

*Environmental Toxicology*

## CROSS-TOLERANCE IN AMPHIBIANS: WOOD FROG MORTALITY WHEN EXPOSED TO THREE INSECTICIDES WITH A COMMON MODE OF ACTION

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**Abstract**—Insecticide tolerance and cross-tolerance in nontarget organisms is often overlooked despite its potential to buffer natural systems from anthropogenic influence. We exposed wood frog tadpoles from 15 populations to three acetylcholine esterase-inhibiting insecticides and found widespread variation in insecticide tolerance and evidence for cross-tolerance to these insecticides. Our results demonstrate that amphibian populations with tolerance to one pesticide may be tolerant to many other pesticides. *Environ. Toxicol. Chem.* 2013;32:xxx–xxx. © 2013 SETAC

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## INTRODUCTION

The evolution of pesticide resistance in target species has become a severe problem, costing over \$1.5 billion each year [1,2]. Pesticide resistance is particularly costly when it confers increased survival to organisms across different pesticides (known as cross-tolerance [3]). Cross-tolerance is frequently observed in pest species and most commonly among chemicals with similar modes of action, although cross-tolerance can also occur among pesticides with different modes of action [4]. Despite the evidence for cross-tolerance in pest species, cross-tolerance and its implications for nontarget organisms have been largely overlooked (but see Brausch and Smith [5]).

The possibility of cross-tolerance in nontarget species has substantial ecological and conservation implications. Pesticides can impose strong selection that affects both the traits involved in conferring tolerance and correlated traits. With pleiotropic effects, pesticide tolerance may carry a fitness cost that will reduce the health of populations after exposure [6,7]. Additionally, pesticide-imposed selection can reduce the genetic diversity of populations, which may hamper their ability to respond to changing environments (i.e., the multiple stressor hypothesis, see Jansen et al. [8]). Cross-tolerance to pesticides may dampen these negative effects of pesticide-imposed selection by reducing the episodes of selection experienced by populations. Thus, investigating cross-tolerance is important for understanding the ecological and conservation ramifications of pesticide inputs into natural systems.

Small ponds and pools provide excellent systems in which to study patterns of pesticide tolerance in nontarget species. These systems are abundant, have well-defined populations of organisms, and receive variable amounts of anthropogenic stress, including insecticides [9,10]. Furthermore, ponds and pools are the preferred habitat of many amphibians, which are experiencing worldwide population declines for a variety of hypothesized reasons, including exposure to insecticides [11,12]. Amphibian populations are often exposed to a number

of insecticides that vary in their chemical and toxic properties [13]. Moreover, amphibian tolerance to pesticides can be highly enigmatic, with tremendous variation across amphibian families [14], among species within families, and among populations [15]. This population variation in tolerance allows for the unique opportunity to investigate whether populations that are tolerant to a particular insecticide are also tolerant to other insecticides.

Given the number of available pesticides and their diverse effects across amphibians, determining general trends of tolerance of even the most commonly used pesticides can be a formidable task. However, by grouping pesticides by their modes of action, it is possible to generalize the effects of multiple pesticides [16]. Acetylcholine esterase (AChE)-inhibiting insecticides (e.g., carbaryl, malathion, and chlorpyrifos) are a group of pesticides that are often used to control pest insects [17]. These insecticides function by reversibly or irreversibly binding to AChE. With AChE inhibited, acetylcholine accumulates and causes an overstimulation of neurons and eventually mortality [18]. With the broad similarity in function across insecticides in this group, mechanisms conferring tolerance are likely to be similar. To our knowledge, the phenomenon of cross-tolerance to insecticides among amphibian populations has not been investigated.

We analyzed whether amphibian larvae show cross-tolerance to insecticides that have the same mode of action. We did this by testing the tolerance of 15 populations of wood frog (*Lithobates sylvaticus* [formerly *Rana sylvatica*]) to three commonly used AChE-inhibiting insecticides (carbaryl, chlorpyrifos, and malathion) and then determining whether population patterns of tolerance were correlated among the three insecticides.

## MATERIALS AND METHODS

*Pesticide background*

We used three commonly applied insecticides carbaryl, chlorpyrifos, and malathion. These insecticides vary in their toxicity and chemical classes but share the same mode of action. Chlorpyrifos and malathion are organophosphates, and carbaryl is a carbamate. All three of these insecticides kill organisms by

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inhibiting AChE activity and are used in the agricultural sector as well as for residential and public pest control. Moreover, all three are found in aquatic systems as a result of direct application, drift, and runoff. Although lower concentrations are more common, maximum concentrations detected in nature are carbaryl = 2,500 ppb, chlorpyrifos = 5.8 ppb, and malathion = 583 ppb [19–21].

#### *Animal collection and husbandry*

Animals were collected as early-stage embryos (Gosner stages 10–12; 10 clutches from each population) from 15 ponds across western Pennsylvania, USA (Supplemental Data, Table S1) [22]. Wood frogs typically move less than 300 m from their natal pond, and the genetic neighborhood is generally within ~1 km of the breeding pond [23–25]. The two closest ponds in our study are separated by 4 km, so it is very unlikely that frogs collected from different ponds were from the same population. All clutches were collected within a 7-d period. To avoid the confounding effects of developmental stage and size on sensitivity to insecticides, we manipulated temperature to standardize hatching time [26]. Clutches collected prior to April 11, 2011, were raised outdoors in 100-L pools filled with 90 L of aged well water (air temperature ranged from  $-1^{\circ}\text{C}$  to  $27^{\circ}\text{C}$ ). Clutches collected on April 11, 2011, were initially held indoors in 14-L plastic containers filled with 10 L of filtered water at a constant temperature of  $20^{\circ}\text{C}$  for 3 d. These indoor temperatures were warm enough to allow for faster embryonic development but are within the range of temperatures experienced by tadpoles in nature. When developmental stages of all clutches had converged, they were transferred to 100-L pools, and development continued outdoors. All 15 populations hatched within a 20-h period. To avoid density-dependent variation during the initial stages of larval development, we transferred 300 tadpoles from each population (Gosner stage 25; a safe tadpole-handling stage) to common garden pools (100-L pools filled with 90 L of aged well water) and fed them 5 g of rabbit chow weekly until they were used in the experiment (about two weeks). Wood frog mass ranged from  $0.5 \pm 0.1$  to  $0.8 \pm 0.2$  g and did not differ across the 15 different populations at the start of the experiment ( $F_{14,150} = 0.647$ ,  $p = 0.822$ ).

#### *Cross-tolerance experiment*

We used a completely randomized, factorial design with 15 populations of wood frogs crossed with four insecticide treatments (water control, 6 ppm carbaryl, 1.75 ppm chlorpyrifos, or 10 ppm malathion). We chose these concentrations of insecticides based on pilot studies; although these concentrations are unrealistically high relative to what is generally found in nature, our objective was to ensure that the tadpoles experienced some mortality from each insecticide so that we could determine whether population tolerance was correlated across the three insecticides. To make insecticide solutions, we first prepared stock solutions by diluting technical-grade chemicals (purchased from Chem Service) in ethanol (stock solution concentrations in ppm: carbaryl = 5,000, chlorpyrifos = 2,024, 2,024, malathion = 5,000). We then prepared working solutions of each insecticide by adding 7.8, 5.8, or 13  $\mu\text{l}$  of stock solution to 6.5 L of carbon-filtered, UV-irradiated water for carbaryl, chlorpyrifos, and malathion, respectively. Samples (500 ml) of each working solution were sent to an independent laboratory (Mississippi State Chemical Laboratory) to ascertain actual concentrations for each insecticide. Actual concentrations were 1.9, 0.3, and 0.4 ppm for carbaryl, chlorpyrifos, and malathion, respectively. Samples were assessed three months after the

experiment; thus, although they were stored according to established analytical methods [27], it is likely that the samples experienced some degradation prior to testing [28,29]. Solvent (ethanol [EtOH]) concentrations in working solutions were below the American Society for Testing and Materials solvent standard (0.1 ml/L EtOH) for aquatic test species (EtOH concentrations in our working solutions: carbaryl = 1.2  $\mu\text{l/L}$ , chlorpyrifos = 0.9  $\mu\text{l/L}$ , and malathion = 2  $\mu\text{l/L}$ ). We chose not to incorporate a solvent control because past studies have demonstrated that even higher solvent concentrations do not decrease tadpole survival [30].

Experimental units were Petri dishes (100 mm diameter by 20 mm height) filled with 70 ml of control or insecticide solution and 10 tadpoles. The 60 treatments were replicated five times for a total of 300 experimental units. We assessed tadpole mortality every 4 h over an 80-h period and removed the dead tadpoles from dishes at each assessment. We determined death by gently prodding tadpoles with a pipette and looking for movement. From these data, we calculated time to death (TTD) for tadpoles exposed to insecticides. Control survival was high, at 99%; thus we did not calculate TTD for the controls. For the 42% of the tadpoles that remained alive in the insecticide treatment after 80 h, we assigned them a TTD value of 80 h. Among the 42% of tadpoles that survived, 17, 40, and 43% were exposed to carbaryl, malathion, and chlorpyrifos, respectively. These static systems were renewed with treatment water every 24 h. In accordance with standard toxicity protocols, tadpoles were not fed during the experiment. All work was approved by the University of Pittsburgh's Institutional Animal Use and Care Committee (protocol 12050451).

#### *Statistical analysis*

We used generalized linear mixed models (GENLINMIXED in SPSS) to determine whether populations, insecticide treatments, and their interaction affected TTD of tadpoles. If a population-by-insecticide interaction was found, we used separate mixed models to assess whether populations differed in tolerance within each insecticide. All models included Petri dish as a random effect to account for the fact that individuals within a dish are not independent. We also used a gamma distribution with a log link function to account for the rightward skew in the data. A sequential Bonferroni adjustment was used to control  $\alpha$  for pairwise comparisons. To test for cross-tolerance, we used a Pearson's correlation test of the mean population TTD of tadpoles from the insecticide treatments. A one-tailed test was used because we had an a priori expectation that the correlation would be positive because of the insecticides sharing the same mode of action.

## RESULTS

We found a significant effect of insecticide treatment ( $F_{2,2202} = 40$ ,  $p < 0.001$ ), population ( $F_{14,2202} = 7.5$ ,  $p < 0.001$ ), and an insecticide-by-population interaction ( $F_{28,2202} = 1.9$ ,  $p = 0.003$ ) on tadpole TTD. Because there was an insecticide-by-population interaction, we conducted separate mixed models to assess population-level variation in tolerance for each insecticide. Populations varied greatly in their tolerance to carbaryl ( $F_{14,735} = 5.7$ ,  $p < 0.001$ ), chlorpyrifos ( $F_{14,735} = 2.5$ ,  $p = 0.002$ ), and malathion ( $F_{14,732} = 12.6$ ,  $p < 0.001$ ; Fig. 1). Tadpole TTD ranged from  $58.1 \pm 3.2$  to  $78.4 \pm 2.3$  h (mean  $\pm$  standard error),  $66.9 \pm 8.7$  to  $80 \pm 2.3$  h, and  $68.86 \pm 6.4$  to  $80 \pm 2.3$  h for carbaryl, chlorpyrifos, and malathion,

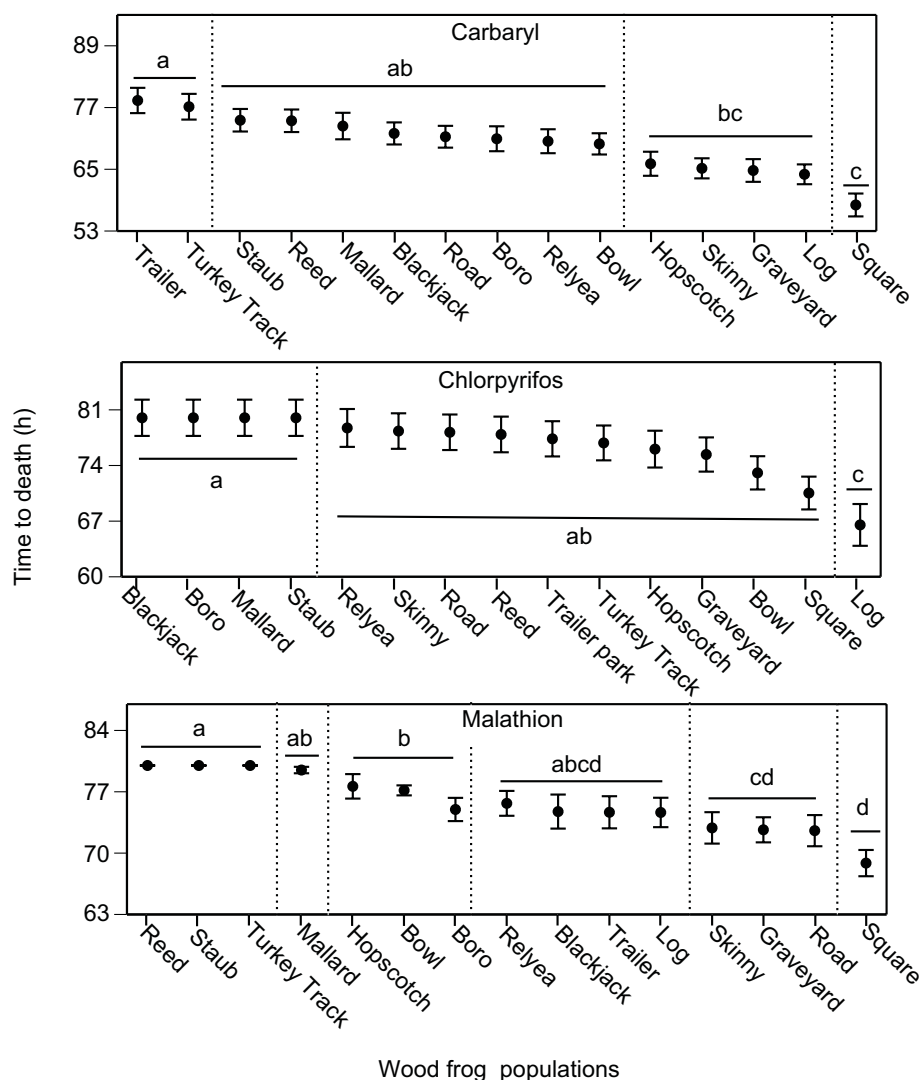


Fig. 1. Effects of carbaryl, chlorpyrifos, and malathion on tadpole time to death. Data are mean  $\pm$  1 standard error. Populations sharing similar letters did not differ in sensitivity to insecticides based on Tukey's pairwise comparisons ( $p > 0.05$ ).

respectively. In summary, there was population-level variation in sensitivity to the insecticides.

Tadpole TTD was positively correlated for two of the three pairwise combinations of insecticides (Fig. 2). Tadpole TTD with exposure to carbaryl was positively correlated with TTD with exposure to chlorpyrifos across populations ( $r = 0.56$ ,  $p = 0.015$ ). Similarly, TTDs of tadpoles exposed to carbaryl and malathion were positively correlated ( $r = 0.64$ ,  $p = 0.005$ ). In the third comparison, the TTD correlation of tadpoles exposed to malathion and chlorpyrifos was also positive, but the correlation was not quite significant ( $r = 0.36$ ,  $p = 0.09$ ).

## DISCUSSION

Cross-tolerance to multiple insecticides is hypothesized to be prevalent when insecticides share a similar mode of action [1], yet there are few examples of cross-tolerance in nontarget species [5] and no examples in amphibians. We found that wood frog populations varied in their sensitivity to three commonly used insecticides and that population-level patterns of tolerance were correlated between carbaryl and both chlorpyrifos and malathion. Although chlorpyrifos and malathion are both organophosphates with the same mode of action, the correlation

in tolerance between these two insecticides was not quite significant.

Despite sharing similar modes of action (AChE inhibition), the degree of cross-tolerance varied among carbaryl, chlorpyrifos, and malathion. In pest species, tolerance to AChE-inhibiting insecticides can develop through genetic mutations, metabolic modifications, or behavioral changes. Specifically, mutations that alter AChE target site or metabolic modifications that upregulate AChE are commonly associated with insect tolerance to carbamates and organophosphates [1,31]. Insecticides of the same modes of action can have unique consequences for different organisms [32]; thus, to better understand cross-tolerance in nontarget organisms, a critical challenge for toxicologists is exploring the mechanisms driving these varying effects.

Cross-tolerance should increase the likelihood that amphibian populations that are tolerant to one insecticide will be able to survive subsequent exposures to other insecticides that have the same mode of action. This is important because amphibians can be exposed to a wide range of insecticides during their development, with each insecticide potentially causing selection for tolerance [15]. Cross-tolerance to insecticides should reduce the episodes of selection experienced by populations and thus

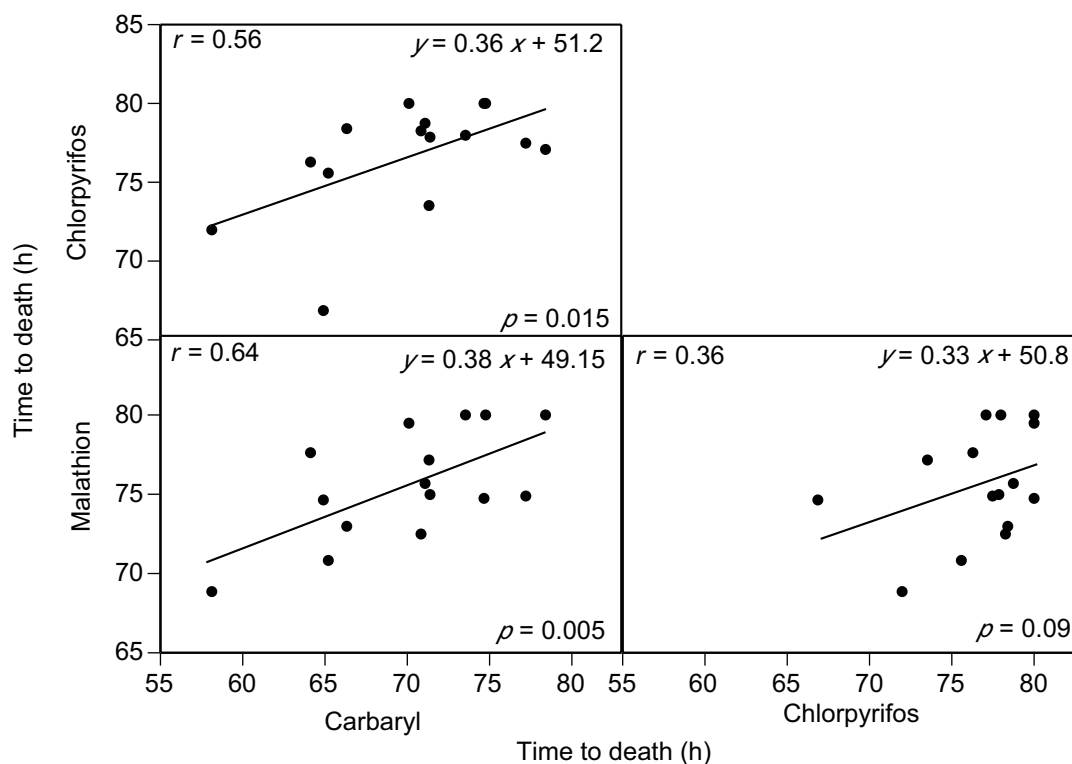


Fig. 2. Tadpole time to death correlations across carbaryl, chlorpyrifos, and malathion for 15 wood frog populations.

minimize both negative pleiotropic effects and eliminate the erosion of genetic variation in the population [33,34]. In terms of amphibian conservation, our results suggest that amphibians with cross-tolerance not only are more tolerant to the insecticide causing selection, but also may be tolerant to a wide range of similarly acting insecticides. With amphibians worldwide experiencing unprecedented declines and some of these declines being associated with insecticide use [35], quantifying the existence and prevalence of cross-tolerance may contribute important insights for conservation efforts.

#### SUPPLEMENTAL DATA

**Table S1.** (102 KB DOC).

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