promega Corp.) for 10 min and rinsed in $_{dd}H_2O$ for 5 min at room temperature. After digital acquisition of gel images, quantification of protein bands was performed with ImageQuant™ for Macintosh.

2.8. Statistical analyses

Statistical significance of differences in CYP1A mRNA expression levels was determined after normalization against the expression of the 18S rRNA by one-way ANOVA followed by Dunnett's t-test with Bonferroni correction to α of $P < 0.05$. Relationships between doses of the metals and CYP1A mRNA expression was tested by simple regression analysis. The Dunnett's t-test with Bonferroni correction was also used to evaluate differences among CYP1A protein expression levels.

A two-factor ANOVA was performed to determine if co-exposure to $NaAsO_2$ or K_2CrO_4 had an effect on the time course of B[a]P-induced hepatic CYP1A mRNA expression. When significant effects of either treatment, or time, or a combination of the two was significant, the results were broken down to individual factors and analyzed by one-factor ANOVA, followed, if necessary, by the Duncan's multiple comparisons test. The significance of differences in hepatic CYP1A mRNA expression between adult Hudson River tomcod acutely exposed to either B[a]P or co-exposed to B[a]P and $NaAsO_2$ or K_2CrO_4 at the same time point was determined by Student's t-test. ANOVA was performed using SuperANOVA x 1.11 6/20/91 from Abacus Concepts, Inc. (Berkeley, CA).

3. Results

3.1. Hepatic CYP1A mRNA expression

Basal levels of hepatic CYP1A mRNA expression were low, although detectable by slot blot hybridization of 1 μg of total RNA in corn oil-treated adult Hudson River and Miramichi River tomcod. In Hudson River fish i.p. injected with B[*a*]P (10 mg/kg), hepatic CYP1A mRNA expression was significantly induced six-fold compared to the vehicle-treated negative controls ($P<0.01$). Similarly, PCB77 (10 mg/kg) induced hepatic CYP1A mRNA expression four-fold in Miramichi River tomcod ($P<0.01$).

In Hudson River tomcod, co-treatment with 2.5, 5, and 10 mg/kg $CdCl_2$ suppressed hepatic CYP1A mRNA expression induced by 10 mg/kg B[*a*]P (Fig. 1). Injection of $CdCl_2$ alone (0.5 and 50 mg/kg) did not significantly alter basal levels of CYP1A mRNA expression in liver. At the doses of 2.5, 5, and 10 mg/kg, co-administration of $CdCl_2$ significantly decreased levels of hepatic CYP1A mRNA expression induced by B[*a*]P by 93.1, 75.6, and 100.3%, respectively ($P<0.05$ versus B[*a*]P alone). Regression analysis showed significant reduction in B[*a*]P-induced CYP1A mRNA with increasing dose of $CdCl_2$ ($r^2=0.310$; $P<0.01$). Due to high inter-individual variability, results from co-exposure to 1.25 mg/kg $CdCl_2$ and B[*a*]P were inconclusive and consequently excluded from any further analysis.

In Hudson River tomcod co-exposed to $NiCl_2$ and 10 mg/kg B[*a*]P, the mean levels of hepatic CYP1A mRNA expression were decreased by 7.6, 44.8, 74.8 and 99.0% at 6.25, 12.5, 25, and 50 mg/kg $NiCl_2$, respectively, compared to mean expression in fish treated with 10 mg/kg of B[*a*]P alone (Fig. 2). Regression analysis showed significant reduction between B[*a*]P induced CYP1A mRNA induction and dose of $NiCl_2$ used ($r^2=0.227$; $P=0.0104$). Administration of $NiCl_2$ alone (5 and 50 mg/kg) did not modify levels of basal CYP1A mRNA expression.

Co-treatment with K_2CrO_4 inhibited B[*a*]P- and PCB77-induced hepatic CYP1A mRNA expression in Hudson River (Fig. 3) and Miramichi River (Fig. 4) tomcod, respectively. In Hudson River fish, co-treatment with K_2CrO_4 significantly decreased B[*a*]P-induced CYP1A expression by 85.3, 114.4, and 96.3% at 6.25, 12.5, and 25 mg/kg, respectively ($P<0.01$ versus B[*a*]P alone). In Miramichi River tomcod, a dose of 6.25 mg/kg K_2CrO_4 did not significantly modify

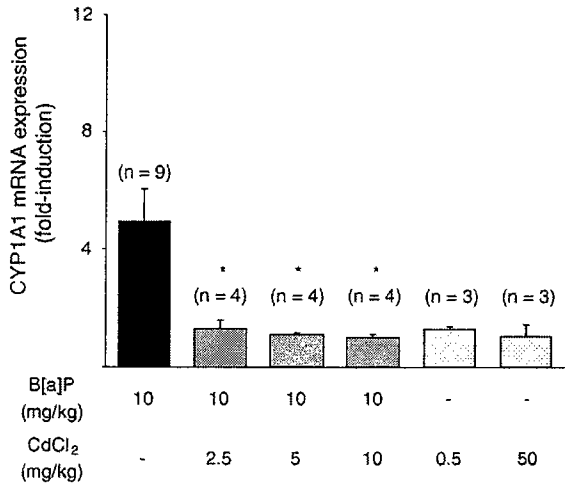

Fig. 1. Effects of graded doses of $CdCl_2$ on basal and B[*a*]P (10 mg/kg)-induced hepatic CYP1A mRNA expression in adult Hudson River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). Statistically significant differences vs. B[*a*]P alone are indicated by $^*P<0.05$. The number of individuals in each experimental group is indicated in parenthesis.

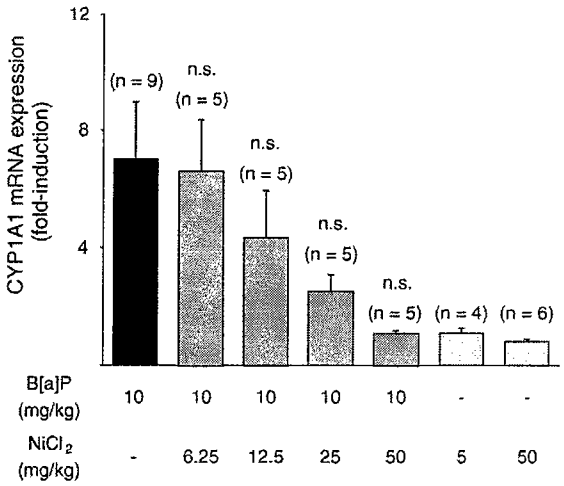

Fig. 2. Effects of grades doses of $NiCl_2$ on basal and B[*a*]P (10 mg/kg)-induced hepatic CYP1A mRNA expression in adult Hudson River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). n.s.: not significant vs. B[*a*]P alone. The number of individuals in each experimental group is indicated in parenthesis.

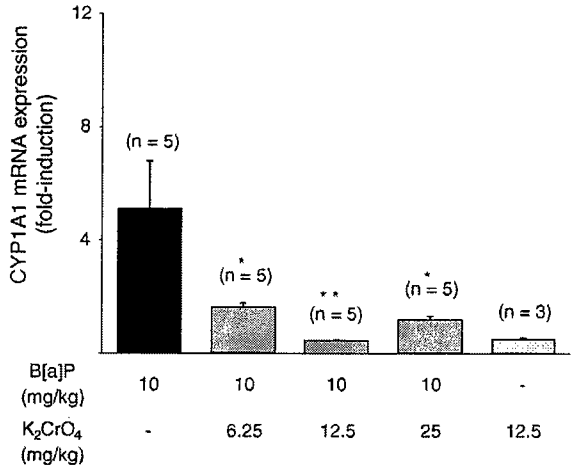

Fig. 3. Effects of graded doses of K_2CrO_4 on basal and B[*a*]P (10 mg/kg)-induced hepatic CYP1A mRNA expression in adult Hudson River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). Statistically significant differences vs. B[*a*]P alone are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. The number of individuals in each experimental group is indicated in parenthesis.

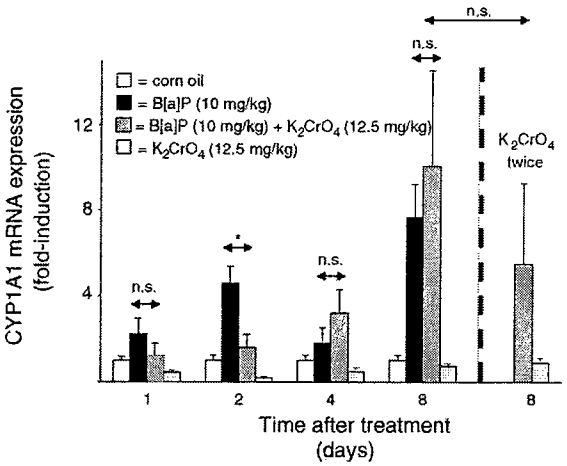

Fig. 5. Effects of single and repeated co-treatments with K_2CrO_4 (12.5 mg/kg) on basal and B[*a*]P-induced hepatic CYP1A mRNA expression in adult Hudson River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). In all groups, $n=3$–4. Student's *t*-test was used to compare, at each time point, groups exposed to 10 mg/kg B[*a*]P alone or in combination with 12.5 ppm K_2CrO_4. The same test was used to analyze the difference between single and repeated co-treatments with K_2CrO_4. Statistical significance of differences between groups are indicated by $^{*}P<0.05$; n.s.: not significant.

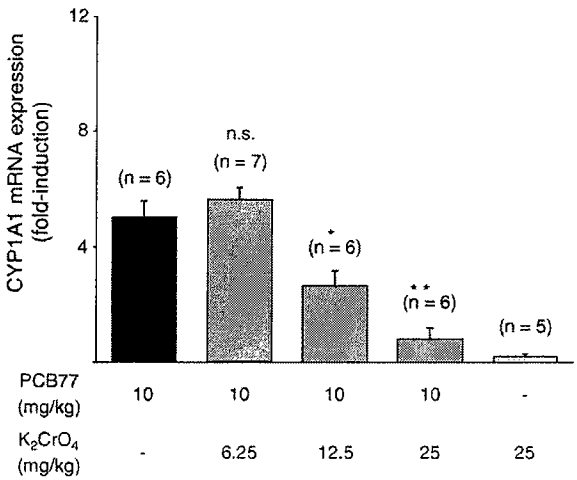

Fig. 4. Effects of graded doses of K_2CrO_4 on basal and PCB77 (10 mg/kg)-induced hepatic CYP1A mRNA expression in adult Miramichi River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). Statistically significant differences vs. PCB77 alone are indicated by $^{*}P<0.05$ and $^{**}P<0.01$; n.s.: not significant. The number of individuals in each experimental group is indicated in parenthesis.

PCB77-induced hepatic CYP1A expression (Fig. 4). However, at the doses of 12.5 and 25 mg/kg K_2CrO_4, CYP1A mRNA levels were 58.6% ($P<0.05$) and 104.7% ($P<0.01$) lower than those in fish treated with PCB77 alone, respectively. Regression analysis showed that the inhibition of PCB77-induced hepatic CYP1A mRNA expression was significantly related to dose of K_2CrO_4 ($r^2=0.598$; $P=0.0001$). Administration of K_2CrO_4 alone did not significantly alter basal CYP1A mRNA expression.

The time-course of hepatic CYP1A mRNA expression induced by B[*a*]P (10 mg/kg) showed that maximal mRNA levels were reached after 48 h and remained at similar levels till 8 days post-injection, the entire duration of the experiment (Fig. 5). Although fluctuations in levels of gene expression were observed at the 2-, 4-, and 8-day time-points, one-way ANOVA showed that these differences were not statistically significant ($P>0.05$). Consistent with the dose–response data, co-exposure to B[*a*]P and K_2CrO_4 (12.5 mg/kg) at 48 h resulted in hepatic CYP1A mRNA levels that were significantly lower than those measured in fish

injected with the same dose of B[*a*]P alone (−65.8% versus B[*a*]P alone, Student's *t*-test, $P < 0.05$).

At 4 and 8 days after treatments, the levels of hepatic CYP1A mRNA expression in fish co-injected with B[*a*]P and K_2CrO_4 were higher than the corresponding B[*a*]P-treated controls by 75.6 and 31.4%, respectively. However, these differences were not statistically significant compared to the corresponding time-matched positive controls treated with B[*a*]P alone. Levels of hepatic CYP1A mRNA expression in the group that received B[*a*]P and two injections of K_2CrO_4 (at 0 and 4 days) were lower than those in fish that received either B[*a*]P alone or B[*a*]P and one injection of K_2CrO_4, although these differences were not significant.

Co-exposure to $NaAsO_2$ did not significantly inhibit B[*a*]P-induced hepatic CYP1A mRNA expression in Hudson River (Fig. 6) or Miramichi River tomcod (Fig. 7). Although levels of hepatic CYP1A mRNA expression were 64.4, 69.5, and 85.6% lower in Hudson River tomcod co-treated with 1.25, 2.5, and 5 mg/kg $NaAsO_2$ than in fish that received 10 mg/kg of B[*a*]P alone, these differences were not statistically significant ($P > 0.05$). Nonetheless, simple regression analysis indicated a negative relationship between the levels of CYP1A mRNA expression and the dose

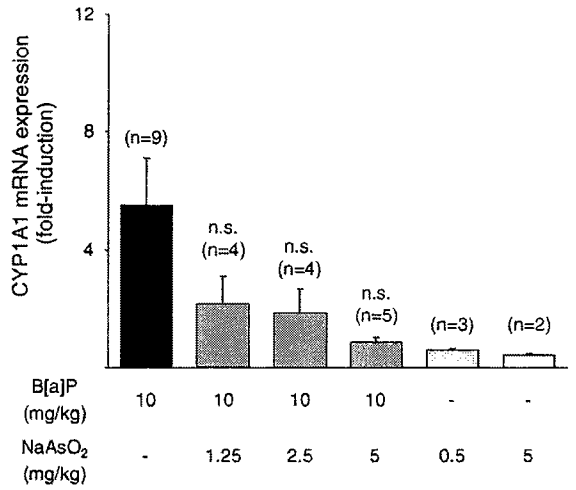

Fig. 6. Effects of graded doses of $NaAsO_2$ on basal and B[*a*]P-induced hepatic CYP1A mRNA expression in adult Hudson River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). n.s.: not significant vs. B[*a*]P alone. The number of individuals in each experimental group is indicated in parenthesis.

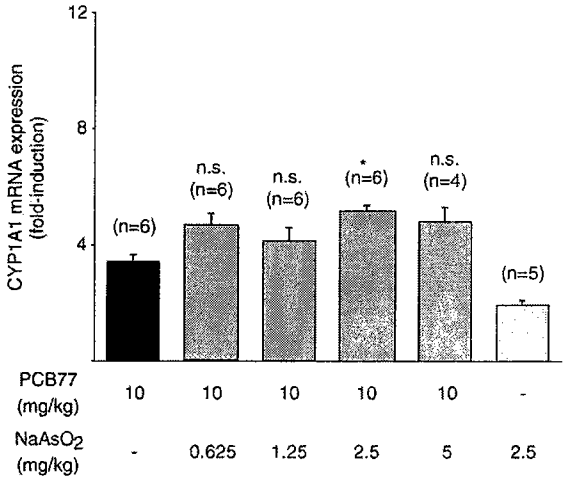

Fig. 7. Effects of $NaAsO_2$ on basal and PCB77-induced hepatic CYP1A mRNA expression in adult Miramichi River tomcod. Results are reported as mean (+S.E.M.) fold-induction compared to negative controls (corn oil). Statistically significant differences vs. PCB77 alone are indicated by $^*P < 0.05$; n.s.: not significant. The number of individuals in each experimental group is indicated in parenthesis.

of $NaAsO_2$ in co-exposed Hudson River tomcod ($r^2 = 0.241$; $P < 0.05$).

In Miramichi River tomcod (Fig. 7), PCB 77-induced hepatic CYP1A mRNA expression was not significantly altered by co-treatment with any of the doses of $NaAsO_2$ except 2.5 mg/kg. At this dose, levels of hepatic CYP1A mRNA expression were significantly higher ($P < 0.05$) than in B[*a*]P-treated controls. Simple regression analysis did not show any relationship between $NaAsO_2$ dose and PCB77-induced hepatic CYP1A mRNA expression ($r^2 = 0.127$; $P > 0.05$).

3.2. *Kinetics of $NaAsO_2$ modulation of B[a]P induction of CYP1A*

Consistent with previous results in Hudson River tomcod i.p. injected with B[*a*]P (10 mg/kg) (Courtenay et al., 1999), hepatic CYP1A mRNA induction expression was low (3.1-fold induced) at 8 h, was highest (10.2-fold) at 48 h after treatment, and was still elevated (8.2-fold) after 120 h (Fig. 8). In fish co-treated with 2.5 mg/kg $NaAsO_2$ and B[*a*]P, CYP1A mRNA expression was 0.8-, 2.0-, and 13.4-fold induced at 8, 48, and 120 h, respectively. Hepatic CYP1A mRNA expression induced by B[*a*]P alone was significantly

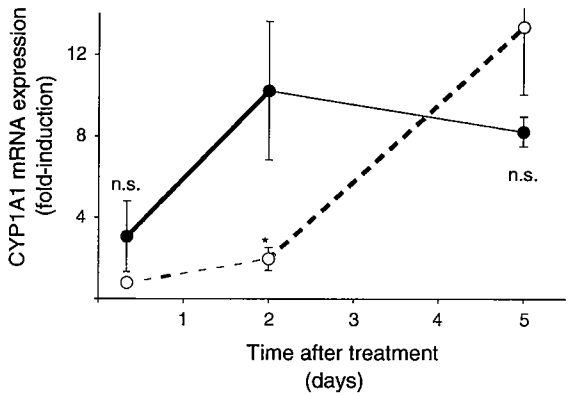

Fig. 8. Effects of co-treatment with 2.5 mg/kg $NaAsO_2$ on the kinetics of hepatic CYP1A mRNA expression induced by 10 mg/kg B[*a*]P in adult Hudson River tomcod. Solid line with solid circles represents fish treated with 10 mg/kg B[*a*]P alone. Broken line with empty circles represents fish co-treated with 10 mg/kg B[*a*]P and 2.5 mg/kg $NaAsO_2$. Results are reported as mean (±S.E.M.) fold-induction vs. negative controls (corn oil). At each time point, statistical significance of differences between groups is indicated by $^*P<0.05$; n.s.: not significant. In all groups, $n=4$.

higher at 48 and 120 h compared to 8 h ($P<0.05$). Further, hepatic CYP1A mRNA expression in Hudson River tomcod treated with a combination of B[*a*]P and $NaAsO_2$, was significantly higher at 120 h than at 8 or 48 h ($P<0.01$). Between treatments (B[*a*]P + $NaAsO_2$ versus B[*a*]P alone), the only significant difference occurred at 48 h ($P<0.05$).

3.3. Hepatic CYP1A protein expression

For the detection of CYP1A protein, the NP-7 mouse monoclonal antibody directed against Atlantic cod CYP1A was used. In microsomal extracts from livers of adult tomcod, the antibody specifically detected one single band corresponding to an inducible protein with an expected molecular weight of approximately 65,000 Da. Accordingly, the NP-7 antibody showed specific cross-reactivity to CYP1A of Hudson River tomcod and could reliably be used to study the effects of metals on B[*a*]P-induced CYP1A protein expression. As expected, in the liver of corn oil-treated and 12.5 mg/kg K_2CrO_4-treated Hudson River tomcod, CYP1A protein expression was low, but detectable. Injections of B[*a*]P (10 mg/kg b.w.) significantly induced CYP1A protein expression levels in livers of adult Hudson River tomcod (four-fold increase; $P<0.01$). Consistent with the CYP1A mRNA data, co-treatment with B[*a*]P (10 mg/kg b.w.) and K_2CrO_4 (12.5 mg/kg b.w.) resulted in lower hepatic CYP1A protein levels compared to tomcod treated with B[*a*]P alone (−45.2%; $P<0.05$).

4. Discussion

4.1. Hepatic CYP1A inducibility is modulated by co-exposure to metals in tomcod

For the first time, we have demonstrated that in vivo co-exposure of a fish to metals can modulate CYP1A inducibility at the transcriptional level. In addition our results suggest that the magnitude of this effect may differ among populations. The doses of $CdCl_2$ (0.1–20 ppm), $NiCl_2$ (0.5–100 ppm), K_2CrO_4 (0.2–40 ppm), and $NaAsO_2$ (0.1–20 ppm) used for acute treatments of tomcod in this study were environmentally relevant because they were based on actual tissue concentrations of these metals in composite samples of whole fish (although not tomcod) from the Hudson River (Wall et al., 1998) and other less contaminated locales (Alam et al., 2000; Cohen et al., 2001). Acute co-exposure to environmentally relevant doses of Ni and Cd significantly inhibited B[*a*]P-induced hepatic CYP1A mRNA expression in tomcod from the Hudson River. Acute co-exposure to K_2CrO_4 significantly inhibited both B[*a*]P- and PCB77-induced hepatic CYP1A mRNA expression in adult tomcod from the Hudson River and Miramichi River, respectively. Effects of acute co-exposure of tomcod to K_2CrO_4 and B[*a*]P on hepatic CYP1A expression were confirmed at the protein level. CYP1A protein was induced, as expected in Hudson River tomcod exposed to B[*a*]P alone, and induction was inhibited by co-treatment with 12.5 mg/kg K_2CrO_4.

Arsenite did not significantly inhibit hepatic CYP1A mRNA expression induced by B[*a*]P in Hudson River fish, nor PCB77 in Miramichi River tomcod. However, the levels of CYP1A expression were lower in Hudson River tomcod co-exposed to B[*a*]P and arsenite than in tomcod that were treated just with B[*a*]P, and there was a dose-responsive relationship between dose of $NaAsO_2$ and decrease in levels of hepatic CYP1A

mRNA expression. In addition, 48 h after treatment, a comparison of hepatic CYP1A mRNA levels between adult Hudson River tomcod treated with 10 mg/kg B[*a*]P alone and a combination of 10 mg/kg B[*a*]P and 2.5 mg/kg $NaAsO_2$ showed a statistically significant difference. These observations support the hypothesis that co-occurrence of several metals in the environment can modulate some of the biological effects of exposure to AHs by altering, at the transcriptional level, the inducibility of CYP1A and, possibly, other genes whose expression is activated by the AHR pathway. In addition, these results suggest the possibility that the Hudson River and Miramichi River tomcod populations differ in their sensitivities to the modulatory effects of co-exposure to Cr VI and possibly arsenite on hepatic CYP1A mRNA inducibility by AHs.

4.2. Modulation of AH-induced gene expression by metals in mammalian models

The inhibitory effects in tomcod of co-exposure to $CdCl_2$, $NiCl_2$, K_2CrO_4, and, in part $NaAsO_2$, on AH-induced CYP1A transcription were consistent with most of the results in the mammalian literature. In primary rat hepatocytes, incubation with arsenite (5 μM) reduced 3-MC induced CYP1A protein and enzymatic activity, and also decreased CYP1A mRNA inducibility, although to a lesser extent: −55% for CYP1A protein and ethoxyresorufin *O*-deethylase (EROD) activity and −25% for mRNA expression levels (Jacobs et al., 1999). The ability of co-exposure to arsenite to inhibit CYP1A inducibility observed in Hudson River tomcod was also observed in human hepatoma Hep3B cells in which CYP1A induction by 3-MC, B[*a*]P, and TCDD was inhibited by As_2O_3 at the transcriptional level. In that study, the effects of arsenite were abrogated by incubation of the cells with *N*-acetylcysteine which blocked arsenic uptake and increased cellular efflux through multidrug-resistance associated proteins (Vernhet et al., 2003). In agreement with our results, in mouse Hepa-1 cells, Cr VI (50 μM) significantly inhibited CYP1A and nicotinamide adenine dinucleotide phosphate-quinoneoxidoreductase (Nqo-1) mRNA expression induced by TCDD (Maier et al., 2000). However, Cd and arsenic did not alter CYP1A mRNA levels, but instead increased TCDD-induced levels of Nqo-1. This contrasts with the inhibitory effect of Cd on hepatic CYP1A mRNA inducibility in Hudson River tomcod, but is consistent with the lack of As-induced inhibition observed in Miramichi River tomcod.

One of the mechanisms often associated with the toxic effects of exposure to metals is the generation of oxidative stress. In this regard, changes in redox conditions modulated DNA-binding of purified AHR complex in vitro (Ireland et al., 1995) and in intact cells (Xu et al., 1998). In addition, CYP1A1 transcription mediated by dioxin response elements (DREs) in a reporter gene assay was suppressed by H_2O_2 (Xu and Pasco, 1998). Maier et al. (2000) dismissed oxidative stress, interference with the binding of AHR complex to DREs and alteration of mRNA stability as possible mechanisms of the inhibitory activity of Cr instead suggesting a direct interaction of Cr with DNA or with DNA-binding proteins, causing disruption of normal chromatin architecture. Of particular mechanistic interest is a recent report that confirms the inhibitory effect of Cr VI on AHR-dependent in vitro gene expression and indicates that this effect is likely caused by alterations in histone acetylation (Wei et al., 2004). Incubation with sodium butyrate, a histone deacetylase inhibitor, reversed the inhibitory effect of Cr VI on B[*a*]P induced expression of a luciferase reporter gene driven by the mouse CYP1A gene promoter stably integrated in mouse hepatoma Hepa-1 cells. This suggests that Cr VI interacts with transcriptionally poised chromatin and blocks some features of initiation and/or progression of transcription from inducible promoters. Furthermore, Feng et al. (2003) report that Cr VI forms binary and ternary Cr–DNA adducts and Cr–amino acid adducts, the presence of which increase the likelihood of PAH metabolites adducting DNA at mutational hotspots.

4.3. Modulation by metals of induced CYP1A expression in fish models

Our results at the transcriptional level in tomcod were consistent with most previous in vitro and in vivo studies in fish at the translational level. For example, in black sea bass (*Centropristis striata*), i.p. co-treatment with 1.4 mg/kg $CdCl_2$ significantly decreased microsomal hydroxylase activity induced by 0.075–7.5 mg/kg B[*a*]P (Fair, 1986). Similarly, in European flounder (*Platichtys flesus* L.), s.c. injection of 1 mg/kg $CdCl_2$

decreased hepatic levels of CYP1A protein and EROD activity induced by B[*a*]P or PCB156 (Sandvik et al., 1997). In primary hepatocytes from black seabream (*Spondyliosoma cantharus*) and rainbow trout, co-exposures to cadmium, copper, zinc, and lead inhibited CYP1A mRNA, CYP1A protein expression, and EROD activity induced by 3-MC (Risso-de Faverney et al., 1999, 2000a). Furthermore, the effects of co-exposure to these metals on 3-MC induction of CYP1A were reversed by the nitroxide stable radical TEMPO, a cell-permeable agent possessing antioxidant properties (Risso-de Faverney et al., 2000b) suggesting that TEMPO might have protected by scavenging free radicals and prevented their inhibitory effects on CYP1A induction. Furthermore, treatment of the *Poeciliopsis* hepatoma cell line (PLHC-1) with $CdCl_2$, $Co(NO_3)_2$, $CuSO_4$, $Ni(NO_3)_2$, $Pb(NO_3)_2$, or $ZnCl_2$ significantly decreased 3-MC induced CYP1A protein expression and EROD activity (Bruschweiler et al., 1996).

4.4. Effects of metals on basal levels of hepatic CYP1A expression

Unlike in mammals, constitutive CYP1A mRNA expression can usually be detected by hybridization techniques in fish liver including Atlantic tomcod. This permitted a determination of the effects of metals on basal levels of gene expression. We saw no significant effect of any of the four metals on basal levels of hepatic CYP1A mRNA expression in tomcod from either population. Ueng et al. (1996) also reported a lack of effect of metals on basal expression of CYP1A in male tilapia hybrids (*Oreochromis niloticus* female × *O. aureus* male). They observed that i.p. injection of $CdCl_2$ did not alter basal CYP, B[*a*]P hydroxylase, or EROD, or ECOD activities measured after 24 h in liver microsomes (Ueng et al., 1996).

However, our results at the transcriptional level differ with most earlier studies in fish, albeit at the protein or activity levels. For example, in rainbow trout, in vivo basal levels of ECOD and EROD activities decreased in liver and kidney 4 days after the first of two injections of $CdCl_2$ (Förlin et al., 1986). In plaice (*Pleuronectes platessa*), a single i.p. injection of $CdCl_2$ (0.1–1 mg/kg) decreased uninduced EROD activity probably due to a decrease in CYP1A protein levels (George, 1989).

Other studies showed an increase in basal and/or induced levels of CYP1A protein or enzymatic activities following exposure to metals. For example, waterborne pre-exposure to $CdNO_3$ for 25 days increased the effects of B[*a*]P on cytochrome P450 both at the protein level and at the enzymatic activity (EROD) level (Lemaire-Gony and Lemaire, 1992). However, exposure to $CdNO_3$ did not alter basal levels of expression. Also, in juvenile European sea bass (*Dicentrarchus labrax*), 15-day exposure to waterborne cadmium increased both basal and B[*a*]P-induced EROD activity in liver (Lemaire-Gony et al., 1995). Also, Tully et al. (2000) used the human hepatoma HepG2 cells to screen the ability of As V, Pb II, Cd II, Cr III, and Cr VI to transcriptionally activate various signal transduction pathways. In summary, they concluded that all of the metals tested could induce rather than inhibit the expression of genes in the AHR battery and others above the basal level.

4.5. Differences between populations in their sensitivities to metals

Although CYP1A inducibility was modulated by metals in tomcod from the two populations, the specifics and magnitude of the effect varied between the two locales. Part of the variation between populations almost certainly resulted from the different AH inducers used and the different times after treatment that the fish were sacrificed. For both populations, fish were sacrificed at times at which maximal induction was previously quantified in livers from adult tomcod for that AH (B[*a*]P: 2 days, PCB77: 7 days) (Courtenay et al., 1999). But because the metals are not as persistent as lipophilic AHs such as PCBs, their concentrations within the livers of AH treated fish probably decreased over time, reducing their effects over the longer exposure period in PCB77 than in B[*a*]P treated fish.

There is a large inter-species variability in the magnitude and kinetics of bioaccumulation of Cr in fish. These characteristics have not been tested in tomcod. Two studies with rainbow trout found whole body bioaccumulation factors of 1.3 and 3 (Fromm and Stokes, 1962; Calamari et al., 1982) and the elimination rate appeared to be biphasic, with 66% of total Cr eliminated in the first 24 h and the remaining with a half-life of 26 days (Tenholder et al., 1978). In contrast, the levels of Cr in the muscle and in the

liver of brown bullhead (*Ictalurus nebulosus*) from the Schuylkill River, PA were, respectively, 1822 and 2622 times higher than in the river water (Eisler, 1986). High levels of Cr can be reached in the gills and in the liver of fish, such as bullhead that bioaccumulate Cr, perhaps resulting from their slow rate of elimination (Buhler et al., 1977; Avenant-Oldewage and Marx, 2000).

In general, As tends to bioaccumulate in fish species and marine mammals, with relatively high levels present in the liver (Sorensen et al., 1985; Kubota et al., 2001). However, there are species such as *Tilapia mossambica* and *Oryzia latipes* in which 90 and 70% of the accumulated whole body burden of arsenic were depurated within the first 24 h after exposure (Suhendrayatna et al., 2002a, 2002b). Thus, there appears to be large interspecific and perhaps tissue-specific variability in the extent of bioconcentration and rates of depuration of these two metals.

We showed that acute co-treatment with $NaAsO_2$ can inhibit hepatic CYP1A mRNA expression induced by B[*a*]P in Hudson River tomcod. Conversely, there was no evidence of inhibition of PCB77 induced CYP1A by co-treatment with $NaAsO_2$ in Miramichi River fish. In fact, in Miramichi River fish, co-treatment with PCB77 and $NaAsO_2$, may have induced slightly, but consistently higher levels of CYP1A mRNA expression than in fish treated with PCB77 alone. Similarly, in PCB77-treated Miramichi River tomcod, the lowest dose of K_2CrO_4 tested (6.25 mg/kg) had no effect on CYP1A inducibility, whereas the same dose significantly inhibited B[*a*]P-induced hepatic CYP1A mRNA expression in Hudson River tomcod.

4.6. Mechanistic basis of metals effects on CYP1A inducibility

We found that any of four metals usually significantly decreased inducibility of hepatic CYP1A mRNA in tomcod by either of two AH compounds in both populations. However, we observed no impact of any of the metals on basal levels of CYP1A mRNA expression in tomcod. These contrasting results may provide some initial insights into the mechanistics basis of how metals decrease CYP1A mRNA inducibility in this model. Basal and inducible expression of CYP1A mRNA may be under independent mechanisms of regulation. Inducible expression of CYP1A mRNA is mediated by binding of AHR to XRE enhancer elements in the 5′ promoter region of CYP1A and other genes in the AHR battery. In contrast, basal expression of CYP1A although in part regulated by AHR (Schmidt et al., 1996) is probably primarily regulated by basal transcriptional elements in the 5′ CYP1A promoter region such as a TATA box, a G box, and NFI/CTF sites (Jones and Whitlock, 1990; Piechock and Hines, 1998). We speculate that function of the AHR pathway, but not that of basal transcriptional elements is impacted by metals exposure. We can envision several steps in AHR mediated transcriptional activation that are potential targets to disruption by metals including, ligand binding, nuclear transformation of AHR, or binding of AHR to XREs. To our knowledge, the effects of metals on these processes have yet to be directly investigated in any model. However, it is known that CYP1A transcription is suppressed by oxidative stress (Morel and Barouki, 1998). Cys427 of the cofactor NFI/CTF is localized in the transactivation domain of this protein and is the critical sensor of oxidative status and can potentially modulate the functional synergism between NFI/CTF and AHR. NFI/CTF interacts with basal transcription factors, and appears to interact also with histones H1 and H3, destabilizing chromatin structure (Morel and Barouki, 2000).

4.7. Evidence of impacts of metals on higher level AH-induced effects

Modulation of induced CYP1A expression by metals should be considered when interpreting results from monitoring programs using this biomarker. Our results show that this effect can be detected at levels of metals that are in the range of those seen in environmentally exposed fish from contaminated sites. Because these modulatory effects of metals are likely mediated through AHR, is there evidence in the literature that metals impact higher level biological effects from exposure to AH contaminants? In this regard, we previously reported that co-exposure of tomcod embryos and larvae to chromium can modulate levels of binding of B[*a*]P metabolites to DNA (Sorrentino et al., 2004). Furthermore, Hu et al. (2004) demonstrated that co-exposure of Chinese hamster ovary (CHO) cells to Cr VI and BPDE (a major mutagenic metabolite of B[*a*]P) significantly increased cytotoxicity and levels of mutations at the *APRT* locus compared to BPDE alone. Because the cells were treated with a metabolite

of B[*a*]P, rather than the parent compound, this impact was not mediated by AHR and as the authors suggested was probably caused by inhibition of nucleotide excision repair by Cr VI. Whether an increase in DNA adducts or mutations will be seen in vivo in animals exposed to the parent B[*a*]P compound has yet to be addressed, although our results in this study suggest that may not be the case. In contrast, we observed that co-exposure to metals did not impact the severity or frequency of teratogenecity induced by B[*a*]P or PCB77 in early life stages of tomcod, but did impact time to hatching of embryos and survivorship (Sorrentino, 2004). While, co-exposure to metals almost certainly modulates many of the toxic effects of AH compounds, the extent of these changes, ramifications on the population level, and their mechanistic bases need further clarification.

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References

Alam, S.K., Brim, M.S., Carmody, G.A., Parauka, F.M., 2000. Concentrations of heavy and trace metals in muscle and blood of juvenile Gulf sturgeon (*Acipenser oxyrinchus desotoi*) from the Suwannee River, Florida. J. Environ. Sci. Health 35, 645–660.

Avenant-Oldewage, A., Marx, H.M., 2000. Bioaccumulation of chromium, copper, and iron in the organs and tissues of *Clarias gariepinus* in the Olifant River, Kruger National Park. Water SA 26, 569–582.

Bruschweiler, B.J., Wurgler, F.E., Fent, K., 1996. Inhibitory effects of heavy metals on cytochrome P4501A induction in permanent fish hepatoma cells. Arch. Environ. Contam. Toxicol. 31, 475–482.

Buhler, D.R., Stokes, R.M., Caldwell, R.S., 1977. Tissue accumulation and enzymatic effects of hexavalent chromium in rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 34, 15–19.

Calamari, D., Gaggino, G.F., Pacchetti, G., 1982. Toxicokinetics of low levels of Cd, Cr, Ni, and their mixtures in long-term treatment on *Salmo gairdneri* Rich. Chemosphere 11, 59–70.

Chan, Y.L., Gutell, R., Noller, H.F., Wool, I.G., 1984. The nucleotide-sequence of a rat 18 S ribosomal ribonucleic-acid gene and a proposal for the secondary structure of 18 S ribosomal ribonucleic-acid. J. Biol. Chem. 259, 224–230.

Cohen, T., Que Hee, S.S., Ambrose, R.F., 2001. Trace metals in fish and invertebrates of three California coastal wetlands. Mar. Pollut. Bull. 42, 224–232.

Cormier, S.M., Racine, R.N., Smith, C.E., Dey, W.P., Peck, T.H., 1989. Hepatocellular carcinoma and fatty infiltration in the Atlantic tomcod, *Microgadus tomcod* (Walbaum). J. Fish Dis. 12, 1051–1116.

Courtenay, S., Grunwald, C.M., Kreamer, G.-L., Fairchild, W.L., Arsenault, J.T., Ikonomou, M., Wirgin, I., 1999. A comparison of the dose and time response of CYP1A1 mRNA induction in chemically treated Atlantic tomcod from two populations. Aquat. Toxicol. 47, 43–69.

Dey, W.P., Peck, T.H., Smith, C.E., Kreamer, G.-L., 1993. Epizoology of hepatic neoplasia in Atlantic tomcod (*Microgadus tomcod*) from the Hudson River estuary. Can. J. Fish. Aquat. Sci. 50, 1897–1907.

Eisler, R., 1986. Chromium hazards to fish, wildlife, and invertebrates: a synoptic review. Biological Report 85 (1.6). Contaminant Hazard Reviews Report No. 6. U.S. Fish and Wildlife Service.

Elskus, A.A., Monosson, E., McElroy, A.E., Stegeman, E.E., Woltering, D.S., 1999. Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance? Aquat. Toxicol. 45, 99–113.

Fair, P.H., 1986. Interaction of benzo(a)pyrene and cadmium on GSH S transferase and benzo(a)pyrene hydroxylase in black sea bass *Centropristis striata*. Arch. Environ. Contam. Toxicol. 15, 257–263.

Feng, Z., Hu, W., Rom, W.N., Costa, M., Tang, M.-S., 2003. Chromium (VI) exposure enhances polycyclic aromatic hydrocarbon-DNA binding at the *P53* gene in human lung cells. Carcinogenesis 24, 771–778.

Förlin, L., Haux, C., Karlsson-Norrgren, L., Runn, P., Larsson, A., 1986. Biotransformation enzyme activities and histopathology in rainbow trout, *Salmo gairdneri*, treated with cadmium. Aquat. Toxicol. 8, 51–64.

Fourney, R.M., Miyakoshi, J., Day III, R.S., Paterson, M.C., 1988. Northern blotting: efficient RNA staining and transfer. Focus 10, 5–6.

Fromm, P.O., Stokes, R.M., 1962. Assimilation and metabolism of chromium by trout. J. Water Pollut. Con. F. 34, 1151–1155.

George, S.G., 1989. Cadmium effects on plaice liver xenobiotic and metal detoxification systems: dose–response. Aquat. Toxicol. 15, 303–310.

Ghosh, M.C., Ghosh, R., Ray, A.K., 2001. Impact of copper on the biomonitoring enzyme ethoxyresorufin-*o*-deethylase in cultured catfish hepatocytes. Environ. Res. 86, 167–173.

Ho, I.C., Lee, T.C., 2002. Arsenite pretreatment attenuates benzo[*a*]pyrene cytotoxicity in a human lung adenocarcinoma cell line by decreasing cyclooxygenase-2 levels. J. Toxicol. Environ. Health A 65, 245–263.

Hu, W., Feng, Z., Tang, M.S., 2004. Chromium (VI) enhances (±)-*anti*-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene-induced cytotoxicity and mutagenicity in mammalian