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Lack of Pesticide Toxicity to *Echinostoma trivolvis* Eggs and Miracidia

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ABSTRACT: Pesticides can elevate trematode infections in amphibians. However, direct adverse effects of pesticides on embryos and free-living stages of trematodes have not been thoroughly explored, despite the potential for these effects to reduce amphibian trematode infections. We measured the effects of atrazine, glyphosate, carbaryl, and malathion on embryo and miracidium (free-living stage) survival of *Echinostoma trivolvis*, a common trematode of amphibians. We found no evidence of biologically relevant effects of these pesticides at ecologically relevant concentrations.

Agricultural contaminants can affect aquatic communities in complex and often indirect ways, including by influencing parasitic infection (Lafferty and Kuris, 1999; Rohr and Crumrine, 2005; Rohr et al., 2006). This might be especially important for amphibians, which are in decline globally (Stuart et al., 2004) and are threatened by both pesticides and emerging infectious diseases (Davidson et al., 2002; Rohr et al., 2003; Rachowicz et al., 2006). Even low levels of pesticides can increase infection by amphibian trematode parasites by suppression of the host immune system (Kiesecker, 2002; Rohr, Raffel et al., 2008). High burdens of the trematode parasites *Ribeiroia ondatrae* and *Echinostoma* spp. can cause substantial mortality to tadpoles (Johnson et al., 1999; Holland et al., 2007). Thus, increases in infection due to agrochemical exposure could have significant effects on amphibian populations.

Though the effects of pesticides on amphibian immunity have been studied in some detail (Taylor et al., 1999; Kiesecker, 2002; Gilbertson et al., 2003), much less is known about the direct effects of pesticides on the parasites themselves. This question is especially challenging for amphibian parasites with complex life cycles, like *E. trivolvis*, which might experience varying effects of pesticides at different life history stages. *Echinostoma trivolvis* enters aquatic environments in the feces of various birds and mammals, which serve as definitive hosts (Kanev et al., 1995). The eggs develop for approximately 10 days, at which point they release the free-swimming miracidia stages, which infect the molluscan first intermediate hosts (Kanev et al., 1995). The cercaria stage, which infects a variety of second intermediate hosts, including amphibians, emerges from these snails in large numbers due to asexual reproduction within the snail (Schmidt and Fried, 1996). The life cycle is completed when an infected amphibian is eaten by a definitive host, usually a wading bird.

The effects of pesticides on cercariae of *E. trivolvis* and on amphibian susceptibility and survival have been examined (Rohr et al., 2003, 2004; Koprivnikar, Forbes et al., 2006; Griggs and Belden, 2008; Rohr, Raffel et al., 2008), but effects on the egg and miracidium stages have not been investigated. Four common agricultural pesticides have been shown to increase tadpole susceptibility to *E. trivolvis* (Rohr, Raffel et al., 2008), but only atrazine had any detectable effects on cercaria survival or infectivity at ecologically relevant (15–200 μg/l) concentrations (Koprivnikar, Forbes et al., 2006, 2007; Griggs and Belden, 2008; Rohr, Raffel et al., 2008), and effects of technical-grade atrazine on cercariae appeared minor relative to effects on amphibian susceptibility (Rohr, Raffel et al., 2008). This combination of increased amphibian susceptibility, coupled with little to no effect on the parasite, could contribute to observed increases in amphibian trematode infections in agricultural ponds (Koprivnikar, Baker et al., 2006; Rohr, Schotthoefer et al., 2008). However, reduction of miracidia hatching or survival by pesticides could counteract the effect of agrochemicals on amphibian susceptibility by reducing the prevalence of infected snails and thus reducing the overall number of cercariae released.

To obtain a source of *E. trivolvis* eggs, metacercariae were taken from the kidney of a naturally infected *Planorbella trivolvis* snail collected from a pond near Harrisburg, Pennsylvania (40°4′56.0″N, 76°46′2.0″W). They were placed in 50% Locke's solution and 50 metacercariae were inserted via gavage into the stomachs of 2 adult female Golden hamsters (*Mesocricetus auratus*). Trematode eggs were obtained by placing the hamsters in metabolism cages overnight and collecting their feces in artificial spring water (ASW, as described by Cohen et al., 1980). Trematode identity was confirmed at the end of the study by killing the hamsters to obtain adult specimens, which were stained with Ehrlich's hematoxylin, mounted in Canada balsam, and identified as *E. trivolvis* based on morphological characters, e.g., number of spines, cirrus shape, and their ability to infect *P. trivolvis* and a mammalian host (Kanev et al., 1995). A voucher specimen was deposited in the U.S. National Parasite Collection (USNPC 101920). All procedures involving hamsters were approved by the Penn State Institutional Animal Care and Use Committee.

We examined the effects of ecologically relevant concentrations of 4 common agricultural pesticides, i.e., atrazine, glyphosate, malathion, and carbaryl, on the eggs and miracidia of *E. trivolvis*. Simultaneously studying 4 pesticides, i.e., 2 herbicides (atrazine, glyphosate) and 2 insecticides (malathion, carbaryl), representing a variety of modes of action, allowed us to draw more general conclusions regarding our pesticide effects (Clements and Rohr, 2009). Pesticide concentrations (201 μg/L atrazine, 3,700 μg/L glyphosate, 33.5 μg/L carbaryl, or 9.6 μg/L malathion) were selected to represent the upper range of ecologically relevant concentrations based on the highest concentration of each pesticide that was recorded in the U.S. Geological Survey's National Water Quality Assessment Data Warehouse for stream systems between 1991 and 2002. Highest concentrations detected in pond systems often exceed highest concentrations detected in streams (Allran and Karasov, 2000). Since glyphosate was not commonly tested for by the USGS between 1991 and 2002, we chose the highest expected environmental concentration for glyphosate suggested by Giesy et al. (2000). Testing environmentally relevant concentrations of multiple pesticides, rather than the traditional approach of testing multiple concentrations of a single pesticide, has been recommended as a more efficient way to deal with, and draw conclusions about, the vast number of registered pesticides (Clements and Rohr, 2009). Pesticide levels higher than an expected environmental concentration are of questionable ecological relevance and pesticides with no effects at this concentration are unlikely to have worse effects at lower concentrations. The primary assumption of this approach, i.e., that responses to pesticides will be monotonic with respect to concentration (Clements and Rohr, 2009), has usually held for the pesticides used in this study (Relyea and Mills, 2001; Relyea, 2005; Relyea and Hoverman, 2008; Rohr, Raffel et al., 2008). We used technical grade pesticides (>98% purity, ChemService, West Chester, Pennsylvania) to avoid the potential for confounding effects of "inactive" ingredients added to commercial pesticide formulations. Our stock solutions were all within 15% of the nominal concentrations (196.3 μg/L atrazine, 3,685.3 μg/L glyphosate, 29.4 μg/L carbaryl, and 9.2 μg/L malathion), as verified by the Mississippi State Chemical Laboratory (Mississippi State, Mississippi).

Our first experiment was designed to test for effects of pesticides on trematode egg hatching success. After gently breaking apart the hamster feces in ASW using a mortar and pestle, *E. trivolvis* eggs were obtained by filtering the feces through a graded series of nitex filters (500-μm and 75-μm mesh sizes) followed by sedimentation. Eggs were transferred to 96-well, flat-bottom tissue culture plates, with 5 eggs per well in 100 μl ASW. Once all wells had been filled with eggs, the experiment was initiated by adding to each well 100 μl of ASW (water control), solvent alone (solvent control, 0.0002% acetone), or 100 μl of ASW containing any 1 of the 4 pesticide treatments plus solvent. Wells were assigned to treatments using a stratified randomized design, with 2 wells from each of 11 rows assigned to each treatment for a total of 22 replicates per treatment.

Following addition of pesticides, plates were kept at 25 C on a 24-hr day/night light cycle and the status of each egg (dead, embryonated, or hatched) was recorded daily for 26 days, at which time no change had been

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TABLE I. Results of the egg hatching experiment. For each response variable, an *F*-ratio statistic is reported for the overall treatment effect based on ANOVA with pooled water and solvent controls. *P* values are also presented for differences between the water and solvent treatments, the block effect of row, and for multiple comparisons of all 4 pesticide treatments with the controls using Dunnett's tests. (Atr, atrazine; Car, carbaryl; Gly, glyphosate; Mal, malathion).

	Solvent	Row	Treatment		Multiple comparisons (*P*)			
Response	*P*	*P*	$F_{(df)}$	*P*	Atr	Car	Gly	Mal
Hatching proportion	0.317	<0.001	$1.04_{(4, 117)}$	0.39	0.32	1.00	0.97	1.00
Hatching rate	0.240	<0.001	$1.35_{(4, 117)}$	0.25	0.33	1.00	0.74	1.00
Embryo proportion	0.369	0.019	$0.21_{(4, 117)}$	0.93	1.00	0.90	0.99	0.94
Embryonation rate	0.183	0.007	$0.34_{(4, 117)}$	0.85	1.00	0.77	0.95	1.00
Embryo hatching	0.305	0.001	$0.59_{(4, 99)}$	0.67	0.49	0.99	1.00	0.94
Embryo mortality	0.092	0.013	$0.69_{(4, 99)}$	0.60	0.48	0.97	1.00	0.89

recorded for 3 days. Eggs were considered embryonated when a distinct mass formed in the center, surrounded by a transparent region. Previously embryonated eggs, which had transitioned to a completely opaque state, were recorded as dead, and those that had emptied (usually associated with a swimming miracidium) were considered hatched.

We ran a second experiment to test for effects of pesticides on miracidium survival. *Echinostoma trivolvis* eggs were isolated from hamster feces as described for the first experiment and incubated in the dark at room temperature in six 100-mm Petri dishes containing ASW, at a density of approximately 10 eggs per Petri dish. Dishes were exposed to light on day 15 to encourage hatching, and miracidia already present were discarded to ensure that all miracidia used in the experiment were exposed to pesticides within 15 min of hatching. Miracidia were transferred individually into wells of a 96-well tissue culture plate in 100 µl of ASW, which were treated by addition of 100 µl of the appropriate pesticide or control solution within 5 min of filling each row of 12 wells. Treatments were assigned to wells as described for the egg hatching experiment, with 2 replicates of each pesticide or control treatment in each of 24 rows, for a total of 48 replicates of each treatment. Since miracidia have short life spans following hatching, survival was recorded 2 hr following pesticide addition and at subsequent 2-hr intervals until all miracidia were dead (no longer moving or responding to stimuli).

All statistics were calculated using R statistical software (www.r-project.org). There were no differences between the water and solvent controls for any analyses ($P > 0.09$), so we pooled the control treatments for each analysis. All proportion data met assumptions of normality and homogeneity of variance following arc-sine square root transformations. For each analysis, we blocked by tissue culture plate row and used Dunnett's tests to compare individual pesticides to the pooled controls (package "multcomp").

Egg hatching and embryonation success were each analyzed using 2 approaches. The first was to analyze the proportions of eggs that had hatched or embryonated using analysis of variance (ANOVA). The second used ANOVA to test for differences in the estimated rate of hatching or embryonation (1/average time to hatching or embryonation) obtained from a censored survival analysis with Gaussian errors for each well (package "survival" in R statistical software). Wells in which no eggs hatched or embryonated were assumed to have an embryonation or hatching success of zero. These results were found to approximate a normal distribution following a 1/(rate + 1) transformation. This survival analysis approach has the benefit of incorporating information about both the proportion hatched and the time to hatching.

Miracidia survival was analyzed with an uncensored survival analysis using the "extreme" distribution, a generalization of the exponential distribution that allows the survival to vary with time (package "survival"). This model provided a better model fit (AIC = 1,241.6) than models using exponential (AIC = 1,546) or Gaussian (AIC = 1,268) distributions. Significance of variables to the model was assessed using the change in deviance for removal of each variable from the full model, which should approximate a chi-square distribution.

There were no detectable effects of any of the 4 pesticides on any measure of embryonation or hatching success for *E. trivolvis* eggs, either in the overall ANOVA or in the Dunnett's tests comparing individual pesticides with the controls (Table I; Fig. 1). The average times to embryonation and hatching were 16.2 ± 2.6 (SD) and 20.2 ± 1.0 days, respectively, after excluding eggs that did not successfully embryonate or hatch.

There was no significant effect of pesticides on miracidia survival (Fig. 2; chi-square = 7.4, 4 df, $P = 0.117$), and no significant effects of individual pesticides in the Dunnett's test (atrazine, $P = 0.79$; carbaryl, $P = 0.075$; glyphosate, $P = 0.15$; malathion, $P = 0.42$; Fig. 2). Further, the trends were in the direction of positive effects of the pesticides (Fig. 2). The average lifespan of a miracidium was 5.36 ± 2.23 (SD) hr.

We found no evidence that any of the pesticides influenced *E. trivolvis* hatching success or miracidium survival at the high, but ecologically relevant, test concentrations. Previous studies found direct effects of atrazine on cercaria survival at similar concentrations, but no effects of the other 3 pesticides (Rohr, Raffel et al., 2008). Certainly, any potentially undetected effects of atrazine on the egg and miracidium stages of *E. trivolvis* appear to be less pronounced than its effects on the cercaria stage, which were found to be significant using similar sample sizes (Rohr, Raffel et al., 2008). The relative scarcity of toxicological studies on trematode eggs or miracidia makes it difficult to derive general conclusions about the relative sensitivity of eggs, miracidia, and cercariae to contaminants (Pietrock and Marcogliese, 2003). To our knowledge, the only other trematode species for which comparable data have been obtained for all 3 of these developmental stages is *Schistosoma mansoni* (Morley et al., 2003; Pietrock and Marcogliese, 2003). In this species, cadmium and zinc caused high mortality to cercariae at concentrations that had no significant effects on eggs or miracidia (Mecham and Holliman, 1975; Holliman and Esham, 1977; Morley et al., 2001), similar to the apparent pattern for atrazine effects on *E. trivolvis*. High sensitivity of cercariae to contaminants relative to miracidia might be caused by longer exposure to pesticides due to longer life spans (Rohr, Raffel et al., 2008). Alternatively, different stages might differ in the speed of contaminant uptake, since cercariae utilize external sources of carbohydrates (Uglem, 1980; Morley et al., 2003), whereas miracidia rely almost exclusively on internal glycogen stores (Boyunaga et al., 2001).

The effects of atrazine on cercaria survival have been suggested to counteract the immunosuppressive effects of atrazine on the amphibian hosts, at least in response to commercial grade (40.8% active ingredient) atrazine (Koprivnikar et al., 2007), but studies with technical-grade atrazine have found this effect too small to substantially reduce the effect of host immune suppression (Rohr, Raffel et al., 2008). Since we were unable to detect atrazine effects on miracidia or eggs, despite using the same pesticide concentration and comparable statistical power as that of Rohr, Raffel et al. (2008), it seems unlikely that direct effects on these stages will have biologically significant impacts on *E. trivolvis* infection in natural ponds. However, it remains possible that "inactive" ingredients in commercial formulations of these pesticides could have significant effects on the eggs or miracidia of this parasite.

The results of this study fill an important gap in our knowledge of pesticide effects on different stages of the *E. trivolvis* life cycle and are consistent with mounting evidence that pesticides increase larval trematode infection of amphibians in experiments and natural ponds by increasing amphibian susceptibility and snail biomass (Kiesecker, 2002; Rohr, Raffel et al., 2008; Rohr, Schotthoefer et al., 2008). Though sub-lethal effects of pesticide exposure on miracidia infectivity for snails

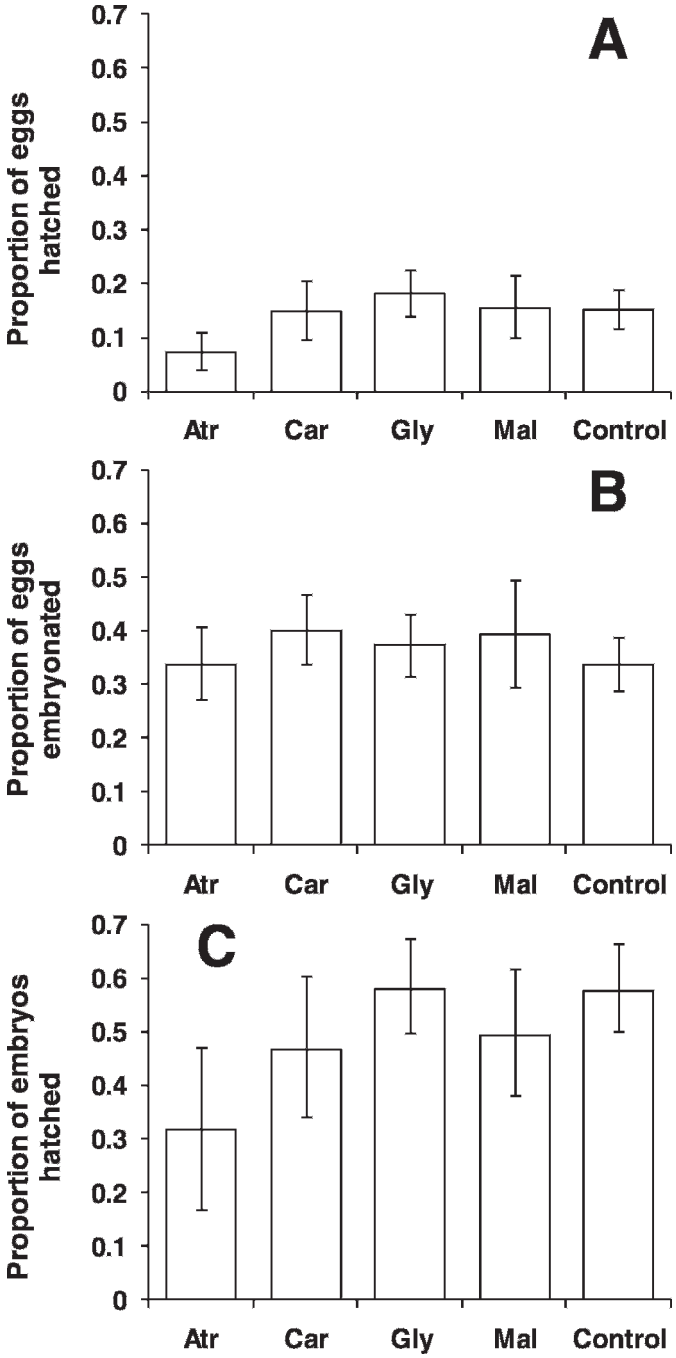

FIGURE 1. Results of the egg hatching experiment, showing (**A**) the proportion of eggs that hatched, (**B**) the proportion of eggs that embryonated, and (**C**) the proportion of embryonated eggs that hatched. Atr, atrazine; Car, carbaryl; Gly, glyphosate; Mal, malathion; Control, combined water and solvent control treatments. Means and error bars were back-transformed from the averages and standard errors of the data used in the analysis for each parameter (arc sine transformation).

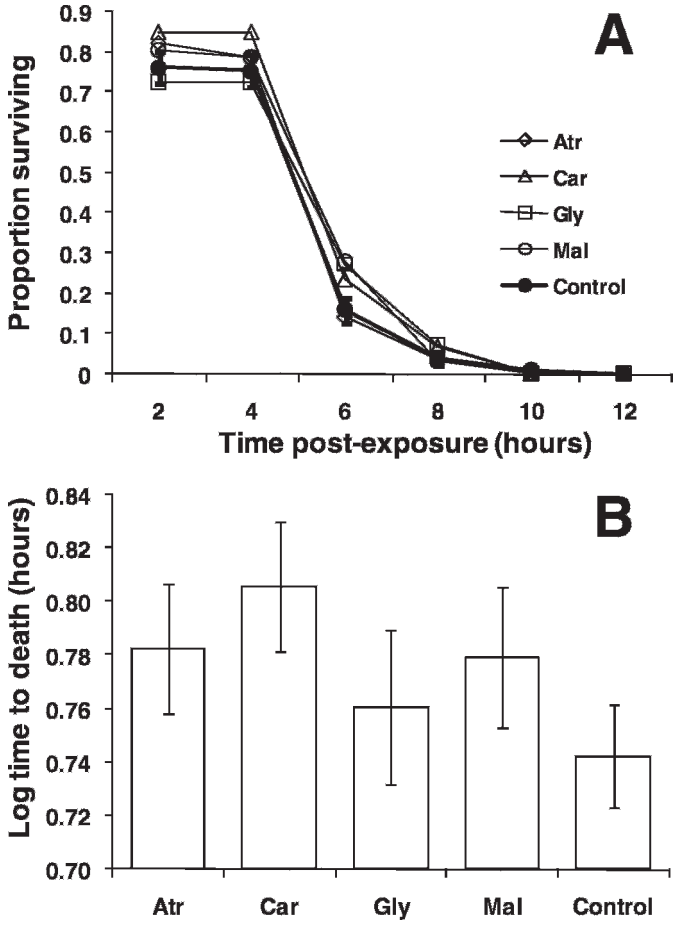

FIGURE 2. Results of the miracidial survival experiment, showing (**A**) the time series of miracidial survival and (**B**) the average log-transformed time to death for the 5 treatments (Atr, atrazine; Car, carbaryl; Gly, glyphosate; Mal, malathion; Control, combined water and solvent control treatments). Error bars = SE and in panel A are presented only for the control for clarity; standard errors were similar for the pesticide treatments.

remain to be tested, there is now information available on the effects of these pesticides on trematode hatching rates, miracidium survival, cercaria survival, and cercaria infectivity on snail survival, growth, and reproduction, and on frog survival and susceptibility (Rohr, Raffel et al., 2008; Rohr, Schotthoefer et al., 2008). This brings us closer to gaining a full mechanistic understanding of how pesticides influence larval trematode infection of amphibians.

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