Arch. Environ. Contam. Toxicol. 27, 20–24 (1994)

ARCHIVES OF
Environmental
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Enhanced Uranium Tolerance of an Exposed Population of the Eastern Mosquitofish (*Gambusia holbrooki* Girard 1859)

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Received: 29 October 1993/Revised: 20 January 1994

Abstract. Genetic differences between populations of mosquitofish from a uranium(U)-contaminated stream were identified by starch gel electrophoresis. Fish collected from the uncontaminated mainstream of Upper Three Runs Creek (South Carolina, USA) exhibited greater genetic variability than those collected from the contaminated Tims Branch. Lower genetic variation displayed in Tims Branch fish could reflect selection associated with toxicant stress, a genetic bottleneck due to low population numbers at the contaminated site, or random genetic drift. A toxicity assay was performed to determine if these genetically distinct mosquitofish also displayed enhanced U tolerance. Times to death were compared for fish from an uncontaminated site and offspring of fish taken from the U-contaminated Tims Branch. After 7 days of exposure to 2.57 mg/L of U as uranyl nitrate, 98% and 96% of the naive population had died in the replicate tanks. In contrast, the final mortality for the offspring from the population previously exposed to U were 25% and 57% in the replicate tanks. Fish derived from the U-contaminated site were more tolerant than those from the uncontaminated site. Because these were second generation fish, this tolerance likely has a genetic basis.

Enhanced tolerance by organisms chronically exposed to pollutants can result from physiological acclimation or genetic adaptation (Weis and Weis 1989). Physiological acclimation occurs when organisms acquire a degree of tolerance after exposure to sublethal concentrations. However, their offspring will not be tolerant unless they too are pre-exposed to the contaminants. Genetic adaptation occurs when populations acquire genetically based resistance through natural selection. Genetically based tolerance is inherited by offspring regardless of whether they are reared in a polluted or clean environment.

Genetically based tolerance is difficult to assess directly in natural populations; consequently, investigators have sought surrogate genetic measures of population response to environmental contamination. Enzyme electrophoresis provides a rapid method for obtaining genetic data for populations. Researchers have compared enzyme genotype frequencies between populations from contaminated and uncontaminated sites (Nevo *et al.* 1984; Gillespie and Guttman 1989). Differences were often found, but identification of the underlying mechanisms remained problematic. Differences may have resulted from random processes or selection against intolerant genotypes. Consequently, tolerance may or may not have been associated with the measured allozymes. For example, studies in our laboratory have detected a significant correlation between a rare homozygous genotype (Gpi-$2^{38/38}$) of the glucosephosphate isomerase-2 locus (Gpi-2) and earlier times-to-death (TTDs) in mosquitofish during exposure to arsenate or inorganic mercury (Diamond *et al.* 1989; Newman *et al.* 1989; Heagler *et al.* 1993). Furthermore, a low frequency of the Gpi-2^{38} allele in a population exposed to elevated mercury was consistent with predictions drawn from laboratory studies (Heagler *et al.* 1993). Kramer *et al.* (1992) reported that the Gpi-$2^{38/38}$ genotype had a distinct shift in glycolytic metabolites during mercury stress that may be associated with intolerance.

Our investigation was undertaken to determine if a population of mosquitofish chronically exposed to U was genetically distinct from nearby populations and if the population had enhanced tolerance to U. To determine if populations from contaminated and uncontaminated areas differed genetically, allozyme frequencies in mosquitofish in and around the U-contaminated region were surveyed. To assess U tolerance, a toxicity assay was done on mosquitofish from an uncontaminated environment and from a U-contaminated site in the Tims Branch region of Upper Three Runs Creek. Specifically, the tolerance assay tested the null hypothesis that offspring from a population of mosquitofish previously exposed to U for many generations and reared in an environment free of metal toxicants did not differ in tolerance to U compared to a naive population of mosquitofish with no known previous exposure.

Materials and Methods

Population Survey

A genetic survey of mosquitofish (*Gambusia holbrooki*, Girard 1859) was conducted between July and September 1990 from areas with and

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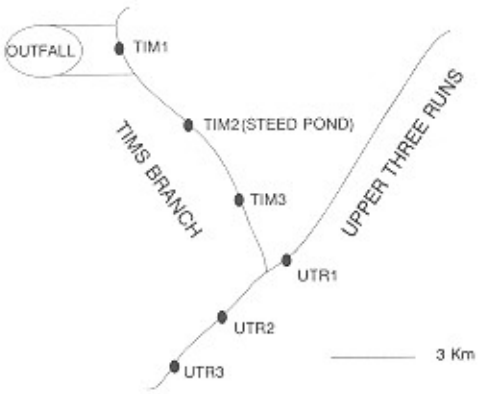

Fig. 1. Locations of six sample sites relative to the uranium outfall

without a history of U contamination. The contaminated sites on Tims Branch received releases of depleted U associated with production of fuel and target assemblies for nuclear reactors at the U.S. Department of Energy Savannah River Site (SRS) near Aiken, South Carolina. Wastewater generated during the manufacturing processes included dissolved and suspended U (Evans *et al.* 1992). Approximately 43,500 kg of U were released during operations that began in the early 1950s. The greatest deposition of U was between the region of release (TIM1) and Steed Pond (TIM2) (Figure 1), resulting in sediment U concentrations as great as 6,165 μg/g dry wt (Evans *et al.* 1992). (Background concentrations of U are approximately 14 μg/g dry wt for the Savannah River watershed.) Evans *et al.* (1992) estimated that roughly 70% of the released U was deposited in Steed Pond sediments with relatively minor deposition in the mainstream of Upper Three Runs Creek. Only 20 kg/yr entered the mainstream, which has a relatively high flow rate. Water column concentrations of U decreased from 4.2 μg/L in Tims Branch to 0.2 μg/L at the confluence of Tims Branch with the mainstream of Upper Three Runs Creek (Evans *et al.* 1992).

Approximately 60 mosquitofish were taken by dip net from each site on Tims Branch (TIM1, TIM2, TIM3) and from sites at its confluence with the mainstream of Upper Three Runs Creek (UTR1, UTR2, UTR3). Wet weight, standard length, and sex were noted for each fish. Any individual 20 mm or less in standard length was classified as a juvenile if sex could not be determined externally. Fish were stored at −70°C for later electrophoresis.

Exposure Study

Sampling and Maintenance: Approximately 100 mosquitofish were collected by dip net from Steed Pond in 1990 to establish an experimental population. The fish were allowed to reproduce for approximately 1 year in an outside, above-ground pool free of toxicants. A reference population of mosquitofish was collected during the summer of 1991 from Risher Pond, an isolated farm pond with no known history of contamination. Risher Pond is 15 km from the contaminated Tims Branch population in a noncontiguous watershed. Prior to exposure, the reference population was maintained in a 520-L tank (Living Streams® Model LS700) at 18°C under a 12:12 h light:dark cycle. The tank was aerated, and 50% of the water was replaced every other day with fresh stream water. Fish were fed Tetramin® tropical fish food three times daily during the holding period and were periodically treated with methylene blue and uniodized salt to control fungal infections.

Toxicity Testing Procedure: Risher Pond fish and the offspring of Steed Pond fish were exposed for 7 days to uranyl nitrate ($UO_2(NO_3)_2$) with a static renewal system. Exposure water was collected from Upper Three Runs Creek. Each test tank contained 60 fish in 25 L of water. Duplicate test tanks were prepared for each experimental population. A control tank for each population contained 5 L of water and 14 fish. Tanks were placed randomly on two racks and were aerated. Test organisms were randomly allocated to tanks. Fish were allowed to acclimate for 2 days in the tanks prior to exposures. Feeding was discontinued 24 h before dosing. Test tanks were spiked every 24 h to a nominal concentration of 4 mg/L U. Exposure concentration was based on results from a previous range-finding test that indicated 50% mortality would occur within 96 h for Risher Pond mosquitofish.

Mortality was monitored at 12-h intervals; however, unscheduled observations were also made. Fish were considered dead when no movement was evident after gentle prodding. Dead fish were sexed, weighed, and placed into plastic centrifuge tubes for later electrophoresis. Upon completion of the experiment, control fish and survivors were killed by immersion in ice water and handled in the same manner.

Water Chemistry: All water chemistry measurements were performed daily. Temperature and dissolved oxygen concentration were determined with a Hydrolab Surveyor II®. However, dissolved oxygen could not be determined in the shallow control tanks. Total alkalinity (potentiometric titration, APHA 1980) and pH measurements were made with an Orion Ionanalyzer 1901 equipped with an Orion 8130 Ross combination electrode. Calcium, Mg, K, and Na were measured by flame atomic absorption spectrophotometry (Hitachi Model 180-80 with Zeeman background correction). Prior to anion analysis, samples were passed through Sep-Pak® reverse-phase columns to remove most of the dissolved organic carbon. Concentrations of SO_4, Cl, and NO_3 were measured with a Dionex 4020i ion chromatograph with a conductivity detector and an HPIC-AS4A separator column (0.424 g/L Na_2CO_3; 0.126 g/L $NaHCO_3$ eluant). Total U was analyzed with an inductively coupled plasma spectrometer (ICP) by a contracting laboratory (General Engineering Laboratories, Charleston, SC). All cation, anion, and U analyses were judged acceptable using procedural blanks, sample spikes, and replicate analyses.

Electrophoresis

Tissue samples from the population survey and the exposure study were ground in their storage tubes with a glass rod and approximately 0.2 ml of cold grinding solution (0.01 M Tris, 0.001 M EDTA, 0.05 M NADP buffer; pH 7.0). Ground samples were centrifuged, and paper wicks were dipped into the supernatant fluid, blotted on filter paper, and then inserted into 12.5% (w/v) horizontal starch gels. The following enzymes and buffers were selected for analysis by electrophoresis: 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), Tris citrate, pH 8.0 (Selander *et al.* 1971), fumarate hydratase (FH, E.C. 4.2.1.2), leucylglycylglycine peptidase (lgg-PEP, E.C. 3.4.1.1-), and phenylalanylproline peptidase (pp-PEP, E.C. 3.4.1.1.-) Tris-EDTA-borate, pH 8.0 (Selander *et al.* 1971). These enzymes were previously studied in mosquitofish and are known to be polymorphic in mosquitofish populations (Diamond *et al.* 1989; Newman *et al.* 1989; Heagler *et al.* 1993). Electrophoresis was carried out at 35 mA for Tris-citrate gels and 25 mA for Tris EDTA borate gels for approximately 16 h. Gels were stained as described in Selander *et al.* (1971) and Harris and Hopkinson (1976). Enzymes were numbered in order of decreasing anodal mobility in the multilocus systems. Allozyme mobilities were determined relative to the most common allozyme for each locus, which was designated 100. For example, an individual homozygous for the common Icd-1 allele was given the designation Icd-$1^{100/100}$ The designation Icd$^{134/100}$ indicates a heterozygous individual.

Data Analysis

Electrophoretic data were analyzed by the BIOSYS-1 program (Swofford and Selander 1981). Genotype frequencies, average individual

Table 1. Frequency of alleles measured in survey fish

Population location	N	Frequency of alleles (%)																		
		ADA		FH		ICD-1			ICD-2		lgg-PEP			MDH		MPI		GPI-2		
		100	77	100	81	134	116	100	161	100	123	100	83	118	100	109	100	100	66	38
TIM1	60	100	0	100	0	28	0	72	0	100	18	82	0	0	100	8	92	100	0	0
TIM2	60	99	1	100	0	29	0	71	0	100	18	82	0	0	100	4	96	100	0	0
TIM3	59	100	0	98	2	14	0	86	0	100	38	62	0	0	100	6	94	100	0	0
UTR1	63	100	0	99	1	27	1	72	28	72	10	90	0	8	92	40	60	70	25	5
UTR2	60	100	0	100	0	26	0	74	13	87	3	96	1	6	94	34	66	79	14	7
UTR3	64	100	0	100	0	38	0	62	9	91	6	94	0	11	89	44	56	87	13	0

heterozygosity (estimated by averaging the total number of heterozygous individuals for each locus/total number of individuals in the sample over all scored loci), and fit of the data to Hardy-Weinberg expectations in the surveyed populations and the exposure populations were determined.

Survival analysis procedures as implemented by the SAS LIFEREG procedure (SAS Institute 1987) were used to test for significant differences in TTD between fish from the two sources. Briefly, these procedures develop predictive models of TTD as a function of one or more covariates. Several covariates including sex, size (ln wet wt), replicate tank, and genotypes were incorporated into the models. Details regarding these methods including examples can be obtained from Dixon and Newman (1991) and Newman and Aplin (1992).

Specifically, the Weibull, log normal, normal, gamma, and logistic functions were assessed as candidate functions in the survival (accelerated failure time) models. A log likelihood statistic was computed for each model and used to compare goodness-of-fit (Dixon and Newman 1991). However, because the number of estimated parameters varied among candidate models, the log likelihood statistic was not used directly for model comparison. Akaike's Information Criterion (A.I.C.) was estimated from the log likelihood statistic to adjust for variation in the number of estimated parameters among models. The model providing the best fit was that with the smallest A.I.C. (Atkinson 1980; Newman *et al.* 1993).

$$\text{A.I.C.} = -2\ (\text{log likelihood}) + 2\ (\text{number of parameters})$$

The Weibull distribution best fit these data. Consequently, a maximum likelihood method (SAS Institute 1987) assuming a Weibull distribution was used to fit these data. Associated χ^2 statistics were used to test for significant effects of covariates on TTD. Herein, an α of 0.05 was used to indicate a significant χ^2.

Results

Population Survey

The percentage of polymorphic loci and mean heterozygosity were nearly twice as great at the Upper Three Runs Creek sites (UTR1, UTR2, and UTR3) relative to the Tims Branch sites (TIM1, TIM2, and TIM3) (Tables 1 and 2). Distinct differences in allele frequencies were also apparent between the two regions. For example, three alleles (Icd-2^{161}, Gpi-2^{66}, and Gpi-2^{38}) were not present in Tims Branch samples but were found in Upper Three Runs Creek samples. The Mpi^{109} allele frequency increased approximately sevenfold in Upper Three Runs samples relative to Tims Branch samples. Two departures (ICD-1 and PGI-2) from Hardy-Weinberg equilibrium were observed at the UTR1 site. Both significant deviations were associated with a deficiency of heterozygous genotypes relative to the expected number.

Table 2. Frequency (%) of polymorphic loci and mean heterozygosity in surveyed fish

Population location	N	Polymorphic loci (%)	Mean individual heterozygosity (%)
TIM1	69	44.4	10.0
TIM2	60	44.4	8.9
TIM3	59	55.6	8.4
UTR1	63	88.9	21.6
UTR2	60	77.8	20.4
UTR3	64	77.8	18.9

Exposure Study

The mean measured U concentration for the experimental tanks was 2.57 mg/L (SD = 0.38). Except for NO_3, pH, alkalinity, and Ca, water chemistry variables were similar for all tanks (Table 3). Elevated NO_3 concentrations in the experimental tanks were due to uranyl nitrate being used as the toxicant, but were assumed to contribute no additional toxic stress because the observed concentrations are generally considered nontoxic to aquatic organisms (Russo 1985). For example, a 96-h LC50 value of nitrate for a guppy *(Poecilia reticulata)* was reported at 180–200 mg/L (Rubin and Elmaraghy 1977). In exposure tanks, the slight change in pH associated with spiking likely increased dissolved Ca and total alkalinity concentrations.

Only two of 28 control fish died during the experiment, one as a result of being siphoned into a draining hose accidently. In the exposure study, Risher Pond fish had significantly shorter TTD than the Steed Pond fish population as indicated by the associated β estimates in Table 4. Mortality in the replicate tanks with Risher Pond fish were 96% and 98% compared to 25% and 56% in the tanks with Steed Pond fish (Figure 2). There was also a significant effect of exposure tank assignment on TTD, although smaller than the effects of population source, sex, or size (Table 4). These models were generated again with the inclusion of allozyme genotypes as covariates. Only fumerase hydratase genotype had a significant effect ($p < 0.05$) on TTD.

Overall, fish population source had the strongest effect on TTD (Table 4). The negative β estimated for fish from Risher Pond (-0.385) relative to the assigned β of 0 for Steed Pond fish indicated that the Risher Pond fish were more sensitive to U toxicity than the Steed Pond fish. The positive β (0.172) estimated for the female sex indicated that males had shorter TTD than the females. Likewise, the positive β for the influence of ln fish weight (0.181) indicated that smaller fish have shorter

Table 3. Summary of water chemistry in exposure and control tanks (n = 7)[a]

Tank/source of fish	Temperature (°C)	Dissolved O_2 (mg O_2/L)	pH[b]	Total alkalinity (mg/L as $CaCO_3$)	Cl (mg/L)	SO_4 (mg/L)	NO_3 (mg/L)	Mg (mg/L)	Ca (mg/L)	Na (mg/L)	K (mg/L)	U (mg/L)
Tank 1	19.7	9.0	6.87	3.2	1.9	0.7	1.8	0.4	1.8	1.5	0.3	2.51
(Risher)	(2.3)	(0.5)	(6.77–6.91)	(0.4)	(<0.1)	(0.3)	(0.1)	(<0.1)	(0.2)	(0.1)	(0.1)	(0.43)
Tank 2	19.0	9.0	6.83	3.1	1.9	0.7	1.7	0.4	1.7	1.5	0.3	2.61
(Risher)	(2.4)	(0.5)	(6.71–6.91)	(0.3)	(<0.1)	(0.2)	(0.1)	(<0.1)	(0.2)	(0.1)	(0.1)	(0.29)
Tank 3	19.0	9.1	6.92	3.8	1.9	0.6	1.7	0.4	1.8	1.4	0.3	2.61
(Steed)	(2.4)	(0.5)	(6.67–6.97)	(0.4)	(<0.1)	(0.1)	(0.1)	(<0.1)	(0.2)	(<0.1)	(0.1)	(0.43)
Tank 4	19.4	9.1	6.88	3.6	1.9	0.6	1.8	0.4	1.7	1.4	0.3	2.56
(Steed)	(2.3)	(0.5)	(6.77–6.94)	(0.2)	(<0.1)	(0.1)	(<0.1)	(<0.1)	(0.2)	(<0.1)	(0.1)	(0.43)
Control	19.7	N/A	7.21	6.6	2.1	0.6	0.7	0.4	3.2	1.6	0.4	
(Risher)	(2.3)		(6.90–7.26)	(0.9)	(0.1)	(<0.1)	(0.4)	(<0.1)	(0.6)	(0.1)	(0.1)	<0.01
Control	19.3	N/A	7.24	6.0	2.2	0.6	0.1	0.4	2.7	1.6	0.3	
(Steed)	(2.4)		(6.98–7.33)	(0.7)	(0.4)	(0.1)	(0.2)	(<0.1)	(0.2)	(0.2)	(0.1)	<0.01

[a] Mean (standard deviation)
[b] Median (range)

Table 4. Summary of survival model using the Weibull distribution

Variable	Class	Parameter estimate(S.E.)	χ^2	Probability associated with the calculated χ^2
Population (β_s)	Risher	−0.385 (0.034)	131	0.0001
Source	Steed	0		
Replicate (β_r)	1	0.097 (0.031)	10	0.0016
	2	0		
Sex (β_{sex})	Female	0.172 (0.035)	25	0.0001
	Male	0		
Ln weight (β_w)		0.181 (0.033)	30	0.0001
Intercept (μ)		5.330 (0.072)	5487	<0.0001
Scale (σ)		0.168 (0.012)		

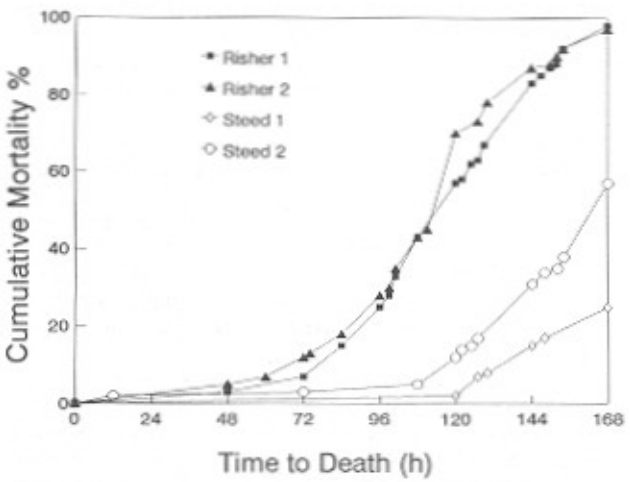

Fig. 2. Cumulative mortality for uranium exposures of mosquitofish from Risher (naive) and Steed (contaminated) Ponds

TTD than larger fish. The following survival-time model derived from these data was used to predict median time-to-death (MTTD):

$$MTTD = e^{\mu} e^{\beta_w \ln wt + \beta_{sex} sex + \beta_s source + \beta_r replicate} e^{\sigma w}$$

where

μ = model intercept (5.330)
β_w = β for the effect of wet weight
ln wt = the ln of wet wt (g)
β_{sex} = β for the effect of sex; sex = 1 if female or 0 if male
β_s = β for the effect of population source; source = 1 if Risher or 0 if Steed
β_r = β for replicate; replicate = 1 if replicate 1 or 0 if replicate 2
σ = model scale parameter (0.168)
W = 50th percentile of the standardized distribution assumed for the error, which is −0.3665 for the Weibull distribution

With this equation, 0.1 g male fish from Risher Pond replicate tank 2 would have a predicted MTTD of 87 h, whereas similar fish from Steed Pond would have a predicted MTTD of 128 h.

Discussion

We rejected the null hypothesis that offspring from a population of mosquitofish previously exposed to U but reared in a clean environment did not differ in tolerance to U compared to a population of mosquitofish with no history of exposure. Mosquitofish derived from the U-contaminated Steed Pond population displayed enhanced tolerance relative to the Risher Pond fish. Because the Steed Pond population was allowed to grow

for a year in clean water, the tolerance exhibited by the offspring in this experiment was likely genetically based and not due to physiological acclimation. These results suggest this population of mosquitofish had undergone genetic adaptation to its contaminated environment. However, slight differences between rearing conditions prior to exposure may have affected the results (Lee *et al.* 1992).

Recent studies of exposures to inorganic mercury and arsenate (Diamond *et al.* 1989; Newman *et al.* 1989; Heagler *et al.* 1993) showed that earlier TTD in mosquitofish was associated with a rare homozygous genotype for the glucosephosphate isomerase-2 (GPI-2) locus. We were unable to assess this genotype effect for the toxicity experiment because the $Gpi\text{-}2^{38}$ allele was not present in the Steed Pond sample and was at low frequency in the Risher Pond fish. Newman *et al.* (1989) reported that a rare heterozygous genotype for fumerate hydratase, Fh^{100}/Fh^{81}, may be sensitive to arsenate. This genotype for fumarate hydratase had an overall effect in this toxicity experiment; however, its significance relative to the field population survey cannot be evaluated due to a small sample number for each population.

Allozyme frequencies for mosquitofish sampled along Tims Branch differed from those of mosquitofish taken from the Upper Three Run sites. Differences in allozyme frequencies between these populations could be a result of natural selection against genotypes that were more sensitive to the contaminants in Tims Branch. However, allozyme frequency differences have been reported for mosquitofish populations in different reaches of a single river or stream in the absence of toxicants (Smith *et al.* 1983). Although differences have been associated with contamination, more inclusive studies considering normal biogeography (*e.g.*, Hernandez-Martich and Smith 1990) are required prior to definitive attribution of such an observation to toxicant-induced natural selection. Alternatively, a genetic bottleneck may have occurred in Tims Branch with a consequent drop in heterozygosity. The bottleneck could have been associated with natural processes or a toxicant-induced reduction in effective population size. Regardless, our findings indicate genetic shifts in the Tims Branch fish were associated with contamination. Offspring of the genetically distinct mosquitofish from Steed Pond displayed enhanced tolerance to U during laboratory exposure.

Acknowledgments. This work was supported by Contract DE-AC09-76SR00-819 between the U.S. Department of Energy and the University of Georgia's Savannah River Ecology Laboratory. We are grateful to Dr. Michael H. Smith for allowing us to use his laboratory to conduct the electrophoresis analyses. Thanks to Anne Chazal for assistance with field work. Also, we are appreciative of Dr. M. Gay Heagler and John McCloskey for assistance in the field and in the laboratory. We also thank Dr. Carl Strojan and two anonymous reviewers for insightful review of an earlier version of this manuscript.

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