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ALLOZYME GENOTYPE IN MOSQUITOFISH, *GAMBUSIA HOLBROOKI*, DURING MERCURY EXPOSURE: TEMPORAL STABILITY, CONCENTRATION EFFECTS AND FIELD VERIFICATION

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Abstract – Genotype frequencies at nine enzyme loci were examined in a population of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure at three concentrations. Genotype at one locus, glucose phosphate isomerase-2 (*Gpi-2*), was correlated with time to death (TTD) at the low (0.83 mg/L) mercury concentration, but genotypes at none of the nine loci were related to TTD at the medium (1.07 mg/L) or the high (1.13 mg/L) mercury concentration. A survey of mosquitofish from a mercury-contaminated canal was undertaken to determine if the results of laboratory exposures could be used to predict accurately the genetic profile of mercury-contaminated field populations. Mosquitofish collected from the contaminated canal had a significantly lower frequency of the $Gpi\text{-}2^{38}$ allele than mosquitofish collected from the adjacent noncontaminated river. The *Gpi-2* allozymes may be useful as an indicator of pollutant stress if used in conjunction with a thorough understanding of the structure and history of the population.

Keywords – Mosquitofish *Gambusia holbrooki* Mercury toxicity Genetics Tolerance

INTRODUCTION

Recently, a number of studies have linked enhanced tolerance to specific allozyme genotype [1–3]. These studies suggested that contaminants may select against sensitive allozyme genotypes and that allozyme polymorphisms could be useful genetic indicators of population responses to pollution stress. Laboratory studies have corroborated this suggestion by showing that shifts in genetic frequencies observed in the field can also be observed in the laboratory [4,5]. However, these studies characteristically fail to fully define the qualities and limitations of differential tolerance. Before using the relationship between allozymes and a population's response to toxicant stress as genetic indicators, it is necessary to define the limits of this relationship.

We have undertaken a series of studies to clarify the relationship between allozyme genotype and differential tolerance [6–9] in a naive population of mosquitofish, *Gambusia holbrooki*. Diamond et al. [6] noted significant relationships between tolerance during an acute mercury exposure and allozyme genotypes at three of eight tested loci. Fish with the following genotypes of isocitrate dehydrogenase-1 ($Icd\text{-}1^{134}/Icd\text{-}1^{134}$), malate dehydrogenase-*1* ($Mdh\text{-}1^{100}/Mdh\text{-}1^{100}$; $Mdh\text{-}1^{118}/Mdh\text{-}1^{118}$), and glucosephosphate isomerase-2 ($Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$) had significantly earlier time to death (TTD) than fish with other genotypes at these loci. Using arsenate as an alternate toxicant, Newman et al. [7] suggested that differences in TTD associated with allozyme variation may be either toxicant-specific (sensitive to one metal) or nonspecific responses (sensitive to two metals). Specific responses were seen for the fumarate hydratase (FH) genotypes in the arsenate exposure, whereas MDH-1 and ICD-1 genotypes seemed to be specific for the mercury exposure. The same response was seen with the GPI-2 genotypes for both arsenate and mercury: The $Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$ genotype was associated with significantly shorter TTDs. In addition, Diamond et al. [6] and Newman et al. [7] demonstrated that TTD during toxicant exposure was significantly correlated with the size and sex of the fish; smaller fish and male fish had consistently shorter TTDs than larger fish and female

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fish. Kramer et al. [8] examined the differential inhibition of GPI and MDH allozymes by measuring Krebs cycle and glycolysis metabolite concentrations in mosquitofish exposed to inorganic mercury. Fish with the $Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$ genotype responded to mercury intoxication with enhanced glycolytic activity, as indicated by higher concentrations of lactate and glucose-6-phosphate metabolites relative to those of fish with other *GPI-2* genotypes.

We continue to explore the relationship between allozyme genotype and population response to toxicants by examining the temporal stability and the effect of concentration on differential tolerance. To be useful as indicators, the correlations between allozyme genotype and TTD should be temporally stable; they should not arise due to undefined or temporally variable population structure. For example, lineages differing in mercury or arsenate tolerance may also differ in allozyme frequencies, which could lead to the observed correlations [9], although there may be no underlying relationship between genotype and TTD. We refer to this as a population structure effect; it reflects the possibility of unintentionally biased sampling of various lineages during fish collection. In addition, the genotype responses detected by Diamond et al. [6] could have been limited to a range of toxicant concentrations. If toxicant concentration were a critical factor in the relationship between genotype and response to a toxicant, the utility of allozymes as indicators might be limited. Furthermore, field verification is needed to determine if the predictions made from the laboratory studies are applicable to field populations experiencing toxicant stress.

We undertook two studies to address these questions. The first study, an acute mercury exposure similar to that of the Diamond et al. [6] study, was designed to examine the effect of population structure and the effect of concentration on the relationship between differential tolerance and allozyme genotype. The repetition of the earlier experiment allowed us to determine if genotype correlations were temporally stable. The random association of genotypes within various lineages would be expected to change significantly within the 24 months (approximately six mosquitofish generations) between the two studies. Therefore, if previous correlations reflected population structure and not an underlying relationship between allozymes and TTD, different genotype correlations would be expected. However, if similar genotype responses were detected, then the previous genotype correlations with TTD would be temporally stable and less likely to have been due to population structure. Further, by using three concentrations of inorganic mercury, which bracketed the concentration used in the previous study [6], the effect of concentration on the genotype response could be assessed. If the genotype correlations with TTD in the three treatments were similar to each other and to those of the first mercury experiment [6], then toxicant concentration would not affect the genotype correlation with TTD. The second study, a survey of a mercury-contaminated canal, was undertaken to determine if the results of the laboratory exposures were applicable to field situations and if demographic characteristics and allozyme frequencies were useful as indicators of pollutant impacts. The laboratory toxicity tests suggested a relationship between mercury tolerance and fish size, sex, and genotype at the *GPI-2* locus. Therefore, we would predict that fish inhabiting a mercury-contaminated environment would be larger, the sex ratio of the population would be more female biased, and the frequency of the $Gpi\text{-}2^{38}$ allele would be lower relative to mosquitofish in nearby noncontaminated areas.

MATERIALS AND METHODS

Acute toxicity study

Collection and sampling. The fish were collected from Risher Pond (Barnwell County, SC), the source of mosquitofish used in previous studies [6–8], as this population has no known history of mercury or other toxicant exposure. Although we have previously referred to mosquitofish from this area as *Gambusia affinis*, the convention based on the reclassification of mosquitofish in the southeastern United States as *G. holbrooki* is now followed [10]. The fish were caught by dip net between September and November 1989 and placed into plastic 48-L coolers filled with aerated pond water. Stress Coat™ (Aquarium Pharmaceuticals, Chalfont, PA), a commercial fish product containing aloe vera that replaces the protective slime coat, was added to the water before transport back to the laboratory. The fish were held in a 520-L tank (Living Streams™ model LSW-700; Toledo, OH) or a 720-L tank (Living Streams model LSW-900) at 15 to 17°C until the exposure began. During the holding period, the fish were fed a mixture of Tetramin® (Tetrawerke, Germany), Tetra Brine Shrimp Treat™ (Tetrawerke), and freeze-dried bloodworms.

Exposure. Two 25-L reference tanks were supplied with a continuous flow of nine tank volumes per day of water from a nearby stream, Upper Three Runs Creek (Aiken County, SC) (5–15 ng/L Hg; M.C. Newman, unpublished data) in which temperature was maintained with a 50-W aquarium heater. Six 167-L exposure tanks (two per concentration) were supplied with a continuous flow of 2.3 tank

volumes per day of Upper Three Runs Creek water spiked to realized concentrations of 0.83, 1.07, or 1.13 mg/L Hg (added as $HgCl_2$). Temperature was maintained in the tanks using a chiller unit to circulate water in a bath containing the tanks. All the tanks were aerated and covered with screening.

The toxicant solution was prepared from 100-L aliquots of water measured into 200-L plastic barrels. Either 100, 120, or 140 ml of a 1.0-g/L Hg solution was added to obtain the desired concentration. Mercury solutions were renewed in the reservoirs every 3 h, and the toxicant was delivered to the appropriate set of tanks with a peristaltic pump.

Fish were randomly assigned to the two reference tanks and the six exposure tanks to obtain densities of approximately 1.8 fish per liter for the exposure tanks and 3.7 fish per liter for the reference tanks. The mean (SD) wet weight of the fish was 0.225 g (0.145) and did not differ significantly among the tanks. Fish were acclimated for 24 h before the exposure tanks were dosed with the appropriate volume of mercury solution and the flow of spiked water was started. The tanks were checked for dead fish every 3 h, for a total of 240 h of exposure, and individuals were scored as dead if they displayed no movement after repeated gentle prodding. After death, each fish was weighed, sexed, and placed into a plastic tube for later electrophoresis. The tissues were stored on dry ice for no longer than 48 h before they were placed in a −70°C freezer. Any fish surviving 240 h of exposure were killed and processed as described above.

Water chemistry. Daily measurements of dissolved oxygen concentration and temperature were made with a Hydrolab Surveyor II (Model L SVR 2-SU, Hydrolab Corp., Austin, TX). The pH was measured daily with an Orion (Boston, MA) Research Microprocessor Ionanalyzer 901 and an Orion 8102 Ross combination electrode. Water samples for other chemistry variables were collected daily and stored at 4°C. Water samples for mercury analysis were acidified using 0.5 ml distilled nitric acid per 500 ml.

Total alkalinity was determined by potentiometric titration [11], and specific conductance was determined using a Sybron (Sybron-Barnstead, Dubuque, IA) PM-70CB conductivity bridge and a Fisher (Fisher Scientific, Fairlawn, NJ) cell (cell constant 0.102/cm). Samples and standards for major cation and anion analysis were passed through Sep-Pak® (Milford, MA) reversed-phase columns and analyzed with a Dionex (Sunnyvale, CA) 4020i ion chromatograph equipped with a conductivity detector. Major cation concentrations (Mg^{2+}, Ca^{2+}, Na^+, and K^+) were determined using an HPIC-CS3 (Dionex, Sunnyvale, CA) separator column, and anions (Cl^- and SO_4^{2-}) were determined using an HPIC-AS4A (Dionex) separator column. Total mercury was determined by a cold vapor technique [11], using a Perkin Elmer (Norwalk, CT) 50A AA spectrophotometer.

Electrophoresis. Horizontal starch gel electrophoresis was used to determine the following enzyme systems: isocitrate dehydrogenase (ICD-1, ICD-2; E.C. 1.1.1.42), malate dehydrogenase (MDH-1; E.C. 1.1.1.37), mannosephosphate isomerase (MPI; E.C. 5.3.1.8), glucosephosphate isomerase (GPI-2; E.C. 5.3.1.9), adenosine deaminase (ADA; E.C. 3.5.4.4), fumarate hydratase (FH; E.C. 4.2.1.2), leucylglycylglycine peptidase (lgg-PEP; E.C. 3.4.11.−), and phenylalanylproline peptidase (pp-PEP; E.C. 3.4.11.−). ADA was added to the examined loci, but otherwise the same enzyme systems and notation as those in the previous studies were used [6,7], as they are polymorphic in the source population. The isozymes were numbered in order of decreasing anodal mobility in multilocus systems. Allozyme mobilities were determined relative to the most common allozyme for each locus, which was arbitrarily designated 100. Therefore, the designation $Mdh\text{-}1^{100}/Mdh\text{-}1^{100}$ indicated an individual homozygous for the common *Mdh-1* allele, whereas $Gpi\text{-}2^{100}/Gpi\text{-}2^{66}$ indicated a heterozygous individual.

Data analysis. Genotype frequencies and fit of these data to Hardy–Weinberg expectations were determined with Swofford and Selander's [12] FORTRAN program, BIOSYS-1. Contingency table chi-square statistics were used to test for homogeneity of allele distributions among the reference and exposed fish.

The mortality data were initially analyzed using the survival analysis techniques implemented by the SAS® version 6.03 LIFEREG procedure [13], the same techniques used by Diamond et al. [6] and Newman et al. [7]. The LIFEREG procedure fits parametric models to failure-time data (i.e., TTD) that can contain survivors. However, when (ln (−ln (1−proportion dead))) of these data was plotted against ln (TTD), the resulting curves were not linear, indicating that the assumption of an underlying Weibull distribution was inappropriate. Similarly, the lognormal and the exponential distributions were tested for their goodness of fit to these data [14] and were also found to be inappropriate. Therefore, a Cox semiparametric proportional hazards model was used. In this model the effects of the predictor variables are multiplicative on the underlying hazard function, but no specific distribution type is assumed for the underlying hazard function. The model is described as follows:

$$h(t,x) = h_0(t)e^{\beta' x} \quad (1)$$

where $h(t,x)$ is the predicted hazard rate for a vector of independent variables, x, associated with a particular survival time, t, and β' is the vector of regression coefficients [14]. This model is similar to the model used by Steadman et al. [15] to examine the survivorship of rainbow trout exposed to fuel oil. It is important to note that the β values generated by Cox's proportional hazards model will have the opposite sign as those generated by the models used previously. For example, smaller β values in Diamond et al. [6] indicated increased sensitivity to mercury poisoning, whereas larger β values indicate increased sensitivity to mercury poisoning in the Cox model. The new method was first tested with the TTD–genotype data from the previous studies [6,7] to ensure consistency in the our comparisons using the Cox proportional hazards model to past conclusions. The conclusions drawn from the Cox proportional hazards model were identical to those generated by the parametric proportional hazards model; therefore, the findings from the Cox model were assumed to be adequate for our intended comparisons between studies.

Two chi-square tests were used to compare the β values generated by the Cox model for each locus. The overall chi-square tested whether there was a difference among the genotypes at each locus, and the individual chi-square of the genotypes tested whether a genotype was significantly different from the reference genotype. If warranted, these tests were supplemented by comparisons of specific genotypes that represented tolerant and intolerant genotypes in the previous studies.

Field verification

Collection and sampling. Gambusia holbrooki was collected within a six-d period during September 1990 from eight locations along the Savannah River: four locations within a mercury-contaminated canal that connects to the Savannah River (C0, C1, C2, C3), and two river sites upstream (SU1, SU2) and two sites downstream (SD1, SD2) of the canal (Fig. 1). The genetic characteristics of fish from this portion of the river have been studied and reported previously [16,17]. Approximately 60 fish were collected by dip net from each sample location and transported on ice to the laboratory, where fish were measured for standard length, weighed, sexed, placed into plastic tubes, and stored at −70°C for electrophoresis. Before electrophoresis, five or more large fish (≥0.250 g) from each site were selected and the section posterior to the anal fin moved, placed into a separate tube, and fr subsequent mercury analysis, whereas the section was retained for electrophoresis.

Mercury analysis. Due to the small amou sue available for mercury analysis, two or th of similar wet weight from each site were po fore digestion. The tissue was digested method of Kivalo et al. [18], and total r was determined by cold vapor technique [1 a Perkin Elmer 50A AA spectrophotometer termine the recovery and digestion efficienci cury-contaminated bass tissue as well as blanks (5.0 μg Hg/L) were digested and an

Electrophoresis. The same notation and systems, with the addition of aspartate amin ferase (AAT-1, AAT-2; E.C. 2.6.1.1.) and erophosphate dehydrogenase (α-GPD; E.C. 1 were used in this study as those used in the tory exposure described above and in the p studies [6,7]. Although MDH-1 has been r in the earlier studies, reliable scoring of the M locus was not possible due to an unexplainab ing interference in the fish collected for this therefore, MDH-1 was dropped from the a

Data analysis. Swofford and Selande FORTRAN program, BIOSYS-1, was use termine genotype frequencies and fit of the to Hardy–Weinberg expectations and to det the percentage of polymorphic loci and the a heterozygosity as descriptors of population variability. In addition, contingency tab square statistics were used to test for homo of allele frequency distributions among the ent sample sites. The proportion of polym loci (P) was calculated by summing the num observed polymorphic loci and then dividing total number of scored loci (11), and the me erozygosity (H) was determined by calculat proportion of heterozygous loci per fish at ea

The SAS [13] General Linear Models (procedure, one-way analysis of variance (AN model $d.f. = 7$) was used to analyze allele freq heterozygosity, mean weight, and mean sta length data to detect differences between car river fish. The model compared fish from th cury-contaminated canal (four samples) to fis the river (four samples) to determine if ther significant differences between the fish expo elevated levels of mercury and the nonexpose Before analysis, the sex ratios were transf using the ln(number of males/number of fe to normalize for the difference in sample s tween the populations.

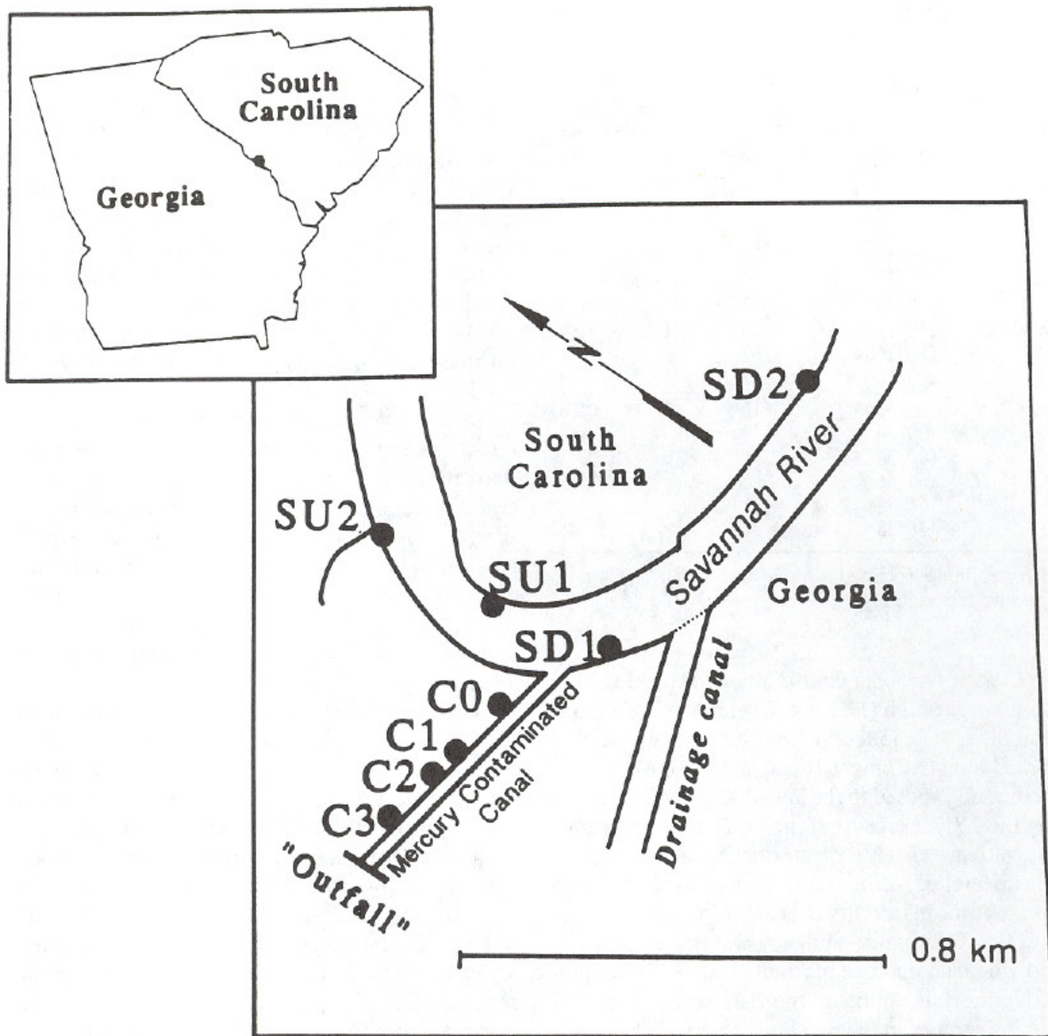

Fig. 1. Collection sites of mercury-contaminated canal and adjacent Savannah River sites. The canal sites are designated C0, C1, C2, and C3; the upstream sites are designated SU1 and SU2, and downstream sites are designated SD1 and SD2. "Outfall" indicates the most likely source of mercury to the canal.

RESULTS

Laboratory exposure

Water quality. Water-quality variables were similar for all tanks throughout the exposure period, except for mercury concentrations (temperature: mean = 17.3°C; dissolved oxygen: mean = 9.6 mg/L; pH: median = 6.52, range = 6.10–6.93). The mean (SD) measured mercury concentrations were 0.83 (0.06) mg/L for the low-concentration tanks, 1.07 (0.01) mg/L for the medium-concentration tanks, and 1.13 (0.04) mg/L for the high-concentration tanks. The mercury concentrations for the reference tanks were <0.001 mg/L.

Genetics. The genotype distributions for the nine enzyme loci for the 2,008 fish were consistent with Hardy–Weinberg expectations (Table 1) and were similar to the distributions found in the previous studies [6,7]. There were no significant ($\alpha = 0.05$) deviations from random assignment of genotypes, sexes, or fish sizes among the reference and treatment tanks, and contingency chi-square tests confirmed that alleles were homogeneously distributed among all experimental tanks.

Table 1. Distributions of genotypes for loci that were previously correlated with time to death (TTD) in mosquitofish for all fish in the laboratory toxicity test (reference and exposure fish) (other enzyme loci were also consistent with random mating expectations)

Locus	Genotype	Distribution	χ^2	p[a]
ICD-1	134/134	8	4.26	0.235
	134/116	8		
	134/100	237		
	116/100	56		
	100/100	1,699		
MDH-1	118/118	179	1.95	0.162
	118/100	797		
	100/100	1,031		
GPI-2	100/100	512	3.79	0.285
	100/66	729		
	100/38	296		
	66/66	256		
	66/38	176		
	38/38	31		

[a]Probability associated with χ^2 test of fit to random mating expectations.

Cox proportional hazards model. All 625 fish exposed to the high (1.13 mg/L) mercury concentration died, 98% (588 of 603) of the fish exposed to the medium (1.07 mg/L) died, and 75% (445 of 591) of the fish exposed to the low (0.83 mg/L) died during the 240 h exposure (Fig. 2). The difference between treatments was determined to be greater than the difference within treatments by using *t* tests to compare mean mercury concentrations of each tank and the Kolmogorv–Smirnov test to compare the individual cumulative mortality curves. The cumulative mortality plot of the fish exposed to 0.99 mg/L mercury in the previous experiment [6] falls between the low- and medium-concentration cumulative mortality plots of this experiment. During the experiment only three of 189 reference fish died; consequently, these reference mortalities were excluded from statistical consideration.

The genotypes at nine loci, size (wet weight), and sex data were used in the Cox proportional hazards model to test the null hypothesis that the effects of size, sex, and single locus genotype were not concentration dependent. These results were then used to assess the temporal stability of the genotype effects by comparing them to the results of our earlier studies [6,7]. As there were minimal differences between water chemistry, mercury concentrations, and genotype frequencies, data from the two exposure tanks for each concentration were pooled before statistical analysis.

Size had a significant effect on TTD in all the mercury treatment concentrations (low: $\chi^2 = 21.09$, $p < 0.001$; medium: $\chi^2 = 4.48$, $p = 0.034$; high: $\chi^2 = 21.45$, $p < 0.001$), with the smaller fish having shorter TTDs than the larger fish. At the low and high mercury concentrations, sex had significant effects on TTD (low: $\chi^2 = 5.26$, $p = 0.022$; high: $\chi^2 = 12.18$, $p < 0.001$), with the females having significantly longer TTDs than males. In addition, there was a significant size·sex interaction in the low and high mercury concentrations (low: $\chi^2 = 5.66$, $p = 0.017$; high: $\chi^2 = 6.79$, $p = 0.009$), which indicated there were sex-specific effects of weight on TTD.

In the low mercury concentration tanks, the $Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$ genotype was found to be more sensitive to mercury poisoning than the $Gpi\text{-}2^{100}/Gpi\text{-}2^{100}$ genotype ($p = 0.041$). This result is consistent with that of the previous mercury experiment [6]. However, when the results from the medium and high concentrations were analyzed, none of the genotypes at the nine loci were found to affect the TTD of the mosquitofish significantly ($\alpha = 0.05$).

To assess further the effect of concentration on the genotype response, the genotypes at nine loci, size (wet weight), and sex data from the three exposure concentrations were combined and used in the Cox proportional hazards model. By including each concentration as a separate stratum, the model was able to predict a separate baseline hazard for each concentration group and to adjust for differences in the baseline hazard between groups. When the three concentrations were pooled in this manner, fish size, sex, and the size·sex interaction were significantly correlated with TTD ($\chi^2 = 35.07$, $p < 0.001$, $d.f. = 1$; $\chi^2 = 8.69$, $p = 0.003$, $d.f. = 1$; $\chi^2 = 5.12$, $p = 0.024$, $d.f. = 1$, respectively) (Table 2). In addition, the probabilities associated with the overall χ^2 statistics indicated that the *GPI-2* genotype had a significant effect on TTD during mercury exposure ($\chi^2 = 13.93$, $p = 0.016$, $d.f. = 5$). The β estimates suggest that the $Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$ genotype is more sensitive to mercury intoxication than the $Gpi\text{-}2^{100}/Gpi\text{-}2^{100}$ genotype. However, the chi-square statistic for this specific comparison was not statistically significant ($\chi^2 = 2.62$, $p = 0.106$) at $\alpha = 0.05$. The β values for the other genotypes were intermediate between those of the $Gpi\text{-}2^{38}/Gpi\text{-}2^{38}$ and the $Gpi\text{-}2^{100}/Gpi\text{-}2^{100}$ genotypes. No additional loci were significantly correlated with TTD in the analysis of the pooled data or in the analysis of the separate data.

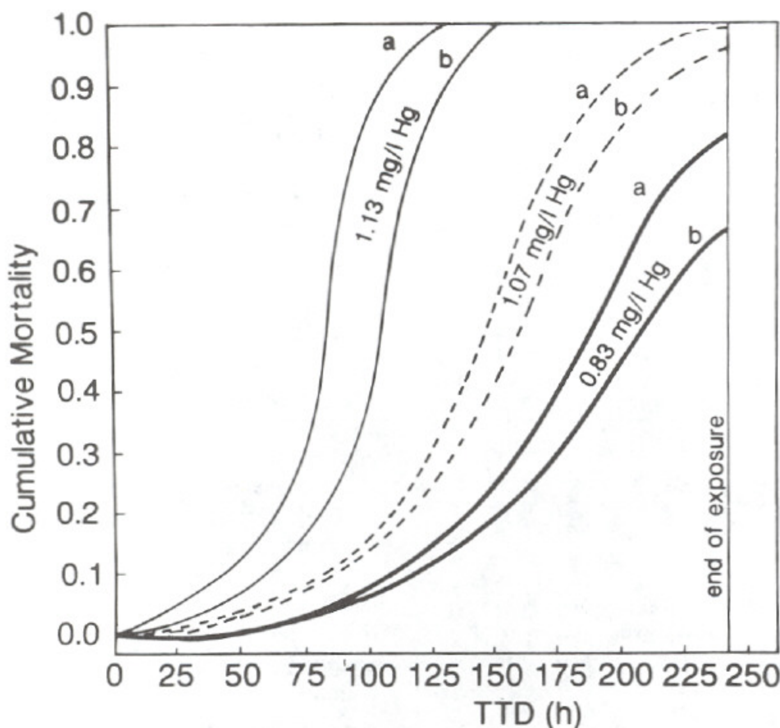

Fig. 2. Cumulative mortality during the 240-h exposure period. Replicate tanks for each concentration are designated **a** and **b**. The cumulative mortality curve for the fish exposed to 0.99 mg/L mercury in the previous experiment [6] falls between the low- and medium-concentration cumulative mortality curves of this experiment.

Field verification

Mercury analysis. Mercury concentrations of the fish from the canal were greater than those from the river. Mean (SD) mercury concentrations ranged from 0.651 (0.290) to 0.934 (0.132) μg/g wet weight in the canal and 0.054 (0.040) to 0.462 (0.088) μg/g wet weight in the river. The site immediately downstream of the canal had a slightly elevated mercury concentration (0.462 μg/g), as well as one of the upstream sites (SU2, 0.291 μg/g). This is not unexpected as the city of Augusta, Georgia, and a sewage treatment plant that discharges its treated effluent 2 km above the first upstream sample site (SU2) are located on the west bank of the river. The mercury-contaminated bass tissue had a mean (SD) mercury concentration of 0.583 (0.024) μg/g wet weight, and the spiked blanks had a mean (SD) mercury concentration of 4.7 (0.50) μg/L (94% recovery). The mean dry-to-wet-weight ratio was 0.264 (SD = 0.031, $n = 3$).

Genetics. Allele frequencies and the genotype distributions for the 11 electrophoretic loci at the eight sampling locations were consistent with Hardy–Weinberg expectations. Deviations from Hardy–Weinberg expectations were observed for *α-Gpd* at sites C2 ($p = 0.01$) and SU2 ($p < 0.001$); however, two deviations were not more than expected by chance in 88 chi-square tests. In addition, the allele frequencies were similar to allele frequencies previously seen in this part of the Savannah River by Wooten et al. (unpublished data). The percentage of polymorphic loci (P) and the mean number of alleles (A) per locus did not differ significantly between the canal ($P = 100.0$; $A = 2.4$) and river fish ($P = 100.0$; $A = 2.4$). Further, mean heterozygosity (H) did not differ significantly between the river ($H = 0.169 \pm 0.052$) and canal (0.165 ± 0.056) samples (Table 3).

One-way ANOVA model. The means of the mercury concentration, weight, and standard length as well as the observations of sex, GPI-2 allele frequencies, and heterozygosity (Table 3) from each sample location were compared using the one-way ANOVA model. The model was used to compare fish from the canal to fish from the river.

Table 2. Summary of Cox proportional hazards analysis: pooled data

Variable	Label	$d.f.$	β (SE)	χ^2	$p > \chi^2$
Size		1	−3.438 (0.581)	35.07	<0.001
Sex		1		8.69	0.003
	Female	1	−0.447 (0.152)	8.69	0.003
	Male	0	0 (0)		
Size·sex		1		5.12	0.024
	Female	1	1.390 (0.614)	5.12	0.024
	Male	0	0 (0)		
ICD-1	Overall	4		4.44	0.350
	134/134	1	−0.064 (0.413)	0.02	0.877
	134/116	1	0.388 (0.383)	1.02	0.311
	134/100	1	−0.011 (0.078)	0.02	0.889
	116/100	1	−0.287 (0.157)	3.35	0.067
	100/100	0	0 (0)		
MDH-1	Overall	2		2.90	0.234
	118/118	1	0.064 (0.092)	0.49	0.485
	118/100	1	−0.070 (0.053)	1.76	0.184
	100/100	0	0 (0)		
GPI-2	Overall	5		13.92	0.016
	100/100	1	−0.331 (0.205)	2.62	0.106
	100/66	1	−0.203 (0.203)	1.00	0.317
	100/38	1	−0.120 (0.209)	0.33	0.565
	66/66	1	−0.169 (0.210)	0.65	0.421
	66/38	1	−0.026 (0.216)	0.01	0.906
	38/38	0	0 (0)		

Reference genotypes or sex are denoted by $d.f. = 0$. The individual genotypes are compared to the reference genotype with χ^2 tests. Greater β values indicate increased sensitivity to mercury poisoning. ICD-1, MDH-1, and GPI-2 were significantly correlated with time to death (TTD) in the previous mercury study [6]. The other six loci were not significantly correlated with TTD in this study.

Table 3. Summary of statistical analysis—one-way ANOVA comparing mean values of the canal fish to the mean values of the river fish

Variable	Canal	River	p
Hg (μg/L)	0.786	0.226	0.002
Wt−female	0.168	0.256	0.010
Wt−male	0.142	0.213	0.045
SL−female	19.52	22.69	0.012
SL−male	19.30	20.31	0.069
Sex ratio (M:F)	1:3.7	1:1.6	
Ln ratio	−1.308	−0.470	0.047
Allele frequency *Gpi-2*^{38} (%)	1.3	4.1	0.024
Mean heterozygosity	0.165	0.169	0.943

Wt refers to the mean wet weight (g), SL refers to mean standard length (mm), and p indicates the probability associated with the F statistic from the ANOVA.

Mercury concentrations in the fish were significantly different between the canal and river ($F = 25.59$, $p = 0.002$, $d.f. = 7$; Table 3). The mercury concentrations of the fish in the canal were approximately four times greater than those of the river fish.

A significant difference in weight for female fish was found between the canal and the river ($F = 13.90$, $p = 0.010$). In addition, the standard length of the female fish differed significantly between the canal and river sites ($F = 12.54$, $p = 0.012$). The female fish tended to be heavier and longer at the sites in the river. For male fish, weight was significantly different between the canal and river sites ($F = 6.37$, $p = 0.045$), with the fish being heavier in the river sites. Length was not significantly different for male fish in the canal and river.

The sex ratio was significantly different between the canal and the river ($F = 6.19$, $p = 0.047$); the ratio of males to females was 1:3.7 in the canal populations and 1:1.6 in the river populations. This bias toward females was consistent with the report of Snelson [19] that the sex ratio in mosquitofish populations is often female biased.

No fish with the *Gpi-2*^{38}/*Gpi-2*^{38} homozygous genotype were found in the river or the canal, but heterozygotes containing the *Gpi-2*^{38} allele were found in both locations. The *Gpi-2*^{38} allele frequency was significantly greater in the river (4%) than in the canal (1%) ($F = 9.05$, $p = 0.024$). Allele frequencies of other loci did not differ significantly between the canal and the river ($p \geq 0.086$).

The one-way ANOVA model was also used to compare the fish collected from the canal to the fish collected from the west bank (sites SU2 and SD1, with slightly elevated mercury concentrations) and the east bank of the river (sites SU1 and SD2). The results were similar to those generated by the previous model. The fish from the canal differed from the fish collected from the east and west banks ($\alpha = 0.025$, $p < 0.005$). However, the fish from the east riverbank were not significantly different from the fish from the west riverbank ($\alpha = 0.025$, $p = 0.0274$).

DISCUSSION

This study adds to our understanding of differential tolerance and its relationship to enzyme genotype by attempting to define its limitations. By comparing the differences and similarities between the genotype effects seen in this study and the previous mercury study [6], some insight is provided into the temporal stability and the effect of concentration on genotype responses to toxicant stress. In

addition, the field survey begins to determine if the results of the laboratory exposures accurately predict field situations and if allozymes are useful as indicators of population-level responses to pollutant stress.

The sex and size of a fish can influence TTD during toxicity studies [20] and were significantly correlated with mosquitofish TTD in the previous mercury study [6], in the arsenate study [7], and in the present study. Size had a significant effect on TTD, regardless of mercury concentration, with the smaller fish dying before the larger fish. Sex and the size · sex interaction had significant effects on TTD at the low and high mercury concentrations and in the pooled data set. However, for reasons that are not apparent, sex and the size · sex interaction did not significantly affect TTD in the medium mercury concentration. In addition, Diamond et al. [21] found that size and sex had a significant effect on TTD in an acute exposure at a higher mercury concentration (1.16 mg/L Hg). Thus, populations of mosquitofish experiencing mercury contamination might be expected to be female biased and composed of larger and older fish than populations from noncontaminated environments.

The *Gpi-2*^{38}/*Gpi-2*^{38} genotype was significantly correlated with TTD at the low mercury concentration (0.83 mg/L) and at a concentration of 0.99 mg/L in the previous study [6]. However, the *Gpi-2*^{38}/*Gpi-2*^{38} was not significantly correlated with TTD in the medium mercury concentration (1.07 mg/L); the high mercury concentration (1.13 mg/L); or, in a previous study, a higher mercury concentration (1.20 mg/L) [21]. The lack of a genotype correlation at the higher concentrations suggests that the genotype responses may be concentration dependent and that genotype effects may be overwhelmed and obscured at high concentrations. Therefore, the use of electrophoretic data as indicators of pollutant stress may be of limited value when the pollutant stress is extreme or highly variable. The tested concentration range was very narrow and weighted toward the higher end of the tolerance range, thus the effects seen at higher concentrations might not accurately have reflected effects seen at lower levels of exposure. Therefore, we caution against extrapolation of these acute results to effects seen at lower concentrations, as additional testing is necessary to determine the effect of concentration on the genotype correlation with TTD at more chronic levels of exposure.

The scored genotype distributions of this study were similar to the distributions found in the previous studies [6,7]. However, genotype effects at two of the loci (ICD-1 and MDH-2) found to be significant in the previous mercury study were not detected again, and no additional loci were significantly correlated with TTD, which suggest that genotype effects at these loci are not temporally stable. The results seen for MDH-1 and ICD-1 in the previous mercury study could have been temporary correlations due to structuring within the population that has changed in the intervening years. This conclusion is supported by the work of Lee et al. [9], which demonstrated that mosquitofish from the same brood had more similar TTDs than fish from different broods. Mosquitofish from a single brood share a common genetic and environmental background; therefore, correlations between differential tolerance (TTD) and allozyme genotype are not unexpected. These correlations are not stable but become mixed as fish breed and different lineages gain dominance. Further, the specificity of the FH correlation with TTD during arsenate exposure reported by Newman et al. [7] could have been a response specific to arsenate, or it could have been a temporary correlation with population structure. Repetition of the arsenate experiment would be necessary to determine the true nature of this correlation. Clearly, an understanding of the temporal consistency of genotype responses to toxicant stress is necessary before allozymes are used as indicators of pollutant stress.

The GPI-2 locus was found to be significantly related to TTD in the low mercury concentration of this experiment and in the previous mercury experiment [6], with the *Gpi-2*^{38}/*Gpi-2*^{38} genotype being the most sensitive. Also, when the data from all the exposure concentrations were pooled, the *Gpi-2*^{38}/*Gpi-2*^{38} genotype was more sensitive to mercury poisoning than the *Gpi-2*^{100}/*Gpi-2*^{100} genotype. The relationships of the GPI-2 genotypes on TTD are consistent with the hypothesis that the genotype effect for this locus is temporally stable and probably not associated with patterns in population structure. The consistency of this response implies either that the *Gpi-2*^{38}/*Gpi-2*^{38} genotype itself may be more sensitive than the other genotypes or that alleles at a closely linked locus are associated with increased sensitivity. The *Gpi-2*^{38}/*Gpi-2*^{38} genotype was also significantly related to TTD in the arsenate exposure study [7], and Chagnon and Guttman [5] have correlated GPI-2 genotype with differential survival of *G. holbrooki* during acute exposures to copper and cadmium. The *GPI*-2 response is a nonspecific effect, as it has been reported in studies involving at least four metals. The nonspecificity of GPI-2 to metal toxicity could limit the

use of this allozyme as an indicator for population-level responses to specific pollutants; yet, it may be useful as a general indicator of pollutant stress.

By examining mercury-contaminated field populations of mosquitofish and determining if laboratory results accurately predict field situations, we can begin to assess better the use of demographic characters and allozyme genotypes as potential indicators of pollutant stress. For example, size and sex effects were seen in previous studies of mosquitofish and mercury toxicity [6,21], with the smaller fish and the male fish consistently having shorter TTDs than larger fish and female fish. In the field survey, weight and standard length of mosquitofish differed significantly between the canal and river, but not as predicted by the laboratory mercury exposures; both males and females were heavier and longer in the river. Although these results appear inconsistent with previous predictions, our predictions were based on acute laboratory toxicity tests using fish from an uncontaminated source. In contrast, fish taken from the field might have experienced mercury stress for their entire lives, which might lead to a smaller size at maturity. Moreover, local habitat effects, independent of mercury exposure, might have limited growth in the canal. We cannot exclude these possibilities, and long-term toxicity studies would be needed to assess these size differences better.

The sex ratio differed between the canal and the river, with a greater proportion of females than males in the canal. This result was consistent with the prediction of the laboratory mercury toxicity experiments [6,7] that female fish have a greater survivorship during acute mercury exposure. Feder et al. [22] also reported an increase in the proportion of female fish for *Gambusia* collected along a thermal gradient.

A number of studies have reported a relationship between electrophoretically determined heterozygosity and performance in variable environmental conditions [4,22,23]. It has been suggested that individuals that are more heterozygous can tolerate a wider range of unfavorable environmental conditions than more homozygous individuals [24]. Diamond et al. [6] found that multiple-locus heterozygosity had a significant effect on TTD during mercury exposure, with more heterozygous individuals exhibiting greater TTD. However, Newman et al. [7] found no significant relationship between interlocus heterozygosity and survival during arsenate exposure for female fish but noted an inverse relationship for male fish. This finding and reanalysis of the data from the Diamond et al. [6] study led Newman et al. [7] to conclude that the effect of multiple-locus heterozygosity on mosquitofish tolerance to mercury or arsenate reflected the summation of single locus effects. The present study is consistent with Newman et al. [7], as there was no difference in mean heterozygosity of fish from the canal or the river ($F = 0.01$, $p = 0.943$), nor were there differences in mean heterozygosity for adults and juveniles at either the river or the canal sites ($\chi^2 = 3.71$, $p = 0.447$).

The *Gpi-2*^{38} allele frequency differed between canal and river fish, with the *Gpi-2*^{38} allele being at a lower frequency in the canal. Although the frequency of the *Gpi-2*^{38} allele was 13% in the laboratory studies, it was <5% in the present study, and individuals that are homozygous for this allele are expected to be rare in the canal and river. The laboratory study [6] and the present study suggest that a loss of the *Gpi-2*^{38}/*Gpi-2*^{38} genotype and, therefore, a decrease in the *Gpi-2*^{38} allele would be expected during mercury exposure. The results of the field survey were consistent with these predictions. It is tempting to speculate that mercury concentration could be associated with the decline in the *Gpi-2*^{38} allele frequency from the river to the canal. However, other factors that were not measured, such as temperature [23], presence of another toxicant [7], reproductive isolation [25], or random loss of rare alleles, could lead to a similar pattern of allele frequencies. No differences in allele frequency were detected for the other 10 loci; thus, random loss would seem a less likely explanation. Moreover, no significant variation over time was found for GPI-2 frequencies in mosquitofish sampled from a nearby pond over an eight-week period (J. Novak, personal communication), and McClenaghan et al. [17] found that allelic frequencies are relatively stable over time for *G. holbrooki* inhabiting the Savannah River drainage. Therefore, the difference in *Gpi-2* allele frequency is consistent with a response to toxicant stress.

Allozymes and demographic characters (sex) may be useful as indicators for populations inhabiting polluted environments. However, it is clear that the approach is not simple and will be most profitably applied with a thorough understanding of the history and dynamics of the impacted population (i.e., samples taken before and after a pollution event or upstream and downstream of a point source). Especially when these methods are used in conjunction with other methods of assessing pollutant impacts, they can provide valuable data on population-level responses to stress not otherwise available.

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