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## Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl

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**Abstract** One of the major unanswered questions in the study of global amphibian declines is why only some species or populations suffer declines. A possible explanation is that species and populations vary in the genetic basis of their tolerance to environmental stress such as chemical contamination. The presence of genetic variation in tolerance to chemicals and in fitness traits of amphibians is essential for persistence of species populations through survival and successful reproduction in contaminated environments. We tested for the presence of genetic variation in the tolerance of amphibian larvae to the insecticide carbaryl using gray treefrog tadpoles (*Hyla versicolor*). We also assessed whether tolerance of tadpoles is negatively associated with larval performance traits directly related to adult fitness, thereby providing a test of the “cost of tolerance” hypothesis. Our results demonstrate significant variation in tolerance of tadpoles to the insecticide carbaryl within a single population of the gray treefrog, *Hyla versicolor*. Our half-sibship design indicates that variation among sires explains a significant amount of the variation in chemical tolerance thereby suggesting a heritability genetic basis. Our results also indicate the presence of a fitness tradeoff with tolerance to the chemical carbaryl being negatively correlated, or traded off, with survival of tadpoles reared in the field in the absence of the chemical. Knowledge of genetic tradeoffs with chemical tolerance under realistic environmental conditions will be important for predicting the rate of adaptation and potential for persistence of species. Finally, the partitioning of environmental and genetic variation in tolerance to chemicals is critical to identifying which species are most susceptible, the amount of genetic variance present, the potential for ad-

aptation to contaminants, and the presence of fitness tradeoffs. Such information is necessary to clearly understand the persistence of populations, and ultimately, the processes leading to species declines.

**Key words** Amphibian · Growth · *Hyla* · Insecticide · Survival

### Introduction

A recent review of the role of genetic variation in ecotoxicology has revealed two major concerns: (1) that exposure of natural populations to contaminants may result in the loss of genetic diversity, and (2) that selection for tolerant genotypes may come at the “cost of reduced fitness” in the absence of contaminant exposure (Forbes 1999). The loss of genetic diversity is expected to compromise the potential of populations to persist and respond adaptively to future episodes of selection. Fitness costs (= selection for genotypes that are less fit in other ways) associated with tolerance are less obvious and differ among those systems studied in detail, but the potential for limiting the spread of tolerant genotypes to uncontaminated areas and for reducing evolutionary responses is apparent (e.g., in plants, Shaw 1999). Both concerns have important implications for evolutionary potential and therefore, to conservation issues such as the probability of extinction and maintenance of biodiversity.

Studies of genetic variation in ecotoxicology are applicable to the globally urgent issue of species loss and population declines among amphibians (Barinaga 1990; Blaustein and Wake 1990; Blaustein et al. 1994). In a summary of the current status of amphibian declines it was noted that “airborne chemical contaminants are implicated in amphibian declines” and that “basic issues of population biology are central to an understanding of species loss and decline” (Wake 1998). Thus, questions related to the persistence of natural populations in the face of chemical contamination are quite relevant to amphibian declines.

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One of the major unanswered questions concerning amphibian declines is why only some species or populations suffer declines. A possible explanation is that species and populations vary in the genetic basis of their tolerance to environmental stress such as chemical contamination. In a recent study, Bridges and Semlitsch (2000a) found that nine species of tadpoles in the family Ranidae varied significantly in tolerance to the insecticide carbaryl thereby suggesting that species can differ in susceptibility to contaminants. Bridges and Semlitsch (2000a) also demonstrated that within a single species, *Rana sphenocephala*, there was significant variation in insecticide tolerance among geographic populations. Thus, for at least one group of amphibians, genetic variation in chemical tolerance is a possible explanation supporting patterns of decline reported in the literature.

Because populations are the primary unit of evolutionary change (Futuyma 1998), describing the presence of genetic variation for tolerance within populations in contaminated environments is ultimately important for understanding the potential for local adaptation and persistence. Although several studies report variation among or within populations of amphibians in acid tolerance (Pough 1976; Pough and Wilson 1977; Dunson and Connell 1982; Tome and Pough 1982; Cook 1983; Karnes 1983), which could reflect the presence of genetic variation, few studies have actually controlled for environmental effects using quantitative genetic designs (but see Pierce and Sikand 1985). Only one study has reported significant variation in insecticide tolerance among both full- and half-sibship families of *Rana sphenocephala* (Bridges and Semlitsch 2000b), and additionally, provided an estimate of heritability for tolerance of  $h^2=0.285$ . However, no data are available on the effects of chemical contamination on amphibian reproduction and survival in natural populations or on how amphibian populations might respond to such contamination, although the potential to adapt to chemical contamination has been well-documented in some plant (Shaw 1999) and fish (Weis et al. 1999) populations.

We address the question of whether genetic variation in chemical tolerance exists for another taxonomic group of anurans (gray treefrog, *Hyla versicolor*; Hylidae). Such data may corroborate previous results found with *Rana sphenocephala* suggesting that detectable levels of genetic variation are present in amphibian populations, and increase understanding of the basis of adaptive responses to future contamination. We also assess whether tolerance of treefrog tadpoles is negatively associated with larval performance traits directly related to adult fitness, thereby providing a test of the "cost of tolerance" hypothesis. Finally, we discuss our results in terms of the potential for adaptation, persistence of populations, and therefore general conservation issues for global amphibian declines.

## Materials and methods

### Collection of frogs and artificial fertilizations

Between 24 April and 13 May 1998, we collected 24 calling male *Hyla versicolor* from a natural population at the Baskett Wildlife Area, Boone County, Mo. On 16 May 1998, 10 female *H. versicolor* in breeding condition (i.e., in amplexus) were collected from the same population. Each female was artificially mated with two different males. Artificial fertilizations were performed on the night that females were collected. We made sperm suspensions for each male by crushing both testes in pond water in a petri dish. Ova from each female were gently squeezed into the sperm suspensions of the two males. To avoid confounding order of ovulation with effect of paternity, we placed a few ova at a time into each sperm suspension, alternating between the two males. After 12 h embryos were transferred to larger containers. On 17 May, we collected two additional breeding females and performed fertilizations in the same manner described above, thus generating a total of 12 maternal half-sibships (=24 full-sibship families, hereafter families). However, only 22 families produced enough tadpoles to be included in the experiment. All tadpoles were maintained in well water in 8-l plastic tubs and fed ground TetraMin fish flakes ad libitum until the time of testing.

### Insecticide carbaryl

Carbaryl is a carbamate insecticide that inhibits acetylcholinesterase, therefore its mode of action is similar to many currently used pesticides (e.g., organophosphates) and can serve as a model chemical with which to examine the effects of many pesticides on amphibians. Carbaryl is widely used in agricultural and forestry practices throughout the United States and Canada. It can also be found on the shelves of gardening centers under the trade name Sevin, where it is the active ingredient in many common garden insecticides and flea powders. Carbaryl has been widely used because it quickly breaks down after application (Liu et al. 1981), thus minimizing the exposure risk to non-target organisms such as amphibians. However, persistence of carbaryl varies with soil type, amount of rainfall, and pH, and its potency is known to increase directly with temperature (Boone and Bridges 1999). Aquatic habitats can become contaminated with carbaryl directly from drift from aerial spraying as well as indirectly through run-off from gardens or agricultural fields. Concentrations as high as 4.8 mg/l have been found in natural aquatic habitats (Norris et al. 1983).

We created a carbaryl stock solution by dissolving 6.018 g of powdered carbaryl (99.8% purity) in 100 ml of technical grade acetone. The measured concentration of this stock, as determined by high-pressure liquid chromatography (HPLC), was 95% of the nominal concentration. Thus, all nominal values reported in this experiment are based on the measured stock solution concentration.

### Testing tolerance (time-to-death)

The most common toxicological test used to determine tolerance to a contaminant is the  $LC_{50}$ . Using several concentrations of a chemical,  $LC_{50}$ s 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 lethal concentration of a chemical across time (Newman and Dixon 1996). We previously have demonstrated a strong significant positive correlation between TTD assays and traditional 24- or 48-h  $LC_{50}$ s of tadpoles ( $r=0.88$ ,  $P=0.009$ ,  $r=0.83$ ,  $P=0.02$ , respectively; Bridges 1999); that is, individuals with high TTDs also demonstrate high  $LC_{50}$ s, indicating that TTD assays yield qualitatively similar results. Thus, time-to-death studies are useful because they can accurately resolve which individuals are most tolerant, and thus variation *within* populations, while using fewer animals than  $LC_{50}$  tests.

Time-to-death was determined by placing individual tadpoles in 250-ml glass beakers containing 200 ml of a 30 mg/l carbaryl solution. 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 CaCO<sub>3</sub>); alkalinity 258 mg/l CaCO<sub>3</sub> in each beaker. Additional beakers filled with 200 ml well water and containing 0.1 ml technical-grade acetone served as solvent controls. Preliminary studies indicated that tadpole survival did not differ between water and solvent controls (C.M. Bridges, unpublished data); therefore, only solvent controls were used. All beakers were arranged in a water bath and maintained at 22±1°C. The large number of tadpoles in our experiment precluded testing all individuals at once. Therefore, five replicates were tested during each time period for two days, and test periods were used as blocks in the statistical analysis. All tadpoles were at stage 25 (Gosner 196019) and were not fed during exposure. No tadpoles died in the control beakers during the testing period in either block. Each of the 22 families was replicated 10 times (=10 individual beakers with one tadpole each). Mortality was determined at 3, 6, 9, 12, 18, 24, 36, 48, and 60 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.

#### Tadpole performance in field enclosures

A sample of tadpoles from 20 of the families was also reared in field enclosures in a natural pond at the Baskett Wildlife Research Area in Boone County, Missouri. Although the history of chemical contamination in this pond is unknown, the pond currently received little or no chemical contamination. Tadpoles were reared at either high (15 tadpoles) or low (5 tadpoles) density in cylindrical screen mesh (2.0 mm mesh) enclosures (15 cm diameter, 60 cm height, approx. 10 l volume). Enclosures were placed in the pond on 27 May 1998. Tadpoles from each family were randomly assigned to three replicate enclosures at each density on 29 May 1998 (total of 120 enclosures). Enclosures were checked daily for metamorphosis starting on 27 June 1998 and mean days to metamorphosis, mean body mass at metamorphosis, and percentage survival in each enclosure was recorded.

All three measures of larval performance are related to adult fitness in amphibians. High growth rates enable tadpoles to metamorphose quickly at a small size to escape drying in ephemeral ponds (Smith 1983; Newman 1988a, b), or alternatively to maximize size at metamorphosis in more permanent ponds (Wilbur and Collins 1973). Larger size at metamorphosis can result in better physiological and locomotor performance in the terrestrial environment (Pough and Kamel 1984; Goater et al. 1993), higher juvenile survival, earlier first reproduction, and larger size at first reproduction (Berven and Gill 1983; Smith 1987; Semlitsch et al. 1988; Berven 1990). Survival to metamorphosis is most strongly related to fitness because of its direct effect on juvenile recruitment and the potential for population growth (e.g., Berven 1990).

#### Data analyses

Mean time-to-death in hours was used to measure the tolerance of tadpoles from the different families to a lethal concentration of carbaryl. Time-to-death was logarithmically transformed to increase additivity of effects and equality of the variance (Snedecor and Cochran 1980). Because of variation in the size of tadpoles among families, possibly due to maternal effects, and slight differences resulting from mortality, and subsequently density-dependent growth rates, wet mass of tadpoles was used as a covariate in all analyses to control for any differences in tolerance due to body size. Also, because two males were mated to each female, males (sires) were therefore nested within females (dams). Further, initial analyses indicated that the variance between blocks (representing 2 test days) was strongly unequal and could not be reduced by transformation, thus the data were analyzed separately by block. The final model for analysis of the data was a random model (to account for the random sample of sires and dams), nested analysis of covariance. Because our breeding design was not a complete factorial of dams and sires, we could not test the dam by sire interaction. We considered the test of dam effects to represent genetic plus environmental maternal effects and the test of sire nested within dam effects to represent only genetic effects. Some portion of the sire within dam effect could be due to the unique combining ability of dam and sire genotypes, and hence their interaction, however this could not be partitioned from the total variance. However, the presence of a significant sire effect was assumed to be indicative of heritability greater than zero (Falconer 1981; Newman 1988a). Once we obtained a significant sire effect by analysis of variance, we estimated "broad-sense" heritability using the mean-square variance associated with sires (Cockerham 1963; Falconer 1981). Broad-sense heritability contains both additive and dominance genetic variances, but is useful for determining the upper limit of an adaptive response in the presence of selection, such as mortality due to chemical contamination.

Mean performance of tadpoles (mass at metamorphosis, days to metamorphosis, and survival) from each family reared in the field enclosures was correlated to mean time-to-death in the lethal concentration of carbaryl in the laboratory. Mean time-to-death of tadpoles was corrected for body size however mean mass at metamorphosis in field enclosures was not. Pearson's correlations were performed separately for high and low densities. The two families from one dam were not available for field rearing and thus could not be included in the correlation analyses.

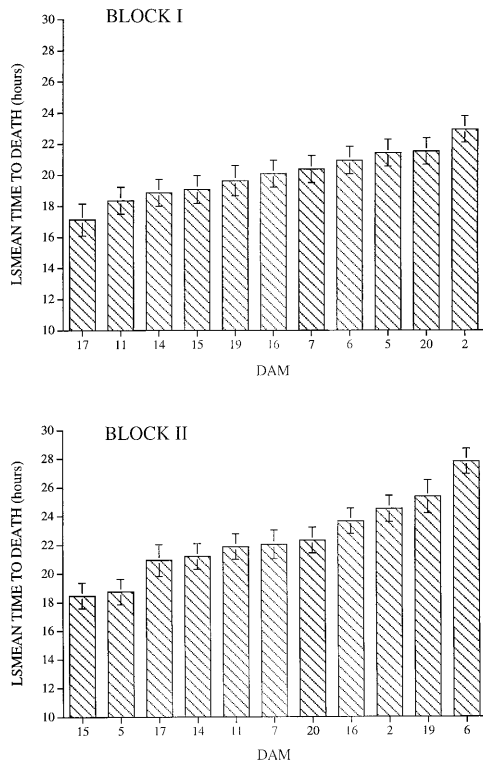
## Results

### Variation in time-to-death

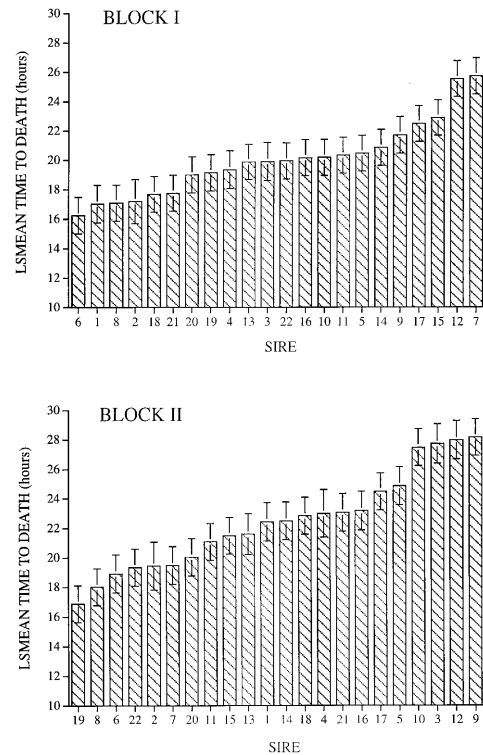
Tolerance varied from 12 to 33 h in time-to-death across the 218 individual tadpoles tested. The analysis of covariance by blocks revealed that body mass accounted for a

**Table 1** Summary of the mixed model, nested analysis of covariance of time-to-death for *Hyla versicolor* tadpoles exposed to 30.0 mg/l of carbaryl. Wet body mass of tadpoles was used as the covariate. Blocks represent two different time periods in which the tests were performed (see Materials and Methods). Type III mean squares were used to control for unequal sample sizes within treatment cells

Source of variation	Numerator		Denominator		F-value	P-value
	df	MS	df	MS		
Block I						
Body mass	1	0.159403	86	0.019204	8.30	0.0050
Dam	10	0.061599	11	0.084761	0.73	0.6888
Sire (dam)	11	0.086764	86	0.019204	4.52	0.0001
Block II						
Body mass	1	0.019403	86	0.016281	1.19	0.2780
Dam	10	0.142719	11	0.063266	2.26	0.0976
Sire (dam)	11	0.064843	86	0.016281	3.98	0.0001



**Fig. 1** Variation in time-to-death among all offspring of *Hyla versicolor* dams tested in two blocks. Values plotted are least-square means  $\pm$  one standard error

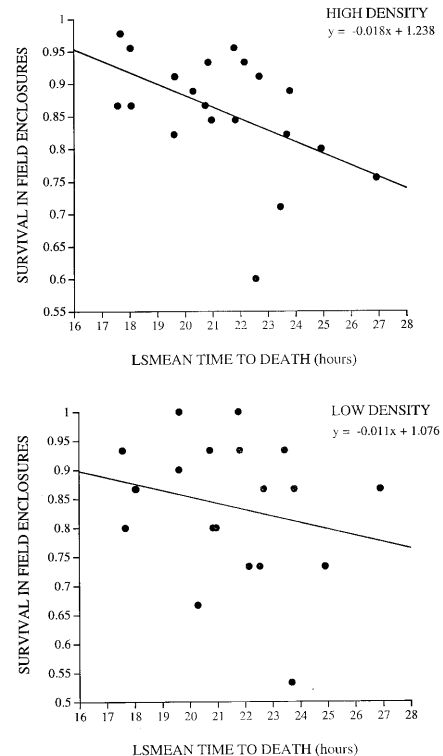


**Fig. 2** Variation in time-to-death among all offspring of *Hyla versicolor* sires tested in two blocks. Values plotted are least-square means  $\pm$  one standard error

significant amount of variation in TTD of tadpoles for block I but not for block II (Table 1). A regression analysis of body mass with TTD for both blocks combined indicated a significant positive relationship ( $F=9.45$ ,  $0.0024$ ,  $df=217$ ), but this relationship had low predictability ( $r^2=0.04$ ). Differences among dams, independent of body mass, did not account for significant variation in TTD in either block (Table 1 ; Fig. 1). However, after accounting for variation in tadpole body mass and maternal effects there was a highly significant effect of sire within dam on TTD in tadpoles for both blocks (Table 1). Because males contribute only genetic material to offspring, the significant differences among sires can be attributed to the presence of genetic variation for tolerance to the insecticide carbaryl (Fig. 2). Estimates of broad-sense heritability for both blocks yielded values of  $H^2=0.38$  and  $0.68$ .

**Tradeoff in fitness**

Mean performance of tadpoles in field enclosures varied substantially in traits directly related to adult fitness: days to metamorphosis (low density: 32–49 days, high density: 35–82 days), body mass at metamorphosis (low density: 177–339.5 mg, high density: 167–242 mg), and survival (low density: 20–100%, high density: 0–100%). The correlation analysis of larval fitness traits with TTD



**Fig. 3** Relationship between mean tadpole survival in field enclosures reared at high and low density and least-square mean time-to-death in lethal concentrations of carbaryl among offspring of 20 *Hyla versicolor* paternal families

indicated a significant negative correlation with survival of tadpoles in field enclosures, but only at the high density ( $r=-0.49$ ,  $P=0.0266$ ,  $\text{power}=0.50$ ; Fig. 3). The correlation of TTD with survival of tadpoles at low density was also negative but not statistically significant ( $r=-0.24$ ,  $P=0.3038$ ,  $\text{power}=0.52$ ; Fig. 3). No other larval traits at either density were significantly correlated with TTD, however, all but one was negative (days to metamorphosis; low density,  $r=-0.005$ ,  $P=0.9818$ ; high density,  $r=-0.07$ ,  $P=0.7667$ ; body mass at metamorphosis; low density,  $r=-0.17$ ,  $P=0.4864$ ; high density,  $r=0.06$ ,  $P=0.8155$ ).

## Discussion

Our results demonstrate significant variation in tolerance of tadpoles to the insecticide carbaryl within a single population of the gray treefrog, *Hyla versicolor*. Our maternal half-sib design indicates that maternal effects, including those due to body size, are less important than genetic effects in explaining variation in tolerance. The genetic variation we describe is independent of the confounding effect of variation in body size among tadpoles due to the environmental maternal contributions as well as other effects (e.g., egg size or yolk quality) because our analysis partitions variances due to body size (covariate) and maternal identity. Although body size does have a positive relationship to TTD and hence tolerance, and it could be important in natural populations, the relationship we found had low predictability ( $r^2=0.04$ ). Among the other studies using half-sibship designs, one found acid tolerance in *Rana sylvatica* tadpoles to be largely due to maternal effects (Pierce and Sikand 1985), whereas one found insecticide tolerance in *Rana sphenoccephala* to be due largely to paternal genetic differences (Bridges and Semlitsch 2000b). However, in the study by Pierce and Sikand (1985), confounding effects due to tadpole body size were apparently not considered. Although results are limited, it is possible that environmental maternal factors such as egg quality are more important for some contaminants (perhaps due to metabolism of internal food sources for longer periods) whereas genetic factors might be more important during exposure to an insecticide (carbaryl) as used in our study and in Bridges and Semlitsch (2000b). In addition, because amphibian species vary in the amount of yolk material deposited in eggs, the importance of maternal effects may vary with the species examined. Thus, it is unknown whether intrapopulation variation in tolerance of amphibians to chemicals is generally based on genetic variation, variation in environmental maternal factors, or both. This suggests that careful partitioning of environmental and genetic variances as well as the study of more species exposed to chemicals is critical to understanding the evolutionary potential of amphibians in contaminated environments.

Further, our results indicate the presence of a fitness tradeoff. We found that tolerance to the chemical carbar-

yl is negatively correlated, or traded off, with survival of tadpoles reared in the field in the absence of the chemical. Although this tradeoff occurs only with one of the three traits measured, survival to metamorphosis is strongly related to fitness because it affects juvenile recruitment into the breeding population and the potential for population growth (Berven 1990). A significant correlation with this important trait is surprising given that correlations were based only on 20 families yielding low power (approx. 0.50). This might explain the absence of statistical significance of survival at low density, rather than the general lack of biological importance. It is also important to note that the tradeoff or negative relationship of survival with TTD is unlikely to be due to confounding effects of body size since both TTD and survival would be positively related to body size. Thus, although the data are not overwhelming, they appear to support the hypothesis that genotypes tolerant to chemical contamination are less fit in other ways. For example, exposure to contamination may select for tolerant genotypes and thereby select against genotypes of higher competitive ability, resulting in an overall reduction of fitness relative to uncontaminated (or pre-contaminated) populations. Survival under the stresses of field conditions integrates all physiological and behavioral processes affecting growth and development, ultimately leading to metamorphic climax. It is unclear which processes led to the lower survival. However, the presence of the significant fitness tradeoff only among tadpoles reared at the high density suggests that the environmental stress of low per capita food resources (perhaps via exploitative competition) was involved. The effect this limitation may have in natural populations is unknown, but the fitness tradeoff is likely to be stronger when high density or food limitations occur and weaker when abundant food or low density conditions prevail. It is known that successful metamorphosis and recruitment for pond-breeding amphibians in uncontaminated areas is episodic over time with favorable growth conditions for each species occurring only periodically (Semlitsch et al. 1996). The presence of a chemical contaminant that also reduces the algal food supply (e.g., a broad spectrum herbicide) would likely exacerbate the fitness tradeoff by reducing growth and development rates (e.g., Fioramonti et al. 1997). Knowledge of genetic tradeoffs with tolerance under realistic environmental conditions will be important for predicting the rate of adaptation and the potential for persistence of species.

The biological link between ecotoxicological studies and conservation lies in determining how environmental contaminants can disrupt the natural regulation and persistence of species populations. A reasonable hypothesis is that variation in natural environmental factors and genetic variation among populations can interact with anthropogenic stressors, such as sublethal levels of chemicals, to cause local or regional declines of amphibian populations. When considering how chemical contamination can disrupt natural processes it is important to recognize that pond-breeding amphibians exhibit complex life

cycles with aquatic larval stages for growth and development, and terrestrial adult stages for reproduction and dispersal (Wilbur 1980). Regulation of populations most likely occurs in the aquatic larval stage, hence direct application and runoff of chemicals that can find their way into aquatic habitats are potential stressors on critical regulatory processes. For example, metamorphosis from the aquatic larval habitat to the terrestrial environment is the critical step for individuals to be recruited into the breeding adult population (e.g., Berven 1990; Semlitsch et al. 1996). Because growth and development leading to metamorphosis are extremely sensitive to environmental factors, any factor – biotic or abiotic – that reduces survival or impedes the process of metamorphosis (e.g., low food, low temperature, high larval density, rapid pond drying, chemical contaminants) can reduce juvenile recruitment and the probability of population persistence. It is also important to understand that in natural populations of amphibians only 3–5% of all offspring produced annually reach metamorphosis (Semlitsch et al. 1996). Thus, anthropogenic stress from chemical contamination may be harsh enough to reduce recruitment even further in those years where metamorphosis are produced or can increase the time interval between bouts of successful recruitment. Either effect could lead to population declines and local extinctions which could be further exacerbated if adult reproductive lifespan is short or chemical contamination is persistent. Thus, partitioning environmental and genetic variation in tolerance to chemicals is critical to identifying which species are most susceptible, the amount of genetic variance present, the potential for adaptation to the contaminants, and the presence of fitness tradeoffs. Such information is necessary to clearly understand the persistence of populations, and ultimately, the processes leading to species declines.

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