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FISH (*FUNDULUS HETEROCLITUS*) POPULATIONS WITH DIFFERENT EXPOSURE HISTORIES DIFFER IN TOLERANCE OF CREOSOTE-CONTAMINATED SEDIMENTS

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Abstract—Prior studies suggest that field-collected fish (*Fundulus heteroclitus*) from a creosote-contaminated Superfund site (Atlantic Wood Industries site, Elizabeth River, VA, USA) have enhanced tolerance to local, contaminated sediments. This study was designed to test whether other populations in the Elizabeth River at less contaminated sites also show similar tolerance and whether this tolerance is heritable. To test this, *F. heteroclitus* populations were sampled from four sites within the Elizabeth River with varying sediment polycyclic aromatic hydrocarbon (PAH) concentrations (3.9–264 ng PAH/g dry wt·10^3) and one reference site in a nearby, uncontaminated estuary (York River, VA, USA; 0.27 ng PAH/g dry wt·10^3). Embryo assays were performed to quantify population differences in teratogenic effects during contaminated sediment exposure. Atlantic Wood sediment was mixed with reference sediment to achieve a range of sediment concentrations. Minimal differences were observed in teratogenic effects among fish taken from sites within the Elizabeth River; however, embryos of fish collected from a nearby, uncontaminated York River site and exposed to contaminated sediments had a significantly higher proportion of embryos with cardiac abnormalities than those from the Elizabeth River sites. Embryos from wild-caught and laboratory-reared Elizabeth River *F. heteroclitus* were simultaneously exposed to contaminated sediments, and no significant tolerance differences were found between embryos from fish taken directly from the field and those reared for a generation in the lab. Differences between fish populations from the two estuaries were larger than differences within the Elizabeth River, and these differences in tolerance were heritable.

Keywords—*Fundulus heteroclitus* Adaptation Polycyclic aromatic hydrocarbons Cardiovascular abnormalities Growth

INTRODUCTION

Populations inhabiting contaminated sites can adapt to pollutants. In the presence of sufficient variability within a population, selection against individuals with lowest tolerance to the pollutant results in genetic adaptation. Such adaptation was documented for a wide range of species, including plants [1,2], oligochaetes [3], isopods [4], soil arthropods [5], and minnows [6,7] for both metals and organic contaminants.

Despite its general occurrence, genetic adaptation to pollutants can be difficult to detect in field populations because exposed individuals can also acclimate to pollutants. Adaptation is the genetic process by which a population changes to accommodate environmental factors. Acclimation is the physiological changes an individual makes to minimize the effects of stressors. For example, loss of enhanced tolerance in field-trapped mosquitofish with initially high tolerance to lead after they were kept for 34 d in clean water is evidence of individual acclimation. A nonheritable basis existed for the initial resistance [8]. Studies of enhanced tolerance should be designed to distinguish between individual physiologic acclimation and genetic adaptation.

Several factors contribute to genetic differentiation among populations, including migration, genetic drift due to geographic isolation, species life history characteristics, and natural selection. Pollutant effects on populations must be assessed in the context of all these processes in order to use genetic differentiation as an indicator of genetic adaptation [5,9–12].

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The mummichog, *Fundulus heteroclitus*, is an ideal animal for examining tolerance enhancement and genetic differentiation associated with contaminants [13]. This small fish is common and widespread in Atlantic Coast estuaries from Canada to Florida, USA. This hardy species is easily trapped in large numbers at contaminated habitats. The mummichog can be a good indicator of local environmental conditions because it tends to exist in local subpopulations or demes. They are amenable to laboratory culture and experimental manipulations [14] and demographic analysis [15]. Mummichog embryos have qualities valuable for toxicity testing: Large numbers of embryos can be obtained from most fish enabling better replication and data analysis, the development time is short, and the transparency of the chorion allows detailed examination of the embryo [16,17]. Because of these qualities, mummichogs are common subjects of population biology and genetics study (e.g., Mitton and Koehn [18], DiMichele et al. [19], and Gonzalez-Villasensor and Powers [20]), including those involving pollutants (e.g., Weis and Weis [17], Munns et al. [21], and Nacci et al. [22]).

Williams [23] described the tolerance of a mummichog population from a site heavily contaminated with PAHs. This Atlantic Wood site (AW) is adjacent to a creosote treatment plant on the South Branch of the Elizabeth River (VA, USA) (Fig. 1). Previous studies at this site focused on the plausible relationship between mummichog liver pathology and sediment contamination [24,25]. At AW, adult mummichog had a 50% liver cancer prevalence and exhibited extrahepatic neoplasms [26]. Williams [23] did a series of laboratory experiments with embryos and juveniles, suggesting that *F. heteroclitus* from this location had enhanced tolerance to pollutants relative to

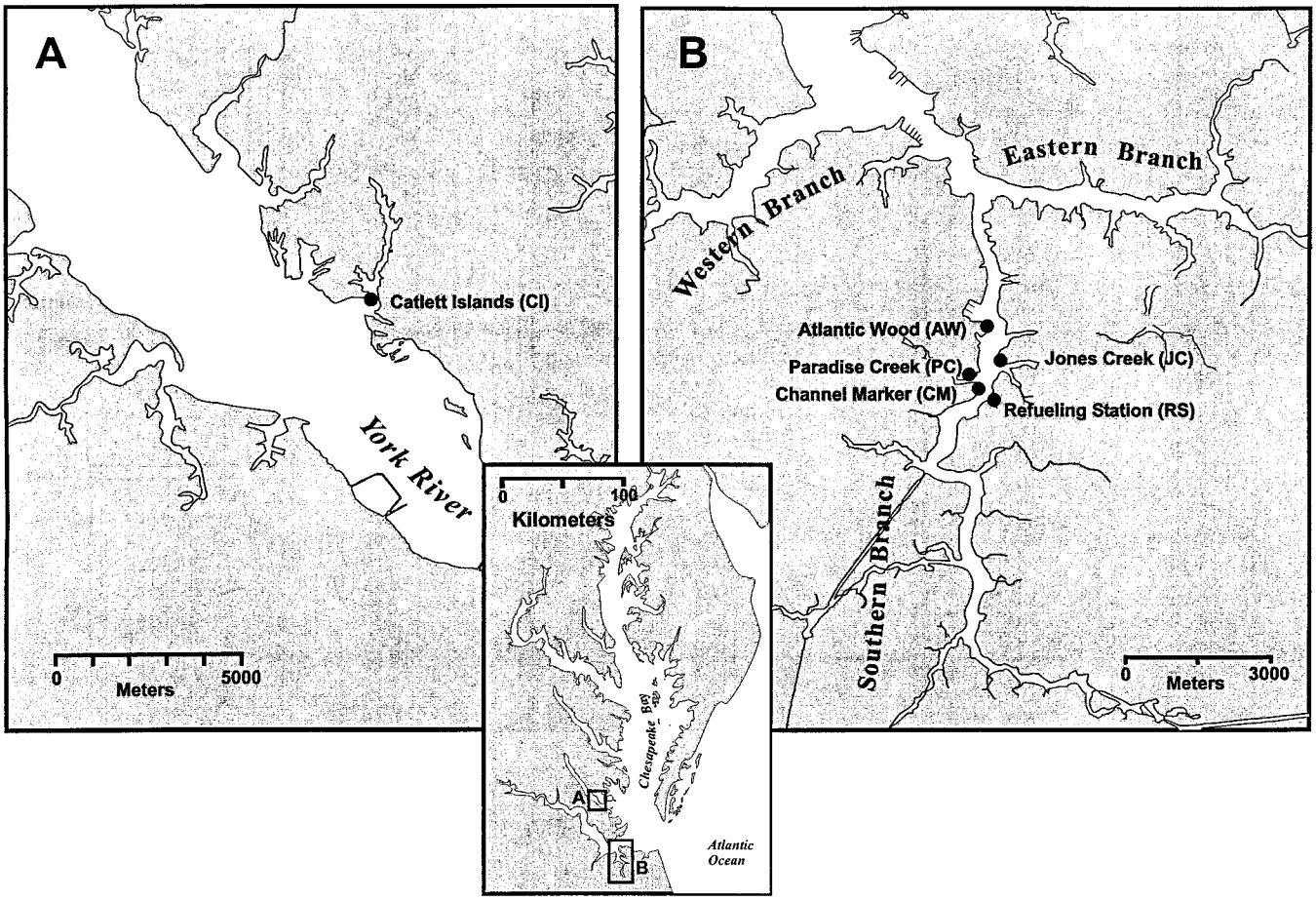

Fig. 1. Population sampling sites on the Elizabeth and York Rivers (VA, USA).

mummichogs taken from Catlett Islands (CI) in the neighboring York River. Armknecht et al. [27] measured elevated glutathione *S*-transferase activity in AW mummichog relative to mummichog from a York River reference site, suggesting that this phase 2 detoxification enzyme might be associated with enhanced tolerance to creosote-contaminated sediments. Elevated levels of P-glycoprotein were found in liver and liver tumors of mummichog from the AW site [28].

In the tolerance studies by Williams [23] described previously, reference populations compared to the AW population were from an adjacent estuary. Little was known about differences in population tolerances within the Elizabeth River. So, definitive statements could not be made about the relationship between AW sediment contamination and enhanced tolerance. Also, the studies described previously gauged differences in mummichog tolerance using a teratogenicity test with eggs and sperm stripped from field-caught adults. Associated experiments could not exclude the possibility of a nongenetic basis for the enhanced tolerance. A nonheritable effect is plausible because Hall and Oris [29] found maternal transfer of anthracene to eggs by fathead minnows and consequent teratogenic effects to embryos. In addition to contaminants, fish can transfer mRNA and proteins directly to embryos [30,31].

The objectives of this study were to evaluate the tolerance of several Elizabeth River mummichog populations relative to a York River reference population and to assess whether the tolerance differences among populations were heritable. Specifically, the goals were to confirm and expand on the differences in tolerance to creosote-contaminated sediments between a highly contaminated site (AW) on the Elizabeth River and a control site (CI) on the York River as seen by Williams, to evaluate differences in population tolerance within the Southern Branch of the Elizabeth River, and to determine if the enhanced tolerance within the Elizabeth River is heritable. To test these hypotheses, field-caught and laboratory-raised mummichog embryos from sites in both estuaries were exposed to creosote-contaminated sediments from the AW site and evaluated for teratogenic effects.

METHODS

Sediments

Based on results from a previous survey, test sediments were selected from two sites on the Elizabeth River: AW and Paradise Creek (PC) (Fig. 1). Sediments from these sites were chosen for their similarities in texture and organic content but contrasting PAH concentrations (Table 1). Sediments were collected in prewashed 20-L high-density polyethylene (HDPE) buckets from each site using a ponar grab in March 1999. The sediment was transported to the Virginia Institute of Marine Science (VIMS, Gloucester Point, VA, USA), passed through a 1-cm^2 sieve, mixed manually to homogenize, and stored in clean, glass jars at −20°C until used for the teratogenicity tests.

Embryo tolerances between estuaries

Reproductively mature (>55 mm in length) *F. heteroclitus* were collected from the AW and CI sites in the early summer

Table 1. Sediment quality at test sites ± standard deviation (n = 2)

Site[a]	PAH concn.[b] (ng/g dry wt·10³)	Clay %	Silt %	Sand %	TOC %[c]
Atlantic Wood (AW)	264 ± 115	14.2 ± 6.6	33.89 ± 11.9	47.86 ± 23.6	6.83 ± 4.28
Refueling Station (RS)	96.1 ± 35.7	7.3 ± 2.1	31.58 ± 8.5	51.09 ± 11.6	12.17 ± 11.6
Jones Creek (JC)	3.9 ± 3.2	2.7 ± 2.4	6.51 ± 1.06	90.55 ± 3.3	0.88 ± 0.02
Paradise Creek (PC)	1.8 ± 0.2	6.9 ± 1.6	15.79 ± 0.15	74.18 ± 5.8	1.76 ± 0.17
Channel Marker 2 (CM)	9.7 ± 1.8	1.7 ± 0.6	2.48 ± 1.0	95.04 ± 2.0	0.26 ± 0.06
Catlett Islands (CI)	0.27 ± 0.6	38.6 ± 12	32.65 ± 9.0	28.83 ± 21	2.00 ± 0.35

[a] All sites are on the Elizabeth River (VA, USA) except for Catlett Islands, which is on the York River (VA, USA).
[b] Polycyclic aromatic hydrocarbon concentration based on list from appendix of Horness et al. [35].
[c] TOC = total organic carbon.

of 2000, 3 d before the full moon. They were kept overnight in tanks receiving a continuous flow of York River water. The following morning, females were anesthetized with MS-222 and their eggs stripped by gently pressing on their abdomen. Eggs were placed into aerated 18‰ Instant Ocean water® (Aquarium Systems, Mentor, OH, USA) (IO water). Males were killed by MS-222 overdose and their testes removed. Testes were placed in a drop of 18‰ IO water and minced with a razor blade to release the milt. The milt from one male was used to fertilize the eggs from three to seven females (~300–400 eggs). The milt remained in contact with the eggs for approximately 1 h, at which time the eggs were washed with IO water three times before being left under a 1-cm layer of IO water in culture dishes overnight in an unilluminated, 27°C incubator. After approximately 20 h, most embryos had reached stage 16, which is characterized by the blastoderm extending over the surface of the yolk, the germ ring advancing ahead of the extraembryonic membrane, and the embryonic shield and embryonic axis increasing in size [32]. Any dead and unfertilized eggs were discarded.

Sediments were prepared by removing AW and PC sediment from the freezer and allowing them to thaw to room temperature. The sediments were mixed by manually inverting the jar 60 to 80 times. Sediments were weighed into cleaned glass jars and mixtures prepared such that a range of AW sediment blends were available (0, 12, 25, 50, 75, and 100% weight/weight [w/w] AW/PC sediment). After the blends were prepared, they were mixed on a magnetic stirrer for 15 min before aliquots for the test were removed. Four-gram aliquots of each mixture for each fish line were weighed into 150-ml beakers and covered until the test began. The remaining sample was kept at −20°C for chemical analysis.

A sediment slurry was prepared by adding 50 ml of IO water to each 150-ml beaker and mixing by swirling. A 20-ml aliquot was placed into each of two replicate exposures. Presorted embryos (stage 19 ± 1) from each line were then randomly placed in the culture dishes to initiate the test exposure. Stage 19 embryos have a well-defined embryonic axis with some condensation of tissue along the lateral margins [32]. Embryos (n = 70/dish) were placed in duplicate culture dishes for the six exposure concentrations. All culture dishes were covered and returned to the 27°C incubator for 96 h. The embryos were removed from the sediments after 96 h and placed into clean aerated IO water. Embryo condition was evaluated by quantifying cardiovascular deformities on day 5 after the test exposure began, using a modified version of the cardiovascular index (CVI) developed by Weis et al. [33]. In our index, 0 = normal heart, 1 = slight defect in structure or function, 2 = tube heart with some degree of chamber development and a definite pulse, 3 = tube heart with no chamber development and no true pulse, and 4 = beating tissue but no heart structure.

Embryo tolerances within the Elizabeth River

To assess differences in tolerance of Elizabeth River mummichog, four sites on the Southern Branch were selected that spanned a range of sediment PAH concentrations (Table 1). The Atlantic Wood site was next to a closed creosote treatment plant and had the highest sediment PAH concentrations. Refueling Station (RS) was located approximately 2.2 km upstream on the opposite bank. Jones Creek (JC) was 1.8 km upstream from AW and located near a railroad trestle. Channel Marker 2 (CM) was a vegetated area directly across the river from RS (Fig. 1). Reproductively mature *F. heteroclitus* were collected from the four sites (AW, CM, JC, and RS) in the summer of 2000, 3 d before the full moon. This embryo test was conducted in the same manner as described previously to quantify any tolerance differences among the Elizabeth River sites. Duplicate culture dishes were exposed with 40 embryos per dish for CM, 48 for JC, 67 for AW, and 98 for RS.

Embryo tolerance from field-caught and laboratory-reared adults

Additional embryos were produced from fish collected in the fall of 1999 and the spring of 2000 to be used as parents in a comparison of tolerance for embryos spawned from exposed (field-captured) and nonexposed (laboratory-raised) parents from the same population. The embryos were hatched by immersion in York River water, and the resulting juveniles (laboratory-raised fish) were reared on a mixed diet of *Artemia* nauplii, cut squid, flake food (special diet #1, Anchor Bay Distributing, New Baltimore, MI, USA), and trout chow (4-mm pellets with Romet 22.2 #/Ton, Ziegler Bros., Gardners, PA, USA) in tanks receiving a continuous flow of York River water during winter months and in outdoor flow-through tanks April to November. Temperature varied with season. In the spring of 2001, additional fish were collected from the five sites (AW, RS, JC, CM, and CI). Eggs and milt were taken from these laboratory-reared and field-caught mummichogs as described earlier. Embryos from laboratory-reared fish (AW, RS, JC, and CM) were exposed concurrently with those from field-caught fish (AW, RS, JC, CM, and CI) to PC and AW sediment. To test for differences among fish lines, the power of the teratogenicity test was increased by using triplicate culture dishes for all lines, instead of duplicates, that produced 80 embryos or more. Because the CI site had low sediment tPAH concentrations (0.2–0.3 ng/g·10³) and water quality fluctuated in a similar manner to our flow-through system in the

laboratory, we did not raise a laboratory line from this site. Notionally, the CI treatment class reflected the response of a population with insignificant PAH-contaminated sediment exposure history.

Chemical and physical analyses

Test sediment PAH concentrations were measured according to the VIMS protocol for toxic organic chemicals [34]. Briefly, sediments were freeze-dried, spiked with surrogate standards, and extracted with dichloromethane by accelerated solvent extraction. The resulting extracts were fractionated by sequential gel permeation and silica gel chromatographies and analyzed for aromatic or heterocyclic compounds by capillary gas chromatography with flame ionization detection and gas chromatography/mass spectrometry in the full-scan electron ionization mode. Aliquots of each sediment sample were also analyzed for grain size and total organic carbon. Blanks, duplicates, and standard reference materials were analyzed simultaneously with environmental samples to ensure data quality. Total selected PAHs (tPAH) were based on the list presented in the appendix of Horness et al. [35] that included, for example, naphthalene, methyl-naphthalene compounds, phenanthrene, pyrene, benzo[*a*]- and [*e*]-pyrene, and fluoranthene.

Statistical analyses

Statistical analyses were performed with the SAS statistical package (SAS® Institute, Cary, NC, USA). A general linear model procedure (PROC GLM) was used to produce nested analysis of variance (ANOVA) models for concentration and site differences. Pairwise *t* tests were used for testing differences in site means at individual concentrations. The Dunnett, one-tailed *t* test was used to evaluate differences between AW and other Elizabeth River sites. Duncan's multiple range test was used for differences between sites at different concentrations.

RESULTS

Three teratogenicity tests quantified the proportion of developing embryos with cardiovascular deformities caused by AW sediment exposure. The first test exposed embryos to a series of AW and PC sediment mixtures (total selected PAH concentrations ranged from 5–650 ng/g dry wt·10^3) in order to compare tolerances of embryos spawned from Elizabeth River AW and York River CI mummichogs. Embryos with cardiovascular index scores of 1 to 4 were defined as having cardiovascular abnormalities. A significant difference (paired *t* test, $p = 0.0007$) was observed in the proportion with cardiovascular abnormalities at all concentrations except the control concentration (Fig. 2, bottom).

The second test exposed embryos from four sites on the Elizabeth River to the same series of concentrations to define the variation among Elizabeth River populations. Again, embryos with CVI scores of 1 to 4 were summed and divided by the total number of exposed embryos in the culture dish to get the proportion with cardiac abnormality. No significant differences were observed among sites at any concentration; however, the power of this experiment was lower than the test about to be described. An increase was observed in cardiac abnormalities with increasing PAH concentration, but this too was not significant in this test ($\alpha = 0.05$; Fig. 2, top).

The third embryo test tested whether embryo tolerance was a consequence of parental exposure or a heritable factor. This

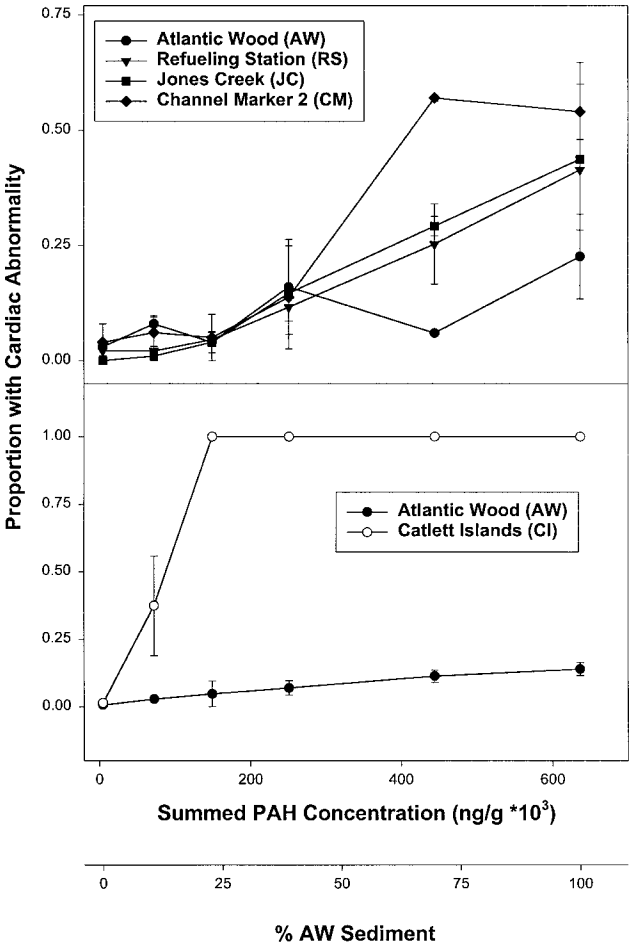

Fig. 2. Effect of polycyclic aromatic hydrocarbon (PAH) concentration on proportion of embryos with cardiac abnormalities. Means of duplicate treatments ± standard error with $n = 67$, 98, 48, and 40 embryos per dish for Atlantic Wood, Refueling Station, Jones Creek, and Channel Marker sites on the Elizabeth River (VA, USA), respectively (**top**), and $n = 70$ embryos per culture dish for both Atlantic Wood and Catlett Islands (York River, VA, USA) (**bottom**). Some error bars are smaller than figure symbols.

test was designed to have more power and was able to detect significant differences among lines within the Elizabeth River that were not evident in the second, less powerful embryo test. Embryos spawned from parents that were laboratory raised were used in a tandem comparison with embryos spawned from newly field-caught mummichogs. Laboratory-raised fish were reared in clean York River water and were unlikely to retain PAH-induced maternal effects potentially present for embryos derived from wild-caught mummichog. Embryos from field-caught and laboratory-reared parents showed similar levels of teratogenic effect over the sites tested (Fig. 3). Field-caught CI fish were used as a positive control. A nested model (SAS PROC GLM) was used to test the effect of history (laboratory-reared vs field-collected parents) and site (AW, RS, JC, CM, and CI populations) on cardiovascular defects. A significant effect of site ($p = 0.0003$) but not of history ($\alpha = 0.05$) was observed. Based on results from a one-tailed *t* test, both CM embryos from field-caught and laboratory-raised had a significantly ($p = 0.0015$ and 0.0005, respectively) higher prevalence of cardiovascular defects than did AW embryos from parents with the same exposure history. Differences were seen only at the $\alpha < 0.10$ level in prevalence of cardiovascular

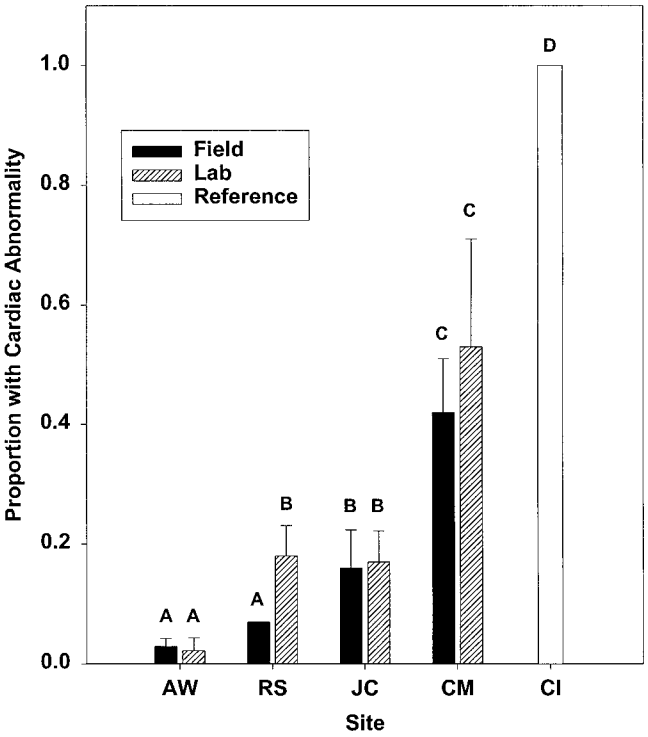

Fig. 3. Effect of parental history (field caught or laboratory reared) on proportion with cardiac abnormalities for four Elizabeth River (VA, USA) sites (Atlantic Wood [AW], Refueling Station [RS], Jones Creek [JC], and Channel Marker [CM]) and a York River (VA, USA) reference site (Catlett Islands [CI]). Mean of three replicates (except RS-Field with two replicates) ± standard error with 40 embryos per replicate (AW-Lab $n = 30$ and RS-Field $n = 15$). Letters represent groups significantly different from each other.

abnormalities between JC from field-caught ($p = 0.095$), JC and RS embryos from laboratory-raised parents ($p = 0.072$ and 0.060, respectively), and AW embryos with the same respective exposure history. The lines that were significantly different with both parental histories came from sites with tPAH concentrations in the range of 4 to 10 ng/g·10^3.

DISCUSSION

Tolerances of several Elizabeth River mummichog populations relative to a York River reference population were evaluated, and the heritability of tolerance was assessed. Mummichog embryos from sites on both estuaries were exposed to contaminated sediments and evaluated for teratogenic effects.

Embryos exposed to contaminated sediment exhibited different degrees of cardiovascular abnormalities. These teratogenic effects were similar to those described by Weis and Weis [33] and Williams [23]. Tube hearts, reduced circulation, or pericardial swelling have been observed in fish embryos exposed to mercury, methylmercury [36], *N*-nitroso compounds [37], naphthalene, 2,4-dinitrophenol, and produced water [38]. Some authors have differentiated between a primary cardiac malformation in true tube hearts and a secondary cardiac malformation of stretched hearts. Our metric of proportion with cardiac abnormality was a straightforward sum of individuals with any type of cardiac abnormality and could be considered a general metric of individual fitness relative to teratogenicity. Cardiac abnormalities are a valuable toxic endpoint because they can be observed within a few days of fertilization and are precursors to fish fitness and hatch success and therefore individual viability. Chemicals found in creosote-contaminated sediments, such as naphthalene, phenanthrene, pyrene, and benzo[*a*]pyrene, have been found to accumulate quickly in embryos of zebrafish, cod, herring, and turbot [39]. Possible mechanisms by which diffusible creosote compounds exert their toxic effects to fish early life stages include cytotoxicity, DNA damage, and induction of cyctochrome P4501A causing alteration in endothelial tissues and consequent edema [40].

Williams [23] found that embryos from AW were significantly more tolerant of PAH-contaminated sediments than embryos from CI. Our results were consistent with her findings (Fig. 2). She suggested that AW mummichog were unique in their tolerance to PAHs. We tested embryos from four Elizabeth River sites to test whether other populations within that estuary were also tolerant to contaminant effects. No statistically significant differences were observed among sites for the formation of cardiac abnormalities in the initial intrariver study. After the power of the teratogenicity test was increased by adding more replicates to each treatment class (Fig. 3), statistically significant differences were detected among Elizabeth River sites. Results from the embryo teratogenicity tests suggested that AW mummichog had the highest tolerance of the site populations tested relative to the formation of cardiac abnormalities. Other mummichogs from the Elizabeth River populations were also more resistant to the formation of cardiovascular deformities than those from the CI population.

Our results suggested that tolerance differences were heritable. In the parental history embryo test, significant differences were observed between the effects of contaminated sediments on embryos from different sites, but embryos from laboratory-reared mummichog had similar tolerance relative to embryos produced by field-caught fish. Williams did a series of crosses to produce embryos with eggs from AW females and milt from CI males. She found that these embryos were as tolerant as embryos from two AW parents [23]. These tests did not rule out the possibility of eggs being influenced by the exposure history of their field-caught parents because fish slowly eliminate PAHs [41], PAH adducts decrease slowly in exposed mummichogs [42], mRNA and proteins can be directly transferred to embryos [30,31], and maternal effects occur in PAH-exposed, egg-bearing fish [29]. Here, the heritability of tolerance was tested by raising mummichogs from birth in clean laboratory waters and comparing the teratogenic effects of field sediments on their embryos to those to embryos spawned directly from field-caught parents. A gradient of tolerance was observed that increased as sediment PAH concentrations increased.

The results of this study indicate that Elizabeth River and York River mummichogs have distinct tolerances to PAH-contaminated sediments that were correlated with the exposure history of the parent populations. Elizabeth River mummichogs raised from embryos in clean water maintained their tolerance to contaminated sediments. Different prevalences of teratogenic abnormalities on exposure to contaminated sediments varied among Elizabeth River populations, and the relative levels of tolerance corresponded to the level of sediment contamination. This difference was heritable, suggesting that natural selection has occurred.

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