Arch. Environ. Contam. Toxicol. 22, 284–287 (1992)

Archives of Environmental Contamination and Toxicology

Time to Death of Mosquitofish (*Gambusia holbrooki*) during Acute Inorganic Mercury Exposure: Population Structure Effects[1]

Charles J. Lee, Michael C. Newman,[2] and Margaret Mulvey

University of Georgia, Savannah River Ecology Laboratory, P.O. Drawer E, Aiken, South Carolina 29801, USA

Abstract. Times-to-death (TTD) of mother and offspring mosquitofish (*Gambusia holbrooki*) were examined during acute exposure to mercury concentrations of approximately 1.0 mg/L. Median TTD were more similar for fish sharing a common mother (defined herein as a sibship) and microhabitat during maturation than between sibships. Field populations may exhibit structure similar to that of these sibships. Correlations between broods (or other population subunits) and allozyme genotype could be responsible for transient, genotype effects noted during electrophoretic surveys attempting to measure population level response to toxicants.

Researchers continue to search for effective biomarkers of population level responses to toxicants. Shifts in allozyme frequencies have become the focus of many such studies (Mulvey and Diamond 1991 and references therein) because correlations between tolerance to toxicants and allozyme genotype have been noted for a variety of species (Nevo *et al.* 1978, 1981, 1984; Lavie and Nevo 1982, 1986). However, the exact relationship between contaminant stress and allele frequency as well as the characteristics of genotype effects remain incompletely defined in most studies (Newman *et al.* 1989a). This is unfortunate as a clear understanding of the nature of such differential tolerance is essential to the effective use of allozyme genotypes as unambiguous biomarkers.

Several characteristics of such genotype effects have been previously defined in our laboratory using mosquitofish tolerance to inorganic mercury as a model. For example, time-to-death (TTD) during acute mercury exposure was correlated with allozyme genotype at three loci: isocitrate dehydrogenase-1 (ICD-1), malate dehydrogenase-1 (MDH-1), and glucosephosphate isomerase-2 (GPI-2) (Diamond *et al.* 1989). Acute exposure of mosquitofish to an alternate toxicant, arsenate, suggested that some genotype effects (GPI-2) may not be specific to a toxicant (Newman *et al.* 1989a). However, genotype effects at two loci (ICD-1 and MDH-1) on TTD during mercury exposure were not observed for the arsenate exposure (Newman *et al.* 1989a). This suggested that these genotype effects could have been specific for mercury or that the effects were temporally unstable. A second acute mercury exposure was undertaken two years later to determine the temporal stability of the ICD-1, MDH-1 and GPI-2 effects. The only consistent genotype effect was that associated with the GPI-2 locus (Heagler *et al.* submitted). Biochemical examination suggested that the most sensitive genotype ($GPI\text{-}2^{38/38}$) was least tolerant due to its distinct shift in Krebs cycle metabolites during mercury stress (Kramer *et al.* in press).

In contrast to the consistent GPI-2 effects, the ICD-1 and MDH-1 effects were not detected again indicating that the relationships between TTD and genotype at these particular loci were unstable. Undefined population structure was identified as a possible source of this instability as mosquitofish populations can exhibit significant temporal and spatial structuring (Kennedy *et al.* 1985). Structuring can be caused by a number of factors. For example, mosquitofish exhibit nonrandom dispersal (Kennedy *et al.* 1985). The tendency of young mosquitofish to avoid predation by hiding in groups within clumps of macrophytes could also contribute to population structure. In the initial mercury study (Diamond *et al.* 1989), fish allozyme genotype and TTD might have been correlated with undefined lineages or spatially distinct, population subunits that differed in tolerance to mercury. The ICD-1 and MDH-1 effects on TTD could then have been correlations produced during unintentionally biased sampling of a structured population. The present study examined the hypothesis that the temporally unstable genotype effects noted earlier for ICD-1 and MDH-1 loci resulted from undefined population structure. Mosquitofish from an artifically structured population were used to address this hypothesis.

Materials and Methods

Collection Site and Sampling

Mosquitofish were taken from Risher Pond on the U.S. Department of Energy's Savannah River Site in South Carolina, the source of mosqui-

[1] By acceptance of this article the publisher recognizes that the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or to allow others to do so, for U.S. Government purposes.

[2] To whom correspondence should be addressed.

tofish in our previous studies (Diamond *et al.* 1989; Newman *et al.* 1989a; Kramer *et al.* in press; Heagler *et al.* submitted). To our knowledge, fish in this 1.1 ha pond have never been exposed to elevated levels of any toxicant.

The fish were dip-netted in June 1990 and placed into 120-L, plastic coolers filled with pond water and Stress Coat™, an Aloe Vera stress treatment. Twenty-one, gravid females were taken to the laboratory, assigned identification numbers, and transferred to individual brooding chambers held in 40-L tanks.

Broods were reared separately to simulate the isolation thought to contribute to population structuring in Risher Pond. Upon birth, the offspring of individual mothers were transferred to individual 100-L, 30 cm deep plastic pools. To provide seeds for algal and macroinvertebrate populations and a relatively natural habitat, each pool was filled with approximately 250 g of dry leaves and pine straw gathered from a Carolina bay (Flamingo Bay) and with water pumped from Upper Three Runs Creek, both on the Savannah River Site. This stream has water chemistry similar to that of Risher Pond (Newman 1986). The offspring remained in the pools until the females reached sexual maturity as determined by the presence of a dark abdominal spot. This isolation of sibships (defined herein as offspring from a common mother) into similar rearing conditions simulated the structuring of fish populations that could occur in Risher Pond. The maintenance of the pools consisted of refilling with Upper Three Runs Creek water to compensate for evaporation. The fish were fed once a day with Tetramin™ Tropical Fish Food until beginning the exposure period. To eliminate sex effects on TTD (Diamond *et al.* 1989), only female offspring were used for exposure.

Exposure System

Individual exposure chambers were made from 4-L, plastic jugs and mesh screening. The tops and 75 mm × 100 mm patches from the four sides were removed and covered with mesh to allow free flow of water through the chamber. The seventeen, surviving mothers or their offspring were placed in their individual chambers. These chambers were numbered and put into a 720-L tank (Living Streams™ Model LS-700). Fish were allowed to acclimate to the exposure temperature 24 h prior to exposure.

The exposure system was supplied with prechilled, Upper Three Runs Creek water (18 C) from a Living Streams™ tank until the initiation of the exposure. Two 112-L exposure tanks were supplied with a continuous 2/3 tank-volume-per-day flow of creek water spiked with mercuric chloride to a nominal concentration of 1.0 mg/L. The water used in preparing the toxicant solution was pumped from the Living Streams™ tank into a plastic barrel which was spiked with a 1.0 g/L Hg solution. The solution was delivered to the exposure tanks by a peristaltic pump. The exposure tanks were aerated and cooled to 17 to 18 C with a refrigeration unit.

Exposure

Exposure chambers containing fish were transferred directly to the exposure tanks. Each of the exposure tanks was spiked by thoroughly mixing in the 1.0 g/L Hg solution to immediately bring the mercury concentration to exposure concentrations. The toxicant supply barrel was filled with water spiked with the 1.0 g/L solution as well. Fish were not fed during the exposure period. Every three hours, fish that appeared dead were examined for ventilation or fin movement, and gently prodded three to four times. Fish that still showed no movement after prodding were scored as dead and removed. Weight, standard length, number of eggs and embryos, and time-to-death to the nearest 3 h were recorded. Exposure duration (10 days) was determined by the time necessary to kill all fish.

Two sets of exposures were run, one with the mothers approximately three weeks after they produced their broods, and one was run on their sexually mature, female offspring. The exposure system was identical for both exposures except that the mothers were placed in individual chambers whereas offspring from each brood were exposed together in a single chamber.

Water Quality

Water samples for subsequent analysis were collected every 8 h and stored at 4°C. Total alkalinity was determined by the potentiometric titration method and specific conductance was determined with a Sybron PM-70CB conductivity bridge and a Fisher cell (cell constant = 0.102 cm^{-1}). Samples and standards for major cation and anion analyses were passed through Sepak™ reverse-phase columns and analyzed with a Dionex 4020i ion chromatograph equipped with a conductivity detector. Major cation concentrations (Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}) were determined with an HPIC-CS3 separator column and a dual eluant technique with a 48 mM HCL/8 mM DAP (2,3 diaminopropionic acid monohydrochloride) eluant and a 12 mM HCL/0.50 mM DAP eluant. The technique consisted of pumping the dilute eluant for 8 min and then pumping the more concentrated eluant through the column for the remainder of the 20 min run. Anions (Cl^{-} and SO_4^{2-}) were determined with an HPIC-AS4A separator column and a 0.42 g/L Na_2CO_3, 0.126 g/L $NaHCO_3$ eluant. Acid-extractable mercury concentrations (USEPA 1983) were determined by a cold vapor technique using a Perkin-Elmer 50A atomic absorption spectrophotometer (APHA 1980).

Data Analysis

The TTD data were analyzed with the SAS statistical package (Version 6.04; SAS Institute 1987). These analyses described the relationship of mother TTD with offspring TTD as well as the relationship between offspring TTD and the variables, brood and individual fish weight. During initial model development, the confounding effects of fecundity and fish age were examined also.

Variation in phenotype (tolerance) is comprised of several components. Hartl and Clark (1989) defined phenotypic variance with the general equation, $P = \mu + G + E$ where P = the phenotype of an individual, μ = the population mean for the character, G = the deviation from μ resulting from the individual's genotype, and E = the deviation from μ resulting from the microenvironment of the individual (or consequences of development). If genetic and environmental effects are not additive then an interaction term can be included.

Under the assumption of no significant genotype-environment covariance, the total phenotypic variance (σ^2_p) in a characteristic such as TTD is equal to the sum of the variance due to genotype (σ^2_g) and the variance due to the environment (σ^2_e). The genetic variance can be broken down into σ^2_a (sum of additive genetic effects over all pertinent genes), σ^2_i (epistatic variation due to gene interactions) and σ^2_d (dominance variance which is not subject to individual selection).

Hartl and Clark (1989) defined "narrow sense" heritability to be the additive genetic variance divided by the total phenotypic variance (σ^2_a/σ^2_p). "Narrow sense" heritability (h^2) was calculated with the equation $b = h^2/2$ where b is the slope of the regression line of mother TTD versus average brood TTD (Hartl and Clark 1989). Several estimates of average offspring response were explored during initial analysis of heritability. Relationships between mother TTD versus mean offspring TTD, mother TTD versus median offspring TTD, and logarithms of mother TTD versus logarithms of median offspring TTD were assessed.

As discussed above, additional factors involving σ^2_e (microhabitat or developmental events), genotype-environment interactions and genetic variance other than σ^2_a can contribute to variation in mercury tolerance (σ^2_p). Possible brood effects more inclusive than those associated with "narrow sense" heritability were explored using analysis of

Table 1. Summary of water quality in exposure tanks

Variable	Mother			Offspring		
	Mean[c]	SD[a,c]	N[b]	Mean[c]	SD[c]	N
Temp (°C)	17.92	0.47	10	17.18		1
D. oxygen (mg O_2/L)	9.43	0.47	10	9.37		1
pH	6.83	6.26–7.05	17	6.72	6.59–6.83	4
S. conductance (μS)	0.039	0.012	10	0.035		1
Total alkalinity (mg/L as $CaCO_3$)	5.05	2.01	17	5.50	1.41	4
SO_4 (mg/L)	4.16	1.57	17	2.72	1.24	7
Cl (mg/L)	2.85	0.01	17	2.87	0.19	4
Na (mg/L)	0.85	0.15	13	1.41	0.12	4
K (mg/L)	0.08	0.07	13	0.36	0.10	4
Mg[d] (mg/L)	0.19	0.12	13	0.52	0.19	4
Ca (mg/L)	1.86	0.26	13	3.00	1.84	4
Hg (mg/L)	1.04	0.15	101	1.06	0.09	18
Hg breakdown for mothers:						
Days 1–4	0.93	0.14	45			
Days 5–10	1.12	0.01	56			

[a] Standard deviation
[b] Number of samples analyzed
[c] Median and range used for pH
[d] Mean and standard deviation of the mother fish were estimated using two values that were below detection limit of 0.014 (mg/L). The technique of Gleit (1985) as implemented by UNCENSOR (Newman *et al.* 1989b) was used for this purpose

Table 2. Summary of ANCOVA results

Source	df	Sums of squares	Mean square	F value	Pr > F
Model	17	8877.07	522.18	2.98	0.0003
Error	115	20134.32	175.08		
Corrected Total	132	29011.39			
Brood	16	8290.88 (Type I)	518.18	2.96	0.004
Wet weight	1	586.19 (Type I)	586.19	3.35	0.070

covariance (ANCOVA) as implemented in the SAS GLM procedure. The fish wet weight was included in the analysis. When fish length, fecundity (number of eggs and embryos within the female) and age were included in the model initially, none of the covariates had associated p-values approaching significance ($\alpha = 0.05$).

Results

Exposure

For the mother fish, the average mercury exposure concentration was approximately 1.04 mg/L (Table 1). However, the concentration for the first four days averaged 0.93 mg/L and was increased to 1.12 mg/L for the remainder of the exposure. No fish died during the first four days. For the offspring exposure, the mercury concentrations averaged 1.05 mg/L for the duration of the experiment. All treated fish died during the exposure period.

Brood Effect

There was a significant brood effect on TTD of offspring (Table 2). An ANCOVA model employing the seventeen, exposed broods had an r^2 of 0.306 ($P = 0.0003$). The specific brood from which the individual originated, indicative both of the genetic and environmental background of an offspring, had a significant effect on the individual's TTD ($P = 0.004$). The covariate, wet weight showed no significant effect on TTD.

"Narrow Sense" Heritability

Ten mother-offspring pairs were used for estimating "narrow sense" heritability. Several mothers that died before testing or had too few female offspring ($N < 5$) could not be included in the analysis. Regression of mother TTD on brood TTD or transformations of these variables failed to yield statistically significant slopes ($\alpha = 0.05$). Inclusion of the covariates, size (wet weight or length), fecundity or fish age failed to generate significant results. Consequently, no statistically significant "narrow sense" heritability was detected.

Discussion

The contribution of population structure to differential mercury tolerance was examined to determine if structure could have contributed to the previously observed relationships between

mercury tolerance and allozyme genotype. The brood from which an offspring originated had a significant effect on when it died during mercury exposure. Offspring of a brood were raised in a common environment, isolated from other broods. All environments (100-L plastic pools) were prepared and maintained in general proximity and with identical water and food sources to eliminate extreme environmental differences between broods. Thus, small differences in microhabitat from pool to pool (e.g., random differences in algal growth or temperature), genetic factors and genetic-environment interactions likely accounted for the significant differences observed between broods for tolerance to mercury. These differences may have been further complicated by maternal effects (Meffe 1987, 1990). These differences remained after adjusting for variations in the fish sizes between broods.

"Narrow sense" heritability of mercury tolerance was explored to determine if differences in TTD were strongly and directly influenced by additive genetic qualities. After normalizing for weight, length, age, and fecundity effects, the data indicated no statistically significant "narrow sense" heritability. This absence of detectable heritability may be attributed to a lack of statistical power. A larger sample number of both mothers and offspring may have shown significant heritability. Regardless, broods sharing similar genetic backgrounds and common rearing environments did show significant differences in TTD. Factors contributing to these differences included those associated with "broad sense" heritability (σ^2_g/σ^2_p) and σ^2_e (Hartl and Clark 1989).

Nonrandom dispersal of mosquitofish within an area (Kennedy *et al.* 1985), the tendency for broods to remain together, and microhabitat differences can lead to population structuring. Relative to our previous studies of acute mercury exposure, this undefined structure likely contributed to the transient nature of the ICD-1 and MDH-1 genotype effects on TTD. Likely, these effects reflect this structuring and, consequently, are useless as biomarkers of population level response to toxicants.

These findings have broader implications regarding the interpretation and design of studies examining allozyme genotype effects on contaminant tolerance. Because population structuring can be significant in wild populations (Snyder *et al.* 1991) and between laboratory stocks (Baird *et al.* 1991, Snyder *et al.* 1991), consideration should be given to the potential for similar effects in other studies. If tolerance and allozyme genotype are not related *per se*, but covary within population structure, the use of shifts in allozyme genotype frequencies as biomarkers may not be as straightforward as is presently assumed.

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. Thanks to Philip Dixon for statistical advise, Vincent Kramer for assistance with field work and exposures, Gay Heagler for assistance in the laboratory and exposures, and Anne Chazal for assistance with water chemistry analyses and exposures.

References

APHA (1980) American Public Health Association, American Water Works Association, and Water Pollution Control Federation: Standard methods for the examination of water and wastewater, 15th ed. APHA, Washington, DC

Baird DJ, Barber I, Bradley M, Soares MVM, Calow P (1991) A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Straus. Ecotoxicol Environ Saf 21:257–265

Diamond SA, Newman MC, Mulvey M, Dixon PM, Martinson D (1989) Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. Environ Toxicol Chem 8:613–622

Gleit A (1985) Estimation for small data sets with detection limits. Environ Sci Technol 19:1201–1206

Hartl DL, Clark AG (1989) Principles of population genetics, 2nd ed. Sinauer Associates, Sunderland, MA, pp 435–440

Heagler MG, Newman MC, Mulvey M, Dixon PM (submitted) Allozyme genotype and time to death of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure: Temporal stability and concentration dependence. Environ Toxicol Chem (in press)

Kennedy PK, Kennedy ML, Smith MH (1985) Microgeographic genetic organization of populations of Largemouth Bass and two other species in a reservoir. Copeia 1:118–125

Kramer VJ, Newman MC, Mulvey M, Ultsch GR (In press) Glycolysis and Krebs cycle metabolites in mosquitofish, *Gambusia holbrooki*. Girard 1859, exposed to mercuric chloride: Allozyme genotype effects. Environ Toxicol Chem (in press)

Lavie B, Nevo E (1982) Heavy metal selection of allozymes in marine organisms: prediction and verification in nature. Mar Biol 71:17–20

—— (1986) Genetic selection of homozygote allozyme genotypes in marine gastropods exposed to cadmium pollution. Sci Total Environ 57:91–98

Meffe GK (1987) Embryo size variation in mosquitofish: Optimality vs plasticity in propagule size. Copeia 3:762–768

—— (1990) Offspring size variation in Eastern mosquitofish (*Gambusia holbrooki*: Poeciliidae) from contrasting thermal environments. Copeia 1:10–18

Mulvey M, Diamond SA (1991) Genetic factors and tolerance acquisition in populations exposed to metals and metalloids. In: Newman MC and McIntosh AW (eds) Metal ecotoxicology: Concepts and applications. Lewis Publishers, Chelsea, MI, p 399

Nevo E, Shimony T, Libini M (1978) Pollution selection of allozyme polymorphism in barnacles. Experimentia 34:1562–1564

Nevo E, Perl T, Belies A, Wood D (1981) Mercury selection of allozyme genotypes in shrimps. Experimentia 37:1152–1154

Nevo E, Ben-Shlomo R, Lavie B (1984) Mercury selection of allozymes in marine organisms: Prediction and verification in nature. Proc Natl Acad Sci USA 81:1258–1259

Newman MC (1986) Comprehensive cooling water report. Vol 2: Water quality, Savannah River Ecology Laboratory Publ. No. 28-II, Univ of Georgia, Aiken, SC

Newman MC, Diamond SA, Mulvey M, Dixon P (1989a) Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute toxicant exposure: A comparison of arsenate and inorganic mercury. Aquat Toxicol 15:141–156

Newman MC, Dixon PM, Looney BB, Pinder JE, III (1989b) Estimating mean and variance for environmental samples with below limit observations. Water Resour Bull 25(4):905–916

SAS Institute (1987) SAS/STAT guide for personal computers. Version 6 ed. SAS Institute, Inc., Cary, NC

Snyder TP, Switzer KM, Keen RE (1991) Allozymic variability in toxicity-testing strains of *Ceriodaphnia dubia* and in natural populations of *Ceriodaphnia*. Environ Toxicol Chem 10:1045–1049

USEPA (1983) Methods for chemical analysis of water and wastes. US Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, OH

Manuscript received October 9, 1991 and in revised form December 7, 1991.