UNDERSTANDING THE NET EFFECTS OF PESTICIDES ON AMPHIBIAN TREMATODE INFECTIONS

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Abstract. Anthropogenic factors can have simultaneous positive and negative effects on parasite transmission, and thus it is important to quantify their net effects on disease risk. Net effects will be a product of changes in the survival and traits (e.g., susceptibility, infectivity) of both hosts and parasites. In separate laboratory experiments, we exposed cercariae of the trematode *Echinostoma trivolvis*, and its first and second intermediate hosts, snails (*Planorbella trivolvis*) and green frog tadpoles (*Rana clamitans*), respectively, to one of four common pesticides (atrazine, glyphosate, carbaryl, and malathion) at standardized, ecologically relevant concentrations (201.0, 3700.0, 33.5, and 9.6 µg/L, respectively). We measured effects of pesticide exposure on six mechanisms important to this host–parasite interaction: (1) survival of *E. trivolvis* cercariae over 26 hours, (2) tadpole survival over two weeks, (3) snail survival over four weeks, (4) snail growth and fecundity, (5) cercarial infectivity, and (6) tadpole susceptibility to a fixed number of cercariae. Pesticides, in general, caused significantly greater mortality of *E. trivolvis* cercariae than did control treatments, but atrazine was the lone chemical to significantly reduce cercarial survival (LC50 value = 267 mg/L) and then only at concentrations greater than commonly found in aquatic ecosystems ($\geq$200 µg/L). None of the pesticides significantly enhanced *E. trivolvis* virulence, decreased tadpole survival, or reduced snail survival, growth, or fecundity. Sublethal exposure of the cercariae to the pesticides (4 h) did not significantly affect trematode encystment in *R. clamitans*. In contrast, sublethal exposure of *R. clamitans* to each of the four pesticides increased their susceptibility as measured by the percentage of cercariae that encysted. The reduction in exposure to trematodes due to pesticide-induced cercarial mortality (a density-mediated effect) was smaller than the pesticide-induced increase in amphibian susceptibility (a trait-mediated effect), suggesting that the net effect of exposure to environmentally realistic levels of pesticides will be to elevate amphibian trematode infections. These findings highlight the importance of elucidating the lethal and sublethal effects of anthropogenic factors on both hosts and parasites to understand the mechanisms underlying changes in parasite transmission and virulence, an approach that is especially needed for amphibians, a taxon experiencing global disease-related declines.

Key words: atrazine; carbaryl; Echinostoma trivolvis *trematode; indirect effect, density-mediated or trait-mediated; glyphosate; herbicide; insecticide; malathion; parasite;* Planorbella trivolvis *snail;* Rana clamitans *amphibian, decline.*

INTRODUCTION

Human and wildlife diseases are emerging at an unprecedented rate (Daszak et al. 2000, Wolfe et al. 2007). Since this is primarily due to changes in the ecology of hosts and parasites (Schrag and Wiener 1995), understanding the environmental factors driving these changes will be essential to designing effective control strategies. Environmental change can have complex effects on parasite transmission that depend on the specific interactions among hosts, parasites, and biotic and abiotic conditions (Lafferty and Kuris 1999). Factors affecting host and parasite ecology can be

dichotomized into those that are lethal or sublethal (Bolker et al. 2003). Lethal effects directly reduce the density of hosts and/or parasites. Sublethal effects can alter host and/or parasite traits, such as parasite infectivity or host immunity, which can subsequently affect host–parasite interactions and disease dynamics (Werner and Peacor 2003, Relyea and Hoverman 2006, Rohr et al. 2006a). Importantly, environmental factors can have concurrent positive and negative effects on parasite transmission, underscoring the importance of quantifying their net effect on disease emergence. For instance, an environmental factor might directly kill parasites while simultaneously increasing host susceptibility to the survivors.

Understanding the net effects of environmental factors on parasite transmission is especially important for amphibians because their global declines appear to

be partly due to interactions between environmental change and emerging parasites and pathogens (Daszak et al. 2003, Pounds et al. 2006). Of particular concern have been trematode infections of amphibians, such as the trematode species *Ribeiroia ondatrae* and *Echinostoma trivolvis* (Johnson et al. 2004). Both species are considered emerging parasites driven by anthropogenic environmental change (Johnson and Sutherland 2003, Skelly et al. 2006). *R. ondatrae* has received substantial research attention because of the grotesque limb malformations it causes (Sessions and Ruth 1990, Johnson et al. 2004). However, we know much less about the ecology or epidemiology of *E. trivolvis*, a more cosmopolitan amphibian trematode (Sorensen and Minchella 1998, Thiemann and Wassersug 2000) that can cause substantial amphibian mortality related to kidney damage (Martin and Conn 1990, Schotthoefer et al. 2003, Belden 2006, Holland et al. 2007).

One group of abiotic environmental factors that is common, widespread, and likely to affect disease prevalence and intensity is pesticides (Relyea and Hoverman 2006, Rohr et al. 2006*a*). Pesticides are often directly toxic to hosts and free-living stages of parasites (Lafferty and Kuris 1999, Morley et al. 2003). In addition, pesticides frequently alter the traits of organisms, such as behaviors (Weis et al. 2001, Rohr et al. 2003, 2004, Rohr and Palmer 2005) and immune responses (Voccia et al. 1999), which can affect parasite transmission and virulence (Lafferty and Kuris 1999, Thiemann and Wassersug 2000, Koprivnikar et al. 2006*b*). Furthermore, since the direction and magnitude of lethal and sublethal effects of pesticides can vary (Relyea and Hoverman 2006, Rohr et al. 2006*a*), there is often no defensible a priori reason to believe that chemical contaminants, in general, should increase or decrease parasite prevalence or intensity. Thus, for a given species, certain chemicals might increase risk of infection whereas others might decrease this risk.

Since there are over 80 000 chemicals registered for use in the United States, one of the major challenges is to prioritize which ones are more likely to influence parasite transmission. While it is possible to compare the effects of pesticides on hosts and parasites across studies, this can be problematic because of different environmental conditions in most experiments. Thus, progress towards understanding the effects of chemicals on disease emergence will be slow unless researchers simultaneously consider multiple pesticides likely to influence parasite transmission. Here we test the hypothesis that four common pesticides have lethal and sublethal effects on *E. trivolvis* cercariae and its first and second intermediate hosts, planorbid snails (*Planorbella trivolvis*) and green frogs (*Rana clamitans*), respectively, that affect *E. trivolvis* transmission and virulence. Specifically, we predicted that pesticides would (1) reduce the survival of trematodes (e.g., Lafferty and Kuris 1999, Morley et al. 2003), snails, and amphibians (e.g., Rohr et al. 2004, 2006*b*, Storrs

and Kiesecker 2004, Relyea 2005*b*), (2) decrease cercarial infectivity (e.g., Reddy et al. 2004), and (3) increase tadpole susceptibility to infections (e.g., Kiesecker 2002, Christin et al. 2004, Brodkin et al. 2007). By manipulating whether *E. trivolvis*, *P. trivolvis*, or *R. clamitans* were exposed to the pesticides and quantifying the survival and traits of each species, we determined whether changes in the density or traits of the hosts or parasite were responsible for any changes in transmission or virulence.

Pesticide background

We studied two herbicides and two insecticides at standardized concentrations to facilitate comparisons across chemicals. The herbicides were glyphosate and atrazine, the two most commonly used pesticides in the United States (Kiely et al. 2004). Both are used heavily on field crops, especially corn. Glyphosate is a broad-spectrum herbicide that inhibits the synthesis of plant amino acids and atrazine is a broadleaf herbicide that inhibits photosynthesis (Solomon et al. 1996, Giesy et al. 2000) The half-life of the most popular formulation of glyphosate, Roundup®, is 7–70 days (Giesy et al. 2000). The half-life of atrazine is highly variable but can exceed 100 days (Diana et al. 2000).

The insecticides we studied were malathion and carbaryl, broad-spectrum insecticides that kill by inhibiting acetylcholinesterase. Malathion is the most commonly used insecticide in the United States (Kiely et al. 2004) and is often applied directly to wetlands to control mosquitoes that harbor pathogens such as malaria and West Nile virus. The half-life of malathion is between two and 26 days (Wang 1991). Carbaryl is a commonly used, short-lived (hours to weeks) insecticide that has received substantial attention from amphibian ecologists (e.g., Boone and Semlitsch 2002, Boone et al. 2005).

MATERIAL AND METHODS

Selecting pesticide concentrations

We selected standardized, "worst case" but ecologically relevant, concentrations to compare our four pesticides. Specifically we selected the highest concentration of these pesticides recorded in the U.S. Geological Survey's National Water Quality Assessment Data Warehouse between 1991 and 2002. This database contains concentrations of chemicals detected at 6100 surface water sites from 42 watersheds across the United States and is probably the most comprehensive record of freshwater pollution in the United States. Although glyphosate is now the most commonly used pesticide in the United States, its use rose recently and thus it was not commonly tested for by the USGS between 1991 and 2002. Consequently, we chose the highest expected environmental concentration for glyphosate suggested by Giesy et al. (2000). Hence, we used the following technical grade concentrations in all experiments containing the four focal pesticides: 201 µg/L of atrazine, 3700 µg/L of glyphosate, 33.5 µg/L of carbaryl, and 9.6

µg/L of malathion (purchased from ChemService, West Chester, Pennsylvania, USA; all purity above 98%). In addition, we included both water and acetone (0.0002%) solvent controls in these experiments. Our stock solutions were tested by the Mississippi State Chemical Laboratory (Mississippi State University, USA). The actual concentrations were 2.4%, 0.4%, 13.9%, and 4.3% lower than the nominal (i.e., targeted) concentrations for atrazine, glyphosate, carbaryl, and malathion, respectively.

Animal collection and maintenance

Echinostoma trivolvis cercariae were obtained from naturally infected *P. trivolvis* snails that were placed in 75 mL of artificial spring water (ASW, as described by Cohen et al. [1980]) under a fluorescent light source to promote cercarial release. Approximately 250 snails were collected for all the experiments. These snails were collected from Harrisburg, Pennsylvania, USA (40°4′56.0″ N, 76°46′2.0″ W). When not being used to obtain cercariae, snails were maintained, at room temperature, in aerated 37.85-L aquaria containing ASW and fed boiled lettuce and fish flakes ad libitum.

In our experiments, we used green frog tadpoles as the intermediate amphibian host for *E. trivolvis* for several reasons. Unlike many tadpole species in Pennsylvania (e.g., wood frogs, spring peepers, northern leopard frogs, American toads), green frog tadpoles hatch and develop later in the season (June–August) and thus are in ponds during peak *E. trivolvis* cercariae shedding by snails (Sapp and Esch 1994, Schmidt and Fried 1997). In addition, green frog tadpoles often overwinter in ponds in this region (Berven et al. 1979) and thus can have two seasons of exposure to *E. trivolvis* cercariae.

For the cercarial infectivity experiment (see *Experiment 4* below), 96 green frog tadpoles between Gosner stages 25 and 39 (Gosner 1960) were collected in October 2005 from a pond in the Pennsylvania State Game Lands #176 (40°45′52.6″ N, 78°0′43.6″ W). Tadpoles in this pond were free of *E. trivolvis* infections based on the lack of evidence of suitable intermediate snail hosts and 12 tadpole dissections confirming the absence of *E. trivolvis*. Further evidence that these tadpoles were free of *E. trivolvis* was that none of the 84 tadpoles exposed to a fixed number of *E. trivolvis* cercariae had more *E. trivolvis* cysts than the number of cercariae to which they were exposed. Tadpoles were held at room temperature in aerated 37.85-L aquaria containing dechlorinated tap water and fed ground alfalfa pellets (rabbit chow) and fish flakes ad libitum until the experiment.

For the tadpole pesticide exposure experiment (see *Experiment 5* below), six green frog egg masses were collected in June 2006 from the same pond from which green frog tadpoles were collected for the cercarial infectivity experiment. They were allowed to hatch in outdoor wading pools covered with shade cloth. After hatching, tadpoles from each egg mass were mixed to homogenize genetic variation. Once the tadpoles were approximately Gosner stage 24 (Gosner 1960), they were brought into the laboratory for the experiment and maintained as described in the previous paragraph for the cercarial infectivity experiment. Both the snails and the tadpoles were maintained in the same room on a 12:12 hour day:night cycle, and all experiments were conducted at room temperature.

Experiment 1: snail pesticide exposure experiment

Reductions in adult snails or snail reproduction could reduce the densities of first intermediate hosts of *E. trivolvis* potentially reducing infection risk for amphibians, whereas changes in snail behavior might affect snail interactions with free-living stages of trematodes. Thus, this experiment was designed to test whether the focal pesticides reduced *P. trivolvis* survival or reproduction or affected their behaviors.

We placed 120 adult *P. trivolvis* into individual glass jars containing 350 mL of ASW. The six pesticide treatments were assigned randomly to jars generating a sample size of 20 for each treatment. The snails were fed boiled lettuce and fish flakes ad libitum, their ASW was changed twice a week, and the pesticides were reapplied after each water change for the first 14 days of the experiment. After this pesticide exposure period, all the snails were transferred to ASW only (no pesticides) for another 14 days to assess whether there were any post-pesticide exposure effects (e.g., Rohr et al. 2006b). Three times a week we quantified snail survival, vertical location in the water column (score of 1–3 for bottom to top third of water column), and number of egg masses. Adult snails were weighed at the start and end of the exposure and recovery periods (first 14 days and second 14 days, respectively), and the number of eggs per mass during the second week of the exposure and recovery periods were counted.

Experiment 2: cercarial mortality experiment

This experiment was designed to assess whether the focal pesticides were directly lethal to *E. trivolvis* cercariae, which in turn would reduce the number of cercariae available to infect tadpoles (a density-mediated effect of pesticides). Cercariae were collected from eight infected snails in July 2005, and 10 cercariae were pipetted into each well of four 24-well plates (960 cercariae total, $N = 16$ replicates for each of the six treatments). Artificial spring water (ASW) and pesticide stock solutions were added to the wells to ensure that each well had 1 mL of solution and the appropriate concentration and type of pesticide. Treatments were applied within two hours of cercarial emergence to ensure similar cercarial ages within and across treatments. A stratified random design was used to assign the six pesticide treatments to the six wells within each row of each plate. The randomization restriction was to have an even distribution of the treatments across the six columns of the plates to ensure that we controlled for
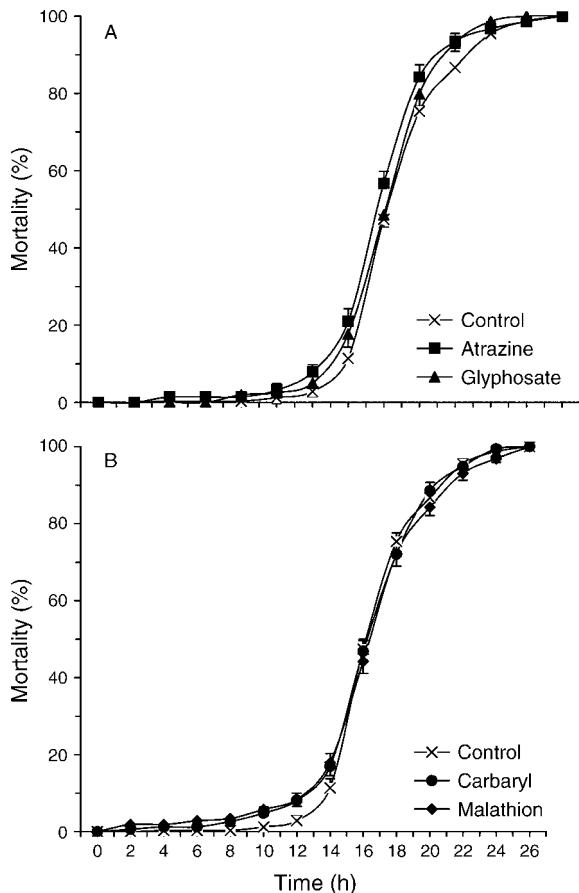
FIG. 1. Cumulative mortality of *Echinostoma trivolvis* cercariae when exposed to water or solvent controls (combined because there was no significant difference in mortality between the two controls; $N = 32$ replicates) relative to (A) herbicides (atrazine at 201 μg/L and glyphosate at 3700 μg/L; $N = 16$ replicates) and (B) insecticides (carbaryl at 33.5 μg/L and malathion at 9.6 μg/L; $N = 16$ replicates). Shown are means (±SE) connected by smoothed lines. The herbicides and insecticides are shown on separate panels simply to reduce the number of overlapping treatments and lines. Panels A and B represent the results of a single experiment.

any spatial effects. Live and dead cercariae were counted every two hours for 26 hours without knowledge of which treatments were assigned to the wells. The life span of cercariae of this species is ~24 h (Fig. 1). Cercariae were considered dead when they did not respond to nudges with a probe, as described previously (Reddy et al. 2004).

Experiment 3: atrazine dose–response experiment

Because *E. trivolvis* cercariae were sensitive to 201 μg/L of atrazine (Fig. 1A), we designed an experiment to evaluate the relationship between atrazine concentration and cercarial survival. We used the same methods as described for the cercarial mortality experiment except that we had six concentrations of atrazine (0, 2, 20, 110, 200, and 2000 μg/L). Only two 24-well plates were used

in this experiment providing eight replicates of each of the six concentrations.

Experiment 4: cercarial infectivity experiment

This experiment was conducted to evaluate whether sublethal exposure to the focal pesticides influenced cercarial encystment in green frog tadpoles (a trait-mediated effect of pesticides). In October of 2006, 84 tadpoles were exposed to cercariae that experienced sublethal pesticide exposure, providing a sample size of 14 for each of the six treatments. Tadpoles were size-matched within each of these 14 blocks to reduce variability attributed to size.

The same methods described for the cercarial mortality experiment were used to expose cercariae to the same pesticides and concentrations with the following exceptions. We used 12-well, rather than 24-well, plates and placed 20, rather than 10, cercariae per well. In addition, the cercariae were exposed to the treatments for four, rather than 26, hours (i.e., sublethal exposure) to ensure enough time after exposure for the cercariae to successfully infect the tadpoles before their natural death prevented infection (see Fig. 1).

After the 4-h exposure period, the cercariae were transferred to cups containing individual green frog tadpoles and 75 mL of ASW so that each tadpole was exposed to 20 cercariae. The small volume of water used to house the tadpoles prevented them from avoiding the cercariae. No cercariae remained in the cups after 24 hours, verifying that all the cercariae had entered the tadpoles, so we assumed that any difference in the number of cercariae that encysted across treatments was a product of the sublethal pesticide exposure for the cercariae and the physiological defenses of individual tadpoles. After the 24-h cercarial exposure, the tadpoles were transferred to larger cups containing 800 mL of dechlorinated tap water and fed ground alfalfa and fish flakes ad libitum. Seven days later, the tadpoles were euthanized by immersion in 1% benzocaine. They were fixed in 10% buffered formalin, massed, and staged (Gosner 1960).

To facilitate counting metacercariae in the kidneys of the tadpoles, we cleared and stained each tadpole using a modified method described by Hanken and Wassersug (1981). The kidneys were then removed and examined under a compound scope to ensure accurate cyst counts. *E. trivolvis* was not found in any other organs. All trematode counts were performed by researchers blind to the experimental groups to which the tadpoles belonged.

Experiment 5: tadpole pesticide exposure experiment

This experiment was designed with two objectives. First, we set out to determine whether the focal pesticides were directly lethal to green frog tadpoles, which could in turn reduce host availability for the cercariae (a density-mediated effect of pesticides). Second, we wanted to assess whether sublethal pesticide

exposure affected the susceptibility of green frog tadpoles to *E. trivolvis* cercarial infections (a trait-mediated effect of pesticides).

In July of 2006, 60 glass jars each received 300 mL of ASW, four green frog tadpoles (Gosner stages 24–26), and one of the six pesticide treatments (assigned randomly). Mortality was quantified daily for seven days. On day eight, we transferred two tadpoles from each jar into separate glass jars with 75 mL of ASW, so that we had two jars each with one tadpole. One of the two jars received 30 *E. trivolvis* cercariae (<2 h old) and the other received a similar volume of ASW without cercariae. In the cases where only one tadpole survived the first seven days of the experiment, that survivor was exposed to cercariae on the eighth day. Twenty-four hours passed to allow the cercariae to infect the tadpoles. Then, we reapplied the same pesticide treatments to which the tadpoles were exposed the previous week. Thus, the tadpoles were exposed to the pesticides before and after cercariae exposure, but the cercariae were not exposed to the pesticides.

Tadpoles were fed fish flakes ad libitum throughout the experiment, and we recorded tadpole mortality daily for seven more days after exposure to cercariae. We then made blood smears for each animal, euthanized the survivors in 1% benzocaine, and fixed all the tadpoles in 10% buffered formalin. Unfortunately, the tadpoles were too small to obtain enough blood for accurate leukocyte counts and thus reliable immune data is not available. Metacercariae in kidneys were counted using the procedures described in the cercarial infectivity experiment.

Statistical analyses

For each experiment in which multiple pesticides were tested, we first tested for a difference in response between the water and solvent controls. If there was no difference between these treatments, they were pooled for subsequent analyses. If there was a difference between the solvent and water control, we focused on comparisons between pesticides and the solvent control because the solvent control was the most appropriate control in this work. This is a common statistical approach for managing water and solvent controls (e.g., Rohr et al. 2003, Rohr and Palmer 2005). After examining the control treatments, we tested for differences between the controls and the pooled pesticide treatments followed by a test for differences among the five treatments. If there was a significant main effect of treatment, we used a one-tailed Dunnett's test to determine which pesticide treatments were different from the controls. All analyses were conducted using the container (i.e., wells, cups, or jars) as the replicate.

For the snail pesticide exposure experiment, we used analysis of variance (ANOVA) to test for the main effects of pesticide treatment, time (exposure vs. post-exposure period; a within subject effect), and their interaction on percentage gain in mass (arcsine square-root transformed), eggs laid, and mean vertical location in the water column. We used the generalized linear model with a binomial error distribution and a probit link to test for effects of pesticide treatments on snail survival at the end of the experiment.

In the cercarial mortality experiment, we used an ANOVA to compare the known duration of cercarial survival among the pesticide treatments within each row of the test plates (i.e., blocking by the 16 rows). We chose this statistical approach for three reasons: (1) the design did not lend itself to standard survival analyses because of multiple individuals per well; (2) most of the cercariae died within the 26-h experiment (99%); and (3) the remaining cercariae were moribund and thus unlikely to be infective.

For the atrazine dose-response experiment, we conducted a regression analysis with probit-transformed (i.e., normal standard deviate of the proportion) mortality as the dependent variable, log-transformed atrazine concentration as the continuous predictor, and the rows of the test plates as a blocking variable. We also calculated an LC50 (lowest concentration to kill 50% of the cercariae) value and a 95% confidence interval for the 14–18 h exposure period using the Schneider-Orelli formula (Schneider-Orelli 1947) to adjust for mortality in control wells. Averaging survival during the first 12 hours of the experiment with survival during hours 14–18 would greatly overestimate the LC50 because there was almost no death across all treatments during the first 12 h. The most relevant time period for calculating the LC50 is when there is the greatest difference among the concentrations, which is why hours 14–18 were chosen.

For both the tadpole pesticide exposure experiment and the cercarial infectivity experiment, we tested for differences in the proportion of cercariae that successfully encysted (arcsine square-root transformed) among the pesticide treatments using an analysis of covariance with tadpole stage as the covariate. In the tadpole pesticide exposure experiment, we used the generalized linear model with a normal error distribution and identity link to test for the effects of pesticide treatment on tadpole survival during the first seven days of the experiment and used a binomial error distribution and probit link to test for the effect of pesticide treatment, cercariae exposure, Gosner stage, and their interaction on tadpole survival during the last seven days of the experiment (using the jar as the replicate). A normal distribution was used in the first week because we could calculate mean survival of the four tadpoles in each jar, whereas in the second week there was only one tadpole per jar.

Although this study was not designed to directly and effectively compare the magnitude of trait- and density-mediated effects of trematode transmission, we did want to qualitatively compare their effect sizes to gain insight into the predicted effects of pesticides on disease risk for amphibians. To calculate effect sizes, we first controlled

TABLE 1.　Effects of the pesticides atrazine, glyphosate, carbaryl, and malathion on adult snail (*Planorbella trivolvis*) survival, growth, fecundity, and vertical location.

| | Treatment | | | | | | | | | | | |
| | Control† | | Atrazine | | Glyphosate | | Carbaryl | | Malathion | | | |
Response	Mean (±SE)	N	Mean (±SE)	N	Mean (±SE)	N	Mean (±SE)	N	Mean (±SE)	N	Statistic	P
Survival (%)	90.00	40	89.47	19	84.21	19	90.00	20	100.00	20	4.77‡	0.312
Gain in mass (%)	10.53 (1.30)	36	9.21 (2.02)	15	9.10 (1.95)	16	9.88 (1.90)	17	11.04 (1.75)	20	0.22§	0.929
Eggs laid per day	27.83 (2.60)	36	23.79 (3.90)	16	29.93 (3.90)	16	28.53 (3.79)	17	28.40 (3.49)	20	0.35§	0.840
Location score‖	1.88 (0.04)	38	1.97 (0.06)	17	1.96 (0.06)	17	1.89 (0.06)	17	2.00 (0.06)	20	1.00§	0.410

Notes: There were no significant effects of pesticides on any response variable examined. Probability values are for the main effect of pesticide treatment, with each pesticide treated as a separate level of this fixed effect.

† The water and solvent controls were pooled because there was no significant difference between them ($P > 0.281$).

‡ Chi-square statistic from logistic regression.

§ F statistic for the main effect of treatment from a model including treatment, time (exposure and recovery periods as within-subject effects), and their interaction. None of the interaction terms was significant ($P > 0.353$).

‖ Snail location was recorded as in the (1) bottom, (2) middle, or (3) top third of the water column.

for the blocks and covariates described above. We then took the residuals from these analyses and calculated Cohen's d and effect size r (Cohen 1988) for the pooled pesticide vs. control groups for both the cercarial mortality and tadpole pesticide exposure experiments.

RESULTS

In the snail pesticide exposure experiment, there was no significant difference between the water and solvent controls for any response variable (Table 1), and thus they were pooled for all subsequent analyses. Two snails, one each from the atrazine and glyphosate treatments, were injured during a water change and thus were removed from all analyses. Only 12 of the remaining 118 snails died during the experiment. We did not analyze time to death because of this low mortality. We found no evidence that the pesticide treatments significantly affected snail survival, growth, fecundity, or vertical location in the water column (Table 1). Additionally, there was no significant interaction between pesticide treatment and time (time being exposure vs. post-exposure periods; Table 1).

We noted some interesting life history relationships and trade-offs, despite substantial variation in life history traits among snails. Larger snails laid more eggs per day ($R = 0.233$, $F_{1,116} = 6.66$, $P = 0.011$) and did so by increasing the number of eggs per mass ($R = 0.254$, $F_{1,112} = 7.76$, $P = 0.006$) rather than by increasing the number of egg masses ($R = 0.013$, $F_{1,116} = 0.019$, $P = 0.891$). Furthermore, there was a significant negative relationship between eggs laid per day and percent mass gain, indicating a trade-off between growth and reproduction ($R = 0.199$, $F_{1,102} = 4.21$, $P = 0.043$).

In the cercarial mortality experiment, there was no significant difference in the duration of cercarial survival between the water and solvent controls ($F_{1,15} = 0.57$, $P = 0.462$), which were therefore pooled for subsequent analyses. Most cercariae died between hours 14 and 18 and almost all were dead by 26 hours (Fig. 1). Combining all four pesticides into a single category and comparing them to the controls revealed that

pesticides, in general, reduced the duration of cercariae survival ($F_{1,79} = 6.65$, $P = 0.012$). Further, when each of the pesticides and pooled controls was treated as a level in an ANOVA, there was a significant main effect of treatment on survival ($F_{4,76} = 2.96$, $P = 0.025$; Fig. 1, Fig. 2A). However, atrazine was the only treatment that had a significantly shorter survival than the controls ($P = 0.003$; Fig. 2A).

In the atrazine dose-response experiment, most cercariae again died between hours 14 and 18 and almost all were dead by hour 26. Atrazine concentration was a positive predictor of cercariae mortality for hours 14–18 (full model, $R^2 = 0.524$, $F_{8,39} = 5.40$, $P < 0.001$; Fig. 3). Only 200 and 2000 µg/L of atrazine caused significantly greater cercarial mortality in this time period than the controls ($P = 0.014$, $P = 0.003$, respectively; Fig. 3). The LC50 value for $\log_{10}$-transformed atrazine was 5.43 (95% confidence interval: 1.38–13.25), which translates to 269.927 mg/L of atrazine.

In the cercarial infectivity experiment four animals were excluded from the analysis, two because they escaped from their cups and two due to tissue damage that prevented adequate clearing and staining. We found no significant difference in the percentage of cercariae that encysted per tadpole between the water and solvent controls ($F_{1,24} = 0.29$, $P = 0.592$) and thus pooled these control treatments. The ability of pesticide-exposed cercariae to encyst was not significantly affected by tadpole stage ($F_{1,73} = 0.12$, $P = 0.730$) or pesticide treatments (all five treatments, $F_{4,73} = 0.47$, $P = 0.760$; pooled pesticides vs. controls, $F_{1,76} = 0.01$, $P = 0.911$; Fig. 2B). In summary, these pesticides may have subtle lethal effects on *E. trivolvis* cercariae but we found no evidence supporting changes in parasite infectivity that would lead to trait-mediated changes in infection risk.

In the tadpole pesticide exposure experiment, there was no significant difference in survival between the water and solvent control during the first week (df = 1, 18, $\chi^2 = 1.36$, $P = 0.243$) or second week (df = 1, 36, $\chi^2 = 1.60$, $P = 0.206$) of pesticide exposure, and thus we pooled the control treatments for subsequent analyses.
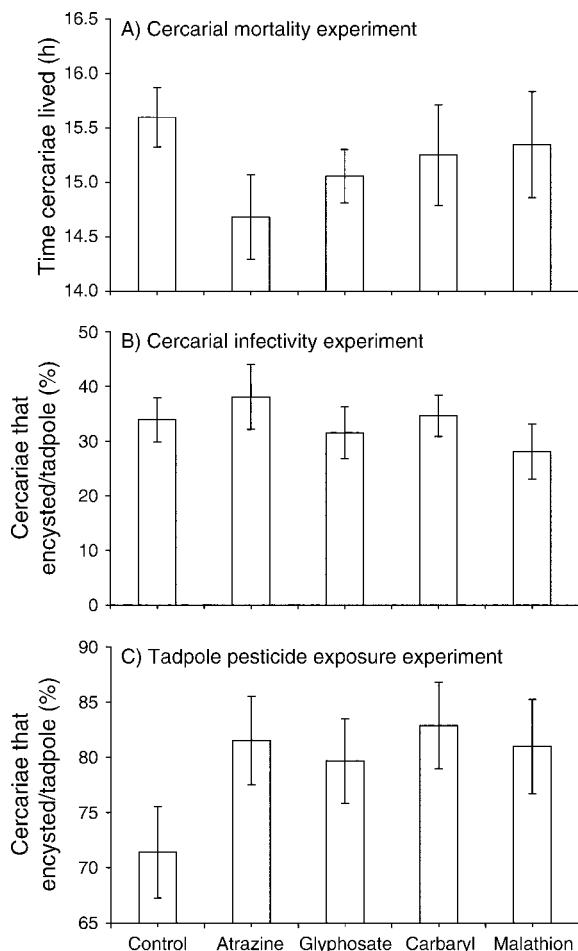
(Gosner stage; 24.79 ± 0.05 [mean ± SE]) than those that lived until the end of the experiment (25.61 ± 0.13). Survival was not significantly affected by any interactions among treatments ($P > 0.489$), indicating that there was no synergistic effect of pesticides and *E. trivolvis* infection on survival.

Jars with greater tadpole mortality during the first seven days of the experiment tended to have fewer cercariae encyst in the surviving tadpoles when exposed during the second seven days ($F_{1,42} = 10.51$, $P = 0.002$), suggesting that there was a positive relationship between susceptibility to non-parasite mortality factors and susceptibility to *E. trivolvis* infections. There was no significant effect of Gosner stage on rates of cercarial encystment ($F_{1,42} = 2.69$, $P = 0.108$). Tadpoles exposed to each of the four pesticides had higher percentages of encysted cercariae than tadpoles exposed to the solvent control (Fig. 2C), resulting in a significantly higher percentage of encysted cercariae for the pesticide-treated than for solvent-treated tadpoles (0.813 ± 0.019 vs. 0.714 ± 0.040 [mean ± SE]; $F_{1,42} = 5.66$, $P = 0.011$; Fig. 2C).

In summary, the tested pesticides and concentrations did not appear to alter *E. trivolvis* transmission by directly reducing the densities of *P. trivolvis* or *R. clamitans*, but we did find evidence that pesticide exposure enhanced transmission by compromising the physiological defenses of *R. clamitans*. Further, the effect size (pesticide vs. control) for the effect of pesticides on tadpole susceptibility to trematodes ($r = 0.953$) was more than 2.5 times larger than that for the effect of pesticides on cercarial mortality ($r = 0.356$),
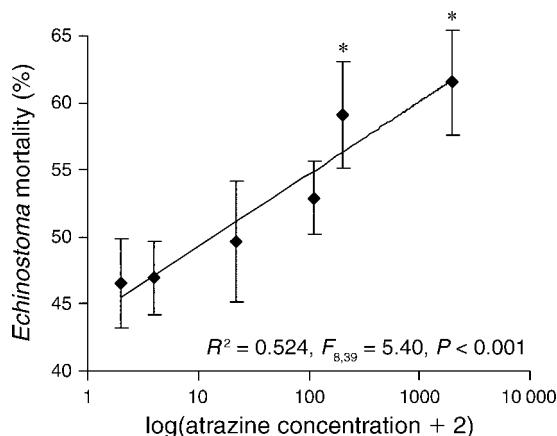
FIG. 2. (A) Known number of hours that *Echinostoma trivolvis* cercariae were alive when exposed to control treatments (water + solvent controls; $N = 32$ replicates) relative to the herbicides atrazine and glyphosate (201 μg/L and 3700 μg/L, respectively; $N = 16$ replicates) and the insecticides carbaryl and malathion (33.5 μg/L and 9.6 μg/L; $N = 16$ replicates). Atrazine was the only pesticide that significantly differed from the pooled control treatments ($P = 0.003$). (B) Percentage of *E. trivolvis* cercariae that successfully encysted (i.e., metacercariae) in *Rana clamitans* tadpoles after only the cercariae were exposed to the control and pesticide treatments for four hours. Values in panels (A) and (B) are weighted means (±SE). (C) Percentage of *E. trivolvis* cercariae that encysted per *R. clamitans* tadpole after only *R. clamitans* was exposed to solvent control and each of the pesticides for seven days. Panel (C) displays least-squares means (±SE) adjusted for pre-trematode exposure mortality. See *Materials and methods: Experiments 4 and 5* for sample sizes in panels (B) and (C).

We found no effect of pesticides on tadpole survival during the first week (df = 4, 55, $\chi^2 = 2.56$, $P = 0.634$) or second week (df = 4, 91, $\chi^2 = 5.31$, $P = 0.257$) of the experiment. Tadpole mortality was significantly greater when exposed to cercariae (34% vs. 18% in second week; df = 1, 91, $\chi^2 = 6.61$, $P = 0.010$) and earlier in development (df = 1, 91, $\chi^2 = 18.79$, $P < 0.001$). That is, tadpoles that died during the experiment were significantly less developed at the start of the experiment

FIG. 3. Relationship between log-transformed atrazine concentration and mean (±SE) mortality of *Echinostoma trivolvis* cercariae during exposure hours 14 through 18 ($N = 8$ replicates). Hours 14 through 18 were chosen because this was the time period when the greatest difference in survival among the concentrations occurred and is thus most relevant to LC50 calculations. The asterisks represent concentrations that were significantly different from the 0 μg/L concentration as determined by a Dunnett's test. The statistics are for the relationship between log-transformed atrazine concentration and probit mortality when blocking by the eight spatial blocks (rows of the welled plates).

suggesting that trait-mediated effects of pesticides on infection risk were greater than density-mediated effects.

DISCUSSION

We revealed both positive and negative effects of pesticide exposure on trematode transmission, but the net outcome of these combined lethal and sublethal effects shows great potential to elevate trematode infections in tadpoles. Environmentally realistic concentrations and exposure durations of pesticides tended to cause greater mortality of *E. trivolvis* cercariae than controls, but only atrazine significantly reduced cercarial survival. Although none of the pesticides directly induced significant snail or tadpole mortality, *E. trivolvis* infections did cause significant mortality for young tadpoles, consistent with previous research (Schotthoefer et al. 2003, Holland et al. 2007). Pesticide-driven reductions in cercarial density in the absence of pesticide-induced changes in snail and tadpole density should reduce amphibian exposure to trematodes. However, parasite loads are a product of both exposure and susceptibility, and pollution can affect susceptibility by altering the traits of the parasite or hosts. In this study, sublethal exposure to pesticides had no effect on cercarial infectivity but did increase green frog susceptibility to infections. The effect of pesticides on cercarial mortality, a density-mediated effect, was two and a half times smaller than the pesticide-induced increase in tadpole susceptibility to infections, a trait-mediated effect. This suggests that pesticide exposure should elevate trematode infections in amphibians, a result which could help explain the emergence of certain amphibian trematode diseases (Johnson and Sutherland 2003, Skelly et al. 2006). Other research also suggests that pesticides elevate amphibian infection risk, such as the work of Kiesecker (2002) and a recent study showing that the herbicide atrazine was the best of over 240 plausible predictors of larval trematode abundance in northern leopard frogs, *Rana pipiens* (Rohr et al. 2008).

There are additional consistencies between our results and those of other studies examining the effects of pesticides on amphibians, snails, parasites, and their interactions. For instance, the concentration and exposure duration of atrazine used here have rarely caused direct amphibian mortality (Diana et al. 2000, Allran and Karasov 2001), consistent with our findings. However, more prolonged atrazine exposures have been shown to cause amphibian mortality at concentrations lower than used in this study (Storrs and Kiesecker 2004, Rohr et al. 2006b). Commercial formulations of pesticides containing glyphosate can be extremely toxic to amphibians, but evidence suggests that it is not glyphosate that causes the mortality but a commonly accompanying surfactant in the formulation (Howe et al. 1998, Mann and Bidwell 1999, Relyea 2005c). Thus, the lack of a glyphosate effect on tadpole survival is not surprising. Likewise, similar concentrations of malathion and carbaryl as used in this study have rarely had

significant direct effects on amphibian survival (e.g., Rohr et al. 2003, Relyea 2004, Boone et al. 2005). Consistent with our results, Relyea (2005a) found little evidence that comparable concentrations of glyphosate, carbaryl, or malathion had effects on adult *P. trivolvis*.

We are unaware of any studies that have examined the direct effects of glyphosate, carbaryl, or malathion on trematodes, but other researchers have found similar effects of atrazine on cercarial survival. Koprivnikar et al. (2006a) demonstrated that atrazine reduced *E. trivolvis* cercarial survival at concentrations similar to those causing direct mortality in this study (also see Griggs and Belden [2008] for effects of a mixture of atrazine and metolachlor). However, their use of the wrong probit value for their LC50 test and their failure to account for mortality in the control treatment in their LC50 calculation makes it difficult to compare their LC50 value to the value calculated in this study (267 mg/L at 14–18 h exposure). Caution should also be used when interpreting our LC50 value because none of our test concentrations induced 50% mortality relative to the controls and thus the associated confidence interval is large. Nevertheless, the estimated LC50 value is well above atrazine concentrations recorded in freshwater ecosystems (Solomon et al. 1996), and we found no significant atrazine-induced mortality at concentrations commonly found in surface water (<200 μg/L; Fig. 3). Further, cercarial efficacy tends to be greatest within the first eight hours after shedding and declines precipitously thereafter (Morley et al. 2003), and we saw no significant effect of atrazine on cercarial survival during this most infectious period (Fig. 1). Thus, while elevated concentrations of atrazine may be capable of reducing amphibian exposure to *E. trivolvis* by reducing cercarial survival, it seems unlikely that this occurs regularly in nature.

Very few studies have investigated whether pesticides have sublethal effects on parasites or pathogens that alter infection risk for hosts, and we are unaware of any studies examining the effects of the widespread pesticides glyphosate, carbaryl, or malathion on parasite or pathogen traits. However, previous studies have found effects of atrazine on viral and trematode infectivity. The survival of salamanders exposed to the combination of atrazine and *Ambystoma tigrinum* virus was higher than when exposed to the virus alone, suggesting that atrazine reduced viral efficacy and emphasizing the importance of considering the effects of pollution on both the pathogen and the host (Forson and Storfer 2006a). While we did not detect any effects of atrazine on cercarial infectivity, Koprivnikar et al. (2006a) found a significant reduction in *E. trivolvis* infectivity following atrazine exposure using a similar concentration of atrazine as we used. There were, however, important differences in methodology between these two studies. We exposed *E. trivolvis* cercariae to atrazine for four hours and then provided 24 hours for them to infect *R. clamitans* tadpoles taken from multiple clutches. Koprivnikar et al. (2006a) exposed *E. trivolvis* cercariae to

atrazine for two hours and provided only 30 minutes for them to infect *R. clamitans* tadpoles from a single clutch. The limited amphibian genetic diversity used by Koprivnikar et al. (2006a) makes it difficult to generalize beyond their single clutch, and 30 minutes is likely too short to ensure that all of the cercariae entered the tadpoles (T. R. Raffel and J. R. Rohr, *personal observation*). Therefore, behavioral avoidance of cercariae by tadpoles may well have played a significant role in preventing infection (Koprivnikar et al. 2006b). The experimental design of Koprivnikar et al. (2006a) emphasizes the ability of trematodes to successfully find and enter a host, whereas our experiment emphasized the ability of trematodes to circumvent host immune responses, since sufficient time was provided for all cercariae to enter the tadpoles.

Thus far we have discussed how pesticides can affect parasite transmission by altering the densities of parasites and hosts and by modifying parasite traits. However, pollution-induced changes in host traits are also likely to be important to parasite transmission. Much research on this topic has focused on understanding the immunosuppressive effects of contaminants that facilitate the spread of disease (Carey et al. 1999, Voccia et al. 1999). Immunosuppression appears to be the most likely explanation for the pesticide-induced increase in tadpole susceptibility because the cercariae all seemed to enter each tadpole. Various studies have demonstrated the immunosuppressive effects of atrazine on intermediate hosts of trematodes (amphibians, Kiesecker 2002, Forson and Storfer 2006b, Brodkin et al. 2007; gastropods, Russo and Lagadic 2004, Sandland and Carmosini 2006), and there is also evidence that the other pesticides tested are immunosuppressive. Glyphosate suppressed various cell-mediated and humoral immune responses in *Tilapia nilotica* (El-Gendy et al. 1998), and carbaryl can suppress innate immune responses of amphibians (Davidson et al. 2007). There is field and laboratory evidence that malathion is immunosuppressive to amphibians (Kiesecker 2002, Gilbertson et al. 2003), which elevated trematode and *Aeromonas hydrophila* bacterial infections (Taylor et al. 1999, Kiesecker 2002). Moreover, several studies have discovered that pesticide mixtures containing at least one of the four pesticides we tested immunosuppressed amphibians and led to higher parasite infection intensities (Christin et al. 2003, Gilbertson et al. 2003, Hayes et al. 2006). All of these studies are consistent with our finding that the tested pesticides increased the susceptibility of *R. clamitans* to *E. trivolvis* cercariae.

The results of this study should not be taken as the definitive effect of these four pesticides on *E. trivolvis* transmission to *R. clamitans* for several reasons. First, traits that we did not quantify could affect transmission. For example, motor activity of hosts has been shown to be an important anti-infection strategy against cercariae (Thiemann and Wassersug 2000, Koprivnikar et al. 2006b), and tadpole growth rates and timing of metamorphosis might influence how long they are exposed to cercariae. Second, non-host and non-parasite species, such as competitors and predators of hosts and parasites, can alter host and parasite activity, growth rates, and densities that can influence infection risk (e.g., Thiemann and Wassersug 2000, Ostfeld and Holt 2004). Likewise, many pesticides can affect activity levels, growth rates (e.g., Rohr et al. 2004, Rohr and Palmer 2005), food resources, and predators and competitors of trematode hosts, which could in turn affect the probability of infection (Rohr et al. 2003, 2004, Rohr and Crumrine 2005). Finally, not all hosts or life stages were tested. Our unpublished research indicates that these same pesticides and concentrations have no direct effects on the egg and miricidial stages of *E. trivolvis* (T. R. Raffel and J. R. Rohr, *unpublished data*), but other hosts, such as other amphibians or definitive hosts, could be more sensitive to pollution than the hosts studied here.

Although numerous researchers have studied the effects of pollution on parasite transmission (Lafferty 1997, Lafferty and Kuris 1999, Morley et al. 2003), researchers have seldom dissected the effects of pesticides on the densities and traits of both parasites and hosts. In addition, most of the readily available research on pollution and trematodes has focused on the effects of metals on transmission (Morley et al. 2003), despite recent evidence that agricultural pollution might be a more important driver of the emergence of certain trematode diseases (Johnson et al. 2002, Kiesecker 2002, Johnson and Sutherland 2003, Johnson and Chase 2004, Johnson et al. 2007). One of the challenges ecologists face is determining which of the thousands of contaminants pose the greatest threat to wildlife. Tackling this challenge requires simultaneously testing the effects of multiple common contaminants at standardized, realistic concentrations, a strategy that is rarely employed (Rohr et al. 2003). However, an emphasis on direct effects of pollution alone will not suffice. It is becoming clear that pollution is altering important species interactions, such as interactions between hosts and parasites, which might facilitate population declines (Relyea and Hoverman 2006, Rohr et al. 2006a). For instance, there is evidence that pesticides are compromising amphibian immunity and increasing ranavirus and *Batrachochytrium dendrobatidis* infections, pathogens linked to amphibian declines (Forson and Storfer 2006b, Davidson et al. 2007). Consequently, it is imperative that research efforts be targeted at studying the net effects of anthropogenic alterations on disease risk, especially for amphibians given their global disease-related declines.

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