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A Comparison of Cytochrome P4501A (CYP1A) mRNA Inducibility in Four Species of Atlantic Coast Anadromous Fishes

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ABSTRACT: Quantification of levels of cytochrome P4501A (CYP1A) gene expression in sentinel species of fishes has been proposed as a management tool to evaluate contamination of aquatic systems. Based on preliminary studies, we hypothesized that differences in CYP1A mRNA inducibility among individuals, populations, or species might lead to spurious conclusions when using this approach in environmental monitoring programs. To address this possibility, we quantitated and compared CYP1A mRNA induction levels in four species of common Atlantic Coast estuarine fish: smooth flounder, hogchoker, striped bass, and Atlantic tomcod, which were treated with model chemicals (beta naphthoflavone (β-NF), or benzo[a]pyrene at 10 ppm) known to induce CYP1A mRNA, or were exposed to contaminated environments. Species-specific CYP1A DNA probes were generated from PCR (polymerase chain reaction) amplification of genomic DNA using conserved oligonucleotide primers, and, along with cloned rainbow trout and Atlantic tomcod CYP1A cDNA probes were used to quantify CYP1A mRNA levels in northern blot analyses. Successful PCR amplification of CYP1A hybridizable DNA fragments was observed for all four species. Results from northern blot analyses showed large differences in CYP1A mRNA induction among species; only Atlantic tomcod exhibited significant induction of CYP1A mRNA for both chemically treated (97-fold) and environmentally exposed fish (34-fold). Significant, although lower, levels of induction were observed in β-NF treated (14-fold) smooth flounder, but not in environmentally exposed smooth flounder. Only low levels (not significant) of CYP1A gene induction were detected in hogchokers and striped bass. We conclude that CYP1A mRNA inducibility differed significantly among fish taxa perhaps due to differences in regulation of gene expression, suggesting that careful selection of sentinel species should be exercised prior to the use of CYP1A mRNA induction in environmental monitoring programs. However, the significance of differences in CYP1A mRNA inducibility in relation to higher level biological endpoints has yet to be determined.

Introduction

Historically, the Hudson River supported important commercial fisheries for anadromous fishes such as striped bass (*Morone saxatilis*), American shad (*Alosa sapidissima*), and Atlantic sturgeon (*Acipenser oxyrinchus*), and it also provides a nursery area for these and other estuarine species (Smith 1985). In proximity to vast northeastern United States urban centers, it serves occasionally as a domestic water supply and is used widely for recreational activities. For the past half century, the Hudson River has received anthropogenic inputs that are of environmental concern from atmospheric release, domestic waste, and industrial point sources. Organic xenobiotics discharged into the river include polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins, and dibenzofurans (Squibb et al. 1991). Sediment concentrations of these xenobiotics at selected sites within the Hudson River estuary are among the highest in estuaries within the United States (National Status and Trends Program 1988). Bioaccumulation of these pollutants has also been documented in organisms at various trophic levels within the Hudson River ecosystem (Pruell et al. 1990; Rappe et al. 1991). Adverse consequences from these inputs probably impact populations of aquatic organisms and human user groups. For example, populations of American oysters, at one time abundant in the estuary, are now severely depleted (Mackenzie 1992), Atlantic tomcod exhibit an unusually high prevalence of liver tumors (Dey et al. 1993), and fisheries for some resident and migratory species within the river have been closed or limited due to tissue contaminant levels that exceed FDA guidelines (Armstrong and Sloan 1988). For migratory species such as striped bass, which

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use the Hudson River estuary as spawning and nursery areas, elevated tissue levels of contaminants have occasionally resulted in the closure of nearby coastal fisheries (Fabrizio et al. 1991). Methods are needed to document quantitatively the temporal trends in xenobiotic bioavailability within the estuary and to evaluate the biological effects of these agents on key species, aquatic food chains, and human populations.

Traditionally, sediment concentrations of xenobiotics have been used to evaluate contaminant exposure; however, this approach does not provide information on bioavailability to target organisms. Quantification of tissue residues of xenobiotics is not informative as to whether the agent induces a biological response and does not provide a temporal window of exposure. For example, many halogenated aromatic hydrocarbons (HAHs) are resistant to metabolic transformation based on the degree and position of halogen substitution and P450 isozyme patterns and levels in target organs (Safe 1992); therefore, tissue loads may reflect long-term accumulation. At the opposite extreme, PAHs are metabolized so rapidly in fishes that it is impossible to directly measure tissue concentrations (Varanasi et al. 1987).

The use of biomarkers in sentinel species has been proposed as an alternative means to evaluate exposure to xenobiotics and their biological effects. Biomarkers have been developed and evaluated which identify and quantitate endpoints on different levels of biological organization: the molecular, cellular, tissue, organismic, population, or community (Huggett et al. 1992). Molecular biomarkers may offer the advantage of there being few events between exposure and response, and as a result, modulation of the biomarker response is minimized (Haasch et al. 1993). Thus, the molecular biomarker response may most accurately reflect the exposure experience. Molecular biomarkers also offer the promise of sensitivity, dose-responsiveness, and cost effectiveness.

An initial consideration in the implementation of a biomarker approach is the choice of target species. Selection of the sentinel species to be used in estuarine systems such as the Hudson River should be based on several considerations, including abundance, residence time within the estuary, position in the water column, foraging strategy, position in the food chain, physiology, commercial or recreational importance, and demonstrated sensitivity to environmental perturbation. Based on preliminary studies, we suspect that genetic differences among individuals or higher taxonomic units may limit or accentuate the response potential (Courtenay et al. 1994).

Levels of expression of environmentally responsive genes have been used as molecular biomarkers to quantitate exposure to xenobiotics (Stegeman et al. 1992; Stegeman and Hahn 1994). Exposure to selected xenobiotics has been shown to increase (induce) expression of these genes and that expression may be accurately quantified at the pretranslational (mRNA) or translational levels (protein concentration or enzyme activity). One of the most frequently used and best characterized molecular biomarkers in aquatic vertebrates is induction of cytochrome P4501A (CYP1A), which encodes for monooxygenase enzymes (Stegeman and Lech 1991; Goksøyr and Förlin 1992). CYP1A is induced in fish from exposure to exogenous substrates, such as PAHs, some PCBs, dioxins, and dibenzofurans; however, levels of gene expression may be modulated by endogenous factors such as steroid hormones. CYP1A enzyme activity is beneficial in that it converts xenobiotics to polar forms, which may be conjugated and excreted from the body (Stegeman and Hahn 1994). However, CYP1A enzyme activity also results in the metabolic conversion of environmental procarcinogenic PAHs to forms which covalently bind to cellular macromolecules, such as proteins and DNA, and form adducts (Stegeman and Lech 1991). It is believed that the generation of DNA adducts at carcinogenically critical genes, such as cellular oncogenes and tumor suppressor genes, is a necessary step in the initiation of chemical carcinogenesis (McMahon 1994). Furthermore, CYP1A-mediated metabolites of some HAHs may be required for selection and clonal expansion of previously initiated cancer cells (Safe 1992; Maronpot et al. 1993).

Induction of CYP1A gene expression by xenobiotics is mediated by activation of the aromatic hydrocarbon receptor (AhR) pathway. Complexing of the xenobiotic agent (ligand) to the AhR molecule in the cytoplasm initiates a cascade of cellular events, including dissociation of the AhR molecule from heat shock protein 90 (hsp90), translocation of the AhR-ligand complex to the nucleus, association of the AhR complex with the aromatic hydrocarbon nuclear translocation factor (ARNT), and induction of CYP1A transcription by binding of the AhR-ligand complex to enhancer elements (AhREs) upstream of the CYP1A gene (Stegeman and Hahn 1994). Presence of an AhR-like protein has been confirmed in several species of elasmobranch and teleost fish (Hahn et al. 1992) and AhREs have been characterized in tomcod (Roy et al. 1996); however, other components of the pathway have yet to be identified and described in lower vertebrates. It is also believed that activation of the AhR pathway is a necessary early event in initiating most, if not all, toxic and teratogenic con-

sequences resulting from exposure to aromatic hydrocarbons. Therefore, induction of CYP1A mRNA is an easily quantifiable and early surrogate biomarker for most toxic effects of exposure to aromatic hydrocarbon compounds.

The use of CYP1A gene expression as a biomarker in aquatic systems has been validated in many environmental studies in which gene expression was significantly induced in fish from contaminated sites, including point sources of discharge from bleached kraft mill effluents (BKME) and from nonpoint sources such as wastes from urban or industrial centers (Goksøyr and Förlin 1992). Furthermore, for several fish species, controlled laboratory studies have been conducted to calibrate the induction response seen in environmentally exposed organisms, and in some cases, to identify the inducing agents. For example, we have demonstrated that levels of CYP1A mRNA are significantly higher in Atlantic tomcod collected from the Hudson River than in more pristine rivers (Wirgin et al. 1994), and that levels of CYP1A mRNA were induced in tomcod caged in BKME at a pulp and paper mill in comparison to other sites on the Miramichi River, New Brunswick (Courtenay et al. 1993). Furthermore, kinetic studies of gene induction and clearance (Kreamer et al. 1991; Grunwald unpublished data) and use of other biomarkers (Wirgin et al. 1994) have demonstrated that PAHs were likely inducers of increased CYP1A gene expression in Hudson River tomcod but not in tomcod from the Miramichi River (Courtenay et al. 1993).

A feature common to our studies of CYP1A mRNA induction in Atlantic tomcod has been the high among-individual variation in levels of gene induction in environmentally exposed fish and in fish treated with model chemicals under controlled laboratory conditions (Courtenay et al. 1994). In environmental studies, this variation may reflect interindividual differences in exposure histories. Additionally, biological parameters such as sex, developmental stage, and reproductive status significantly affect levels of gene expression in fish (Förlin and Haux 1990), including tomcod (Courtenay et al. 1994). However, even when exposure and these biological factors are controlled in laboratory experiments, levels of interindividual variation in gene expression are still high. We hypothesize that this variation represents underlying genetic differences in the expression or structure of components of the AhR pathway, CYP1A regulatory regions, or in processing of transcript (Roy et al. 1995). Variation among target organisms in response to xenobiotic exposure is important because it presents difficulties in testing for statistical significance among exposure or treatment groups, and it may indicate genetic differences in susceptibility to disease or toxicity. In this study, we attempted to quantitate the extent of interspecific differences in CYP1A gene induction among several common anadromous species of Atlantic Coast fish which might be candidates for selection as sentinel species in marine and/or estuarine environmental monitoring programs. We also evaluated the use of conserved oligonucleotides as primers in PCR amplification of the CYP1A gene from the same fish species. Successful amplification would provide the opportunity to generate species-specific CYP1A DNA probes that could be used to evaluate gene expression in other fish species.

Methods

Sample Collections and Treatments

Two of the species, tomcod (*Microgadus tomcod*) and hogchoker (*Trinectes maculatus*), were chosen for analyses because they share a common ecological niche in the Hudson River (bottom dwelling and euryhaline). Tomcod from the Hudson River exhibit an unusually high prevalence of hepatic tumors, whereas hogchokers do not. Smooth flounder (*Pleuronectes putnami*) was selected because of genetic relatedness to hogchoker (both flatfishes) and consideration of its use as a sentinel species in the Miramichi River. Striped bass (*Morone saxatilis*) were selected because of their abundance and commercial importance within the Hudson River.

Tomcod from the Hudson River were collected in March off New York City (river mile 5, RM 5) with bottom trawls or in January off Garrison (RM50), New York, with box traps. Hogchokers were collected with bottom trawls in Haverstraw Bay (RM 35), New York, in August and off New York City (RM 5) in June. Striped bass juveniles were obtained from a hatchery in Verplank (RM 40), New York, for which broodstock are obtained annually from the Hudson River and which uses a stone quarry and the Hudson River as water sources. An additional sample of environmentally exposed striped bass was collected with hook and line in late June at the Troy Dam (RM 155), an area of the Hudson River in which fish (aggregated species) contain elevated tissue concentrations of total PCBs (Rohmann and Lilienthal 1987). A second set of tomcod and a sample of smooth flounder were collected in October with a bottom trawl at Chatham, New Brunswick, which is 20 km downriver of the pulp and paper mill on the Miramichi River (Courtenay et al. 1993).

For tomcod, smooth flounder, and hogchokers, a subset of fish collected from the natural environment was immediately sacrificed (n = 4–9 per species). All striped bass collected from the natural environment were immediately sacrificed. The re-

maining fish were transported to the laboratory where they were depurated in clean laboratory water for >20 d and i.p. injected with 10 ppm benzo[a]pyrene (B[a]P), with 10 ppm beta-naphthoflavone (β-NF), both standard PAH inducers in fish (Haasch et al. 1989), or with corn oil vehicle in 0.1–0.5 ml volumes. Although, corn oil may oxidize into potent CYP1A-inducing agents, we have consistently observed no difference in CYP1A mRNA expression levels between uninjected and corn-oil-treated tomcod. Treated fish were sacrificed 48 h after injection. Livers were excised from all fish (avoiding the gall bladder), frozen in liquid nitrogen, and stored at −70° C until processing. Blood to be used as a source of DNA was obtained from striped bass, hogchokers, and tomcod by caudal puncture and stored at −70° C.

DNA Isolation and Analysis

Total DNA was isolated from whole blood or homogenized liver using AT solution (1N NH_4OH and 0.2% Triton X-100) as described by Wirgin et al. (1990). Total DNA from each species was amplified using the polymerase chain reaction (PCR) for an 800–1200 base pair (bp) fragment of the putative CYP1A gene (Berndston and Chen 1994) using 20 base oligonucleotide primers (5′ GTTGCCATGATCACCGTGTG 3′; and 5′ GCTTCCTGTCCTCGCAGTGG 3′) chosen from 5′ CYP1A sequences conserved between rainbow trout (*Oncorhynchus mykiss*; Heilmann et al. 1988) and Atlantic tomcod (Roy et al. 1995). Amplification procedures were identical for each species and reaction concentrations followed manufacturer's recommendations (Perkin-Elmer). Approximately, 0.2 μg to 0.8 μg of template DNA were used per reaction. Thermal cycling conditions were as follows: initial denaturation for 5 min at 95° C; 25 cycles of 1 min denaturation at 94° C; 2 min primer annealing at 54° C; 2.5 min extension at 72° C; and a final 5 min extension at 72° C.

The putative CYP1A PCR products from each species were electrophoretically separated in 1.3% agarose gels, stained in ethidium bromide solution and photographed, Southern blotted (Southern 1975) to Zetabind membranes (Cuno, Meriden, Connecticut), and fixed by vacuum baking. Membranes were prehybridized and hybridized at 65° C (Wahl et al. 1979) with dried milk as a blocking agent (Wirgin et al. 1990) to ^{32}P-radiolabelled (Rigby et al. 1977) probe DNAs. Probes were the plasmid, pfP_1450-3′, containing rainbow trout 3′ CYP1A cDNA sequences (Heilmann et al. 1988), an Atlantic tomcod full length (2.6 kb) CYP1A cDNA probe developed from a β-NF induced fish (Roy et al. 1995), and a PCR product containing tomcod 5′ CYP1A cDNA sequence. The tomcod PCR probe was amplified from β-NF induced cDNA and contains 800 bp of 5′ sequence. The rainbow trout CYP1A cDNA probe was developed from a 3-methylcholanthrene (3-MC) treated fish (Heilmann et al. 1988) and it contains 1.4 kb of 3′ CYP1A sequence. Membranes were washed in a final solution of 1.0 × SSC/0.1% SDS at 65° C for at least 1 h, and autoradiography at −70° C with intensifying screens for 1–3 d was used to visualize DNA fragments.

RNA Isolation and Analysis

Total RNA was isolated from hepatic tissue from individual fish using the method of Chomczynski and Sacchi (1987) with RNAzol reagent (Biotecx, Houston, Texas) as described by Kreamer et al. (1991). Two 5 μg aliquots of each sample were heat-denatured and electrophoretically separated in two 1.0% denaturing agarose gels (Fourney et al. 1988), stained in ethidium bromide solution, and photographed to evaluate RNA integrity and equivalency of loading. RNAs were transferred to Nytran membranes (Schleicher and Scheull, Inc.) overnight (Southern 1975), membranes were vacuum baked, prehybridized, and hybridized exactly as described in Kreamer et al. (1991). Each RNA sample was hybridized to three probes: 1) rainbow trout cDNA, 2) tomcod cDNA, and 3) species-specific CYP1A hybridizable PCR products. Final wash conditions were 1.0 × SSPE/0.1% SDS at 65° C for at least 30 min. Autoradiography was performed with intensifying screens at −80° C for 1–7 d and levels of CYP1A mRNA were quantified from autoradiographs using the Whole Band Analysis package in the Millipore BioImage system. Following quantification of CYP1A mRNA levels, CYP1A DNA probes were stripped off membranes by several immersions in boiling water and membranes were rehybridized as described above to a constitutively expressed housekeeping gene, rat rRNA (Chan et al. 1984). This step ensured that approximately equal concentrations of total RNA were loaded into each lane of gels as determined by visual inspection of autoradiographs. Although Haasch et al. (1992) reported differences in levels of rRNA between acrylamide-treated and nontreated trout, we have observed little variation in rRNA expression among environmentally exposed or chemically treated tomcod.

Nonparametric test statistics (Mann-Whitney U, Kruskal-Wallis H; Zar 1984) were used to compare control, environmentally-exposed, and chemically-treated groups. For all comparisons, the alpha level of significance was 0.05. All fold induction estimates (Table 1) are given as group means relative to the control mean. For comparisons where control fish had no detectable expression, fold induc-

TABLE 1. Comparisons of CYP1A mRNA induction for control, environmentally exposed, and chemically exposed anadromous fish species. B[a]P = benzo[a]pyrene, β-NF = beta-naphthoflavone. Sample collection sites and treatments given in text.

Species	Comparison (N)	Fold Induction
Hogchoker (*Trinectes maculatus*)	Control (5) vs. B[a]P (8)	2.6
	Control (5) vs. Environment (4)[b]	1.8
	Control (5) vs. Environment (4)[c]	0
Smooth flounder (*Pleuronectes putnami*)	Control (10) vs. β-NF (10)	13.7*[a]
	Control (10) vs. Environment (5)	0
Striped bass (*Morone saxatilis*)	Control (6) vs. B[a]P (6)	1.3[a]
	Control (6) vs. Environment (6)	1.6*[a]
Atlantic tomcod (*Microgadus tomcod*)	Control (11) vs. B[a]P (11)	97**
	Control (11) vs. Environment (15)	34**

* Denotes significance of $p < 0.05$.
** Denotes significance of $p < 0.001$.
[a] Minimum estimate as control fish were not induced.
[b] Haverstraw Bay, Hudson River.
[c] New York City, Hudson River.

tion was reported as the mean CYP1A mRNA level of the chemically treated or environmentally exposed group, and should be considered as a minimum estimate of induction.

Results

PCR products were obtained for all four species using the conserved CYP1A primers. When these upstream PCR products were analyzed in Southern blot hybridizations, single DNA fragments hybridized with the tomcod 5′ CYP1A DNA probe (Fig. 1) and their sizes were as follows: tomcod (1150 bp), striped bass (800 bp), hogchoker (800 bp), and smooth flounder (800 bp). In each case, the same dominant DNA fragment visualized by ethidium bromide staining hybridized to the tomcod probe. Little hybridization was seen with the trout 3′ cDNA probe.

Northern blot analyses were used to quantitate and compare CYP1A mRNA levels among depurated (controls), environmentally exposed, and chemically-treated representatives of the four fish taxa (Table 1). Similar results were obtained for each of the three probes; however, the strength of the hybridization signal varied among species. Basal levels of CYP1A expression in control individuals were low in all four species. In Atlantic tomcod, basal levels of CYP1A mRNA were detectable in almost all depurated individuals and, in hogchokers, basal expression was detectable in several individuals; however, in the other species, there was little or no detectable expression in control fish. As a result, estimates of fold-induction over controls should be considered minimum values.

Among species, the tomcod consistently had much higher levels of induction in either chemically treated (97-fold) or environmentally exposed groups (34-fold). Striped bass (1.3-fold and 1.6-fold) and hogchoker (2.6-fold and 1.8-fold) showed only low levels of induction (not significant) in either chemically treated or environmentally exposed fish, respectively. Smooth flounder exhibited induction with β-NF (13.7-fold), but not in environmentally exposed fish (Table 1). In statistical comparisons of control versus environmentally exposed and chemically exposed groups within each species, only tomcod exhibited highly significant induction (Table 1). The smooth flounder, however, did show significant induction of the β-NF injected fish. In an overall comparison among all four species of chemically treated fish (relative to controls), there was a significant difference among species (Kruskal-Wallis H test, $p < 0.01$), which is not surprising given the high fold-induction observed in tomcod.

We also compared absolute levels of CYP1A mRNA in female tomcod ($n = 5$) and female smooth flounder ($n = 9$) that were collected in the same bottom trawl at Chatham on the Miramichi River. The median level of CYP1A mRNA was significantly 9.1-fold higher ($p < 0.01$) in tomcod than in smooth flounder.

Discussion

Our results demonstrate that mRNA hybridizable to rainbow trout and Atlantic tomcod CYP1A cDNA probes or species-specific CYP1A genomic DNA probes was not equally inducible in species of estuarine fishes treated with model chemicals or by exposure to contaminated natural environments. In all four species, basal levels of CYP1A mRNA were low in depurated samples; however, basal levels of gene expression were usually detectable in tomcod, sometimes in hogchoker, and rarely in the two other species. In contrast, large differences in the magnitude of CYP1A mRNA induction among species were observed when fish were exposed to xenobiotics. There was little induction of CYP1A mRNA in either chemically treated or environmentally exposed hogchoker or

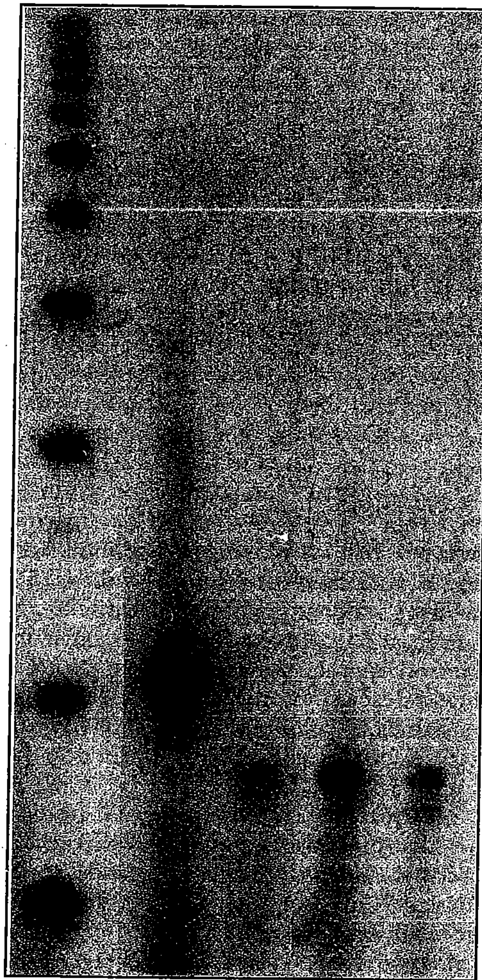

Fig. 1. PCR-amplified products from genomic DNA of four Atlantic Coast fish species hybridized on a Southern blot to a full length Atlantic tomcod CYP1A cDNA probe. AT = Atlantic tomcod (1150 bp), SB = striped bass (800 bp), HC = hogchoker (800 bp), SF = smooth flounder (800 bp). The standard in the first lane is a one kilobase ladder (KBL; Gibco BRL), and the three smallest fragments are 520 kb, 1018 kb, and 2036 kb, respectively.

striped bass. Smooth flounder exhibited statistically significant induction in chemically treated, but not in environmentally exposed fish. CYP1A mRNA was highly induced in both B[a]P and Hudson River exposed Atlantic tomcod. For example, CYP1A mRNA was induced almost 100-fold in B[a]P-treated Atlantic tomcod, and levels of CYP1A gene expression were also very high in fish collected from the Hudson River off New York City (34-fold induction) compared to depurated controls. Tomcod collected from the Miramichi River also exhibited high induced levels of CYP1A mRNA, while smooth flounder collected in the same trawl did not. For comparison, levels of CYP1A mRNA were only induced 2-fold to 4-fold in killifish (*Fundulus heteroclitus*) collected from highly contaminated Newark Bay in the lower Hudson River estuary compared to killifish from a clean site in central New Jersey (Haasch et al. 1993). Furthermore, an absence of CYP1A enzyme induction was reported in flatfish also collected from the Miramichi River by Addison et al. (1991). These results indicate that CYP1A mRNA expression in Atlantic tomcod is highly responsive to exposure to environmentally borne xenobiotics.

We sought to determine if our inability to detect significant induction of CYP1A mRNA in chemically treated or environmentally exposed striped bass and hogchokers was due to insufficient homology between induced CYP1A mRNAs and the rainbow trout, tomcod, or species-specific probes, or interspecific differences in gene inducibility. However, we have previously demonstrated that sufficient homology exists between the trout probe and hogchoker and striped bass genomic DNA to visualize a single intense DNA fragment for each species in Southern blot analysis (Kreamer et al. 1991). Furthermore, Haasch et al. (1989) reported that the trout CYP1A cDNA probe successfully hybridized to β-NF induced mRNA in a distantly related fish taxon, the scup (*Stenotomus chrysops*), and even in higher terrestrial vertebrates such as garter snakes (*Thamnoplis* sp.) and Sprague-Dawley rats. We further addressed the possibility of insufficient homology by using full length and upstream Atlantic tomcod CYP1A cDNA probes which harbor conserved 5′ CYP1A sequences in northern hybridizations. When using these probes we also observed an absence of CYP1A mRNA induction in hogchokers and striped bass. In addition, we observed little evidence of CYP1A mRNA induction in these species when using the PCR-generated, species-specific CYP1A genomic DNA probes. Although identity of the PCR probes was suggested by their abilities to hybridize to the Atlantic tomcod CYP1A cDNA probe in Southern blot analyses, absolute confirmation of their identity awaits determination of their actual DNA sequences.

An absence of CYP1A gene induction in xenobiotic and environmentally exposed fish could reflect biological and chemical factors such as interspecific variation in the kinetics or chemical specificity of CYP1A induction; sex, season, and reproductive state, which have previously been shown to significantly modulate levels of CYP1A induction in fishes; prior exposure to HAHs, which has been demonstrated to inhibit further induction of CYP1A protein and mRNA in HAH-treated fish; variation in toxicokinetic parameters, which could

impact on tissue concentrations of these xenobiotics; or interspecific differences in CYP1A mRNA inducibility, reflecting genetic differences in expression or structure of Ah pathway molecules or CYP1A regulatory sequences.

Differences in gene expression in chemically treated fish could reflect interspecific variation in the kinetics or chemical specificity of CYP1A gene induction. For example, we demonstrated that maximum induction in tomcod i.p. injected with a single dose of β-NF or B[a]P was 72 h and 48 h, respectively, following treatment and that CYP1A mRNA was 180-fold induced with 10 ppm B[a]P in range-finding dose experiments (Grunwald et al. unpublished data). However, time course studies of CYP1A mRNA induction in β-NF treated killifish and rainbow trout demonstrated that maximum induction occurred more rapidly, between 18 h and 40 h after treatment (Haasch et al. 1989; Kloepper-Sams and Stegeman 1989). Furthermore, studies in a zebrafish (*Brachydanio rerio*) liver cell line demonstrated that immunoreactive CYP1A protein expression and EROD activity were highly induced by exposure to TCDD but not by β-NF (Miranda et al. 1993). Thus, our results with these exposures to nonhalogenated aromatic compounds may not be entirely reflective of responses in fish treated with other aromatic hydrocarbon compounds or for different periods of time.

Inherent biological factors such as sex or reproductive status significantly modulate induction of gene expression. For example, studies in other fish species have demonstrated that prior to and during spawning periods, levels of CYP1A protein (Elskus et al. 1992) and enzyme activity (Förlin and Haux 1990) are significantly reduced in females, including tomcod (Courtenay et al. 1994). For all four species used in this study, all chemical treatments and, with the exception of striped bass, all environmental exposures were conducted several months postspawning. Thus striped bass (spring spawners) and hogchokers (early summer spawners) were tested in early fall, and smooth flounder (late winter spawners) were evaluated in late spring and early fall. Thus, it is unlikely that induction of CYP1A mRNA in this study was significantly diminished because of seasonal reproductive effects.

Variation in CYP1A mRNA induction could reflect toxicokinetic differences in the bioaccumulation of inducing agents. For example, it has been demonstrated that Atlantic tomcod have unusually high hepatic lipid concentrations and that Atlantic tomcod from the Hudson River have even higher levels than tomcod from cleaner rivers (Cormier et al. 1989). Thus, it is likely that tomcod from the Hudson River would have higher liver concentrations of xenobiotics than the other species tested. However, the relationship between hepatic contaminant concentrations and CYP1A induction is not clear. In controlled laboratory experiments in which hatchery-reared Atlantic cod (*Gadus morhua*) and rainbow trout were treated with equivalent doses of TCDD, trout exhibited higher levels of CYP1A gene induction than cod despite their lower hepatic TCDD levels (Hektoen et al. 1994).

It has been demonstrated that prior exposure to HAH-contaminated environments may inhibit further induction of CYP1A gene expression in chemically treated fish. For example, Monosson and Stegeman (1991) reported inhibition of CYP1A protein induction and its associated ethoxyresorufin (EROD) enzyme activity in winter flounder (*Pleuronectes americanus*) collected from a PCB-polluted estuary in Rhode Island and treated with the coplanar PCB congener 3,3′,4,4′-tetrachlorobiphenyl (TCB). Similarly, we found that CYP1A mRNA was not inducible in Atlantic tomcod collected from the Hudson River and treated with TCB or TCDD (Wirgin et al. 1992) despite extensive depuration for up to 305 d (Wirgin unpublished data). In controlled laboratory experiments, Celander and Förlin (1995) reported decreased responsiveness of CYP1A mRNA and protein induction in rainbow trout after prolonged exposure to PCBs. Both the hogchokers and smooth flounder used in our study were collected from HAH-polluted environments and thus it is possible that prior exposure to environmentally-borne PCBs, dioxins, and furans in the Hudson River and BKME in the Miramichi River might contribute to a lack of CYP1A mRNA induction. However, the noninducibility of CYP1A mRNA in chemically treated striped bass is probably not due to prior exposure history because these fish were reared and maintained under clean hatchery conditions with the exception that diluted Hudson River water was used as a water source at later stages of development.

It is surprising that differences in CYP1A mRNA among fish species does not always follow expectations based on phylogeny. For example, we did not detect significant induction of CYP1A mRNA in two flatfish species (*Pleuronectiformes*), the hogchoker or smooth flounder, that were exposed to contaminated environments. In contrast, levels of CYP1A protein induction and EROD activity were significantly induced in winter flounder collected at contaminated sites off the New England and Canadian coasts (Monosson and Stegeman 1994). Similarly, significant differences in induction of CYP1A enzyme activities and glutathione S-transferase has been observed between two Pacific Coast flatfish species, English sole (*Parophrys vetulus*) and starry flounder (*Platichthys stellatus*), from

Puget Sound that were exposed to contaminated environments (Collier et al. 1992).

Interspecific differences in CYP1A mRNA inducibility could result from genetic variation at several points in the pathway leading to CYP1A transcription. These could include the AhR or other cytoplasmic proteins involved in ligand binding or translocation of activated AhR complex to the nucleus, proteins involved in the binding of activated AhR complex to CYP1A enhancer elements, or in regulatory elements controlling CYP1A transcription. For example, recent studies have demonstrated differences in levels of expression of AhR and ARNT mRNA among humans which were associated with levels of CYP1A induction (Hayashi et al. 1994) and have found polymorphisms in the mouse AhR which could result in amino acid substitutions (Chang et al. 1993). Furthermore, although CYP1A regulatory elements have been identified in tomcod (Roy et al. 1996), screening for polymorphisms has yet to be attempted. Comparative investigations of the number, position, and structure of CYP1A enhancer and other regulatory elements may provide insights into mechanisms of variation in CYP1A mRNA inducibility and perhaps to susceptibility to aromatic hydrocarbon mediated disease.

Implications from this study can be viewed from both the perspective of development of sensitive and representative biomarkers and the relationship of CYP1A mRNA inducibility to higher level biological effects. 1) If the goal of an environmental monitoring program is to most sensitively assess contamination levels, then the choice of highly responsive sentinel species is critical. Our results highlight the need to conduct pilot studies to select sentinel species that are sensitive, dose-responsive models for use in monitoring programs. It should also be cautioned that results from a sensitive sentinel species may not be representative of the response of the typical member of the ecosystem. Maybe, the highly responsive species is the aberration. 2) Are interspecific differences in CYP1A gene inducibility predictive of variation in susceptibility to higher level toxicological endpoints? Interspecific differences in the biotransformation and toxicity of TCDD have been reported among species of freshwater fish (Kleeman et al. 1988). However, the relationship of CYP1A mRNA induction to higher level biological effects at the organismic or population level is still open to investigation. A correlation between induction levels of CYP1A gene expression and histologically observed toxic effects has been reported in rainbow trout exposed to relatively low doses of TCDD (van der Weiden et al. 1992). Similarly, some studies have shown a correlation between induction of CYP1A gene expression and reproductive impairment in feral fish populations exposed to BKME (Munkittrick et al. 1991, 1992); however, other studies failed to find this association (Hodson et al. 1992).

Induced levels of CYP1A expression in feral fish populations have been correlated with cancer-related endpoints. For example, induced levels of CYP1A protein and enzyme activity have been correlated with higher level effects such as elevated levels of hepatic DNA adducts and increased prevalence of hepatic neoplasia in English sole from contaminated sites in Puget Sound (Stein et al. 1992). Consistent with this association is the low level of CYP1A gene induction and a low prevalence of hepatic tumors in starry flounder from contaminated sites in Puget Sound despite their sympatric distribution with cancer-prone English sole (Collier et al. 1992). Furthermore, tomcod from the Hudson River exhibit elevated levels of CYP1A mRNA expression, high levels of hepatic DNA adducts (Wirgin et al. 1994), an activated K-*ras* oncogene (Wirgin et al. 1989), and an unusually high prevalence of liver neoplasia (Dey et al. 1993).

However, exceptions to the association between CYP1A gene induction and cancer-related endpoints have also been observed. For example, hogchokers, which are sympatric with tomcod in the Hudson River show no evidence of CYP1A mRNA induction or hepatic liver tumors. Yet, levels of hepatic DNA adducts in hogchokers from a contaminated site in the Hudson River are comparable to those in tomcod (Stein and Wirgin unpublished data). Similarly, inconsistencies in a cascade of biomarker responses has been reported in oyster toadfish (*Opsanus tau*), which are exposed to unusually high levels of sediment-borne PAHs in the Elizabeth River, Virginia (Collier et al. 1992). The uptake and biotransformation of these compounds is confirmed by elevated levels of PAH metabolites in bile and high levels of DNA adducts. However, hepatic CYP1A protein concentrations and enzyme associated activities were extremely low in fish from contaminated sites, at levels associated with relatively clean control sites. Furthermore, oyster toadfish are highly resistant to hepatocarcinogenesis; hepatocyte-derived tumors have yet to be observed in this species despite the frequent distribution of toadfish in aromatic hydrocarbon-polluted waters.

If the paradigm is true that CYP1A-encoded monooxygenase activity is required for the biotransformation of environmental procarcinogens to reactive metabolites, which can adduct to DNA and at times initiate neoplasia, how can species (such as hogchoker and toadfish) living in PAH-contaminated sites exhibit high levels of DNA adducts in the absence of CYP1A gene induction? Are other alternative non-CYP1A enzyme mediated pathways operative in the transformation of these

compounds? Based on these results, induction of CYP1A gene expression may not always be predictive of higher level effects.

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