

Population-level variation in infection outcomes not influenced by pesticide exposure in larval wood frogs (*Rana sylvatica*)

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Abstract

1. There is increasing evidence that populations of non-target wildlife species can evolve tolerance to pesticides. As ecosystems become increasingly exposed to chemical contaminants globally, it is important to consider not only the immediate consequences of contaminant exposure but also the potential costs associated with evolved responses. Theory predicts there may be trade-offs, including increased susceptibility to parasites, associated with evolved pesticide tolerance. It remains unclear, however, how environmental context (i.e. presence/absence of pesticides in the contemporary environment) interacts with evolved pesticide tolerance levels to influence infection outcomes.
2. Several studies have demonstrated that wood frog (*Rana sylvatica*) populations close to agriculture, where frequent exposure to pesticides is more likely, show higher baseline tolerance to pesticides than do populations far from agriculture. Using eight wood frog populations from across an agricultural gradient, we explored patterns of variation in susceptibility to parasites associated with a population's proximity to agriculture (a proxy for pesticide tolerance), and how these patterns are influenced by experimental exposure of tadpoles to the insecticide carbaryl. We did this by first placing tadpoles in an environment containing the pesticide carbaryl (1 mg/L) or in a pesticide-free control environment for 5 days, and subsequently exposing tadpoles to trematodes (*Echinostoma trivolvis*) or rana-virus (frog virus 3).
3. We found that variation in trematode susceptibility was related to the tadpole populations' proximity to agriculture. Individuals from populations located close to agriculture were modestly more susceptible to trematode infections than individuals from populations farther from agriculture. Ranavirus susceptibility was not associated with proximity to agriculture.
4. Surprisingly, exposure to carbaryl increased the survival rates of tadpoles infected with ranavirus. There were no other significant effects of carbaryl exposure on the measured disease outcomes.
5. This study provides evidence for a potential trade-off between trematode resistance and putative pesticide tolerance. We show that host populations can vary significantly in their susceptibility to pathogens, but that pesticide exposure does

not always increase parasite susceptibility or uniformly exacerbate disease outcomes. Further research is needed to determine how genetic variation among populations impact susceptibility to parasites, and if these patterns of susceptibility are consistent across space and time.

KEYWORDS

acetylcholinesterase inhibitor, disease ecology, *Echinostoma trivolvis*, Ecotoxicology, frog virus 3

1 | INTRODUCTION

The broad use of synthetic pesticides has been important for disease management, pest control, and agricultural productivity since the mid-20th century. However, the widespread use of pesticides has also negatively impacted non-target wildlife species, particularly those that live near agricultural operations (Pimentel, 2005; Stone, Gilliom, & Ryberg, 2014; Toccalino et al., 2014). Pesticide contamination has the potential to alter physiological traits and ecological interactions of populations that are exposed (Köhler & Triebkorn, 2013). For example, pesticide exposure can negatively influence growth, development, behaviour, immune function, and survival in a variety of taxa (Egea-Serrano et al., 2012; Gill et al., 2012; Köhler & Triebkorn, 2013). It is well-documented that different species can vary dramatically in their sensitivity and responses to pesticides (Bridges & Semlitsch, 2000; Hammond et al., 2012). It is also evident that there can be significant variation in pesticide sensitivity among populations of the same species (Bendis & Relyea, 2014; Brausch & Smith, 2009; Bridges & Semlitsch, 2000; Cothran et al., 2013; Hua et al., 2013). However, the causes and consequences of population-level differences in pesticide sensitivity are not well understood.

Over 900 target species have evolved some degree of pesticide tolerance (Pimentel, 2005). Moreover, there is increasing evidence suggesting that populations of non-target wildlife species can also evolve tolerance to pesticides (Bendis & Relyea, 2014; Brausch & Smith, 2009; Cothran et al., 2013; Hua, Morehouse, et al., 2013). The traditional paradigm under which increased pesticide tolerance is predicted to occur is through natural selection for constitutive (i.e. canalised, always expressed) tolerance over multiple generations, with populations that are consistently exposed to a pesticide (e.g. close to agricultural fields; Declerck et al., 2006) being more likely to evolve tolerance (Brausch & Smith, 2009; Futuyma & Kirkpatrick, 2017). For example, Bendis and Relyea (2014) found that zooplankton populations derived from ponds close to agricultural fields were more tolerant to the insecticide chlorpyrifos than populations derived from ponds farther from agricultural fields. Brausch and Smith (2009) found a similar relationship, where fairy shrimp (*Thamnocephalus platyurus*) derived from ponds in agricultural catchments were less sensitive to multiple pesticides than fairy shrimp derived from ponds surrounded by native grasslands. Though evolved responses to pesticides may allow organisms to persist when exposed to these contaminants, evolved responses to anthropogenic changes may

also involve nonadaptive fitness trade-offs or beneficial correlated traits that alter interspecific interactions (Alout et al., 2016; Mateos-Gonzalez et al., 2015). For example, populations of *Daphnia magna* selected for higher tolerance to the insecticide carbaryl are more susceptible to parasitic infections than control populations (Jansen et al., 2011). Thus, it is important to consider not only the immediate consequences of pesticide exposure but also the potential costs and trade-offs associated with evolved responses.

Wood frogs (*Rana sylvatica*) present an excellent system to explore potential trade-offs associated with pesticide tolerance based on recently observed spatial patterns of pesticide tolerance (Cothran et al., 2013; Hua et al., 2013, 2015a; Hua et al., 2014). Over the past decade, several studies have demonstrated that wood frog populations close to agriculture, where frequent exposure to pesticides is more likely, show higher baseline tolerance to pesticides than do populations far from agriculture (Cothran et al., 2013; Hua et al., 2015b). Based on these spatial patterns, Hua et al., (2017) conducted a study with 15 wood frog populations to investigate how evolved pesticide tolerance to the pesticide carbaryl influenced their susceptibility to two common amphibian parasites, trematodes and ranavirus, in the absence of pesticide exposure. This study found that a population's proximity to agriculture and pesticide tolerance influenced infection outcomes, demonstrating that evolved pesticide tolerance can indirectly influence host-parasite interactions. It remains unclear, however, how environmental context (i.e. pesticide presence/absence in the contemporary environment) interacts with pesticide tolerance levels to influence infection outcomes.

Here, we examine population-level variation in susceptibility to parasites in wood frog tadpoles, and how these patterns are influenced by experimental exposure of tadpoles to the insecticide carbaryl. We used eight populations that vary in their distance to the closest agricultural field, ranging from under 10 m away to over 1,500 m away from the closest field. We first exposed larvae to one of two treatments: a sublethal carbaryl concentration (1 mg/L) or a pesticide-free control. Then, we conducted experimental parasite exposures, using either trematodes (*Echinostoma trivolvis*) or ranavirus (frog virus 3), to determine how pesticide exposure influences infection outcomes within and among populations. First, we predicted that in the absence of pesticide exposure, tadpoles from populations close to agriculture would be more susceptible to parasites than tadpoles from populations far from agriculture

due to potential nonadaptive associated with evolved pesticide tolerance. Second, we predicted an interactive effect between a population's distance to agriculture and pesticide exposure on infection outcomes, with infection outcomes of populations close to agriculture being less influenced by pesticide exposure than populations far from agriculture because closer populations are generally less sensitive to pesticides. Third, we predicted that, overall, pesticide-exposed individuals would have exacerbated disease outcomes relative to the no-pesticide control individuals based on the documented immunosuppressive effects of carbaryl and other pesticides on amphibians (Christin et al., 2003; Davidson et al., 2007; Rohr et al., 2008).

2 | METHODS

2.1 | Model pesticide background

We used the carbamate insecticide carbaryl (98.1% pure; ChemService, West Chester, PA, U.S.A.) for this study. First registered in 1959, carbaryl is a reversible acetylcholinesterase inhibitor that has been heavily used for agricultural and residential pest control (Atwood & Paisley-Jones, 2017; Čolović et al., 2013). The half-life of carbaryl is 10 days at a pH of 7. Carbaryl has been found at concentrations up to 4.8 mg/L in aquatic ecosystems but is typically found at concentrations below 1 mg/L (Norris et al., 1983; Peterson et al., 1994). Carbaryl has documented lethal (wood frog LC50 values ranging from 1.2 to 22 mg/L) and sublethal effects (e.g. altered activity levels, reduced predator avoidance behaviour) on wood frogs and other amphibians (Boone & Bridges, 1999; Bridges & Semlitsch, 2000; Relyea, 2003; Relyea & Mills, 2001; Rohr et al., 2003).

2.2 | Model parasite background

Echinostomes (family: Echinostomatidae) are a diverse group of generalist trematode parasites. They have a complex life cycle, using larval amphibians, snails, and fish as secondary intermediate hosts. Amphibians are infected by the free-swimming cercarial life stage, which encysts in the kidneys. Pathology is dose-dependent with oedema, reduced growth, and mortality occurring at high parasite loads (Johnson & McKenzie, 2008). Ranaviruses are a common pathogen in North American amphibian communities and have been implicated in die-off events of larvae and recently metamorphosed individuals (Duffus et al., 2015; Miller et al., 2011). Ranaviruses cause cell death in the liver, kidney, and spleen, often leading to mortality in 7–10 days (Gray et al., 2009). Pesticide exposure can increase susceptibility to both pathogens (Pochini & Hoverman, 2017; Rohr, Schotthoefer, et al., 2008). However, the degree to which pesticide exposure differentially influences disease outcomes among populations of the same species remains unclear.

2.3 | Animal collection and husbandry

We collected wood frog egg masses from eight populations (range: 7–10 masses/population; see Table S1 for site coordinates) in north-western Pennsylvania from 31 March to 7 April 2019. All populations were separated by >5 km, beyond the demonstrated c. 1 km genetic neighbourhood of wood frogs (Berven & Grudzien, 1990; Semlitsch, 2000). The ponds used in this study varied in their proximity to agriculture (range: 9.8–1,609 m). Distance to agriculture was measured as the linear distance from each pond to the nearest agricultural field using Google Earth (2019, v. 7.3.2). Additional details for how proximity to agriculture was measured are presented in Hua et al. (2015b). After collection, we transported the egg masses to the Purdue Wildlife Area (PWA) in West Lafayette, IN. We distributed the egg masses from each population into 180-L plastic outdoor culturing pools containing c. 150 L aged well water, placing one egg mass in each pool. After hatching, tadpoles were fed rabbit chow (Purina) ad libitum. Tadpole health (i.e. body condition, water quality) was checked daily until the start of experiments. All protocols and procedures employed were ethically reviewed and approved by the Purdue University IACUC (protocol 1701001530).

2.4 | Phase 1: Sublethal pesticide exposures

On 9 May 2018, we transferred a subset of wood frog tadpoles from each population to the laboratory to acclimate to indoor conditions for 24 hr (12:12 light cycle, 23°C). We euthanised (via MS-222 overdose) and preserved 10 haphazardly selected tadpoles from each population to assess initial size and developmental stage prior to the start of the experiment (stage: median = 27, range = 26–28; snout-vent length (SVL): mean = 6.5 mm $\pm$ 0.76 SD). On 10 May 2018, we haphazardly chose 200 lab-acclimated individuals from each population and assigned them to one of two pesticide treatments: 1 mg/L carbaryl or pesticide-free control (UV-irradiated, carbon-filtered well water; Figure 1). These two groups represented the pesticide-exposed and pesticide-naïve tadpoles, respectively.

We used 14-L plastic containers containing 7 L of treatment solution as the experimental units and assigned 100 individuals from each population to both treatments. There were four containers per treatment per population for a total of 64 containers ($N = 25$ tadpoles/container). We made a 20 g/L stock solution by dissolving technical grade carbaryl in 100% ethanol. We then added 350 μ L of carbaryl stock solution to the appropriate experimental units. Sorption of carbaryl by plastic experimental units is minimal (approx. 0.1%; (Bridges, 2000)). We then added 350 μ L of 100% ethanol to each pesticide-free control experimental unit (0.005%) as sham exposures to account for solvents. The carbaryl solution was not renewed and we conducted no water changes over the 5-day exposure period. On 15 May 2018, tadpoles were transferred into clean 14-L bins containing 7 L of UV-irradiated, carbon-filtered well water. Tadpoles were maintained in these containers for 2 days prior to the

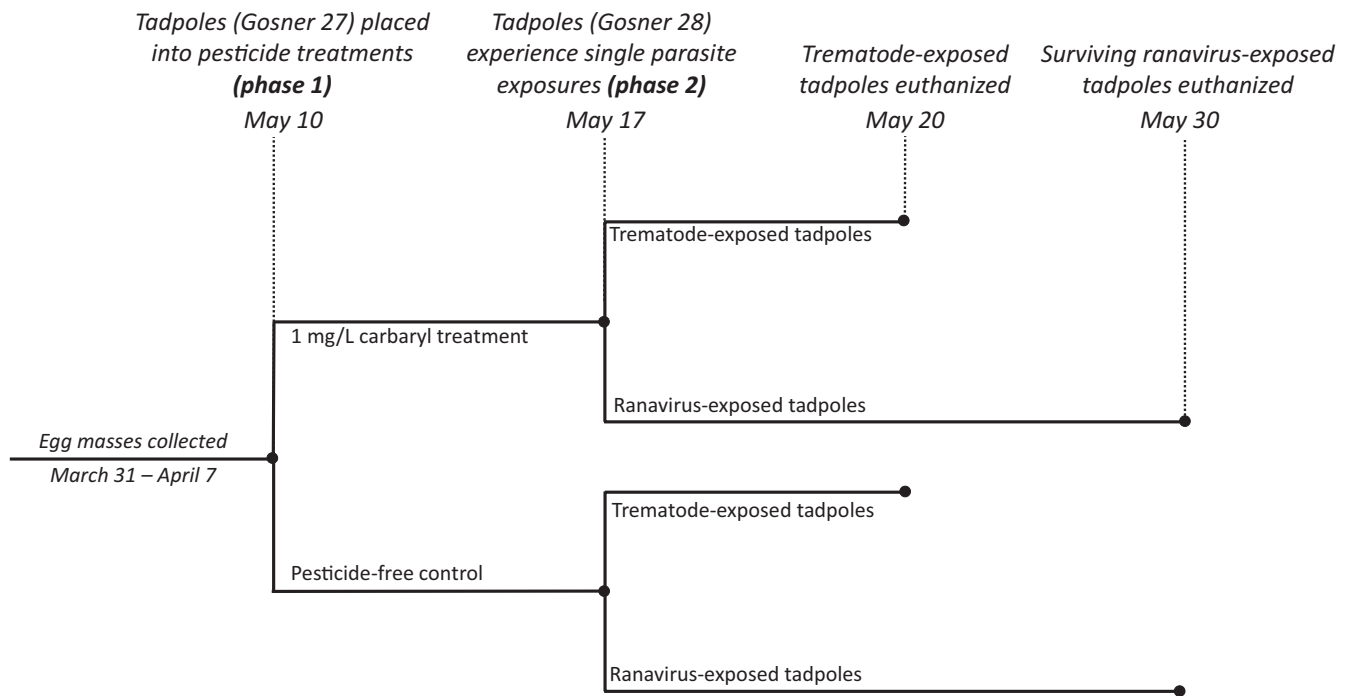


FIGURE 1 Schematic representation of the experimental timeline

beginning of the parasite exposure. Tadpoles were fed rabbit chow ad libitum during phase 1 and no mortalities were observed.

2.5 | Phase 2: Parasite exposures

We investigated the effects of pesticide exposure on the susceptibility of tadpoles to two common amphibian parasites via two separate parasite exposure experiments (Figure 1). Prior to the start of the parasite exposures on 17 May, we aggregated the 100 tadpoles from each phase-1 treatment by population in preparation for haphazard selection of individuals for experiments. For both experiments, experimental units were 130-ml cups containing 100 ml UV-irradiated well water. Each experimental unit was assigned one tadpole. The units for the experiments were housed on two separate shelving units in the same room across three vertical shelves in a randomised block design. We fed all tadpoles rabbit chow ad libitum throughout the experiments.

2.5.1 | Trematode experiment

To obtain echinostome cercariae, we collected c. 100 adult ramshorn snails (*Helisoma trivolvis*), the first intermediate host of echinostome cercariae, from a large permanent pond at the PWA on 4 May 2018. To screen the snails for infection, we isolated them individually in 50-ml tubes containing 35 ml of UV-irradiated well water and placed them 10 cm under a light source (100-W light bulbs) for 1 hr to induce cercarial shedding (Szuroczi & Richardson, 2009). We identified echinostome-infected snails ($n = 25$) by placing cercariae on

slides under a compound scope following Schell (1985). To reduce shedding prior to the experiment, we individually isolated infected snails in 2-L cups containing 1.5 L UV-irradiated well water and stored them at 7°C. Two days prior to the experiment, the snails were acclimated to 23°C. Snails were fed a mixture of rabbit chow and spirulina powder ad libitum.

We haphazardly chose 25 tadpoles from each phase-1 treatment for each population. On 17 May 2018, we individually exposed 20 of the 25 tadpoles from each treatment $\times$ population combination to echinostome cercariae. This produced a total of 16 treatments (eight populations $\times$ two pesticide treatments) replicated 20 times for a total of 320 experimental units. The remaining five tadpoles were assigned as no-trematode controls to assess background mortality (i.e. 80 additional experimental units).

We used an aliquot method to administer the cercariae (Buss & Hua, 2018; Tucker et al., 2001). To do this, we first shed the 25 echinostome-infected snails individually in 50-ml tubes containing 35 ml of UV-irradiated well water under a light source for 1 hr. We then confirmed that each snail was shedding echinostome cercariae under a dissecting microscope and homogenised the water from each tube in a 1-L beaker. To quantify cercarial density, we gently mixed the water and took five 1-ml subsamples. The subsamples had an average of 28.8 cercariae/ml $\pm$ 3.27 SD. Based on this, we dispensed 2 ml of cercariae slurry into each experimental unit for an approximate density of 57.6 cercariae/experimental unit. We used the aliquot method of counting to ensure that cercariae were added to experimental units within 2 hr of shedding to control for cercarial age between experimental units.

We checked for tadpole mortality daily, and no water changes were conducted throughout the experimental exposure. We ended

the experiment after 3 days (20 May), as this is enough time for echinostome cercariae to successfully encyst in the kidneys as metacercariae without allowing parasite clearance to begin (Hoverman et al., 2013). We euthanised (MS-222 overdose) and preserved tadpoles in 10% buffered formalin. We weighed, staged, and measured SVL and total length of individuals prior to dissection. To estimate trematode load, we first dissected the kidneys of each tadpole, placed them between two microscope slides, and then counted the total number of metacercariae under a compound microscope (Schothoefer et al., 2003). We then examined the rest of the body for metacercariae; however, all cysts were found in the kidneys.

2.5.2 | Ranavirus experiment

The ranavirus exposure experiment began on 17 May 2018. We used a ranavirus strain isolated from an infected green frog (*Rana clamitans*) at the PWA. The virus was cultured on fathead minnow cells and Eagle's minimum essential medium containing 5% foetal bovine serum to a titre of 2.07×10^6 plaque-forming units [PFU]/ml.

We conducted a factorial experiment that crossed 20 tadpoles from each phase 1 treatment, by population, with either the presence or absence of ranavirus exposure (10^3 or 0 PFU/ml) for a total of 32 treatments and 640 experimental units. To achieve a concentration of 10^3 PFU/ml, we added 48 µl of the virus to each experimental unit assigned to the virus treatment. We then added 48 µl of minimum essential medium to each experimental unit assigned to the ranavirus-free control treatment.

We conducted mortality checks to determine if virus-induced mortality rates differed by treatment and population. Checks were conducted daily prior to 23 May 2018 and every 6 hr beginning at 6:00 a.m. on 23 May. At each check, dead individuals were preserved individually in 70% ethanol and their time-to-death was recorded. Water changes were conducted every 3 days. The experiment was terminated on 30 May 2018 at 6:00 a.m. after 12 days, which is sufficient time to observe disease outcomes from ranavirus exposure in wood frogs (Hoverman et al., 2011). We euthanised all surviving individuals and individually preserved them in 70% ethanol to quantify infection status and viral load.

We weighed, staged, and measured SVL and total length of individuals prior to dissection. To quantify infection status and viral load, we dissected the liver and kidneys of each tadpole, combined the tissues in a 1.5 ml microcentrifuge tube, and stored the sample at -80°C for later DNA extraction and quantitative polymerase chain reaction (qPCR) analysis. To prevent cross-contamination, all tools were soaked in 10% bleach for ≥ 5 min and gloves were changed between dissections.

We conducted DNA extractions using DNeasy Blood and Tissue Kits (Qiagen). We used qPCR to determine infection status and viral load of individuals following the methods of Wuerthner et al. (2017). Each reaction contained 6.25 µl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 0.1125 µl of forward and reverse primers at 10 pmol/µl each (rtMCP-F [5'-ACA

CCA CCG CCC AAA AGT AC-3'] and rtMCP-R [5'-CCG TTC ATG ATG CGG ATA ATG-3']), 0.0313 µl of fluorescent probe rtMCP-probe (5'-CCT CAT CGT TCT GGC CAT CAA CCA-3'), 3.49 µl of reverse osmosis water, and 2.5 µl of template to a final volume of 12.5 µl. We used a CFX Connect™ (Bio-Rad Laboratories, Hercules, CA, U.S.A.) to conduct qPCR. We included a standard curve and a negative control containing reverse osmosis water as template. We used a synthetic double-stranded DNA standard by synthesising a 250-bp fragment of the major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies, Coralville, IA, U.S.A.), which is conserved among *Ranavirus* species. We prepared a log-based dilution series (4.014×10^7 – 4.014×10^4 viral copies/µl) for the standard curve. Each standard curve sample and unknown sample were run in duplicate. For each unknown sample, we calculated viral load (viral copies/ng DNA) by dividing the number of copies of ranavirus DNA (viral copies/µl) by the total DNA present in the sample (ng DNA/µl). All duplicate unknown samples that peaked before cycle 40 were considered positive. We did not detect discrepancies between replicates (i.e. both replicates were either positive or both were negative).

2.6 | Pesticide analysis

We collected one 25-ml water sample from each of the experimental units the day they were dosed with carbaryl to verify pesticide concentrations from the phase-1 exposures and stored them at -20°C prior to analysis. Briefly, each pesticide sample was spiked with a known concentration of internal standard (carbaryl-d7), extracted by pouring through 3-cc Oasis SPE cartridges (Waters INC, Milford, MA, U.S.A.), and then eluting with 3 ml of acetonitrile prior to delivering to Purdue Bindley Bioscience Center (West Lafayette, IN, U.S.A.) for confirmation. One sample was lost prior to analysis (RMD replicate 1). The mean carbaryl concentration of the 23 samples from the phase-1 treatments was $0.94 \text{ mg/L} \pm 0.19 \text{ SD}$ (range: 0.56–1.34 mg/L).

2.7 | Statistical analysis

We performed all statistical analyses using R version 4.0.2 (R Core Team, 2020).

2.7.1 | Effect of pesticide treatment on tadpole traits

To minimise the total number of animals sacrificed for this study, we used the trait measurements of individuals euthanised at the end of the trematode exposure as a proxy for size prior to parasite exposure and following pesticide exposure (trait measurements summarised in Table S1). This is a reasonable estimate of size differences following pesticide exposure because of: (1) the short duration from the time of parasite exposure to the end of the experiment (3 days); and (2) previous research demonstrating that exposure to a moderate number of echinostome cercariae, such

as in this experiment, has no impact on growth or development of larval amphibians (Koprivnikar et al., 2008; Orlofske et al., 2009). The response variables were SVL, mass, and developmental stage. For each analysis, we used population and phase 1 treatment as the predictor variables. We used a generalised linear model (GLM) to analyse the effects of the predictor variables on each response variable (*glm* function, *stats* package).

2.7.2 | Disease outcome analyses

For each analysis, we assessed whether there were population-level differences in disease outcomes generally, and whether disease outcomes were influenced by pesticide exposure. We compared models with different combinations of predictor variables and selected the best-fit model by comparing the Akaike information criterion (AIC) and selecting the model with the lowest AIC (Nakagawa & Cuthill, 2007). We then substituted distance to agriculture for population to assess if differences in disease outcomes were related to a population's distance to agriculture. For models with distance to agriculture as the predictor variable, we included population as a random effect (1|population) to account for the non-independence of individuals from egg masses collected at the same pond. We applied a logarithmic transformation to measures of distance to agriculture. Where appropriate, we used the ANOVA function (*car* package; Fox & Weisberg, 2011) to estimate P-values. Tukey post-hoc tests were used to determine significant differences among the populations (*cld* function, *multcomp* package; Hothorn et al., 2008).

Trematode experiment analyses

We analysed the response variable, trematode infection load (number of metacercarial cysts/tadpole), using a GLM with a negative binomial distribution (*glm.nb* function, *MASS* package; Venables & Ripley, 2002). We considered population, phase 1 treatment, final SVL (i.e. a proxy for size differences at the time of exposure), and their interactions as potential predictor variables. We considered final SVL as a predictor variable because tadpole size can influence trematode susceptibility (Marino et al., 2017). The best-fit model as determined by AIC (Table S2) included population, phase 1 treatment, and final SVL as predictor variables (model structure: trematode load ~ population + phase 1 treatment + SVL). We then substituted distance to agriculture for population (model structure: trematode load ~ ln(distance to agriculture) + phase 1 treatment + SVL + (1|population)) and analysed trematode load using a generalised linear mixed model (GLMM) with a negative binomial distribution (*glmer.nb* function, *lme4* package; Bates et al., 2015). We used negative binomial distributions to account for overdispersion in the data, as is common for trematode infection load data (Jones et al., 2019). We did not analyse tadpole survival data because only three of 320 individuals (0.92%) died.

Ranavirus experiment analyses

The response variables for the ranavirus experiment were infection prevalence, time-to-death, and infection load. We first

analysed differences in infection prevalence using a GLM with a binomial distribution (logit link function; 1 = infected, 0 = uninfected; *glm* function, *stats* package). We considered population, phase 1 treatment, and their interaction as potential predictor variables in model selection. The best-fit model as determined by AIC (Table S3) included population and phase 1 treatment as predictor variables, but not their interaction (model structure: infection prevalence ~ population + phase 1 treatment). We then substituted distance to agriculture for population (model structure: infection prevalence ~ ln(distance to agriculture) + phase 1 treatment + (1|population)) and analysed infection prevalence using a GLMM with a binomial distribution (*glmer* function, *lme4* package). We did not consider final SVL as a predictor variable, as it is not an accurate proxy for initial size because infected individuals died at different times over the 12-day experiment. As a precaution, however, we assessed whether differences in mean initial size between groups (using mean final SVL of individuals from the trematode exposures as a proxy; Table S1) had an effect on infection prevalence using a GLM with a binomial distribution (model structure: infection prevalence ~ mean final SVL + population + phase 1 treatment). There was no significant effect of mean population SVL on ranavirus infection prevalence (estimate: 0.129 ± 0.390 , $z = 0.331$, $p = 0.741$); therefore, we excluded initial SVL from analysis.

Next, we analysed differences in time-to-death using Cox proportional hazards models (*coxph* function, *Survival* package; Therneau, 2020). For this analysis, we considered only individuals with detectable ranavirus infections (210 out of 320 total observations). We considered population, phase 1 treatment, and their interaction as potential predictor variables in model selection. The best-fit model as determined by AIC (Table S4) included population and phase 1 treatment as predictor variables (model structure: time-to-death ~ population + phase 1 treatment). We then substituted distance to agriculture for population (model structure: time-to-death ~ ln(distance to agriculture) + phase 1 treatment + (1|population)) and analysed time-to-death using a mixed-effects Cox proportional hazards model (*coxme* function, *coxme* package; Therneau, 2020).

Next, we analysed differences in ranavirus load (ln-transformed) using a GLM with a normal distribution (*glm* function, *stats* package). We considered population, phase 1 treatment, survival status (1 = died, 0 = survived), and their interactions as predictor variables. We considered survival status as a predictor variable to account for the c. 5 times higher average viral load of individuals that died (c. 2.7×10^7 viral copies/ng of DNA) versus those that survived (c. 5.4×10^6 viral copies/ng of DNA). The best-fit model as determined by AIC (Table S5) included population, phase 1 treatment, survival status, and the interaction between population and survival status as predictor variables (model structure: viral load ~ population + phase 1 treatment + surv.status + population:surv.status). We then substituted distance to agriculture for population (model structure: viral load ~ ln(distance to agriculture) + phase 1 treatment + surv.status + ln(distance to agriculture):surv.status + (1|population)) and analysed viral load using a GLMM with a normal distribution (*glmer* function, *lme4* package).

3 | RESULTS

3.1 | Effect of pesticide treatment on tadpole traits

There was no effect of phase 1 carbaryl treatment on SVL (estimate = -0.01 ± 0.11 SE, $t_{311} = -0.087$, $p = 0.931$) or mass (estimate = -2.95 ± 4.76 SE, $t_{311} = -0.621$, $p = 0.535$). There was, however, a very small but significant effect of phase 1 carbaryl treatment on developmental stage (estimate = -0.438 ± 0.169 SE, $t_{311} = -2.590$, $p = 0.01$); individuals from the carbaryl treatment were at a slightly earlier mean Gosner stage (28.6) than individuals from the control treatment (29.0). There were also small but statistically significant differences in SVL, mass, and Gosner stage among tadpoles from the eight populations not related to pesticide exposure (Table S1).

3.2 | Trematode experiment

Overall, 99.1% of the trematode-exposed individuals survived and 100% of the no-trematode control individuals survived. The average trematode load across all individuals was 29.5 (c. 51.2% encystment success based on estimated exposure of 57.6 cercariae/individual). There was a significant effect of population on trematode load ($F_{7,310} = 2.2$, $p = 0.033$, Table S6) with a c. 41% greater infection load in individuals from the most susceptible population (LOG; 33.9 cysts/tadpole) as compared to the least susceptible

population (CRK; 24.1 cysts/tadpole). There was also a significant positive effect of SVL on trematode load (estimate = 0.087 ± 0.016 SE, $t_{310} = 5.52$, $p < 0.001$). There was a small but significant negative relationship between a population's distance to agriculture and trematode load (estimate = -0.020 ± 0.009 SE, $t_{310} = -2.18$, $p = 0.029$ Figure 2). Including an interaction between distance to agriculture and pesticide treatment did not improve model fit, and therefore was removed. There was no effect of pesticide exposure on trematode load (estimate = 0.010 ± 0.030 SE, $t_{310} = 0.332$, $p = 0.74$).

3.3 | Ranavirus experiment

There was 100% survival in the no-virus control individuals (i.e. no background mortality). Therefore, they were excluded from further analyses. Across all ranavirus-exposed individuals, 66% of individuals became infected, 40% of ranavirus-infected individuals died, and average viral load of infected individuals was c. 1.36×10^7 viral copies/ng of DNA.

The first set of analyses assessed differences in infection prevalence following ranavirus exposure. There were significant differences in infection prevalence among the eight populations ($\chi^2 = 21.6$, $df = 7$, $p = 0.003$; Figure 3, Table S7). However, there was no effect of a population's distance to agriculture on infection prevalence (estimate = -0.053 ± 0.107 SE, $z = -0.491$,

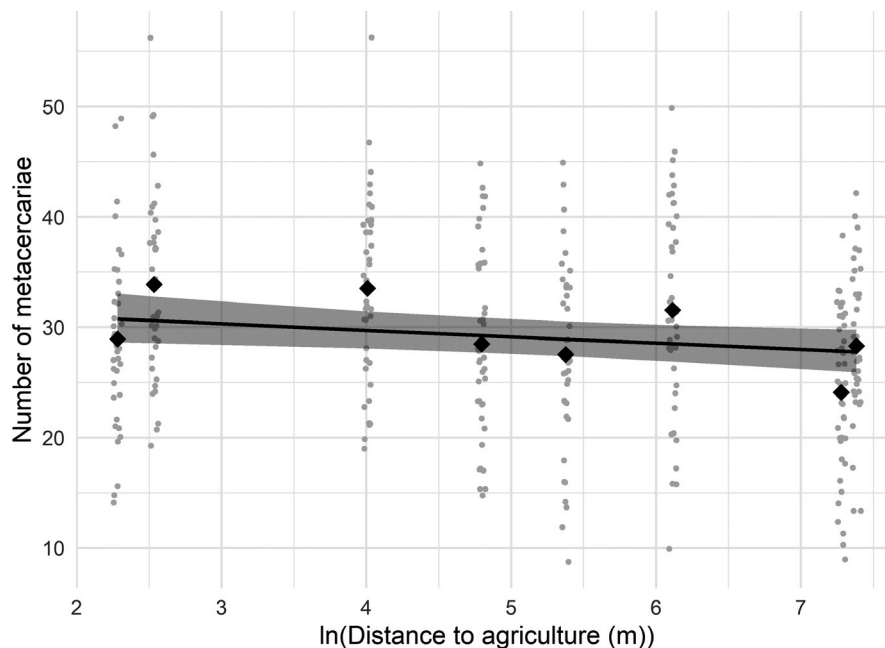


FIGURE 2 Relationship between a wood frog population's linear distance to the closest agricultural field and the number of metacercariae infecting each tadpole following individual lab exposures to echinostome cercariae ($n = 320$ [eight populations, 40 individuals/population], negative binomial generalised linear mixed model; estimate = -0.020 ± 0.009 SE, $t = -2.18$, $p = 0.029$). The line represents the negative binomial generalised linear mixed model fit and the shading represents the 95% confidence interval. Black diamonds (◆) represent population-wise means and small grey circles represent infection loads of individual tadpoles. Individual data points are jittered horizontally to make them more distinct. This plot contains individuals from the 1 mg/L carbaryl and pesticide-free control phase 1 treatments, as carbaryl exposure had no effect on trematode infection load (estimate = 0.010 ± 0.030 SE, $t = 0.332$, $p = 0.74$)

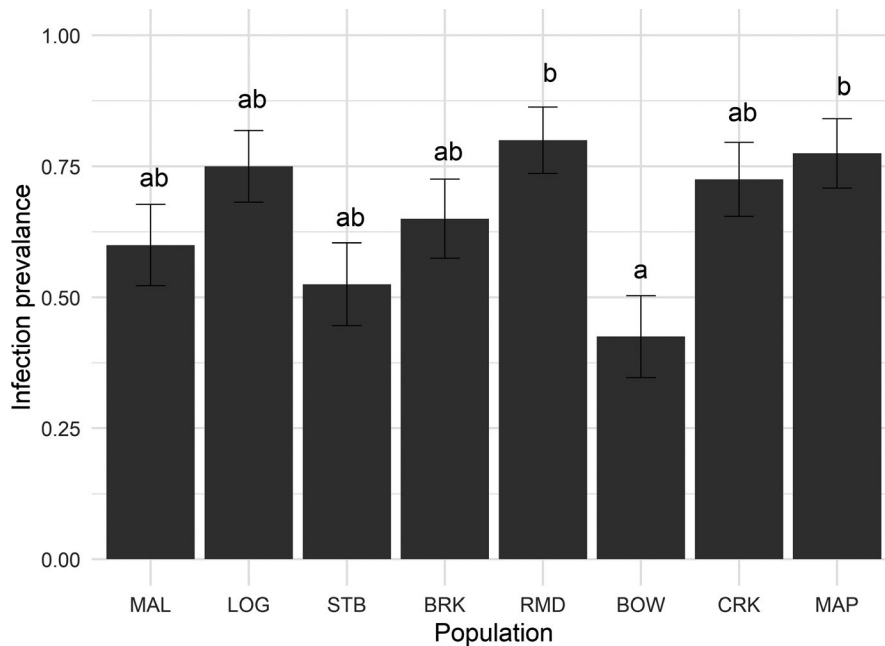


FIGURE 3 Population-level variation in infection prevalence (proportion infected) in larval wood frogs following individual lab exposures to ranavirus ($n = 320$ [eight populations, 40/population]; $\chi^2 = 21.6$, $df = 7$, $p = 0.003$). The x-axis is rank-ordered from the closest (left) to farthest (right) population from an agricultural field. Data (estimated marginal means $\pm$ SE) include individuals from the 1 mg/L carbaryl and no-pesticide control phase 1 treatments, as carbaryl exposure had no effect on infection prevalence (estimate = -0.059 ± 0.244 SE, $z = 0.244$, $p = 0.807$). Populations sharing lower case letters are not significantly different from one another (Tukey HSD)

$p = 0.624$). There was no effect of pesticide exposure on infection prevalence (estimate = -0.059 ± 0.244 SE, $z = 0.244$, $p = 0.807$).

The second set of analyses assessed differences in mortality risk of ranavirus-infected individuals from the eight populations. There was no significant effect of population on risk of mortality ($\chi^2 = 6.07$, $df = 7$, $p = 0.532$; Table S8). However, there was a significant negative effect of pesticide treatment on risk of mortality (coefficient = -0.440 ± 0.224 , $z = -1.966$, $p = 0.049$; Figure 4); carbaryl-exposed individuals experienced lower mortality risk than control-exposed individuals. There was no effect of a population's distance to agriculture on risk of mortality (coefficient = 0.063 ± 0.060 , $z = 1.06$, $p = 0.29$).

The final set of analyses assessed differences in viral load following ranavirus exposure. There were significant differences in viral loads among the eight populations ($F_{7,193} = 2.73$, $p = 0.01$; Figure 5, Table S9). As expected, individuals that died from infection had significantly higher infection loads than infected individuals that did not die by day 12 (estimate = 3.388 ± 1.590 SE, $t_{193} = 2.131$, $p = 0.034$). There was also a significant population $\times$ survival status interaction ($F_{7,193} = 2.788$, $p = 0.009$); in all populations, individuals that died had a higher infection load than survivors, but the magnitude of this difference varied among the populations. There was no effect of pesticide exposure on viral load (estimate = -0.618 ± 0.426 SE, $t_{193} = 2.131$, $p = 0.149$). There was no effect of a population's distance to agriculture on viral load (estimate = 0.141 ± 0.218 SE, $t = 0.644$, $p = 0.536$).

4 | DISCUSSION

Using tadpoles derived from eight wood frog populations, we explored patterns of variation in susceptibility to parasites associated with a population's proximity to agriculture (a proxy for pesticide tolerance), and how these patterns are influenced by experimental exposure of

tadpoles to the insecticide carbaryl. We did this by exposing tadpoles to the pesticide carbaryl (1 mg/L) or a pesticide-free control, followed by exposure to one of two common amphibian parasites. We found a negative relationship between a population's distance to agriculture and susceptibility to trematode infections. We also found significant population-level variation in ranavirus infection prevalence and viral load of surviving tadpoles, although this variation was not related to a population's proximity to agriculture. Interestingly, we found that ranavirus-infected individuals that were previously exposed to carbaryl experienced a lower mortality risk than individuals from the no-pesticide control treatment. There were no other main effects of pesticide exposure on patterns of infection beyond this.

We found significant population-level variation in trematode susceptibility, with a c. 41% greater infection load in individuals from the most susceptible population (LOG; 33.9 cysts/tadpole) compared to the least susceptible population (CRK; 24.1 cysts/tadpole). This is consistent with previous studies, which have found significant population-level variation in echinostome susceptibility among wood frog populations (Hua et al., 2017). Moreover, we found that this variation in trematode susceptibility was related to a population's distance to agriculture, with individuals from populations close to agriculture being more susceptible to infection with trematodes than individuals from populations far from agriculture. These findings are consistent with our prediction that in the absence of pesticide exposure, tadpoles from populations close to agriculture, where evolved tolerance is most likely to occur, would be more susceptible to trematode infection than tadpoles far from agriculture. This result is consistent with much of existing theory, which suggests that increased tolerance to anthropogenic stressors can indirectly influence interspecific (e.g. host-parasite) interactions via genetic or energetic trade-offs of tolerance, and that resistant phenotypes should be deleterious if the environment is in the untreated state (Gassmann et al., 2009; Hamilton et al., 2017; Jansen

FIGURE 4 Survival curves of ranavirus-infected wood frog tadpoles that were exposed to a no-pesticide control or a sublethal concentration of carbaryl (1.0 mg/L) in phase 1 of the experiment ($n = 210$ infected individuals [control treatment = 104 individuals, carbaryl treatment = 106 individuals]). Carbaryl-exposed individuals experienced lower mortality following infection with ranavirus than control-exposed individuals (Cox regression: coefficient = -0.440 ± 0.224 , $z = -1.966$, $p = 0.049$). Individuals from all eight populations are pooled in each curve, as there was no significant effect of population on mortality ($\chi^2 = 6.07$, $df = 7$, $p = 0.532$)

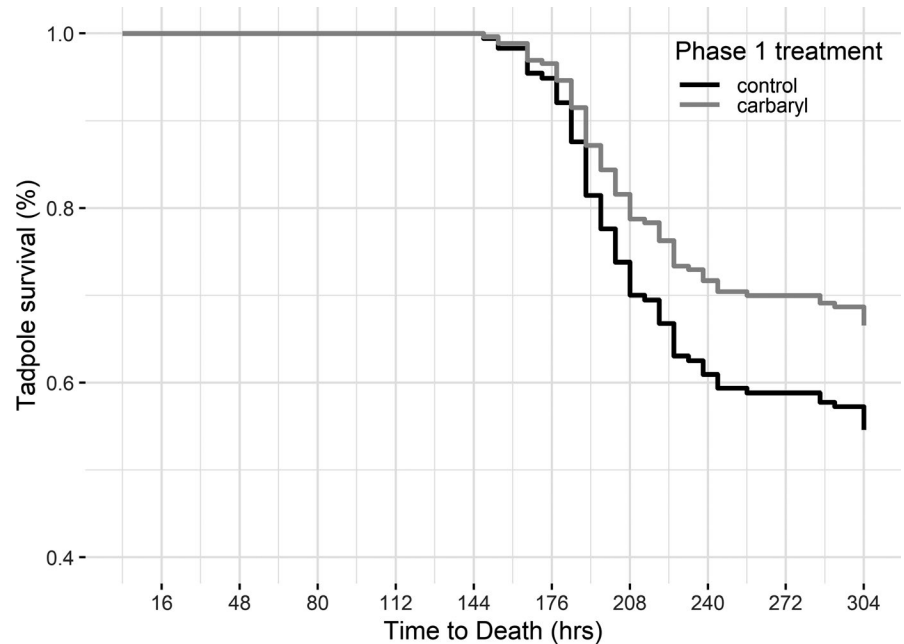
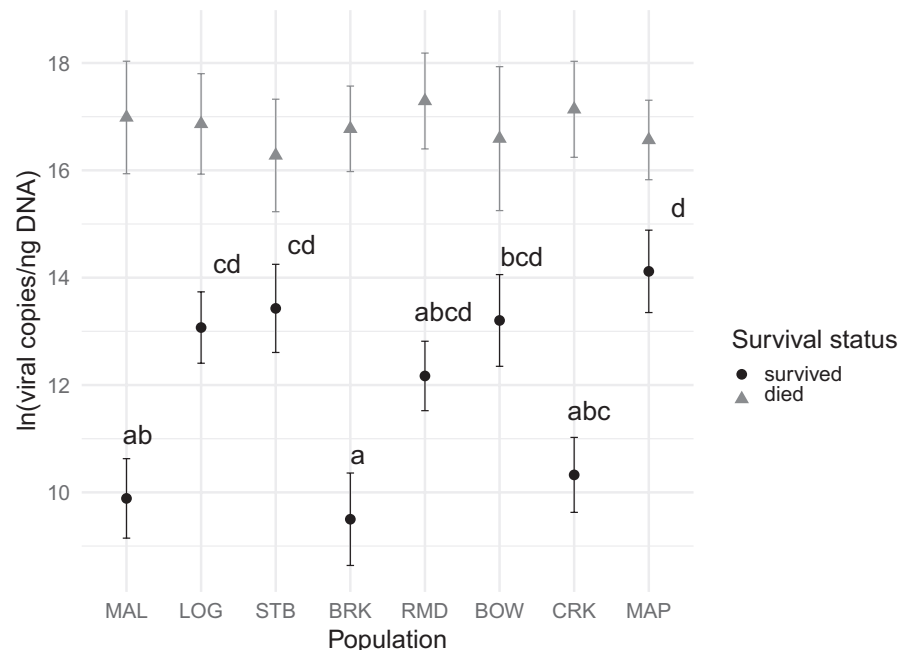


FIGURE 5 Population-level variation in viral load (estimated marginal means ± 1 SE, ln-transformed) of ranavirus-infected wood frog tadpoles following individual lab exposures to ranavirus ($n = 210$ infected individuals; $F_{7,193} = 5.39$, $p < 0.001$). The x-axis is rank ordered from the closest (left) to farthest (right) population from an agricultural field. Individuals are separated by survival status (dead/alive at the end of the experiment), as tadpoles that died had significantly higher viral loads (estimate = 3.388 ± 1.590 SE, $t = 2.131$, $p = 0.034$). Populations sharing lower case letters are not significantly different from one another (Tukey HSD)



et al., 2011; Kliot & Ghanim, 2012; Shi et al., 2004). Although the mechanisms underlying these findings remain speculative in this system, populations frequently exposed to contaminants may have an upregulation of metabolic activity and detoxification enzymes (e.g. cytochrome P450 1A [CYP1A]), which can be energetically costly (Hamilton et al., 2017; Kliot & Ghanim, 2012). It is noteworthy that this result seemingly conflicts with the findings of Hua et al., (2017) that populations closer to agriculture were less susceptible to infection with echinostome trematodes than populations far from agriculture. However, the 15 populations used in Hua et al., (2017) have relatively well-established measures of pesticide tolerance (see Hua et al., 2015b) which were reduced into a single predictor variable incorporating both distance to agriculture and pesticide tolerance via

Principal Axis Factoring. When the regression analysis conducted in Hua et al., (2017) is limited to consider only a population's proximity to agriculture as the predictor variable rather than the first principal component of distance to agriculture and pesticide tolerance, the relationship is weaker but remains qualitatively unchanged ($|r|$ reduced from 0.55 to 0.49; $p = 0.0583$; data from Hua et al., 2017). Thus, the differences in the analysis do not explain the qualitatively different results found in Hua et al., (2017) and the present study. The fact that both studies identified effects related to proximity to agriculture, despite their conflicting nature, is intriguing and warrants further study to determine if evolved responses to other environmental factors (e.g. naturally occurring pathogen communities, predators, temperature, interactions between pesticide presence and parasite

abundance [Rumschlag et al., 2019]) play an important role in these observed patterns.

Consistent with previous work, we found that larval wood frogs were highly susceptible to ranavirus, with 66% ($n = 210$) of individuals that were exposed to ranavirus becoming infected. However, there were significant differences in infection prevalence among the eight populations (range: 42.5%–80.0%). This finding demonstrates that intraspecific variation in infection susceptibility can be nearly as dramatic as interspecific variation (Hoverman et al., 2011). Additionally, we found that there was significant population-level variation in the viral load of survivors (range: 9.3–14.0 ln viral copies/ng of DNA). In contrast, we found little population-level variation in viral load of dead individuals (range: 16.4–17.3 ln viral copies/ng of DNA), consistent with previous studies demonstrating that amphibian mortality is highly likely to be beyond a threshold viral load (Hua et al., 2017; Wuerthner et al., 2017). Together, these results suggest that although wood frogs are highly susceptible to ranavirus infections, individuals and populations can vary widely in susceptibility and subsequent infection outcomes. Indeed, Brunner et al., (2017) argue that wood frog tadpoles can be highly heterogeneous in their susceptibility to ranavirus and that these heterogeneities drive transmission. It remains unclear, however, what factors drive individual- and population-level variations in virus susceptibility. Future studies should explore whether differences in innate or adaptive immunity influence these interpopulation differences in virus susceptibility and if there are differences in the biotic or abiotic environment between sites that may influence these differences.

Pesticide exposure did not increase the severity of any measured disease outcome. Surprisingly, however, we found that carbaryl-exposed individuals that were infected with ranavirus had a lower risk of mortality than control-exposed individuals. In other words, previous carbaryl exposure appears to have had a positive effect on the survivorship of tadpoles infected with ranavirus. This result is surprising given the documented negative effects of carbaryl and other pesticides on amphibian immunity (Rohr et al., 2003; Rohr, Schotthoefer, et al., 2008; Christin et al., 2003). Moreover, a previous study using the ranavirus–wood frog system found that exposure to carbaryl (1 mg/L) prior to exposure to ranavirus resulted in faster death in the carbaryl-exposed individuals relative to the control individuals (Pochini & Hoverman, 2017). Despite these previous findings, however, the positive effects of pesticide exposure on animal performance found in the present study are not without precedent. For example, Forson and Storfer (2006) found that exposure to the pesticide atrazine decreased the susceptibility of long-toed salamanders (*Ambystoma macrodactylum*) to ranavirus, although the exact mechanisms (e.g. pesticide-induced immunostimulation vs. viral attenuation) are unclear. More generally, exposure to a sublethal stressor can have preparative carryover effects that are beneficial in the face of a later stressor, commonly known as hormesis (Berry & López-Martínez, 2020). For example, prior exposure to a sublethal pesticide concentration, cues from a predator, and a simulated stressor (i.e. exposure to stress hormones) can all increase the tolerance of wood frog tadpoles to pesticides (Billet

& Hoverman, 2020; Hua et al., 2014; Jones et al., 2017). It remains unclear why pesticide exposure in some situations can exacerbate the severity of parasitic infections but in other situations ameliorate them. Future studies should manipulate pesticide concentration, exposure duration, host condition, host identity, and parasite identity to clarify how pesticide exposure influences parasitic infections in different contexts.

We found no effect of pesticide exposure on susceptibility to trematodes relative to control individuals: infection outcomes were relatively consistent within a population, regardless of the pesticide exposure history. This is surprising given that wood frogs are sensitive to carbaryl (Bridges, 2000; Relyea & Mills, 2001; Rohr et al., 2003) and that other studies have demonstrated that prior exposure to 1 mg/L carbaryl affects susceptibility to parasitic infection in other amphibian species (Pochini & Hoverman, 2017). The overall echinostome infection success (c. 51% encystment rate) was high relative to other studies (Holland et al., 2007; Orlofske et al., 2013; Hua et al., 2017), which could indicate that either these wood frog populations are highly susceptible to echinostome cercariae, or that the cercariae used in this study were particularly infective. Alternatively, high infection rates could be due to the size of the experimental units. Because each tadpole in the experiment was housed in a relatively small volume of water (100 ml, compared to 500 ml in Hua et al., 2017), the experimental design limited the effectiveness of behavioural parasite avoidance, an important strategy for preventing trematode infections (Koprivnikar et al., 2006). Thus, the study may be more representative of differences in host immune resistance between groups. The results of this study suggest that carbaryl exposure of up to 1 mg/L does not significantly alter the tadpole immune response to trematodes. However, because behavioural avoidance can play a critical role in preventing echinostome infections and carbaryl can alter tadpole behaviour (Bridges, 2000; Daly & Johnson, 2011; Koprivnikar et al., 2006), future studies should address how a sublethal carbaryl dose can influence behavioural responses to parasites and how that influences infection success.

While it is well-documented that responses to pesticides and parasites can vary among amphibian species (Bridges & Semlitsch, 2000; Hoverman et al., 2011; Gahl et al., 2011; Hammond et al., 2012; Gervasi et al., 2013, 2017; Blaustein et al., 2018), there is considerably less literature experimentally exploring this variation at the population level. We found significant interpopulation differences in susceptibility to two common amphibian parasites, ranavirus and trematodes. Moreover, we found that differences in trematode susceptibility were related to a population's distance to agriculture, with individuals from populations close to agriculture being more susceptible to infection with trematodes than individuals from populations far from agriculture. Further research is needed to parse out how environmental differences among populations, including land-use patterns, as well as evolutionary history impact susceptibility to parasites, and if these patterns of susceptibility are consistent across space and time. To this end, long-term studies that track population-level variation in

parasite susceptibility through time will greatly increase our understanding of these trends.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data associated with this paper are deposited in the Purdue University Research Repository: <https://purr.purdue.edu/publications/3679/1> (Hoverman and Billet, 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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