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Compound- and mixture-specific differences in resistance to PAHs and PCB-126 among *Fundulus heteroclitus* subpopulations throughout the Elizabeth River estuary (Virginia, USA)

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Abstract

Atlantic killifish (*Fundulus heteroclitus*) inhabiting the Atlantic Wood Industries Superfund Site (Elizabeth River, Portsmouth, VA, USA) are resistant to the acute toxicity and cardiac teratogenesis caused by high levels of polycyclic aromatic hydrocarbons (PAHs) from creosote. The resistance is linked to down regulation of the aryl hydrocarbon receptor (AHR) pathway. We investigated the association between CYP1 activity, as a marker of potential AHR pathway suppression, and contaminant resistance in killifish subpopulations from sites throughout the estuary that varied significantly in PAH contamination level. Adult killifish and sediments were collected from seven sites across approximately 13.7 kilometers in river length within the estuary and from a nearby reference site. Sediment PAH levels were determined using gas chromatography mass spectrometry. Embryos obtained via manual spawning were exposed to individual AHR agonists and PAH mixtures 24 hours post fertilization (hpf); CYP1 activity was determined by *in ovo* ethoxyresorufin-o-deethylase (EROD) 96 hpf and cardiac deformity severity was scored 144 hpf. The total PAH levels measured among the sites varied from approximately 200–125,000 ng/g dry sediment. Overall, the resistance to teratogenesis was strongest in the subpopulations from sites in or closest to the major PAH contamination sites, but even embryos from less-contaminated sites within the Elizabeth River demonstrated at least partial resistance to many challenges. Surprisingly, all of the subpopulations tested were highly resistant to PCB-126 (3,3',4,4',5-pentachlorobiphenyl). However, the degree of CYP1 activity response varied significantly among subpopulations and did not always correlate strongly with resistance to teratogenesis; some subpopulations resisted the cardiac teratogenesis caused by the challenges at doses that still elicited strong EROD induction. Our results suggest that there is variation in the adaptive phenotype exhibited by laboratory-spawned embryos from killifish subpopulations throughout the estuary. Furthermore, the results show that contaminants have affected killifish subpopulations throughout the estuary, even in sites with lower levels of PAHs.

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Supporting Information Available

Seven additional pages containing one table and three figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

The Elizabeth River (ER) is a highly industrialized sub-estuary in the southern part of the Chesapeake Bay estuary (Virginia, USA) greatly influenced by anthropogenic contaminants.¹ In particular, several former wood treatment facilities contaminated portions of the river with creosote, a complex mixture consisting primarily of unsubstituted polycyclic aromatic hydrocarbons (PAHs) and some heterocyclic and phenolic PAHs. At the Atlantic Wood (AW) Industries Superfund site, total PAH concentrations of 100–500 µg/g dry sediment were reported.^{1–3}

PAHs are generated by incomplete combustion of organic compounds and enter the environment through natural sources such as forest fires or through anthropogenic activities such as fossil fuel use. PAHs are ubiquitous, and their levels in the environment track human population growth and burning of fossil fuels.^{4–7} Estuarine habitats are vulnerable to PAH contamination via a variety of routes, including oil shipping and refining, industrial outfalls, wastewater discharges, urban runoff, and atmospheric deposition.⁸

PAHs are carcinogenic, immunosuppressive, and non-specific narcotic toxicants contaminants. Some are agonists for the aryl hydrocarbon receptor (AHR), while others are antagonistic or have low affinity for the receptor.^{9–11} In addition, environmentally-relevant concentrations of some PAHs cause early life stage toxicity and teratogenesis in fish. This toxicity manifests as cranio-facial and cardiac malformations, and pericardial and yolk-sac edema similar to the effects observed with certain dioxin-like compounds (DLCs) that are potent AHR agonists (e.g., 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB-126)).^{12–14}

Fundulus heteroclitus (the Atlantic killifish or mummichog; hereafter referred to as killifish) is a small teleost found in estuaries from Newfoundland to Florida, including the ER.¹⁵ Killifish are one of the most abundant intertidal fishes and a major component of 10 estuarine food webs.^{16–18} Despite wide distribution, individual killifish have small home ranges;^{19, 20} their high site fidelity and limited migration make them ideal for studying the impacts of local contamination and other stressors.²¹

The killifish population inhabiting the AW Superfund site is chronically exposed to PAH-contaminated sediments, but has developed remarkable resistance to the acute toxicity and teratogenesis caused by ER sediments, PAHs, and PCB-126.^{22–24} Ownby et al.²⁴ compared the embryotoxicity caused by PAH-contaminated sediments among killifish embryos from subpopulations inhabiting four ER sites, including the AW site, and a York River, VA reference population. Exposed embryos from a reference population suffered from a variety of cardiac abnormalities, but embryos from AW parents were resistant to these effects. Embryos from other ER subpopulations exhibited an intermediate degree of resistance to the same effects. The level of resistance was associated with the total PAH levels measured at the sites of collection.

In addition to resisting toxicity, AW killifish are recalcitrant to the induction of cytochrome P450 (CYP1) metabolic enzymes by AHR agonists, such as certain PAHs and PCB-126.^{22, 23, 25, 26} Recalcitrant CYP1 induction is concomitant with marked resistance to

the toxic effects of DLCs in several other fish populations from polluted sites.^{27–31} However, inhibition of CYP1A does not actually convey resistance; in fact, both chemical inhibition and gene knockdown of CYP1A have been demonstrated to dramatically enhance the cardiac teratogenesis caused by certain PAHs and extracts from the AW sediments.^{32–34} Because CYP1 induction is generally considered to be a marker of AHR pathway activation,³⁵ recalcitrance to CYP1 induction may instead indicate that suppression of AHR pathway activity is an important component of resistance. Furthermore, gene knockdown of AHR2 demonstrated that teratogenesis caused by certain PAHs and DLCs in fish is mediated at least in part through the AHR pathway.^{36–38} However, some PAHs appear to cause teratogenesis independent of the AHR.^{39, 40} Ownby and co-workers²⁴ did not investigate AHR pathway induction in their experiments; with the exception of the AW subpopulation, the role of AHR pathway suppression in the resistance of ER killifish subpopulations had not been investigated previously.

We hypothesized that suppression of CYP1 activity, as a potential marker of AHR pathway suppression, would be associated with contaminant resistance in killifish subpopulations throughout the ER estuary. We compared CYP1 induction and cardiac teratogenesis generated by PAHs and PCB-126, individually and in mixtures, in embryos from killifish subpopulations from sites that varied widely in PAH contamination level.

The results revealed that the role of AHR pathway suppression in resistance to cardiac teratogenesis varied significantly among subpopulations, suggesting that killifish subpopulations throughout the estuary exhibit a multi-faceted mechanism of resistance to PAH contamination.

MATERIALS AND METHODS

Fish

In summer 2008, adult killifish were collected from four sites in the south branch of the ER, which flows north into the Chesapeake Bay, and one site on the east branch (Figure 1). The AW site is located at the Atlantic Wood Industries Superfund site (36°48'27.2" N, 76°17'38.1" W). Scuffeltown Creek (SC) (36°48'33.9" N, 76°17'04.1" W) is directly across the river, about 0.8 km from AW. Jones Creek (JC) (36°48'05.5" N, 76°16'43.8" W) is about one km into a small tributary that enters the ER about one km south (upriver) of the AW site. Previous work utilized killifish collected closer to the mouth of JC.^{3, 24, 42} Pescara Creek (PC) (36°50'02.7" N, 76°16'38.4" W) is on the eastern branch, approximately 5.5 river km from the AW site, near a shipyard in an area that may have been the historical site of a wood treatment facility (Michael Unger, Virginia Institute of Marine Science, personal communication). Mains Creek (MC) is approximately 8 km south of the AW site, upstream of the major sites of creosote contamination and close to the river's junction with the Intracoastal Waterway (36°45'13.5" N, 76°24'58.9" W). Reference killifish were collected at King's Creek (KC), a relatively-uncontaminated tributary of the Severn River (37°18'16.2"N, 76°24'58.9"W). Subsequently (summer 2010), two PAH-contaminated sites were added. The Republic site is approximately 1.5 km south of AW and is the site of the former Republic Creosoting company (36°47'39.65"N, 76°17'31.94"W). The Hess site is approximately 3 km south of the AW site. It is at the site of the former Eppinger and Russell

creosote facility. Hess fish were sampled in an area (36°46'59.64"N, 76°18'5.88"W) where sediment remediation and wetland restoration was begun in summer 2009.

Fish were maintained in 20‰ artificial sea water (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA) at 23–25°C, with a 14:10 light:dark cycle, and were fed pelleted fish feed (Aquamax ® Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, USA). Adults were maintained in clean water for a minimum of two months to allow depuration of PAHs. Eggs were obtained from all fecund females from a given subpopulation (typically >75% of approximately 150–200 females) and sperm from approximately 30 males from the same population. The eggs and sperm were mixed and allowed to fertilize for a minimum of 1 hour, then washed briefly with 0.3% hydrogen peroxide in ASW. Care and reproductive techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A234-07-08).

Chemicals and sediment extraction

β-naphthoflavone (BNF), ethoxyresorufin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), and fluoranthene (FI) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Absolute Standards, Inc. (Hamden, CT, USA) and 3,3',4,4',5–23 pentachlorobiphenyl (PCB-126) was purchased from AccuStandard (New Haven, CT, USA). All stocks were prepared in DMSO. Coal tar (standard reference material 1597a), a standard mixture of PAHs containing 4363.83 mg total PAHs/L, was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The coal tar standard is a mixture consisting of 92 different PAHs and substituted PAHs. For a detailed information on the concentration of the individual PAHs present in the coal tar standard reference material, please see Wise et al.⁴³ ER sediment extract (ERSE) was prepared from sediment collected in the ditches on the north side of the AW Superfund Site. Around 2 kg of sediment were collected from each of three points along two east-to-west transects and stored in glass jars in the laboratory at 4°C for approximately 30 days. Sediment and deionized water (20–25 mL each) were combined and shaken vigorously for 1 minute, then centrifuged at 10000 g for 25 minutes. Supernatant from multiple extractions was decanted, combined and mixed, and frozen at –80°C in individual aliquots.

Determination of PAHs in sediments

Sediments were collected coincident with fish collection in the summer of 2008 at AW, SC, JC, PC, MC, and KC. Sediments were not collected at the Republic or Hess sites coincident with fish capture, but the sediment PAHs were quantified previously by Vogelbein and Unger (see Table 1).² Sediment (2–3 kg total) was collected at six locations along a transect within each site. Sediment from the six collection locales within each site was homogenized to provide a representative sediment sample and stored at 4°C. Wet sediment (approximately 0.6 g) was ground with Na₂SO₄, spiked with a surrogate standard mix containing four deuterated PAH standards: d8 2-methylnaphthalene, d10fluorene, d10fluoranthene and d12perylene. Recoveries of surrogate standards ranged from 68–103% with the exception of 2-methylnaphthalene, for which recoveries were near 36%. Samples were extracted in 50/50 dichloromethane (DCM) and hexane (v/v) using an accelerated solvent extractor (ASE 300,

Dionex, Sunnyvale, CA). Extracts were concentrated using rapid evaporation under N₂ (Turbo Vap, Caliper LifeSciences, Hopkinton, MA, USA) to approximately 0.5 mL, and cleaned with alumina column chromatography using 6% deactivated alumina (4 g) eluted with 50 mL 50/50 DCM/hexane. Purified extracts were concentrated under N₂ and HCl-cleaned copper turnings were added during concentration to remove sulfur. All extractions were conducted in triplicate. Blanks (n=5) and standard reference materials (n=3, SRM 1944, National Institute of Standards and Technology) were included with extractions and PAH levels in the sediments were blank-corrected. Levels of PAHs in the blanks and SRM and method detection limits are reported in Table S1. PAHs were analyzed using a gas chromatograph mass spectrometer (Agilent GC 6890N, MS 5975, Newark, DE) in electron impact mode using selected ion monitoring and splitless injection (250°C). Analytes were separated on a DB-5 column (30 m, 250 µm nominal diameter, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) using an oven temperature program with a thermal gradient (40°C for 0.6 min, increase to 280°C over 14.6 min, hold at 280°C for 24 min). Sediment moisture content was measured gravimetrically by weighing approximately 1.3 g wet sediment before and after drying at 105°C for 16 hours. Moisture content was calculated as (moist weight – dry weight)/dry weight, and used to correct PAH concentrations to dry weight.

Embryo dosing, EROD activity, and deformity assessment

For challenges with individual hydrocarbons we chose the following: BNF, a model PAH frequently used to study fish teratogenesis; BkF, a high molecular weight PAH that is commonly found in the ER and can induce deformities by itself; and PCB-126, a polychlorinated biphenyl that causes cardiac deformities and has been studied in AW fish.^{38, 41} For the mixture challenges, we chose the following: BaP and FI, which are both present in high quantities in the ER and which we have shown to synergistically cause cardiac teratogenesis; ERSE, a porewater extract of sediments from the AW Superfund site, and coal tar, a standard reference material consisting of a complex pyrogenic mixture of PAHs similar to those found in creosote.

The embryo-toxicity experiments were designed to compare the deformity and EROD responses to a given compound across the subpopulations and to compare the response to various compounds within an individual subpopulation. For that reason, the experiments included treatment groups from all of the subpopulations (and the reference population) treated with each of the six compounds or mixtures. Because of the large total number of individuals needed for simultaneous comparison of the various compounds in all of the populations, it was necessary to choose individual challenge doses that provided both EROD induction and caused deformities based on previous laboratory experience,^{22, 23, 32, 34, 38} rather than to determine simultaneous dose responses.

In the first experiment, embryos from KC, AW, SC, JC, PC, and MC parents were exposed to 10 µg/L BNF, 300 µg/L BkF, 1 µg/L PCB-126, a mixture of 100 µg/L BaP and 500 µg/L FI, 10% (v/v) ERSE, 0.005% (v/v) coal tar or DMSO vehicle control. Dosing solutions were made in 20" ASW. A second experiment was conducted to compare EROD response in embryos from KC, AW, SC, JC, PC, and MC parents exposed to equimolar (3 nM) doses of

BNF (0.82 µg/L), BaP (0.76 µg/L), BkF (0.76 µg/L), and PCB-126 (0.98 µg/L). After collection from Hess and Republic in 2010, a final experiment was conducted comparing the responses of Hess and Republic embryos to those of AW and KC embryos using 300 µg/L BkF, 1 µg/L PCB-126, and 10% (v/v) ERSE.

Embryos were dosed individually with 10 mL of dosing solution in 20-mL glass scintillation vials (VWR, West Chester, PA) beginning at 24 hpf. Dosing solutions also contained 21 µg/L ethoxyresorufin, a substrate for the ethoxyresorufin-*o*-deethylase (EROD) assay. Embryos were maintained in dosing solution in the incubator at 27°C. Exposures consisted of three experimental replicates with n=10 embryos per treatment group per experiment with two exceptions; the equimolar exposures and the BaP plus FI exposures consisted of three experimental replicates with n=8 embryos per treatment group per experiment.

EROD activity and cardiac teratogenesis were assessed at 96 and 144 hpf, respectively. CYP1 activity was measured via the in ovo EROD assay modified from Nacci et al.⁴⁴ (detailed description in Matson et al.³⁴). EROD data were not collected for the BaP and FI mixture exposure because the dose of FI used reduces CYP1 activity to very low levels (data not shown). All EROD values are expressed as percent of the King's Creek (reference) population DMSO dosed control group response unless otherwise noted. Cardiac teratogenesis was scored blind under light microscopy (40X magnification). Deformity severity was scored as a 0 (normal), 1 (moderate deformity), or 2 (severe deformity) as described previously.^{34, 38} The primary cardiac abnormalities observed were heart elongation, improper atrial-ventricular alignment, and pericardial edema.

Data analysis

All analyses were performed using JMP 8.0 (SAS Institute Inc, Cary, NC, USA). For these analyses, the individual embryo was the unit of replication. The experiments were replicated three times. To determine if the experimental replicates could be combined, we first tested to see if there was a main effect of experiment. Because there was not, we combined the experimental replicates yielding n=24 (BaP/FI and equimolar experiments) or n=30 (all other challenges) individually dosed embryos per treatment group. Non-parametric deformity and EROD data were rank-transformed and analyzed by analysis of variance (ANOVA) followed by least square means (LSMeans) procedures. For post hoc comparisons, Tukey-adjusted pairwise comparisons were conducted to determine the significance of differences among groups. Statistical significance was accepted at $p = 0.05$ for all tests.

RESULTS

PAHs in sediments

The total sediment burdens of the targeted PAHs (ng/g dry sediment) are shown in Table 1. Data for the Hess and Republic sites are from Vogelbein and Unger.² AW, Hess, and Republic sediments had the greatest total PAH levels, whereas SC, PC, and JC had lower total PAH levels that were 4–12 times greater than the total PAH level observed at the reference site. The KC (reference) site had lower total PAHs than all of the ER sites except

for MC. The concentrations (ng/g dry sediment) of individual measured PAHs at each of five ER sites and the KC site are displayed in Figure S2. The profiles of the KC and MC sites were somewhat shifted toward lower molecular weight PAHs, whereas the profiles of the AW, JC, and SC sites showed a greater prevalence of higher molecular weight PAHs. The PAH profile of PC site was shifted even more toward higher molecular weight PAHs.

Response of ER subpopulations and reference population to PAH and PCB-126 exposures

The mean deformity score and EROD activity induced by the PAHs and PCB-126 in the five ER subpopulations and the reference population tested in the first experiment are shown in Figure 2 and Figures S2 and S3. As expected, KC (reference) embryos were highly sensitive to both cardiac teratogenesis and induction of EROD activity for all exposures. In contrast, AW embryos were highly resistant to both cardiac teratogenesis and EROD induction for all exposures except coal tar, which induced EROD activity slightly ($207 \pm 29\%$; $p=0.0452$) but did not cause deformities.

In general, the responses of SC embryos were similar to those of AW embryos. None of the exposures induced statistically significant cardiac teratogenesis in SC embryos, although BkF and ERSE caused a slight elevation in deformity score. EROD activity was induced in SC embryos by BkF ($627 \pm 60\%$; $p<0.0001$), coal tar ($502 \pm 44\%$; $p<0.0001$) and ERSE ($480 \pm 53\%$; $p<0.0001$).

The JC subpopulation demonstrated an intermediate response. JC embryos did not exhibit statistically significant teratogenesis from BNF, BkF, or PCB-126, but suffered mild deformities due to exposure to BaP+FI (0.47 ± 0.13 ; $p=0.0391$), coal tar (0.60 ± 0.13 ; $p=0.0001$) and ERSE (0.57 ± 0.14 ; $p=0.0016$). The EROD response was elevated in JC embryos exposed to BkF ($630 \pm 50\%$; $p<0.0001$), coal tar ($346 \pm 25\%$; $p=0.0001$), and ERSE ($325 \pm 30\%$; $p<0.0001$).

PC embryos also demonstrated an intermediate response. PC embryos were resistant to teratogenesis caused by BNF, BkF, PCB-126, BaP+FI, but suffered mild deformities from coal tar (0.60 ± 0.13 ; $p=0.0011$) and intermediate deformities from ERSE (1.13 ± 0.13 ; $p<0.0001$). Additionally, EROD activity was induced in PC embryos for all exposures tested ($p<0.006$).

MC embryos were the most responsive of the ER subpopulations and exhibited a similar response to the reference population for several exposures. MC embryos suffered intermediate deformities from BkF (0.87 ± 0.09 ; $p<0.0001$) and severe deformities from coal tar (1.33 ± 0.13 ; $p<0.0001$), and ERSE (1.43 ± 0.13 ; $p<0.0001$). In addition, the deformity score was elevated but not statistically different from control for exposure to BNF (0.47 ± 0.09 ; $p=0.0542$) and PCB-126 (0.44 ± 0.12 ; $p=0.2876$). Furthermore, EROD activity was induced in MC embryos for all exposures tested ($p<0.0001$).

Response of ER subpopulations and reference population to equimolar concentrations of individual PAHs and PCB-126

To further compare the AHR pathway responsiveness of each subpopulation to different agonists, EROD activity was assessed in response to equimolar (3 nM) concentrations of

BaP, PCB-126, BNF, and BkF (Figure 3). The reference population exhibited the highest EROD response to BaP ($631 \pm 66\%$), PCB-126 ($822 \pm 130\%$), BNF ($1440 \pm 83\%$), and BkF ($1310 \pm 95\%$). The AW subpopulation exhibited very little response ($<40\%$ of KC DMSO response for all exposures). The SC subpopulation had a very similar EROD response to that of the highly resistant AW population. The JC subpopulation also exhibited low response to most exposures. The JC EROD response ($261 \pm 59\%$) to BkF exposure was not statistically different from that of the DMSO-dosed control ($p=0.4013$). The EROD responses caused by PCB-126 ($180 \pm 55\%$) and BNF ($139 \pm 48\%$) in the PC subpopulation were also not statistically different from control ($p=0.9844$ and $p=1.000$, respectively). However, BkF did induce EROD significantly in the PC subpopulation ($490 \pm 60\%$; $p<0.0001$). The MC subpopulation response was similar to the other ER subpopulations for BaP, but its EROD response was statistically elevated for exposure to PCB-126 ($657 \pm 150\%$; $p<0.0001$), BNF ($720 \pm 160\%$; $p<0.0001$), and BkF ($691 \pm 91\%$; $p<0.0001$).

Republic and Hess response to select BkF, PCB-126, and ERSE

Like the AW subpopulation, both the Hess and Republic subpopulations were highly resistant to deformities caused by BkF, PCB-126, and ERSE (Figure 4). However, EROD activity was significantly induced in both Hess and Republic embryos by BkF ($875 \pm 142\%$ and $1030 \pm 114\%$, respectively; $p<0.0001$ for both) and ERSE ($430 \pm 57\%$ and $440 \pm 39\%$, respectively; $p<0.0001$ for both).

DISCUSSION

In the current study, ER killifish exhibited subpopulation-specific patterns of response to PAHs and PCB-126. Furthermore, even subpopulations from sites with relatively low sediment PAH concentrations exhibited strong resistance to some compounds. The variation in responses suggested that while the adapted phenotype is found across a great distance within the estuary (~ 14 km in river length), the pattern of response is not uniform among the subpopulations, perhaps indicating that the adaptive phenotype is not uniformly distributed in subpopulations throughout the estuary.

Variation in adaptive response of ER subpopulations

The response of ER killifish to PAHs and PCB-126 varied greatly among subpopulations. In addition, the observed pattern of response was often specific to the individual subpopulation (Figure 2). The deformity scores for each subpopulation are compiled in Figure S2 and the EROD responses in Figure S3 for additional visualization of the response patterns.

As stated previously, the reference population was the most sensitive to cardiac teratogenesis across all contaminants, the AW, Hess, Republic, and SC subpopulations were the least sensitive, and the JC, PC, and MC subpopulations exhibited intermediate sensitivity. Two of the intermediately sensitive populations, JC and PC, were affected more by PAH mixtures than individual hydrocarbons. Interestingly, the PC subpopulation was more sensitive to ERSE than coal tar; the other subpopulations had a consistent response to the two complex mixtures. Finally, the MC subpopulation was nearly as susceptible as the

reference to cardiac teratogenesis generated by all of the exposures tested, with the dramatic exception of their resistance to PCB-126 and the BaP and FI mixture.

Notably, all of the ER subpopulations were quite resistant to cardiac teratogenesis generated by PCB-126 and the simple mixture of BaP and FI. Also, most of the ER subpopulations showed a greater deformity response due to BkF than PCB-23 126, despite the frequent presence of BkF in ER sediments. This lack of sensitivity to PCB-126 has been reported in previous studies of the AW subpopulation.²³ Nacci et al.⁴¹ demonstrated that AW killifish were less sensitive to PCB-126 than other adapted killifish from sites, such as New Bedford Harbor, MA, with much greater PCB contamination than in the ER (201 ng PCBs/g sediment at AW vs. 22,666 ng/g at New Bedford Harbor). The United States Environmental Protection Agency (USEPA) reported a lack of PCBs in recent sediment samples taken in the AW Superfund Site.⁴⁵

Several of the subpopulations exhibited EROD induction with exposures that did not cause cardiac deformities in those subpopulations at the dose tested. For example, EROD activity was strongly induced in MC embryos exposed to PCB-126, despite their relative resistance to PCB-induced teratogenesis. Even the Hess and Republic embryos demonstrated an intermediate EROD response to BkF and ERSE, despite being highly resistant to the cardiac teratogenesis caused by these exposures in reference fish. This differences in relative sensitivity to EROD induction with respect to resistance to cardiac teratogenesis among some of the subpopulations is more apparent when each subpopulation's EROD response is normalized to its own DMSO response instead of that of the reference population (fold change or relative EROD inducibility, Figure S3B). For example, the fold change in EROD response in SC, JC, and PC embryos exposed to BkF was almost identical to that of reference embryos despite all three subpopulations resisting the cardiac teratogenesis generated by BkF. Likewise, the fold change in EROD response of PC and MC embryos exposed to PCB-126 was similar to that of reference embryos, yet both subpopulations were resistant to PCB-126-induced cardiac teratogenesis. Additionally, the fold change in EROD response generated by ERSE and coal tar was disproportionately high in SC embryos; SC embryos were nearly as resistant to cardiac teratogenesis generated by ERSE and coal tar as were AW fish. Finally, the fold change in EROD activity of the PC subpopulation was greater than or very similar to that of reference fish for all exposures, possibly indicating that the PC subpopulation's AHR pathway is as inducible as that of reference fish but has a lower basal (and perhaps maximal) EROD response.

One of the limitations of the current study is the lack of full dose-response curves for each challenge in each of the subpopulations. As described previously, the challenge doses were picked based on published literature and previous laboratory experience with the goal of providing doses that both induced EROD activity and caused deformities in non-adapted fish. However, we are unable to determine if the observed differences are due to shifts in the overall dose response curve for one or both of the endpoints (i.e., a change in the EC50 for EROD activity) or shifts in the maximum response to a given challenge for each endpoint. In addition, the in ovo EROD assay is limited in that it measures the accumulation of a product of CYP1 activity, but not the kinetic rate of reaction. Furthermore, the EROD reaction can be inhibited by the inducing compound or by CYP inhibitors, such as

fluoranthene, present in exposure mixtures. Therefore, EROD activity might not be a fully accurate indicator of CYP induction. Overall, further work is needed to determine if differential responses to the challenges among the subpopulations occur across a range of doses.

A number of interesting conclusions are suggested by the variation in CYP1 induction and resistance to cardiac teratogenesis exhibited by the ER subpopulations. While heritable resistance to PAHs clearly occurs throughout the ER estuary, the underlying adaptive phenotype may not be uniform among the killifish subpopulations. If the adaptation were simple and identical among subpopulations, one might expect to see a pattern of resistance that varied only in magnitude across all exposure types based on the percentage of individuals within the population that carried the heritable adaptation. Instead, two subpopulations (MC and PC) exhibited AHR pathway inducibility similar to that of reference fish for all challenges and others (SC and JC) for only some exposures. Likewise, some subpopulations (AW, Hess, Republic, and SC) were highly resistant to cardiac teratogenesis generated by all of the exposures tested, while others (JC, PC, and MC) were highly resistant to cardiac teratogenesis due to some exposures but only moderately resistant to others.

As described previously, cardiac teratogenesis caused by DLCs and some PAHs in fish is mediated at least in part through the AHR pathway,^{36–38} and downregulation of the AHR appears to underlie resistance in multiple fish populations adapted to DLCs (reviewed by Wirgin and Waldman⁴⁶). Understanding these phenomena is complicated by the fact that fish have multiple AHRs and their individual roles in toxicity and development are still being determined, although we have shown that AHR2 mediates the teratogenic effects of some PAHs and PCB-126 in killifish.^{38, 47} The role of allelic variation in AHR in contaminant adaptation is not clear. For example, resistance of Hudson River tomcod (*Microgadus tomcod*) has been attributed to changes at a single locus, the deletion of six bases in AHR2.⁴⁸ In contrast, Hahn *et al.* demonstrated that AHR1 allele frequencies differed between dioxin-sensitive and resistant killifish populations, but the representative alleles did not differ functionally.⁴⁹ Work is ongoing in our laboratory and others to determine the genomic variation associated with contaminant adaptation in killifish from multiple populations on the Atlantic coast.

Overall, the fact that some ER subpopulations exhibit resistance to teratogenicity at doses that induce significant CYP1 activity suggests that suppression of the AHR pathway response to the degree exhibited by the highly resistant AW subpopulation is not required for resistance to embryotoxicity. This observed variability in subpopulation response supports previous findings that contaminant resistance in ER killifish is associated with multiple adaptations. Alterations in several xenobiotic metabolism and excretion pathways are associated with resistance in AW killifish, including elevated levels of glutathione S-transferase,⁵⁰ UDP-glucuronosyl transferase (UGT),⁵¹ sulfotransferase,⁵¹ and hepatic P-glycoprotein (Pgp)⁵² and upregulated antioxidant defenses.^{53, 54} However, only the heritability of the upregulated antioxidant defenses has been investigated and confirmed in ER killifish.⁵³ The GSTs, UGTs, and sulfotransferases aid in increasing the water solubility and excretion of PAHs; polymorphic variation in these enzymes and differences in their

expression are associated with inter-individual differences in response to PAHs.⁵⁵ The Pgps are membrane transport ATPases that are involved in the efflux of compounds. Although they have been identified in a variety of normal tissues, they are also frequently elevated in multidrug-resistant cell lines and chemotherapy-resistant tumors.⁵⁶

Association of resistance with PAH contamination and proximity to highly-adapted subpopulations

PAH contamination clearly has an estuary-wide effect on ER killifish. However, it is not clear how the pollution is driving adaptive changes among the subpopulations. One possibility is that the lower levels of PAHs found outside of the major contamination sites are sufficient to drive adaptation of isolated individual subpopulations of killifish. Alternatively, adapted subpopulations in the most contaminated areas may be acting as sources of adaptive genetic material for other subpopulations.

In general, the overall sensitivity to PAH-mediated teratogenesis of the subpopulations studied here followed the order KC(ref)>MC>PC>JC>SC>Hess≈Republic>AW. This pattern was roughly the inverse of the total PAH levels found at the sites, which followed the order MC≈KC(ref)<JC<PC≈SC<Republic≈Hess≈AW. However, PAH levels do not seem to be fully explanatory. Plotting the log₁₀ of the total PAHs measured for each site against all deformity responses for the subpopulations only weakly supports an inverse relationship between PAH contamination level and deformity score ($y = -0.3224X + 1.622$, $R^2 = 0.2101$). This may be driven primarily by a few of the subpopulations that do not fit this relationship. For example, the total PAHs (4493±557 ng/g dry sediment) measured at PC were very similar to those measured at the SC site (6328±1238 ng/g dry sediment), yet the PC subpopulation was among the more responsive. One possible explanation is that the sediment contamination at the PC site may be from an older source than elsewhere (Michael Unger, Virginia Institute of Marine Science, personal communication), perhaps allowing for greater weathering of the PAHs or altering bioavailability. In fact, examination of the PC PAH profile in Figure S1 shows a greater burden of higher molecular weight PAHs relative to some of the sites. The PC subpopulation might represent a formerly highly-adapted subpopulation “recovering” from PAH contamination. A greater relative burden of higher molecular weight PAHs also tracks an enrichment of PAHs that are AHR agonists; this might be expected to increase toxicity unless bioavailability decreased concurrently. In contrast to the PC subpopulation, the JC subpopulation was subject to lower total PAH contamination but was relatively resistant to teratogenesis generated by PAHs and PCB-126. Interestingly, Ownby *et al.*²⁴ also found that a killifish subpopulation collected at the mouth of JC, approximately 900 meters from our collection site, was much more resistant to cardiac teratogenesis than another subpopulation collected in an area with higher total PAHs in sediments. The most striking example of a disassociation between sediment contamination and resistance to PAHs in our study was exhibited by the MC subpopulation. MC killifish resided in an area with relatively low sediment PAH levels, yet were highly-resistant to several hydrocarbon exposures.

Another possible explanation for the disconnect between sediment PAH measurements and resistance is that the resistance is driven by contaminants other than PAHs. The Elizabeth

River is a highly industrialized and urbanized watershed, so there are other potential sources of pollutants. Although the USEPA has detected some metals, dioxin, and PCBs at the AW Superfund site, they state that the contamination in the river sediment is dominated by PAHs, and dioxin and PCBs were not found in recent sediment samples.⁴⁵ In comparison to another site with highly adapted killifish, Nacci *et al.* report a concentration of 201 ng total PCBs/g dry sediment in the AW site compared to 22,666 ng/g in New Bedford Harbor, MA.⁴¹ In ongoing work, we are investigating the presence of chlorinated hydrocarbons and other contaminants at the various collection sites used herein. We are performing toxicity identification assays to further isolate the source(s) of the teratogenic effects caused by the ERSE and preliminary results support the high molecular weight PAHs as causative agents of cardiac teratogenesis (unpublished data).

Mulvey and coworkers used allozyme frequency³ and mtDNA haplotype⁴² to investigate the genetic structure of subpopulations from the ER estuary, including those from the AW, SC, and PC sites studied here. They also examined fish from JC (collected in the same sites described by Ownby *et al.*,²⁴ about 900 m from where we collected in JC). They found significant heterogeneity in allozyme frequency for 3 of the 15 loci examined (*Idh-2*, *Ldh-B*, and *Gpi-1*), although there was no consistent relationship between allozyme frequency and sediment PAH concentration. With both methods, they found that the AW subpopulation was genetically distinct from the other subpopulations. In both studies, they found that genetic distance was significantly correlated with PAH contamination, but not with geographic distance. This correlation between genetic distance and contamination level observed by Mulvey and coworkers contrasts somewhat with the results of the current study. In part, this is probably due to their use of relatively neutral markers (mtDNA) and genes that were almost certainly not under selection (allozymes). Because it seems highly likely that resistance to the acute toxicity is a major component of adaptation to PAHs, the gene or genes underlying this resistance are the probable units of selection. However, in the current study the pattern of resistance phenotypes, reflecting the underlying genotypes actually under selection, did not correlate strictly with contamination level.

As stated previously, one possible explanation for poor correlation of resistance to contaminant level is that the highly-resistant AW, Hess, and Republic subpopulations act as a source of resistance genes for other subpopulations. This would be logical given the responses of the proximate, highly-resistant but relatively less exposed SC and JC subpopulations. The varied response of the MC subpopulation might also provide evidence in support of movement of resistance genes. MC killifish responded similarly to the reference population for most exposures, as might be expected for a subpopulation exposed to the lowest PAH levels we measured in the ER. However, unlike reference fish, they were highly resistant to teratogenesis from PCB-126 and the mixture of BaP and Fl. This could indicate that the MC subpopulation has some components, but not others, of a multigenic PAH-adaptation. Mulvey and coworkers did not find evidence of gene flow of this nature, but they did find that migration among localities in the ER was 9.6 juveniles and 17.5 adults per generation. This is more than sufficient to move the resistance genes among the sites. However, the resistance genes would only remain in the individual subpopulations if they conveyed a competitive benefit or at least did not hinder performance. There is evidence that

AW fish do not survive as well under clean conditions,⁵⁸ perhaps indicating that migrants from a highly-adapted subpopulation would be at a competitive disadvantage in a less-contaminated locale.

Several other studies have described the spatial distribution of resistance to contaminants in fish. Yuan et al.⁵⁹ found that Atlantic tomcod collected in sites spanning 144 km of the Hudson River are resistant to CYP induction by PCBs and polychlorinated dibenzo-*p*-dioxins. Nearly 320 km of the Hudson River, New York, is a Superfund site due to PCB contamination originating upstream. Fernandez et al.⁶⁰ showed that the pattern of PCB burden in tomcod, although not the overall level, was associated with proximity to the original PCB sources. Despite possible differences in exposure history, tomcod from throughout the estuary did not vary significantly in their degree of resistance.⁵⁹ This is likely because tomcod move throughout the Hudson River during their life cycle and represent a single population. Unlike the Hudson River tomcod, PCB tolerance of killifish was predicted by the level of sediment PCBs at the site of collection in populations distributed from 0–100 km from the New Bedford Harbor, MA Superfund site.⁶¹ A subsequent study added sites from Maine to Virginia, including the AW and KC sites and heavily PCB-contaminated sites in Bridgeport, CT, Newark, NJ, and Jamaica Bay, NY.⁴¹ A significant correlation between sediment PCBs and resistance was observed; however, the AW population was somewhat of an outlier in this relationship because it was by far the most resistant to the effects of PCB-126 but had much lower PCB levels than the other sites with highly resistant fish. These studies demonstrate how the spatial distribution of contamination, more than the movement of adapted individuals, can drive changes in populations at a geographic scale.

The occurrence of spatially-extensive resistance to contaminants shown in the current study demonstrates a heritable effect of anthropogenic contamination across an entire ecosystem. Regardless of whether landscape level changes in population parameters are driven directly by contamination or by movement of adaptive genes throughout the estuary, the fact that anthropogenic contamination exerts effects at the metapopulation scale may have wider consequences. Because killifish are an integral part of Atlantic estuarine ecosystems, alterations of multiple subpopulations could result in ecosystem-wide consequences that require further exploration. Furthermore, only a few fitness costs associated with PAH adaptation in AW killifish have been identified,⁵⁸ but it is likely that the heritable adaptation involves tradeoffs, which warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Location of King's Creek and Elizabeth River collection sites

The expanded map shows the location (black dots) of the seven Elizabeth River collection sites. Circles mark the approximate location of three former creosote facilities that have contributed major contamination to the river.

Figure 2. Mean deformity score and ethoxyresorufin-o-deethylase (EROD) response of five Elizabeth River killifish subpopulations and a reference population exposed to PAHs and PCB-126

Mean deformity score (shown in bars and on the left vertical axis) and ethoxyresorufin-o-deethylase (EROD) response (shown in lines and on the right vertical axis) of embryos from five Elizabeth River subpopulations and the reference population exposed to A) 10 $\mu\text{g/L}$ β -naphthoflavone (BNF), B) 300 $\mu\text{g/L}$ benzo[k]fluoranthene (BkF), C) 1 $\mu\text{g/L}$ 3,3',4,4',5-pentachlorobiphenyl (PCB-126), D) 100 $\mu\text{g/L}$ benzo[a]pyrene (BaP) plus 500 $\mu\text{g/L}$ fluoranthene (FI), E) 0.005% (v/v) coal tar or F) 10% (v/v) Elizabeth River sediment extract (ERSE). Embryos were exposed individually at 24 hours post fertilization (hpf); EROD was measured at 96 hpf and deformities assessed at 144 hpf. White bars and dashed lines represent DMSO-dosed groups and grey bars and solid lines represent PAH- and PCB-126-dosed groups. Error bars represent $\pm\text{SEM}$. Bars not marked by the same letter are

significantly different at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). EROD values marked by * are significantly different from the population-matched DMSO-dosed control at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). $n = 30\text{--}45$ individuals per treatment group except for BaP plus FI where $n = 24\text{--}30$ individuals per group. Sites are arranged with the reference and highly adapted populations first and then by distance from the Atlantic Wood site. Abbreviations: AW (Atlantic Wood), KC (King's Creek), SC (Scuffeltown Creek), JC (Jones Creek), PC (Pescara Creek), and MC (Main's Creek).

Figure 3. Ethoxyresorufin-*o*-deethylase (EROD) response of five Elizabeth River killifish subpopulations and the reference population (King's Creek) exposed to 3 nM benzo[a]pyrene, 3,3',4,4',5-pentachlorobiphenyl, β -naphthoflavone, or benzo[k]fluoranthene

Ethoxyresorufin-*o*-deethylase (EROD) response of embryos from five Elizabeth River subpopulations and the reference population (King's Creek) exposed to dimethyl sulfoxide control (DMSO, dark grey bars), benzo[a]pyrene (BaP; hatched bars), 3,3',4,4',5-pentachlorobiphenyl (PCB-126; black bars), β -naphthoflavone (BNF; white bars), or benzo[k]fluoranthene (BkF, light grey bars). Embryos were exposed individually at 24 hours post fertilization (hpf) and EROD activity was measured at 96 hpf. Error bars represent $\pm$ SEM. Bars not marked by the same letter are significantly different at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). $n = 30$ individuals for all treatment groups. Sites are arranged with the reference and highly adapted populations first and then by distance from the Atlantic Wood site. Abbreviations: AW (Atlantic Wood), KC (King's Creek), SC (Scuffeltown Creek), JC (Jones Creek), PC (Pescara Creek), and MC (Main's Creek).

Figure 4. Mean deformity score and ethoxyresorufin-o-deethylase (EROD) responses of the Hess, Republic, and AW subpopulations and the reference population after exposure to 3,3',4,4',5-pentachlorobiphenyl, benzo[k]fluoranthene, or Elizabeth River sediment extract

Mean deformity score (shown in bars and on the left vertical axis) and ethoxyresorufin-o-deethylase (EROD) response (shown in lines and on the right vertical axis) of embryos from five Elizabeth River subpopulations and the reference population exposed to 300 µg/L benzo[k]fluoranthene (BkF), 1 µg/L 3,3',4,4',5-pentachlorobiphenyl (PCB-126), or 10% (v/v) Elizabeth River sediment extract (ERSE). Embryos were exposed individually at 24 hours post fertilization (hpf); EROD was measured at 96 hpf and deformities assessed at 144 hpf. Dark grey bars and closed circles represent the King's Creek (KC; reference) response. Black bars and open circles represent the Atlantic Wood (AW) response. Light grey bars and open triangles represent the Republic response. White bars and Xs represent the Hess response. Error bars represent $\pm$ SEM. Bars marked by # are significantly different from the population-matched DMSO-dosed control at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). EROD values marked by * are significantly different from the population-matched DMSO-dosed control at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). $n = 24$ –30 individuals per treatment group.

Table 1
Total selected PAH concentrations from seven Elizabeth River sites and King's Creek (reference)

Total concentration (ng PAH/g dry sediment) of measured PAHs from seven sites in the Elizabeth River and the King's Creek site (reference). See Figure S2 for concentrations of individual PAHs that these totals comprise and Table S1 for SRM and blank values, internal standard recoveries, and method detection limits. For Atlantic Wood, Scuffeltown Creek, Jones Creek, Pescara Creek, and Mains Creek, sediments were collected from six locations along a transect within the site and homogenized to provide a single representative sample of sediment from the site.

Site	Total selected PAHs (ng/g dry sediment)
King's Creek (reference)	526 ± 624
Atlantic Wood	122,665 ± 16,854
Scuffeltown Creek	6,328 ± 1,253
Jones Creek	1,910 ± 518
Pescara Creek	4,493 ± 557
Mains Creek	186 ± 201
Hess (Eppinger and Russell)	124,381 ¹
Republic Creosoting	113,886 ¹

¹ Data from Hess and Republic are from Table 7 in Vogelbein and Unger.²