

Genetic Variation in Insecticide Tolerance in a Population of Southern Leopard Frogs (*Rana sphenocephala*): Implications for Amphibian Conservation

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Currently, conservation efforts are devoted to determining the extent and the causes of the decline of many amphibian species worldwide. Human impacts frequently degrade amphibian habitat and have been implicated in many declines. Because genetic variance is critical in determining the persistence of a species in a changing environment, we examined the amount of genetic variability present in a single population for tolerance to an environmental stressor. We examined the amount of genetic variability among full- and half-sib families in a single population of southern leopard frogs (*Rana sphenocephala*) with respect to their tolerance to lethal concentrations of the agricultural chemical, carbaryl. Analysis of time-to-death data indicated significant differences among full-sib families and suggests a large amount of variability present in the responses to this environmental stressor. Significant differences in responses among half-sib families indicated that there is additive genetic variance. These data suggest that this population may have the ability to adapt to environmental stressors. It is possible that declines of amphibian populations in the western United States may be attributed to low genetic variability resulting from limited migration among populations and small population sizes.

AS reports of worldwide amphibian declines continue to increase (Blaustein et al., 1994; Wake, 1998), much evidence has accumulated implicating, directly or indirectly, various causal factors (see review in Alford and Richards, 1999), including anthropogenic stressors such as the introduction of exotic species (Hayes and Jennings, 1986; Bradford, 1989; Kupferberg, 1997), chemical contamination (Kirk, 1988), and habitat destruction (Petranka et al., 1993; Means et al., 1996; Demaynadier and Hunter, 1998). However, reasons for many declines and extinctions remain elusive (e.g., *Bufo perigrinus*, Pounds and Crump, 1994; *Rheobatrachus silus*, Tyler, 1991; but see Kaiser, 1999). In fact, there are few places where human presence has not altered amphibian habitats in some manner. Although reduction or elimination of human practices that vitiate the environment may be an ideal solution to improving habitat quality, enforcement and implementation of these reductions or eliminations may be impossible. Therefore, the ability of organisms to adapt to such anthropogenic environmental changes is critical for them to persist in evolutionary time.

Darwin (1859) observed that, for a population to adapt to a changing environment, a trait must be phenotypically variable. In addition, phenotypic variation must have a heritable genetic basis. The amount of genetic variation and its long-term maintenance is critical to under-

standing adaptation for changing environmental conditions. Mechanisms such as the maintenance or generation of genetic variability (i.e., additive genetic variance) must be evaluated to understand the evolutionary potential of populations (Lande and Shannon, 1996). Addressing the potential for a species' persistence in a changing environment will require quantifying genetic variability in populations exposed to such environments.

It is possible to determine the degree of phenotypic variation within a population by using a number of full-sibling families. However, full-sibling analyses do not allow variances to be partitioned into additive, nonadditive, and maternal components (V_A , V_D , V_M , respectively); analysis of the potential for a population to adapt to an environment requires that each of these causal variance components be examined separately and is accomplished using a number of paternal half-sibling families (Falconer and Mackay, 1996). Because paternal half-sibling families share the same mother and have different fathers, differences among half-sibling families are solely a result of the genetic contribution of the father. Such quantitative genetic analyses are required to determine whether genetic variation exists for increased tolerance of amphibians to environmental stressors. Populations with more additive genetic variation may have an increased ability to adapt to a stressful environment. Alternatively, if little additive ge-

netic variance is present, the population may not have the ability to adapt to environmental changes, and the consequences of environmental stressors may be more severe.

We investigated the amount of genetic variability in tolerance to an environmental stressor within a single population of the southern leopard frog (*Rana sphenocephala*) by using both full- and half-sibling families. We chose the insecticide carbaryl as a stressor. Carbaryl is widely used throughout the United States and Canada and can contaminate amphibian habitats via drift from spray during aerial application or through runoff from adjacent agricultural fields or gardens. Carbaryl acts by inhibiting release of the neurotransmitter acetylcholinesterase, a common mode of action among insecticides. Thus, carbaryl can serve as a model chemical with which to examine amphibian responses.

MATERIALS AND METHODS

On 20 March 1997, 17 *R. sphenocephala* full-sibship (hereafter full-sib) families were collected as freshly laid egg masses from a roadside ditch along the Missouri River floodplain in Cole County, Missouri. We collected egg masses spaced far enough apart (≥ 1 m) to increase our confidence that they were from distinct breeding pairs. In the case that egg masses from a single pair of frogs were sampled more than once, any differences observed in our experiment would result in a conservative estimate of the variation present in the population.

To create half-sibship (hereafter half-sib) families, one gravid *R. sphenocephala* female and 23 males were collected on 20 and 21 April 1999 from the same population as the full-sib egg masses in 1997. On 22 April 1999, sperm suspensions were created from each male by macerating both testes in approximately 2 mL of distilled water. Eggs were stripped from the female and placed into 23 separate Petri dishes (20–30 eggs per dish). A sperm suspension from a single male was used to fertilize the eggs in a single Petri dish; this process was repeated for each male. Embryos remained in the Petri dishes until hatching.

Once eggs from half-sib families had hatched, tadpoles were reared in the laboratory on a 12:12 L:D cycle in separate 4-liter containers filled with well water. Free-swimming tadpoles were fed fish flakes (TetraMin) ad libitum and were maintained in fresh well water at 22 ± 1 C until the time of testing. Egg masses from full-sib families were maintained in a similar manner in separate 4-liter containers throughout development. Because *Rana blairi* is also found in this

population and is known to hybridize with *R. sphenocephala*, the identity of each adult used in creating half-sib families as well as a sample of individuals from each full-sib egg mass was confirmed by electrophoresis at three diagnostic allozyme markers (i.e., *Ldh-I*, *Pgm-I*, *EAP*; for methods, see Parris et al., 1999).

The most common toxicological test used to determine sensitivity to a contaminant is the LC50. Using several concentrations of a chemical, LC50s represent the concentration at which 50% of a test population dies (i.e., the mean lethal concentration). In our experiments, we used time-to-death (TTD) assays, which examine mortality at a single concentration of a chemical across time (Newman and Dixon, 1996). Time-to-death studies are useful because they can accurately resolve which individuals are most sensitive while using a minimum number of animals. We have previously demonstrated a significant positive correlation between TTD and 24- or 48-h LC50s; individuals with high TTDs also demonstrate high LC50s (Bridges, 1999), indicating that either test is suitable for determining rank-order sensitivity.

Carbaryl stock solutions were created by dissolving 6.018 g of powdered carbaryl (99.8% purity) in 100 mL of technical grade acetone. The measured concentration of these stocks as determined by high-pressure liquid chromatography (HPLC) was 85% and 109% (1997 and 1999, respectively) of the nominal (i.e., expected) concentration. No HPLC analyses were carried out on any other carbaryl solutions; thus, although the reported values are nominal, they are based on a measured stock solution concentration. Because separate stocks were created each year, data between the two years are not directly comparable. Time-to-death was determined by placing individual tadpoles in 250-mL glass beakers containing 200 mL of a 30 mg/L carbaryl solution. Although 30 mg/L is greater than expected field concentrations (≤ 4.8 mg/L; L. A. Norris, H. W. Lorz, and S. V. Gregory, USFS, 1983, unpubl.), it is only slightly higher than the average 24-h LC50 values for a number of ranid species (e.g., *R. clamitans*, 22.55 mg/L; Boone and Bridges, 1999; *R. sphenocephala* 20.5 mg/L and *R. blairi* 16.5; Bridges, 1999). By exposing tadpoles to such a high concentration, we were able to elicit mortality (an experimental endpoint) within a short time (i.e., ≈ 48 h). Although by using high concentrations we sacrificed realism, we have observed variability among tadpoles exposed to more environmentally relevant concentrations (i.e., 2.5 mg/L; Bridges and Semlitsch, 2000), which suggests that variability in sensitivity exists regardless of

chemical concentration. The carbaryl solution was created by adding 0.1 mL of carbaryl stock solution to the 200-mL well water (pH 7.8; hardness 286 mg/L CaCO₃; alkalinity 258 mg/L CaCO₃) in each beaker. Additional beakers were filled with 200-mL well water and contained 0.1-mL technical grade acetone to serve as solvent controls. All beakers were arranged in a water bath and maintained at 22 ± 1 C. All tadpoles were at stage 25 (Gosner, 1960) and were not fed during exposure. Each family was replicated 10 times, except in three half-sib families in which fewer individuals were available (two families had seven replicates, one family had six replicates). Mortality was determined at 3, 6, 9, 12, 18, 24, 36, and 48 h after the beginning of a test and was defined as the absence of all movement after repeated prodding. After each tadpole had died, it was weighed (wet weight) to the nearest 0.1 mg. In the full-sib portion of the experiment, lack of adequate laboratory space precluded testing all individuals at once. Therefore, five replicates were tested during each day for two days, and test days were used as statistical blocks. There was no mortality among tadpoles in any control treatment.

Time-to-death data were found to be normally distributed using a Shapiro-Wilk test ($W > 0.05$). Analysis of variance (ANOVA) determined whether TTDs differed significantly among families, which was used as a random-effects variable. Because tadpoles were of slightly different sizes when used in the tests, tadpole mass was initially used as a covariate. Although mass was significant in the half-sib analysis, it was not significant in the full-sib analysis and was removed from the model. Similarly, the effect of blocking factor "day," in the full-sib analysis was not significant and was removed from the model. Significant differences among families in both the full- and half-sib experiment were determined using LSD means comparison tests. Pearson's correlations were performed for both the half- and full-sib families to determine whether mean tadpole mass and mean TTD for each group were correlated.

RESULTS

In the full-sib experiment, significant differences existed in TTD attributable to family ($F_{16,153} = 1.72$; $P = 0.0481$). Although most of the families had similar TTDs, there were a few families that were significantly more sensitive than others (Fig. 1). Similar significant differences were observed among families in the half-sib experiment ($F_{22,198} = 2.60$; $P = 0.0002$; Fig. 1).

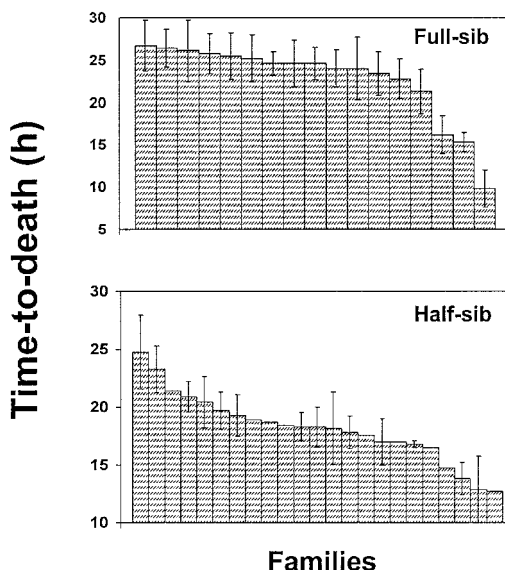


Fig. 1. Vertical bars depict the mean time to death for each family. Top graph represents full-sib families, bottom graph represents half-sib families. Vertical lines on each bar represent ± 1 standard error. Tadpoles in families with no error bars all died at the same time.

No significant correlation existed between TTD and mass ($r = -0.0385$, $P = 0.6180$) for full-sib families (Fig. 2). There was, however, a significant negative correlation among half-sib families between TTD and tadpole mass; larger tadpoles were more sensitive (i.e., had lower TTDs; $r = -0.2878$; $P < 0.0001$; Fig. 2). Tadpoles used in the full-sib analysis were more similar in size to one another than tadpoles in the half-sib portion of the experiment (Fig. 2), which may account for no significant correlations being observed among full-sibs.

Heritability of carbaryl tolerance in this population was calculated from half-sib families by using the equation $h^2 = V_A/V_T$ (Falconer and Mackay, 1996). V_A is the additive genetic variance and was derived from the variance associated with differences among the families in the half-sib experiment and V_T is the total variance observed. Heritability from our study population was calculated to be 0.285.

DISCUSSION

The causes of declining amphibian populations have been difficult to establish (Wake, 1998). Curiously, in some instances only a single species is declining while sympatric species are thriving (e.g., McAllister et al., 1994); in other instances, some populations within a species are

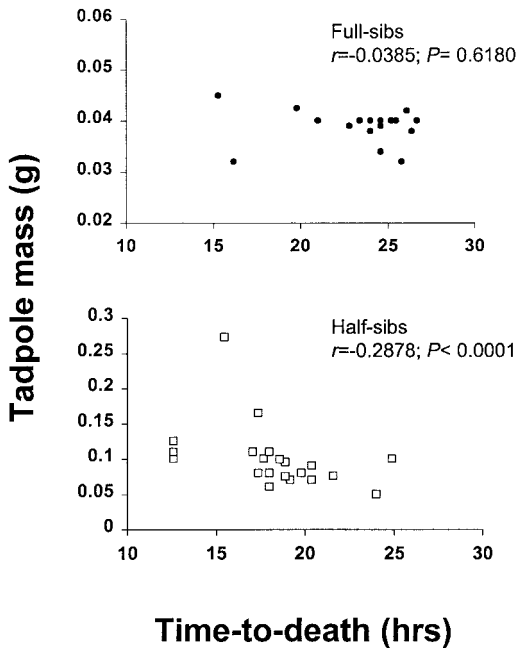


Fig. 2. Correlation between tadpole mass and time to death for full-sibs (top graph) and half-sibs (bottom graph).

declining while others remain unaffected (e.g., *Rana pipiens*, Corn and Fogleman, 1984; *Rana muscosa*, Bradford, 1991). Because not all populations or species have been affected to the same degree, it is important to identify causes for the discrepancies among populations whose environments appear physiographically similar; that is, what makes some species and populations vulnerable and others resistant? An important question is whether the observed differential declines are the result of local environmental conditions [e.g., chemical contamination, chytrid fungal infections (Berger et al., 1998), or interaction of multiple stressors] or to genetic variation among individuals, populations, and species.

Gosner and Black (1957) were the first to demonstrate that the distribution of amphibians was influenced by pH, and that individuals exposed to low pH had limited survival within the naturally acidic waters of the New Jersey Pine Barrens. Since then, many have observed that larval amphibians from populations in areas of historically low environmental pH demonstrate increased tolerance to acidic conditions (Karnes, 1983; Clark and LaZerte, 1987; Pierce and Harvey, 1987). In regions where differential sensitivity is observed, it is important to resolve whether this is the result of phenotypic or genetic variation. In other words, is decreased sen-

sitivity merely the outcome of physiological tolerance resulting from previous exposure to the stressors (natural or anthropogenic), or is it the result of natural selection for tolerance? We have documented significant variation among and within populations of *R. sphenoccephala* with respect to tolerance to an insecticide (Bridges and Semlitsch, 2000). However, it is unknown whether these differences are attributable to local adaptation to an environmental stressor rather than random effects or to geographic variation associated with other traits. Local adaptation would require that (1) environmental concentrations of insecticides were not high enough to elicit mortality of all frogs in the population, (2) insecticide tolerance within populations has a heritable genetic basis (addressed in the present study), (3) stressors persist in the environment between generations, and (4) there are fitness differences between tolerant and sensitive genotypes.

The population from which our tadpoles originated is situated on the floodplain of the Missouri River and likely receives runoff from adjacent agricultural fields. If this population has recently undergone strong and rapid selection caused by an agricultural chemical, it would be predicted that little additive genetic variance would remain within the population, and only the most resistant frogs would have survived and been present in our sample. As a consequence, this population might have a limited ability to adapt further. However, we found a significant amount of genetic variation for tolerance to carbaryl among half-sib families in this experiment, suggesting that this population may have the ability to persist in the presence of carbaryl contamination. Furthermore, because carbaryl has a mode of action that is common among insecticides (e.g., carbamates, organophosphates), it is likely that this population would respond to many chemicals in a similar manner. The relatively low heritability of carbaryl tolerance ($h^2 = 0.28$) observed in this experiment suggests that some selection may have occurred in this population as a result of insecticide exposure, thus decreasing the remaining additive genetic variance present.

Although our breeding design did not allow us to determine how much variance in response was the result of maternal effects, it is possible that factors such as egg size or egg quality may affect tolerance. Maternal effects such as these have been shown to be responsible for variation in larval traits such as growth rate (Travis, 1981; Kaplan, 1985; Newman, 1988). Pierce and Sikand (1985) have conducted the only study to our knowledge that has examined the genetic

basis for differential sensitivity of amphibians to chemicals by proportioning genetic variance. They observed that significant variation in the pH sensitivity of larval amphibians was attributable to environmental maternal effects and, to a lesser extent genetic effects, although their experimental design precluded further partitioning of the components of genetic variation (e.g., additive and nonadditive genetic variance).

The significant variation among full-sib families found in our experiment has important implications for the field of ecotoxicology. Toxicity tests are often conducted using individuals from a minimum number of egg masses (i.e., limited genetic variation), under the supposition that one sample is representative of the entire population. The significant variation observed among families in our experiment suggests that, when tadpoles from only one or a few egg masses are used, the ability to extrapolate the results to an entire population is limited. The use of laboratory cultured species (e.g., *Rana pipiens*, *Xenopus laevis*) further hinders the ability to make accurate inferences because of limited genetic variation through captive breeding. Therefore, we advocate using native amphibian species and including individuals from a number of families, and even a number of populations, whenever possible when studying the effects of stressors on individuals or populations (Clark and LaZerte, 1987; Whiteman et al., 1995).

We also advocate the use of TTD rather than LC50 tests when attempting to discern relative pesticide tolerance among groups. It has been demonstrated that there is a significant positive correlation between values derived from TTD and both 24- and 48-h LC50 values (Bridges, 1999). Furthermore, that TTD assays are relatively easy to conduct and are of shorter duration than LC50s. Additionally, because these tests require only a few individuals (one animal per replicate), TTDs are preferable over LC50s in determining sensitivity when sample sizes are necessarily small, as in the case of the half-sib families in this experiment, or when testing threatened or endangered species.

Although it would seem that larger tadpoles would be more tolerant to insecticide exposure, our data indicate that smaller individuals are more tolerant. Sanders (1970) also reported a negative correlation between size and DDT tolerance in tadpoles. Schlueter et al. (1995, 1997) noted that smaller fish are more tolerant to copper than larger fish and speculated that small fish may have size-dependent allozymes that, when present, make smaller fish more resistant to copper. If tadpoles were to have similar size-

dependent allozymes, we might expect to see decreased insecticide tolerance with increasing size. Because of the significant negative correlation between tadpole mass and TTD, it is important to expose tadpoles of similar sizes (e.g., as in the full-sib portion of our experiment) and to use tadpole mass as a covariate for all data analyses.

Increasingly, anthropogenic factors are altering habitats and causing amphibian populations to decline worldwide. To adapt to such alterations, amphibians must possess additive genetic variance for tolerance to environmental changes, because it is this variance that ultimately fuels evolutionary change and dictates population persistence. Although genetic responses may be inadequate when environmental changes are too rapid or severe, populations with larger amounts of additive genetic variance that inhabit environments experiencing slow changes may have enhanced ability to adapt to these changes. Knowledge of the amount of genetic variance within a population may help identify high-risk populations before numbers decline and may be useful in making conservation decisions, such as in restoration efforts. Because amphibians are amenable to quantitative genetic experiments, amphibian conservation efforts including determination of genetic variances should aid in predicting population persistence.

Hayes and Jennings (1986) reported that nearly every ranid frog species in the western United States has undergone declines to some degree. It has been suggested that, in regions with a high density of amphibian populations, if one population were to go extinct, it is likely to be rescued via migration from nearby populations (Wake, 1991; Blaustein et al., 1994). In the western United States, where the majority of declines have been reported, aquatic habitats for amphibians occur at a density lower than in the eastern United States and, therefore, may be more localized or fragmented, prohibiting recolonization. Furthermore, migration can be a chief source of new genetic variation for a population. Therefore, populations maintaining a high degree of migration cannot only be recolonized in instances of local extinction but will likely maintain the genetic variance necessary to cope with environmental changes. Consequently, it is possible that the effects of changing environmental conditions may be exacerbated by a low degree of genetic variance in some western U.S. amphibian populations; this may perhaps explain why these populations are declining while others are remaining stable.

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