J. N. Am. Benthol. Soc., 1990, 9(3):271-276

Relationship of allozyme genotype to survivorship of mayflies (*Stenonema femoratum*) exposed to copper

MICHAEL J. BENTON AND SHELDON I. GUTTMAN

Department of Zoology, Miami University, Oxford, Ohio 45056 USA

Abstract. The relationship of allozyme genotype to survivorship of *Stenonema femoratum* (Say) (Ephemeroptera:Heptageniidae) exposed to copper was tested. Mayflies were exposed to 1.6 mg/L copper sulfate for 126 hr, and individual times to death (TTD) were recorded. All individuals were then analyzed electrophoretically at three polymorphic loci: glucose phosphate isomerase (GPI); phosphoglucomutase (PGM); and malate dehydrogenase (MDH). TTD was significantly shorter in two GPI genotypes, although one of those genotypes was extremely rare. Reduction in genetic variability by differential elimination of sensitive genotypes may reduce the ability of impacted populations to recover from additional impacts or adapt to slowly changing environmental conditions. Periodic genetic sampling may be a sensitive method for monitoring the stability of natural populations.

Key words: allozyme, genotype, copper toxicity, differential survivorship, electrophoresis, genetic variation, *Stenonema*.

Certain electrophoretically defined genotypes and levels of heterozygosity have been associated with differential resistance to the effects of environmental contaminants, or have been found in higher frequencies than other genotypes in impacted areas. Ben-Shlomo and Nevo (1988) reported differential survivorship among phosphoglucomutase (PGM) genotypes in the marine shrimp, *Palaemon elegans*, specific for cadmium, mercury, and their interaction. Differential tolerance to copper (Lavie and Nevo 1982), zinc and cadmium (Lavie and Nevo 1986a, 1986b), and detergent/crude oil mixtures (Lavie et al. 1984) in marine snails has also been shown. Allelic frequencies of isozymes in the mummichog, *Fundulus heteroclitus*, and the largemouth bass, *Micropterus salmoides*, have been found to differ significantly between populations from artificially heated and unheated waters (Mitton and Koehn 1975, Smith et al. 1983). Gillespie and Guttman (1989) have shown a lower frequency of certain PGM genotypes in populations of the central stoneroller, *Campostoma anomalum*, collected from radionuclide-impacted sites than in populations upstream of the impact. In the laboratory, individual fish with these PGM genotypes and a particular malate dehydrogenase genotype were more sensitive to the toxic effects of copper sulfate than other genotypes. Diamond et al. (1989) reported that genotypes at three of eight loci, and heterozygosity at those eight loci, had a significant effect on time to death of mosquitofish, *Gambusia affinis*, exposed to acute mercury toxicity.

While fish and marine invertebrates have been commonly used in these field and laboratory studies, freshwater invertebrates have been largely ignored. Benthic invertebrates occupying lotic habitats are especially appropriate for this type of study given that their environment is so often the depository for agricultural and industrial waste. This study was designed to test the relative sensitivity to copper toxicity of allozyme variants at several electrophoretically detectable loci in the heptageniid mayfly *Stenonema femoratum*. This species was chosen because of its wide distribution, the known sensitivity of mayflies to heavy-metal contaminants, and the importance of mayflies in energy flow of aquatic systems.

Methods

Stenonema femoratum (Say) (Heptageniidae: Ephemeroptera) larvae were collected from the undersides of rocks in Indian Creek, a third-order stream in southwestern Ohio, and returned to the laboratory where they were placed in aerated reconstituted soft water (pH = 7.2-7.6, Hardness = 40-48 mg $CaCO_3$/L, Alkalinity = 30-35 mg $CaCO_3$/L) (USEPA. 1975). Mayflies were acclimated to laboratory conditions (20°C, 12:12 LD) for 48 hr, then placed into 2-L crystallizing dishes containing 1 L copper sulfate

solution. This solution was mixed, using reconstituted soft water as the diluent, at a concentration previously determined to produce approximately 80% mortality over 96 hr (1.6 mg Cu/L). Twenty replicates of 9 to 25 mayflies (n = 416) and four control treatments (n = 79) were used. The copper sulfate solution was replaced every 24 hr, and containers were checked for mortalities every 2–3 hr around the clock. Dead larvae were removed and frozen at −70°C, and day and time of death recorded. At the conclusion of the experiment (extended to 126 hr in order to obtain the desired level of mortality) survivors were also frozen. Prior to electrophoresis, the head width of each larva was measured. The pH, hardness, alkalinity, and copper concentration of the stock solution were tested prior to and after the 126-hr test period.

For electrophoresis, individual mayflies were manually ground in an equal volume of 0.25 M sucrose in 2% (V/V) 2-phenoxyethanol solution. Horizontal starch gel (13–15% Sigma starch, Sigma Chemical Co., St. Louis) electrophoretic techniques were employed for the resolution of genetic loci. Lithium hydroxide (Selander et al. 1971) gels were used to resolve glucose phosphate isomerase (GPI), and amine citrate (Clayton and Tretiak 1972) gels were used for the resolution of phosphoglucomutase (PGM) and malate dehydrogenase (MDH). These three enzyme systems were found to be polymorphic in a preliminary electrophoretic survey of *S. femoratum*. Banding patterns were interpreted by classifying the fastest migrating allozyme as "A", the next fastest as "B", and so on. No appreciable secondary banding as a result of the 2 to 3-hr delay in freezing of some dead individuals was observed. Preliminary experiments showed that GPI, PGM, and MDH from dead mayflies were still active and scorable up to 12 hr at 20°C.

Data were analyzed by first using BIOSYS-1 (Swofford and Selander 1981) to determine allelic frequencies and test genotype frequencies and heterozygosity for conformity to a Hardy-Weinberg distribution. The effects of genotype, heterozygosity level, and body size on time of death (TTD) were then tested using the SAS LIFEREG procedure (SAS Institute Inc. 1985), which fits parametric models to failure-time and right-censored (i.e., survivorship) data. This procedure first constructs a "reference organism", which is defined by assigning certain values to each independent variable (head width, heterozygosity level, and PGM, GPI, and MDH genotypes in this analysis). The effect of each independent variable on the dependent variable (TTD in this analysis) is then tested individually while the remaining independent variables are held constant. Thus, any reported significant genotypic effect is independent of other genotypes, heterozygosity level, or head width (see also Diamond et al. 1989). LIFEREG also employs a log-likelihood ratio method, which is robust for small sample sizes (SAS Institute Inc. 1985).

Results

A total of 366 treated mayflies (88%) were dead at the end of 126 hr. Only 6 control mayflies (7%) died. Control mortalities are believed due to handling as all but one death occurred within the first 48 hr.

Electrophoretic analysis revealed 9 alleles and 15 genotypes for GPI, 6 alleles and 12 genotypes for PGM, and 3 alleles and 3 genotypes for MDH (Table 1). Seven GPI, 6 PGM, and 1 MDH genotypes occurred in less than one percent of the sample and were considered rare (Table 1). The GPI-I allele was determined to be null as evidenced by heterozygotes with the GPI-D allele, which appeared as two bands of equal intensity. Normally, dimeric heterozygotes appear as three bands, with the middle band approximately twice as dark or as dense as the upper and lower bands.

GPI and PGM genotype frequencies deviated from a Hardy-Weinberg distribution (GPI: Chi-square = 540.9, PGM: Chi-square = 123.3; $p <$ 0.001 for each), apparently due to the absence of certain rare-allele recombinants. When homozygotes and heterozygotes were each pooled, they did not differ from expected frequencies with respect to number of homozygotes for the most common allele, ratio of common to rare homozygotes, or number of rare homozygotes and other heterozygotes (GPI: Chi-square = 0.534, $p > 0.40$; PGM: Chi-square = 2.33, $p > 0.10$). MDH genotype frequencies did conform to a Hardy-Weinberg distribution (Chi-square = 0.323, $p > 0.90$).

TTD was found to differ significantly among GPI genotypes (Tables 1 and 2). The EE genotype (n = 1) survived a significantly shorter period than did all other genotypes (Chi-square = 28.7–56.8, $p < 0.0001$ for all). The FF genotype

TABLE 1. *S. femoratum* allozyme genotype numbers and frequencies, and median times to death for GPI genotypes.

GPI	FREQ	*n*	TTD (hr)	PGM	FREQ	*n*	MDH	FREQ	*n*
AD	0.003	(1)	56.0	AD	0.023	(8)	AB	0.006	(2)
BB	0.045	(16)	46.3	BB	0.006	(2)	BB	0.886	(312)
BD	0.263	(93)	44.2	BC	0.014	(5)	BC	0.108	(38)
BE	0.006	(2)	43.7	BD	0.092	(32)			
BF	0.003	(1)	57.8	CC	0.009	(3)			
CC	0.006	(2)	62.6	CD	0.112	(39)			
CD	0.031	(11)	45.8	DD	0.706	(245)			
DD	0.499	(176)	45.7	DE	0.006	(2)			
DE	0.042	(15)	41.7	DF	0.020	(7)			
DF	0.059	(21)	46.1	EE	0.006	(2)			
DI*	0.003	(1)	46.2	FF	0.003	(1)			
EE	0.003	(1)	3.6	FG	0.003	(1)			
FF	0.014	(5)	33.5						
GG	0.020	(7)	56.6						
HH	0.003	(1)	61.7						

* I allele is null.

($n = 5$) survived a significantly shorter time than did the BB, DD, DF, and GG genotypes (Chi-square = 3.8–5.2, $p < 0.05$ for all) and significantly longer than the EE genotype (Chi-square = 35.6, $p < 0.0001$). TTD was also positively and significantly affected by head width (Chi-square = 14.95, $p < 0.001$). There were no significant differences in TTD among PGM or MDH genotypes, or among levels of heterozygosity (0–3 loci).

Because of the relative rarity of GPI-EE, FF, GG, and HH genotypes, and because the GPI-E, F, G, and H alleles do not commonly occur in the heterozygous condition (Table 1), the presence of a second species might be suspected. Should a second species indeed be present, it would be indicated by the association of these rare GPI genotypes with rare PGM and MDH homozygotes. However, an examination of these loci revealed that of the 14 individuals that represent the 4 rare GPI genotypes (1 GPI-EE, 5 GPI-FF, 7 GPI-GG, 1 GPI-HH), none were homozygous for unique PGM or MDH genotypes (Table 3). Thus, the available electrophoretic data support the morphological evidence that all individuals used in this experiment are of a single species.

Discussion

This study produced evidence for differential survivorship among allozyme genotypes of *S. femoratum*. Statistically significant differences in TTD were observed among GPI genotypes, although those involving one (GPI-EE) genotype may be spurious because it occurred in only a single mayfly. Allozyme genotypes have been previously correlated with response differences to various environmental factors, including pollution (Lavie et al. 1984, Lavie and Nevo 1986a, 1986b, Diamond et al. 1989, Chagnon and Guttman 1989a, Gillespie and Guttman 1989, Newman et al. 1989, Benton and Guttman, unpublished data). Field studies have also demonstrated that those resistant genotypes may occur in much higher frequencies in impacted habitats (Gillespie and Guttman 1989). This suggests that differential selection for resistant genotypes occurs in contaminated areas, reducing natural levels of genetic variability. Considering the ubiquity of anthropogenic pollutants in marine and freshwater systems, genetic variability in populations that occupy those habitats may be crucial to their long-term survival. Reduced variability may lessen the likelihood that populations can withstand additional episodes of natural or anthropogenic stress, or adapt to slowly changing conditions.

Mechanisms by which certain allozyme genotypes survive longer under polluted conditions are not yet fully understood. In the case of copper contamination, it has been shown that enzymes may be inactivated by the competitive inhibition of heavy-metal ions (Milstein 1961).

TABLE 2. Pairwise comparisons of differences in time to death among GPI genotypes. * = $p < 0.05$; ** = $p < 0.001$; NS = not significant.

—	AD	BB	BD	BE	BF	CC	CD	DD	DE	DF	DI	EE	FF	GG	HH
AD	—	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS
BB	NS	—	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	*	NS	NS
BD	NS	NS	—	NS	NS	NS	NS	NS	NS	NS	NS	**	*	NS	NS
BE	NS	NS	NS	—	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	NS
BF	NS	NS	NS	NS	—	NS	NS	NS	NS	NS	NS	**	NS	NS	NS
CC	NS	NS	NS	NS	NS	—	NS	NS	NS	NS	NS	**	NS	NS	NS
CD	NS	NS	NS	NS	NS	NS	—	NS	NS	NS	NS	**	NS	NS	NS
DD	NS	NS	NS	NS	NS	NS	NS	—	NS	NS	NS	**	*	NS	NS
DE	NS	NS	NS	NS	NS	NS	NS	NS	—	NS	NS	**	NS	NS	NS
DF	NS	NS	NS	NS	NS	NS	NS	NS	NS	—	NS	**	*	NS	NS
DI	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	—	**	NS	NS	NS
EE	**	**	**	**	**	**	**	**	**	**	**	—	**	**	**
FF	NS	*	*	NS	NS	NS	NS	*	NS	*	NS	**	—	NS	NS
GG	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	—	NS
HH	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS	—

Differential sensitivity may be a result of certain genetically determined enzyme configurations (allozymes) which are more or less susceptible to inhibition. Chagnon and Guttman (1989b), by adding copper sulfate to electrophoretic stain mixtures, found that two PGM phenotypes differed substantially in staining intensity, suggesting that these two isoalleles are differentially sensitive to copper. Electrophoretic staining of esterases was inhibited in caddisflies that were exposed to mercury, but not inhibited in untreated caddisflies unless mercury was added to the stain mixture (Benton and Guttman, unpublished data). These in vitro results suggest that there may also be in vivo interference of esterase activity by mercury. Diamond et al. (1989) proposed that electrophoretically defined sensitive or resistant loci may be genetically linked to other loci that produce the differential response.

We found no evidence for TTD differences among PGM or MDH genotypes, or among levels of heterozygosity. Because enzymes are known to differ in temperature optima, substrate specificity and sensitivity to inhibitors, differential sensitivity of PGM and MDH genotypes in *S. femoratum* may be observable only under different experimental conditions. For example, negative results for these loci may have been due, in part, to the limited size range of test animals. Head widths of 0.8–1.9 mm only were tested, which probably represented early-middle to ultimate instars, but differential susceptibility may be present or detectable only in earlier instars or in the egg stage of *S. femoratum*. Alternatively, differential response may be resolvable only at higher or lower copper concentrations. The experimental copper concentration, while chosen based on accepted bioassay guidelines and preliminary toxicological testing, may not necessarily be biologically relevant. If susceptible and resistant genotypes are present in *S. femoratum*, they may be physiologically distinct (and therefore experimentally detectable) only over a prolonged period at lower concentrations, or over brief periods under more highly toxic conditions. It also may be that differential susceptibility is not manifested as differential survivorship in *S. femoratum*, but rather as genotype-specific shifts in life-history or reproductive parameters. Or it may be that "pre-adapted" susceptible/resistant genotypes simply do not exist at these loci. Further studies using various contaminant types and contaminant levels, and many different taxa are needed to determine how individual genetic composition influences the susceptibility of benthic invertebrates, and how anthropogenic stressors affect benthic invertebrate population genetic structure.

We believe that studies like ours may lead to new methods of monitoring aquatic populations. Shifts in genotype and allele frequencies may precede more obvious phenotypic or nu-

TABLE 3. Numbers of GPI-PGM genotype pairs.

	PGM											
GPI	AD	BB	BC	BD	CC	CD	DD	DE	DF	EE	FF	FG
AD	0	0	0	0	0	0	1	0	0	0	0	0
BB	1	0	0	1	0	2	12	0	0	0	0	0
BD	1	2	1	10	0	10	65	0	3	0	1	0
BE	0	0	0	1	0	0	1	0	0	0	0	0
BF	0	0	0	0	0	0	1	0	0	0	0	0
CC	0	0	0	0	0	0	2	0	0	0	0	0
CD	0	0	1	1	2	0	6	0	0	0	0	0
DD	6	0	3	16	0	17	118	2	3	1	0	0
DE	0	0	0	1	0	2	10	0	0	0	0	0
DF	1	0	0	0	0	3	16	0	0	0	0	0
DI	0	0	0	0	0	0	1	0	0	0	0	0
EE*	0	0	0	0	0	0	0	0	0	0	0	0
FF	0	0	0	0	0	1	2	0	1	0	0	1
GG	0	0	0	0	1	0	6	0	0	0	0	0
HH	0	0	0	0	0	0	1	0	0	0	0	0

* Corresponding PGM genotype not resolvable.

merical changes, and may serve as warning signals of population instability. Early identification of low-level contamination is imperative for preventing genetic bottlenecks or the outright decimation of natural populations at risk.

Acknowledgements

The authors thank D. Dean and L. Holton for their assistance in the laboratory and in the field. We also thank J. Oris for his advice on toxicological methodology, and M. Newman for his assistance with statistical analysis. The advice and suggestions of E. Benfield, B. Sweeney, and two anonymous reviewers is greatly appreciated. This research was supported by Academic Challenge and Research Challenge grants from Miami University.

Literature Cited

BEN-SHLOMO, R., AND E. NEVO. 1988. Isozyme polymorphism as monitoring of marine environments: the interactive effects of cadmium and mercury pollution on the shrimp, *Palaemon elegans*. Marine Pollution Bulletin 19:314-317.

CHAGNON, N. L., AND S. I. GUTTMAN. 1989a. Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper or cadmium. Environmental Toxicology and Chemistry 8:319-326.

CHAGNON, N. L., AND S. I. GUTTMAN. 1989b. Biochemical analysis of allozyme copper and cadmium tolerance in fish using starch gel electrophoresis. Environmental Toxicology and Chemistry 8:1141-1147.

CLAYTON, J. W., AND D. N. TRETIAK. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. Journal of the Fisheries Research Board of Canada 29:1169-1172.

DIAMOND, S. A., M. C. NEWMAN, M. MULVEY, P. M. DIXON, AND D. MARTINSON. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. Environmental Toxicology and Chemistry 8:613-622.

GILLESPIE, R. B., AND S. I. GUTTMAN. 1989. Effects of contaminants on the frequencies of allozymes in population of the central stoneroller. Environmental Toxicology and Chemistry 8:309-317.

LAVIE, B., AND E. NEVO. 1982. Heavy metal selection of phosphoglucose isomerase allozymes in marine gastropods. Marine Biology 71:17-22.

LAVIE, B., AND E. NEVO. 1986a. The interactive effects of cadmium and mercury pollution on allozyme polymorphisms in the marine gastropod, *Cerithium scabridum*. Marine Pollution Bulletin 17:21-23.

LAVIE, B., AND E. NEVO. 1986b. Genetic selection of homozygote allozyme genotypes in marine gastropods exposed to cadmium pollution. Science of the Total Environment 57:91-98.

LAVIE, B., E. NEVO, AND U. ZOLLER. 1984. Differential variability of phosphoglucose isomerase allozyme genotypes of marine snails in nonionic de-

tergent and crude oil-surfactant mixtures. Environmental Research 35:270–276.

MILSTEIN, C. 1961. On the mechanisms of activation of phosphoglucomutase by metal ions. Biochemical Journal 79:574–584.

MITTON, J. B., AND R. K. KOEHN. 1975. Genetic organization and adaptive response of allozymes to ecological variables in *Fundulus heteroclitus*. Genetics 79:97–111.

NEWMAN, M. C., S. A. DIAMOND, M. MULVEY, AND P. DIXON. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute toxicant exposure: a comparison of arsenate and inorganic mercury. Aquatic Toxicology 15:141–156.

SAS INSTITUTE INC. 1985. SAS/STAT guide for personal computers. Version 6 Edition. Cary, North Carolina.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old field mouse (*Peromyscus polionotus*). Studies in Genetics VI. University of Austin Press, Austin.

SMITH, M. W., M. H. SMITH, AND R. K. CHESSER. 1983. Biochemical genetics of mosquitofish. I. Environmental correlates, and temporal and spatial heterogeneity of allele frequencies within a drainage. Copeia 1983:193–197.

SWOFFORD, D. L., AND R. K. SELANDER. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. Journal of Heredity 72: 281–283.

USEPA. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. EPA-660/3-75-009.

Received: 28 December 1989
Accepted: 23 April 1989