Induced Tolerance from a Sublethal Insecticide Leads to Cross-Tolerance to Other Insecticides

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S Supporting Information

ABSTRACT: As global pesticide use increases, the ability to rapidly respond to pesticides by increasing tolerance has important implications for the persistence of nontarget organisms. A recent study of larval amphibians discovered that increased tolerance can be induced by an early exposure to low concentrations of a pesticide. Since natural systems are often exposed to a variety of pesticides that vary in mode of action, we need to know whether the induction of increased tolerance to one pesticide confers increased tolerance to other pesticides. Using larval wood frogs (*Lithobates sylvaticus*), we investigated whether induction of increased tolerance to the insecticide carbaryl (AChE-inhibitor) can induce increased tolerance to other insecticides that have the same mode of action (chlorpyrifos, malathion) or a different mode of action (Na⁺ channel-interfering insecticides; permethrin, cypermethrin). We found that embryonic exposure to sublethal concentrations of carbaryl induced higher tolerance to carbaryl and increased cross-tolerance to malathion and cypermethrin but not to chlorpyrifos or permethrin. In one case, the embryonic exposure to carbaryl induced tolerance in a nonlinear pattern (hormesis). These results demonstrate that that the newly discovered phenomenon of induced tolerance also provides induced cross-tolerance that is not restricted to pesticides with the same mode of action.
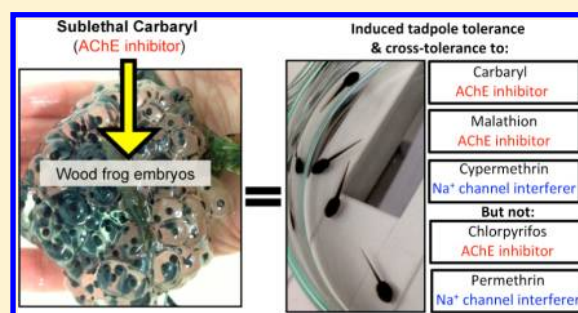
INTRODUCTION

Pesticides are important tools for disease prevention and agricultural production. However, as the use of pesticides continues to grow, understanding their effects on natural systems will become increasingly important.[1] An issue that has received substantial attention is the evolution of insecticide tolerance in targeted pest species.[2] While insecticide tolerance in pest or vector species causes over $1.5 billion of losses each year,[3] the same phenomenon may positively affect nontarget species by facilitating population persistence following insecticide contamination.[4] Interestingly, despite the conservation implications of increased insecticide tolerance, our understanding of tolerance in nontarget organisms is extremely limited.

Insecticide tolerance is almost exclusively described as a constitutive trait that arises from the microevolution of decreased susceptibility over time.[3,5,6] However, another process that allows for increased tolerance is the induction of tolerance through phenotypic plasticity, which is defined as the capacity of a single genotype to exhibit variable phenotypes in different environments.[7] In this scenario, exposure to sublethal insecticide concentrations can induce increased tolerance to a lethal concentration of the same pesticide later in life. Past studies have demonstrated that exposure to other contaminants (i.e., heavy metals) early in development can lead to positive effects later in life, but this phenomenon has rarely been considered in the context of pesticides.[8] Currently this phenomenon is only known to occur in one species of

invertebrate (i.e., a mosquito [*Aedes aegypti*])[9,10] and one species of vertebrate (wood frogs [*Lithobates sylvaticus*]).[11] It is possible that many other species have this ability, but little investigation has been conducted, likely because induced tolerance does not fit the current paradigm of constitutive pesticide tolerance. In the few studies that have been conducted, researchers have found that the concentration of initial sublethal exposure and the proximity of the population to agricultural fields (i.e., a proxy for the frequency of pesticide exposure) can affect the existence of induced tolerance.[11]

Given the enormity of available pesticides (over 1055 registered active ingredients),[12] induced pesticide tolerance would be particularly beneficial to nontarget species if it were to confer increased tolerance not only against the pesticide it first experienced but also against many other pesticides (e.g., induced cross-tolerance). Constitutive cross-tolerance is frequently observed in targeted pest species[13,14] and has been documented in a few nontarget species.[15,16] It is most common among pesticides with similar modes of action, although constitutive cross-tolerance can also occur among pesticides with different modes of action.[17,18] This raises the possibility that induced tolerance might also result in induced cross-tolerance to other pesticides. Such cross-tolerance would have

major conservation implications for populations exposed to multiple pesticides. We are not aware of any study that has tested for pesticide-induced cross-tolerance in any species.

Amphibians are excellent model organisms to study the possibility of induced cross-tolerance. Amphibians are able to respond plastically to various stressors in their environment (i.e., competitors, predators, pesticides) by altering behavioral and morphological traits.[19−21] Particular to pesticides, wood frog populations living far from agricultural fields respond to sublethal concentrations of the insecticide carbaryl by inducing increased tolerance to lethal concentrations of carbaryl later in life. Indeed, wood frogs can be induced both as embryos and as newly hatched tadpoles. Further, carbaryl is an inhibitor of acetylcholine esterase (AChE), and induced wood frogs that survive a high dose of carbaryl (18 mg/L) have increased AChE concentrations.[11] In contrast, wood frog populations living close to agricultural fields do not exhibit induced tolerance.

Amphibian populations likely encounter a number of insecticides that differ in mode of action,[22,23] and past studies have found that noninduced wood frog populations (i.e., animals that did not receive an initial sublethal exposure to carbaryl) exhibit cross-tolerance to different insecticides that share the same mode of action.[16] Thus, it is possible that amphibians induced by sublethal concentrations of one insecticide might induce increased tolerance to many other insecticides.

Using larval wood frogs, the goal of our study was to determine whether an embryonic exposure to sublethal concentrations of carbaryl, which induces higher tolerance to lethal concentrations of carbaryl later in life, could also induce higher tolerance to other insecticides that possess either the same or different modes of actions. Since the upregulation of AChE is a likely mechanism by which amphibians might induce increased tolerance to AChE inhibiting insecticides,[11] we hypothesized that embryonic exposure to sublethal concentrations of an insecticide would induce higher tolerance to a later exposure of insecticides with the same mode of action but would not induce tolerance to a later exposure of insecticides with a different mode of action.

■ METHODS AND MATERIALS

Insecticide Background. To induce tolerance, we used carbaryl, an insecticide that dominates home insecticide sales and is commonly applied in agricultural settings for pest control and disease prevention.[24] The half-life of carbaryl is 10 d at a pH of 7, and the range of concentration reported in aquatic systems is 0.73−1.5 mg/L (Table S1).[25] Carbaryl operates by reversibly binding to AChE. Inhibition of AChE causes acetylcholine to accumulate, leading to overstimulation of neurons and eventually mortality.[26,27]

To investigate the possibility of induced cross-tolerance, we chose four other commonly applied insecticides: chlorpyrifos, malathion, cypermethrin, and permethrin.[23,28] Chlorpyrifos and malathion are organophosphates, and they have the same mode of action as carbaryl (i.e., an inhibitor of AChE). However, while carbaryl binds reversibly, chlorpyrifos and malathion both bind irreversibly to AChE.[29] Cypermethrin and permethrin are pyrethroids; their mode of action is to interfere with sodium channel function.[30] Thus, the mode of action of the two pyrethroids differs from carbaryl.

Experimental Design. Using a two-phase experiment similar to that of Hua et al.,[11] we tested for induced cross-tolerance in wood frogs collected from two populations that

have been previously shown to exhibit induced tolerance when exposed to sublethal concentrations of carbaryl (Hopscotch Pond and Square Pond).[11] In phase 1 of the experiment, we exposed wood frogs to a control and two sublethal carbaryl treatments to induce tolerance. In phase 2 of the experiment, we tested whether the sublethal exposures to carbaryl induced an increase in tolerance to carbaryl and the other four insecticides later in life. We assessed tolerance by using a time to death (TTD) assay and survival analysis, which is commonly used for assessing relative tolerance among different experimental groups.[31−33]

Due to differences in oviposition timing, we collected 15 and 7 newly oviposited egg masses from Hopscotch and Square Ponds on 2 and 8 April 2013, respectively. For both populations, we immediately placed the egg masses into plastic buckets filled with ~9 L of carbon-filtered, UV-treated water (Gosner stage 3).[31] To control for the differences due to oviposition timing between the two populations, we conducted separate experiments for each population.

Phase 1- Inducing Higher Tolerance. For both populations, within 2 h of collection, we isolated 1,200 individual embryos (Gosner stage 4) by individually separating an equal number of embryos from each of the egg masses. In doing so, we took care to keep the jelly coat of each embryo intact. We then distributed individual eggs into a control or one of two sublethal carbaryl exposures (nominal concentrations: 0.5 or 1 mg/L of carbaryl; Sevin 22.5% active ingredient; CAS 63-25-2). We chose these concentrations because past studies indicate they induce tolerance without causing mortality.[11] We replicated each exposure five times each for a total of 15 experimental units. Our experimental units were 500-mL plastic containers filled with 450 mL of well water and 80 eggs per container. We reared the embryos in the laboratory at a constant temperature of 20 °C on a 16:8 light dark cycle, and the insecticide solutions were not renewed. Once all individuals reached Gosner stage 19 (Hopscotch Pond: 6 April; Square Pond: 11 April), we transferred the hatchlings to 14-L containers filled with 7-L of insecticide-free, UV-irradiated, carbon-filtered well water and made sure that we kept all individuals from each experimental unit together. The hatchlings were held in clean water until all individuals reached Gosner stage 25. Hatchlings were not fed because they were still living on their yolk reserves.

Phase 2- Lethal Exposure to Assess Induced Tolerance. *Hopscotch Pond.* Once tadpoles from Hopscotch Pond reached Gosner 25 (10 April), we crossed the three sublethal treatments from phase 1 with a control and five lethal insecticide treatments in a TTD assay. When conducting TTD assays, the objective is to cause some mortality but not complete and immediate mortality.[30] Thus, to discriminate whether prior carbaryl exposure led to increased tolerance, we chose different lethal concentrations for each insecticide based on past studies[11] and our own pilot data: 0 mg/L control, 15 mg/L of carbaryl (Sevin 22.5% active ingredient), 5 mg/L of chlorpyrifos (technical grade; CAS 523-15-07-8), 15 mg/L of malathion (technical grade; CAS 121-75-5), 0.03 mg/L of cypermethrin (Hot Shot 26% a.i.; CAS 523-15-07-8), and 0.1 mg/L of permethrin (technical grade; CAS 526-45-53-1). Using a factorial, completely randomized design, this produced 18 treatments replicated five times each, for a total of 90 experimental units.

The experimental units were 100-mL, glass Petri dishes filled with either 70 mL of water (control) or 70 mL of the lethal
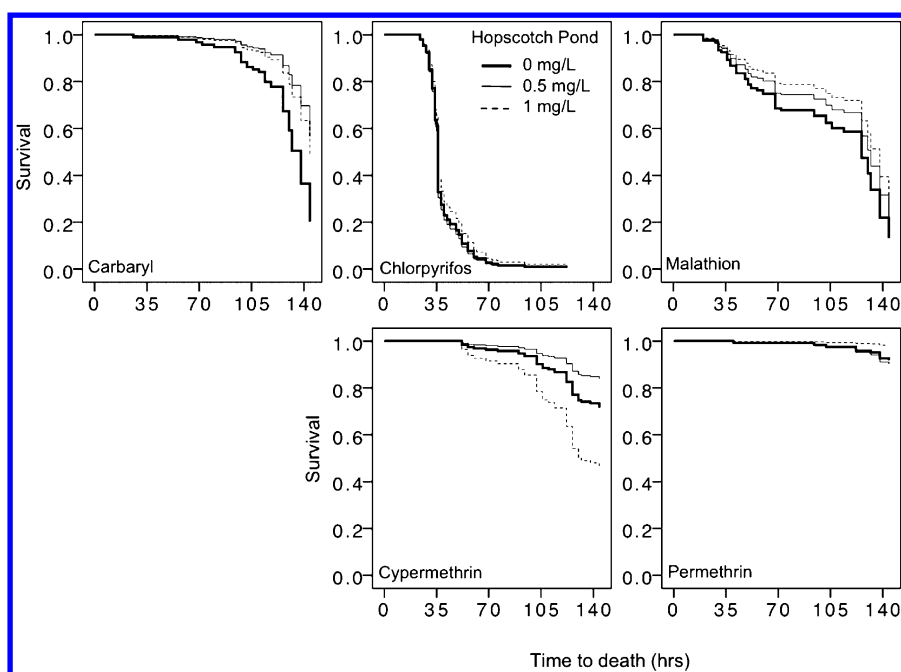
Figure 1. The survival of wood frog tadpoles from Hopscotch Pond over time after being exposed to different sublethal concentrations of carbaryl as embryos and a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin as tadpoles.

insecticide solution. Keeping individuals from phase I replicates together, we haphazardly assigned 10 tadpoles to either the no-insecticide control or a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin. We conducted water changes every 24 h with a renewal of the pesticide concentrations at each water change. To assess tadpole tolerance using TTD, we monitored tadpole mortality every 4 h and terminated the experiment after 120 h. In accordance with standard toxicity tests, tadpoles were not fed during the test (ASTM 2008). The hatchling tadpoles had food reserves in the form of yolk as evidenced by the low mortality observed in animals exposed to the no-insecticide control in the TTD assay (Hopscotch Pond = 0%; Square Pond = 2%). All methods were approved by the University of Pittsburgh's IACUC (protocol 12050451).

Square Pond. Once tadpoles from Square Pond reached Gosner stage 25 (16 April), we conducted a TTD assay using similar methodology described for tadpoles from Hopscotch Pond. However, we made two modifications. First, due to faster mortality rates of tadpoles from Square Pond, we terminated the experiment after 96 h rather than 120 h. Second, because 0.1 mg/L of permethrin did not cause sufficient mortality in the TTD assay for Hopscotch Pond (<7% mortality), we increased the concentration of permethrin from 0.1 to 0.5 mg/L.

Insecticide Applications. For phase 1 (induction of tolerance), we created a working solution by dissolving a commercial grade solution of carbaryl (22.5% Sevin) in filtered water (pH = 7). To achieve 0.5 and 1 mg/L of carbaryl, we added 7.5 and 15 ul of commercial grade carbaryl to 3.5 L of filtered water, respectively. We added 450 mL of the carbaryl solution to each of the 500-ml experimental units.

For phase 2, we first dissolved technical-grade insecticides (malathion, chlorpyrifos, and permethrin) into an ETOH vehicle (Table S2) to create stock solutions. We did not include an ETOH vehicle control in this study since past studies have demonstrated that solvent concentrations higher than we used do not affect tadpole mortality.[34] To prepare the working

solutions of each insecticide, we added the concentrated stock solutions of malathion, chlorpyrifos, and permethrin or the formulated product of carbaryl and cypermethrin to filtered water (Table S2). We then added 70 mL of these working solutions to each of the Petri dishes. After adding the insecticide solutions, we added ten tadpoles to each Petri dish. Finally, we used filtered water to create the control solutions.

Insecticide Testing. To determine the actual concentrations of insecticides used in this study, we collected 500-mL samples of each working solution after embryos were added in phase 1 and after tadpoles were added into Petri dishes at phase 2. Actual concentrations recovered from the samples in phase 1 were close to the nominal concentrations (Table S3), so when describing our results we will refer to 0, 0.5, or 1.0 mg/L carbaryl when describing the induction treatments. In contrast, despite using appropriate pesticide mixing protocols[35] and storing samples in accordance to established analytical methods,[36] all samples from phase 2 except for the 15 ppb carbaryl treatment experienced degradation. For nominal 15 ppm carbaryl, 5 ppm chlorpyrifos, 15 ppm malathion, 0.03 ppm cypermethrin, 0.1 ppm permethrin (Square), and 0.5 ppm permethrin (Hopscotch), we recovered 13 ppm, 2 ppm, 5 ppm, 0.008 ppm, 0.06 ppm, and 0.2 ppm. Despite adding enough of each pesticide to achieve nominal concentrations (Table S2), the discrepancy between actual and nominal concentrations renders the exact concentrations used relatively uncertain. One explanation for this discrepancy is sample degradation which can occur through a variety of biological and chemical processes.[35,37] However, despite this variation in actual and nominal concentrations, all concentrations used in phase 2 caused mortality as intended; thus we were still able to determine whether exposure to sublethal carbaryl during phase 1 induced increased tolerance to lethal concentration of carbaryl and cross-tolerance to lethal concentrations of chlorpyrifos, malathion, cypermethrin, and permethrin. Thus, to prevent confusion, we will refer to these treatments simply as

Table 1. Hazard Ratios for Tadpoles from Hopscotch Pond That Had Been Previously Exposed to Three Sublethal Concentrations of Carbaryl As Embryos (0, 0.5, or 1.0 mg/L) and Then Exposed As Tadpoles to a Lethal Concentration of Carbaryl, Chlorpyrifos, Malathion, Cypermethrin, or Permethrin[a]

	embryonic carbaryl exposure hazard ratios; percent censored (p-value)		
insecticide	0 mg/L vs 0.5 mg/L	0 mg/L vs 1 mg/L	0.5 mg/L vs 1 mg/L
carbaryl	−1.02; 41% (**<0.001**)	−0.77; 35% (**0.002**)	0.22; 55% (0.47)
chlorpyrifos	0.04; 0.7% (0.84)	−0.13; 0% (0.52)	−0.25; 1% (0.22)
malathion	−0.24; 11% (0.27)	−0.5; 20% (**0.03**)	0.23; 25% (0.31)
cypermethrin	−0.64; 77% (0.15)	0.86; 59% (**0.008**)	1.5; 65% (**<0.001**)
permethrin	0.2; 91% (0.77)	−1.4; 95% (0.2)	−1.65; 92% (0.13)

[a]In each comparison, a negative hazard ratio indicates that the first embryonic concentration made tadpoles less tolerant than the second embryonic concentration. A positive hazard ratio indicates the opposite phenomenon.
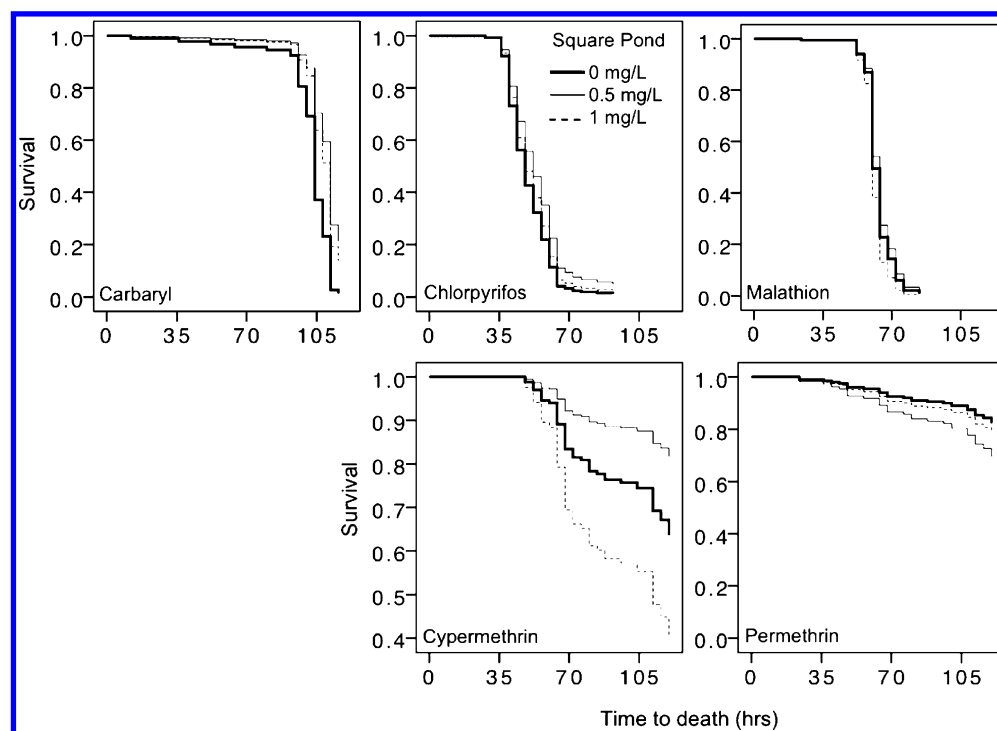

Figure 2. The survival of wood frog tadpoles from Square Pond over time after being exposed to different sublethal concentrations of carbaryl as embryos and a lethal concentration of carbaryl, chlorpyrifos, malathion, cypermethrin, or permethrin as tadpoles.

lethal carbaryl, lethal chlorpyrifos, lethal malathion, lethal cypermethrin, or lethal permethrin.

Statistical Analysis. To test for the presence of induced tolerance and induced cross-tolerance, we compared rates of tadpole survival in the TTD assay when previously exposed to three sublethal carbaryl concentrations. We separately analyzed the data for each lethal insecticide treatment using Cox's proportional hazards model for each population (SPSS).[38] Using this method of survival analysis, we then used the TTD of individual tadpoles to determine hazard ratios, which examine the probability of mortality in animals previously exposed to sublethal carbaryl concentrations (0.5 and 1 mg/L) and in phase 1 compared to animals previously exposed to 0 mg/L of carbaryl in phase 1.[11] A hazard ratio <0 indicates a decrease in the probability of mortality if the animals were previously exposed to sublethal carbaryl, whereas a hazard ratio >0 indicates the reverse outcome. Finally, we also used the Cox regression analysis to compare the probability of mortality during the TTD assay of animals previously exposed 0.5 mg/L versus 1 mg/L of carbaryl. Here a hazard ratio <0 indicates a decrease in the probability of mortality of the animals

previously exposed to 0.5 mg/L relative to 1 mg/L or carbaryl; a hazard ratio >0 indicates the reverse outcome.

■ RESULTS

Hopscotch Pond. Relative to embryonic exposure to 0 mg/L, the Cox regression analysis found that embryonic exposure to both 0.5 and 1 mg/L of carbaryl induced higher tolerance to a lethal dose of carbaryl as tadpoles (both $p < 0.001$). Hazard ratios indicate that tadpoles exposed to 0.5 and 1 mg/L of carbaryl were both more tolerant to a lethal concentration of carbaryl than tadpoles that were not exposed to carbaryl as embryos (Figure 1; Table 1). When comparing TTD of tadpoles exposed to 0.5 vs 1 mg/L of carbaryl as embryos we found no difference ($p = 0.47$; Table 1).

We then examined two insecticides that share the same mode of action with carbaryl: chlorpyrifos and malathion. Using chlorpyrifos, embryonic exposure to 0.5 and 1 mg/L of carbaryl did not significantly affect tadpole TTD compared to embryonic exposures to 0 mg/L of carbaryl ($p = 0.84$; $p = 0.52$). Using malathion, embryonic exposures to 0.5 mg/L did not significantly affect tadpole TTD when exposed to a lethal

Table 2. Hazard Ratios for Tadpoles from Square Pond That Had Been Previously Exposed to Three Sublethal Concentrations of Carbaryl As Embryos (0, 0.5, or 1.0 mg/L) and Then Exposed As Tadpoles to a Lethal Concentration of Carbaryl, Chlorpyrifos, Malathion, Cypermethrin, and Permethrin[a]

	embryonic carbaryl exposure hazard ratios; percent censored (p-value)		
insecticide	0 mg/L vs 0.5 mg/L	0 mg/L vs 1 mg/L	0.5 mg/L vs 1 mg/L
carbaryl	−1.06; 11% (**<0.001**)	−0.72; 9% (**0.001**)	0.26; 20% (0.25)
chlorpyrifos	−0.04; 2.9% (0.06)	−0.14; 2% (0.48)	0.23; 3% (0.26)
malathion	−0.15; 0% (0.45)	0.31; 0% (0.13)	0.46; 0% (**0.03**)
cypermethrin	−0.8; 73% (**0.05**)	0.69; 52% (**0.02**)	1.5; 61% (**<0.001**)
permethrin	0.63; 76% (0.14)	0.23; 80% (0.61)	−0.39; 74% (0.33)

[a]In each comparison, a negative hazard ratio indicates that the first embryonic concentration made tadpoles less tolerant than the second embryonic concentration. A positive hazard ratio indicates the opposite phenomenon.

concentration of malathion ($p = 0.27$) compared to embryonic exposures to 0 mg/L of carbaryl. However, embryonic exposure to 1 mg/L of carbaryl increased tadpole TTD when exposed to a lethal concentration of malathion compared to embryonic exposures to 0 mg/L of carbaryl ($p = 0.03$; Figure 1; Table 1). When comparing TTD of tadpoles exposed to 0.5 vs 1 mg/L of carbaryl as embryos we found no significant effect of chlorpyrifos ($p = 0.22$) or malathion (p 0.32; Table 1).

We also examined two insecticides that have different modes of action than carbaryl: permethrin and cypermethrin. Using permethrin, we found that embryonic exposure to 0.5 and 1 mg/L concentrations of carbaryl did not affect tadpole TTD when exposed to a lethal concentration of permethrin ($p = 0.77$; $p = 0.17$, respectively) compared to tadpoles that received an embryonic exposure to 0 mg/L of carbaryl. Using cypermethrin, embryonic exposure to 0.5 mg/L of carbaryl did not affect tadpole TTD when exposed to a lethal concentration of cypermethrin compared to tadpole given an embryonic exposure to 0 mg/L of carbaryl ($p = 0.14$). However, embryonic exposure to 1 mg/L of carbaryl did affect tadpole TTD when exposed to a lethal concentration of cypermethrin ($p = 0.006$). The hazard ratio analysis indicated that tadpoles exposed to 1 mg/L of carbaryl as embryos were *less tolerant* to a lethal concentration of cypermethrin compared to tadpoles exposed to 0 mg/L of carbaryl (Figure 1; Table 1). Finally, when comparing TTD of tadpoles embryonically exposed to 0.5 vs 1 mg/L of carbaryl, we found no difference when tadpoles were exposed to permethrin ($p = 0.13$), but there was a difference when tadpoles were exposed to cypermethrin ($p < 0.001$). Hazard ratio analysis indicated that tadpoles exposed to 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of cypermethrin than tadpoles exposed to 1 mg/L of carbaryl as embryos (Table 1).

Square Pond. Compared to embryonic exposures to 0 mg/L, embryonic exposure to both 0.5 and 1 mg/L of carbaryl induced higher tolerance to lethal concentrations of carbaryl as tadpoles ($p < 0.001$; $p = 0.001$, respectively). Similar to Hopscotch Pond, hazard ratios indicated that tadpoles exposed to 0.5 and 1 mg/L of carbaryl were more tolerant to a lethal concentration of carbaryl than tadpoles that were not exposed to carbaryl as embryos (Figure 2; Table 2). When comparing TTD of tadpoles exposed to 0.5 vs 1 mg/L of carbaryl as embryos we found no significant effect ($p = 0.25$; Table 2).

For insecticides that share the same mode of action with carbaryl, the Cox regression analysis found that embryonic exposure to 0.5 and 1 mg/L of carbaryl did not affect TTD relative to embryonic exposure to 0 mg/L when exposed to a lethal concentration of chlorpyrifos ($p = 0.06$; $p = 0.48$) or malathion ($p = 0.45$; $p = 0.13$). When we compared TTD of

tadpoles exposed to 0.5 vs 1 mg/L of carbaryl as embryos, we found no effect of embryonic exposures with chlorpyrifos ($p = 0.26$), but there was an effect of embryonic exposures with malathion ($p = 0.03$; Figure 2; Table 2). Hazard ratios indicated that tadpoles exposed to 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of malathion compared to tadpoles exposed to 1 mg/L carbaryl as embryos.

For insecticides that have different modes of action than carbaryl, the Cox regression analysis found that embryonic exposure to 0.5 and 1 mg/L concentrations of carbaryl did not significantly affect TTD when exposed to a lethal concentration of permethrin ($p = 0.13$; $p = 0.61$, respectively). In contrast, embryonic exposure to both 0.5 and 1 mg/L of carbaryl significantly affected tadpole TTD when exposed to a lethal concentration of cypermethrin ($p = 0.045$; $p = 0.018$, respectively). The analysis of hazard ratios indicated that tadpoles exposed to 0.5 mg/L of carbaryl as embryos were significantly more tolerant to a lethal concentration of cypermethrin compared to tadpoles with no previous exposure (Figure 2; Table 2). However, similar to Hopscotch Pond, tadpoles exposed to 1 mg/L of carbaryl as embryos became *less tolerant* to a lethal concentration of cypermethrin compared to tadpoles not exposed to carbaryl as embryos (i.e., a hormetic dose response).[39] Finally, when comparing TTD of tadpoles exposed to 0.5 vs 1 mg/L of carbaryl as embryos, we found no effect with permethrin ($p = 0.33$), but there was an effect with cypermethrin ($p < 0.001$). Hazard ratio analysis indicated that tadpoles from Square Pond exposed to 0.5 mg/L of carbaryl as embryos were more tolerant to a lethal concentration of cypermethrin than tadpoles exposed to 1 mg/L of carbaryl as embryos (Table 2).

■ DISCUSSION

Similar to the first study that discovered that sublethal exposure to carbaryl induced increased tolerance to carbaryl in wood frogs,[11] this study also demonstrated that embryonic exposure to sublethal concentrations of carbaryl induced increased wood frog tadpole tolerance to carbaryl. This was a necessary first step in testing for cross-tolerance. For tadpoles from Hopscotch Pond, the sublethal concentrations that induced tolerance (0.5 and 1 mg/L) to a subsequent lethal dose of carbaryl were consistent with the results of Hua et al.[11] In the current study, tadpoles from Square Pond exposed to 0.5 and 1 mg/L of carbaryl as embryos both exhibited higher tolerance to a lethal dose of carbaryl. In contrast, Hua et al. found that while 0.5 mg/L of carbaryl induced higher tolerance, 1 mg/L of carbaryl induced lower tolerance to carbaryl.[11] Despite the variation in the concentration of carbaryl that induces tolerance, we show

that the general phenomenon of induced higher tolerance is repeatable.

Given that an early exposure to carbaryl can induce higher tolerance to a lethal exposure to carbaryl, we hypothesized that it might also induce cross-tolerance to insecticides that share the same mode of action (i.e., malathion and chlorpyrifos). Indeed, sublethal exposures to carbaryl induced a higher tolerance to a lethal concentration of malathion. In a recent study we found that populations of wood frog tadpoles that were not exposed in the laboratory to an early sublethal concentration of carbaryl but were constitutively tolerant to carbaryl were also constitutively cross-tolerant to malathion.[16] However, the current study is the first to demonstrate that cross-tolerance can also occur through the process of induction.

The pattern of induced cross-tolerance to malathion differed between the two populations. Embryonic exposure to 1 mg/L carbaryl induced cross-tolerance to malathion in tadpoles from Hopscotch Pond but not in tadpoles from Square Pond. Due to a number of factors including population history of insecticide exposure, wood frog populations can vary in their constitutive and induced tolerance.[11,32,33] To understand the contribution of cross-tolerance in buffering nontarget organisms from chemical contaminants, future studies expanding upon the population-level factors that drive this phenomenon are an important next step.

Despite the fact that chlorpyrifos shares a similar mode of action with carbaryl, we found no evidence that sublethal embryonic exposure to carbaryl induced cross-tolerance to chlorpyrifos. The TTD of tadpoles from all phase 1 treatments exposed to lethal concentrations of chlorpyrifos was 73% and 53% earlier than the TTD of tadpoles exposed to lethal concentrations of carbaryl for Hopscotch and Square Ponds, respectively. These lower TTD values suggest that the concentration of chlorpyrifos used in our TTD assay may have been too high to allow the observation of induced tolerance. As noted earlier, the objective of a TTD assay is to cause some mortality but not immediate mortality.[30] Exposure to higher concentrations can overwhelm the ability of organisms to tolerate the insecticide.[2,40] Thus, future studies using lower concentrations of chlorpyrifos would be helpful in more fully examining whether induced cross-tolerance occurs with chlorpyrifos.

For the two insecticides that have a different mode of action than carbaryl, we hypothesized that embryonic exposure to sublethal carbaryl would not induce higher cross-tolerance. Surprisingly, for tadpoles from Square pond, we found that embryonic exposure to 0.5 mg/L of sublethal carbaryl induced higher tolerance to cypermethrin. Although not statistically significant, tadpoles from Hopscotch Pond followed a similar pattern with tadpoles exposed to 0.5 mg/L of carbaryl having the highest tolerance to cypermethrin. Although constitutive cross-tolerance to insecticides with different modes of action has been documented in several targeted pest species,[17,18] this is the first study to demonstrate that induced cross-tolerance to insecticides can occur for insecticides with different modes of action.

The process of induced cross-tolerance, which happens within just a few days, has critical implications for nontarget species population persistence. In particular, amphibian populations are declining worldwide for a number of reasons, including a hypothesized link to pesticides.[41−43] With growing human populations and increased dependence on pesticides,[1] the ability to induce higher tolerance and cross-tolerance has

significant conservation implications for the persistence of populations unintentionally exposed to pesticides. Although TTD assays examine differences in pesticide tolerance by measuring differences in survival when exposed to a high pesticide concentration over a few days, the differences in tolerance are likely to also be expressed in other traits at lower concentrations. As a result, we can hypothesize that tadpoles induced to have higher tolerance to an insecticide (as measured by survival) would also have improved performance (e.g., less impaired behaviors) when subsequently exposed to low concentrations of the insecticide. Collectively, knowledge of induced tolerance and cross-tolerance may alter how we think about and manage insecticide tolerance in target and nontarget species alike.

The induction of cross-tolerance to cypermethrin in tadpoles from Square Pond did not follow a typical dose-dependent pattern. Instead, sublethal concentrations exhibited a nonlinear hormetic response. A hormetic response occurs when exposure to a low concentration of a chemical agent or environmental factor is beneficial to an individual but a higher concentration is damaging to the individual.[44,45] Such responses are commonly found in response to environmental toxins.[46] In this study, tadpoles from Square Pond exposed to the 0.5 mg/L of carbaryl as embryos induced a higher tolerance to cypermethrin, while tadpoles exposed to 1 mg/L of carbaryl as embryos induced a lower tolerance to cypermethrin. For Hopscotch pond, though not statistically significant, the classic inverted "J" pattern of tadpole tolerance exposed to 0, 0.5, and 1 mg/L of carbaryl as embryos (low concentration inducing highest tolerance, control concentration inducing intermediate tolerance, and high concentration inducing lowest tolerance) is suggestive of a hormetic pattern.[39] Collectively, these results indicate that the concentration of insecticide inducing higher cross-tolerance may be confined to a narrow range for cypermethrin. An important challenge toward understanding the phenomenon of induced cross-tolerance is to identify the range of concentrations that lead to the induction of increased versus decreased pesticide tolerance.

Finally, although exposure to 0.5 mg/L carbaryl induced higher tolerance to cypermethrin, we found no evidence of induced cross-tolerance to permethrin, which has the same mode of action as cypermethrin. However, only 7% to 24% of tadpoles from the two populations died from exposure to permethrin. Such low mortality makes it difficult to reliably assess whether prior exposures to carbaryl affect the subsequent tolerance to permethrin. To determine the possibility of induced cross-tolerance to permethrin, future studies should use higher concentrations of permethrin to more accurately access differences in tolerance later in life caused by exposure to different sublethal treatments early in life.

In summary, the goal of this study was to investigate whether embryonic exposure to sublethal concentrations of one insecticide can induce cross-tolerance to other insecticides that have the same or different modes of actions. This is the first study to demonstrate the induction of cross-tolerance. Further, we show that induced cross-tolerance is not just limited to insecticides of the same mode of action. The patterns of induced cross-tolerance varied across the two populations, which highlight the need for future studies to consider the factors driving population-level variation in the inducibility of tolerance. Finally, there was one case where sublethal concentrations of carbaryl initiated a nonlinear hormetic dose response, demonstrating that the inducibility of cross-tolerance

may be confined to a narrow range of concentrations for some pesticides. The rapid process of the induction of higher tolerance and cross-tolerance has promising implications for the persistence of amphibian populations in the face of chemical contaminants.

With the discovery of induced higher tolerance and cross-tolerance, the critical next step is to determine the relative contribution of this phenomenon to the persistence of nontarget species exposed to insecticides. Toward this goal, we need to (1) determine the generalizability of induced tolerance across different taxa, (2) pinpoint the biotic and abiotic factors that may lead to the induction of increased versus decreased tolerance, (3) investigate the length of time that induced tolerance is retained, and (4) identify potential trade-offs associated with inducing increased tolerance. Further, this study uses survival as an indicator population persistence; future studies should also explore other indicators of postmetamorphic fitness (i.e., mass at and time to metamorphosis) as these factors have also been shown to affect amphibian population persistence. Thus, future studies should consider whether population induced to have higher tolerance for survival also have higher tolerance in other measures of performance. Addressing these factors will not only aid in conservation efforts for nontarget populations but also have broad multidisciplinary applications to the understanding of ecological and evolutionary mechanisms shaping patterns of species abundances in response to anthropogenic contaminants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information regarding insecticide sampling, chemical analyses, and actual insecticide concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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