

# Relative Toxicity of Malathion to Trematode-Infected and Noninfected *Rana palustris* Tadpoles

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Received: 27 November 2007 / Accepted: 11 March 2008 / Published online: 10 April 2008  
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**Abstract** Amphibian populations around the world are facing threats that include disease and pollution. Although the effect of environmental contaminants on susceptibility to infection has been demonstrated for several amphibian species, to our knowledge, the opposite interaction, infection status affecting contaminant susceptibility, has not been studied. We conducted standard 48-h toxicity tests to compare susceptibility to malathion, a widely used organophosphate insecticide, of uninfected pickerel frog (*Rana palustris*) tadpoles and tadpoles infected with two levels (10 or 30 cercariae) of the trematode *Echinostoma trivolvis*. Trematode encystment rates were high (>90%) in both trematode treatment groups. LC<sub>50</sub> values ranged from 16.5 to 17.4 mg/L, within the range reported for other amphibian species. However, we found no differences in susceptibility to malathion among parasite treatments. Although we detected no effect of parasites on pesticide susceptibility in this system, it is important to investigate this question using other pesticides, parasites, and amphibian hosts before dismissing this potentially threatening interaction.

## Introduction

Amphibian populations are declining around the world (Stuart et al. 2004; Wake 1991), and both disease (Berger

et al. 1998; Daszak et al. 1999, 2003; Kiesecker et al. 2004) and environmental contaminants (Berger 1989; Bishop et al. 1999; Davidson 2004) have been implicated in some of the declines. Not only are contaminants and disease individually important, but they might act in combination to have a larger cumulative impact on amphibian populations (Beasley et al. 2003; Kiesecker 2002). Environmental contaminants have been correlated with decreases in immune function (Christin et al. 2003, 2004; Gilbertson et al. 2003; Kiesecker 2002) and increases in prevalence of parasite infection (Christin et al. 2003; Lewis et al. 2003). Furthermore, pesticides might indirectly increase susceptibility to parasite infection by decreasing activity patterns (Bridges and Semlitsch 2000) because tadpoles can avoid free-swimming parasites by moving away or swimming in erratic patterns (Koprivnikar et al. 2006; Thiemann and Wassersug 2000).

Although the influence of pollution on disease susceptibility has been studied in amphibians, the reverse scenario has, to our knowledge, never been tested. However, the influence of parasite load on susceptibility to contaminants has been studied in other taxa. For example, fish species acting as second intermediate hosts to parasites were more susceptible to heavy metals than noninfected conspecifics (Boyce and Yamada 1977; Ewing and Ewing 1982; Pascoe and Cram 1977). Coho salmon fry were more susceptible to oil, naphthalene, and toluene when infected with parasitic glochidia of freshwater mussels (Moles 1980). Similarly, tadpoles generally play the role of second intermediate hosts for many parasites, especially trematode species; yet, the effects of parasites on amphibian susceptibility to contaminants has not been studied.

Using a common parasite and pesticide, our study addressed the effects of parasite infection on the susceptibility of amphibians to contaminants. Malathion (diethyl

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[(dimethoxyphosphinothioyl)-thio] butanedioate) is the primary organophosphate (OP) used to combat mosquitoes, the most utilized agricultural insecticide, and the sixth most heavily used of all pesticides in the United States (Kiely et al. 2004). It is commonly found at low concentrations in rivers, streams, and drinking water (Hoffman et al. 2000; Larson et al. 1999). *Planorbella trivolvis* snails are widely distributed throughout North America (Friesen 1981) and are first intermediate hosts for the trematode *Echinostoma trivolvis*, which leaves the snail host as a cercariae to encyst as metacercariae in the kidneys of *Ranid* tadpoles (Huffman and Fried 1990). Definitive hosts for *E. trivolvis* include semiaquatic birds and mammals (Huffman and Fried 1990). We used standard LC<sub>50</sub> methods (ASTM 2005) to compare the toxicity of malathion among *E. trivolvis*-infected and noninfected pickerel frog, *Rana palustris*, tadpoles.

## Materials and Methods

### Animal Husbandry

Pickerel frogs are found throughout the eastern United States. In early spring, females lay clutches of ~2,500 eggs, which hatch in 7–14 days, depending on temperature (Conant and Collins 1998). Pickerel frogs used in this experiment were collected from a pond in rural Botetourt County, Virginia. The pond did not contain *P. trivolvis* snails. On March 25, 2007, we collected nine clutches of recently laid eggs (before Gosner stage 12; Gosner 1960) and transported them in a cooler to our laboratory in Blacksburg, Virginia. Eggs were immediately separated by hand, keeping jelly coats intact. To ensure similar genetic composition across replicates, 11 eggs from each clutch were combined to form 16 lots of 99 eggs and each lot was allowed to hatch in 1.5 L of water in the laboratory. The water used was a 75/25 mix of dechloraminated town water and well water, respectively. Previous research demonstrated the available well water was extremely hard (364 mg/L CaCO<sub>3</sub>) and caused spinal malformations in developing wood frogs, *Rana sylvatica* (unpublished data). Town water (62 mg/L CaCO<sub>3</sub>) was dechloraminated with ChlorAm-X (AquaScience). This mix was necessary to bring the hardness to acceptable levels (172 mg/L). Fifty-percent water changes were carried out every 2 days prior to hatching.

Hatching success was assessed on April 4, 2007. Sixty-five well-formed hatchlings from each of the 16 lots were acclimated to mesocosm temperatures, then slowly transferred to 16 corresponding 1,500-L polyethylene outdoor mesocosms at the Virginia Tech aquaculture center. In

early March, mesocosms were filled with ~475 L of well water and 475 L of dechloraminated city water. Because the mesocosms received natural precipitation as well as biological material (see below), the 50/50 mix achieved desirable hardness (190 mg/L CaCO<sub>3</sub>). Each mesocosm received 1 kg of air-dried deciduous leaf litter, 17 g of finely ground Purina Rabbit Chow<sup>®</sup>, and two 1.5-L spikes of pond water on March 14, 2007 and April 2, 2007. The pond water, taken from a small permanent pond on the Virginia Tech property, was filtered through a 200- $\mu$ m sieve to remove potential predators before addition to the tanks. To decrease the variability in initial phytoplankton and zooplankton communities, 18-L buckets of water were exchanged among mesocosms in a set pattern such that each mesocosm donated and received three buckets (54 L) of water. To provide shade and exclude predators and competitors, mesocosms were covered with black mesh lids. In three randomly selected replicates, we measured conductivity, pH, temperature, and dissolved oxygen (DO) weekly at 7:30 am and 7:30 pm (approximate coolest and warmest daily water temperatures, respectively).

### Parasite Exposure

The experiment was conducted in three separate 4-day runs over the course of 7 days, later combined to form three complete replicates as stipulated by ASTM (2005). The runs were overlapped to minimize tadpole size differences among runs. For each of the three runs, 14 tadpoles were haphazardly selected from each of the 16 mesocosms. Animal rearing conditions met requirements of ASTM (2005) and the National Resource Council (Browne and Zippel 2007; NRC 1974). At the time of the runs, tadpoles were 33, 36, and 38 days posthatch and at approximately Gosner stage 26 (Gosner 1960). The tadpoles were acclimated in the laboratory for 24 h in bulk containers while their water was slowly changed to reconstituted water (hardness: 45 mg/L CaCO<sub>3</sub>; ASTM 2005). Tadpoles were then placed individually in plastic cups with 90 mL of reconstituted water and randomly assigned to control, low (10 cercariae), or high (30 cercariae) parasite treatments. *P. trivolvis* snails were collected from a pond in Riner, Virginia and then screened for infection by placing the snails under a heat lamp. The four infected snails were induced to shed cercariae under a heat lamp again for each run. Cercariae were immediately counted under a dissecting microscope and then added to the tadpole cups with a pipette (Belden 2006). Cercariae were given 24 h to encyst in the tadpoles, which is the normal life span for the free-swimming cercariae. After 24 h, the tadpoles were removed from their parasite exposures and randomly assigned to pesticide concentrations.

## Pesticide Exposure

A stock solution was prepared by diluting pure malathion (Chem Service, West Chester, PA) with methanol. The pesticide treatments included five test concentrations ranging between 5.2 and 40 mg/L, each 60% of the next higher concentration, a water control, and a solvent control equal to the highest test concentration of methanol (0.4%). Concentrations were verified in duplicate by the Virginia Tech Pesticide Residue Laboratory (Table 1). Ten tadpoles from each parasite treatment were randomly assigned to each of the 7 exposure treatments, for a total of 21 experimental containers per run (i.e., 3 parasite manipulations crossed with 7 pesticide manipulations). Three replicates of each parasite–pesticide combination were formed by running the experiment three times. The test containers consisted of 2-L glass beakers and were randomly assigned to a location within a temperature-controlled chamber. We used 48-h static tests that were conducted at 17°C so that ASTM temperature-specific mass/volume limits were met. The tadpoles received 16 h light and 8 h dark.

Mortality was assessed every 8 h for the duration of the 48-h tests by gently stirring the jars and checking for movement. Unresponsive tadpoles were examined further for signs of life. Dead tadpoles were frozen in individual microcentrifuge tubes for later verification of parasite encystment levels. For all three parasite treatments (0, 10, and 30 cercariae), temperature, DO, and pH were measured at 0, 24, and 48 h in the control, low, medium, and high concentrations as stipulated by ASTM (2005). The 10 tadpoles in the malathion control treatment (0 ppb) were weighed at the end of each run for each of the three parasite treatments. All surviving tadpoles were euthanized in MS-222 and frozen for subsequent dissection to verify parasite encystment levels.

## Parasite Dissections

Under a dissecting scope, the tadpole kidneys (pronephros and mesonephros) were removed from each tadpole with forceps and placed on a slide. A cover slip was gently pressed onto the tissue to produce a thin layer. Metacercarial

cysts were counted at 100× using a compound microscope. Fifteen control (no parasite) tadpoles from each of the three runs were dissected to verify that they were infection-free. For each run, six parasite-infected tadpoles from each pesticide treatment were randomly subsampled to confirm infection levels. All parasite-infected tadpoles at the concentration (14.4 mg/L) with partial mortality were dissected. In total, 66% of the 420 low- and high-parasite tadpoles and 20% of the 210 noninfected tadpoles were dissected.

## Statistical Analysis

To compare tadpole masses among treatments and runs, Kruskal–Wallis tests were used because mass data were not normally distributed (Shapiro–Wilk  $P < 0.05$ ). We compared the overall proportion of parasites encysting between the 10- and 30-cercariae exposure treatments using Wilcoxon's two-sample test because the proportions were not normally distributed. To compare the mean proportion encysting among runs and cercariae treatments, we conducted a nonparametric two-way ANOVA, the Scheirer–Ray–Hare extension of a Kruskal–Wallis test. To compare proportion encystment among pesticide concentrations and cercariae treatments, we ran a second Scheirer–Ray–Hare analysis. Because we only had one concentration with partial mortality, we used Spearman–Karber methods to estimate an  $LC_{50}$  for each run followed by a Kruskal–Wallis test to compare  $LC_{50}$ 's among runs because  $LC_{50}$  values were not normally distributed. At the concentration with partial mortality, ANOVA was used to compare parasite encystment levels of surviving tadpoles to those that died for both parasite infection treatments. The experimentwide  $\alpha$  was set at 0.05.

## Results

All three runs met ASTM water quality standards. Mean DO was 96.8%, 89.4%, and 86.3% after 0, 24, and 48 h, respectively, surpassing the ASTM guideline of minimum 60% DO. The minimum measured DO over all three runs was 79.3%. The average temperature ( $16.57 \pm 0.04^\circ\text{C}$ ) approximated the selected test temperature (17°C). Temperatures met ASTM standards for allowable variation among replicates and runs. The average pH was 7.29, falling within ASTM guidelines and the range in which malathion is stable.

There were no differences in tadpole mass among parasite treatments within each run ( $P = 0.25, 0.77, 0.71$ ) although mass did differ significantly across the three runs ( $F = 6.03, df = 2, 87, p = 0.004$ ). Mean tadpole mass ( $\pm 1$  SE) for the runs were 0.172 (0.009), 0.200 (0.009), and

**Table 1** Nominal and actual malathion concentrations used in  $LC_{50}$  tests

Nominal concentration	Sample 1	Sample 2	Mean
5.2 mg/L	5.4	4.6	5.0
14.4 mg/L	14	15	14.5
40.0 mg/L	40	40	40

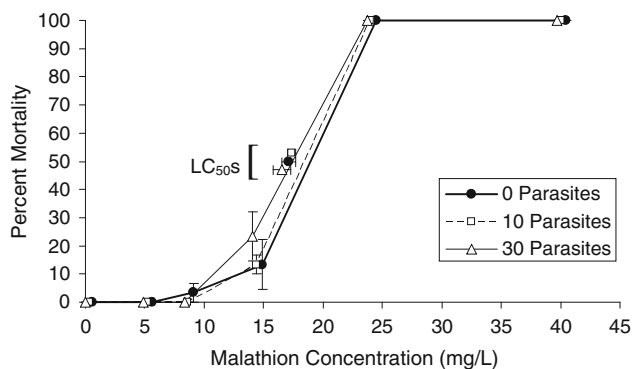
Note: Concentrations were verified in duplicate by the Virginia Tech Pesticide Residue Laboratory

0.215 (0.009) g, respectively. The consistency of toxicity results and nonsignificant linear relationship between mass and  $LC_{50}$  value ( $F = 2.5$ ,  $df = 1$ ,  $P = 0.16$ ) suggested this increase of 0.043 g between runs 1 and 3, although statistically significant, did not influence sensitivity to malathion. The overall proportion of cercariae encysting was high (over 91%) and did not vary between the 10- and 30-cercariae treatments ( $P = 0.83$ ). There was no significant effect of run ( $\chi^2 = 5.95$ ,  $df = 2$ ,  $P = 0.051$ ), cercaria treatment ( $\chi^2 = 0.05$ ,  $df = 1$ ,  $P = 0.83$ ), or the interaction of run and cercaria treatment ( $\chi^2 = 5.27$ ,  $df = 2$ ,  $P = 0.07$ ) on the mean proportion encysting. Similarly, the mean proportion encysting did not differ among malathion concentrations ( $\chi^2 = 5.00$ ,  $df = 6$ ,  $P = 0.54$ ), cercaria treatment ( $\chi^2 = 0.004$ ,  $df = 1$ ,  $P = 0.95$ ), or the interaction of these two factors ( $\chi^2 = 1.83$ ,  $df = 6$ ,  $P = 0.93$ ).

The toxicity tests also met ASTM requirements for mortality. There were no mortalities in the control, solvent, and lowest malathion concentration during all three runs, and complete mortality occurred in the highest two concentrations (Fig. 1).  $LC_{50}$  estimates were similar among runs and treatments (range: 15.2–17.7 mg/L) and did not differ significantly by parasite treatment (means: 16.5–17.4 mg/L,  $P = 0.49$ ). In the one concentration with partial mortality (14.4 mg/L), parasite loads did not differ significantly between the tadpoles that survived and died in either the 10-cercariae (survived:  $9.6 \pm 0.5$ ; died:  $9.8 \pm 0.6$ ) or 30-cercariae (survived:  $27.7 \pm 0.8$ ; died:  $25.4 \pm 1.3$ ) treatments ( $P = 0.91$  and 0.16, respectively).

## Discussion

We found no difference in the  $LC_{50}$ 's of malathion among parasitized and unparasitized tadpoles, although future research examining higher parasite loads in this system or



**Fig. 1** Mean percent mortality ( $\pm 1$  SE, vertical) across malathion concentrations and  $LC_{50}$  values ( $\pm 1$  SE, horizontal) for the three parasite treatments. Points are staggered on both axes for clarity

another amphibian species are needed before dismissing this possible interaction. The maximum and minimum  $LC_{50}$ 's only differed by 5% and a power analysis revealed that 18 additional replicates of each treatment would be needed to detect such slight differences among treatments. This power analysis bolstered our conclusion that parasite infection did not influence  $LC_{50}$ 's, rather than a lack of detection due to insufficient sample size. Our conclusion was further supported by the similar parasite loads of tadpoles that survived and died at the 14.4-mg/L concentration. This latter comparison has not been reported in related fish literature (Boyce and Yamada 1977; Ewing and Ewing 1982; Moles 1980; Pascoe and Cram 1977), but it could be a useful post hoc test for understanding subtle differences in host susceptibility to contaminants attributable to parasite infection.

Our  $LC_{50}$  results differ from previous studies with fish hosts that reported large effects of parasite load on toxicity (Boyce and Yamada 1977; Ewing and Ewing 1982; Moles 1980; Pascoe and Cram 1977), although neither malathion nor any other OP was previously tested with fish in this manner. For example, Coho salmon, *Oncorhynchus kisutch*, fry artificially infected with *Anodonta oregonensis* glochidia were 4.2, 3.0, and 4.6 times more susceptible to naphthalene, toluene, and crude oil, respectively, than uninfected fry (Moles 1980). Three-spined stickleback, *Gasterosteus aculeatus*, infected with the cestode *Schistocephalus solidus* had decreased survival times (up to 555 h) compared to uninfected fish when exposed to cadmium (Pascoe and Cram 1977). The mechanisms by which both internal (cestode) and external (glochidia) parasites affected the tolerances of these fish species to toxicants as diverse as heavy metals, aromatic hydrocarbons, and crude oil are unknown (Boyce and Yamada 1977; Moles 1980; Pascoe and Cram 1977).

Without further knowledge of the mechanisms explaining these susceptibility differences in infected fish, it is difficult to say why we failed to see a similar response in our amphibian study, but several possibilities exist. Our parasite exposures of only 24 h might have been too short to affect contaminant susceptibility. It is also possible that our infection level might have been below the level necessary to impair kidney function (e.g., Belden 2006) or compound damage from malathion exposure. *E. trivolvis* infection intensity in natural tadpole populations can exceed those used in this study by 10-fold (Fried and Bradford 1997). Up to 1650 cysts per individual have been documented in natural ponds (Skelly et al. 2006), and those higher-parasite burdens might affect contaminant susceptibility. However, the number of cysts a tadpole can withstand is also a function of its developmental stage and size (Schotthoefer et al. 2003), and our preliminary data suggested that 10–30 cercaria were within the appropriate

sublethal range for the size and stage of tadpoles used in this study. Finally, it is plausible that differences between our study and previous studies on fish might relate to species-specific differences in the host–parasite systems studied.

Our LC<sub>50</sub> values (16.5–17.4 mg/L) fell within the wide range of known values of malathion toxicity for other aquatic wildlife. The US Environmental Protection Agency (EPA) lacks sufficient data to classify the toxicity of malathion for aquatic amphibians (US EPA 2006), although preliminary data suggest aquatic invertebrates and fish are more susceptible than many, but not all, amphibian species. Malathion is classified as very highly toxic to fish, with a LC<sub>50</sub> value of 30 µg/L for bluegill sunfish, *Lepomis macrochirus*. It is also highly toxic to aquatic invertebrates, with an EC<sub>50</sub> of 1.0 µg/L (US EPA 2006). Although differences in testing protocols might cause some variation in toxicity results and preclude comparison, other *Ranid* frogs have LC<sub>50</sub>'s as high as 40 mg/L for *Rana tigrina* tadpoles (Mohanty-Hejmadi and Dutta 1981) and as low as 0.6 µg/L for *Rana hexadactyla* hatchlings (Khangarot et al. 1985). Malathion is moderately toxic to *Rana limnocharis* tadpoles, which have an LC<sub>50</sub> of 2.2 mg/L (Pan and Liang 1993). Other anuran genera might be orders of magnitude more susceptible to malathion; *Bufo woodhousei fowleri* and *Pseudacris triseriata triseriata* tadpoles have LC<sub>50</sub> values of 420 and 200 µg/L, respectively (Sanders 1970). Studies suggest that acetylcholinesterase-inhibiting pesticides, a class that includes malathion, can decrease the condition and survival of aquatic salamander larvae by decreasing food resources (Metts et al. 2005; Relyea 2005), but more data on the toxicity of malathion to larval salamanders are needed.

Although we found no effect of parasite load on malathion toxicity for amphibian hosts, individual effects of both disease and toxicants are well established in amphibians (Berger et al. 1998; Bishop et al. 1999; Daszak et al. 1999, 2003; Davidson 2004), as are the effects of toxicants on amphibian susceptibility to disease (Beasley et al. 2003; Bridges and Semlitsch 2000; Christin et al. 2003, 2004; Gilbertson et al. 2003; Kiesecker 2002). In ecological systems modified by human disturbances, the incidence and intensity of parasite infections in amphibians and other hosts might increase in the future. Increased densities of snails, the primary hosts of trematode parasites, have been linked to eutrophication (Johnson and Chase 2004; Johnson et al. 2007). As eutrophication expands, the intensity and prevalence of trematode infection might rise, increasing the need for toxicity testing using infected organisms. Furthermore, global climate change might increase the range and infectivity of many parasite species (Marcogliese 2001). In the current era of climate change, cultural eutrophication, and pesticide use, it is important to

investigate the effects of parasite load on toxicity using other pesticides, parasites, and amphibian hosts before dismissing this potentially threatening interaction.

**Acknowledgments** The authors thank S. Orlofske, J. Griggs, and C. Culp for their valuable assistance and advice during the project. Michael Edwards provided access to his property for egg collection. Methods were in compliance with Virginia Polytechnic Institute and State University's animal care and use protocols. Financial support was provided to WAH by Virginia Polytechnic Institute and State University in the form of new faculty startup funds and by the National Science Foundation (NSF grant No. IOB-0615361).

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