POPULATION GENETIC STRUCTURE OF A NONMIGRATORY ESTUARINE FISH (FUNDULUS HETEROCLITUS) ACROSS A STRONG GRADIENT OF POLYCHLORINATED BIPHENYL CONTAMINATION

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Abstract—Populations of the estuarine fish Fundulus heteroclitus indigenous to contaminated sites exhibit heritable resistance to some of the toxic effects of early life-stage exposure to polychlorinated biphenyls (PCBs). This evolved tolerance provides evidence of strong selection by PCBs, and it suggests other potential genetic effects of these stressors on resident populations. Environmental contaminants have the potential to affect the genetic structure of populations and to reduce genetic diversity, but species life-history traits, particularly patterns of migration and dispersal, also influence the distribution of genetic variation among populations. Therefore, the present work was conducted to determine whether genetic diversity or genetic structure is altered in populations of F. heteroclitus indigenous to 18 sites in Massachusetts (USA) and Rhode Island (USA), representing a steep gradient of sediment PCB contamination and culminating in a Superfund site at New Bedford Harbor (NBH; MA, USA). Allele frequencies at enzymatic loci were used to assess genetic structure and diversity. Selection experiments using a highly toxic PCB congener (3,3',4,4',5-pentachlorobiphenyl) were conducted to determine if genetic patterns at field sites could be associated with contaminant exposures. Although allele frequencies clearly reflected a pattern of isolation by distance, the results indicated neither significant loss of genetic diversity nor alteration of allele frequencies for populations of F. heteroclitus in NBH.

Keywords—Fundulus sp. Allozymes Genetic diversity Adaptation Polychlorinated biphenyls

INTRODUCTION

Adaptation to contaminants results from directional selection for chemical tolerance, eliminating individuals at the lower end of the range of tolerance. Thus, selection, though it increases the mean tolerance of the surviving population, may be accompanied by a loss of genetic variation. Therefore, the long-term effect of the adaptation may be detrimental if the variants or genotypes that are eliminated through adaptation to a toxicant are beneficial in the absence of the toxicant. Adaptation also may reduce the adaptive potential of the population if the variants or genotypes that are eliminated by adaptation to one contaminant are important for adaptation to other contaminants or stressors [1,2].

In addition to selection, environmental stressors, including toxic chemicals, may alter the natural patterns of genetic structure and diversity through other mechanisms. Population size may be reduced by elimination of habitat, changes to ecosystem structure and function, and random mortality. Patterns of migration and, hence, of gene flow may be altered by creation of areas of unsuitable habitat or other barriers to interpopulation movement. Populations with smaller size and reduced migration are more subject to loss of genetic diversity through genetic drift and reduced fitness because of inbreeding [3]. In sum, the outcome of environmental degradation involves the interaction of natural selection, genetic drift, gene flow, mutation, population demographics, and life-history traits. Therefore, it is difficult to predict accurately the genetic effects of chemical stress on wild populations.

To improve our understanding of the relationship between genetic change and anthropogenic stress, we examined genetic patterns of a nonmigratory fish species having phenotypic evidence of genetic adaptation to local conditions. Fundulus heteroclitus, an estuarine killifish, is an extremely abundant littoral species that occurs in a discontinuous band along the Atlantic coast of North America and resides in sites of extremely varying environmental quality. The linear population structure, characteristic limited dispersal, large and dense populations, and high genetic diversity of this species have allowed the evolutionary development and maintenance of clinal variation at several allozyme loci associated with the strong temperature gradient along the Atlantic coast [4]. These characteristics may have contributed to the ability of this species to adapt to localized environmental conditions, including chemical contamination.

Chemical adaptation has been demonstrated in populations of F. heteroclitus from some of the most contaminated locations along the Atlantic coast [5,6], including Newark (NJ, USA) [7–9], the Elizabeth River (VA, USA) [10–14], and New Bedford Harbor (NBH; MA, USA) [15–17]. Tolerance in each of these populations is marked by strongly suppressed induction of cytochrome P4501A (CYP1A), a monooxygenase typically induced in response to exposure to aromatic hydrocarbon–receptor agonists, including polycyclic aromatic hydrocarbons (PAHs), and dioxins as well as dioxin-like compounds (DLCs), including some of the most toxic polychlorinated biphenyl (PCB) congeners [18,19]. Evidence from several multigeneration experimental studies demonstrates that the ob-
erved contaminant resistance has a heritable component; however, in at least one population, the resistance also results from physiological acclimation and, possibly, maternal effects [5,6,12,13].

In the present study, we focused on populations of *F. heteroclitus* indigenous to areas both in and around NBH (Fig. 1). New Bedford Harbor is designated as a Superfund site because of PCBs, but other local contaminants include polychlorinated dibenzo-p-dioxins, polychlorinated dibenzo furans, PAHs, and several trace metals [20,21]. Resident biota and sediments are highly contaminated. In 1996, mean dry weight of total PCBs in the livers of *F. heteroclitus* was 324 μg/g in fish from the most contaminated portion of the upper harbor, 163 μg/g in fish from the midharbor, and 2.4 μg/g in fish from West Island (MA, USA), a reference site approximately 10 km outside NBH [16].

The main goal of the present study was to determine whether populations adapted to local conditions in an estuary highly contaminated with PCBs have a reduction in genetic diversity or exhibit significant changes in population genetic structure. We considered how processes of natural selection, gene flow, and genetic drift as well as species-specific traits, including migratory patterns, home range, and population density, have interacted with sediment contamination and habitat modification to affect population genetic patterns. In the present study, we compared population genetic patterns and genetic diversity of the nonmigratory fish *F. heteroclitus* in NBH and in neighboring sites in Massachusetts (USA) and Rhode Island (USA). In another study [22], we addressed population genetic diversity and genetic structure in the migratory species *Menidia menidia* in NBH and neighboring sites in Rhode Island. As part of each study, we conducted controlled laboratory exposures to determine if sediment contamination might be responsible for differences in allozyme frequencies at reference and control sites.

Our first objective was to determine if populations of *F. heteroclitus* in NBH exhibited reduced genetic diversity or differences in allele frequencies compared with nearby populations from less contaminated sites. We predicted that continuous exposure to contaminated sediments in NBH would have altered allele frequencies and reduced genetic diversity, because directional selection for contaminant resistance would have eliminated or reduced the frequency of PCB-sensitive genotypes from the population. In addition, because previous research [17] suggested that the populations inside NBH generally were more resistant to exposure to DLCs than those outside NBH, we hypothesized that the hurricane barrier (Fig. 1) limited migration in and out of NBH, effectively restricting gene flow and enhancing genetic differentiation of NBH populations.

Our second objective was to determine if *F. heteroclitus* embryos exposed to DLCs in the laboratory exhibited differential survivorship among individuals with different allozyme genotypes. Because previous multigeneration experiments with *F. heteroclitus* from NBH suggested that DLC tolerance was heritable [16], we predicted that sensitive and resistant individuals would have different allozyme genotypes.

**MATERIALS AND METHODS**

*Fish collection*

*Fundulus heteroclitus* were collected from 18 sites in Massachusetts and Rhode Island. Six sites were within NBH, eight were in Massachusetts but outside NBH, and four were located in Rhode Island (Fig. 1). Sampling locations were chosen by accessibility, potential presence of contaminants, available habitat, and previous use as collection sites for this species [16,17]. Total PCB concentrations, as measured from a single sample from the top centimeter of sediment in the intertidal zone, were available for 10 of these sites. Sediment PCB concentrations ranged from 2 to 22,666 ng/g dry weight [17] (Table 1). In addition, DLC responsiveness of *F. heteroclitus* populations resident at the same 10 sites had been characterized as the concentration required to produce lethal effects in 20% of the exposed population (LC20) in embryonic exposure assays with PCB congener 3,3',4,4',5-pentachlorobiphenyl (PCB 126) [17]. To facilitate spatial analysis of genetic patterns, sites were grouped into regions according to geographic location (Table 1).

Fish were collected using conical, galvanized steel minnow traps baited with chopped squid. A minimum of four traps were set at each site, with a spacing of approximately 10 m. When possible, similar numbers of fish were collected from all traps. We attempted to select fish from each trap randomly to avoid selection by size or sex. Fish were anesthetized with ethyl 3-aminobenzoic methanesulfonate (MS-222), individually bagged and labeled, frozen on dry ice, shipped to Miami University (Oxford, OH, USA), and stored in a −80°C freezer until electrophoresis was performed at Miami University. All fish were handled in accordance with Miami University Animal Care Protocol 437.
Genetic structure of *F. heteroclitus* along a PCB gradient

Environ. Toxicol. Chem. 24, 2005 719

Selection experiment

Previous studies [16,17] have used survivorship of early life-stage exposure to PCB 126 as an indicator of population-level DLC tolerance. We adapted this early life-stage exposure model for use in selection experiments. Nominal concentrations were chosen from survivorship curves for *F. heteroclitus* at West Island with the intention of achieving approximately 90% mortality. To identify specific genotypes or alleles associated with DLC tolerance or sensitivity, bioassays were conducted on a large number of embryos from a single spawning event. Fertilized embryos were obtained from populations of wild-caught adults from West Island that had been maintained in flow-through conditions at the U.S. Environmental Protection Agency (U.S. EPA) laboratory in Narragansett (RI, USA) for about a year. Procedures for collection and exposure of embryos have been described elsewhere [16,17,23]. Briefly, normal embryos were selected at 1 d postfertilization and transferred to individual glass, 20-ml vials. Acetone (0.04% by volume) or PCB 126 dissolved in acetone was transferred to each vial. After 7 d, the embryos were removed and placed into 75- × 150-mm glass dishes (*n* = 100 embryos each) filled with seawater and then gently aerated. After hatching began, larvae were fed *Artemia nauplii* daily. When the fish reached approximately 2 cm in length, they were transferred to larger flow-through aquaria and allowed to grow for about a year before being euthanized for genetic analyses.

*Fundulus heteroclitus* from two separate exposure experiments were used for genetic analyses. In the first exposure, 450 individuals were treated with a nominal PCB 126 solution of 500 ng/L, and 150 individuals were used as controls. In the second exposure, 300 individuals were dosed with a nominal PCB 126 solution of 200 ng/L, and 100 individuals were used as controls. Because nominal concentrations were used in these selection experiments, survivorship data are comparable only within the present study.

Genetic analyses

Horizontal starch gel electrophoresis was used to determine the genotype of each individual at 10 presumptive enzymatic loci (Table 2) for each population sample and for controls and survivors of selection experiments. Methods followed standard procedures for starch gel electrophoresis and biochemical staining [24].

Observed genotype frequencies were compared to those expected under Hardy–Weinberg equilibrium conditions (i.e., random mating) using the Markov Chain Monte Carlo (MCMC) method [23], which uses a randomization procedure to generate an approximation of the exact probability distribution of the data. The test was performed using the Tools for Population Genetics Analysis (TFPGA) genetic analysis software (Ver 1.3; http://bioweb.usu.edu/mpmbio) [26], which includes a batching procedure to calculate standard errors of the estimated *p* values.

Allele frequencies at each locus were compared among populations using a MCMC randomization procedure [27]. This test was performed using TFP GA, which again uses a batching procedure to generate standard errors of the estimated *p* values. The MCMC method is used to compare allelic distributions at each locus independently, but it does not generate a probability for multilocus comparisons. To determine the significance of differences among populations across multiple loci, the TFP GA software uses Fisher’s combined probability test [26,27], also referred to as Fisher’s method [28], in which the

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### Table 1. Site information for *Fundulus heteroclitus* collections

<table>
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<tr>
<th>Site name</th>
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<th>Site information for <em>Fundulus heteroclitus</em> collections</th>
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<tr>
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<td>NBH superfund site S</td>
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<td>Suckett's Road</td>
<td>Mattapoisett, MA, USA</td>
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*NA* = not available; *NBH* = New Bedford Harbor; *PCB* = polychlorinated biphenyl.

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## Notes

1. Measured as the shortest distance over water.
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sum of the negative of twice the natural log of the $p$ value ($-2 \ln p$) for each test is compared to a chi-square distribution with degrees of freedom equal to twice the number of $p$ values being combined.

The $F$ statistics are used in population genetic studies to describe the partitioning of genetic variation among individuals, populations, and regions [3]. The method described by Weir and Cockerham [29] for estimating $F$ statistics is commonly used, because it does not make assumptions about numbers of populations, sample sizes, or heterozygote frequencies. Weir and Cockerham used the notation $F$, $\theta$, and $f$, which are analogous to the original symbols, $F_{ST}$, $F_{ST}$, and $F_{IS}$, respectively. The TFPGA software uses the method described by Weir and Cockerham to estimate $F$ statistics and uses jackknife and bootstrapping across loci to generate variance estimates and confidence intervals, respectively. In the present study, a hierarchical design was used to estimate values of $\theta$ for populations ($\theta_k$) and regions ($\theta_k$).

To determine whether population genetic structure reflected a pattern of isolation by distance, Mantel tests [30] were used to correlate geographic distance among sites and genetic distance among populations. In addition, regarding the 10 sites from NBH, linear regression also was performed with site 15 as an outlier. Analysis of variance and regression were conducted with the MINITAB® statistical software package (student version, release 12; Minitab, State College, PA, USA).

### RESULTS

#### Population genetic data

Genotype frequency data and sample size for each locus and each population are listed in Appendix 1 [SETAC Supplemental Data Archive, Item ETC-24-03-002; http://etc.allenpress.com]. Comparisons of observed genotype frequencies to those expected under Hardy–Weinberg equilibrium conditions were made for each population on all loci exhibiting multiple alleles (163 comparisons). Deviations ($p < 0.05$) occurred in only seven tests and were distributed among seven populations (sites 1, 2, 3, 4, 11, 15, and 17) and five loci (phosphoglucomutase $[PGM-1^*]$, lactate dehydrogenase $[LDH-2^*]$, glucose-6-phosphate isomerase $[GPI-1^*]$), phosphogluconate dehydrogenase $[PGDH^*]$, and mannose phosphate isomerase $[MPI^*]$). For populations sampled in both 1999 and 2000 (sites 2, 3, 17, and 18), Hardy–Weinberg equilibrium was tested separately for each year.

Allele frequency distributions of populations differed significantly between 1999 and 2000 at $PGDH^*$ at site 3 and at malate dehydrogenase (MDH-2*) at sites 17 and 18. Combined probability tests across loci were nonsignificant ($p > 0.05$) in all four repeated-year samples. Because few significant differences were found in allele frequencies between 1999 and 2000, data from the two years were pooled for all subsequent analyses.

The heterozygous loci within individuals differed significantly among populations (analysis of variance, $F = 4.77, df = 17, p < 0.001$) (Fig. 2). Tukey's pairwise comparisons ($\alpha = 0.05$) indicated no significant differences in HLWI among populations within NBH (sites 1–6), but HLWI at site 9 was significantly greater than that at sites 2 and 7. Populations at sites in southern Rhode Island had higher HLWI than many other sites: HLWI at site 17 differed significantly from HLWI at sites 2, 3, 7, and 13, and HLWI at site 18 differed from HLWI at sites 1, 2, 3, 4, 7, 11, 12, 13, and 14.

To assess the potential relationship of HLWI and sediment contamination, sediment PCB data (log dry wt) [17] were compared with heterozygosity data (Fig. 2). Linear regression indicated no significant relationship of HLWI and total PCB concentration ($r^2 = 0.022; df = 1, 8; F = 0.18; p = 0.680$). The PCB contamination at site 15 in northern Rhode Island was highly localized and, presumably, did not originate from NBH, linear regression also was performed with site 15 removed, but the result was still nonsignificant ($r^2 = 0.06; df = 1, 7; F = 0.45; p = 0.525$). Finally, to address the rela-
The six populations within NBH differed only at frequencies within each of the six regions identified only three regions were relatively homogeneous; comparisons of allele frequencies among regions (as defined in Table 1). Populations within each region were run for Massachusetts sites only. Again, no significant relationship was detected when all 18 sites were included (linear regression). The relationship of HLWI and distance from NBH (Fig. 2) was positive when all 18 sites were included (linear regression; \( r^2 = 0.53; df = 1, 14; F = 18.21; p = 0.001 \)). Removing all Rhode Island sites from the analysis resulted in a nonsignificant relationship.

To quantify differences in genetic composition of populations, allele frequencies at each locus were first compared across all populations. Differences were highly significant \( p < 0.0001 \) for eight loci (PGM-1*, PGM-2*, LDH-2*, MDH-2*, IDH*, MPI*, GPI-1*, GPI-2*, PGDH*; and IDH*) and were nonsignificant for two loci (LDH-1* and isocitrate dehydrogenase [IDH-1*]). Allele frequencies also were compared within and among regions (as defined in Table 1). Populations within each region were relatively homogeneous; comparisons of allele frequencies within each of the six regions identified only three cases in which allele frequencies at a locus differed among populations. The six populations within NBH differed only at \( MDH-2* \) \( (p = 0.019 \pm 0.002 \text{ mean } \pm \text{ standard error}) \), the three Massachusetts populations southwest of NBH only at PGDH* \( (p = 0.011 \pm 0.002) \), and the two populations in southern Rhode Island only at PGDH* \( (p = 0.031 \pm 0.004) \). Results of combined probability tests to compare populations across loci were not significant in any of the six regions.

Although allele frequency differences were rare within regions (defined in Table 1), pairwise comparisons among regions indicated significant genetic differentiation in each case. The combined probability for region 1 versus region 2 was \( p = 0.011 \); for region 1 versus region 4, \( p = 0.0002 \); for region 2 versus region 3, \( p = 0.0041 \); and for region 2 versus region 4, \( p = 0.015 \). All other combined probability values were highly significant \( (p < 0.0001) \). Because this involved 15 separate comparisons, a conservative correction for multiple comparisons was made by dividing \( \alpha = 0.05 \) by 15, thus reducing \( \alpha = 0.0033 \). Following this correction, allele frequencies of populations from region 1 and region 2 and of populations from region 2 and region 3 were not significantly different. In summary, comparisons of allele frequencies within and among populations and regions clearly indicated genetic differentiation among regions, especially between Rhode Island and Massachusetts, whereas very little differentiation was evident among populations within each region.

Results of comparisons of allele frequency distributions suggested regional differences. However, \( F \) statistics are the traditional method for assessing population genetic structure and are useful for hierarchical comparisons. The \( F \) statistics for each allele and each locus are reported in Table 3. Means, standard deviations, and 95\% confidence intervals generated using jackknifing and bootstrapping procedures are reported. Values for \( F \), \( \theta_s \), and \( \theta_h \) were generally similar within each locus, but among-loci values ranged from \(-0.005 \) to \(0.191 \). For two loci, \( LDH-2* \) and \( MDH-2* \), all values for \( F \), \( \theta_s \), and \( \theta_h \) were greater than 0.1, indicating considerable differentiation at multiple levels. The estimate for \( f \) was much smaller (0.012 and 0.028 for \( LDH-2* \) and \( MDH-2* \), respectively). Together, the \( F \) statistics for \( LDH-2* \) and \( MDH-2* \) indicate genetic differentiation at the population and regional levels but homogeneity within populations. In contrast, for \( IDH* \) and \( LDH-1* \), the estimate for each parameter was very close to zero, indicating no genetic differentiation at any level.

The \( F \) statistics also were calculated separately for region 1 (six sites within NBH) and region 2 (three sites immediately outside NBH) to distinguish between large- and small-scale patterns of genetic structure. Estimates for \( \theta_s \) and \( \theta_h \) were very low (0.001 and \(-0.001 \), respectively), indicating very little
genetic differentiation among populations or regions 1 and 2. Estimates of $F$ and $f$ were 0.032 and 0.033, respectively, indicating the presence of some within-population substructure at these sites. In summary, the $F$ statistics for regions 1 and 2, a scale of less than 20 km, indicate that essentially all the genetic variation in these regions occurs within populations rather than among populations or regions. This contrasts with the results of $F$ statistics as estimated across all populations and regions, a scale of approximately 100 km, which indicate significant genetic variance among populations and regions.

Sediment PCB concentration [17] was not significantly related to distance from NBH when all 10 sites were included. Removing site 15 in northern Rhode Island resulted in a significant negative relationship ($r^2 = 0.48; df = 1, 7; F = 8.32; p = 0.024$). The relationship also was significant with all Rhode Island sites removed ($r^2 = 0.60; df = 1, 6; F = 11.27; p = 0.015$).

Modified Rogers’ genetic distance was calculated for each pair of sites, and Mantel tests were used to correlate genetic distance with geographic distance, the square root of the difference in sediment PCB concentration, and the difference in sensitivity to PCB 126 (LC20 data [17]) for each pair of sites. The relationship of geographic distance and genetic distance across all sites was highly significant ($r^2 = 0.84, p = 0.001$) (Fig. 3). The relationship of genetic distance and geographic distance across sites 1 through 9, however, was not significant. No relationship was found between genetic distance and the difference in PCB concentration or LC20 values.

**Laboratory experiments**

Survivorship of exposed individuals in the two experiments was 1.8% in the 500 ng/L treatment and 6.7% in the 200 ng/L treatment. Genetic analysis was performed on all exposure survivors and a sample of each control group. Because no significant differences in allele frequencies were identified between the control groups or between the exposure survivors, genetic data from the experiments were pooled. Deviations of observed genotype frequencies from Hardy–Weinberg equilibrium expectations ($p < 0.05$) occurred only at $PGM^*$ in the control group. This deviation was present in one group before pooling as well. No significant differences in heterozygosity or in allele or genotype frequencies were detected at any loci between exposure survivors and controls for either experiment, whether separately or combined. Genotype frequency data for samples from the laboratory experiment are listed in Appendix 1.

**DISCUSSION**

Concern exists that selection by environmental contaminants may reduce genetic diversity and alter genetic structure in affected populations, but the results of studies designed to assess population genetic patterns at contaminated sites have been inconsistent [32]. This probably is because the determinants of population genetic patterns are complex and because the genetic structure of populations is affected by natural as well as anthropogenic influences. Researchers studying the effects of contaminants on population genetic structure and diversity need to distinguish between natural genetic variation and that resulting from anthropogenic influence. It also is important to understand the interactive relationship of natural and anthropogenic processes in shaping genetic patterns.

In the present study, we used allele frequencies at 10 enzymatic loci to assess patterns of genetic structure and diversity in 18 populations of *F. heteroclitus* across six regions that ranged from undeveloped to industrially contaminated. The most contaminated region included a Superfund site where resident populations exhibit a marked resistance to DLCs, which may be at least partially heritable. The cleanest region, located approximately 75 km from the Superfund site (a distance many times larger than the species home range [33]), included uncontaminated sites where fish are not resistant to DLCs. Our results from these field samples showed regional differences among populations. However, sediment PCB concentrations and DLC sensitivity are inversely correlated, and PCB concentrations decrease with distance from the PCB source in NBH. Therefore, interpreting whether genetic patterns result from DLC tolerance or from isolation by distance requires caution. To address this issue directly, we compared results from controlled laboratory experiments with genetic data from field sites to look for parallel patterns. In our analysis of populations at field sites, we sought to identify not just patterns associated with NBH but also patterns associated with NBH that appeared to deviate from the natural patterns of variation at surrounding sites.

The results of the laboratory selection experiments did not reveal any significant relationships between allozyme genotype and PCB survivorship. In our experiment, 28 individuals combined from two selection experiments constituted the surviving population. If strong associations between genotype and survivorship existed, these 28 individuals would have had a high proportion of the tolerant genotype. However, no significant differences were detected between controls and exposures, suggesting that allozyme genotype at the loci surveyed in the present study was not strongly correlated with DLC survivorship.

Although concern exists that contaminant exposure and adaptation may cause reduced genetic diversity, no relationship between heterozygosity and sediment PCB concentration was detected in the present study. Across all sites, heterozygosity increased with increasing distance from NBH. This pattern was driven by high heterozygosity at the two southern Rhode Islands.
Island sites, and removal of these sites eliminated the significant relationship. Relatively higher heterozygosity in Rhode Island sites resulted from differences in allele frequencies at $LDH-2^*$, $MDH-2^*$, and $PGDH^*$, all loci for which previous allozyme studies have identified large-scale spatial variation and/or clinal patterns in populations of $F. heteroclitus$ [34,35]. Considering only Massachusetts sites, a few differences in heterozygosity were detected, but no relationship with distance from NBH or sediment contamination was evident. Therefore, data from the present study do not support the prediction that genetic diversity (measured as heterozygosity) would be lower in $F. heteroclitus$ populations in NBH.

To distinguish between natural and anthropogenic causes of genetic variation in populations in NBH and surrounding sites, we considered patterns of DLC tolerance, sediment contamination, and geographic distance in relation to genetic structure. Previous research [17] demonstrated that DLC sensitivity during early life stages was related closely to PCB sediment concentration, although populations immediately outside NBH were somewhat more tolerant than sediment concentrations might predict. The greatest difference in PCB concentration and in DLC sensitivity of $F. heteroclitus$ populations occurred between NBH sites (region 1 in the present analysis) and sites immediately outside NBH (region 2). The DLC tolerance of populations inside NBH was two orders of magnitude greater than that of the West Island population (site 9), which is approximately 16 km away from the PCB source. West Island fish were four- to fivefold more tolerant than those at distant reference sites in southern Rhode Island (site 17), located approximately 70 km southwest, and fourfold more tolerant than those at Mattapoisett (site 10), located approximately 10 km northeast (Table 1) [16,17]. Therefore, we predicted that genetic patterns at allozyme loci, if associated with PCB tolerance, would follow a similar pattern. That is, allele frequencies at loci associated with tolerance would exhibit greater differences in frequency between collective NBH populations (region 1) and those in the region immediately outside NBH (region 2) than between regions immediately outside NBH and those at more distant Massachusetts and Rhode Island sites (regions 3–6).

Genetic patterns did not fit our predictions. Allele frequency differences were highly significant among regions but varied little within regions. Among regions, genetic differences generally increased with increasing distance. Correcting for multiple comparisons, populations in the NBH region did not differ from those immediately outside NBH, despite differences in DLC tolerance of two orders of magnitude. The $F$ statistics revealed patterns similar to contingency table comparisons. At some loci, regional differentiation resulted in large values for $\theta_S$ and $\theta_V$. However, $F$ statistics calculated only for NBH and sites immediately outside indicated no significant genetic differentiation among populations or regions. Therefore, genetic differences follow a pattern of isolation by distance that differs from the pattern of PCB contamination and DLC resistance of $F. heteroclitus$ populations.

The results also do not support the hypothesis that the hurricane barrier limits gene flow between $F. heteroclitus$ populations inside and outside NBH, potentially increasing the rate of genetic divergence of populations. These results are surprising, because isolation of NBH populations was hypothesized to be important for maintaining the high tolerance to DLCs relative to that in populations outside NBH [17]. It is important to note, however, that our analysis might reflect historical population connectivity and that the long-term isolating affect of the hurricane barrier (erected in 1964) has not become evident in the allele frequencies of these populations.

In a study similar to ours, genetic diversity and genetic structure of $F. heteroclitus$ at contaminated sites in the Elizabeth River were measured using protein electrophoresis [36] and mitochondrial DNA [37]. Allozyme frequencies for three loci in juveniles and one locus in adults at the contaminated site were distinct from those at all other sites. No relationship was detected between genetic diversity or frequencies of specific allozyme loci and sediment contamination. However, in contrast to the present study, no significant correlation of geographic and genetic distance was identified. Rather, genetic distance was significantly related to the difference in sediment PAH concentration. The difference in the correlation of genetic distance and geographic distance in these studies may be explained by considering the spatial scale of each study. Mulvey et al. [36] sampled populations at nine sites over approximately 12 km on the southern branch of the Elizabeth River as well as several additional sites on the York River and eastern branch of the Elizabeth River, covering a total distance of approximately 50 km. In the present study, $F. heteroclitus$ populations were sampled over a range of approximately 100 km. If their study had covered a larger geographic area, a significant relationship of genetic and geographic distance might have been apparent. Similarly, in the present study, consideration of only sites 1 through 9, separated by less than 20 km, yielded no significant relationship of genetic and geographic distance.

Differences in habitat connectivity and gene flow may be partially responsible for the differences in the results from the present study and those of Mulvey et al. [36] regarding the correlation of sediment contamination and genetic distance. The $F_{ST}$ values were 0.0254 for juveniles and 0.0141 for adults on the Elizabeth River at sites separated by approximately 12 km. In the present study, measures analogous to $F_{ST}$ values, $\theta_S$ and $\theta_V$, estimated for regions 1 and 2 (a scale of ~20 km) in the present study were very low (0.001 and ~0.001, respectively). These results indicate that across similar spatial scales, Elizabeth River populations are more heterogeneous than those of NBH. Mulvey et al. [36] noted that populations in the Elizabeth River inhabit locally discrete habitats separated by large areas of unsuitable habitat. In contrast, although unquestionably fragmented, much of the eastern side of NBH appears to provide suitable habitat for this species. Habitat fragmentation could be more limiting to gene flow in the Elizabeth River, allowing local selective pressure to cause genetic divergence of populations. Habitat heterogeneity, including heterogeneity of contaminant mixtures, also might be greater in the Elizabeth River than in NBH. The combination of reduced gene flow and increased divergent selection would facilitate greater population differentiation.

In addition to the present study using allozymes, whole genome and gene target approaches have been applied to assess population genetic changes in $F. heteroclitus$ of NBH. For example, McMillan et al. [38] used amplified fragment length polymorphisms to compare genetic structure of populations in NBH with that of neighboring sites. Preliminary analyses of these data indicate the presence of some microgeographic genetic structure but no strong differences between contaminated and reference sites. In another study examining $F. heteroclitus$ from NBH and a distant reference site, a comparison of DNA sequence variation at a major histocompatibility locus [39] identified differences in functional regions of the gene that
may be related to local selective pressures. However, to our knowledge, the functional association between tolerance to PCBs or other (presumably immunogenic) stressors is not known. Therefore, despite evidence that observed DLC tolerance is heritable, these studies have not provided evidence of erosion of genetic diversity in *F. heteroclitus* of NBH.

A possible explanation for the apparent disparity of the results of the present study and those suggesting a genetic basis for the enhanced DLC tolerance of *F. heteroclitus* at contaminated sites is that the enhanced tolerance results from genetic changes at very few loci. Mechanistic studies suggest that a simple modification to the aromatic hydrocarbon-receptor pathway or another regulator of CYP1A transcription might be responsible for the observed effect [5,6,10,15,16]. In this case, allele frequencies at other loci, including the enzymatic loci surveyed in the present study, might be unaffected. Even if genetic bottlenecks and an accompanying loss of genetic diversity occurred many generations ago, when PCBs were first introduced to NBH, subsequent gene flow from unaffected populations may have restored any lost diversity. The demographics of *F. heteroclitus*, then, may contribute to the apparent lack of an effect on genetic structure and diversity. Within NBH, this species exists in very large, dense populations, and despite a reputation for a small home range and limited migration, gene flow appears to be high among adjacent populations. If *F. heteroclitus* populations were smaller and more isolated, perhaps the predicted effects of genetic adaptation (erosion of genetic diversity) would be more likely to be realized.

Lack of evidence of reduced genetic diversity in *F. heteroclitus* from NBH does not necessarily suggest that loss of diversity with adaptation to contaminants is not a concern, but it does illustrate the need for understanding the limitations of studies using genetic markers. If we had conducted this research in the absence of the survivorship data from previous studies [16,17], we might have concluded that populations in NBH had not adapted to contaminants, because no genetic change was evident. These results therefore emphasize the importance of considering survivorship data in addition to molecular results when considering the potential for contaminant adaptation.

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Genetic structure of *F. heteroclitus* along a PCB gradient

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