**Evolved pesticide tolerance in amphibians:
Predicting mechanisms based on pesticide novelty and mode of action.**

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ABSTRACT

We examined 10 wood frog populations distributed along an agricultural gradient for their tolerance to six pesticides (carbaryl, malathion, cypermethrin, permethrin, imidacloprid, and thiamethoxam) that differed in date of first registration (pesticide novelty) and mode-of-action (MOA). Our goals were to assess whether: 1) tolerance was correlated with distance to agriculture for each pesticide, 2) pesticide novelty predicted the likelihood of evolved tolerance, and 3) populations display cross-tolerance between pesticides that share and differ in MOA. Wood frog populations located close to agriculture were more tolerant to carbaryl and malathion than populations far from agriculture. Moreover, the strength of the relationship between distance to agriculture and tolerance was stronger for older pesticides compared to newer pesticides. Finally, we found evidence for cross-tolerance between carbaryl and malathion (two pesticides that share MOA). This study provides one of the most comprehensive approaches for understanding patterns of evolved tolerance in non-pest species.

Capsule

Using 10 wood frog populations, we detected evidence for evolved tolerance, found that the evolved tolerance depends on insecticide novelty, and found evidence for cross-tolerance.

Key words: *Lithobates sylvaticus*, Organophosphates, carbamates, pyrethroids, neonicotinoid.

INTRODUCTION:

Populations face rapidly changing environments caused by multiple and diverse anthropogenic stressors including pesticides (Christensen et al., 2006; Gilliom, 2007; Rhind, 2009). While pesticides are vital tools in crop management and disease prevention, their persistent use to control pest populations has led to frequent observations of pest species evolving increased tolerance and cross-tolerance (Georghiou, 1990). To combat the evolution of pesticide tolerance and cross-tolerance, there is an increasing push to develop new pesticides with novel modes of action to slow the evolutionary process in pest populations (Moffat, 1993). Consequently, non-target populations are increasingly exposed to a broad range of pesticides that vary in mode of action and frequency of use (Grube et al., 2011; Newman, 2010; Ritter, 2009). Given the complex chemical milieu that non-target populations frequently encounter in nature, there is a need to explore how pesticides influence evolutionary processes in these populations (Jansen et al., 2011).

The evidence for evolved tolerance in non-target populations continues to accumulate (Bendis and Relyea, 2014; Brausch and Smith, 2009a; Cothran et al., 2013). Indeed, past studies have documented high levels of variation in pesticide tolerance among non-target populations. For example, (Bridges and Semlitsch, 2000) demonstrated that populations of leopard frogs (*Lithobates sphenoccephalus*) vary in their tolerance to the insecticide carbaryl. Similarly, Cothran et al. (2013) and Hua et al. (2013a) found that populations of wood frogs (*L. sylvaticus*) vary substantially in their tolerance to the insecticides chlorpyrifos, carbaryl, and malathion. Further, consistent with predictions of evolved tolerance, Bendis et al. (2014) and Cothran et al. (2013) demonstrated that populations of waterfleas (*Daphnia pulex*) and wood frogs living farther from agriculture were less tolerant to a single, commonly applied insecticide (i.e.

chlorpyrifos) than populations living closer to agriculture (Jansen et al., 2011). While the evidence for evolved tolerance in non-target populations continues to accumulate, our ability to provide generalizations regarding patterns of evolved tolerance in response to multiple pesticides remains limited. Here, we investigated whether distance to agriculture could be used as a tool to predict spatial patterns in pesticide tolerance using a suite of commonly used insecticides.

In addition to demonstrating spatial patterns of evolved tolerance, we need to increase our ability to make *a priori* predictions regarding the likelihood of evolved tolerance to different pesticides (Pimentel, 2005). Because the evolution of tolerance to pesticides requires multiple generations, the likelihood of evolved tolerance is greater for pesticides that have been used for longer periods relative to more novel pesticides (Georghiou, 1990). Thus, pesticide novelty (i.e. time since registration) might be a useful predictor about the likelihood of evolutionary responses of populations to pesticides. Toward this goal, the U.S. EPA's pesticide first registration records, which indicates when the U.S. EPA registered the first product containing a particular active ingredient (EPA, 2014), provides a well-documented record of when populations might have been first exposed to certain pesticides. Using these records, we examined whether pesticide novelty was associated with the likelihood of evolved pesticide tolerance.

In the United States, there are over 600 different active ingredients that are currently registered for pesticide use (EPA, 2010). Given the diversity of chemicals that populations could potentially face, pesticide tolerance would be particularly beneficial if it also conferred cross-tolerance to other chemicals (i.e. cross-tolerance). Theory and past empirical work predicts that populations with pesticide tolerance should be similarly tolerant to pesticides that share a similar mode of action (Hua et al., 2013a; Newman, 2010). For instance, (Hua et al., 2013a)

demonstrated that populations of tadpoles that are tolerant to the acetylcholine esterase (AChE)-inhibiting insecticide carbaryl are also cross tolerant to other AChE-inhibiting insecticides (malathion and chlorpyrifos). In contrast, predictions of cross-tolerance are less straightforward across pesticides that differ in mode of action. One perspective suggests that tolerance between insecticides with different modes of action should be negatively related because adaptations to a particular pesticide can lead to energetic costs inhibiting cross-tolerance to pesticides with different modes of action (Kanga et al., 1997; Rivero et al., 2011). Alternatively, tolerance between pesticides that differ in mode of action should be positively related if tolerance is achieved via a shared detoxification mechanism. For example, (Brausch and Smith, 2009a, 2009b) demonstrate that cross-tolerance in fairy shrimp between methyl parathion (AChE-inhibiting insecticide), Cyfluthrin (Na⁺ inhibitor), and DDT (interferes with Cl⁻ channel function) was due to an increase in metabolizing enzyme production of cytochrome P450s and hydrolases, which are involved in detoxification of contaminants. If cross-tolerance to multiple pesticides is indicative of shared mechanisms for achieving tolerance, investigating patterns of cross-tolerance among insecticides that share and differ in mode of action can elucidate potential mechanisms of pesticides tolerance in populations (Georghiou, 1990; Kanga et al., 1997; Nkya et al., 2014).

We investigated patterns of pesticide tolerance in 10 wood frog populations that varied in their distance to agriculture. We selected six common insecticides (carbaryl, malathion, cypermethrin, permethrin, imidacloprid, and thiamethoxam) that varied in their novelty (i.e. date of first registration) and mode of action. Our objectives were to explore: (1) the evidence for evolved tolerance to different insecticides (i.e. a negative relationship between population tolerance and distance to agriculture), (2) whether patterns of evolved tolerance are related to the

novelty of insecticides, and (3) whether evolution has resulted in cross-tolerance between insecticides that share and differ in mode of action,

METHODS:

Model system

Aquatic systems provide an excellent model for studying the evolutionary response of populations to pesticides (De Meester et al., 2005). They have well defined boundaries, are widely distributed worldwide including within agricultural systems, and are exposed to a diversity of pesticides (Declerck et al., 2006; EPA, 2014; Gilliom, 2007). Our focal species for examining pesticide tolerance was the wood frog (*L. sylvaticus*). Wood frog populations vary in their naïve tolerance to the insecticides chlorpyrifos and carbaryl, with populations living far from agriculture having higher tolerance compared to populations close to agriculture (Cothran et al., 2013; Hua et al., in press). Further, wood frogs are explosive breeders, which facilitates the collection of many individuals across multiple populations at a similar age, mass, and developmental stage (Cothran et al., 2013; Hua et al., 2013a; Relyea, 2005).

Animal collection and husbandry

We collected wood frogs from 10 populations located in western Pennsylvania, USA (Table A2). Because the genetic neighborhood for amphibians is generally within ~1 km of the breeding pond (Berven and Grudzien, 1990; Semlitsch, 2000, 1998), we chose wood frog populations that were separated by at least 4 km to minimize genetic relatedness. Early-stage embryos (i.e. egg clutches, Gosner stage 3-7; Gosner 1960) were collected from each population within a 7-d period and reared separately by population (Table A2). To control for the effects of developmental stage and size, which can affect sensitivity to pesticides, we manipulated temperature to standardize hatching time (Cothran et al., 2013; Hua et al., 2013a). Initially, all

wood frog clutches were raised outdoors in 100-L pools filled with ~90 L of aged well water (air temperature ranged from 1°C to 21°C). On 13 April, clutches collected before 7 April were chilled in a walk-in cooler to 1.6°C to slow development while clutches collected after 7 April remained in 100-L pools where they experienced warmer outdoor conditions (air temperature ranged from 11°C- 26°C). These temperature are well within the range that natural wood frog embryos encounter (Frisbie et al., 2000). After 34 hrs, embryonic development of clutches collected after 7 April equaled those collected before 7 April and they were moved back into their outdoor pools. The embryos from all 10 populations hatched (Gosner stage 20) within a 20-h period on 21 April and reached the larval stage (Gosner stage 25) on 5 May. From each population, we haphazardly selected 300 tadpoles for inclusion in the experiment. The tadpoles were transferred indoors, held in 14-L plastic containers filled with 10 L of UV filtered water (150 tadpoles/plastic container), and fed rabbit chow *ad libitum*.

Pesticide background

We chose to work with six pesticides that vary in their mode of action and first registration date (EPA, 2014). We selected two acetylcholine esterase (AChE) inhibitors (carbaryl and malathion), two pyrethroid Na⁺ channel disruptors (cypermethrin and permethrin), and two neonicotinoid acetylcholine disruptors (imidacloprid and thiamethoxam; Table A1). All pesticides are used in agricultural and residential settings (Fossen, 2006; Grube et al., 2011; Main et al., 2014).

Experimental design

On 6 May, we conducted a time-to-death (TTD) assay, which is an established toxicological measure of relative tolerance, to assess the pesticide tolerance of each population (Bridges and Semlitsch, 2000; Cothran et al., 2013; Semlitsch et al., 2000). For each population,

we exposed tadpoles to seven treatments (control or a lethal concentration of carbaryl, malathion, cypermethrin, permethrin, imidacloprid, or thiamethoxam). These 70 treatments (7 chemical treatments x 10 populations) were replicated four times for a total of 280 experimental units. Our experimental units were 100-mL, glass Petri dishes filled with either 70 mL of water (control) or 70 mL of the lethal carbaryl, malathion, cypermethrin, imidacloprid, and thiamethoxam solutions. Because our objective for the TTD assays was to cause moderate mortality over time (Newman, 2010), we chose different lethal concentrations for each pesticide based on past studies (Hua et al., 2013b) and pilot data; the concentrations were as follows: carbaryl = 25 mg/L, malathion = 9.5 mg/L, cypermethrin = 30 mg/L, permethrin = 40 mg/L, imidacloprid = 95 mg/L, and thiamethoxam = 38 mg/L. The expectation is that TTD assays, which use relatively high concentrations to assess the relative sensitivities to different insecticides, provide information regarding sublethal effects (Newman, 2010).

Keeping individuals from each population together, we randomly assigned 10 tadpoles to each experimental unit. The experimental units were held in the laboratory at 20°C on a 16:8 hr light-dark cycle with a water temperature of $19.3^{\circ}\text{C} \pm 0.03$ and pH of 8.4 ± 0.01 . We changed the water every 24 hrs with a renewal of the pesticide concentration. To assess tadpole tolerance, we monitored tadpole mortality every 2 hrs for the first 12 hrs, every 4 hrs after 12 hours, and terminated the experiment at 72 hrs. In accordance with standard toxicity tests, tadpoles were not fed during the test (ASTM 2008). We observed low mortality (0.07%) in animals exposed to the no-pesticide control in the TTD assay.

Pesticide applications

To create working solutions, we used commercial-grade chemicals for all pesticides except permethrin (Table A1). During our pilot studies, we did not cause any tadpole mortality

with concentration over 400 mg/L using commercial grade permethrin. In contrast, pilot studies using 35 mg/L of technical grade permethrin caused some mortality. Since our objective for the TTD assays was to cause moderate mortality (Newman, 2010), we chose to work with technical grade permethrin. For pesticide application details, refer to the Appendix.

To determine the actual concentrations of pesticides used in this study, we created an additional 3-L solution of each pesticide treatment using the exact methods as the experiment. We then collected and sent a 500-mL sample to the University of Connecticut's Center for Environmental Sciences and Engineering (Storrs, CT). The actual concentrations for carbaryl, malathion, cypermethrin, imidacloprid, thiamethoxam, and permethrin were 24.6, 8.1, 23.7, 107.5, 29.6, 4.1 mg/L, respectively (reporting limit = 0.5 ug/L; Table A1). Excluding permethrin, our actual concentrations were on average within 9% (1.6% to 22%) of nominal concentrations. For permethrin, the actual concentration was an order of magnitude below nominal. Permethrin has an aquatic half life ranging from 6 to 27 hrs (Toynton et al., 2009; WHO, 1990). While we stored samples according to established analytical methods (Sherma and Beroza, 1980), the samples could have experienced some degradation prior to testing (Maštovská and Lehotay, 2004; OECD, 2007). Because our experimental procedures standardize the permethrin dosage across the 10 populations, we were still able to assess the relative tolerance of the populations to permethrin.

Statistical analysis

We quantified insecticide tolerance in each population by measuring TTD when exposed to each of the six insecticides. We analyzed these TTD values by conducting a separate Cox regression analysis (SPSS 21) for each insecticide and calculating a hazard regression coefficient (b) for each of the 10 populations (Cox, 1972). Populations with larger coefficients are more

likely to experience mortality when exposed to a particular insecticide compared to populations with lower coefficient values (Walters, 2009).

To address our first objective, we conducted correlation analyses between our measure of a population's tolerance to an insecticide (regression coefficient [b]) and the population's distance to agriculture (Bendis and Relyea, 2014; Brausch and Smith, 2009b; Coors et al., 2009; Cothran et al., 2013; Nkya et al., 2014). We used Google Earth (2013, v. 7.1.2) to measure the linear distance (meters) from each pond (at the location egg masses were collected) to the nearest agricultural field. Additional details regarding how proximity to agriculture was measured are presented in the Appendix and (Hua et al., in press). Assuming that populations close to agriculture are frequently exposed to insecticides whereas populations far from agriculture are infrequently exposed, we expected a negative relationship (r_a) between population tolerance and distance to agriculture (Cothran et al., 2013). We conducted a correlation analysis for each of the six insecticides.

Our second objective was to assess whether patterns of evolved tolerance were related to the insecticide novelty. For this analysis, we used the correlation coefficients (r_a) from the first analysis as our estimates of evolved tolerance for each insecticide. The correlation coefficient, r_a , provides a measure of the strength of the relationship between tolerance and distance to agriculture for each insecticide. We calculated a one-way Pearson's correlation (r_b) on the relationship between r_a and date of first registration for each insecticide. Given that older insecticide have had more time to enact selective pressures on amphibian populations, we predicted that evolved tolerance would be more likely for older insecticides relative to newer insecticides.

Our final objective was to determine whether populations with evolved tolerance to an insecticide would also display cross-tolerance to the other insecticide with the same mode of action. We conducted a correlation analysis between insecticides that share mode of action (SPSS 21). Because we had an *a priori* prediction that populations with increased tolerance to one insecticide should have increased tolerance to other insecticides with the same mode of action, we calculated a one-way Pearson's correlation coefficient (r_c). Next, we examined the relationship between insecticides that differ in mode of action by conducting correlation analysis for all other pairwise comparisons the insecticides with different modes of action. Because we did not have an *a priori* prediction for the direction of the relationship in these analyses, we calculated a two-way Pearson's correlation coefficient (r_c). Additionally, to account for the larger number of comparisons between insecticides that differ in mode of action, we also report Bonferroni corrected (12 total; $p = 0.05/12 = 0.004$) results to account for the potential for obtaining false-positive results (type I errors; Bland and Altman, 1995).

Results

Tadpoles from each of the 10 populations varied in their tolerance to the different insecticides (survival curves of each of the populations exposed to the six insecticides are reported in Appendix Fig. A1). The strength of the relationship between TTD and distance to agriculture varied among the insecticides with different modes of actions but was similar between insecticides with similar modes of action. The relationship was the strongest for tadpoles that were exposed to the AChE-inhibiting pesticides ($r_{\text{carbaryl}} = -0.6$; $r_{\text{malathion}} = -0.5$) followed by the nAChR-inhibitors (neonicotinoid pesticides; $r_{\text{imidacloprid}} = -0.3$; $r_{\text{thiamethoxam}} = -0.2$), then the Na^+ channel inhibitors (pyrethroids; $r_{\text{cypermethrin}} = 0.2$; $r_{\text{permethrin}} = 0.3$; Fig. 1). The relationship between distance from agriculture and tolerance to carbaryl and malathion were

significant ($p = 0.03$) and marginally significant ($p = 0.09$), respectively, whereas the relationship was not close to significant for any of the other insecticides.

Using these correlation coefficients (r_b) to assess the relationship between TTD and distance to agriculture for each insecticide, we found that patterns of evolved tolerance were related to insecticide registration dates ($r^2 = 0.8$, $p = 0.02$; Fig. 2). More specifically, there was a stronger correlation (r_b) between TTD and distance to agriculture for older insecticides compared to newer insecticides.

Finally, we examined the potential of cross-tolerance between insecticides that share and differ in mode of action. When comparing the relationship between insecticides that share mode of action, we found evidence for cross-tolerance between the two AChE-inhibiting insecticides (carbaryl vs. malathion; Fig. 3A). In contrast, we found no evidence of cross-tolerance between the two Na^+ channel inhibitors (cypermethrin vs. permethrin) or between the two nAChR inhibitors (imidacloprid vs. thiamethoxam; Fig. 3A). There was limited evidence for cross-tolerance between insecticides that differ in mode of action. Of the 12 possible comparisons, there was only a significant positive relationship ($p = 0.04$) between malathion and imidacloprid ($p = 0.04$; Fig 3B). However, this relationship was not significant following Bonferroni correction ($p < 0.004$).

Discussion

Populations face rapidly changing environments caused by anthropogenic stressors such as pesticides (Christensen et al., 2006; Rhind, 2009). While the ability to evolve tolerance to pesticides can allow non-target populations to persist, our understanding of patterns of tolerance stems primarily from responses to single pesticides (Georghiou, 1990). In this study, we investigated patterns of tolerance in 10 wood frog populations using six common pesticides that

vary in date of first registration and mode of action. Our results suggest that patterns of wood frog tolerance to carbaryl and malathion are consistent with predictions of evolved tolerance. Additionally, we discovered that pesticide novelty accurately predicted patterns of tolerance in populations to multiple pesticides. Finally, we found limited evidence of cross-tolerance between insecticides that both share and differ in mode of action and we suggest that the presence and absence of cross-tolerant relationships may be useful to narrowing down or eliminating potential mechanisms of pesticide tolerance.

Despite the wealth of studies examining pesticide tolerance in pest species (Georghiou, 1990; Pimentel, 2005), far fewer studies have considered pesticide tolerance in non-target taxa (Cothran et al., 2013; Jansen et al., 2011; Weston et al., 2013). We found that the likelihood of evolved tolerance (i.e. magnitude and direction of the relationship between distance to agriculture and tolerance) varied significantly for each of the six pesticides. Specifically, we demonstrated that the oldest class of insecticides, the AChE inhibitors (carbaryl and malathion) displayed the strongest relationships between tolerance and distance to agriculture. We have previously shown that tolerance to carbaryl in wood frog populations is correlated with distance to agriculture (Hua et al. in press). However, this is the first study to explore whether the same pattern holds for other pesticides and provides one of the most comprehensive assessments of insecticide tolerance in a non-target species to date. As human reliance of pesticides continues to grow, understanding the potential for evolutionary responses of non-target taxa inadvertently exposed to pesticides has broad conservation implications (Cothran et al., 2013).

Pesticides that have been in use for decades have had more time to influence the evolution of populations compared to newer pesticides; thus, we predicted that pesticide novelty could be used to predict the likelihood of evolved tolerance in populations. Indeed, we detected a

strong relationship between evolved tolerance and the date of first registration. This novel finding suggests that the date of first registration can be used as a tool for making evolutionary predictions regarding the responses of populations to pesticide contaminants. With this discovery, a critical next step is to consider whether these patterns are repeatable across other species of amphibians, other taxonomic groups, and different contaminants. Finally, though this study focused on making predictions about the patterns of tolerance in non-target taxa inadvertently exposure to pesticides, future studies could also consider whether date of first registration can be used as a tool for understanding the evolution of tolerance in target pest populations that are directly targeted by pesticides. For example, since pest populations are directly exposed to pesticides, the predicted negative relationship between likelihood of tolerance and date of first registration may be stronger for pest species. Alternatively, it is possible that more frequent exposure to pesticides can lead to pest populations experiencing faster evolution to novel pesticides, resulting in a potential shift from a negative to a neutral or positive relationship between likelihood of tolerance and date of first registration (i.e. pest species being more tolerant to the novel pesticides relative to the older pesticides).

The design of our study also allowed us to assess patterns of cross-tolerance between the insecticides. We expected populations with tolerance to insecticides of a particular mode of action to be cross-tolerant to other insecticides that also share mode of action (Brausch and Smith, 2009b; Hua et al., 2013a; Saini et al., 1989). Consistent with our predictions and past studies of wood frogs, we found evidence of cross-tolerance between our two AChE-inhibiting pesticides (Hua et al., 2013a). Similar cross-tolerance patterns between carbaryl and malathion have been reported in other taxa (*Tribolium castaneum* and *Nephotettix cincticeps* (Champ and Campbell-Brown, 1970; Iwata and Hama, 1972). In target pest species, tolerance to AChE-

inhibiting insecticides can be achieved by either increasing AChE production or by point mutations that alter the AChE binding site (Charpentier and Fournier, 2001; Nkya et al., 2014). The results of our study, in conjunction with past work in pest species, suggests that future work focusing on AChE may lead to an understanding of the molecular mechanisms associated with tolerance to AChE-inhibiting insecticides.

While we demonstrate cross-tolerance between AChE-inhibiting insecticides, we found no evidence for cross-tolerance between the two Na⁺ inhibiting pesticides (cypermethrin; permethrin) across the wood frog populations. When considering other taxa, evidence for cross-tolerance between these two particular pyrethroid insecticides is equivocal. For example, similar to our study, limited cross-tolerance was detected between cypermethrin and permethrin in the mosquito, *Culex pipiens quinquefasciatus* (Hardstone et al., 2007). In contrast, laboratory selection of the common house fly to permethrin led to a 10,000-fold increase in cross-tolerance to cypermethrin (Liu and Yue, 2000). Pyrethoid insecticides are classified into two categories depending on the presence or absence of an α -cyano substituent (Casida et al., 1983). Pyrethroids, such as permethrin, lack the α -cyano substituent and initiate type I behavioral syndromes (restlessness, incoordination, and paralysis). In contrast, to create cypermethrin, the α -cyano substituent has been incorporated into the chemical structure of permethrin, which leads to type II behavioral syndromes (convulsions and intense hyperactivity; Casida et al., 1983; Vijverberg and Bercken, 1982). Despite having the same modes of action, permethrin and cypermethrin can initiate vastly different responses therefore it is reasonable to expect that evolutionary responses to one may not necessarily confer cross-tolerance to the other.

We also found no evidence for cross-tolerance between the two nAChR-inhibiting (imidacloprid; thiamethoxam) insecticides. In the literature, evidence for cross-tolerance is

highly variable with these chemicals. Similar to our study, no cross-tolerance was detected between imidacloprid and thiamethosam in aphids (*Aphis gossypii*; Shi et al., 2011). In contrast, Alyokhin et al. (2007) demonstrated that populations of Colorado potato beetle (*Leptinotarsa decemlineata*) that are tolerant to imidacloprid are cross-tolerant to thiamethoxam. Studies in pest populations indicate that the mechanisms for tolerance to neonicotinoid insecticides are highly diverse including enhanced metabolism by esterases, carboxylesterases, monooxygenases, target site insensitivity, and behavioral resistance (Alyokhin et al., 2008).

Given the diversity of pesticides available, one approach for simplifying predictions about multiple pesticides is to group chemicals by their mode of action (Hua et al., 2013a; Rohr et al., 2006). However, our results demonstrate that tolerance can vary widely even between chemicals that share the same mode of action. Although additional research is warranted, combining pesticides by their mode of action may not provide the most accurate assessment of tolerance. However, it is likely that the lack of evidence for cross-tolerance between the pyrethroid and neonicotinoid insecticides is due to the limited evidence for the evolution of tolerance to these pesticides. Given their relative novelty, it is possible that cross-tolerance between pyrethroid and neonicotinoid insecticides may develop given broader temporal periods.

We also assessed the potential for cross-tolerance between insecticides that differ in mode of action. One prediction is that there should be a negative relationship between pesticides that differ in mode of action since pesticides enact strong selective pressures on non-target populations, leading to cost when facing pesticides with different in modes of action (Rivero et al., 2011). In contrast, if the mechanism for achieving tolerance provides generalizable protection across multiple pesticides, then there should be a positive relationship. Overall, we found limited evidence for cross-tolerance between insecticides that differ in mode of action suggesting that,

for wood frogs, evolved tolerance to older pesticides (AChE-inhibitors) does not convey protection to the newer generation pesticides.

Summary

Given the diversity of chemicals that non-target populations frequently encounter in nature, there is a need to explore how pesticides influence evolutionary processes in nature (Jansen et al., 2011). This study demonstrated that wood frog populations in close proximity to agricultural fields have evolved greater tolerance to two AChE-inhibiting insecticides (carbaryl and malathion). With increasing evidence demonstrating non-target populations have the capability to evolve tolerance to pesticides (Brausch and Smith, 2009b; Cothran et al., 2013; Weston et al., 2013), a crucial next step is to identify whether patterns of evolutionary responses are generalizable not only across different taxa and pesticides but also in response to different anthropogenic contaminants (i.e. road salts, heavy metals).

We also demonstrated that pesticide novelty predicted patterns of tolerance across multiple pesticides. As evidence for evolved tolerance in non-target populations continues to accumulate (Bendis and Relyea, 2014; Brausch and Smith, 2009a; Cothran et al., 2013), there is a need for the development of tools that will allow us to accurately predict the likelihood that non-target populations will evolve tolerance to chemicals. Our results suggest that considering pesticide novelty may be one method for making *a priori* predictions. However, future studies that include other taxa and chemicals will be necessary to determine the generality of this method. Finally, we found limited evidence for cross-tolerance between pesticides that share and differ in MOA; there was only evidence of cross-tolerance between the oldest class of insecticides (AChE inhibitors). Future studies that continue to monitor cross-tolerance are

imperative as it is possible that over time these populations will develop tolerance to the newer-generation insecticides.

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Figure captions

Figure 1. The relationship (r_b) between the time-to-death of 10 wood frog populations and distance to agriculture (i.e. a proxy for past pesticide exposures) for 6 difference pesticides.

The date of registration for each pesticide is provided under the name of each pesticide.

Figure 2. The relationship between evolved tolerance (Pearson's r_b ; the strength of the correlation between tolerance and distance to agriculture) and the first date of each pesticide's registration for six difference pesticides.

Figure 3. Time-to-death correlations (r_a) of 10 wood frog populations between pesticides that share and differ in mode of action. For comparisons of pesticides that differ in mode of action, we used Bonferonni correction thus relationships are significant only when $p < 0.004$ (12 total comparisons; $p = 0.05/12$).

Figure 1

Figure 2

A. Cross-tolerance between pesticides that share mode of action

B. Cross-tolerance between pesticides that differ mode of action (only significant results represented)

Figure 3.