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Aquatic Toxicology xxx (2005) xxx–xxx

AQUATIC TOXICOLOGY

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Comparison of hepatic and extra hepatic CYP1A induction by graded doses of aryl hydrocarbon receptor agonists in Atlantic tomcod from two populations

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Received 12 August 2005; received in revised form 2 October 2005; accepted 15 October 2005

Abstract

Atlantic tomcod *Microgadus tomcod* from the Hudson River, New York, are exposed to high levels of polycyclic aromatic hydrocarbons (PAHs) and bioaccumulate mixtures of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and polychlorinatedfurans (PCDD/Fs). Previous studies demonstrated that hepatic cytochrome P4501A (CYP1A) mRNA was not inducible in tomcod from the Hudson River treated with single doses of PCB77 or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), but was inducible with PAHs. In this study, we sought to determine if CYP1A mRNA was inducible with higher doses of these and other halogenated aromatic hydrocarbons (HAHs) in Hudson River tomcod and if decreased sensitivity to gene inducibility occurs across all tissues. Tomcod from the Hudson River and the cleaner Miramichi River, New Brunswick, were treated individually with graded doses of TCDD and coplanar PCBs (PCB77, PCB81, PCB126, PCB169) and profiles of hepatic CYP1A mRNA expression were compared between the two populations. CYP1A mRNA inducibility was also compared in multiple tissues of tomcod from the two rivers that were treated with PCB77. Additionally, hepatic CYP1A mRNA was characterized in Miramichi River tomcod treated with pairs of PCB congeners that included aryl hydrocarbon receptor (AHR) agonists and antagonists. Hepatic CYP1A mRNA was significantly inducible by all chemicals in tomcod from the Miramichi River and TCDD and two of four PCBs in tomcod from the Hudson River. CYP1A mRNA was also significantly inducible in four of five tissues of tomcod from the Miramichi River but only in liver of Hudson River tomcod. In summary, CYP1A mRNA inducibility was approximately two orders of magnitude less sensitive in tomcod from the Hudson than in those from the Miramichi River. But when achieved, maximum levels of CYP1A expression were similar in tomcod from the two populations. Co-administration of PCB126 and PCB77 did not produce significantly greater CYP1A mRNA induction than administration of PCB126 alone and co-administration of mono-*ortho*-substituted PCB105 significantly decreased CYP1A mRNA inducibility by PCB77. These results indicate that CYP1A mRNA expression is significantly inducible by HAHs in tomcod from the Hudson River and suggest that all components of the AHR pathway are present and functional, but that the pathway is less sensitive to activation in tomcod

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doi:10.1016/j.aquatox.2005.10.006

from the Hudson River. Our results also indicate that CYP1A mRNA levels in environmentally exposed fish may not reflect additive tissue burdens of PCB congeners.

Keywords: Resistance; PCBs; Dioxins; Hudson River; Atlantic tomcod; Aryl hydrocarbon receptor

1. Introduction

Atlantic tomcod *Microgadus tomcod* is a common, bottom-dwelling, anadromous fish species that spawns in estuaries of the North American Atlantic Coast from the Hudson River to southern Labrador (Bigelow and Schroeder, 1953). Tomcod are resident year-round in their natal estuaries, although they do undergo wintertime intra-estuary migrations to the vicinity of the upriver salt front to spawn (Klauda et al., 1988; McLaren et al., 1988). Tomcod is the only fish species to spawn in the Hudson River Estuary during the winter months making its young-of-the-year (YOY) available there as a dominant piscine food item well before juveniles of other species in the spring (McLaren et al., 1988; Chambers and Witting, in press).

Because of their abundance, benthic existence, lipid-rich livers, and residency in natal estuaries, tomcod have proven to be valuable sentinels of environmental degradation (Wirgin and Chambers, in press). Studies conducted in the 1970s and early 1980s demonstrated that tomcod from the Hudson River exhibited remarkably high prevalences of hepatocellular carcinomas exceeding 90% in 2-year-old fish (Smith et al., 1979; Dey et al., 1993; Cormier and Racine, 1990). Concurrently, the age structure of the Hudson River tomcod population was significantly truncated compared to those in cleaner rivers (Dey et al., 1993).

Tomcod from the Hudson River are exposed to much higher levels of aromatic hydrocarbon (AH) pollutants than tomcod from elsewhere. For example, levels of metabolites of PAHs in bile (a short-term measure of PAH exposure (Krahn et al., 1986)) were significantly higher in adult tomcod from the Hudson River than in those from four cleaner Atlantic coast rivers (Wirgin et al., 1994). Similarly, levels of hepatic bulky DNA adducts (a longer term measure of PAH exposure) were up to 40-fold higher in adult tomcod from the Hudson River than in those from the same four cleaner rivers (Wirgin et al., 1994). Levels of polychlorinated dibenzo-*p*-dioxins/furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) were quantified on a congener-specific basis in livers of adult and juvenile tomcod and in their unfertilized eggs from multiple locations in the Hudson River Estuary including the Hackensack River, Newark Bay, and sites along the main stem Hudson River and in tomcod from two cleaner rivers including the Miramichi River, New Brunswick (Courtenay et al., 1999; Yuan et al., 2001; Roy et al., 2001; Fernandez et al., 2004). Hepatic and egg burdens of PCDD/Fs and PCBs, including coplanar and mono- and di-*ortho*-substituted PCB congeners were much higher, often two orders of magnitude or more, in tomcod from sites in the Hudson River Estuary than in those from the Miramichi River and Margaree River, Nova Scotia.

Hepatic cytochrome P4501A (CYP1A) expression is usually a sensitive, dose responsive biomarker of exposure of fishes to aromatic hydrocarbon (AH) compounds, particularly those that most resemble 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Stegeman and Hahn, 1994). Transcription of CYP1A is mediated by activation of the aryl hydrocarbon receptor (AHR) pathway (Ma, 2001) as are most toxic effects of AH compounds including early life-stage toxicities and hepatic neoplasia. Thus, CYP1A expression is an easily quantifiable measure that often correlates with higher-level toxic effects of AHs. Coplanar PCBs are effective AHR agonists in mammals and fishes. Mono-*ortho*-substituted PCBS are effective AHR agonists in mammals, but not so in fishes (Hesterman et al., 2000). Little is known of the in vivo interactive effects of exposure to mixtures of AH contaminants on levels of CYP1A expression in fishes.

In biomarker studies, hepatic CYP1A mRNA expression was significantly higher in environmentally exposed adult tomcod from the Hudson River compared to tomcod from cleaner rivers and correlated well with levels of bile metabolites of PAHs and hepatic DNA adducts (Wirgin et al., 1994). However, within the Hudson River, levels of CYP1A mRNA expression in juvenile tomcod did not correlate with their hepatic bur-

dens of PCDD/Fs and PCBs expressed as TCDD toxic equivalency quotients (TCDD, TEQs) suggesting that endogenous or exogenous factors modulated inducibility of CYP1A (Yuan et al., 2001). Because tomcod from the Hudson River bioaccumulate complex mixtures of non-*ortho* and mono- and -di-*ortho*-substituted PCBs as well as PCDD/Fs, it is possible that non-additive levels of CYP1A expression occur. In particular, studies in mammalian models have demonstrated that di-*ortho*-substituted PCB congeners are often AHR antagonists and can significantly inhibit CYP1A1 induction and higher-level biological effects of these compounds (Safe, 1997–1998; Suh et al., 2003).

We previously reported that hepatic CYP1A mRNA expression was not significantly inducible in adult tomcod from the Hudson River that were treated with a single dose of coplanar PCB77 (1 mg/kg body wt.) or TCDD (0.5 μg/kg body wt.) (Wirgin et al., 1992; Courtenay et al., 1999) or in juvenile tomcod from seven sites in the Estuary treated with a single dose of PCB77 (10 mg/kg body wt.) (Yuan et al., in press). However, hepatic CYP1A mRNA expression was significantly inducible in Hudson River tomcod treated with two PAHs, benzo[*a*]pyrene (B[*a*]P) (0.5 mg/kg body wt.) or β-naphthoflavone (β-NF) (10 or 100 mg/kg body wt.) (Wirgin et al., 1992; Courtenay et al., 1999). In contrast, hepatic CYP1A was significantly inducible with all four AH compounds in adult (Courtenay et al., 1999) and juvenile tomcod (Yuan et al., in press) from the cleaner Miramichi River. We speculated that an absence of inducibility of CYP1A mRNA with halogenated aromatic hydrocarbons (HAHs) in tomcod from the Hudson River population was representative of its overall resistance to these compounds, a response perhaps mediated by functional impairment of the AHR pathway (Roy and Wirgin, 1997).

In this study, we sought to determine if CYP1A mRNA is not inducible or alternatively is inducible, but significantly less so, in tomcod from the Hudson River than in those from a reference population in the Miramichi River. Thus, tomcod from both populations were treated with graded doses of a variety of AHR agonists and CYP1A mRNA was quantified in hepatic and extra hepatic tissues. We also sought to develop tomcod-specific toxic equivalency factors (TEFs) for coplanar PCB congeners and compare them between tomcod from resistant and sensitive populations. Finally, we sought to initially evaluate the effects of co-exposure to binary mixtures of AHR agonists and antagonists on hepatic CYP1A mRNA expression in tomcod.

2. Materials and methods

2.1. Tomcod collections

Adult tomcod were collected from the Hudson River in mid-January to late February of 1999 and 2000 with unbaited boxtraps set off a bulkhead in Garrison, New York (RM 50). Fish were transferred to NYU where they were maintained at 4 °C in clean laboratory water at a salinity of 5 ppt for more than 21 days for partial depuration.

Adult tomcod were also collected with smelt bag nets from November to late February in 1999 and 2000 from the Miramichi River near Loggieville, New Brunswick. Miramichi River tomcod collected in 1999 were transported to NYU and maintained identically, but separately, from those from the Hudson River. Tomcod collected in 2000 from the Miramichi River were transported to the aquarium facility at the Gulf Fisheries Centre in Moncton, New Brunswick, where they were held in 15 ppt seawater at 5 °C and were treated identically to those of Hudson River origin.

2.1.1. Treatment with coplanar PCBs

Depurated adult tomcod from both the Hudson River and the Miramichi River were i.p. injected with graded doses of TCDD (1 ppt–100 ppb), PCB77 (0.1–100 ppm), PCB126 (1 ppb–10 ppm), and PCB169 (0.1–10 ppm) dissolved in corn oil vehicle. Tomcod from the Miramichi River were also treated with graded doses of PCB81 (0.001–10 ppm). Doses were selected based on our previous studies of CYP1A mRNA expression in tomcod treated with TCDD and PCB77 (Courtenay et al., 1999) and fish toxic equivalency factors reported in Van den Berg et al. (1998). PCB congeners were purchased either from AccuStandard (New Haven, CT) or Ultra Scientific (North Kingstown, RI). Fish were sacrificed 7 days after treatment because previous kinetic experiments with Miramichi River tomcod i.p. injected with TCDD and PCB77 demonstrated maximum levels of hepatic CYP1A mRNA expression at this time after treatment with these chemicals

(Courtenay et al., 1999). For PCB77 treated fish, heart, intestine, kidney, liver and spleen were sampled, snap frozen, and stored at −80 °C until use. For all other treatment groups, only liver tissue was sampled and it too was snap frozen and stored at −80 °C.

2.2. Co-exposure to PCB126 and PCB77

Miramichi River tomcod were i.p. injected with 0–0.1 ppm PCB126 (an AHR agonist) either alone or in combination with 0.5 ppm PCB77 (an AHR agonist), a dose above the CYP1A induction threshold of 0.1 ppm previously documented for tomcod from this population (Courtenay et al., 1999). Fish were sacrificed 7 days after treatment and livers were excised and immediately frozen at −80 °C. This experiment was carried out in each of two separate years with different batches of Miramichi River tomcod.

2.3. Co-exposure to PCB105 and PCB77

Miramichi River tomcod were i.p. injected with 1 or 10 ppm PCB105 (a mono-*ortho*-substituted congener), which is not a CYP1A inducer in fish, including tomcod (Wirgin et al., 1992), either alone or in combination with 0.5 ppm PCB77. Fish were sacrificed 7 days after treatment, livers were excised and immediately frozen at −80 °C. This experiment was carried out in each of two separate years with different batches of Miramichi River tomcod.

2.3.1. Isolation of RNAs

RNAs were isolated with RNAzol or Ultraspec reagent (BIOTECX Laboratories Inc., Houston, TX) as recommended by the manufacturer and described for tomcod in Courtenay et al. (1999). Concentrations and purities of RNA samples were determined by UV spectrophotometry at 260 and 280 nm. All RNA samples were evaluated for the integrity of their 18S and 28S rRNA bands in ethidum bromide stained northern gels as described in Fourney et al. (1988). Samples with degraded rRNA bands were re-isolated or omitted from subsequent analysis.

2.4. CYP1A mRNA quantification

Two micrograms of each RNA sample was denatured and applied under vacuum onto Nytran Nylon Plus membranes (Schleicher & Schuell, Keene, NH) using a 72-well slot blot apparatus (Schleicher & Schuell) as described in Courtenay et al. (1999).

Full-length tomcod CYP1A cDNA isolated from a β-NF treated Hudson River tomcod (Roy et al., 1995) and full-length rat 18S rRNA cDNA (Chan et al., 1984) were used as probes and were ^{32}P-radiolabeled using Random Priming Kits (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer's protocol.

Membranes were prepared, pre-hybridized, and hybridized overnight at 65 °C as described in Courtenay et al. (1999). After hybridizations, the membranes were washed three times in 6× SSPE/0.1% at room temperature for 5 min each and twice in 1× SSPE/0.1% SDS at 65 °C for 1 h in total. Dried membranes were autoradiographed or exposed to Phosphor Imaging screens. The length of time for which membranes were Phosphor Imaged for both CYP1A and 18S rRNA varied among experiments and therefore resulted in differing absolute CYP1A/rRNA OD units among blots. CYP1A mRNA levels were quantified from X-ray films using the Whole Band Analysis Package in the Millipore BioImage Analysis System. CYP1A mRNA levels were quantified from Phosphor Imaging screens using a Storm 860 Scanner and Molecular Dynamics ImageQuant™ for Macintosh software version 1.0 (Sunnyvale, CA).

After quantification of CYP1A mRNA levels, CYP1A probes were stripped off membranes by their immersion in boiling 0.1× SSC/0.5% SDS three times for 20 min each with shaking. The membranes were then prehybridized and hybridized with ^{32}P radiolabeled 18S rRNA probes and quantified as above.

3. Data analysis

3.1. Comparison of gene expression among tissues

Data were converted to their natural logs. Within each tissue and population, responses to different doses of PCB77 were compared by ANOVA followed by Tukey's multiple range test. Significant induction is reported for treatments showing CYP1A mRNA levels significantly higher than controls ($p < 0.05$). Fold induction was calculated from back-transformed means for each treatment group. Induction of CYP1A mRNA of tomcod from the Miramichi River and Hud-

son River populations was compared across doses of PCB77 by two-way ANOVA. Means and standard errors of raw data are presented.

3.2. Comparison of gene expression among congeners

CYP1A mRNA expression in tomcod i.p. injected with graded doses of TCDD, PCB126, PCB77, PCB169 and PCB81 was compared respective to vehicle controls by ANOVA followed by the Tukey's multiple range test (data logged to improve normality).

Mean and 95% confidence interval for fold induction over corn oil vehicle controls were graphed against dose injected (log scale for both variables). Where sufficient data existed to describe the full dose–response curve, estimated minimum fold induction, maximum fold induction and EC50 were calculated using the standard four-parameter logistic regression in SigmaPlot's non-linear curve-fitting module as described in Billiard et al. (2004).

3.2.1. Gene expression in tomcod co-treated with two PCBs

CYP1A mRNA concentrations were measured as integrated optical density (I.O.D.) units and are presented as means ± 1 S.E. Differences among treatment groups were evaluated by ANOVA on log transformed data followed by the Tukey's multiple range test.

4. Results

4.1. Comparison of CYP1A mRNA expression among tissues of tomcod treated with PCB77

Significant dose-responsive CYP1A mRNA induction was observed in all tissues sampled from Miramichi River tomcod that were i.p. injected with PCB77 7 days earlier (Fig. 1; Table 1). The induction thresholds for liver, intestine and heart were similar at ≤0.1 ppm. Kidney was less sensitive, showing first significant induction at 10 ppm, and no threshold was determined for spleen.

Significant dose-responsive CYP1A mRNA induction was also observed in the livers of Hudson River tomcod, but starting at an induction threshold 100 times higher than observed in liver of Miramichi River tomcod: 10 ppm versus 0.1 ppm. No significant induction was observed in other tissues of Hudson River tomcod exposed to 0.1–10 ppm PCB77, so induction thresholds for heart, intestine, kidney, and spleen must all be >10 ppm.

Statistical comparison of CYP1A mRNA expression levels in livers (the one tissue for which data were collected at all doses of PCB77) indicated stronger CYP1A mRNA induction in Miramichi River than Hudson River tomcod (two-way ANOVA, population difference: $F_{1,68} = 8.086$, $p = 0.006$). However, at the two highest doses administered (50 and 100 ppm, data pooled), there was no difference between populations in their levels of CYP1A mRNA ($F_{1,14} = 0.111$, $p = 0.744$). Nevertheless, because basal concentrations of CYP1A mRNA in Hudson River tomcod were 2.5 times higher than in Miramichi River tomcod (Fig. 1), tomcod from the Hudson River still showed lower maximal fold induction than those from the Miramichi River (56-fold versus 147-fold in response to 100 ppm PCB77) (Table 2).

4.2. Comparison of hepatic CYP1A mRNA levels in tomcod treated with TCDD and four PCB congeners

4.2.1. TCDD

The threshold for hepatic CYP1A mRNA induction in Miramichi River tomcod was 100 pptr above which the response increased with dose to a plateau of responses not differing significantly between 5000 and 50,000 pptr ($F_{8,60} = 47.769$, $p < 0.001$; Tukey's test: $p < 0.05$). EC_{50} calculated from four-parameter logistic regression was 739 pptr TCDD (Fig. 2). By contrast, first significant induction for Hudson River tomcod was observed at 5000 pptr, 50 times the induction threshold for Miramichi River tomcod ($F_{6,32} = 20.992$, $p < 0.001$; Tukey's test: $p < 0.05$). Response increased with dose thereafter with no indication that maximal response, which was still lower than the respective response in Miramichi River tomcod ($F_{1,10} = 74.842$, $p < 0.001$), had been achieved by the highest dose administered (50,000 pptr) (Fig. 2). Therefore, an EC_{50} could not be calculated for Hudson River tomcod, but it would appear to be higher than the EC_{50} in tomcod from the Miramichi River by approximately two orders of magnitude.

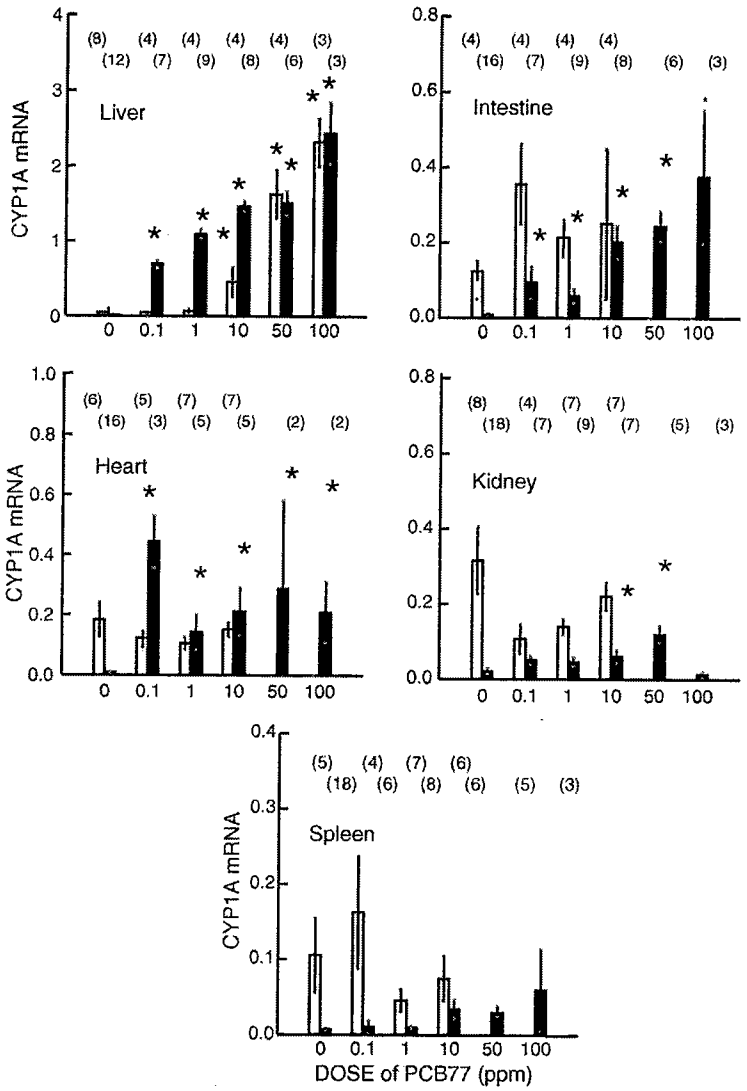

Fig. 1. Comparison of mRNA expression levels in five tissues of Atlantic tomcod from the Hudson River (black bars) or Miramichi River (open bars) i.p. injected with graded doses of PCB77. Mean + 1 S.E. Number in parentheses is the number of specimens analyzed at that dose. Asterisks indicate significant induction over respective controls (0 dose).

4.2.2. PCB126

Dose-responsive induction of hepatic CYP1A mRNA was observed in Miramichi River tomcod from the lowest dose administered (0.001 ppm) ($F_{6,39} = 41.706$, $p < 0.001$; Tukey's test: $p < 0.05$) and rising rapidly to a plateau of similar responses above 0.01 ppm (Fig. 2). About 0.1 ppm PCB126 elicited no response in Hudson River tomcod, but strong induction was seen in response to 1 and 10 ppm ($F_{3,21} = 78.862$, $p < 0.001$; Tukey's test: $p < 0.05$). The EC_{50} calculated from these two dose–response curves were 0.05 and 0.76 ppm for Miramichi River and Hudson River tomcod, respectively.

4.2.3. PCB77

Hepatic CYP1A mRNA was significantly induced by all doses tested in tomcod from the Miramichi River (0.1–100 ppm) ($F_{5,39} = 222.313$, $p < 0.001$; Tukey's

Table 1
ANOVA comparisons of CYP1A mRNA expression levels in tissues of Miramichi River and Hudson River Atlantic tomcod i.p. injected with graded doses of PCB77

Tissue	Population	d.f.	F	p	Tukey's test, p
Liver	Miramichi	5.39	222.3	<0.001	All groups: <0.001
	Hudson	5.21	37.9	<0.001	10, 50, 100 ppm: <0.001 0.1, 1 ppm: >0.05
Intestine	Miramichi	5.43	22.0	<0.001	All groups: ≤0.001
	Hudson	3.12	1.7	0.229	All groups: >0.05
Heart	Miramichi	5.27	25.3	<0.001	All groups: <0.001
	Hudson	3.21	1.5	0.245	All groups: >0.05
Kidney	Miramichi	5.43	5.9	<0.001	10 ppm: 0.030 50 ppm: 0.001 All other groups: >0.05
	Hudson	2.22	2.9	0.055	All groups: >0.05
Spleen	Miramichi	4.50	3.5	0.011	All groups: >0.05
	Hudson	3.18	1.9	0.159	All groups: >0.05

Tukey's test, p refers to comparison of all PCB groups to negative control group for that population.

test: $p<0.05$; Fig. 2). Hudson River tomcod showed dose-responsive induction of hepatic CYP1A mRNA starting two orders of magnitude higher at 10 ppm ($F_{5,21}=37.906$, $p<0.001$; Tukey's test: $p<0.05$). Neither dataset comprised a sufficient portion of the dose–response relationship to calculate an EC_{50}, Miramichi River tomcod lacking information for the bottom portion of the curve and Hudson River tomcod lacking information for the top of the curve.

4.2.4. PCB169

Miramichi River tomcod injected with PCB169 showed significant dose-responsive induction of hepatic CYP1A mRNA starting at a dose of 0.5 ppm ($F_{5,30}=49.642$, $p<0.001$; Tukey's test: $p<0.05$; Fig. 2). By contrast, Hudson River tomcod showed no significant response to doses of PCB169 as high as 10 ppm. Neither dataset included a sufficient portion of the upper dose–response curve to permit calculation of EC_{50}.

Table 2
CYP1A mRNA fold induction over respective negative control in five tissues of Miramichi River and Hudson River Atlantic tomcod i.p. injected with graded doses of PCB77 (0.1–100 ppm)

Dose (ppm)	Liver	Intestine	Heart	Kidney	Spleen
Miramichi					
0.1	**42**	**9**	**69**	3	1
1	**66**	**6**	**14**	3	2
10	**89**	**23**	**26**	**4**	5
50	**90**	**37**	**37**	**9**	7
100	**147**	**52**	**32**	1	10
Hudson					
0.1	1	3	1	1	2
1	2	2	1	1	1
10	**9**	1	2	1	1
50	**38**				
100	**56**				

Numbers in bold represented statistically significant elevations over negative controls. For 50 and 100 ppm PCB77, Hudson River tomcod were sampled only for liver.

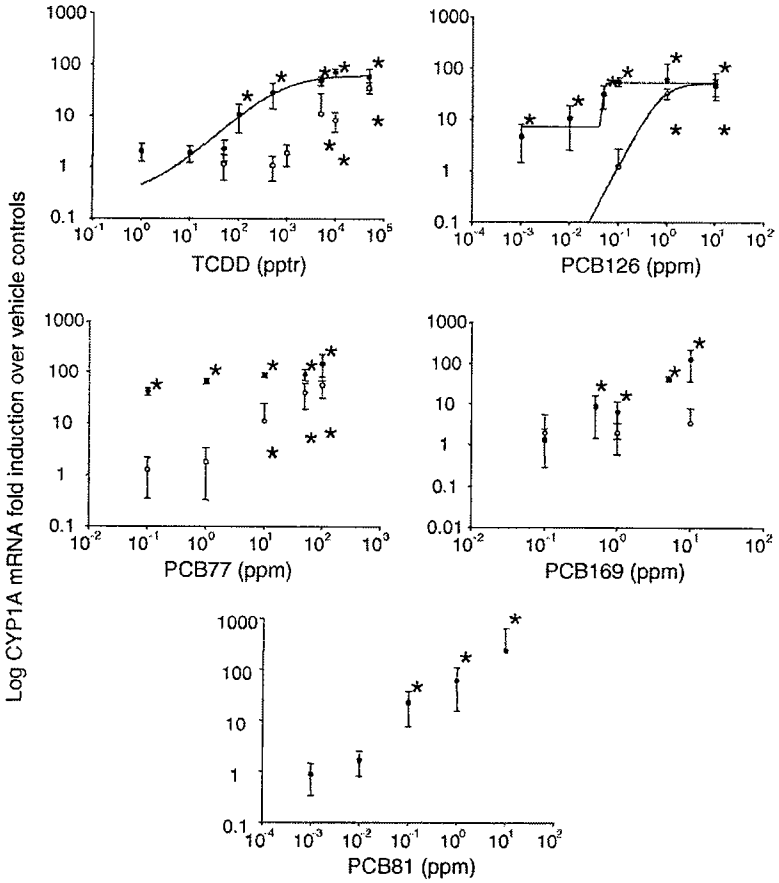

Fig. 2. Comparison of hepatic CYP1A mRNA expression in Miramichi River (filled circles) and Hudson River Atlantic tomcod (open circles) i.p. injected with graded doses of TCDD, PCB126, PCB77, PCB169 and PCB81 (only Miramichi River tomcod for PCB81). Data are mean and 95% confidence interval expressed as fold induction over vehicle controls. Asterisk indicates significant deviation from corn-oil vehicle control. Curves fit by four-parameter logistic regression. TCDD—Miramichi River tomcod, r^2: 79.671%, $F_{3,57}=74.462$, $p<0.001$; parameters, minimum: 0.267, maximum: 63.763, EC_{50}: 738.802, hillslope: −0.880. Hudson River tomcod, insufficient data were obtained to plot dose–response curve. $N=5$–6 fish per point (Hudson), 5–8 (Miramichi except 12 for 5000 ppm). PCB126—Miramichi River tomcod; r^2: 59.0%, $F_{3,36}=17.267$, $p<0.001$; parameters, minimum: 7.290, maximum: 54.490, EC_{50}: 0.050, hillslope: −20.086. Hudson River tomcod, r^2: 70.50%, $F_{3,15}=11.952$, $p=0.003$; parameters, minimum: 0, maximum: 53.72, EC_{50}: 0.761, hillslope: −1.845. $N=4$ fish per point (Hudson except 11 for 1.0 ppm), 4–7 (Miramichi except 10 for 0.10 ppm). PCB77: neither Miramichi River nor Hudson River data could be fit by four-parameter logistic regression. $N=6$–9 fish (Miramichi), 4–5 fish (Hudson) per dose except 3 for 100 ppm (both populations). PCB169—neither Miramichi River nor Hudson River data could be fit by four-parameter logistic regression. $N=4$–5 fish per dose except seven for 0.5 ppm Miramichi. PCB81—Miramichi River data only. Data could not be fit by four-parameter logistic regression. $N=6$–7 fish per dose except 4 for 10 ppm.

4.2.5. PCB81

Response to PCB81 was examined only in Miramichi River tomcod. Dose responsive induction of hepatic CYP1A mRNA began at 0.1 ppm and was still increasing at the highest dose tested (10 ppm) ($F_{5,32}=39.904$, $p<0.001$; Tukey's test: $p<0.05$; Fig. 2) so an EC_{50} could not be calculated. The relative potencies of these HAHs as CYP1A inducers in Miramichi River tomcod were: TCDD > PCB126 > PCB77 > PCB81 > PCB169.

4.3. Hepatic CYP1A in tomcod treated with two AHR agonists: PCB77 and PCB126

PCB77 (0.5 ppm) and all doses of PCB126 produced significant induction of hepatic CYP1A over

controls in Miramichi River tomcod when administered alone ($F_{6,44} = 24.99$, $p < 0.001$; Tukey's test: $p < 0.05$; Fig. 3A). However, combinations of PCB77 and PCB126 did not induce greater CYP1A mRNA expression levels than the individual congeners. Specifically, induction produced by 0.5 ppm PCB77 was not augmented by simultaneous exposure to 0.001 or 0.01 ppm PCB126.

This experiment was repeated with the addition of a treatment of 0.5 ppm PCB 77 plus 0.1 ppm PCB 126. As in the previous experiment, treatment with PCB77 (0.5 ppm) and all doses of PC126 produced significant induction when administered alone ($F_{7,43} = 13.72$, $p < 0.001$; Tukey's test: $p < 0.05$) but co-administration of the two PCBs did not produce significantly higher induction than administration of PCB126 alone at any concentration (Fig. 3B). Only at a dose of 0.1 ppm PCB126 was there any indication of increased CYP1A expression.

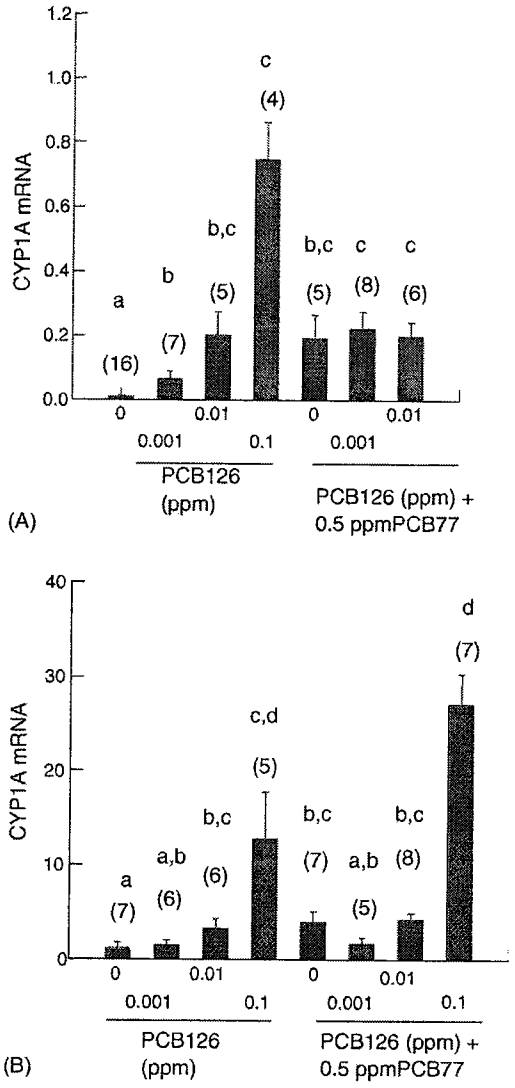

Fig. 3. Hepatic CYP1A mRNA expression in Miramichi River Atlantic tomcod i.p. injected with graded doses of PCB126 alone or in combination with 0.5 ppm PCB77. Bars represented means + 1 S.E. of raw data, sample size is shown above figure. Groups not differing significantly share a common letter (a–d) (Tukey's multiple range test following significant ANOVA on log transformed data). (A) and (B) experiments carried out on different groups of tomcod in different years.

4.4. Hepatic CYP1A in tomcod treated with an AHR agonist and an AHR-antagonist: PCB77 and PCB105

PCB77 (0.5 ppm) administered alone produced significant hepatic CYP1A mRNA induction in Miramichi River tomcod ($F_{5,35} = 30.35$, $p < 0.001$; Fig. 4A), but PCB105 did not at doses of 1 or 10 ppm when administered alone. Response to 0.5 ppm PCB77 appeared to be significantly reduced by simultaneous administration of PCB105 at a dose of 1 ppm, but not 10 ppm.

This experiment was repeated with the addition of treatments of 0.1 ppm PCB105 with and without co-administration of 0.5 ppm PCB77. As in the first experiment, PCB77 produced significant hepatic CYP1A induction in Miramichi River tomcod whereas PCB 105 administered alone did not ($F_{7,47} = 7.68$, $p < 0.001$; Fig. 4B). Also as observed in the first experiment, there was some suggestion that co-administration of low doses of PCB 105 (0.1 or 1.0 ppm), but not a high dose (10 ppm) reduced response to PCB77 though this effect was much less marked than in the first experiment.

5. Discussion

For the first time, we demonstrate that CYP1A mRNA expression is significantly inducible by several HAHs in tomcod from the Hudson River, but expression is two orders of magnitude less sensitive to induction than in tomcod from the cleaner Miramichi River (Figs. 2 and 3). Hepatic CYP1A mRNA expression was

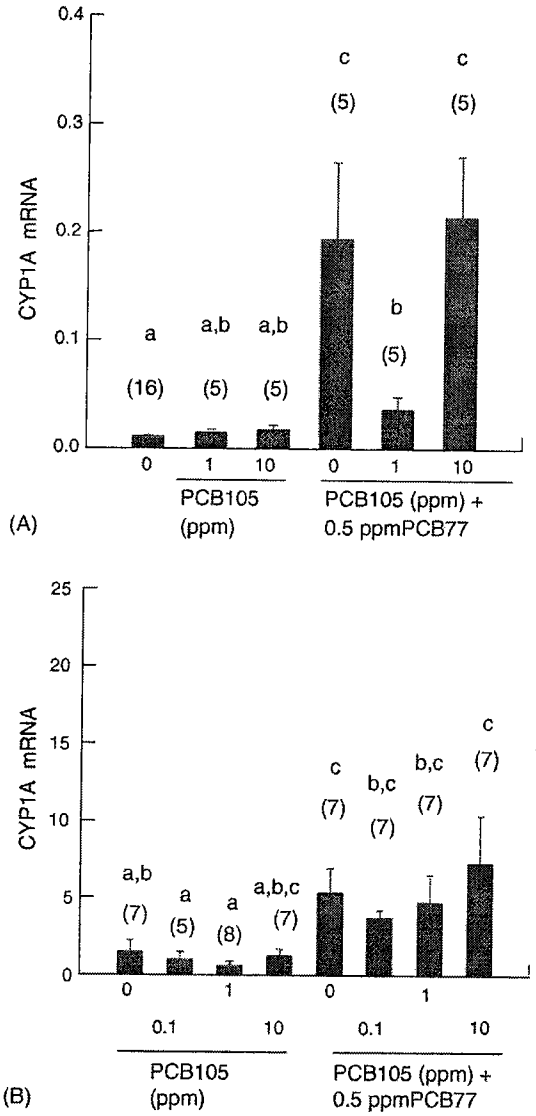

Fig. 4. Hepatic CYP1A mRNA expression in Miramichi River Atlantic tomcod i.p. injected with graded doses of PCB105 alone or in combination with 0.5 ppm PCB77. Bars represented means + 1 S.E. of raw data, sample size is shown above figure. Groups not differing significantly share a common letter (a–c) (Tukey's multiple range test following significant ANOVA on log transformed data). (A) and (B) experiments carried out on different groups of tomcod in different years.

approximately two orders of magnitude less sensitive to induction by TCDD and three coplanar PCBs in tomcod from the Hudson River compared to those from the Miramichi River. However, because of the high doses of PCBs needed to achieve maximum CYP1A1 expression in Hudson River tomcod, we were unable to compare quantitatively EC50 values for PCBs between tomcod from the two populations. This difference in CYP1A mRNA inducibility was also seen across a battery of extra hepatic tissues in tomcod from the Hudson River treated with PCB77 compared to those from the Miramichi River (Fig. 1). But, we were unable to quantify the difference between populations in extra heptic gene inducibility because significant induction of CYP1A was not achieved at the doses tested in tomcod from the Hudson River. Our results indicate that resistance to CYP1A induction may be manifest for all HAH agonists and in all tissues of tomcod from the Hudson River.

Maximum levels of CYP1A mRNA expression were not significantly different between tomcod from the two populations when achieved for both populations in this study with TCDD and PCB126. Because resistance is observed at the mRNA level, and CYP1A transcription is mediated by activation of the AHR pathway in all vertebrates including fishes (Hahn, 1998a), it is likely that alteration of AHR function, down regulation of pathway sensitivity to activation, has occurred in the Hudson River tomcod population. Because CYP1A was significantly inducible and to the same levels in tomcod from the two populations, it is likely that all components of the AHR pathway are present and fully functional in Hudson River tomcod, but are less sensitive to activation by AHR agonists. The fact that CYP1A mRNA inducibility by several PAHs is similar between tomcod from the Hudson River and cleaner locales (Wirgin and Waldman, 2004; Wirgin, unpublished data) supports this hypothesis.

Our results in this study suggest that selection for phenotypes that are less sensitive to CYP1A mRNA induction by HAHs or other AHR mediated toxicities has developed in the Hudson River population. In contrast, our studies with adult tomcod (Wirgin et al., 1992; Courtenay et al., 1999) and tomcod embryos (Yuan et al., in press; Wirgin et al., unpublished data) demonstrate no significant difference in CYP1A mRNA inducibility between tomcod from the Hudson River and two reference populations from cleaner locales that were treated with the PAH, B[*a*]P. It is likely that chronic exposure over the past six decades of Hudson River tomcod to PCBs and/or PCDD/Fs has resulted in the rapid development of genetic adap-

tation(s) in the population that serve to down regulate AHR pathway sensitivity to these compounds. Because PCBs and PCDD/Fs are refractory to metabolism and are bioaccumulated in livers and perhaps extra hepatic tissues, it is probable that exposure to these compounds and activation of AHR in naïve tomcod results in persistent uncoupling of the catalytic cycle of CYP1A and the generation of high levels of toxic reactive oxygen species (ROS) (Schlezinger et al., 1999). Thus, selective pressures in the Hudson River would favor an AHR pathway with reduced sensitivities to these halogenated aromatic compounds. In contrast, because PAHs are so rapidly metabolized in fish tissues including tomcod (Krahn et al., 1986), it is less likely that exposure to them would result in the persistent generation of high levels of ROS. An AHR pathway sensitive to PAH exposures would provide the ability to oxidize PAHs to metabolites that are less acutely cytotoxic than the parent compounds. Thus, we can envision distinctly different selection pressures and molecular responses in environmentally exposed Hudson River tomcod to halogenated versus non-halogenated AHs. We hypothesize that AHR-mediated sensitivity to HAHs is down regulated in the Hudson River tomcod population whereas sensitivity to PAHs is uncompromised compared to tomcod from cleaner locales. This would suggest that more than one molecular pathway mediates toxicities of AH compounds in tomcod.

AHR pathway function may be modulated at several steps in the process of activation of CYP1A transcription including binding of ligand, translocation of AHR-ligand complex to the nucleus, binding of AHR-ARNT complex to distal dioxin response elements (DREs) in the 5′ promoter region of CYP1A, or transactivation of proximal transcriptional elements (Hahn, 1998b). Data from this study do not allow us to distinguish among these possibilities, but our previous results have shown that the sensitivity of hepatic CYP1A mRNA inducibility by β-NF and B[*a*]P does not significantly differ between tomcod from the Hudson River and those from cleaner locales (Miramichi River (Wirgin et al., 1992; Courtenay et al., 1999) or Shinnnecock Bay, New York (Wirgin and Chambers, in press)). Variation between populations in CYP1A inducibility by HAHs, but not PAHs, suggests that population differences may lie in the ability of AHR to bind HAHs or that binding of halogenated ligands differentially alter the conformation of other functional domains downstream of the ligand binding domain in AHR. Hahn (1998b) has pointed out that mutations in AHR could selectively alter its affinity for some ligands, but not others. For example, variation in ligand binding affinity is the mechanistic basis of resistance to hepatic toxicity between sensitive and resistant strains of mouse (Okey et al., 1989). Also, fish and mammals share similar binding affinities for TCDD and coplanar PCBs (Lorenzen and Okey, 1990), but mono-*ortho*-substituted PCBs are potent AHR agonists in mammals, but are not in fishes. If other steps downstream in AHR activation of gene expression were impaired, CYP1A transcription would probably be modulated for all AHR agonists, not HAHs alone. Comparison of AHR cDNA sequences between tomcod from the Hudson River and more sensitive populations as described in Yuan (2003), combined with functional analysis of variant alleles should begin to address this question.

Atlantic killifish populations from several estuaries (New Bedford Harbor, Newark Bay, Hudson River, and Elizabeth River) along the Atlantic coast of North America that are highly contaminated with environmentally persistent HAHs and PAHs (and other contaminants) exhibit decreased inducibility of CYP1A at the transcriptional, translational and enzyme activity levels and are resistant to early life-stage toxicities elicited by these chemicals (Bello, 1999; Nacci et al., 1999; Elskus et al., 1999; Prince and Cooper, 1995a,b; Van Veld and Westbrook, 1995; Ownby et al., 2002). How does the manifestation of resistance, including reduced CYP1A inducibility, compare between tomcod and killifish? Similar to our results in tomcod, resistance to CYP1A mRNA and protein expression was seen across four extra hepatic tissues in killifish from PCB-contaminated New Bedford Harbor (Bello et al., 2001). Furthermore, it was demonstrated that the AHR pathway is fully functional in hepatocytes of killifish from New Bedford Harbor in in vitro experiments with graded doses of two AHR agonists (TCDD and β-NF). Similar to tomcod from the Hudson River, CYP1A expression was one to two orders of magnitude less sensitive to induction than in conspecifics from a non-resistant population (Bello et al., 2001). Maximum levels of in vitro CYP1A expression in primary hepatocytes were not significantly different between killifish from resistant and sensitive populations. However, unlike tomcod, killifish from New Bedford Harbor were resistant to both HAHs (PCB126, TCDD,

and 2,3,7,8-tetrachlorodibenzofuran) and PAHs (3-methylcholanthrene and B[*a*]P) (Nacci et al., 1999, 2002a,b; Bello et al., 2001), but perhaps not as much to another PAH, β-NF (Bello et al., 2001). Killifish from the creosote-contaminated Elizabeth River exhibited similar levels of resistance to a PAH (Meyer et al., 2002) and PCB126 (Meyer and Di Giulio, 2002) despite the absence of sediment contamination with PCBs or PCDD/Fs in the Elizabeth River. This suggests that manifestations of resistance and their molecular bases are often similar, but not identical, between tomcod and killifish. Furthermore, molecular mechanisms of resistance may not be identical even among populations within a single species.

The "resistant hepatocyte model" has been proposed as a physiological strategy for survival in the face of exposure to high levels of hepatotoxic contaminants (Farber, 1990; Farber and Rubin, 1991; Yusuf et al., 1999). These investigators proposed that development of relatively rare, but resistant, hepatic preneoplastic nodules allow for organismic survival in the face of further exposure to a broad array of hepatotoxicants. It was proposed that this evolutionary strategy is beneficial for xenobiotically-challenged populations because it permits the survival and reproduction of individuals prior to the onset of cancer induced mortality (Farber, 1990). For example, Hudson River tomcod spawn at age-1 and cancer-related mortality presumably does not occur until after they have reproduced one time (Dey et al., 1993). But, resistance to CYP1A inducibility in tomcod is significantly decreased in all tissues, not just those that bioaccumulate high levels of HAHs. Resistance in tomcod is also seen in early life-stages to toxicities induced by PCBs and TCDD, not just in tissues of adult fish (Wirgin and Chambers, in press). Thus, resistance in tomcod, in all likelihood represents a genetic adaptation in a pathway central to metabolic processing or toxic response to these xenobiotics rather than an example of physiological acclimation as proposed in the "resistant hepatocyte" model.

Tomcod from the Hudson River Estuary bioaccumulate high levels of mixtures of PCDD/Fs and PCBs in their livers that include congeners that are coplanar and others that are mono- or -di-*ortho*-substituted and are potentially AHR antagonists (Fernandez et al., 2004). We have previously demonstrated that there was no significant relationship between levels of hepatic CYP1A mRNA in environmentally-exposed juvenile tomcod from sites in the Hudson River and their hepatic burdens of TCDD TEQs (a summary measure of exposure to AHR agonists) (Yuan et al., 2001). We hypothesized that this might result from their resistance to AHR inducibility by HAHs, their bioaccumulation of compounds that significantly modulate CYP1A inducibility, or a combination of both factors. Thus, it is possible that environmental mixtures of bioaccumulative PCBs may include congeners that antagonize the response to AHR agonists.

Studies in mammalian cells have shown that CYP1A1 mRNA, protein, and enzyme inducibility by TCDD (Safe, 1997–1998; Chen and Bunce, 2004), and sometimes coplanar PCBs (PCB126 and PCB77) (Suh et al., 2003), are significantly inhibited by co-treatment with di-*ortho*-substituted and less so by mono-*ortho*-substituted PCB congeners. In rainbow trout cells *Oncoryhnchus mykiss*, Zabel et al., (1996) reported that PCB105 along with several other mono-*ortho*-substituted PCBs were partial agonists of CYP1A induction and that congeners acted additively in HAH mixtures. But, Hesterman et al. (2000) observed that PCB105 is an AHR antagonistic in another fish cell line due to both reduced affinity binding to AHR and decreased intrinsic efficacy of activated AHR complex in inducing gene expression.

We show, that in vivo in tomcod, PCB105, a mono-*ortho*-substituted congener can significantly antagonize CYP1A mRNA induction by PCB77, an AHR agonist. As expected, co-treatment of tomcod with PCB126, an AHR agonist did not diminish PCB77 induction. Because of the high levels of mono- and -di-*ortho* substituted PCBs in livers of Hudson River tomcod (Yuan et al., 2001), it is not unreasonable to speculate that bioaccumulation of these compounds has impaired CYP1A inducibility and has contributed to the lack of a significant relationship between hepatic burdens of HAHs expressed as TCDD, TEQs and CYP1A mRNA levels in environmentally exposed tomcod from the Hudson River.

In summary, for the first time we report that hepatic CYP1A mRNA is significantly inducible by coplanar PCBs and TCDD, but two orders of magnitude less so in tomcod from the Hudson River than in those from a cleaner locale. These results suggest that the AHR pathway is fully functional, albeit less sensitive to activation, in tomcod from the Hudson River than those from reference populations. This significant difference