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Altered CYP1A expression in *Fundulus heteroclitus* adults and larvae: a sign of pollutant resistance?

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Abstract

This study addresses the development of resistance to CYP1A induction in a population of *Fundulus heteroclitus* from highly contaminated Newark Bay, NJ. CYP1A is a P450 monooxygenase whose expression is induced in vertebrates by numerous organic contaminants, including polychlorinated biphenyls (PCBs). Reduced expression of CYP1A in highly contaminated populations may represent resistance to chemical stressors. Adult *F. heteroclitus* from Newark Bay and a reference site were held in clean seawater for 6 months to allow them to depurate their contaminant body burdens. These fish were then exposed to a single intraperitoneal injection of PCBs during gametogenesis and CYP1A expression measured in parents and larvae. Hatching success, larval survival and larval growth were also measured; no effect of PCB treatment was observed in these parameters. Fish from the reference population exhibited a strong CYP1A dose-response to PCB treatments. Reference adult males treated with 0, 10 or 100 ppm PCB (kg body weight)$^{-1}$ had hepatic ethoxyresorufin *O*-deethylase (EROD) activities of 132 ± 46.1, 433 ± 59.5 and 345.4 ± 197.5 pmol min^{-1} mg^{-1}, respectively, and CYP1A protein levels of 7 ± 5.6; 6.1 ± 4.8 and 92.6 ± 25 pmol mg^{-1} (means $\pm$ SD) at 7 weeks post-injection. CYP1A expression in 24-h and 8-day post-hatch larvae from PCB-treated reference parents was measured immunohistochemically and revealed a strong and persistent induction that was dose-related. Further, larval organs responded sequentially to increasing parental PCB dose in the following order: hepatocytes, gill, gut and heart; the kidney appeared to express CYP1A constituitively. In contrast to the reference fish, Newark Bay adults and larvae failed to show induction of CYP1A in response to any PCB treatment. These results indicate that the Newark Bay population has acquired an altered response to CYP1A inducers that is both persistent and possibly heritable, that use of CYP1A as a biomarker may be misleading under [illegible] of chronic or extreme exposure, and emphasize the need for carefully designed studies which employ

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indicators in addition to CYP1A as biomarkers of exposure or effects. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Evidence has accumulated demonstrating that non-migratory, indigenous species can develop increased tolerance for contaminants in their environment (Vinson et al., 1963; Klerks and Levinton, 1989; Weis and Weis, 1989; Levinton, 1996). One species which has received particular attention is the marsh minnow, *Fundulus heteroclitus*. Populations of this species have demonstrated resistance to the toxic effects of mercury (Weis and Weis, 1989), polychlorinated biphenyls (PCBs; Nacci et al., 1996), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs; Prince and Cooper, 1995a,b; Bello et al., 1996; Cooper, 1996) and creosote, a source of polynuclear aromatic hydrocarbons (PAHs; Horton-Williams, 1994; Van Veld et al., 1996; Vogelbein et al., 1996). Tolerance in these populations has been measured as reduced mortality (Prince and Cooper, 1995a,b; Nacci et al., 1996), reduced developmental abnormalities (Weis and Weis, 1989; Prince and Cooper, 1995a) and/or altered expression of Phase I and II enzymes (Van Veld et al., 1992; Van Veld and Westbrook, 1995; Prince and Cooper, 1995b; Bello et al., 1996; Nacci et al., 1996; Van Veld et al., 1996).

Identifying tolerant populations is essential to our understanding of the effects of chronic pollution. Adaptation to pollutants may increase an organism's tolerance to those chemicals but reduce its capacity to adapt to other environmental stressors by reducing genetic diversity (LeBlanc, 1994; Fox, 1995). Reduced survival and growth may only become apparent when adapted populations are confronted with additional stresses, such as reduced food availability, temperature extremes, or increased fishing pressure. Such 'costs of tolerance' may provide one explanation for otherwise unexpected occurrences (Benjamin and Klaine, 1995); for example, population decline in a chronically contaminated area, but not in an acutely exposed one, following a sudden and dramatic environmental change.

Several possible mechanisms may underlie resistance to pollutants. Tolerance may be achieved by decreasing contaminant uptake (references cited in Klerks and Bartholomew, 1991), altering detoxication rates (Klerks and Bartholomew, 1991), increasing release (Morrow and Cowan, 1988), or by sequestration (Andreason, 1985; Klerks and Bartholomew, 1991). It also has been suggested that cancer may be an adaptive response (Farber and Rubin, 1991) which is expressed in fish exposed to extremely high levels of PAHs (Van Veld et al., 1992; Johnson and Landahl, 1994; Van Veld and Westbrook, 1995).

CYP1A is a monooxygenase enzyme whose synthesis is induced by numerous organic contaminants, including PAHs, PCDD/Fs and coplanar PCBs. The expression of this enzyme, or its associated catalytic activity, demonstrates a biochemical response to the presence of such compounds. Recent work with *F. heteroclitus* suggests that CYP1A may become refractory to induction in populations chronically exposed to organic pollutants (Van Veld et al., 1992; Prince and Cooper, 1995b; Van Veld and Westbrook, 1995; Bello et al., 1996; Nacci et al., 1996; Van Veld et al., 1996), a response which may seriously compromise or limit its usefulness as a biomarker of chronic exposure to highly contaminated environments. It is important to note, however, that under conditions of acute exposure, or exposure to lesser contamination, CYP1A is a powerful and robust marker of organism response to pollution (Buchneli and Fent, 1995).

This study addresses the development of resistance to CYP1A induction in *F. heteroclitus* from a polluted environment, Newark Bay, NJ. *F. heteroclitus* is a non-migratory omnivore with benthic eggs and a restricted home-range (Bigelow and Schroeder, 1953) making it an ideal sentinel of its

environment. Preliminary work in our laboratory suggested that CYP1A in larval killifish from Newark Bay may be refractory to induction by in situ contaminants (Monosson et al., 1995). These contaminants include PCBs, PCDD/Fs and PAHs, chemicals which are potent CYP1A inducers (Stegeman and Hahn, 1994) and are found in high concentrations in this region (Jones et al., 1989; Squibb et al., 1991; Long et al., 1995; Adams et al., 1998). Lack of CYP1A inducibility has been reported in adults of this species in Newark Bay (Prince and Cooper, 1995b), a response which may partly result from the already high contaminant body burden in this population. In the present study, adult *F. heteroclitus* from Newark Bay were allowed to depurate for 6 months. These fish were then exposed to PCBs during gametogenesis and CYP1A expression was measured in the adults and in their larvae as a first step in determining heritability of reduced CYP1A expression in this population.

2. Materials and methods

2.1. Chemicals

Aroclors 1248 and 1262 were obtained from Ultra Scientific (Hope, RI). All solvents were high purity grade Burdick and Jackson (VWR). A universal immunoperoxidase staining kit (murine) was obtained from Signet Laboratories (Dedham, MA). Silica gel (100–200 mesh) was obtained from Fisher (Springfield, NJ). All other biochemicals were obtained from Sigma (St. Louis, MO).

2.2. PCB analysis

PCB congener distributions were analyzed, in triplicate, in: (1) the ovaries from fresh-caught Newark Bay *F. heteroclitus* to determine an appropriate congener mixture for dosing; and (2) in strip-spawned eggs of experimental females. Briefly, tissue was dried with sodium sulfate (1:4), spiked with IUPAC 29 and 143 (Ballschmiter and Zell, 1980) as internal standards, soxhlet extracted overnight in acetone:hexane (1:1), lipids removed with acid-treated silica gel, and PCBs fractionated on a 5% deactivated silica gel column (2 cm × 20 cm). Prior to injection, samples were spiked with octachloronaphthalene to correct for recovery (recoveries ranged from 53 to 98%). PCBs were analyzed on an HP5890 Series II GC fitted with an electron capture detector and a 30-m DB5 capillary column (J&W Scientific). Samples were injected (2 μl, splitless) at 40°C and held for 2 min with a temperature program of 30°C min^{-1} to 120°C, 2°C min^{-1} from 120 to 240°C, hold for 10 min. Injection port and detector temperatures were 300°C. Tissue PCB content (ppm wet wt.) is based on the summed peak area of 45 congeners. Detection limits for individual congeners ranged from 0.33 (controls) to 2.2 (high dose) ng (g wet tissue wt)$^{-1}$.

2.3. Animals and treatments

Field collected fish were depurated for 6 months prior to experimental treatment. This was done so that their body burdens of PCBs (PCB halflife in fish is 4 months, Nimii and Oliver, 1983) and PCDD/Fs (halflife of 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD, in killifish is < 60 h, Prince and Cooper, 1995b) would be sufficiently low to insure CYP1A expression represented a response to injected PCBs rather than to endogenous CYP1A inducers. Post-spawning adult *F. heteroclitus* were collected via fish traps from Flax Pond, NY (Reference) and the Roanoke Yacht Club in Newark Bay, NJ (Newark Bay) in September 1995. Fish were held in 18‰ Instant Ocean (Royal Pet Supply, Brentwood, NY) static recirculating systems (using biofilters) under ambient light/dark and temperature conditions for a depuration period of 6 months. Fifty percent of the water was renewed each month. Fish were fed daily with Tetramin (Royal Pet Supply, Brentwood, NY) from September through the end of the experiment (May). From April through May this diet was supplemented daily with krill (Royal Pet Supply, Brentwood, NY). One week prior to the onset of gonadal recrudescence, as indicated by the development of breeding colors (March 1996), adults were dosed intraperitoneally with a single injection of vehicle (8.8% heptane in corn oil), 10 mg

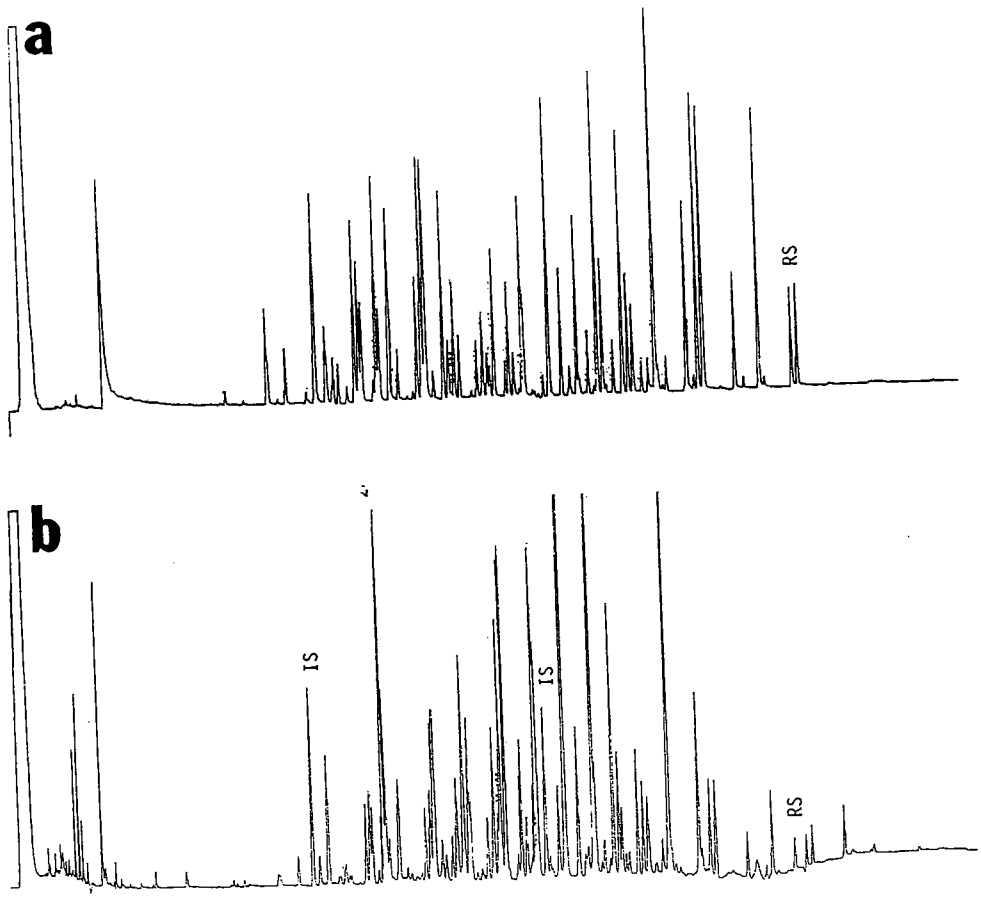

Fig. 1. Gas chromatographic traces of (a) the PCB mixture (1:1.5 A1262:A1248) used to treat depurated killifish, and (b) gonadal PCB distribution in resident Newark fish. IS, internal standards IUPAC 29 and 143, respectively; RS, recovery standard octachloronaphthalene. See Section 2 for details.

(kg body weight)$^{-1}$ (medium dose) or 100 mg (kg b.w.)$^{-1}$ (high dose) of a PCB mixture (1:1.5 A1262:A1248) representative of congener distributions measured in resident Newark Bay *F. heteroclitus* (Fig. 1). It should be noted that the majority of PCB congeners have chlorine atoms substituted in the ortho position, a configuration which reduces their potency both as CYP1A1 inducers (Goldstein et al., 1977) and as toxins (Safe, 1990). Instead it is the non-ortho substituted congeners, such as 3,4,3′,4′-tetrachlorobiphenyl (IUPAC 77), 3,4,5,3′,4′-pentachlorobiphenyl (IUPAC 126) and 3,4,5,3′,4′,5′-hexachlorobiphenyl (IUPAC 169) that are the most potent CYP1A1 inducers and the most toxic of the PCB congeners (Safe, 1990). IUPAC 77, 126 and 169 make up approximately 0.32%, 0.005% and 0%, respectively, of the PCBs present in our dosing solutions. Doses were chosen based on preliminary experiments (Monosson et al., 1995) to achieve ovarian PCB levels similar to those measured in ovaries of Newark Bay field fish. Twelve to 16 females, and six to 16 males were dosed and divided into two replicate tanks per treatment. Fish were allowed to mature naturally with increasing daylength and water temperature.

Since *F. heteroclitus* are lunar spawners, we exposed them over several spawning cycles to insure adequate transfer of injected chemicals to the gametes, and hence to the larvae, during

critical developmental stages. After 7 weeks (two to three spawning cycles, ambient temperature 18°C) fish were strip-spawned and eggs from two to three females were pooled and fertilized with sperm from two to three males. A portion of each egg pool was stored at −80°C for chemical analysis. Livers from males were pooled (three males per pool, three pools per treatment), flash-frozen in liquid nitrogen (−192°C) and stored at −80°C until analyzed (2 months) for CYP1A. Triplicate plates of 30 embryos each per treatment were incubated on a 14-h/10-h light/dark cycle at 20°C until spontaneous hatching occurred (10–12 days). Synchronous embryo hatching was achieved by dousing the embryos with seawater (SW). Five larvae were taken from each plate for length and five fixed in 10% neutral buffered formalin in preparation for paraffin embedding (within 48 h) for immunohistochemical (IHC) analysis of CYP1A; these represent the 24-h post-hatch larvae. Remaining larvae from each plate were placed into 4.5 l buckets at a density of 18–30 larvae/bucket (one bucket had only six larvae), three buckets per treatment and were held in a flow-through SW table (28‰) at 20°C. Larvae were fed EPA Reference artemia (5–10 artemia ml^{-1}; courtesy US EPA/AED) twice daily and held through yolk-sac absorption (8 days). As before, five larvae were taken for length and five fixed for IHC; these represent the 8-day post-hatch larvae.

2.4. Biochemical assays

Due to the known suppression of CYP1A activity in female fish by estradiol (Forlin and Hansson, 1982; Pajor et al., 1990; Elskus et al., 1992), only livers from male fish were analyzed for CYP1A expression and catalytic activity. Hepatic microsomes were isolated as previously described (Stegeman et al., 1979). Microsomal protein was measured using the bicinchoninic acid method of Smith et al. (1985) with bovine serum albumin as the standard. EROD, an activity specifically catalyzed by CYP1A, was measured fluorometrically in microsomes using a Cytofluor 2300 (Millipore) multiwell plate reader, as described by Hahn et al. (1993). Microsomal CYP1A protein was quantified immunochemically using MAb 1-12-3, a monoclonal antibody which recognizes CYP1A in multiple vertebrate species (Kloepper-Sams et al., 1987; Stegeman and Hahn, 1994) using AP-conjugated goat anti-mouse IgG as secondary antibody.

2.5. Immunohistochemistry

Larvae were fixed in 10% neutral buffered formalin for no longer than 48 h prior to paraffin embedding. Immunohistochemical processing was carried out using a modification of the technique of Stegeman et al. (1991). Briefly, 5 micron sections were deparaffinated, dried with ethanol, incubated in 3% hydrogen peroxide for 5 min to block endogenous peroxidase (Polak and Van Noorden, 1983), and hydrated in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS). Sections were incubated in normal goat serum for 5 min to block non-specific binding of the secondary antibody (goat anti-mouse IgG; Polak and Van Noorden, 1983). Sections were washed once with BSA/PBS followed by incubations in MAb 1-12-3, pass 5 (MAb 1-12-3p5; 1 μg protein ml^{-1}, 2 h), goat antimouse IgG (20 min) and peroxidase-linked mouse IgG (20 min). Following each antibody incubation sections were washed with BSA/PBS. Color development was achieved by incubation for 12–16 min in 3-amino-9-ethylcarbazole in acetate buffer. Sections were washed, counterstained with Mayers hematoxylin followed by 0.5% NH_4OH and mounted in Crystalmount (Biomeda, Foster City, CA) and Permount (Fisher Scientific).

Sections of liver from an uninduced and induced fish, the teleost scup, were included in each set as controls for the staining method. Matching serial sections for each larvae were stained using a non-specific IgG (purified mouse myeloma protein, UPC-10, IgG_{2A}, Organon Teknika, West Chester, PA) at 1.7 μg protein ml^{-1} in 1% BSA/PBS (Polak and Van Noorden, 1983). In all cases, staining with MAb 1-12-3p5 was specific; there was no staining with the non-specific MAb.

Immunohistochemical detection of CYP1A was scored both for the occurrence of the stain (number of cells staining) and for the intensity of

staining for each tissue. Tissue scores were averaged over the five larvae per pool for a final mean ± SEM for three pools of five larvae each per treatment. Overall CYP1A response represents the sum of CYP1A scores for all responding tissues in a given larvae.

2.6. Statistical analyses

Data were analyzed using STATVIEW™ (Northbrook, IL). Two-way analysis of variance (ANOVA), followed by Fisher's PLSD and Scheffe's multiple comparison as the post-hoc tests, were used to determine significant differences between sites and between treatments at $P < 0.05$.

3. Results

3.1. PCB analysis

PCB concentrations in the eggs of resident fish from Newark Bay and Flax Pond differed by approximately ten-fold (2.0 ± 0.9 vs. 0.2 ± 0.02 μg PCB (g wet weight)$^{-1}$, respectively; Table 1). During 7.5 months of depuration (6 months depuration followed by 1.5 months post-vehicle injection) Newark Bay fish apparently lost a significant proportion of their PCB body burden. The low PCB values in the eggs of depurated control Newark Bay killifish (0.20 ± 0.08 ppm) relative to those in resident (undepurated) populations (2.0 ± 0.9 ppm) indicate that Newark Bay fish depurated more than 80% of their PCB body burden over 7.5 months (Table 1). PCB levels in Flax Pond fish remained unchanged over the depuration period, being 0.20 ± 0.02 in freshly caught residents and 0.15 ± 0.05 in depurated animals (Table 1).

Table 1
PCB concentrations (ppm wet wt.) in eggs stripped from 6-month depurated *F. heteroclitus* injected i.p. with PCBs versus field-caught resident fish

Site	Treatment	Eggs (μg PCB (g wet)$^{-1}$)
Flax	Corn oil	0.15 ± 0.05 (3)
	10 mg kg^{-1}	2.5 ± 0.5 (3)
	100 mg kg^{-1}	17.0 ± 6.0 (3)[a]
Flax	Resident	0.20 ± 0.02 (3)
Newark	Corn oil	0.20 ± 0.08 (2)
	10 mg kg^{-1}	2.4 (1)
	100 mg kg^{-1}	14.9 ± 7.8 (3)[a]
Newark	Resident	2.0 ± 0.9 (3)

Values represent means ± SD (*n*).
[a] Significantly different from control and medium dose fish at $P < 0.05$.

Seven weeks after *F. heteroclitus* adults were treated with a single injection of PCBs there was a clear relationship between injected dose and the internal dose or PCB body burden (Table 1). Ten-fold differences in PCB dose (0, 10 and 100 mg kg^{-1}) produced approximately ten-fold differences in PCB concentrations in the eggs of both Flax Pond (0.15, 2.5 and 17 ppm) and Newark Bay (0.20, 2.4 and 14.9 ppm) fish. Despite differences in route of exposure, PCB concentrations achieved in the eggs of killifish injected with 10 mg PCB kg^{-1} (2.5 and 2.4 ppm) were similar to PCB levels found in the eggs of resident Newark Bay killifish (2.0 ppm; Table 1).

3.2. Adult male CYP1A protein and activity

Adult males from Flax Pond and Newark Bay displayed strong differences in hepatic CYP1A response to PCB treatment (Table 2). Seven weeks after a single i.p. injection with a high dose of PCBs (100 mg PCB kg^{-1}) Flax Pond males had significantly elevated CYP1A protein and EROD activity levels relative to control and low dose fish. Newark Bay males failed to show CYP1A induction at any PCB dose.

3.3. Embryo/larval survival and growth

There were no site-related or dose-related differences in embryo hatching success, larval length, larval growth or larval mortality. Average hatching success (percent hatched on day 12 after fertilization) ranged from 43% to 87% (Flax fish: 80–97% (controls), 67–97% (10 ppm), 33–87% (100 ppm); Newark Bay fish: 13–80% (controls), 53–67% (10 ppm), 33–50% (100 ppm)). In previ-

Table 2
Hepatic CYP1A protein content and EROD activity levels in adult male *F. heteroclitus* 7 weeks after a single i.p. injection of PCBs

Site	Treatment	Microsomal protein (mg g^{-1})	CYP1A (pmol mg^{-1})	EROD (pmol min^{-1} mg^{-1})
Flax	Corn oil	12.2 ± 0.9	7.0 ± 5.6	132.2 ± 46.1
	10 mg kg^{-1}	16.3 ± 3.2	6.1 ± 4.8	43.2 ± 59.5
	100 mg kg^{-1}	13.3 ± 1.0	92.6 ± 25.0[a]	354.4 ± 197.5[b]
Newark	Corn oil	12.5 ± 1.1	12.8 ± 12.6	162.0 ± 130.5
	10 mg kg^{-1}	17.5 ± 3.3	8.4 ± 2.0	102.9 ± 93.7
	100 mg kg^{-1}	17.6 ± 1.1	4.8 ± 1.8	129.8 ± 45.7

Values represent means ± SD, $n = 3$.
[a] Significantly different from all other groups at $P < 0.05$.
[b] Significantly different from all other groups, except Newark controls, at $P < 0.07$.

ous studies in our laboratory using freshly caught fish, hatching success was much higher, varying between 90 and 95% for killifish from the Flax and Newark Bay populations (Monosson et al., 1995). The wide ranging percent hatch in the present study may reflect some accumulated dietary deficiency after 7 months on laboratory feed.

Larval lengths ranged from 6.9–7.2 mm at 24 h post-hatch, to 9.5–10.0 mm at 8 days post-hatch. One hundred percent of the larvae survived from hatch to 8 days post-hatch in all treatment groups.

3.4. Larval CYP1A expression

3.4.1. Tissue specificity

Immunohistochemical analysis of 24-h and 8-day post-hatch larvae from PCB-treated adult *F. heteroclitus* indicated CYP1A protein expression in five larval tissues: kidney tubules, hepatocytes, gill pillar cells, heart endothelium, and gut epithelium (Fig. 2). With the possible exception of the spleen in one fish, no other larval tissues were observed to express this protein.

3.4.2. Dose-response

Four of the five responding tissues expressed CYP1A in a dose-response manner: hepatocytes, gill, heart and gut (Table 3). Dose-response was defined by both an increase in Mab 1-12-3 staining intensity and an increase in the number of tissues expressing CYP1A with increasing PCB dose. The increased 'recruitment' of CYP1A responding tissues, or gradual appearance of CYP1A expression in additional tissues with an increasing dose, accounts for the overall dose-response observed in larvae from PCB-treated Flax Pond adults (Fig. 3a).

The proximal and distal tubules of the kidney expressed CYP1A protein differentially. CYP1A expression was consistently observed in proximal tubules of Flax Pond larvae although the degree of expression varied between individuals; distal tubules rarely showed any CYP1A staining. In larvae from Flax Pond parents administered corn oil (controls) or 10 mg PCB kg^{-1} (medium dose), the proximal tubules showed weak (control) to moderate (control and medium dose) staining for CYP1A (Fig. 4a). Distal tubules showed no staining in 24-h post-hatch larvae of control and medium dose parents (Fig. 4a) but after 8 days of growth (8-day post-hatch) distal tubules in larvae from medium dose parents did show staining. In the highest dose group (100 mg PCB kg^{-1}), proximal tubules stained strongly and distal tubules showed weak to moderate CYP1A expression (Fig. 4b). In 24-h post-hatch larvae from Newark parents CYP1A expression in proximal tubules varied from no expression to strong expression across all treatment groups including controls; distal tubules did not express CYP1A in any group. Eight-day post-hatch Newark larvae expressed CYP1A consistently in the proximal tubules, distal tubules remained non-expressive. Values for kidney CYP1A (Table 3, Fig. 3) represent expression in kidney proximal tubules.

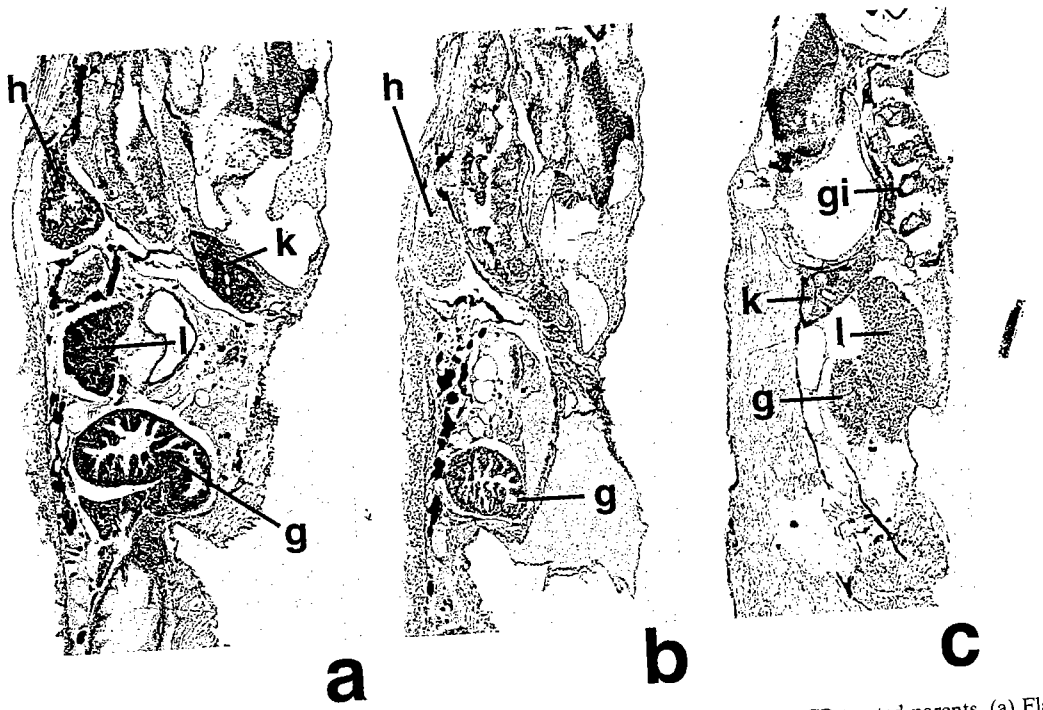

Fig. 2. Immunohistochemical section stained for CYP1A protein in 24 h post-hatch larvae from PCB-treated parents. (a) Flax—100 mg PCB kg^{-1} dose, hybridized with Mab 1-12-3, specific for teleost CYP1A; (b) section adjacent to that shown in (a) but hybridized with UPC, a non-specific Mab; (c) Newark—100 mg kg^{-1} dose, hybridized with Mab 1-12-3. Staining intensity is proportional to amount of protein present. k, kidney; g, gut; l, liver; h, heart; gi, gill. Magnification 100 ×.

3.4.3. Site differences

PCB treatment of parental fish induced CYP1A in larvae from Flax Pond, but not Newark Bay, parents. At both 24 h and 8 days post-hatch, Flax Pond larvae displayed a gradual 'recruitment' of tissues expressing CYP1A with increasing parental PCB dose, additional tissues responding to increasing dose in the following order: hepatocytes, gill, gut and heart (Table 3, Fig. 3a). At 24 h post-hatch, larvae from control parents expressed very low to non-detectable levels of CYP1A. By 8 days post-hatch, CYP1A expression increased slightly in all Flax Pond treatment groups, including controls (Fig. 3c).

In contrast to the strong PCB dose-response exhibited by larvae from Flax Pond parents, CYP1A expression was weak or completely absent in the tissues of larvae from Newark Bay parents. With the exception of the kidney which expressed CYP1A in all Newark Bay larvae regardless of dose, CYP1A protein was absent (controls, medium dose) or expressed at low levels (high dose) in 24-h post-hatch larvae from Newark Bay parents (Fig. 3b). In 8-day post-hatch larvae only the gut and kidney expressed CYP1A but without regard to the dose, being equally expressed in larvae from control and PCB-dosed Newark Bay parents (Fig. 3d).

Summing the CYP1A response of all tissues illustrates the strong overall dose-response of Flax Pond larvae to parental PCB exposure and the complete lack of a dose-response in the Newark Bay larvae, a difference which persists from 24 h to 8 days post-hatch (Fig. 5). This dose-response difference between Flax Pond and Newark Bay larvae is due not to the differences in intensity of CYP1A expression in a given tissue but to the overall number of tissues expressing CYP1A with very few tissues expressing CYP1A in Newark Bay larvae.

Table 3
Immunochemical detection of CYP1A protein in 24-h and 8-day post-hatch larvae from PCB-treated adult *F. heteroclitus*

Age post-hatch	Treatment[a]	Site[b]	N[c]	Hepatocytes	Gill pillar cells	Kidney tubules[d]	Gut epithelium	Heart endothelium
24 h	Corn oil	F	3	0.1 ± 0.1[e]	0	8.2 ± 1.8	0	0.3 ± 0.3
		N	3	0	0	11.0 ± 1.4	0.6 ± 0.4	0
	10 mg kg^{-1}	F	3	5.9 ± 1.7	1.8 ± 1.6	11.0 ± 0.8	0	5.8 ± 2.1
		N	2	0	0	8.5 ± 2.4	0.1 ± 0.1	0
	100 mg kg^{-1}	F	3	11.9 ± 0.8	11.1 ± 2.0	13.9 ± 0.5	6.2 ± 2.0	14.5 ± 0.4
		N	3	0	2.8 ± 2.3	5.4 ± 2.1	0.6 ± 0.2	4.5 ± 4.5
8 day	Corn oil	F	3	3.4 ± 0.1	0.3 ± 0.3	11.4 ± 0.6	4.1 ± 1.6	0
		N	3	1.4 ± 1.3	0	13.0 ± 1.0	5.6 ± 0.7	0
	10 mg kg^{-1}	F	3	5.2 ± 1.7	2.9 ± 1.7	12.7 ± 1.2	4.4 ± 1.8	7.0 ± 1.0
		N	1	0	0	13.8 ± 1.6	2.3 ± 0.8	0
	100 mg kg^{-1}	F	2	12.7 ± 1.6	7.2 ± 2.2	13.1 ± 1.7	5.2 ± 4.4	15.0 (1)
		N	3	0.3 ± 0.3	0	12.0 ± 0.8	6.1 ± 1.3	0

All data are expressed as mean ± SEM.
[a] Injected (mg PCB (kg body weight)$^{-1}$) into adults 7 weeks prior to strip-spawning.
[b] F, Flax; N, Newark.
[c] n = number of pools of five larvae each.
[d] Proximal tubules only.
[e] Relative IHC signal; see Section 2 for description of IHC scoring.

4. Discussion

The results of the present study suggest that a resident, non-migratory Hudson River fish, *F. heteroclitus*, is adapted to in situ contaminant conditions in Newark Bay. We found that CYP1A, an enzyme which is inducible by and catalyzes the metabolism of hydrophobic organic contaminants (Ioannides and Parke, 1990; Stegeman and Hahn, 1994), is refractory to induction by PCBs in this population. CYP1A resistance to PCB induction in both depurated Newark Bay adults and their offspring indicates that the Newark Bay population has acquired an altered response to CYP1A inducers that is both persistent and possibly heritable. The mechanism of this adaptation is not known.

These data are consistent with previous studies suggesting some sort of adaptation or altered CYP1A response in animals collected from sites highly contaminated with CYP1A inducing compounds. In adult Newark Bay killifish CYP1A catalytic activity (EROD) was refractory to induction by the potent inducer, TCDD (Prince and Cooper, 1995b). Decreased CYP1A inducibility in chronically contaminated fish has been reported to be accompanied by an elevation in Phase II detoxication enzymes (Gallagher, 1996; Van Veld et al., 1996) and elevated resistance to PAH, PCB and PCDD/F toxicity (Horton-Williams, 1994; Bello et al., 1996; Cooper, 1996; Gallagher, 1996; Nacci et al., 1996; Van Veld et al., 1996). It should be noted that in some chronically polluted populations, adults have not become tolerant to the toxicity of the contaminants present (Weis and Weis, 1989; Black et al., 1998). Whether these non-tolerant animals had altered CYP1A levels is not known.

That adaptation to toxicity and/or genetic suppression of CYP1A induction appears to occur in response to chronic exposure to a number of different CYP1A-inducing compounds, including TCDD (Prince and Cooper, 1995b), 2,3,7,8-tetrachlorodibenzofuran (TCDF, Bello et al., 1996) and PCBs (Nacci et al., 1996; present study) indicates that this is not a PCB-specific phenomenon. The consistent suppression of CYP1A expression in all cases by compounds which mediate their

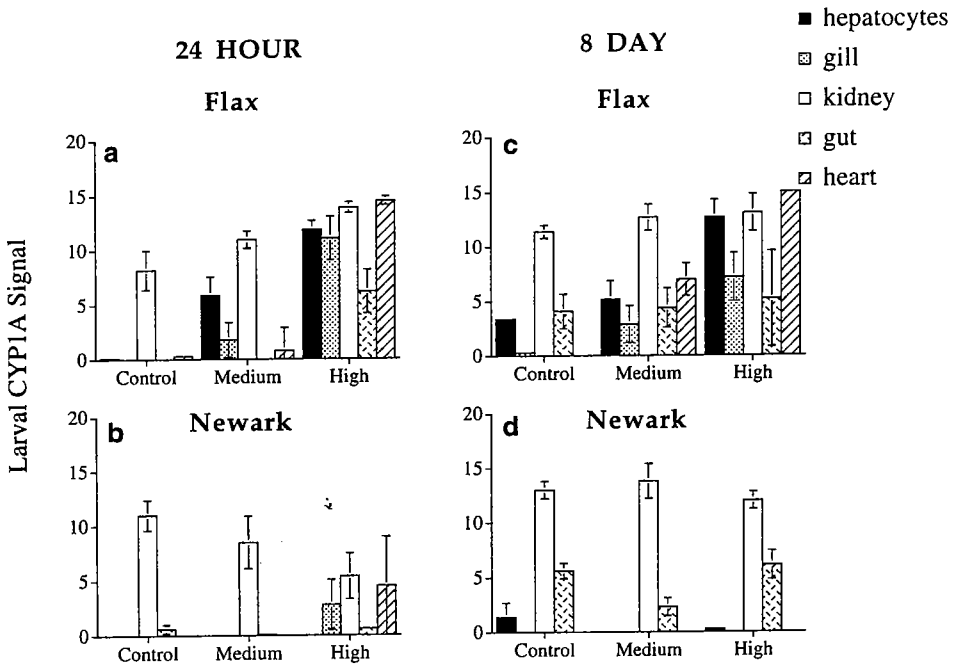

Fig. 3. Immunohistochemical quantification of CYP1A protein in tissues of (a, b) 24-h, and (c, d) 8-day post-hatch larvae from Flax Pond and Newark Bay parents given a single i.p. injection of PCBs. Values represent means ± SEM, $n = 2–3$ pools of five larvae each. See Table 3 for statistical analyses.

induction of CYP1A via binding to the aryl hydrocarbon (Ah) receptor suggests that this apparent 'adaptation' may be, at least in part, an AhR-mediated phenomenon. However, until detailed mechanistic studies are undertaken, the extent of AhR involvement in adaptation cannot be determined.

In light of studies of other chronically exposed, yet robust, fish populations, the similar lack of CYP1A inducibility we observed in the Newark Bay population suggests that depressed CYP1A activity may be beneficial to population survival. The potential benefit arising from a decline in CYP1A-mediated production of reactive metabolites that can bind DNA remains equivocal. Investigation of two co-occurring species of flatfish in Puget Sound found ability to metabolize PAH and formation of DNA adducts to reflect tumor susceptibility, with the species having lower capacity also having a lower incidence of tumors (Stein et al., 1990). However, studies of two other bottom-dwelling fish, the mirror carp and brown bullhead, reported the opposite relationship (tumor-resistant species produced more DNA adducts and metabolized PAHs equally as well as the tumor-prone species, Steward et al., 1989). Studies with cell lines report reduced CYP1A activity is correlated with increased formation of PAH-DNA adducts (Willett et al., 1998). Killifish generationally exposed to extremely high concentrations of PAHs were found to have low CYP1A activities accompanied by high tumor incidence (Van Veld et al., 1992; Van Veld and Westbrook, 1995).

The refractory nature of the Newark Bay population to CYP1A inducers was in direct contrast to that of the reference site (Flax Pond) fish. CYP1A response was clearly dose-related in larvae from Flax Pond, but not Newark Bay, parents. This difference persisted from 24 h through 8 days after hatching, a time when the yolk sac and its associated contaminant load is fully absorbed. This indicates that lack of CYP1A induction in the Newark Bay larvae was not due to a delay in the response or to insufficient exposure to the contaminants. Indeed, PCB concentrations in the

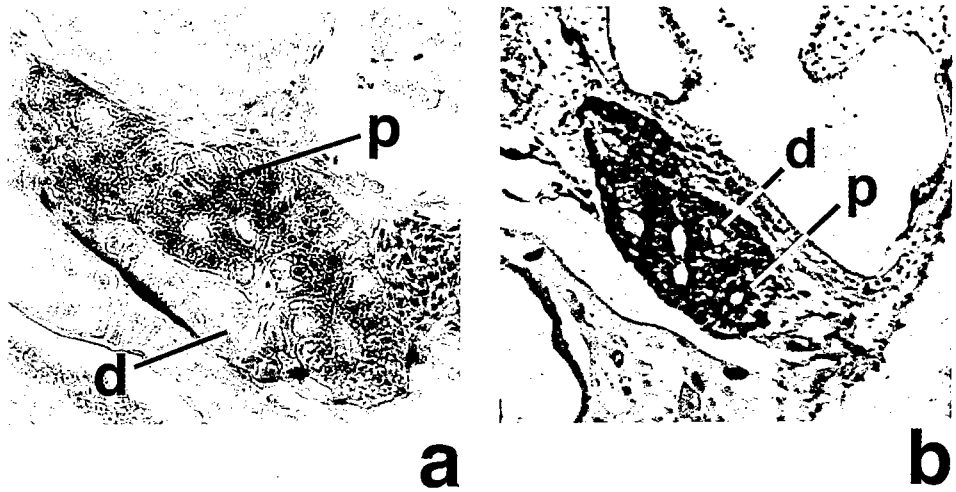

Fig. 4. Immunohistochemical sections depicting relative CYP1A protein expression in kidney tubules of 24 h post-hatch larvae of Flax Pond parents treated with (a) 10 mg PCB kg^{-1} (400 × magnification) or (b) 100 mg PCB kg^{-1} (view of kidney from Fig. 2(a) magnified two-fold). p, proximal tubule; d, distal tubule.

eggs from which these larvae hatched were identical between dose-matched Newark Bay and Flax Pond groups. Further, preliminary studies of larvae from freshly-caught, non-depurated Newark Bay parents showed no expression of hepatic CYP1A at 24 h or 7 days post-hatch (unpublished data), evidence that CYP1A is not strongly expressed even in Newark killifish exposed to in situ mixtures of potent inducers. It should be noted that, although unresponsive to further induction by TCDD, non-depurated adult Newark Bay killifish do show consistently higher EROD levels than reference site fish (Prince and Cooper, 1995b). In the present study with 7-month depurated killifish, however, there was no difference in EROD or CYP1A levels between Newark Bay and reference fish.

In addition to site differences, there were also striking tissue-related differences in larval CYP1A response to parental PCB exposure. In 24-h post-hatch Flax Pond larvae there was a gradual 'recruitment' of tissues expressing CYP1A with increasing dose; not all responsive tissues expressed CYP1A until the highest dose was attained. Additional tissues responded to increasing PCB dose in the following order: hepatocytes, gill, gut and heart. The tissue types and the increase in number of tissues responding with increasing dose are consistent with those of other studies examining CYP1A expression in larval fish (Weidmer et al., 1996). Interestingly, the kidney proximal tubules of most larvae expressed CYP1A to some degree at all doses, including larvae from control parents. The expression of CYP1A in larval kidney not only in controls but in Newark Bay fish, where other tissues in this population showed little to no CYP1A expression, suggests that there may be a level of CYP1A expression in kidney proximal tubules that is 'constitutive.' Such 'constitutive' expression of CYP1A in kidney has recently been observed in adults of this species (Van Veld et al., 1997; Bello, pers. comm.), however the reason for this kidney-specific response is not known.

CYP1A expression in adult fish from Flax Pond, the reference site, reflected their relative exposure to inducers. The modest degree of CYP1A expression, both CYP1A protein and EROD activity, in depurated adult fish from Flax Pond is consistent with an exposure to low levels of inducers. Treatment with a single injection of PCBs induced a CYP1A response in adult Flax Pond fish only at the highest dose (100 mg PCB kg^{-1}). This is in contrast to Flax Pond larval fish which responded to both the 10- and 100-mg PCB kg^{-1} doses. This difference may be due to differ-

ences in the time-course of exposure to PCBs between the adult and larval fish. The sensitivity of the CYP1A system to PCBs in killifish embryos increases nine-fold within 24 h of hatching (Binder and Stegeman, 1984), which suggests that CYP1A expression in the 24-h post-hatch larvae from PCB-treated Flax Pond parents likely represents the maximal larval CYP1A response to this PCB exposure. In contrast, adult CYP1A levels were not measured until 7 weeks post-injection, a time when CYP1A response may be diminishing. Unlike PAHs which produce a rapid but short-lived induction of CYP1A in fish (Kloepper-Sams and Stegeman, 1989), at high doses PCB treatment can lead to prolonged CYP1A expression. In adult trout dosed with 100 mg PCB kg^{-1} (our highest dose) CYP1A levels were elevated for 20 weeks (Celander and Forlin, 1995). Similarly, in the present study adult killifish dosed with 100 mg PCB kg^{-1} had elevated CYP1A levels at 7 weeks post-injection. However, the response of adult *F. heteroclitus* to a ten-fold lower dose (10 mg PCB kg^{-1}, our medium dose) did not differ from that of controls, suggesting that at lower PCB doses, CYP1A response may be diminished by 7 weeks post-injection.

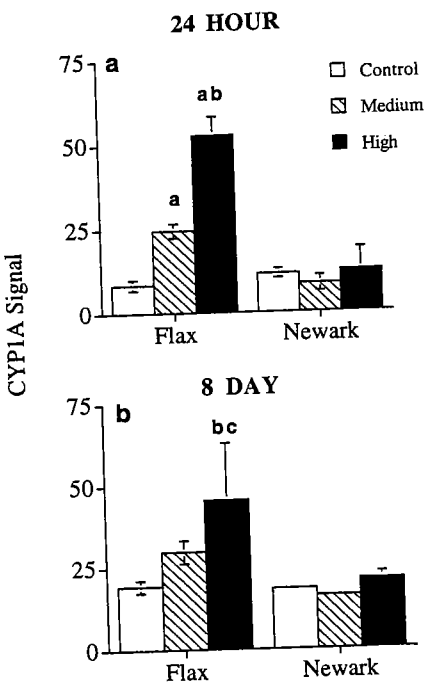

Fig. 5. Overall CYP1A response in (a) 24-h and (b) 8-day post-hatch larvae from control and PCB-dosed *F. heteroclitus* adults. Overalll CYP1A response was evaluated using the sum of CYP1A IHC scores for all responding tissues. Means ± SEM, $n = 2–3$ pools of five larvae each. Significantly different at $P < 0.05$ from: a, all other Flax groups; b, all Newark groups; and c, Flax controls.

Adaptation to chronic contaminant conditions may have consequences for both the adapted species and its predators. Adaptation may increase an organism's tolerance to contaminants, but reduce its capacity to adapt to other environmental stressors (LeBlanc, 1994; Fox, 1995). Further, the ability to survive high body burdens of contaminants, and/or to become cross-resistant to other toxicants may have ecosystem-level effects as resistant prey populations contribute to biomagnification and/or act as sources of toxic food for non-adapted predators (Andreason, 1985). Whether the apparent health of the Newark Bay population is maintained at the expense of decreased tolerance to other environmental stressors cannot be determined without further study, however it is clear that due to their high PCB body burdens the Newark Bay population is a source of toxic prey for aquatic and avian predators.

The mechanism and consequences of reduced CYP1A responsiveness are not known. Increased depuration rate does not appear to play a major role in the adaptation of Newark Bay killifish since PCB-treated fish from Newark Bay and the reference site had identical PCB body burdens at 7 weeks post-injection. Decreased uptake rates also appear unlikely as resident Newark Bay killifish have high PCB body burdens (Table 1). That some CYP1A protein is expressed by the resistant fish from Newark Bay indicates that their CYP1A gene is present and functional, however further work is needed to determine at what level (transcription or translation) CYP1A suppression is occurring.

Adaptation of CYP1A response to common aquatic contaminants has implications for the use of this enzyme as a biomarker. CYP1A is an enzyme whose synthesis is induced in organisms exposed to certain organic pollutants, including PCBs, and thus the presence of this enzyme, and/or its associated catalytic activity, demon-

strates a biochemical response to the presence of such compounds. The finding by ourselves and others that chronically exposed populations in Newark Bay have developed resistance to CYP1A inducers indicates that use of CYP1A as a biomarker may be misleading under conditions of chronic or extreme exposure. Roy and Wirgin (1996) report a similar lack of responsiveness of CYP1A mRNA to chlorinated hydrocarbons, but not to PAH, in Atlantic tomcod from Newark Bay. Their work further emphasizes the need to use indicators in addition to CYP1A as biomarkers of exposure or effects and to carefully design biomarker studies.

5. Conclusion

The results of the present study suggest that resident, non-migratory Newark Bay fish may be enzymatically adapted to in situ contaminants. Thus far, such adaptation has only been detected in fish exposed chronically, possibly for generations, to contaminants which are sometimes at extreme concentrations. The use of a biomarker of exposure under such conditions may not be very informative. Under conditions of acute exposure, or exposure to lesser contamination, such markers of exposure still appear robust. Further studies are needed to determine whether adaptation is beneficial or detrimental to such populations, how wide-spread the development of pollutant resistance is among species, and which biological/biochemical systems are adapting. This study also demonstrates the power of immunohistochemistry to illuminate and illustrate the tissue-specificity of dose response.

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