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GENETIC AND DEMOGRAPHIC RESPONSES OF MOSQUITOFISH (*GAMBUSIA HOLBROOKI*) POPULATIONS EXPOSED TO MERCURY FOR MULTIPLE GENERATIONS

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Abstract—Genetic and demographic responses of mosquitofish were examined after multiple generations of exposure to mercury. Previous studies of acute lethal exposures of mosquitofish to either mercury or arsenic demonstrated a consistent correlation between time to death and genotype at the glucosephosphate isomerase-2 (*Gpi-2*) locus. A mesocosm study involving mosquitofish populations exposed to mercury for 111 d showed significant female sexual selection and fecundity selection at the *Gpi-2* locus. Here the mesocosm study was extended to populations exposed to mercury for several (approx. four) generations. After 2 years, control and mercury-exposed populations met Hardy–Weinberg expectations and showed no evidence of genetic bottlenecks. The mean number of heterozygous loci did not differ significantly between the mercury-exposed and control populations. Significant differences in allele frequencies at the *Gpi-2* locus were observed between the mercury-exposed and control populations. Relative to the initial and control allele frequencies, the $Gpi\text{-}2^{100}$ allele frequency was lower, the $Gpi\text{-}2^{66}$ allele frequency increased, but the $Gpi\text{-}2^{38}$ allele frequency did not change in mercury-exposed populations. No significant differences were found in standard length, weight, sex ratio, or age class ratio between the control and mercury-exposed populations. Allele frequency changes at the *Gpi-2* locus suggest population-level response to chronic mercury exposure. Changes in allele frequency may be useful as indicators of population response to contaminants, provided that the population in question is well understood.

Keywords—Genetics Mercury Allozymes Selection *Gambusia holbrooki*

INTRODUCTION

Allozyme analysis is frequently used to assess the impact of toxicants on populations [1–7]. Many laboratory studies have demonstrated differential sensitivities among allozyme genotypes exposed to toxicants [1–5,7]. Differences in allele frequencies among populations have been correlated with environmental contamination [3,8]. Allozyme analysis has also been used to study the effect of toxicants on populations reared in mesocosms [6]. Of the many isozymes examined in these studies, glucosephosphate isomerase has most consistently been identified as a correlate with differential responses of individuals and populations [1,4–13]. Riddoch [14] reported that *Gpi* allozymes have been correlated with temperature (along altitude and latitude clines), pollutants, salinity, and dissolved oxygen.

A consistent relationship between *Gpi-2* genotype and response to environmental stressors, including toxicants, has been shown in a series of experiments designed to assess the suitability of allozyme analysis for biomonitoring. Mosquitofish (*Gambusia holbrooki*) exposure to mercury served as a model to study allozyme genotypes under toxicant stress. Differential survival of *Gpi-2* allozyme genotypes was found in several acute exposures using mercury concentrations under 1 mg/L [5,8]. Newman et al. [7] also found a relationship between time to death and *Gpi-2* genotype when mosquitofish were acutely exposed to arsenate. This indicated that the relationship at the *Gpi-2* locus was not toxicant specific. Differences in glycolytic and Krebs cycle metabolite concentrations were observed among *Gpi-2* genotypes during acute mercury exposures [15,16]. Glycolytic flux was highest in mosquitofish homozygous for the $Gpi\text{-}2^{38}$ allele, suggesting inefficient use of metabolic resources in comparison to other *Gpi-2* genotypes [16].

Low-level exposures to mercury (weekly pulses of 18 μg/L of Hg for 111 d) also produced effects associated with the *Gpi-2* locus in mosquitofish populations exposed in mesocosms [6]. In this case, the common *Gpi-2* homozygote ($Gpi\text{-}2^{100/100}$) exhibited reduced reproductive fitness. Specifically, a lower percentage of $Gpi\text{-}2^{100/100}$ female mosquitofish were gravid (female sexual selection) relative to other *Gpi-2* genotypes. Also, $Gpi\text{-}2^{100/100}$ females had fewer late-stage embryos (fecundity selection). Differential reproductive fitness under sublethal exposure conditions was consistent with our working hypothesis that differences in metabolic allocation were responsible for the effects associated with the *Gpi-2* locus. In a field survey [8], the $Gpi\text{-}2^{38}$ allele frequency was lower for mosquitofish inhabiting a mercury-contaminated canal than mosquitofish in the adjoining river.

This study tested whether the viability and reproductive effects quantified in previous studies result in an observable shift in *Gpi-2* allele frequencies over several generations of mercury exposure. Allele frequencies at seven polymorphic loci were determined, and demographic data were collected for mercury-exposed and control mosquitofish populations. Comparisons of genetic and demographic data between mercury-exposed and control populations were made to determine the effects of mercury exposure spanning multiple generations.

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MATERIALS AND METHODS

Mesocosms

Eight 7,250-L pools, established by Mulvey et al. [6], functioned as mesocosms in this experiment. At the termination of the experiment, the mesocosms were drained, and the sediments were allowed to dry to ensure elimination of all mosquitofish. The mesocosms were filled with well water. Water levels were maintained by precipitation and by addition of well water as needed. The four control mesocosms from the previous experiment contained unspiked water (<0.1 μg/L of mercury). Mercury-treated mesocosms derived all their mercury from the sediments remaining from the previous experiment. No additional mercury was added for this experiment. In this exposure, the water concentration of mercury remained below detectable levels. Whole-body concentrations of mercury in mosquitofish were used to quantify mercury exposure and assign treatment levels to mesocosms.

Fish populations

The mosquitofish populations in the experimental mesocosms were initiated in June 1994. Mosquitofish were seined at random from the Risher Pond population on three occasions and were transported to the site of the experiment. The initial mosquitofish populations were established by randomly assigning 215 mosquitofish to each of the control and mercury-treated mesocosms. The mosquitofish populations were sampled with a seine during a 3-week period in October 1996. All fish were frozen (-70°C) until electrophoresis was performed. Fish were sexed (adult males, adult females, and juveniles), weighed, and measured (standard length) prior to electrophoretic analysis. Gravid females were dissected to determine the number of eggs and developing embryos. One embryo from each gravid female was taken for electrophoretic analysis.

Electrophoresis

The following allozymes were determined using horizontal starch gel electrophoresis: fumarate hydratase (FUM, E.C. 4.2.1.2), mannosephosphate isomerase (MPI, E.C. 3.5.1.8), glucosphosphate isomerase-2 (GPI-2, E.C. 3.5.1.9), adenosine deaminase (ADA, E.C. 3.5.4.4), malate dehydrogenase-1 (MDH-1, E.C. 1.1.1.37), and isocitrate dehydrogenase-1,2 (ICD-1&2, E.C. 1.1.1.42). Electrophoretic methods were those described previously [5,7,8].

Mercury analysis

Ten fish from each mesocosm were selected for mercury analysis. The wet weight of each fish was recorded prior to digestion. Whole fish were digested in 5 ml of trace-metal-grade nitric acid in closed Teflon™ vessels in a microwave oven digester (CEM, Matthews, NC, USA). The digested samples were diluted with high-purity deionized water, and bromine monochloride was added to prevent oxidation. Total mercury was determined by cold vapor atomic fluorescence spectroscopy using a Model 2 analyzer (Brooks Rand, Seattle, WA, USA). All samples were analyzed within 24 h of digestion.

Mosquitofish were digested in batches of 10, with each digest set containing one blank and one standard reference material (TORT2, lobster hepatopancreas, National Research Council of Canada). All the standard materials analyzed were within the certified range of mercury concentration, and none of the blanks had detectable levels of mercury.

Data analysis

Allozyme frequencies and fit of observed data to Hardy–Weinberg expectations were calculated using BIOSYS-1 [17]. Rare alleles were pooled for *Icd-1* and *Gpi-2* to test for fit to Hardy–Weinberg expectations. The average number of heterozygous loci per individual was calculated by dividing the number of heterozygous loci by the total number of polymorphic loci [6]. Assessment of genetic bottlenecks was accomplished using BOTTLENECK [18], which performs three statistical tests using allele frequency data to detect recent effective population size reductions.

Univariate statistics for standard length and weight were calculated with the MINITAB statistical software package [19]. Differences in standard length and weight between treatments were analyzed with a split-plot analysis of variance (ANOVA) to control the variance due to mesocosm effects with the MIXED procedure of the Statistical Analysis System [20]. The χ^2 tests for nonindependence of allele frequencies between 1994 and 1996 were also performed with the MINITAB statistical software package [19]. Wilcoxon rank-sum tests were employed to test for differences in *Gpi-2* selection coefficients, mercury concentration, and heterozygosity between control and mercury-treated mesocosms with the Statistical Analysis System [20] using the NPAR1WAY procedure. Selection coefficients for the GPI-2 alleles were calculated using the following formula:

$$S = \log\{[P_{1996}/(1 - P_{1996})]/[P_{1994}/(1 - P_{1994})]\} \quad (1)$$

where S = the selection coefficient, P_{1996} = the mesocosm population allele frequency in 1996, and P_{1994} = the allele frequency of the initial population.

RESULTS

Whole-body mercury concentrations (mg/kg wet wt.) were significantly different for fish in control and mercury-treated mesocosms (Tables 1 and 2). Mercury concentrations for fish from control mesocosms ranged between 0.009 and 0.036 mg Hg/kg wet weight. Mercury concentrations for fish from mercury-treated mesocosms ranged between 1.101 and 6.946 mg Hg/kg wet weight.

Allele frequencies for the initial population and the 1996 populations were consistent with Hardy–Weinberg equilibrium expectations. (Allele frequencies for the seven allozyme loci are not reported here but are available from the primary author.) In addition, no evidence of genetic bottlenecks was found for any of the populations. Allele frequencies for the *Icd-1, Icd-2, Mpi, Ada, Mdh,* and *Fum* loci were homogeneous for the initial and 1996 samples. The χ^2 tests of homogeneity of allele frequencies between initial and 1996 samples revealed significant changes in allele frequencies at the *Gpi-2* locus in three mesocosms ($p < 0.03$). The significant change in allele frequencies at the *Gpi-2* locus prompted calculation of selection coefficients for the three *Gpi-2* alleles by mesocosm. Analysis of selection coefficients indicated a decrease in the *Gpi-2*^{100} allele ($p = 0.055$) and a corresponding increase in the *Gpi-2*^{66} allele in mercury-exposed mosquitofish populations. No selection was noted for the rare *Gpi-2*^{38} allele (Table 2). The changes in *Gpi-2* allele frequencies associated with mercury exposure are illustrated in Figure 1. Additionally, all *Gpi-2* allele frequencies displayed greater variance among mercury-exposed populations than control populations (Fig. 1). The allele frequencies for the *Gpi-2* locus are reported in Table 3. The average number of heterozygous loci per individual did

Table 1. Characteristics of mosquitofish in control and mercury exposed populations

	A		B		C		D	
Variable	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Control mesocosms								
Standard length (mm)								
Female	81	31.65 (5.65)	185	30.81 (5.44)	38	26.34 (7.58)	68	21.85 (3.15)
Male	80	23.09 (2.33)	88	22.30 (2.40)	46	18.54 (1.15)	78	18.85 (1.32)
Weight (g)								
Female	81	0.697 (0.389)	185	0.617 (0.361)	38	0.386 (0.438)	68	0.194 (0.118)
Male	80	0.190 (0.083)	88	0.191 (0.069)	46	0.106 (0.025)	78	0.113 (0.028)
Age ratio (adult:juveniles)[a]	864	4.88:1	814	3.4:1	1,794	0.27:1	1,250	1.15:1
Sex ratio (female:male)[a]	717	1.54:1	629	2.02:1	385	3.81:1	670	2.10:1
Hg concn. (mg/kg)	10	0.013 (0.007)	10	0.009 (0.003)	10	0.036 (2×10^{-4})	10	0.032 (0.014)
Mercury-exposed mesocosms								
Standard length (mm)								
Female	185	23.43 (5.05)	127	26.79 (5.85)	110	28.60 (2.76)	67	22.78 (3.97)
Male	69	19.12 (1.88)	98	22.20 (2.40)	69	22.78 (1.54)	67	20.03 (1.62)
Weight (g)								
Female	185	0.267 (0.254)	127	0.400 (0.249)	110	0.402 (0.119)	67	0.223 (0.137)
Male	69	0.119 (0.035)	98	0.188 (0.059)	69	0.179 (0.041)	67	0.128 (0.033)
Age ratio (adult:juvenile)[a]	1,384	0.98:1	1,383	1.40:1	456	227:1	1,304	1.87:1
Sex ratio (female:male)[a]	686	3.16:1	807	1.40:1	454	1.93:1	849	1.57:1
Hg concn. (mg/kg)	10	1.928 (0.314)	10	1.101 (0.150)	10	6.946 (0.670)	10	2.038 (0.417)

[a] Age and sex ratios were calculated from the total number of mosquitofish collected from the mesocosms. The sample size for standard length and weight is a subset of the fish collected.

not differ between the mercury-exposed or the control populations (Table 2).

Weight and standard length exhibited wide variance among mesocosms. For this reason, a split-plot ANOVA did not detect significant effects of mercury treatment on standard length or weight for adult males and females in the 1996 sample.

Age ratios were highly variable among mesocosms (Table 1) but were not significantly different between mercury-exposed and control populations (Table 2). Mesocosm C, with the highest tissue burdens of mercury (6.946 mg Hg/kg), had only two juvenile fish in the entire sample ($N = 456$). The adult-to-juvenile ratio for this mesocosm was higher than any of the other control or mercury-treated mesocosms. It is possible that the high mercury concentration in this mesocosm had an adverse effect on reproduction or recruitment or altered the availability of prey items in a manner that promoted cannibalism. Insufficient data exist to identify a population-level process responsible for the low number of juveniles in mesocosm C. Sex ratios also differed among mesocosms but were female biased in all control and mercury-treated mesocosms. No significant differences in sex ratio were detected between mercury-exposed and control populations (Table 2).

DISCUSSION

Populations categorized as mercury exposed differed in numerous ways, including mercury concentration. Because no evidence was found to suggest a concentration response, statistically significant differences in mercury concentration do not imply biologically significant differences. However, the differences in mercury concentrations between controls and mercury-treated mesocosms were 100-fold, whereas the differences within mercury-treated mesocosms were only sevenfold. The categorization obtained by combining all mercury mesocosms and all control mesocosms was justified by the relative magnitude of the differences in mercury concentrations.

No significant differences were found in adult size, age ratio, and sex ratio between control and mercury-exposed populations. Allele frequencies did not differ between control and mercury-exposed populations at six of the allozyme loci investigated over the two-year exposure. The frequencies of the *Gpi-2*^{66} and *Gpi-2*^{100} alleles in mercury-exposed populations were different between the 1994 and 1996 samples. The *Gpi-2* allozyme frequencies in the control populations did not differ between the 1994 and 1996 samples. Rearing mosquitofish in mesocosms did not affect *Gpi-2* allele frequencies, as evidenced by the constancy of the *Gpi-2* allele frequencies in control populations between the initial and the 1996 samples.

Significant differences in *Gpi-2* allele frequencies between mercury-exposed and control populations in the 1996 sample were revealed by a χ^2 test ($p < 0.001$). The differences in

Table 2. Wilcoxon rank sum tests on *Gpi-2* selection coefficients, heterozygosity, demographic ratios, and mean mercury concentration

Variable studied	One- or two-tailed test	*Z*	Prob > |*Z*|
Gpi-2^{100} selection coefficient	One-tailed	1.597	0.055
Gpi-2^{66} selection coefficient	One-tailed	−1.588	0.056
Gpi-2^{38} selection coefficient	One-tailed	0	0.999
Mean number of heterozygous loci per individual	Two-tailed	−0.144	0.885
Age ratio (adult:juvenile)[a]	Two-tailed	−0.144	0.885
Sex ratio (female:male)[a]	Two-tailed	0.722	0.471
Hg concentration (mg/kg)[a]	Two-tailed	7.694	0.0001

[a] Selection coefficients were not calculated for age ratio, sex ratio, and mercury concentration. These analyses test if the sex ratio, age ratio, and mercury concentrations are the same for control and for mercury-exposed populations.

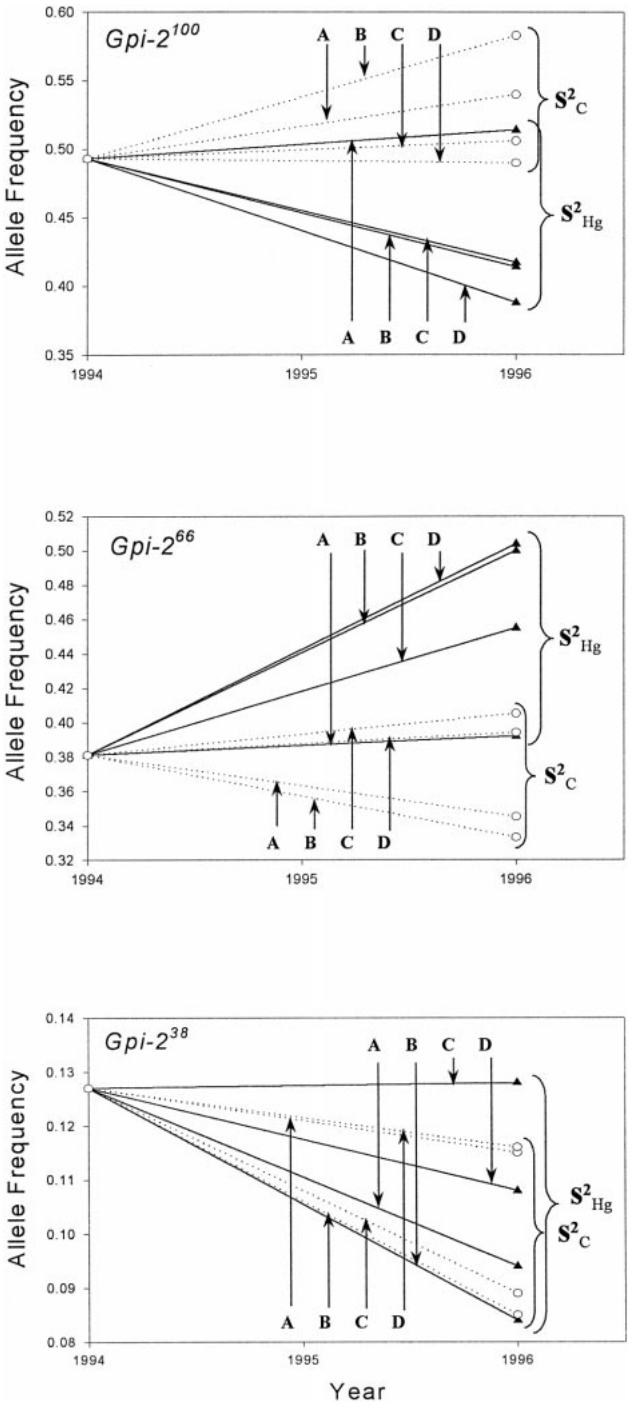

Fig. 1. Change in the frequencies of *Gpi-2* alleles over two years (approx. four generations) in experimental mosquitofish populations. Solid lines with solid triangles denote mercury-exposed populations; dotted lines with open circles denote control populations. Lines are labeled A, B, C, or D, corresponding to mesocosm designations in Table 1. Allele plotted is designated in the upper-left corner of each panel. All alleles exhibit greater sample variance among mercury-exposed populations (s^2_{Hg}) than among control populations (s^2_C).

Gpi-2 allele frequencies between treatments in the 1996 sample, coupled with the significant changes in *Gpi-2* allele frequencies over time in mercury-exposed populations, suggest that mercury can change the genetic composition of mosquitofish populations. Shifts in *Gpi-2* allele frequencies associated with mercury exposure were consistent with previous allozyme studies using the same source population of mosquitofish. For this population, differences in performance among fish of different *Gpi-2* genotypes have been demonstrated during acute mercury exposures [5,7,8], acute arsenate exposure [7], and a low-level, 111-d mercury exposure [6].

Four additional studies using *G. holbrooki* also demonstrate a correlation between *Gpi-2* genotype and response under stressful conditions. Mulvey et al. [21] found that time to reach maturity differed for fish of different *Gpi-2* allozyme genotypes in environments with elevated temperature. Differential survivorship of *Gpi-2* allozyme genotypes in response to acute salinity stress was found in both Australian [10] and U.S. [13] *G. holbrooki* populations. Susceptibility to the organophosphate insecticide, chlorpyrifos, also differed among allozyme genotypes at the *Gpi-2* locus [11]. Although it is possible that the *Gpi-2* locus is a neutral marker for an unknown linked locus affecting fitness, the consistency of *Gpi-2* allozyme genotype differences in response to various stressors argues for its direct involvement in fitness differences.

In the original Risher Pond source population and the control mesocosm populations, the *Gpi-2*^{100} allele was the most common, followed by the *Gpi-2*^{66} allele and then the relatively rare *Gpi-2*^{38} allele. Several generations of mercury exposure produced a pronounced shift in allele frequencies where the *Gpi-2*^{66} allele had become the most common allele, followed by the *Gpi-2*^{100} allele. This shift in allele frequencies was comparable to that predicted with computer simulations of the *Gpi-2* response of mosquitofish populations exposed to mercury [22]. The simulations were based on a complex model that incorporated survival and reproductive effects of mercury that were quantified in previous studies [5–8] as well as genetic drift and immigration. Changes in allele frequencies were predicted in response to periodic short pulses of mercury exposure combined with low-level chronic exposure. The simulations indicated that the *Gpi-2*^{100} allele was replaced by the *Gpi-2*^{66} allele as the most common allele in populations exposed to mercury over several overlapping generations. Simulations also predicted that the *Gpi-2*^{38} allele would be lost from the population under the pulsed-exposure scenario [22]. In the present mercury exposure study, the *Gpi-2*^{38} allele frequency increased in one population and decreased in three populations (Fig. 1).

Because allozyme analysis is being discussed for possible use in biomonitoring, it is important to note these population differences. For example, the frequency of the *Gpi-2*^{100} allele increased in one of the mercury-treated populations and decreased in three populations. One mercury-exposed population did not have a large increase in the *Gpi-2*^{66} allele frequency. Even for *Gpi-2*, which has often been reported to respond to stress, shifts in allele frequency were not consistent for all populations. Assessment of contaminant impact can benefit by including analysis of allozyme frequency data, but allozyme frequency surveys may not be effective as the sole indicator of population response to contaminants.

Shifts in allele frequencies at the *Gpi-2* locus were detected in mercury-exposed populations, but the underlying population-level processes responsible for the observed shifts were not identified. This is a typical problem when allozyme analysis is used to assess population-level effects of toxicants. The process of selection requires genetic variation, differential fitness, and inheritance [23]. Allozyme studies effectively measure genetic variation. However, quantification of fitness differences has not been fully investigated in allozyme studies. Although fitness has several aspects, most allozyme studies

Table 3. Allele frequencies at the *Gpi-2* locus for the 1994 and 1996 samples

	1994	1996							
		Control mesocosms				Mercury-exposed mesocosms			
Allele	Initial frequencies (n = 134)	A (n = 161)	B (n = 272)	C (n = 84)	D (n = 146)	A (n = 254)	B (n = 225)	C (n = 179)	D (n = 134)
Gpi-2^{100}	0.493	0.540	0.583	0.506	0.490	0.514	0.416	0.416	0.388
Gpi-2^{66}	0.381	0.345	0.333	0.405	0.394	0.392	0.500	0.455	0.504
Gpi-2^{38}	0.127	0.115	0.085	0.089	0.116	0.094	0.084	0.128	0.108

quantify only survival, neglecting the aspects of mating ability, fertilizing ability, fertility, and fecundity [22]. A study of mosquitofish populations exposed to mercury in mesocosms indicated female sexual selection and fecundity selection in mercury-treated mesocosms. Females of different *Gpi-2* genotypes differed in the probability of being gravid and the number of embryos carried [6]. The underlying processes are likely similar for mosquitofish populations held for many generations in these mesocosms.

The increasing use of allozyme analysis has brought some criticism concerning experimental design and the presence of confounding ecological and biological factors [22,23]. Elements of design that weaken experimental strength include lack of replication or pseudoreplication and use of reference populations in place of true controls. Newman and Jagoe [22] discussed five problems that contributed to inferential weakness of allozyme studies. One of these has been previously discussed (the quantification of survival vs the reproductive components of fitness). Of the remaining problems, the first involves the disparity between laboratory and field concentrations of toxicants. The second problem is uncertainty in assignment of causation due to reliance on correlation analysis of field data. Allozyme genotype effects ascribed to toxicants could be attributed to biotic processes, including predation, competition, or accidents of genetic history, such as population bottlenecks, founder effects, migration, or preexisting genetic clines. Abiotic factors confounding assignment of causation include incomplete understanding of toxicant exposure (e.g., duration, chemical identification, or route), habitat alteration, and factors such as temperature and dissolved oxygen. The third problem is that covariates influencing phenotype (survival), such as animal size and sex, are often unquantified. The final problem is that the mechanism of genotype effect is frequently undefined and considered only speculatively.

This study has addressed many of the weaknesses associated with allozyme studies. The use of mesocosms allowed us to strengthen the experimental design by enabling replication of controls and treatments. Because all the initial populations came from a naive source population, this study was able to use controls instead of reference populations. The demographics and genetic history of the source population are well documented [5–8], and no evidence of founder effects or genetic bottlenecks has been found. The use of mesocosms eliminated the confounding factors of gene flow and predation present in natural populations while reducing environmental variance by providing similar environments, except for the presence of mercury. The contaminant and the exposure history are well known, thus strengthening the assignment of causation of selection to mercury. Finally, associated studies of metabolite pools and enzyme kinetics suggested a bioenergetic mechanism for differences between *Gpi-2* allozyme genotypes [15,16,24].

The results of this study suggest that shifts in allozyme frequencies can be used to indicate population-level effects of toxicants. This study also indicates that shifts in allozyme frequencies for toxicant-exposed populations are associated with probabilistic outcomes and that deviations from the predicted response are to be expected. For this reason, caution should be used in the application of allozyme analysis to field populations. Effective use of allozyme data requires that attention be paid to the ecology and genetic history of the populations under study so that population response is properly attributed to toxicants instead of confounding ecological and biological factors. Correlation between response and *Gpi* allozyme genotype has been documented for diverse taxa and a wide range of chemicals and environmental stressors [1,4–14,16,21,25–26]. This suggests that the *Gpi* locus may be one of the more reliable genetic markers in indicating population-level effects of toxicants.

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