Immediate and lag effects of pesticide exposure on parasite resistance in larval amphibians

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SUMMARY

Across host–parasite systems, there is evidence that pesticide exposure increases parasite loads and mortality following infection. However, whether these effects are driven by reductions in host resistance to infection or slower rates of parasite clearance is often unclear. Using controlled laboratory experiments, we examined the ability of larval northern leopard frogs (*Lithobates pipiens*) and American toads (*Anaxyrus americanus*) to resist and clear trematode (*Echinoparyphium* sp.) infections following exposure to the insecticide carbaryl. Northern leopard frogs exposed to 1 mg L^{-1} of carbaryl had 61% higher parasite loads compared with unexposed individuals, while there was no immediate effect of carbaryl on parasite encystment in American toads. However, when tadpoles were exposed to carbaryl and moved to freshwater for 14 days before the parasite challenge, we recovered 37 and 63% more parasites from carbaryl-exposed northern leopard frogs and American toads, respectively, compared with the control. No effects on clearance were found for either species. Collectively, our results suggest that pesticide exposure can reduce the ability of amphibians to resist parasite infections and that these effects can persist weeks following exposure. It is critical for researchers to incorporate species interactions into toxicity studies to improve our understanding of how contaminants affect ecological communities.

Key words: agrochemical, carbamate, helminth, disease ecology, acetylcholinesterase inhibitor.

INTRODUCTION

Infectious disease is a central component of ecological communities, influencing host fitness, population dynamics and community composition (De Castro and Bolker, 2004; Smith *et al*. 2006; Johnson *et al*. 2015; Wood and Johnson, 2015). Indeed, disease agents comprise a substantial proportion of biomass in natural systems and perform important functions in food webs (Lafferty *et al*. 2006; Kuris *et al*. 2008). While disease research often focuses on host–parasite interactions in isolation, there is an increasing interest in disease dynamics within the context of complex natural systems. In particular, the interaction of disease and environmental stressors such as climate change, habitat alteration and chemical contamination are pertinent in a progressively human-influenced environment (Bradley and Altizer, 2007; Rohr and Raffel, 2010).

Contamination from pesticides is a stressor of particular concern due to the widespread use of pesticides on agricultural, commercial and residential land. In the USA, ~544 million kg of pesticides (active ingredient) are applied annually to a broad range of habitats (Grube *et al*. 2011). Moreover, these chemicals often enter natural systems, where they can affect non-target organisms (Relyea and Hoverman, 2006; Grube *et al*. 2011; Marcogliese and Pietrock, 2011; Köhler and Triebskorn, 2013; Mason *et al*. 2013). Pesticide exposure has been shown to influence development, immune function, behaviour and survival across vertebrate and invertebrate taxa (Egea-Serrano *et al*. 2012; Gill *et al*. 2012; Brühl *et al*. 2013; Di Prisco *et al*. 2013). Given the sublethal effects of pesticide exposure on host physiology, studies have increasingly explored the consequences of pesticide exposure on disease dynamics. Pesticide exposure has been associated with increased susceptibility to infection, greater pathology and higher parasite abundance in communities (Christin *et al*. 2003; Coors *et al*. 2008; Rohr *et al*. 2008*a*, 2013; Di Prisco *et al*. 2013). While our understanding of this interaction is growing, there is a need to identify the mechanisms by which pesticide exposure influences disease.

When challenged with a parasite, a host can decrease its parasite load using a process known as resistance (Boots, 2008; Read *et al*. 2008). Hosts can resist parasite infections in several ways including behavioural alterations that avoid parasites and immunological responses that function to reduce the parasites' success at initially infecting the host or persisting within the host post-infection (parasite clearance; Råberg *et al*. 2009). Given that natural systems are highly variable, it is likely that resistance mechanisms are affected by environmental conditions and stressors. In particular, pesticide exposure is expected to alter these resistance mechanisms by

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disrupting immune function, causing physiological changes and altering behaviour (Marcogliese and Pietrock, 2011). Pesticides can reduce leucocyte counts, which have been correlated with trematode avoidance and clearance in amphibians (Kiesecker, 2002; Christin *et al*. 2003; Rohr *et al*. 2008*a*; LaFonte and Johnson, 2013). Pesticides can also decrease cholinesterase activity, leading to reduced movement in larval fish, another mechanism of parasite avoidance (Beauvais *et al*. 2001; Koprivnikar *et al*. 2006). Interestingly, pesticides can have a lag effect that increases susceptibiliy to infection weeks after exposure (Budischak *et al*. 2008; Rohr *et al*. 2013). Moreover, these resistance mechanisms are context dependent, are subject to tradeoffs, and exert different selective pressures on hosts and parasites and may thereby be affected by stressors in different ways (Fineblum and Rausher, 1995; Roy and Kirchner, 2010; Rohr *et al*. 2010). As such, there is a need for research that addresses the complexity of these interactions.

Amphibians are an ideal model system for studying pesticide–disease interactions due to the prevalence of pesticide contaminants in wetland environments and because emerging diseases are currently and drastically reducing global amphibian populations (Daszak *et al*. 2003; Relyea and Hoverman, 2006). The interaction between pesticides and pathogens has frequently been studied using echinostomes, particularly *Echinostoma trivolvis* and *Echinoparyphium* spp., because they are widespread and highly prevalent parasites (Kanev *et al*. 1995; Szuroczki and Richardson, 2009). While amphibians are often found surviving with high echinostome loads (>2000 parasites; Schotthoefer *et al*. 2003; Skelly *et al*. 2007; Johnson and McKenzie, 2009; Szuroczki and Richardson, 2009; Rohr *et al*. 2010; Orlofske *et al*. 2013), host exposure to pesticides is known to increase susceptibility to echinostome infection and mortality following infection (Budischak *et al*. 2008; Rohr *et al*. 2008*b*; Koprivnikar, 2010). Moreover, these effects have been documented weeks after pesticide exposure, suggesting that pesticides can have a lag effect on susceptibility to echinostome infection (Budischak *et al*. 2008). However, no studies have explored the effects of pesticides on clearance of echinostomes, and none have examined the effects of pesticides on echinostome avoidance and clearance while incorporating lag effects. Importantly, the use of multiple host species and multiple pesticide concentrations is central in accounting for the context-dependency of these interactions.

Our objectives were to determine whether exposure to the insecticide carbaryl affects resistance to echinostome infection, measured as both initial infection success and clearance of infection, and whether pesticide exposure has a lag effect on resistance for two different species of larval amphibians. If pesticide exposure causes immunosuppression (e.g. reduced leukocyte counts; Christin *et al*. 2003) in hosts and this immunosuppression is maintained 2 weeks following pesticide exposure, we expected to see a reduction in resistance whereby parasite loads increase as pesticide concentration increases from 0 to 1 mg L^{-1}. Likewise, if pesticide exposure causes immunosuppression, we hypothesized that the rate of parasite clearance, irrespective of initial parasite resistance, will decrease as pesticide concentration increases from 0 to 1 mg L^{-1}.

MATERIALS AND METHODS

Species collection and husbandry

We collected northern leopard frogs (*Lithobates pipiens*) and American toads (*Anaxyrus americanus*) from a local pond in West Lafayette, Indiana (40·452245, −87·054992) in April 2013; we collected five partial *L. pipiens* egg masses and ten partial *A. americanus* egg masses. Egg masses were reared outdoors in 100-L pools filled with 70 L of well water and covered with 70% shade cloth. After hatching, tadpoles were fed rabbit chow *ad libitum* until the start of the experiments. Tadpoles were brought inside and acclimated to laboratory conditions (23° C, 12:12 h day:night photoperiod) for 24 h prior to the start of each experiment. Unless noted otherwise, tadpoles were fed Tetramin *ad libitum* every 2 days during each experiment. The Purdue Institutional Animal Care and Use Committee (IACUC) approved all animal husbandry and euthanasia procedures (protocol #1304000846), and the minimal number of animals needed to produce statistically significant results were used.

To obtain *Echinoparyphium*, we collected their intermediate hosts (ramshorn snails, *Helisoma trivolvis*) from local ponds in West Lafayette, IN. Snails were screened for infection by placing individuals in 50-mL tubes filled with 45 mL of filtered, ultraviolet (UV)-irradiated water and allowing them to shed the free-living stage of the parasite (cercariae) under a heat lamp (Hua *et al*. 2016). Infected snails were housed in 15-L tubs filled with 8 L of aged well water at a density of 3 individuals L^{-1} and fed rabbit chow *ad libitum* until the start of the experiments. To obtain *Echinoparyphium* cercariae for experiments, snails were induced to shed parasites as described above. We used a glass pipette and stereo dissection scope to isolate and count cercariae for each experiment. The cercariae were transferred to clean 50-mL tubes filled with 45 mL of water and immediately added to the appropriate experiment unit. For control treatments not assigned *Echinoparyphium*, we repeated this procedure adding the same volume of water from uninfected

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snails. The echinostomes used in these experiments were classified as *Echinoparyphium* sp. based on a genetic analysis of echinostomes collected from the same pond (Hua *et al*. 2016).

Focal pesticide

We used the widespread insecticide carbaryl, an acetylcholinesterase inhibitor, for each experiment. Carbaryl is applied as an agricultural insecticide, with application rates reaching 400 000 kg annually and surface water concentrations measured as high as 4·8 mg L^{-1} in the USA (Norris *et al*. 1983; Baker and Stone, 2015). LC50 estimates of carbaryl range from 7·4 to 9·6 mg L^{-1} for northern leopard frogs and approximately 6·5 mg L^{-1} for American toads (Bridges *et al*. 2002; Relyea, 2003). We selected 0·5 and 1 mg L^{-1} as our experimental concentrations because they were environmentally relevant and sub-lethal. We created a stock solution by adding 1 mL of commercial grade carbaryl (22·5% Sevin) to 9 mL of filtered, UV-irradiated water to achieve a concentration of 23 600 mg L^{-1} of carbaryl. For each experiment, we mixed the stock solution with filtered, UV-irradiated water to create appropriate pesticide concentrations. Although actual carbaryl concentrations were not assessed, our previous work using similar methods has demonstrated that actual concentrations are approximately 75% of nominal concentrations (Pochini and Hoverman, 2016).

Experimental design and sample processing

Separately for each species, we conducted a randomized factorial experiment consisting of three pesticide treatments and four parasite treatments to examine the effects of pesticide exposure on tadpole resistance of echinostome infection. Our three pesticide treatments consisted of a control (0 mg L^{-1}) and exposure to either 0·5 or 1 mg $L^{-1}$of carbaryl for 7 days. Water was changed on day 4 and the pesticide concentrations renewed. Our four parasite treatments consisted of: (1) a control (0 cercariae); (2) exposure to 50 cercariae immediately following pesticide exposure and processed 2 days after cercariae exposure (2-day); (3) exposure to 50 cercariae immediately following pesticide exposure and processed 14 days after cercariae exposure (14-day); and (4) exposure to 50 cercariae 14 days following pesticide exposure and processed 2 days after cercariae exposure (14-day lag effect). All tadpoles were housed in freshwater following pesticide exposure to ensure no confounding effects of the pesticide on the cercariae (Hua *et al*. 2016). We replicated each of our 12 treatments six times for a total of 72 experimental units. Experimental units consisted of 2-L containers filled with 1 L of filtered, UV-irradiated water. Each unit housed one tadpole at Gosner (1960) stage 27 ± 0·069 with mass 0·276 ± 0·007 g (mean ± S.E.) for northern leopard frogs and stage 31 ± 0·211 with mass 0·059 ± 0·002 g for American toads. At the appropriate time for each treatment, tadpoles were euthanized in MS-222 and preserved in 10% formalin for processing. Each individual was weighed, measured for snout–vent length (SVL) and total length, and staged (Gosner, 1960). We removed tadpole kidneys under a dissecting scope, placed them between two slides to create a thin layer of tissue, and counted metacercarial cysts (Hoverman *et al*. 2013). We also searched the remainder of the tadpole body cavity to ensure all cysts were counted.

Statistical analyses

For each parasite treatment, individual one-way analyses of variance (ANOVA) were used to assess differences in the proportion of echinostomes encysted (number encysting out of 50 administered) among pesticide treatments. Individuals in the no-parasite treatment were excluded because no parasites were detected. We analysed data from each species separately. We logit transformed the dependent variable for American toads in the lag effect treatment to meet the assumption of homoscedasticity. Additionally, for each species, we used two-way ANOVA to investigate the effects of pesticide concentration and parasite treatment (2 and 14 days) on the proportion of echinostomes encysted. This was done to determine whether parasite load decreased between 2 and 14 days (i.e. clearance) and whether the pesticide and parasite treatments interactively affect parasite load, which would indicate an effect of the pesticide on parasite clearance (LaFonte and Johnson, 2013). In this way, we could differentiate between the effect of the pesticide on initial resistance and its effect on rate of clearance irrespective of initial resistance. Using Pearson's correlations, we determined whether our size variables (stage, mass, SVL) should be included in our analyses. For American toads, size was not correlated with the proportion of echinostomes encysted ($P \geqslant 0{\cdot}066$). For northern leopard frogs, SVL was negatively correlated with the proportion of echinostomes encysted and was therefore included as a covariate in our analyses ($P \geqslant 0{\cdot}045$). However, all other size variables were excluded ($P \geqslant 0{\cdot}085$). Because developmental stage has been shown to influence parasite resistance (Rohr *et al*. 2010), we also used ANOVA to determine whether stage varied between the 2- and 14-day lag effect treatments and between species. All analyses were performed using SPSS 23·0 (SPSS Inc., Chicago, IL, USA) at $\alpha = 0{\cdot}05$.

RESULTS

For northern leopard frogs, we found a strong positive effect of pesticide concentration on echinostome

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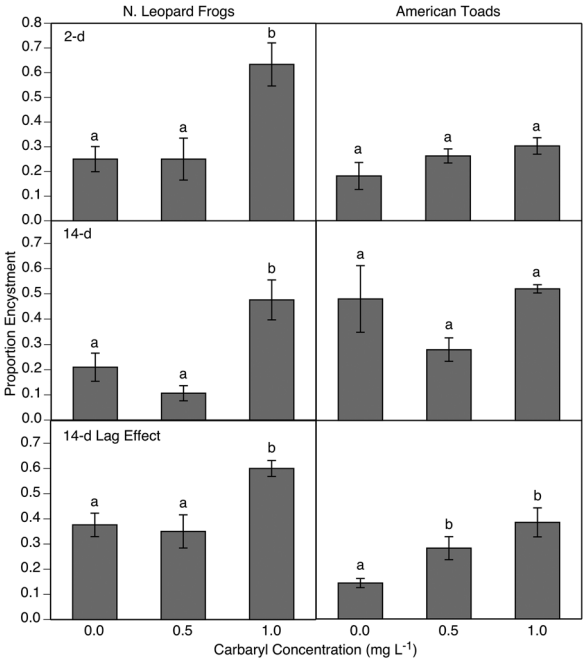

Fig. 1. Proportion of echinostomes encysted across pesticide concentrations and parasite treatments. Tadpoles were exposed to one of three carbaryl concentrations and either immediately exposed to 50 echinostome cercariae and processed 2 or 14 days later (2-day, 14-day) or exposed to 50 echinostome cercariae 14 days following pesticide exposure and processed 2 days later (14-day lag effect). Northern leopard frogs and American toads are represented on separate columns. Data are means ± 1 S.E. Within each panel, treatments sharing lower case letters are not significantly different from each other based on pairwise comparisons using Fisher's LSD (least significant difference) test ($P > 0{\cdot}05$).

encystment for all parasite treatments with 61, 56, and 37% greater encystment in the 1 mg L^{-1} treatment compared with the control for the 2-, 14- and 14-day lag effect treatments, respectively (2-day, $F_{2,14} = 5{\cdot}296$, $P = 0{\cdot}019$; 14-day, $F_{2,13} = 8{\cdot}584$, $P = 0{\cdot}004$; 14-day lag effect, $F_{2,26} = 4{\cdot}365$, $P = 0{\cdot}023$; Fig. 1). However, no differences were found between the 0·5 mg L^{-1} treatment and the control (2-day, $P = 1{\cdot}00$; 14-day, $P = 0{\cdot}243$; 14-day lag, $P = 0{\cdot}730$). For our investigation of parasite clearance, we found no interactive effect of pesticide treatment and parasite treatment (2- and 14-day) on echinostome encystment ($F_{2,28} = 0{\cdot}687$, $P = 0{\cdot}516$) and no effect of parasite treatment on encystment ($F_{1,28} = 1{\cdot}348$, $P = 0{\cdot}213$).

Pesticide concentration did not affect echinostome encystment in American toads for either the 2-day ($F_{2,15} = 1{\cdot}951$, $P = 0{\cdot}177$) or 14-day parasite treatments (14-days, $F_{2,7} = 2{\cdot}152$, $P = 0{\cdot}187$). However, there was a strong effect of pesticide concentration on echinostome encystment for the 14-day lag effect treatment ($F_{2,27} = 7{\cdot}298$, $P = 0{\cdot}003$), with 63% greater encystment in the 1 mg L^{-1} treatment compared with the control ($P = 0{\cdot}001$) and 49% greater encystment in the 0·5 mg L^{-1} compared with the control ($P = 0{\cdot}026$). For our analysis of parasite clearance, there was a weak interactive effect of pesticide treatment and parasite treatment ($F_{2,22} = 2{\cdot}974$, $P = 0{\cdot}072$); however, encystment was higher in the 14-day treatment compared with the 2-day ($F_{1,22} = 9{\cdot}859$, $P = 0{\cdot}005$).

For northern leopard frogs and American toads, respectively, developmental stage was 2 and 6% higher in the 14-day lag effect treatment compared with the 2-day treatment (northern leopard frogs, $F_{1,45} = 4{\cdot}416$, $P = 0{\cdot}041$; American toads, $F_{1,45} = 16{\cdot}165$, $P < 0{\cdot}001$). Additionally, American toads were 21% more developed (i.e. higher stage) than northern leopard frogs ($F_{119} = 535{\cdot}362$, $P < 0{\cdot}001$).

DISCUSSION

Pesticide exposure and infectious disease are two common stressors that may co-occur in natural systems and interactively affect organisms. While research on this interaction often addresses the effects of pesticide exposure on host susceptibility to parasitic infection, more information is needed on how pesticides affect the mechanisms of parasite avoidance and clearance by which hosts resist infection. We found that exposure to the insecticide carbaryl increased initial parasite loads in northern leopard frogs but did not affect clearance. For American toads, carbaryl did not have an immediate effect on either component of resistance. However, for both species, carbaryl had a lag effect, increasing parasite load 2 weeks after exposure. Our results show that pesticide exposure can negatively influence parasite resistance and that these effects can persist through development. Furthermore, our study underscores species-level variation in responses to combined stressors.

Carbaryl exposure had an immediate negative effect on parasite resistance, reducing initial echinostome encystment in one of our two study species. For northern leopard frogs, parasite encystment was 61% higher for individuals exposed to 1 mg L^{-1} of carbaryl compared with the control. However, there was no effect of carbaryl at 0·5 mg L^{-1}, suggesting that 1 mg L^{-1} may represent a threshold concentration for inducing immunosuppression in larval northern leopard frogs. Concentrations of carbaryl as low as 0·03 mg L^{-1} have been shown to increase echinostome encystment in green frogs, but species differences in pesticide sensitivity could account for this disparity (Rohr *et al*. 2008*b*). American toads, on the contrary, exhibited no change in echinostome avoidance with pesticide exposure. Amphibian tolerance to pesticides has been shown to vary phylogenetically, with ranids exhibiting lower tolerance than bufonids (Hammond *et al*. 2012). Moreover, species-level variation in response to multiple stressors has been

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documented for pesticide–predator interactions in amphibians (Relyea, 2003). Our results suggest that the role of phylogeny in pesticide–disease interactions may be an important factor and warrants further investigation.

Our examination of lag effects demonstrated that pesticides can have a lasting effect on disease outcomes and species that may not be immediately affected by pesticides (e.g. American toads) can still experience lag effects of exposure. When tadpoles were allowed 14 days between pesticide and parasite exposures, pesticides decreased their ability to resist infection, with 37 and 63% greater encystment for northern leopard frogs and American toads, respectively, when exposed to 1 mg L^{-1} carbaryl compared with the control. Moreover, American toads were sensitive to the lower concentration of 0·5 mg L^{-1}, exhibiting 49% greater encystment compared to the control. Across amphibian disease systems, there is mounting evidence that pesticides can have lag effects on disease outcomes (Budischak *et al*. 2008; Rohr *et al*. 2013). However, it is largely unknown how long the lag effect of pesticide exposure will last or whether lag effects are influenced by phylogeny or pesticide characteristics (e.g. mode of action, concentration). It is also important to note that due to our experimental timeline, tadpoles in the 14-day lag effect treatment were 14 days older and at a slightly higher developmental stage at the time of echinostome exposure than those in the 2-day treatment. Moreover, American toads were 21% more developed than northern leopard frogs. While we found no relationship between developmental stage and parasite encystment, it is important to understand that stage can affect resistance to trematode infection (Rohr *et al*. 2010). To better understand how lag effects function, particularly across host species, future studies that directly manipulate developmental stage and time since pesticide exposure will be needed.

We found no evidence of parasite clearance over time or an effect of pesticide exposure on clearance for either species. Clearance of trematodes has been documented for echinostomes and the more debilitating *Riberoia ondatrae*. However, clearance appears to be stronger with *R. ondatrae*, presumably because of its subcutaneous encystment and high virulence (LaFonte and Johnson, 2013). In contrast, echinostomes exhibit a relatively low virulence, causing negligible fitness costs at moderate parasite loads (Orlofske *et al*. 2009). Therefore, the low parasite loads used in this experiment may not have been sufficient to prompt parasite clearance. Studies utilizing higher parasite loads may be necessary to evaluate whether pesticides alter clearance rates.

Our results demonstrate that, similar to previous studies (Rohr *et al*. 2008*a*, *b*), carbaryl exposure decreased the ability of hosts to immediately resist echinostome infection; however, we also reveal influences of several understudied factors. Lag effects, for instance, have been documented in amphibian disease systems, but there is a need for research that determines the mechanisms behind these effects and how they influence disease outcomes. Phylogeny is also an important factor in ecotoxicological studies, but phylogenetic approaches have rarely been used to predict and understand responses to multiple stressors and their interactions. Further, in order to fully understand how these interactions affect complex ecological systems, studies need to incorporate variation in factors such as pesticide mode of action, pesticide concentration and parasite species. In particular, comparisons among parasites of varying virulence and pesticides of varying toxicity would be valuable in understanding these systems. Given the ubiquity and regular co-occurrence of pesticide and parasite stressors in natural systems, it is imperative to understand the complex ways in which they interactively affect ecological communities, particularly as anthropogenic stressors become more prevalent and increasingly interact with natural stressors.

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