BIOMARKERS, 2002, VOL. 7, NO. 2, 162–173

Cytochrome P4501A1 is induced by PCB 77 and benzo[*a*]pyrene treatment but not by exposure to the Hudson River environment in Atlantic tomcod (*Microgadus tomcod*) post-yolk sac larvae

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Received 2 July 2001, revised form accepted 9 November 2001

In fish, the embryos and larvae are the life-stages most sensitive to damage from environmentally borne dioxin-like compounds and polycyclic aromatic hydrocarbons (PAHs). Methods are not routinely available to measure the body burdens of contaminants in embryos and larvae, thus precluding the investigation of links between exposure and biological effects. Quantification of expression of cytochrome P4501A1 (CYP1A1) provides an index of relative exposure of natural populations to bioavailable aromatic hydrocarbons (AH) and an initial evaluation of their biological effects. We developed a quantitative approach to standardize total RNA loading and then used competitive reverse transcriptase-polymerase chain reaction (RT-PCR) to quantify the CYP1A1 mRNA expression in environmentally exposed Atlantic tomcod (*Microgadus tomcod*) post yolk-sac larvae (postlarvae) from the Hudson River, New York, and in chemically treated postlarval offspring of controlled laboratory crosses of Hudson River parents. Significant induction of CYP1A1 expression was observed in tomcod postlarvae exposed to waterborne 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) (four-fold) and benzo[*a*]-pyrene (eight-fold) compared with vehicle-exposed controls. In contrast, CYP1A1 was not induced in Hudson River-exposed postlarvae compared with vehicle-exposed controls. This study demonstrates the feasibility of using competitive RT-PCR for the measurement of gene expression in environmentally exposed larvae of sentinel species, and is consistent with the hypothesis that postlarvae exposed to the Hudson River environment have not bioaccumulated sufficient levels of AHs to induce CYP1A1 expression. The high levels of hepatic CYP1A1 mRNA expression previously reported in 5–8 month old juvenile tomcod from the Hudson River coincides with their descent to the benthic habitat and the onset of independent feeding on AH-contaminated benthic prey.

Keywords: competitive RT-PCR, cytochrome P4501A1, PCBs, PAHs, postlarvae, biomarkers.

Introduction

Aromatic hydrocarbons (AHs) are ubiquitous lipophilic contaminants that concentrate in the sediments of contaminated aquatic environments. Persistent halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls

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Biomarkers ISSN 1354–750X print/ISSN 1366–5804 online © 2002 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500110113981

(PCBs), dioxins (PCDDs) and furans (PCDFs), biomagnify in the upper trophic levels of polluted ecosystems, whereas polycyclic aromatic hydrocarbons (PAHs) are so rapidly metabolized in fish livers that they usually cannot be detected (Varanasi and Stein 1991). PCDDs, PCDFs, co-planar PCBs (Van den Berg *et al.* 1998) and PAHs (Diamond *et al.* 2000) are known to elicit toxicity in early life-stages of fish either through maternal transfer of contaminants (Walker *et al.* 1994) or by direct exposure of embryos and larvae to polluted environmental matrices. The early life-stages of many fish species are sensitive to AH-induced mortality and teratogenicity (Elonen *et al.* 1998) and these toxicities have been correlated with decreased recruitment to natural populations (Walker *et al.* 1991, 1994). Based on structure–activity relationships, early life-stage toxicities in fish due to PCDDs, PCDFs and PCBs are believed to be mediated by the aryl hydrocarbon receptor (AhR) pathway (Van den Berg *et al.* 1998).

Cytochrome P4501A1 (CYP1A1) induction is also mediated by the AhR pathway and thus its expression may serve as a surrogate measure for other toxic responses to AHs mediated by AhR. CYP1A1 induction in fishes is widely used as an index of bioavailable AH contamination of aquatic ecosystems, since CYP1A1 expression in naive fishes is usually dose-responsive at the transcriptional, protein and enzymatic activity levels to environmentally relevant concentrations of AHs (Stegeman and Hahn 1994, Courtenay *et al.* 1999). Expression of AhR, induction of CYP1A1 (Wang *et al.* 1998, Roy *et al.* 2001) and bioactivation of PAHs to DNA-reactive metabolites (Fong *et al.* 1993) have all been demonstrated in fish embryos and larvae.

CYP1A1-encoded monooxygenases convert environmental PAHs to reactive electrophiles that can adduct to and damage DNA. Early life-stages of fish are also sensitive to PAH photoactivated toxicity (Diamond *et al.* 2000). PCDDs, PCDFs and PCBs are refractory or very slowly metabolized, but induction of CYP1A1-encoded activities by these persistent HAHs may still elevate levels of damage to DNA and other cellular constituents through the generation of reactive oxygen species (Schlezinger *et al.* 1999).

A competitive reverse transcriptase-polymerase chain reaction (cRT-PCR) method has been developed (Campbell and Devlin 1996) and used (Campbell *et al.* 1996, Campbell and Devlin 1997) to quantify CYP1A1 mRNA expression in salmonid fishes. Using this approach, hepatic and gonadal CYP1A1 expressions were quantified in β-naphthoflavone, coal dust and bleached kraft mill effluent-treated chinook salmon *Oncorhynchus tshawtscha* and in 3-methylcholanthrene-treated pooled ovarian follicles from mature coho salmon *Oncorhynchus kisutch*. This approach was also used to measure expression of CYP1A1 in PCB- and TCDD-treated killifish *Fundulus heteroclitus* larvae (Powell *et al.* 2000) and lake trout *Salvelinus namaycush* embryos and larvae (Guiney *et al.* 2000).

Atlantic tomcod is a bottom-dwelling fish species with a lipid-rich liver that is very common in estuaries along the Atlantic coast of North America from the Hudson River, New York, to southern Labrador. Tomcod move seasonally within their natal estuaries but do not exit into coastal waters. Juvenile and adult tomcod from the Hudson River exhibit very high hepatic levels of PCDDs, PCDFs and PCBs compared with tomcod from elsewhere (Courtenay *et al.* 1999, Yuan *et al.* 2001). Unfertilized eggs from Hudson River females also show higher levels of PCDDs, PCDFs and PCBs than tomcod eggs from cleaner estuaries in

Atlantic Canada, but not as high as those in liver on a wet weight basis (Roy *et al.* 2001).

Tomcod spawn in the middle freshwater reaches of the Hudson River estuary from approximately river mile (RM) 25, (i.e. miles above the southern tip of Manhattan Island) to RM 75 between late December and the end of January, with the epicentre of spawning occurring between RM 47 and RM 55 (Klauda *et al.* 1988, Dew and Hecht 1994). After fertilization, eggs are demersal and have an incubation period from 36–42 days (McLaren *et al.* 1988) to 60–65 days (Dew and Hecht 1994). Tomcod are 5–6 mm at hatching, which begins in early February, peaks in early March and is completed by mid March (Dew and Hecht 1994). Newly hatched larvae appear capable of swimming into the water column and reaching the surface (Peterson *et al.* 1980), although peak densities of yolk sac larvae may occur on the bottom (Dew and Hecht 1994). The proportion of larvae found at the surface increases with age (Dew and Hecht 1994). Maximum densities of post-yolk sac larvae (postlarvae) occur from early to mid April, and transformation to the early juvenile life-stage occurs from late April through to mid May (Klauda *et al.* 1988). The vertical distribution of postlarvae may include the entire water column and their horizontal distribution reflects both passive movements and active migration (Klauda *et al.* 1988). Transition from postlarval to early juvenile stage is defined by the completion of fin ray development. Juveniles are most abundant in the first 3 weeks in May and are restricted to the benthos throughout this and subsequent adult life-stages.

Studies have shown elevated levels (up to 28-fold) of hepatic CYP1A1 mRNA expression in adult tomcod from the Hudson River compared with adult tomcod from four other less contaminated Atlantic coast estuaries (Kreamer *et al.* 1991, Wirgin *et al.* 1994). Further studies revealed significant spatial heterogeneity of hepatic CYP1A1 mRNA levels among samples of juvenile tomcod (5–8 months old) collected from late May to early August from multiple sites in the lower Hudson River estuary (Yuan *et al.* 2001). However, in controlled laboratory experiments, resistance to hepatic CYP1A1 mRNA induction by HAHs (co-planar PCBs and tetrachlorodibenzo-*p*-dioxin [TCDD]), but not by PAHs (Wirgin *et al.* 1992, Courtenay *et al.* 1999, Yuan *et al.* unpublished data) was observed in adult tomcod. Modulation of expression of CYP1A1 in environmentally exposed Hudson River adults may be due to chronic exposure to high levels of AHs and other contaminants (Yuan *et al.* 2001).

In order to determine the effects of AHs on early life-stage survivorship, development, prevalence of liver lesions and adult abundance in tomcod from the Hudson River population, it was necessary to evaluate the bioavailability and early biological effects of AHs on environmentally exposed early life-stages of tomcod from the Hudson River. We postulated that CYP1A1 mRNA expression in larvae will provide a semi-quantitative measure of their exposure to bioavailable AH contaminants. Because of their small size and the limited yield of total RNA, it was necessary to modify available procedures to permit an evaluation of the integrity of RNAs and to express CYP1A1 mRNA levels per concentration of total RNA. We then proceeded to validate the applicability of this approach by quantifying CYP1A1 mRNA expression in tomcod postlarvae that had been chemically-treated with waterborne benzo[*a*]pyrene (B[*a*]P) and 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) or environmentally exposed to the Hudson River environment.

Materials and methods

Tomcod collections and experimental matings

Atlantic tomcod parents were collected from the Hudson River at Garrison, New York (RM 50), in January 2000. Eggs were extruded from four females and fertilized at 6°C with the sperm expressed from four males (1:1) and fertilized eggs were pooled. Embryos and larvae were reared in water of 5 p.p.t. salinity at 6°C for 60 days before exposure to chemicals. Postlarvae were exposed in dishes to B[*a*]P (Sigma, St Louis, Missouri, USA) (1 p.p.m.) and PCB 77 (Ultra Scientific, North Kingstown, Rhode Island, USA) (1 p.p.m.) dissolved in acetone in 200 ml diluted seawater at 6°C for 2 days and 7 days, respectively, and then sacrificed in mid March 2000. Control fish were exposed to water-borne acetone (12 µl in 200 ml seawater) for 2 days and sacrificed. Twenty larvae from each treatment group were flash frozen individually in 1.5 ml microcentrifuge tubes. Additionally, two groups of four to six larvae from each treatment group were pooled and also frozen in 1.5 ml tubes.

Environmentally exposed tomcod postlarvae ($n = 34$) were collected with bongo nets on 1 April 2000 from six sites in the Hudson River between RM 7 and RM 14 and immediately sacrificed. Once collected and sacrificed, postlarvae were placed in individual 1.5 ml microcentrifuge tubes, frozen immediately in liquid nitrogen, and maintained at −70°C until processing.

RNA isolations

RNAs were isolated as described in Roy *et al.* (2001). Briefly, 500 µl of Ultraspec reagent (Biotecx, Houston, Texas, USA) was added to each microcentrifuge tube containing a single larva or multiple larvae, the larvae were homogenized with a micropestle, the homogenates were incubated on ice for 10 min, 100 µl of chloroform was added, and then the homogenates were vortexed and incubated on ice for 10 min and centrifuged at 15,000 *g* for 15 min at 4°C. The aqueous phase was transferred to a new tube, mixed with an equal volume of ice-cold isopropanol, and incubated on ice for 45 min. RNA was pelleted by centrifugation at 15,000 *g* for 15 min at 4°C, pellets were washed twice with ice-cold 70% ethanol, centrifuged at 15,000 *g*, air-dried, resuspended in 50 µl of diethyl pyrocarbonate (DEPC)-treated water and stored at −70°C until use.

Total RNA quantification

We were unable to reliably determine total RNA concentrations from larval samples using ultraviolet spectrophotometry because absorbance values were too low. As an alternative, we used RNA slot blot analysis to quantify 18 S rRNA hybridization intensities in larval RNA samples and compared this with 18 S rRNA hybridization signals on the same membranes for four dilutions of adult tomcod liver samples for which total RNA concentrations had been determined previously by ultraviolet spectrophotometry.

Slot blot hybridizations

Larval and adult liver RNA samples were brought to a volume of 12.5 µl with DEPC-treated H_2O, and incubated in 1 M glyoxal, 50% (v/v) dimethyl sulphoxide and 10 mM sodium phosphate buffer, pH 7.0, at 50°C for 1 h. Samples were cooled on ice for 30 s and 10 × saline sodium citrate (SSC) stock was added to a final concentration of 6 × SSC, and the volume was adjusted to 200 µl with DEPC-treated H_2O. Larval RNAs were then applied with an Schleicher & Schuell (S & S, Keene, New Hampshire, USA) Slot Blot manifold onto S & S Nytran Nylon Plus membranes under vacuum along with four amounts (0.5, 2.0, 5.0 and 12.5 ng) of previously characterized adult hepatic RNAs. Slots were washed three times with 6 × SSC under vacuum, and the membranes were air-dried and then baked under vacuum for 1.5 h.

Membranes were prehybridized for 2 h at 65°C, hybridized overnight at 65°C to a ^{32}P radiolabelled rat 18 S rRNA probe (pHRR118) (Chan *et al.* 1984) and washed exactly as described in Wirgin *et al.* (1994). The hybridization signal for 18 S rRNA for each larval and liver sample was quantified using a Molecular Dynamics Phosphor Imager. A standard curve of total adult liver RNA versus rRNA hybridization signal was generated for each slot blot and used to calculate total RNA concentrations for each larval RNA. This approach allowed us to express CYP1A1 mRNA levels per nanogram of total RNA.

Northern blot hybridizations to evaluate RNA integrity

The integrity of each RNA sample was evaluated by northern blot hybridizations using ^{32}P radiolabelled pHRR118 18 S rRNA probes (figure 1). Each total RNA was electrophoresed in 1.0% denaturing agarose gels at 120 V for 2 h, transferred overnight by capillary action to Nytran Nylon Plus membranes, vacuum baked, prehybridized, hybridized, washed and autoradiographed as described in Courtenay *et al.* (1999). The integrity of the 18 S rRNA bands was qualitatively evaluated from the X-ray films.

cRT-PCR analysis

An internal, truncated, tomcod CYP1A1 mRNA standard was developed as described in Roy *et al.* (2001). The truncated standard mRNA was identical to the native tomcod CYP1A1 mRNA except for a 97 nucleotide deletion extending from nucleotide 1156 to 1252 of the tomcod cDNA sequence (Roy *et al.* 1995). The upstream PCR primer (U2) was designed to anneal to nucleotides in both exons 4 and 5 and span intron 4, and the downstream primer (D2) was exclusively in exon 7. This design prevented the amplification of contaminating genomic DNA.

For each larval sample, four dilutions of RNA were mixed with a fixed amount (0.2 pg) of the truncated CYP1A1 standard in 10 µl volumes. Then 2 µl of random hexamers (Invitrogen) (total 150 pmol) were added, reactions were incubated at 75°C for 5 min to reduce secondary structures, and chilled on ice for 5 min. Reverse transcriptase mixture was added to each reaction to final concentrations of 1 × Moloney murine leukaemia virus (M-MLV) reverse transcriptase buffer, 0.5 mM deoxynucleotides (dNTPs), 4 U of RNasin ribonuclease inhibitor and 20 U of M-MLV reverse transcriptase (Promega), and reactions were incubated at 42°C for 1 h. Reaction products were denatured at 98°C for 5 min, chilled on ice for 5 min and an aliquot was subjected to PCR amplification. PCR reactions were in 25 µl volumes containing 1 × reaction buffer (Boehringer Mannheim, Indianapolis, USA), 0.2 mM dNTPs, 1 µM of each of the CYP1A1 primers derived from the tomcod CYP1A1 sequence reported in Roy *et al.* (1995) (U2: 5′-CACCAGGAGATCAAGG-3′; D2: 5′-CTGCAGATATAGCA-GACAG-3′) and 0.5 U of Taq DNA polymerase (Boehringer Mannheim). Reaction conditions were one cycle for 5 min at 95°C, 30 cycles for 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, and a final extension for 7 min at 72°C.

Reaction products were electrophoresed in 1.2% agarose gels, stained with ethidium bromide and computer imaged with a Kodak Edas 290 camera (figure 2). Band intensities of the truncated control standard and native CYP1A1 bands were determined from computer images using Kodak 1D Image Analysis Software (Kodak 1D 3.5.2 USB).

Calculations of CYP1A1 mRNA in larval samples were made from the relationship $(A/B) \times C$, where A and B are the intensity of the products from total larval RNA and competitive truncated RNA standard, respectively, and C is the picogram amount of the control RNA used in the reverse transcriptase reaction. Consideration was not given to the size of the CYP1A1 mRNA to calculate the number of picograms present.

Data standardization

Relative concentrations of CYP1A1 mRNA were found to be replicable, though estimates of absolute concentrations varied considerably between cRT-PCR runs. That is, in any cRT-PCR run B[*a*]P-treated postlarvae would show much higher levels of CYP1A1 mRNA than controls, but readings overall might be considerably higher in one run than another. Therefore, it was necessary to derive correction factors to compare data collected in different runs. cRT-PCR readings of CYP1A1 mRNA concentrations in wild-caught Hudson postlarvae and laboratory-reared postlarvae exposed to B[*a*]P, PCB 77 or acetone vehicle were standardized with correction factors derived as follows. Laboratory controls and 11 of the 34 wild-caught larvae were analysed simultaneously. Control levels were then made comparable to wild-caught levels by multiplying individual control levels by the ratio of the mean CYP1A1 mRNA concentration in wild-caught postlarvae in the common analysis to the mean RNA concentration in the previous analysis of those same wild-caught postlarvae (i.e. 24.008/4.147 = 5.789). Similarly, CYP1A1 mRNA concentrations in B[*a*]P and PCB 77-exposed postlarvae were made comparable to concentrations in wild-caught postlarvae by multiplying them by the ratio of the control levels, corrected as above, to the levels of controls analysed with the B[*a*]P and PCB 77-exposed postlarvae (i.e. 39.049/1.882 = 20.749).

Statistical comparisons

CYP1A1 mRNA concentrations in wild-caught and laboratory-exposed postlarvae were log transformed to improve normality and then compared by analysis of variance (ANOVA) followed by the Tukey multiple range test. Postlarvae sampled from RM 7–9 were pooled to form a group of six centered around RM 8. Differences between the laboratory control and environmentally exposed or chemically treated groups are reported when $p < 0.05$. Means and 95% confidence intervals for each group were back-transformed for presentation.

Comparability of cRT-PCR and slot blot analyses in quantifying CYP1A1 mRNA

The comparability of cRT-PCR and slot blot analyses in quantifying relative levels of CYP1A1 expression was evaluated in two pooled subsets of each of the chemically treated (B[*a*]P and PCB 77) and control (acetone) larval samples. RNAs from each of the six pools were blotted to a Nytran membrane, hybridized to a ^{32}P radiolabelled, β-naphthoflavone induced, tomcod CYP1A1 cDNA probe (Roy *et al.* 1995) and washed as described in Wirgin *et al.* (1994) and Courtenay *et al.* (1999).

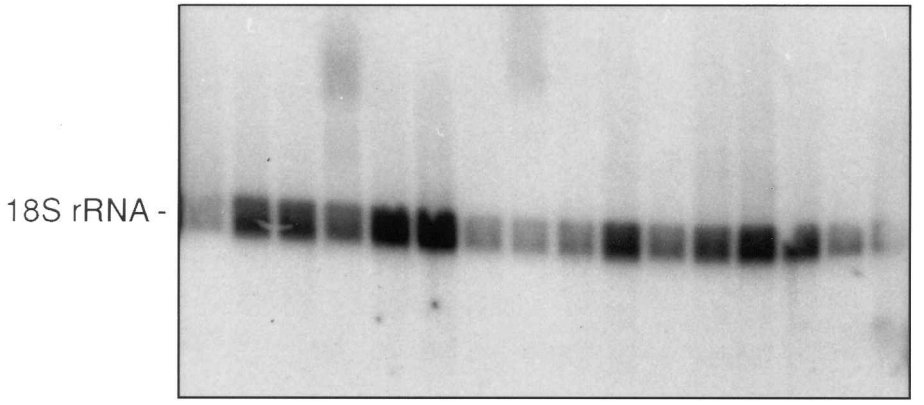

Figure 1. An autoradiograph of a Northern blot hybridization of total RNA from an individual tomcod larva hybridized to a ^{32}P radiolabelled rat 18 S rRNA probe (pHRR118). Hybridizations were done to evaluate the integrity of all larval RNA preparations.

Results

The major problem in the use of young life-stages or very small tissue samples in quantitative gene expression assays is standardization of the total RNA amounts that are used as templates in the cRT-PCR reactions. For example, amounts of total RNA isolated from individual tomcod yolk-sac larvae analysed in an earlier study varied between 765 and 1975 ng, with a mean of 1267.5 ± 149.3 ng (Roy *et al.* 2001). Generally, slot blot analysis requires a minimum of 1–3 mg of total RNA and northern blots considerably more. As a result, we were unable to obtain sufficient amounts of total RNA from individual larvae to use ultraviolet spectrophotometry to reliably quantify total RNA concentrations. Slot blot hybridization with an 18 S rRNA probe to total hepatic RNAs of known concentrations isolated from adult tomcod allowed us to generate membrane-specific standard curves that were used to quantify the amounts of total RNAs in individual larval samples. Validity of this approach is based on the assumption that the relative concentrations of 18 S rRNAs remain constant during development and among treatment groups. Furthermore, the use of northern blot hybridizations with the 18 S rRNA probe allowed us to evaluate the integrity of each RNA sample.

Results from controlled laboratory exposures validated the use of CYP1A1 gene expression in assessing the exposure history of environmentally exposed postlarvae. As assessed by cRT-PCR, basal levels of gene expression in postlarvae reared in the laboratory and exposed to acetone vehicle were uniformly low and showed relatively low levels of interindividual variability (figure 2). Postlarvae reared in the laboratory and exposed in mid March to waterborne B[*a*]P (1 p.p.m.) or PCB 77 (1 p.p.m.) showed significant eight-fold and four-fold CYP1A1 mRNA induction, respectively, over controls exposed to acetone vehicle alone ($F_{6,69} = 38.54$, $p < 0.001$, Tukey test) (figure 3). With the exception of one larva, there was no overlap in the levels of CYP1A1 expression between vehicle-exposed and chemically exposed larvae. In contrast, tomcod postlarvae sampled from the Hudson River between RM 7 and RM 14 on 1 April showed no evidence of CYP1A1 mRNA induction, with levels of gene expression comparable to, or slightly lower than ($p = 0.029$, Tukey test), the laboratory controls.

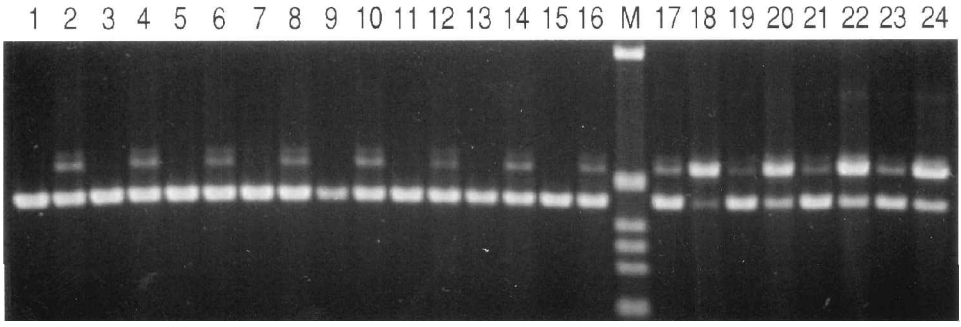

Figure 2. Photograph of an ethidium bromide stained 1.2% agarose gel containing products of cRT-PCR reactions of tomcod post yolk sac larval RNA samples and a truncated tomcod CYP1A1 mRNA standard. Variable amounts of total larval RNA (1 and 10 µl of a 1:20 dilution) and a constant amount (0.2 pg) of truncated CYP1A1 mRNA standard were used in each cRT-PCR reaction. Reactions in lanes 1–8 contained RNA from larvae that were exposed to the Hudson River environment, lanes 9–16 contained RNA from vehicle (acetone)-exposed larvae, and lanes 17–24 contained RNA from B[*a*]P-exposed (1 p.p.m.) larvae. Lane M contains a 1 kb DNA ladder (Life Technologies).

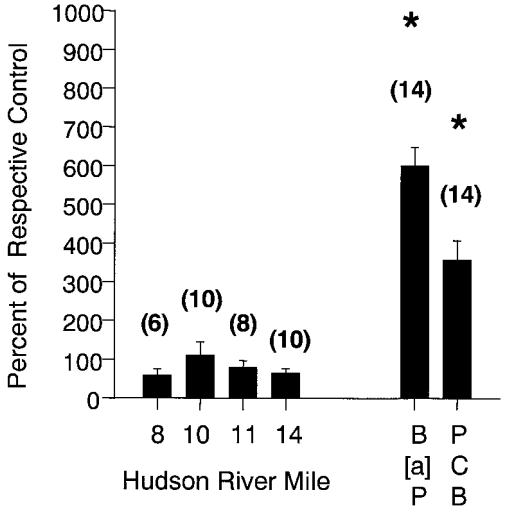

Figure 3. CYP1A1 mRNA concentrations in Hudson River tomcod postlarvae sampled from RM 8–14 of the Hudson River estuary on 1 April 2000, or hatched in the laboratory and exposed in mid March 2000 to waterborne B[*a*]P (1 p.p.m.), PCB 77 (1 p.p.m.) or acetone vehicle alone (control). RM 8 represents pooled samples from RM 7–9. The bars represent the mean CYP1A1 mRNA concentrations for the sample sizes shown over the bars in parentheses, and the vertical lines are 95% confidence intervals around means. Means and confidence intervals are back-transformed from the log-transformed units on which statistical analysis was carried out. Asterisks indicate CYP1A1 mRNA concentrations significantly different from laboratory controls (Turkey test $p < 0.05$, following ANOVA: $F_{6,69} = 38.54$, $p < 0.001$).

Slot blot analysis of CYP1A1 mRNA expression of two pools of 4–6 larvae from each treatment group provided results that were similar to those from cRT-PCR. Mean levels of CYP1A1 expression in the PCB 77- and B[*a*]P-treated groups were 12- and two-fold induced, respectively, over vehicle-exposed controls.

Discussion

The absence of significant gene induction in Hudson River-exposed postlarvae compared with chemically exposed and unexposed control larvae was surprising and may provide initial insights into the timing and routes of exposure that induce CYP1A1 expression and perhaps AhR-mediated toxic responses in environmentally exposed juvenile and adult life-stages. In controlled laboratory experiments, postlarval tomcod just slightly younger than the Hudson River-exposed larvae investigated in this study were capable of expressing significantly induced CYP1A1 mRNA after waterborne exposures to B[*a*]P and PCB 77. These results are consistent with those previously reported in Japanese medaka *Oryzias latipes* and common killifish *Fundulus heteroclitus*, in which laboratory exposures to waterborne 2,3,7,8-TCDD and coplanar PCBs significantly induced CYP1A1 expression in embryos and larvae, respectively (Wang *et al.* 1998, Powell *et al.* 2000). Similarly, significant induction of CYP1A1 mRNA was observed in tomcod yolk sac larvae of Hudson River parentage that were exposed to waterborne B[*a*]P or PCB 77 (Roy *et al.* 2001). Thus, CYP1A1 mRNA expression is apparently inducible by AHs in young life-stages of fish, including tomcod of Hudson River origin. Our results argue that tomcod postlarvae in the Hudson River have not accumulated sufficiently high levels of AH contaminants to induce CYP1A1 expression. This suggests that waterborne concentrations of AHs in the Hudson River or their bioavailability to postlarvae in Hudson River is insufficient to induce CYP1A1.

However, several other factors should be considered that may help explain the absence of induced CYP1A1 in Hudson River-exposed postlarvae. Because PAHs are so rapidly metabolized in fish tissues (Varanasi and Stein 1991), it is possible that environmental exposures to AHs in the Hudson River are episodic and that PAHs were metabolized and depurated several days prior to their collection for this study. Of course, this argument would not apply to the more persistent HAHs that have been observed in high concentrations in the eggs and livers of Hudson River tomcod (Courtenay *et al.* 1999, Roy *et al.* 2001). In addition, it has been demonstrated that hepatic CYP1A1 mRNA inducibility with individual PCB congeners and TCDD in adult Hudson River tomcod is reduced compared with conspecifics from elsewhere (Courtenay *et al.* 1999), perhaps due to a combination of genetic adaptation and physiological acclimation (Roy *et al.* 2001). However, the induction of CYP1A1 in this study with PCB 77 exposure in tomcod postlarvae of Hudson River descent indicates that CYP1A1 inducibility is not significantly compromised in Hudson River postlarvae due to genetic factors. Additionally, results from studies in which fish primary cells (Risso-De Faverney *et al.* 2000) and fish *in vivo* (including tomcod) (Sorrentino and Wirgin unpublished data) were co-exposed to metals and AhR agonists in controlled laboratory experiments demonstrated that CYP1A1 induction was abolished or reduced compared with that for AHs alone. Therefore, it can be suggested that the absence of induced CYP1A1 mRNA in Hudson River-exposed tomcod resulted from modulated CYP1A1 inducibility resulting from physiological acclimation due to chronic exposure to mixtures of contaminants. However, the induction of high levels of hepatic CYP1A1 mRNA in Hudson River-exposed juvenile (Yuan *et al.* 2001) and adult tomcod (Wirgin *et al.* 1994) indicates that the gene is inducible in this population despite its exposure history.

Our results suggest that the maternal contribution of AHs to eggs has been depurated by either mobilization of contaminated lipid reserves in the yolk or diluted by larval growth. Previous analysis has demonstrated that unfertilized eggs from female Hudson River tomcod contain relatively high levels of PCDDs, PCDFs and PCBs compared with tomcod eggs from elsewhere. For example, wet weight concentrations of 2,3,7,8-TCDD TEQs (Toxic Equivalency Quotients) from total PCDDs and PCDFs (2,3,7,8-TCDD TEQs = 8) were eight-fold higher than in tomcod eggs from the cleaner Miramichi River (Roy *et al.* 2001). Based on controlled dose–response experiments with AHs in adults (Courtenay *et al.* 1999), and assuming equivalent CYP1A1 inducibility in post-larvae as in adults, levels of AHs in eggs should be sufficient to induce CYP1A1 expression in postlarvae. Thus, during early embryonic and larval development, burdens of AHs must have decreased to levels that were no longer sufficient to induce CYP1A1 expression.

A comparison of gene expression in the Hudson River-exposed tomcod postlarvae to that previously reported in environmentally exposed older juveniles (5–8 months old) from approximately the same locales in the Hudson River suggests that the transformation between larval and juvenile life-stages is associated with significantly increased exposure to AHs. For example, CYP1A1 mRNA expression was induced 22-fold in juvenile tomcod collected in July from Hudson River RM 10 compared with matched, unexposed juvenile controls that had been collected from the Hudson River and depurated extensively in clean laboratory water for 60 days before being sacrificed (Yuan and Wirgin unpublished data). This increase in the bioavailability of AHs in juvenile tomcod is associated with a shift in the vertical distribution of the tomcod from mid-water to the benthos, which results in direct dermal contact of juveniles with contaminated sediments and presumably a change in diet from planktonic to benthic invertebrates.

Calanoid copepods are the prevalent prey of Hudson River juvenile tomcod (including postlarvae) before July (Nittel 1976, Grabe 1978, 1980, McLaren *et al.* 1988). By July the diet becomes more diverse, encompassing harpacticoid copepods, the infaunal isopod *Cyathura polita*, *Neomysis* species including the opossum shrimp *Neomysis americana*, and amphipods including the *Gammarus*, *Monoculodes* and *Chaoborus* species. The epibenthic sand shrimp *Crangon septemspinosa* may also become important prey in fall. A shift from large prey such as amphipods and sand shrimp back to copepods occurs with the onset of winter and gonadal maturation in December (Grabe 1978, Bulak and Reidinger 1980).

To our knowledge AH levels have not been quantified in any species upon which tomcod prey in the Hudson River. Gagnon *et al.* (1990) conducted congener-specific analyses of PCBs at various trophic levels in the maximum turbidity zone of the contaminated St Lawrence River estuary. As anticipated, they noted an increase in total PCB loading throughout the food chain, extending from water, zooplankton, larval smelt (*Osmerus mordax*), juvenile smelt and tomcod, and finally through adult smelt and tomcod. Not surprisingly for the maximum turbidity zone in a contaminated estuary, five times higher levels of total PCBs were found in the suspended sediments than in the zooplankton.

Previously we have reported significantly reduced hepatic CYP1A1 mRNA inducibility in HAH-treated adult tomcod from the Hudson River compared with similarly treated tomcod from elsewhere (Wirgin *et al.* 1992, Courtenay *et al.* 1999). More recent studies also found decreased CYP1A1 mRNA inducibility in a

variety of non-hepatic tissues, including heart, kidney and intestine, in adult Hudson River tomcod (Yuan *et al.* unpublished data). Similarly, other investigators have reported impaired hepatic CYP1A1 protein or 7-ethoxyresorufin-*O*-deethylase (EROD) inducibility in killifish from AH-contaminated Atlantic coast estuaries, including the Hudson River (Prince and Cooper 1995, Van Veld *et al.* 1996, Elskus *et al.* 1999, Nacci *et al.* 1999, Bello *et al.* 2001). For killifish studies, laboratory-reared F_1 and F_2 descendants from polluted systems continued to exhibit reduced CYP1A1 inducibility compared with offspring from reference populations, indicating a strong genetic component to the resistant phenotype. In contrast, we reported significant induction of CYP1A1 mRNA expression in laboratory-reared F_1 tomcod yolk sac larvae of Hudson River descent treated with PCB 77 (10 p.p.m.) and B[*a*]P (10 p.p.m.), although levels of expression for both chemicals were not as high as in similarly treated Miramichi River yolk sac larvae (Roy *et al.* 2001). Results from the chemically treated postlarvae in the current study are consistent with our earlier results in demonstrating that CYP1A1 mRNA is significantly inducible in the larval stages of Hudson River tomcod, confirming the presence of physiological factors reducing CYP1A1 in Hudson River adults.

Overall, these results indicate that levels of bioavailable AHs in wild Hudson River tomcod postlarvae were insufficiently high to induce CYP1A1 expression. This is surprising given the relatively high burdens of these contaminants in unfertilized eggs from Hudson River females. This suggests that the actual burdens of these contaminants decreases during embryonic and larval development due to growth and/or depuration, and that additional accumulation during this period of larval development was minimal. The contrast in CYP1A1 expression between larvae versus juveniles/adults suggests that diet and/or proximity to contaminated sediments is the major route of exposure of postlarval life-stages to Hudson River-borne AH contaminants.

Acknowledgements

We acknowledge the Hudson River Foundation, the Superfund Basic Research Program – NIEHS Grant ES10344, Northeast Fisheries Sciences Center-NMFS, and NIEHS Center Grant ES00260 for support of this project. We thank M. Mattson, M. Ricci, C. Sweeney, C. Sorrentino and crew members of Normandeau Associates for their assistance in collecting tomcod larvae, and David Witting for his contributions to the captive rearing of tomcod.

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