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Genetic responses of *Isonychia bicolor* (Ephemeroptera:Isonychiidae) to chronic mercury pollution

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Abstract. The relationship between allozyme genotype and survival of nymphs of the mayfly *Isonychia bicolor* (Walker) when exposed to acute mercury concentrations was tested in laboratory experiments. The probability of survival and individual times-to-death were found to be significantly different among genotypes at the glucose phosphate isomerase locus, but not at the phosphoglucomutase locus. This pattern was consistent for both summer and winter temperatures and for populations from 2 separate, unpolluted streams. Subsequent field surveys were conducted to determine if results of these acute laboratory tests could be extrapolated to the more chronic, sublethal conditions present in the South River, Virginia. Genotypes identified as sensitive and tolerant in the laboratory experiments showed no consistent relationship with environmental mercury levels in the South River. In fact, one heterozygote identified as tolerant was found to be more frequent at the reference site than at contaminated sites, and no significant between-site differences were observed in the frequencies of the most sensitive genotype. Consequently, despite fitness differences to mercury exposure among allozyme variants, we were unable to attribute any between-site differences in genetic structure in *I. bicolor* populations to adaptation to mercury pollution.

Key words: mercury, genetic responses, *Isonychia bicolor*, allozymes, bioindicators, adaptation.

Proven methods designed to measure effects of acute pollution have been developed for all levels of biological organization and include indices based on individuals, population, and communities (Kelly and Harwell 1989). However, identifying biological metrics or bioindicators sensitive to more chronic exposure to sublethal concentrations of pollution have proved more difficult for 2 reasons. First, virtually all aquatic species have patchy distributions in nature. As a result, efforts to measure subtle between-site differences associated with exposures to low levels of toxicant using traditional measures, such as abundance, are often unsuccessful due to high sample variances within sites (Allan 1984). Second, most population and community metrics are often unable to detect residual or long-term effects of chronic exposures during times when pollution concentrations are particularly low (e.g., rainy seasons), or during life cycle stages that are more resistant (Luoma and Carter 1991).

These problems have led scientists to search for more effective methods to measure the effect of sublethal exposure to pollution that are less sensitive to sampling design. Genetic markers may be a particularly promising alternative. Hughes (1990) listed several possible advantages of population monitoring using electrophoretically determined genetic data including: detection of sublethal, as well as lethal, effects; detection of effects even after pollution has stopped; and ease and rapidity of the technique. In addition, there is evidence that, for at least some stressors such as metals, changes in allozyme frequencies may be stress-specific. In contrast, changes in most measures of physiology, and population- and community-based parameters represent generalized stress responses, which are difficult to relate to a specific environmental stressor (Bayne and Worral 1980).

Although the significance of allozyme variation continues to be debated, there is considerable evidence that at least some proportion of the allozyme variation observed in natural populations is adaptively significant. Much of the evidence rests in the preponderance of genotype–environment interactions reported. For example, allozyme variation has been correlated with gradients in salt ions (Burton and Feldman 1983, Hilbish and Koehn 1985), temperature (Mitton and Koehn 1975, Watt 1983), and moisture (Parsons 1980, Nevo and Yang 1982). In addition, some efforts have been made to eliminate

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nonselective causes such as genetic drift or gene flow as explanations for significant genotype–environment interactions by establishing direct evidence of molecular differences among allozymes (e.g., Gibson 1970, Koehn et al. 1971, Thorig et al. 1975, De Jong and Scharloo 1976, Johnson 1976, Hickey 1977). These studies provide biochemical evidence for fitness differences among allozymes, as well as strong correlations between environmental variables and allozyme variation.

Allozymes have been used to examine adaptive responses of populations to pollution gradients as well. In particular, allozymes have been successfully used to measure effects of metal pollution on several species of marine invertebrates in the Mediterranean Sea (Nevo et al. 1981, Lavie and Nevo 1982, 1987). In the last decade, the use of allozyme markers has been extended to freshwater systems (Chagnon and Guttman 1989, Gillespie and Guttman 1989, Newman et al. 1989, Benton and Guttman 1990). However, only a few studies have focused on freshwater invertebrates (Benton and Guttman 1990, 1992a, 1992b), and the efficacy of using freshwater organisms has not been fully evaluated.

In a previous study of energetics in the South River, Virginia, we found nymphs of the mayfly *Isonychia bicolor* collected from sites contaminated by mercury were more tolerant of acute exposure to mercury than individuals collected from reference sites (Snyder 1992). In addition, there was evidence that the observed tolerance was genetically based. In this paper we evaluate the potential use of allozyme variants to measure the genetic response of natural populations of *I. bicolor* to sublethal mercury exposure. The specific objectives of the study were 1) to identify allozyme variants that are sensitive or tolerant to mercury, and 2) to measure and compare the frequency of these genotypes among differentially polluted sites. The null hypotheses tested were: 1) there are no differences in percent survival and time-to-death among allozyme genotypes exposed to acute mercury concentrations, and 2) there are no differences in frequency distributions among populations at differentially polluted sites.

Methods

Study sites

Nymphs for laboratory fitness experiments were collected from sites on 2 streams in Virginia. Sinking Creek (Station 1), is a 4th-order tributary of the New River; and Mill Creek (Station 2) is a 2nd-order tributary of the North Fork of the Roanoke River (Fig. 1). Both of these streams have no history of metal pollution and contain large populations of *Isonychia bicolor*.

Field validation surveys were conducted on the South River, a 4th-order tributary of the South Fork of the Shenandoah River in northern Virginia (Fig. 1). Between 1929 and 1950, mercuric sulphate was used as a catalyst in the production of acetate fiber by a factory in Waynesboro. During that period an undetermined amount of mercury was accidentaly released into the South River. Plant officials discovered the leak in 1977 and subsequent surveys by the Virginia State Water Control Board revealed levels of mercury in sediments and fish tissues that were higher than US Environmental Protection Agency action levels. A ban on the consumption of fish caught anywhere on the South River was put in place by the Virginia Department of Health. However, the ban has since been lifted and replaced with a health advisory. More detailed descriptions of the physical and chemical characteristics of the South River can be found in Snyder et al. (1991) and Snyder and Hendricks (1995).

Four sampling stations were selected on the South River (Fig. 1) that represented a gradient in environmental mercury levels in fish and sediments (Lawler, Matusky, and Skelly Engineers 1989). Station 3, the reference site, was about 1.6 river km upstream of Waynesboro at Oak Hill. Station 4 was 16.1 river km downstream of Waynesboro, near Chrimora; and Station 5 was 21.0 river km downstream of Waynesboro, near Grottoes. Environmental mercury levels at stations 4 and 5 were high. Station 6 was about 25 river km downstream of Waynesboro on the South Fork of the Shenandoah River near Lynwood, below the confluence of the South River and the Middle River. At this station, mercury levels in fish and sediments were detectable but much lower than at stations 4 and 5 as a result of dilution from the Middle River (Lawler, Matusky, and Skelly Engineers 1989).

Study organism and preliminary genetic screening

Aquatic insects have relatively short life cycles, high average densities, and high reproductive outputs. Consequently, the potential for

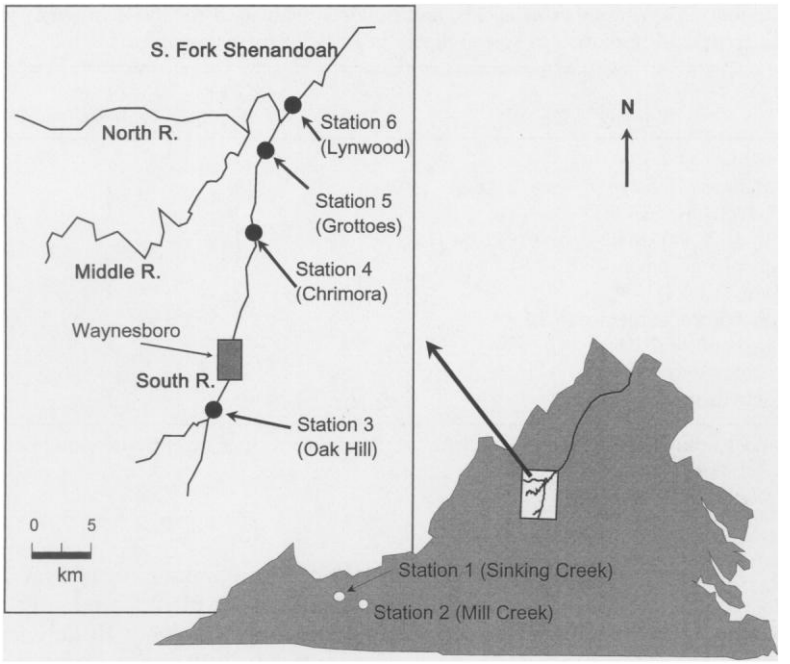

FIG. 1. Map of study sites in Virginia, with an enlargement of the locations of the field validation sites.

rapid adaptive responses to natural or man-made stresses is great. *Isonychia bicolor* (Ephemeroptera:Isonychiidae) was specifically chosen for this study because 1) the genus is widely distributed throughout the United States (Unzicker and Carlson 1982), 2) it is relatively abundant at both reference and contaminated sites on the South River (Hendricks et al. 1995), and 3) it has been shown to be highly sensitive to heavy metals (Clements 1991). In addition, the biology of this species in the South River is well known (Hendricks et al. 1989). Populations in the South River are bivoltine, with a short summer generation which hatches in the spring, grows and develops rapidly through the summer, and emerges in late summer and early fall. The winter generation hatches in late fall/early winter, and emerges in early spring. Growth and development of the winter generation are rapid initially, but slow as temperatures decline. Development appears to cease altogether during the coldest portion of the winter (Hendricks et al. 1989). Nymphs pass winter in a wide range of moderately sized instars.

Preliminary electrophoretic surveys were conducted between October 1989 and June 1990. Nymphs for the survey were collected from 4 sites on 3 streams: 1 site each on Sinking Creek (Station 1) and Mill Creek (Station 2) and (stations 3 and 5). A total of 375 individuals were collected with a D-frame kick net over the 9-mo period. Nymphs were frozen on dry ice in the field, taken to the lab, and stored at −80°C. Allozymes were separated by horizontal starch gel electrophoresis using methods described by Selander et. al. (1971) with modifications outlined in Sweeney et al. (1987). We initially chose allozyme systems important in the Krebs cycle, because environmental stress is generally associated with measurable changes in an individual's energetics (Koehn and Bayne 1989). Secondly, we focused on those systems requiring divalent metals as coenzymes, because metal pollutants have been shown to compete with these coenzymes for active sites on substrate molecules (Luckey et al. 1974, Pellerin-Massicotte et al. 1989). Based on these criteria, 29 enzyme systems were screened. Of these, only 10 were consistently scorable, yielding data on 12 presumptive loci (Table 1). Other loci could not be reliably scored in nymphs because of poor resolution. Three loci were polymorphic: glucose phosphate isomerase (*Gpi*), mannose phosphate isomerase (*Mpi*), and phosphoglucomutase

TABLE 1. Number of loci, number of alleles, and buffer system used for those enzyme systems found to have sufficient activity and resolution to score reliably in *Isonychia bicolor* nymphs.

Enzyme code	No. of loci	No. of alleles	Buffer system[a]
Aldehyde oxidase (1.2.3.1)	2	1,1	P-7[b]
Acid phosphatase (3.1.3.2)	1	1	TC 6.4[c]
Alkaline phosphatase (3.1.3.1)	1	1	TBE 8.6[c]
Glyceraldehyde-3-phosphate-dehydrogenase (1.2.1.12)	1	1	LiOH 8.3[c]
Glucose-phosphate isomerase (5.3.1.9)	1	3	LiOH 8.3
Malic enzyme (1.1.1.41)	1	1	P-7
Mannose-phosphate isomerase (5.3.1.8)	1	2	P-7
Phosphoglucomutase (2.7.5.1)	1	2	P-7
Trehalase (unknown)	2	1,1	P-7
Xanthene dehydrogenase (1.2.3.2)	1	1	TBE 8.6

[a] See Harris and Hopkinson (1976) and Pasteur et al. (1988) for recipes except where otherwise noted
[b] See Sweeney et al. (1987) for recipe
[c] See Pasteur et al. (1988) for recipe

(*Pgm*)(Table 1). However, at the *Mpi* locus allozyme variants were rare (<2%). Therefore, the remaining work focused on *Gpi* and *Pgm*.

Laboratory experiments

Differential survivorship of genotypes was determined by 2 acute bioassays coupled with electrophoretic analyses. The 2 lab experiments differed in duration of exposure, temperature, feeding regime, and analytical methods as outlined below. The design of the 2 experiments contrasted responses of individuals from 2 different populations that were exposed to mercury at 2 different water temperatures. However, the primary motivation for the 2nd experiment was to make certain that factors such as lack of food did not influence or otherwise confound the results of the 1st experiment.

In both experiments, large (1.5 L) crystallizing dishes were used as test containers for acute static bioassays. Flow was maintained in each container using magnetic stirrers. A fiberglass screen was placed over the stir bar to prevent injury to nymphs. Mercuric nitrate was used as the test toxicant, and water collected from reference sites was used as the dilutant. All containers were well aerated with air stones and held at constant temperature in an environmental chamber. Two separate bioassays were conducted using a modified version of methods described by Lavie and Nevo (1982).

Laboratory experiment I.—In the 1st test, nymphs (N = 80) and stream water were collected from Sinking Creek on 21 July 1990. Nymphs were placed randomly into test containers (approximately 10 individuals/container) with 1 L of filtered stream water and allowed to acclimate for 24 h prior to the introduction of mercury. Mercuric nitrate was added to all but 1 container (control) to obtain a nominal concentration of 0.2 mg mercury/L in each dish. A concentration of 0.2 mg mercury/L was found to be acutely toxic (i.e., approximate LC_{50}) in preliminary trials. Acclimation and testing were done at 22°C. Containers were checked every 4 h and dead nymphs were removed and frozen at −80°C for later electrophoresis. When approximately 50% of the nymphs had died, after 76 h, the experiment was terminated and survivors were frozen separately. All nymphs were later electrophoresed as described above and the *Gpi* and *Pgm* genotypes of each individual were determined.

If the probability of surviving acute mercury exposure is independent of genotype, then the frequency of each allele and genotype should be equally represented in the surviving and dead fractions. This null hypothesis was tested for each allele and genotype at both the *Gpi* and *Pgm* loci using the G-test of independence with the Yates's factor for small sample size (Sokal and Rohlf 1981).

Laboratory experiment II.—In the 2nd test, nymphs (N = 140) and stream water were collected from Mill Creek on 13 February 1991. Bioassay procedures were essentially the same as those described above except for 4 factors: 1)

duration of the test was considerably longer (159 h) to ensure higher mortality; 2) water was not filtered, thus allowing filter-feeding nymphs to feed on seston maintained in suspension by the magnetic stirrer and ensuring that mortality would not be attributed to starvation; 3) test solution was renewed every 48 h by replacing about 75% of the test solution with fresh test solution to ensure that most metabolic wastes were eliminated while mercury concentration and food remained stable; and 4) acclimation and testing were done at 12°C.

Containers were checked every 3 h until the end of the experiment. Dead nymphs were removed, placed into 1.5-mL Eppendorf tubes, and frozen for later electrophoresis. Body length was measured and the time-to-death was recorded for each individual (scored as the number of 3-h intervals survived in the presence of mercury). Nymphs were then electrophoresed as described above, and the *Gpi* genotype of each nymph was determined.

For this experiment, the effects of genotype and body size on time-to-death were tested using the SAS LIFEREG procedure (SAS Institute, Cary, North Carolina), which fits parametric models to failure-time (i.e., time-to-death) and right-censored (i.e., time-to-death predicted for individuals that survived to the end of the experiment) data. The effect of each independent variable (body length and *Gpi* genotype in this analysis) is tested individually. Also, LIFEREG employs a log-likelihood ratio method which is robust for small sample sizes. Dixon and Newman (1991) gave a thorough treatment of time-to-death models and their use in toxicity testing.

Field validation surveys

To assess spatial and temporal patterns in allele and genotype frequencies, approximately 50 nymphs were collected approximately monthly from Station 3 (reference site) and Station 5 (contaminated site) on the South River. In October 1991, an additional 2 sites (stations 4 and 6) were sampled to relate spatial patterns to mercury levels in nymphal tissues. Once collected, nymphs were placed in 1.5-mL Eppendorf tubes and frozen on dry ice in the field. Nymphs were later electrophoresed and the *Gpi* genotype was determined for each nymph. A chi-square test was used to test genotype frequency of each population for conformity to Hardy-Weinberg distribution. The G-test of independence (Sokal and Rohlf 1981) was used to test for spatial and temporal differences in allele and genotype frequencies.

Results

Differential survival experiments

Laboratory experiment I.—Of 70 mayflies treated with mercury, 42 (60%) were dead at the end of 76 h. None of the individuals in the control group died during the experiment. We were able to score only 41 nymphs at the *Gpi* locus because of degraded chemicals or procedural error. We scored 3 alleles and 4 genotypes at the *Gpi* locus, and 2 alleles and 2 genotypes at the *Pgm* locus. Neither *Gpi* nor *Pgm* genotype frequencies were found to deviate from Hardy-Weinberg expectations (*Gpi*: $\chi^2 = 0.83$, *Pgm*: $\chi^2 = 0.46$, $p > 0.70$ for each).

Differences in sensitivity to acute mercury exposure were observed among alleles and genotypes at the *Gpi* locus (Table 2). A significantly higher proportion of Gpi^S alleles, and a significantly lower proportion of Gpi^M alleles were found in the survivor fraction than would be expected by chance. Similarly, a significantly higher proportion of individuals homozygous for the Gpi^M allele were found among the dead than would be expected by chance. No such differences were observed at the *Pgm* locus (Table 2).

Laboratory experiment II.—Of 125 mayflies treated with mercury, 104 (83%) had died by the end of 159 h. Two individuals from the control group died early in the experiment. These deaths are believed to be due to stress imposed during transport from the field. Because there were no significant differences in survival among *Pgm* genotypes in Experiment I, only *Gpi* genotypes were scored in this test. *Gpi* genotype frequencies conformed to Hardy-Weinberg expectations ($\chi^2 = 3.25$, $p > 0.40$).

No relationship between body length and time-to-death was observed ($\chi^2 = 0.47$, $p = 0.49$). However, a priori efforts were made to select similar-sized nymphs for the experiment, and lack of differences probably reflect a lack of sufficient size variation or, at least, reduced potential to detect differences due to size.

Time-to-death was found to differ significantly among *Gpi* genotypes ($\chi^2 = 17.43$, $p = 0.002$).

Median time-to-death was lowest in the Gpi^{FS} heterozygote (Fig. 2). However, this genotype was represented by only 1 individual in the Mill Creek population (i.e., 0.8%), and was never observed in the Sinking Creek or South River populations. Among the remaining 4 genotypes, median time-to-death ranged from 49 in the Gpi^{MM} homozygote to 83 in the Gpi^{MS} heterozygote (Fig. 2). The common Gpi^{MM} homozygote survived a significantly shorter period than the Gpi^{MS} and Gpi^{FM} heterozygotes. The other homozygote scored at this locus (Gpi^{SS}) was intermediate in terms of time-to-death. These findings agree with results of Experiment I conducted at summer temperatures using nymphs from another unpolluted stream.

Field validation surveys

Temporal patterns.—Genotype frequency distributions at *Gpi* did not deviate significantly from Hardy-Weinberg expectations on any of the sample dates. This was true for both Station 3 and Station 5 populations (χ^2, $p > 0.26$ for all sample dates). The genotype found to be most sensitive to mercury in the lab experiments, Gpi^{MM}, was the most frequent genotype at both stations throughout the sampling period (Fig. 3). The relatively mercury-tolerant genotype, Gpi^{MS}, was also common at both stations, while the other mercury-tolerant heterozyote, Gpi^{FM}, was rare at Station 3 and was never found at Station 5 (Fig. 3).

Temporal fluctuations in allele and genotype frequencies were observed, especially in the spring and fall. Specifically, in September 1990, both allele and genotype frequencies were significantly different from all other sample dates at Station 5 (Allele: $G_{adj} = 8.20$, $p = 0.02$; Genotype: $G_{adj} = 8.66$, $p = 0.03$). A similar, although not significant deviation was observed in May 1991 at both station 3 and 5, and in September 1990 at station 3. During these time intervals, the frequency of the mercury sensitive Gpi^{MM} genotype was reduced, while the frequency of the 2 more tolerant heterozyotes increased (Fig. 3). A similar pattern was observed in June 1990 but was not statistically significant at either site.

Spatial patterns.—Allele frequency distributions at the *Gpi* locus were not significantly different between sites on the South River on any sample date, although the results obtained in September 1990 are marginal ($G_{adj} = 5.76$, $p = 0.06$). Likewise, between-site differences in genotype frequency distributions were not significant except in June 1990 ($G_{adj} = 8.21$, $p = 0.04$).

Because the frequency of the Gpi^{M} allele was high at all sites, between-site differences in allele and/or genotype frequency distributions were largely determined by between-site differences in the frequency of this allele. As a result, potential between-site differences in the frequency of the 2 rarer alleles might be masked. To test for possible masking, allele and genotype frequencies were pooled over all sample dates within each site. Subsequently, between-site comparisons of the frequencies of each allele and genotype relative to the frequency of all other alleles and genotypes combined were made using the 2 × 2 contingency G-test of independence (Sokal and Rohlf 1981). Pooling of

TABLE 2. Results of Laboratory experiment I using nymphs collected from Sinking Creek, Virginia. The G-test of independence was used to compare allele/genotype frequencies between nymphs that survived and those that died. Frequency of each allele/genotype relative to all others combined was used to generate a 2 × 2 matrix with 1 df. Results for both the *Gpi* and *Pgm* enzyme loci are reported. N refers to the total number of alleles or genotypes observed at each locus. Note for *Gpi*, we were able to resolve the genotype for only a fraction (i.e., 41/80) of the individuals tested due to logistical problems (see text for details).

	Frequency				
	Alive	Dead	N	G_{adj}[a]	*p*-value
Gpi allele					
F	0.06	0.00	2	1.10	0.29
M	0.78	1.00	75	9.94	0.002
S	0.16	0.00	5	6.07	0.014
Gpi genotype					
FM	0.13	0.00	2	1.12	0.29
MM	0.62	1.00	35	8.64	0.003
MS	0.19	0.00	3	2.69	0.10
SS	0.06	0.00	1	0.05	0.82
Pgm allele					
F	0.07	0.08	11	0.004	0.96
S	0.93	0.92	129	0.004	0.96
Pgm genotype					
SF	0.14	0.17	11	0.004	0.96
SS	0.86	0.83	59	0.004	0.96

[a] Used Yates's correction factor for small sample size

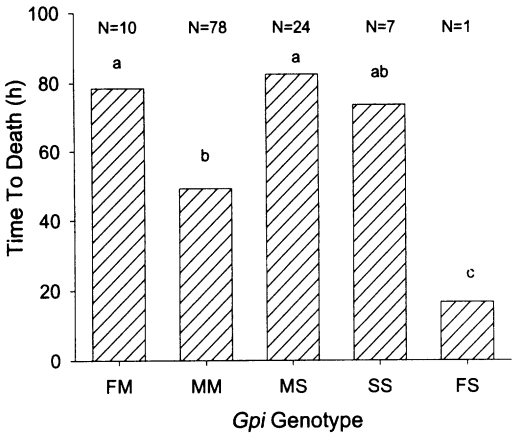

FIG. 2. Results of Laboratory experiment II showing median time-to-death of 5 *Gpi* genotypes of *I. bicolor* nymphs from Mill Creek, Virginia. Genotypes with the same letters at the top of the bar were not significantly different from each other. Number of individuals (N) of each genotype is also shown.

frequency data within each site was justified because no overall temporal differences in allele or genotype frequencies were observed at either site (Fisher's Combined Test, $p > 0.15$ for both stations). Furthermore, in September and May when there were substantial, if not significant, differences from the rest of the year, both sites showed the same pattern.

The frequency of the Gpi^S allele was found to be significantly higher at the contaminated site on the South River (Table 3). Conversely, the frequency of the Gpi^F allele was significantly higher at the reference site, Station 5. Frequency of the Gpi^M allele did not differ significantly between sites. Frequencies of the 2 heterozygotes genotypes (i.e., Gpi^{MS}, Gpi^{FM}) also were significantly different between sites and in opposite directions (Table 3). Conversely, no differences in the frequency of the 2 homozygotes were observed.

Spatial differences in allele and genotype frequencies were observed in the multi-site comparisons as well. However, there was no clear relationship with mercury concentrations in nymphal tissues. Both the Gpi^F allele and the Gpi^{FM} genotype were found only at the reference site (Table 4). In addition, the Gpi^S allele and the Gpi^{MS} genotype increased in frequency in a downstream direction. However, the frequency of both continued to increase even below the confluence with the Middle River (i.e., Station 6) where environmental mercury levels were low (Table 4).

Discussion

Laboratory experiments

Laboratory experiments demonstrated that the probability of survival and mean time-to-death in *Isonychia bicolor* nymphs exposed to acute concentrations of inorganic mercury were significantly different among genotypes at 1 of the 2 enzyme loci examined (Table 2, Fig. 2). Among the 4 genotypes scored at the *Gpi* locus, the lowest probability of survival and shortest mean time-to-death was associated with the most common homozygous genotype (Gpi^{MM}). Conversely, the Gpi^{MS} heterozygote was found to have the highest probability of survival and longest mean time-to-death. These results were consistent at both summer and winter temperatures and in populations from 2 unpolluted sites. Glucose-phosphate isomerase (GPI) has previously been associated with differential responses to acute heavy metal toxicity in fishes (Chagnon and Guttman 1989), marine gastropods (Lavie and Nevo 1982, 1987), and 1 other mayfly (Benton and Guttman 1990). GPI is an important glycolytic enzyme which requires a divalent metal coenzyme such as Mg^{++} or Mn^{++} to be effective. Competitive inhibition of coenzymes by heavy metal pollutants is thought to be a likely explanation for the observed sensitivity of this enzyme to metal pollution (Milstein 1961, Pellerin-Massicotte et al. 1989).

Our results have important implications for natural populations exposed to toxic pollutants. For example, the evolution of tolerance may make it difficult to identify changes in populations using traditional censusing techniques, because adapted populations may be able to maintain high reproductive outputs and mean individual growth rates despite exposure. However, this interpretation is not synonymous with "no effect". Indeed, genetic changes that result in increased tolerance to a specific stressor may be accompanied by increased sensitivity to other stressors (e.g., Ben-Shlomo and Nevo 1988). Furthermore, adaptation may result in a reduction in the amount of genetic variability in affected populations if resistant individuals represent a relatively small fraction of the population prior to exposure (Bradshaw and Hardwick 1989).

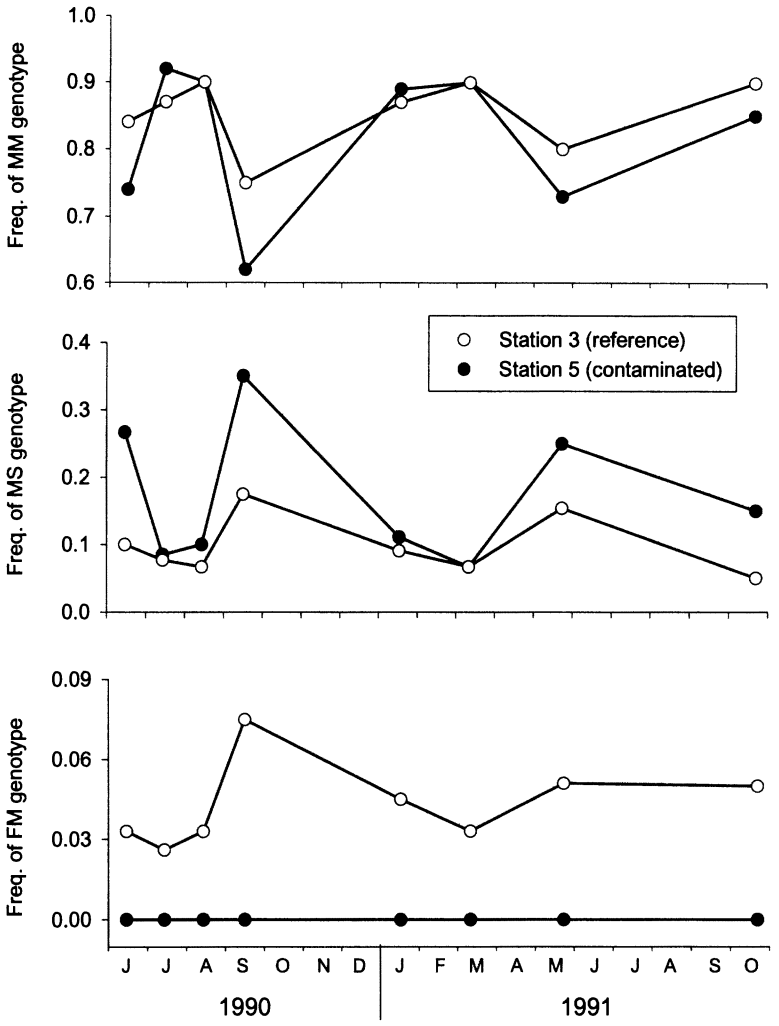

FIG. 3. Frequencies of 3 *Gpi* genotypes at Station 3 (reference) and Station 5 (contaminated) for 8 time intervals. The Gpi^{MM} genotype (top) was found to be most sensitive, and the 2 heterozygotes (Gpi^{MS} and Gpi^{FM}, middle and bottom respectively) were found to be most tolerant to mercury in 2 laboratory experiments.

Such losses may reduce the capacity of populations to change in response to other environmental changes. Thus, genetic monitoring of populations may provide information on the vulnerability of populations to future stresses.

Field validation

The relevance of the results of these acute, short-term exposures to effects during chronic, low-level exposures that are more common in nature is not known. Results of standard acute bioassays, which are used routinely in pollution studies, have been criticized (e.g., Kimball and Levin 1985) because they are often used to make predictions about population responses to chronic exposures without proper field validation. It does not necessarily follow that relative sensitivities to acute exposure may be extended to chronically polluted situations.

In this study, the genetic structure of populations at both reference and contaminated sites remained relatively stable throughout the year. However, significant temporal changes in allele and genotype frequencies at *Gpi* were observed in September 1990 at the contaminated site, and a similar, albeit not statistically significant, pattern was observed in May 1991 (Fig. 3). On

TABLE 3. Results of G-test of independence to test for differences in *Gpi* allele and genotype frequencies between Station 3 (reference) and Station 5 (contaminated) after data were pooled over sample dates (see text). Between-site differences in the frequency of each allele and genotype was compared relative to the frequencies of all others combined (i.e., 2 × 2 matrix). Degrees of freedom equals 1 for all comparisons.

	Station 3		Station 5			
	Frequency	N	Frequency	N	G_{adj}[a]	*p*-value
Gpi allele						
F	0.020	11	0.000	0	11.013	0.001
M	0.913	493	0.897	472	0.587	0.444
S	0.067	36	0.103	54	4.031	0.045
Gpi genotype						
FM	0.041	11	0.000	0	11.106	0.001
MM	0.833	225	0.806	212	0.498	0.481
MS	0.118	32	0.182	48	4.820	0.030
SS	0.008	2	0.011	3	0.884	0.352

[a] Used Yates's correction factor for small sample size

these dates, the frequency of the sensitive Gpi^{MM} genotype was lower and the frequency of heterozygotes higher relative to the rest of the year. These dates also corresponded with emergence/recruitment periods and so the population consisted mainly of small individuals at these times. Results of previous energetics experiments showed small nymphs were more sensitive to mercury (Snyder 1992), and therefore changes in genotype frequencies observed on these dates could reflect the outcome of natural selection for mercury-resistant individuals at their most vulnerable time. However, observed changes in genotype frequencies were short-lived, as frequencies quickly returned to rest-of-the-year levels the following months (Fig. 2). Consequently, if selection for mercury resistance were important during recruitment periods, then either individuals with resistant *Gpi* genotypes were less fit the remainder of the year, or immigration from upstream sites through larval drift may have returned the frequencies to "normal". As stated earlier, patterns of allele and genotype frequencies were similar at the reference site although frequency changes were not statistically significant (Fig. 3). Thus, although no significant temporal patterns were detected for either site overall, the fact that both sites showed parallel deviations during the fall and spring supports the conclusion that these deviations were unlikely the result of chance. However, it also suggests that other factors besides mercury, such as adult females from nearby streams and tributaries laying eggs in the South River, sexual selection of mates during reproduction, or differential survival of adults prior to mating, are primarily responsible for these temporal changes in allozyme frequencies. The larger, statistically significant, changes during those times at the contaminated sites could suggest that mercury may have enhanced those changes in *Gpi* genotype frequencies, but this is purely conjecture.

Between-site differences in *Gpi* allele and ge-

TABLE 4. Mercury concentrations in seston and *Isonychia bicolor* nymphs, and *Gpi* allele and genotype frequencies at 4 sites on the South River. Number of nymphs analyzed for Hg was 40 for each site.

SITE	[Hg] in seston (μg/g)	[Hg] in nymphs (μg/g)	Allele frequency			Genotype frequency			
			(F)	(M)	(S)	(FM)	(MM)	(MS)	(SS)
Station 3	<0.01	<0.01	0.025	0.950	0.025	0.050	0.900	0.050	0.000
Station 4	17.12	2.23	0.000	0.950	0.050	0.000	0.900	0.100	0.000
Station 5	15.22	1.27	0.000	0.925	0.075	0.000	0.850	0.150	0.000
Station 6	2.01	0.12	0.000	0.912	0.088	0.000	0.825	0.175	0.000

notype frequencies were also observed in *I. bicolor* nymphs on the South River. However, there was no clear evidence that differences were related to mercury. Based on the results of the laboratory experiments, a lower frequency of individuals homozygous for the Gpi^{M} allele and a higher frequency of both heterozygotes were expected at contaminated sites. However, the frequency of the sensitive (Gpi^{MM}) genotype was not significantly different between reference and contaminated populations (Table 3). And although the genotype identified as most tolerant in the laboratory experiments (Gpi^{MS}) was consistently more frequent at Station 5 (contaminated site) than Station 3 (reference site) (Fig. 3, Table 3), it was most frequent at Station 6 below the confluence of the Middle River, where mercury levels were low due to dilution (Table 4). Although the longitudinal gradient in the frequency of this genotype does imply some relationship between fitness and some environmental variable or complex, there seems to be no evidence that chronic mercury levels are important.

The other heterozygote at this locus (Gpi^{FM}) was also found to be relatively tolerant to acute mercury exposure. Despite this observed tolerance, the frequency of this genotype was significantly higher at Station 3 (reference site) (Table 3). In fact, this genotype was never observed at any of the contaminated sites (Table 4). These results also do not agree with predictions based on laboratory fitness experiments.

In contrast to our study, work with marine invertebrates has shown striking relationships between results of laboratory fitness experiments and genotype frequencies in metal-polluted environments (Nevo et al. 1984, Nevo et al. 1987). Possible explanations to account for this discrepancy are numerous. Wider variation in metal concentrations may have been present among field sites in these studies, some of which may have been closer to acute levels used in fitness experiments. The authors do not report metal levels in these studies and instead rely on gross comparisons between highly polluted and reference sites (e.g., Nevo et al. 1987).

An alternative explanation for the lack of agreement between laboratory experiments and patterns observed in the field, involves uptake mechanisms. In our study, we dosed the water used for laboratory fitness experiments. However, mercury levels in the South River are associated primarily with sediments and suspended organics (Hendricks et al. 1989, Lawler, Matusky, and Skelly Engineers 1989) and are virtually undetectable in the water. Furthermore, most of the whole-body metal concentrations in aquatic insects inhabiting metal-contaminated environments are associated with the gut and gut contents (Smock 1983, Snyder and Hendricks 1995), implying that uptake via food is most important. Therefore genetic responses observed in our fitness experiments may have been specific to water-contaminated conditions.

Finally, taxonomic considerations may have contributed to the lack of agreement between the laboratory experiments and field surveys. Specifically, we assumed that the various populations we sampled were panmictic. However, it may be that populations at Sinking Creek and Mill Creek, from which individuals were collected for the laboratory fitness experiments, were genetically distinct, though morphologically indistinguishable, from the South River population. Recent evidence suggests that the species *Isonychia bicolor* may actually be a complex, comprising as many as 8 distinct species (Kondratieff and Voshell 1984). Thus, in our efforts to ensure that the gene pool of nymphs used in lab experiments were naive to mercury pollution, we may have compromised the study by introducing taxonomic uncertainty. Jackson and Resh (1997) suggest that morphologically cryptic species are not uncommon among aquatic insects, and that the interpretation of unusual ecological variability may be confounded by the presence of such cryptic species.

In summary, we believe the use of allozyme markers to measure long-term effects of chronic exposure to metal pollution in aquatic populations is promising. Based on our findings and those of numerous other investigators (see above), it is clearly possible to identify genetic markers of metal pollution tolerance. However, for studies designed to detect changes in populations chronically exposed to sublethal concentrations of pollution, it is unlikely that monitoring of a single locus, even if fitness differences among allozyme variants are known, will be sufficient, because other environmental variables may counteract expected changes in allozyme frequencies due to pollution. Analysis of multi-locus complexes (i.e., comparing survival of individuals with various multiple-loci combinations) may be more effective, but the cost

and effort for such evaluations are higher (i.e., much larger sample sizes would be needed to reflect all the possible gene combinations). Furthermore, such analyses may be limited to the adult stages of the insect life cycle when, typically, more enzyme systems can be resolved (David Funk, Stroud Water Research Center, personal communication), thus reducing the possibility of assessing seasonal changes. This is particularly troubling given our finding that gene frequencies fluctuated temporally. That is, frequencies observed during the adult life cycle stage may not necessarily represent genetic structure at other times of the year or other life cycle stages. This could impose a significant compromise on studies designed to use genetic markers to detect impacts of sublethal stresses on natural populations, because one would have to assume such changes would be reflected in the adult stage.

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