

# Population-specific toxicity of six insecticides to the trematode *Echinoparyphium* sp.

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## SUMMARY

The ubiquitous use of pesticides has increased concerns over their direct and indirect effects on disease dynamics. While studies examining the effects of pesticides on host–parasite interactions have largely focused on how pesticides influence the host, few studies have considered the effects of pesticides on parasites. We investigated the toxicity of six common insecticides at six environmentally-relevant concentrations to cercariae of the trematode *Echinoparyphium* from two populations. All six insecticides reduced the survival of cercariae (overall difference between mortality in control *vs* pesticide exposure =  $86.2 \pm 8.7\%$ ) but not in a predictable dose-dependent manner. These results suggest that *Echinoparyphium* are sensitive to a broad range of insecticides commonly used in the USA. The lack of a clear dose-dependent response in *Echinoparyphium* highlights the potential limitations of toxicity assays in predicting pesticide toxicity to parasites. Finally, population-level variation in cercarial susceptibility to pesticides underscores the importance of accounting for population variation as overlooking this variation can limit our ability to predict toxicity in nature. Collectively, this work demonstrates that consideration of pesticide toxicity to parasites is important to understanding how pesticides ultimately shape disease dynamics in nature.

Key words: *Helisoma trivolvis*, carbaryl, malathion, cypermethrin, permethrin, imidacloprid, thiamethoxam.

## INTRODUCTION

Infectious diseases are emerging at an unprecedented rate in plant, wildlife and human populations (Daszak *et al.* 2003; Jones *et al.* 2008; Tompkins *et al.* 2015). While a diversity of factors can drive disease dynamics, there is growing awareness that human actions may be a significant contributor. Human activities have dramatically altered the environment through climate change, habitat fragmentation, and introduced species, which can directly or indirectly influence disease dynamics (Blaustein and Kiesecker, 2002; Morley *et al.* 2003, 2005; Daszak *et al.* 2003; Jones *et al.* 2008; Blaustein *et al.* 2011). Recently, the influence of agrochemicals on disease dynamics has garnered attention. For over 70 years, pesticides have been critical tools for improving human health by enhancing the yield of agricultural systems. However, our increasing reliance on pesticides to control a broad range of pest species has raised concern over whether these chemicals could be playing a role in disease outcomes (Christensen *et al.* 2006; Gilliom, 2007; Rhind, 2009). For instance, the interaction between parasites and pesticides has been proposed as a contributor to

population declines in amphibians, honeybees and salmon (Blaustein *et al.* 2011; Dietrich *et al.* 2014; Doublet *et al.* 2015). As the human population grows and anthropogenic chemicals continue to contaminate natural systems, consideration of the interactions between pesticides and parasites is fundamental to our understanding of potential factors influencing emerging infectious diseases (Marcogliese and Pietrock, 2011).

Mounting evidence suggests that pesticides can influence disease outcome directly (e.g., mortality) and indirectly (e.g., immune suppression; Carey *et al.* 1999; Kiesecker, 2002; Christin *et al.* 2004; Rohr *et al.* 2008). For instance, Coors and De Meester (2008) found that exposure to the insecticide carbaryl had a synergistic effect on *Daphnia magna* by increasing susceptibility to the parasite *Pasteuria ramosa*. Similarly, in amphibians, Rohr *et al.* (2008) found that green frog tadpoles exposed to the herbicide atrazine had significantly higher levels of encysted trematodes (*Echinostoma trivolvis*). Further, Kiesecker (2002) linked pesticide exposure to increased infection and pathology (i.e., limb malformations) in amphibians exposed to the trematode *Ribeiroia ondatrae*. These studies demonstrate that pesticides can influence disease dynamics in complex ways across a wide range of taxa. Investigating pesticide-mediated effects on the transmission phase of host–parasite interactions can

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have broad implications for understanding disease dynamics as well as protecting wildlife populations facing multiple stressors.

While growing evidence suggests that pesticides can influence disease outcomes of hosts (e.g., infection, pathology and mortality), fewer studies have considered how pesticides affect parasites (Koprivnikar *et al.* 2006; Rohr *et al.* 2008). Specifically, many parasites (e.g., trematodes) have free-living stages that are likely exposed to pesticide contamination, which can influence parasite survival or behaviour and affect transmission to the host (Koprivnikar *et al.* 2006). While past evidence suggests that exposure to pesticides during these free-swimming stages may influence parasite mortality, the existing evidence is equivocal. For example, Rohr *et al.* (2008) exposed *E. trivolvis* cercariae to two herbicides (atrazine and glyphosate) and two insecticides (malathion and carbaryl) and found that only atrazine ( $201 \mu\text{g L}^{-1}$ ) caused a significant increase in mortality. Similarly, Koprivnikar *et al.* (2006) found that  $200 \mu\text{g L}^{-1}$  but not  $20 \mu\text{g L}^{-1}$  of atrazine caused an increase in mortality relative to the control. Griggs and Belden (2008) found that  $100 \mu\text{g L}^{-1}$  of atrazine did not cause an increase in mortality until it was mixed with  $85 \mu\text{g L}^{-1}$  of another herbicide (metolachlor). Although these studies suggest that pesticides can be toxic to free-living parasites, our understanding is based on a limited number of pesticides (atrazine, glyphosate, malathion and carbaryl; Koprivnikar *et al.* 2006; Griggs and Belden, 2008; Rohr *et al.* 2008). With over 600 different active ingredients currently registered for use in the USA (EPA, 2010a), there is a need to expand our understanding of how parasites respond to a larger diversity of pesticides that vary in mode of action (Newman, 2010; Grube *et al.* 2011).

Another challenge in pesticide research that may limit our understanding of how pesticides influence disease dynamics is that the toxicity values for different chemicals can show substantial population-level variation. For example, studies across diverse taxa (e.g., water fleas, fairy shrimp, wood frogs and leopard frogs) have found that populations can dramatically differ in their tolerance to pesticides (Bridges and Semlitsch, 2000a; Brausch and Smith, 2009; Cothran *et al.* 2013; Bendis and Relyea, 2014; Hua *et al.* 2015). There is also increasing evidence that such population-level variation can be attributed to proximity to agriculture; populations located closer to agricultural fields tend to have higher pesticide tolerance compared with populations farther from agricultural fields (Bridges and Semlitsch, 2000a; Brausch and Smith, 2009; Cothran *et al.* 2013; Bendis and Relyea, 2014; Hua *et al.* 2015). Collectively, this research suggests that populations can evolve pesticide tolerance, underscoring the need to examine pesticide toxicity across populations.

However, whether similar population-level differences in pesticide tolerance exist for parasites remains relatively unexplored (but see Morley *et al.* 2003).

Traditional toxicological approaches rely on the assumption that organisms respond to pesticides in a dose-dependent manner with increasing pesticide levels causing increased mortality (i.e. 'the dose makes the poison'; Calabrese and Baldwin, 2003). However, growing evidence suggests that pesticides and other toxins commonly initiate a hormetic dose-response curve with lower and higher doses causing higher mortality compared with intermediate doses (Calabrese, 2005). For instance, using heavy metals, Morley *et al.* (2005) demonstrated that early exposure to lower concentrations of metals actually initiated a protective effect on cercariae of *Diplostomum spathaceum*. Yet to date, the majority of studies have focused on narrow ranges of concentrations and most often examine the effects of relatively high concentrations of pesticides (i.e., worst-case scenario concentrations; Koprivnikar *et al.* 2006; Griggs and Belden, 2008; Rohr *et al.* 2008). For pesticides that initiate hormetic patterns of toxicity, overlooking lethal effects of lower concentrations can obscure our understanding of ecologically relevant concentrations of pesticides on disease dynamics. To increase our ability to predict how pesticides will shape disease dynamics, there is a need for studies that test a broad range of ecologically relevant concentrations to quantify dose-response relationships.

Here, we investigated the toxicity of six common insecticides (carbaryl, malathion, cypermethrin, permethrin, imidacloprid and thiamethoxam) at six environmentally relevant concentrations to two populations of the trematode *Echinoparyphium* lineage 3 (Detwiler *et al.* 2010). To date, this study represents the most comprehensive range of concentrations and pesticides, tested on parasites and is the only study that incorporates a range of concentrations large enough to allow for the detection of non-linear responses. Using traditional toxicological experiments, we quantified dose-response curves for parasite survival for each insecticide, compared toxicity across the insecticides, and evaluated the amount of variation in toxicity estimates between the parasite populations.

## METHODS

### *Study system*

This study focused on the cercarial stage of the trematode *Echinoparyphium* lineage 3 (Detwiler *et al.* 2010). The first intermediate host of *Echinoparyphium* are snails (e.g., *Helisoma trivolvis*). *Echinoparyphium* miracidia penetrate the head foot area of the snail and form into a sporocyst (Kanev *et al.* 1995). *Echinoparyphium* then form free-living cercariae, which leave the snails and enter the

aquatic environment where they infect second intermediate hosts, including both gastropods and amphibian larvae. Once in the second intermediate host, the trematode develops into a metacercaria that encysts in the kidney of the amphibian host or the mantle and pericardium organs in molluscs. *Echinoparyphium* is widespread across North America and have also been found in Europe, Asia and Africa (Kanev *et al.* 1995). We chose to focus on the cercarial stage because this free-living stage is most likely in direct contact with pesticides for the longest period of time. Toxic effects of pesticide during this stage may directly influence parasite survival and subsequently transmission to the second intermediate host. Therefore, quantifying these effects on the cercariae stage may help to shed light on how pesticides influence disease dynamics.

#### *Animal collection and husbandry*

On 30 March and 1 April 2015, we collected 25 adult ramshorn snails (*H. trivolvis*) from two local ponds located in West Lafayette, IN, USA: Purdue Wildlife Area (PWA) and Indian Creek Pond (ICP). PWA (2636 m<sup>2</sup>) and ICP (3266 m<sup>2</sup>) are both ponds that are comparable in size and have similar amphibian and snail abundance and diversity (personal observation). PWA is surrounded by forest and ICP is surrounded by agriculture and using Google Earth (2013, v. 7.1.2), we measured the linear distance from each pond centre to the nearest agricultural field using similar methodology described in (Hua *et al.* 2015). PWA is located 278 m from agriculture and ICP is located 50 m from agriculture. Previous research has demonstrated that agricultural practices have the strongest influence on ponds within 150 m of agricultural land (Declercq *et al.* 2006). We screened each snail for *Echinoparyphium* infection by isolating individuals in 50 mL tubes filled with 45 mL of aged well water and induced the shedding of the cercariae by placing the tube under a light source for 1 h (Cohen *et al.* 1980). We selected three snails from each population that shed the highest density of cercariae and housed them separately in 2 L plastic containers with 1.5 L of filtered well water at 7 °C to slow shedding of cercariae until the start of the experiment. Three days prior to the start of the experiment, snails were slowly acclimated to 25 °C and fed rabbit chow *ad libitum*.

#### *Trematode identification*

We identified echinostome cercariae from both ponds through standard molecular sequencing of the ITS1 gene and implementation of Bayesian phylogenetics methods (Supplementary methods in Appendix). Based on our phylogeny (see online Supplementary information; Figure S1), we identified

all of our samples as belonging to *Echinoparyphium* lineage 3 as described by Detwiler *et al.* (2010).

#### *Experimental design*

Our focal pesticides were six insecticides that vary in mode of action and are commonly used in agricultural and residential settings (Fossen, 2006; Grube *et al.* 2011; Main *et al.* 2014). We chose two acetylcholine esterase inhibitors (AChE; carbaryl and malathion), two Na<sup>+</sup> channel disruptors (cypermethrin and permethrin), and two nicotinic acetylcholine receptor disruptors (nAChR; imidacloprid and thiamethoxam). For each of the two parasite populations, our pesticide treatments were a pesticide-free control and six environmentally relevant concentrations of six different pesticides (see Table A3 for concentrations). These 37 treatments were replicated four times for a total of 296 experimental units (37 treatment × 4 replicates × 2 populations). Experimental units were individual wells within a 24-well plate. To prevent cross contamination of pesticides and to limit the time needed to find and add cercariae to the appropriate experimental unit, we used a separate plate for each insecticide for each population. Thus, for a given population (ICP *vs* PWA), a single 24-well plate allowed us to test all four replicates of the six concentrations for any given insecticide (6 concentrations × 4 replicates/concentration). For the pesticide-free control treatments, we created an additional 24-well plate with four pesticide-free wells for each population. Therefore, we used a total of 14-well plates for the experiment.

#### *Pesticide solutions and testing*

To create the pesticide solutions, we first diluted commercial grade insecticide to make the stock solution for each pesticide. We added 1.5 µL of the concentrated commercial grade formulation of each insecticide to 1.5 L of aged filtered water to achieve stock solutions of 0.23, 0.5, 0.25, 0.25, 0.24 and 0.24 µg µL<sup>-1</sup> of carbaryl, malathion, cypermethrin, permethrin, imidacloprid and thiamethoxam, respectively. We then used the dilute stock solution to create the concentrations used in the experiment (working solutions). For details regarding the volume of dilute stock solutions added to create each working solution, refer to Appendix (Table A3). We sent samples of the diluted stock solution used to create each of the working solutions to Purdue Bindley Bioscience Center (West Lafayette, IN) for confirmation. For nominal concentrations of 0.23, 0.5, 0.25, 0.25, 0.24 and 0.24 µg µL<sup>-1</sup> of carbaryl, malathion, cypermethrin, permethrin, imidacloprid and thiamethoxam, actual concentrations were 0.22, 0.4, 0.3, 0.35, 0.28 and 0.28 µg µL<sup>-1</sup>, respectively.

### Cercariae addition

To obtain cercariae for the experiment, we individually placed the six infected snails (three from each population) into 50 mL Falcon tubes filled with 45 mL of aged well water and exposed them to a light source for a total of 1 h to induce the shedding of cercariae. We then mixed together cercariae from the same population. Next, we used a glass pipette to randomly collect ten cercariae for addition to each experimental unit ( $N = 2960$  total cercariae). To prevent cross-contamination among pesticide concentrations, we added cercariae from the lowest concentration to the highest concentration and switched glass pipettes after every concentration. To keep the age of cercariae as consistent as possible within a plate, we added cercariae to all experimental units within a plate before moving on to the next plate (15–20 min per plate). To control for any potential effect of cercariae age across plates, we randomized the order of plates in which we added cercariae and used newly shed cercariae every 30 min by moving snails to a new Falcon tube with 45 mL of fresh water. For the  $16 \mu\text{g L}^{-1}$  treatment of imidacloprid, due to an accidental oversight, we did not add cercariae from PWA to the treated wells.

We terminated the experiment 6 h after the addition of cercariae to the experimental unit and counted the number of cercariae still alive. A common measure of cercariae survival is the lack of movement after mechanical stimulation (i.e., a stimulus that involves pressure or distortion; Reddy *et al.* 2004) which can include nudges with a probe or water agitation (Rohr *et al.* 2008; Griggs and Belden, 2008; Koprivnikar *et al.* 2006). To assess survival in our experiment, we used a glass pipette to direct a stream of water at the cercariae. Similar to described in Rohr *et al.* (2008), cercariae were considered dead if they did not move following mechanical stimulation with the directed stream of water. We chose to terminate the experiment at 6 h because pilot and past studies found that control cercariae mortality outside of a host naturally increases at 6–8 h and cercariae efficacy is highest within the first 8 h after shedding (Rohr *et al.* 2008). In our study, the natural control mortality for cercariae from PWA and ICP at 6 h was 7.5 and 30%, respectively.

### Statistical analysis

To assess the effects of pesticides on cercariae, we first determined whether pesticides caused an increase in mortality relative to the control. For each of the six pesticides, we ran a separate analysis of variance (ANOVA; SPSS 21) examining the effect of concentration, population and their interaction on cercariae mortality. Using established toxicological protocol, each of the pesticides were

compared with a shared control (Newman, 2010). Given that we observed mortality in our control treatments for each population (PWA – 7.5% and ICP – 30%), we conducted subsequent analyses that accounted for background mortality to facilitate comparisons between the populations. For these analyses, we transformed mortality using Abbot's formula, a commonly used tool in toxicology (corrected mortality = uncorrected mortality – control mortality / 1 – control mortality; Healy, 1952). After correcting for control mortality, we then ran ANOVAs to assess the effects of concentration, population and their interaction on cercariae mortality for each pesticide.

For all analyses, we tested whether the assumptions of ANOVA were satisfied. If the assumptions were violated, we ranked transformed the data for analysis (Quinn and Keough, 2002). For all significant main effects, we conducted Tukey's pairwise comparisons for non-transformed data and Student–Newman–Keuls (SNK) means comparison tests for all ranked data. For significant interactions, we conducted Bonferroni adjusted pairwise comparisons (EMMEANS SPSS 21) to better assess the relationship between the mortality of cercariae at each concentration across populations and also the mortality of cercariae at each concentration within a population. We chose to use ANOVA instead of traditional toxicological analyses (i.e., LC50) because none of the environmentally relevant concentrations we used caused 100% mortality (a criteria for LC50s).

## RESULTS

### AChE inhibitors

**Carbaryl.** We found a significant effect on cercariae mortality caused by different carbaryl concentrations ( $F_{5,36} = 17.3$ ;  $P < 0.001$ ), a marginal effect between the two populations ( $F_{1,36} = 4.0$ ;  $P = 0.051$ ), and no significant interaction between these two variables ( $F_{5,36} = 1.2$ ;  $P = 0.33$ ). All concentrations of carbaryl increased cercariae mortality when compared with the control treatment (Fig. 1;  $P < 0.001$ ).

After accounting for control mortality, we found a significant effect of carbaryl concentration ( $F_{5,36} = 2.7$ ;  $P = 0.04$ ), but no effect of population ( $F_{1,36} = 1.9$ ;  $P = 0.17$ ) or the interaction ( $F_{5,36} = 0.74$ ;  $P = 0.60$ ) on cercariae mortality (Fig. 1). Averaged across populations,  $15 \mu\text{g L}^{-1}$  caused marginally lower mortality compared to  $5 \mu\text{g L}^{-1}$  ( $P = 0.07$ ) and  $70 \mu\text{g L}^{-1}$  ( $P = 0.06$ ; Table S2).

**Malathion.** We found a significant effect of population ( $F_{1,36} = 26.4$ ;  $P < 0.001$ ), malathion concentration ( $F_{5,36} = 4.6$ ;  $P = 0.001$ ), and the interaction ( $F_{5,36} = 7.7$ ;  $P < 0.001$ ) on cercariae mortality. In the ICP population, malathion did not influence the mortality of cercariae (Fig. 1; Table A3).



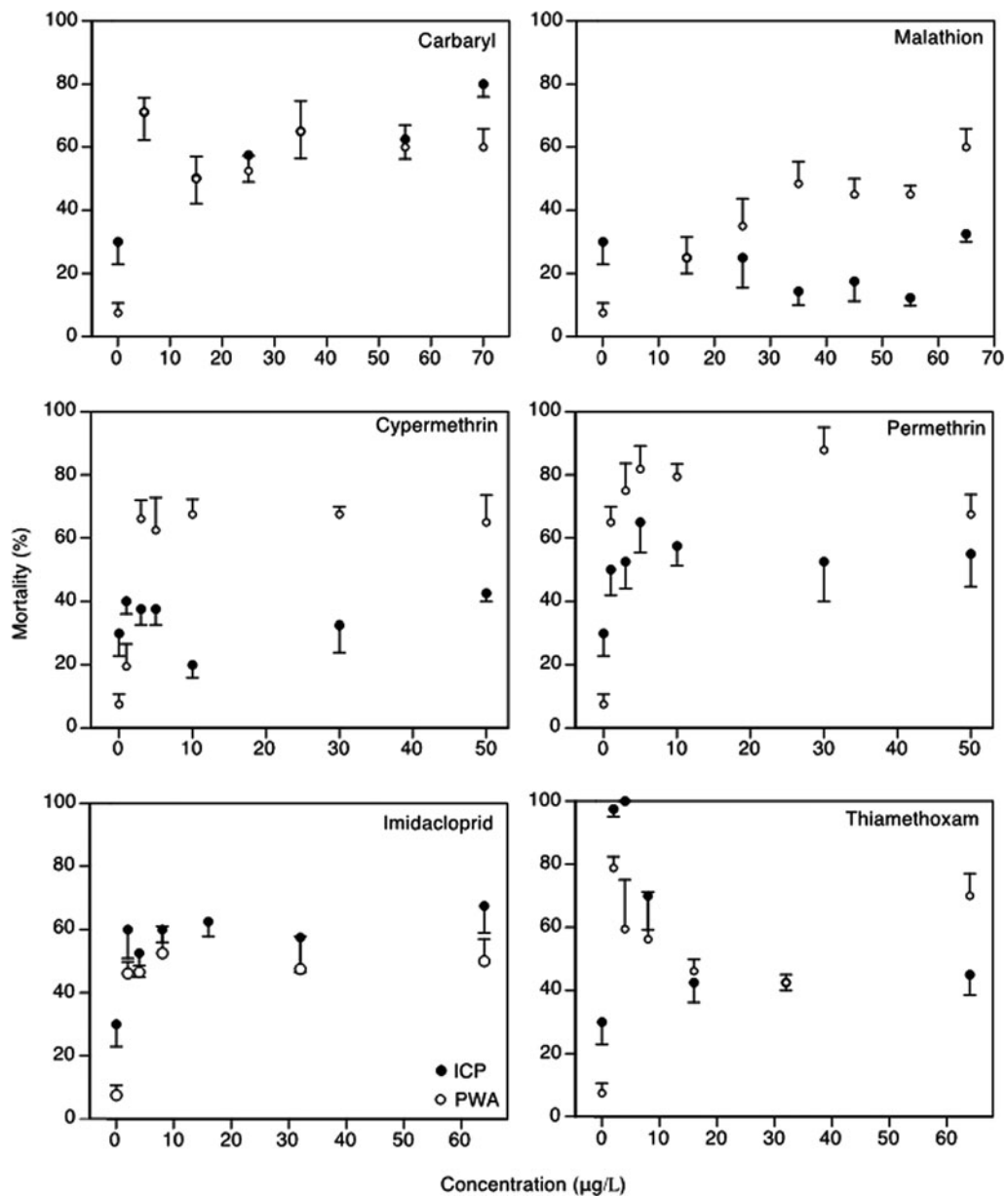


Fig. 1. The mortality (average  $\pm$  S.E.) of two populations of *Echinoparyphium* Lineage 3. cercariae to two acetylcholine esterase inhibiting insecticides (carbaryl and malathion), two Na<sup>+</sup> channel disruptors (cypermethrin and permethrin), and two nicotinic acetylcholine receptor disruptors (imidacloprid and thiamethoxam). Solid symbols represent the ICP population and open symbols represent the PWA population.

In contrast, for cercariae from PWA, all concentrations of malathion (all  $P < 0.001$ ) except for the lowest concentration ( $15 \mu\text{g L}^{-1}$ ;  $P = 0.367$ ; Table A3) caused higher mortality relative to the control.

After accounting for control mortality, we found a significant effect of population ( $F_{1,36} = 149.7$ ;  $P < 0.001$ ), malathion concentration ( $F_{5,36} = 2.8$ ;  $P = 0.029$ ) and the interaction ( $F_{5,36} = 2.9$ ;  $P = 0.02$ ) on cercariae mortality (Fig. 1). Cercariae from PWA were more susceptible to malathion than those from ICP at all concentrations ( $P < 0.04$ ). For cercariae from ICP, mortality was similar across the different malathion concentrations (all  $P = 1.0$ ). In contrast, mortality of cercariae from PWA generally

increased with increasing pesticide concentrations. Cercariae exposed to  $15 \mu\text{g L}^{-1}$  had lower mortality compared with 35, 45 and 55,  $65 \mu\text{g L}^{-1}$  ( $P = 0.02$ , 0.07, 0.07 and  $P < 0.001$ , respectively) and cercariae exposed to  $25 \mu\text{g L}^{-1}$  had significantly lower mortality compared with  $65 \mu\text{g L}^{-1}$  ( $P = 0.01$ ). All other comparisons were not significant (Table S3).

#### Na<sup>+</sup> channel disruptors

**Cypermethrin.** We found a significant effect of population ( $F_{1,36} = 27.6$ ;  $P < 0.001$ ), cypermethrin concentration ( $F_{5,36} = 11.5$ ;  $P < 0.001$ ), and the interaction ( $F_{5,36} = 11.5$ ;  $P < 0.001$ ) on cercariae mortality. We found no difference in the mortality

of cercariae from ICP in any of the cypermethrin treatments relative to the control (Fig. 1; Table A3). In contrast, for cercariae from PWA, all cypermethrin concentrations (all  $P < 0.001$ ; Table A3) except for the lowest concentration ( $1 \mu\text{g L}^{-1}$ ;  $P = 0.367$ ) caused higher mortality compared with the control.

After accounting for control mortality, we found a significant effect of population ( $F_{1,36} = 99.6$ ;  $P < 0.001$ ), cypermethrin concentration ( $F_{5,36} = 2.8$ ;  $P = 0.03$ ) and the interaction ( $F_{5,36} = 4.7$ ;  $P = 0.002$ ) on cercariae mortality (Fig. 1). At  $1 \mu\text{g L}^{-1}$ , there was no difference in mortality between the two populations ( $P = 1.0$ ). However, cercariae from PWA were more susceptible to cypermethrin than those from ICP at concentrations  $> 1 \mu\text{g L}^{-1}$  ( $P \leq 0.001$ ). For cercariae from ICP, mortality was similar across the different cypermethrin concentrations. In contrast, cercariae from PWA exposed to  $1 \mu\text{g L}^{-1}$  had significantly lower mortality compared with all other concentrations ( $P < 0.001$ ; Table S4).

**Permethrin.** We found a significant effect of population ( $F_{1,36} = 13.1$ ;  $P = 0.001$ ), permethrin concentration ( $F_{5,36} = 14.8$ ;  $P < 0.001$ ) and the interaction ( $F_{5,36} = 3.4$ ;  $P = 0.008$ ) on cercariae mortality. For ICP, exposure to  $5 \mu\text{g L}^{-1}$  of permethrin increased mortality compared with the control. There were no differences in mortality among the other treatments (Fig. 1; Table A3). For PWA, all concentrations of permethrin caused increased mortality compared with the control ( $P < 0.001$ ).

After accounting for control mortality, we found a significant effect of population ( $F_{1,36} = 36.9$ ;  $P < 0.001$ ) but no effect of permethrin concentration ( $F_{5,36} = 0.88$ ;  $P = 0.5$ ) or the interaction ( $F_{5,36} = 0.38$ ;  $P = 0.86$ ) on cercariae mortality (Fig. 1). Cercariae from PWA were more susceptible to permethrin compared to cercariae from ICP.

#### *nAChR* disruptors

**Imidacloprid.** We found a significant effect of population ( $F_{1,36} = 10.4$ ;  $P = 0.002$ ) and imidacloprid concentration ( $F_{5,36} = 9.2$ ;  $P < 0.001$ ), but no interaction ( $F_{5,36} = 0.46$ ;  $P = 0.81$ ) on cercariae mortality. In both populations, all concentrations of imidacloprid caused higher cercariae mortality relative to the control ( $P \leq 0.001$ ). After accounting for control mortality, we found no effect of population ( $F_{1,36} = 0.13$ ;  $P = 0.72$ ), imidacloprid concentration ( $F_{5,36} = 0.41$ ;  $P = 0.84$ ), or the interaction ( $F_{5,36} = 0.24$ ;  $P = 0.91$ ) on cercariae mortality (Fig. 1).

**Thiamethoxam.** We found a marginally significant effect of population ( $F_{1,36} = 2.9$ ;  $P = 0.09$ ), a significant effect of thiamethoxam concentration ( $F_{5,36} = 22.1$ ;  $P < 0.001$ ), and a significant interaction ( $F_{5,36} = 3.8$ ;  $P = 0.004$ ) on cercariae mortality. Cercariae

from ICP exposed to  $2 \mu\text{g L}^{-1}$  ( $P < 0.001$ ),  $4 \mu\text{g L}^{-1}$  ( $P < 0.001$ ) and  $8 \mu\text{g L}^{-1}$  ( $P = 0.003$ ) of thiamethoxam had higher mortality compared with the control treatment (Fig. 1). In contrast, for cercariae from PWA, all concentrations of thiamethoxam ( $P < 0.001$ ) caused higher mortality relative to the control.

Finally, after accounting for control mortality, we found a significant effect of population ( $F_{1,36} = 6.2$ ;  $P = 0.02$ ) and thiamethoxam concentration ( $F_{5,36} = 8.1$ ;  $P < 0.001$ ) on cercariae mortality (Fig. 1), but no interaction ( $F_{5,36} = 2.2$ ;  $P = 0.08$ ). Cercariae from ICP were more tolerant of thiamethoxam compared to cercariae from PWA. Cercariae exposed to  $2 \mu\text{g L}^{-1}$  had significantly higher mortality compared to all other concentrations ( $P < 0.04$ ) except for cercariae exposed to 4 and  $8 \mu\text{g L}^{-1}$  and cercariae exposed to  $4 \mu\text{g L}^{-1}$  had significantly higher mortality compared with 16 ( $P = 0.01$ ) and  $32 \mu\text{g L}^{-1}$  ( $P = 0.004$ ).

#### DISCUSSION

As wildlife diseases emerge at unprecedented rates, understanding the contribution of factors such as pesticides to disease dynamics has broad implications. While the majority of studies have examined the influence of pesticides on host susceptibility and pathology, fewer studies have assessed the effects of pesticides on parasites, especially those with free-living stages (Rohr *et al.* 2008). In this study, we investigated the toxicity of six common insecticides at six environmentally relevant concentrations on two populations of *Echinoparyphium* cercariae. At ecologically relevant concentrations, we found that these pesticides reduced the survival of cercariae but not in the expected linear dose-dependent manner. Moreover, we found that cercariae mortality was highly population and pesticide specific.

We examined the toxicity of six insecticides representing three chemical classes to the cercariae of *Echinoparyphium*. We discovered that all of the insecticides increased the mortality of cercariae. Despite the variation between the populations in the amount of mortality, these results suggest that *Echinoparyphium* are sensitive to a broad range of insecticides that are commonly used in the USA (Gilliom, 2007; Stone *et al.* 2014). While several studies have examined the effects of herbicides on cercariae (Koprivnikar *et al.* 2006; Griggs and Belden, 2008; Rohr *et al.* 2008), few have considered insecticides and none have examined the effects on *Echinoparyphium*. Rohr *et al.* (2008) found that  $9.6 \mu\text{g L}^{-1}$  of malathion and  $33.5 \mu\text{g L}^{-1}$  of carbaryl did not increase mortality of *E. trivolvis* cercariae. However, for *Echinoparyphium*, carbaryl and malathion both caused mortality across a broad range of concentrations in one or both of our populations. We also found that cypermethrin, permethrin, imidacloprid and thiamethoxam were toxic to cercariae, which has not been previously reported for these

pesticides. According to the EPA categories of toxicity for aquatic organisms (EPA, 2010b), pesticide with an LC50 value  $<0.1 \text{ mg L}^{-1}$  are considered 'very highly toxic.' While we were unable to calculate LC50 because our highest concentration did not cause 100% mortality, it is likely that cercariae would fall within this 'very highly toxic' category as concentrations we used were below  $<0.1 \text{ mg L}^{-1}$  and many caused  $>50\%$  mortality. Therefore, our results suggest that insecticides at a broad range of ecologically relevant concentrations are generally toxic to *Echinoparyphium* cercariae.

There was also substantial variation between the two cercariae populations in the toxicity of the pesticides. Overall, *Echinoparyphium* cercariae from ICP were more tolerant relative to cercariae from PWA. A growing body of research has found that tolerance is related to distance to agriculture with populations closer to agriculture having higher pesticide tolerance (Brausch and Smith, 2009; Coors *et al.* 2009; Cothran *et al.* 2013; Bendis and Relyea, 2014; Hua *et al.* 2015). Similarly, we found that the more pesticide tolerant population (ICP) was closer to agriculture (50 m) relative to the less tolerant population (PWA, 278 m). While our study is the first to document population-level variation in cercarial susceptibility to pesticides, this pattern is consistent with previous work in amphibians and zooplankton (Cothran *et al.* 2013; Bendis and Relyea, 2014; Hua *et al.* 2015). This study is consistent with the prediction that distance to agriculture plays an important role in driving population responses to pesticides, but future studies that consider these patterns across more populations are necessary. As toxicity may depend on the evolutionary history of the target population, overlooking population-level variation in susceptibility to pesticides can limit our ability to predict toxicity in nature and to make generalizations about pesticide toxicity.

Toxicological theory typically assumes dose-dependent responses such that mortality increases with increasing toxin concentration (Newman, 2010). Surprisingly, for *Echinoparyphium*, we found that cercarial mortality rarely occurred in the expected linear dose-dependent manner. The only insecticide that initiated a linear dose-dependent response curve was malathion. In contrast, for the majority of the other insecticides, mortality typically plateaued as concentrations increased. The plateau response suggests that there are a subset of individuals that are tolerant of a broad range of concentrations and future work that explores a wider range of concentrations are necessary to determine whether this tolerance is generalizable.

While we did not detect a classic dose-dependent response, two insecticides initiated a hormetic-like dose response. Hormesis is a response in which exposure to an intermediate concentration of a chemical agent is less severe relative to lower or higher

concentrations (Hayes *et al.* 2002; Mattson, 2008). Indeed, for both populations of *Echinoparyphium*, we detected a hormetic-like curve when cercariae were exposed to carbaryl and thiamethoxam. An increasing number of studies have reported hormetic responses to environmental toxins challenging the traditional dose-dependent paradigm used in environmental toxicology (Calabrese and Baldwin, 2003; Morley *et al.* 2003; Calabrese, 2005; Costantini *et al.* 2010). This is the first evidence of hormesis in trematode responses to pesticides and this discovery corroborates the evidence suggesting that hormesis is a common phenomenon across a diversity of taxa. Thus, the growing evidence for hormetic-dose responses highlights the value of considering the potential for hormesis (i.e., incorporating and assessing mortality at both low and high doses) in future studies of toxicology (Calabrese, 2005). Although traditional linear dose-dependent responses were not common in our data, it was clear that exposure to environmentally relevant pesticide concentrations increased cercariae mortality and the magnitude of this increase was dependent on insecticide type.

In conclusion, we demonstrated that *Echinoparyphium* are directly susceptible to a diversity of commonly used pesticides. However, *Echinoparyphium* rarely respond to pesticides in the expected linear dose-dependent manner and responses were highly pesticide and population specific. Collectively, we conclude that pesticides can potentially influence disease dynamics by causing direct mortality to the cercariae stage of parasites. In addition to considering the direct effects of pesticides on cercariae survival, future areas of research should consider: (1) how pesticides influence infection success, i.e. the number of metacercariae in the host, (2) more parasite populations and (3) additional parasite species. Further, to begin developing generalizations about how pesticides may directly influence cercariae and ultimately how they may shape disease dynamics in nature, future studies should also consider a broader range of pesticide concentrations and working with insecticides cocktails to explore additive, synergistic or antagonistic effects. Finally, pesticides can have direct and indirect effects on each player in the host-parasite interaction. However, we did not consider the effects of these pesticides on intermediate hosts. As suggested by past studies, pesticides may also cause an increase in host susceptibility (Kiesecker, 2002; Rohr *et al.* 2008). Therefore, despite increased cercariae mortality, pesticide may still have no net influence on disease outcomes (e.g., infection and pathology). Moreover, a number of studies have demonstrate that hosts can vary in susceptibility to both pesticides and parasites (Bridges and Semlitsch, 2000b; Searle *et al.* 2011; Cothran *et al.* 2013; Hua and Relyea, 2014; Bradley *et al.* 2015). Because pesticides can have direct and indirect effects on hosts and parasites, future studies

that continue to tease apart the complex effects of pesticides on the different components of the host–parasite interaction are necessary (Rohr *et al.* 2008).

#### SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0031182015001894>

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