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EFFECTS OF MALATHION ON EMBRYONIC DEVELOPMENT AND LATENT SUSCEPTIBILITY TO TREMATODE PARASITES IN RANID TADPOLES

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Abstract—We investigated the effects of embryonic exposure to the widely used organophosphate malathion (15–600 μg/L) on the early development and latent susceptibility of pickerel frog (*Rana palustris*) tadpoles to the trematode parasite *Echinostoma trivolvis*. The latent effects of contaminant exposure are rarely examined but could have important implications for individual survival and population viability. Malathion decreased hatching success by 6.5% and viability rates by 17% at 600 μg/L, which is a lower concentration than previously documented for anuran embryos. Incidence of malformations increased from 0.5% in controls to 11.2% in the 600-μg/L malathion treatment. The primary malformations documented in the two highest pesticide concentrations were ventralization and axial shortening. After seven weeks of development in water with no malathion, tadpoles previously exposed as embryos for only 96 h to 60 and 600 μg/L malathion suffered increased parasite encystment rates when compared to controls. Our research identifies embryonic development as a sensitive window for establishing latent susceptibility to infection in later developmental stages.

Keywords—Organophosphate *Echinostoma trivolvis* *Rana palustris* Malformation Amphibian

INTRODUCTION

The recent decline of some amphibian populations is a globally recognized crisis [1,2], but the causes of declines and their relative magnitudes are still debated [3]. Factors including overexploitation, habitat loss and fragmentation, invasive species, climate change, increased ultraviolet-B exposure, environmental contaminants, and disease have been cited as potential causes of declines [4,5]. These factors may in many cases work in combination to have interactive impacts on amphibians. For example, increased ultraviolet-B exposure in low-water years increased the susceptibility of *Bufo boreas* eggs to a parasitic water mold [5], and malathion exposure increased the susceptibility of adult *Bufo woodhousi* to a pathogenic bacteria [6].

Although the role of chemical contamination in global amphibian population declines is not well understood, regional amphibian declines, lower population densities, and decreased amphibian species diversity have been correlated with agricultural land use [4,7]. In addition, environmental contaminants reduce immunocompetency in many species [8]. Pesticides with several different modes of action decrease antibody responses [9], lymphocyte proliferation [10], and levels of circulating eosinophilic granulocytes [11] in amphibian larvae and adults. However, little is known about how contaminant exposure early in development can impact the amphibian immune system. The developing immune system may be more susceptible to contaminants [12], and early exposure to foreign compounds could lead to long-term changes in immunocompetency [13].

In general, latent effects of contaminant exposure in amphibians are less well known than the direct lethal and sublethal effects [12]. In one of the few studies of latent effects on amphibians, larval salamanders failed to show water-conserving behavior and had decreased survival 14 months after herbicide exposure [14,15]. Long-term behavioral changes such as this would not be detected by traditional short-term (96-h) toxicity tests. However, understanding latent effects of short-term exposure is important because many of the modern, commonly used pesticides rapidly break down in the environment.

We hypothesized that acute exposure to the organophosphate insecticide malathion during early embryonic development would negatively affect embryonic survival, development, and latent susceptibility to parasitic infection. Malathion is the single most heavily used agricultural insecticide and the sixth most used agricultural pesticide in the United States [16] and affects immunocompetency of several amphibian species [6,17]. We exposed embryos of pickerel frogs, *Rana palustris*, to three environmentally realistic concentrations of malathion for 96 h and then quantified hatchling viability. To investigate the latent effects of early embryonic exposure to malathion, we measured larval susceptibility to a common trematode, *Echinostoma trivolvis*, seven weeks posthatch.

MATERIALS AND METHODS

Species information

Pickerel frogs are found throughout all but the southernmost parts of the eastern United States. They commonly breed in permanent ponds with submerged aquatic vegetation in early spring. Females lay clutches of approximately 2,500 eggs that hatch in 7 to 14 d, depending on temperature. The tadpoles metamorphose in one season, typically within 70 to 80 d [18]. *Echinostoma trivolvis* is a 37-collared-spined digenean trematode parasite in the family Echinostomatidae. It uses the snail, *Planorbella trivolvis*, as a first intermediate host and often uses ranid tadpoles as second intermediate hosts, encysting in

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the kidneys. Definitive hosts include semiaquatic birds and mammals [19].

Pickerel frogs used in this experiment were collected from one pond in rural Botetourt County, Virginia, USA, adjacent to Jefferson National Forest lands and with no historical pesticide application. We surveyed the edges of the pond and submerged woody debris using dip nets and found no evidence of *P. trivolvis*. On March 25, 2007, we collected seven clutches of recently laid pickerel frog eggs and transported them in a cooler to a laboratory in Blacksburg, Virginia, USA. Eggs were immediately separated by hand, keeping jelly coats intact. Eleven eggs from each of the seven clutches were combined to form 24 lots of 77 eggs each. All eggs were initially raised in 1,500 ml of 75/25 mix of dechloraminated city water (ChlorAm-X®, AquaScience Research Group, Kansas City, MO, USA) and well water in the laboratory. The available well water was extremely hard (364 mg/L $CaCO_3$), and previous research demonstrated that it caused spinal malformations in developing wood frogs (S.A. Budischak, unpublished data), so this mix was necessary to bring the hardness to acceptable levels (172 mg/L). Fifty percent water changes were carried out every 2 d.

Pesticide exposure

Three days after collection, the majority of embryos completed neurulation, reaching Gosner stage 14 [20]. The 24 lots were randomly assigned to one of six replicates of the four malathion treatments. Previous studies suggest that exposure to acetylcholinesterase-inhibiting pesticides during later embryonic development is more detrimental than exposure during the first few days of development [21,22], possibly because natural expression of acetylcholinesterase increases exponentially between fertilization and hatching [22]. Thus, we exposed the pickerel frog embryos to malathion from Gosner stage 14 to 18–19 [20], starting approximately 5 d before hatching and ending within a day of hatching.

Embryos were exposed to 2 L of 0, 15, 60, or 600 μg/L malathion (Chem Service, West Chester, PA, USA) for 96 h with one complete solution change after 48 h. Concentrations were confirmed in duplicate at the Virginia Tech Pesticide Residue Laboratory (means = 14.5, 62, and 600 μg/L). Our concentrations represent realistic malathion concentrations in natural waters receiving urban runoff and those exposed to spray drift or runoff from agricultural fields [23]. Malathion may be more likely to enter amphibian breeding habitats, including ponds and wetlands, than many other pesticides because malathion is the only organophosphate insecticide approved for aerial application for adult mosquito control.

After 96 h of pesticide exposure, embryos were transferred to a 75/25 mix of dechloraminated city water and well water until hatching. Hatching began the day after exposures ended, April 2, and continued until April 4. We calculated hatching rates for each replicate and checked all hatchlings for malformations [24]. Hatchling viability was defined as individuals hatching and lacking malformations. Malformed individuals were separated, photographed under a dissecting microscope and stored in 70% ethanol. Forty properly formed tadpoles from each of six replicates of the four malathion treatments were then moved to 24 corresponding pesticide-free outdoor aquatic mesocosms to test for latent effects of embryonic exposure.

Aquatic mesocosms

Replicate aquatic community mesocosms were established in 1,500-L polyethylene stock tanks in Blacksburg. Mesocosms were filled in early March with approximately 475 L of well water and 475 L of dechlorinated city water. A 50/50 mix was used rather than the 75/25 laboratory mix because the mesocosms received natural precipitation as well as biological material (see the following discussion). The resulting water hardness was 190 mg/L. Each mesocosm also received 1 kg of air-dried deciduous leaf litter and 17 g of finely ground Purina Rabbit Chow® (St. Louis, MO, USA). The mesocosms were spiked twice with 1.5 L of pond water from a permanent pond on the Virginia Tech property filtered through a 200-μm sieve. To decrease the variability in initial phytoplankton and zooplankton communities, portions of water were repeatedly exchanged between mesocosms prior to the addition of tadpoles. Mesocosms were covered with black mesh lids to provide shade and exclude predators and competitors. Conductivity, pH, temperature, and dissolved oxygen were monitored weekly at 7:30 AM and 7:30 PM (approximate coolest and warmest daily water temperatures, respectively) in five randomly selected replicates. No pesticides were added to mesocosms.

Parasite exposure

Forty-six days posthatch, 11 tadpoles (~Gosner stage 26 [20]) were haphazardly removed from each of the 24 mesocosms and moved into the laboratory. For unknown reasons, two mesocosms (receiving tadpoles previously exposed to 0 and 600 μg/L malathion) failed to support tadpoles and were excluded from the remainder of the present study, reducing sample size to $n = 5$ in these two treatments. The subsampled tadpoles, grouped by mesocosm, were acclimated to room temperature and reconstituted water [25] over 24 h. Ten tadpoles from each mesocosm were randomly assigned to individual 120-ml plastic cups containing 90 ml of reconstituted water. The cups were coded so that pesticide treatments were unknown. The remaining tadpole from each mesocosm was assigned to a control treatment to verify initial absence of parasite exposure. These parasite-control tadpoles were placed in identical cups with 90 ml of reconstituted water but did not receive any parasites.

Snails (*P. trivolvis*) were collected from a pond in Montgomery County, Virginia, USA. Seven *E. trivolvis* infected snails were induced to shed cercariae under a heat lamp. Sixty freshly shed cercariae were transferred to each tadpole cup with a glass pipette. Because of the limited rate of parasite shedding by the snails, groups of approximately 30 tadpoles were exposed each hour until all received parasites. Subsequent measurements of tadpoles were adjusted accordingly so that all individuals were exposed to cercariae for the same amount of time.

After 48 h of exposure to cercariae, all tadpoles were measured, weighed, and staged [20]. Tadpoles were then euthanized with MS 222 (ethyl 3-aminobenzoate, methanesulfonic acid salt, ACROS Organics, Morris Plains, NJ, USA) and frozen in individual microcentrifuge tubes for subsequent dissection. The microcentrifuge tubes also were coded so that the pesticide treatment of each tadpole was unknown during dissection. Because *E. trivolvis* encysts in the kidneys, the entire kidneys, both pronephros and mesonephros tissues, were removed using forceps under a dissecting scope and placed on a slide. A coverslip was gently pressed onto the tissue to pro-

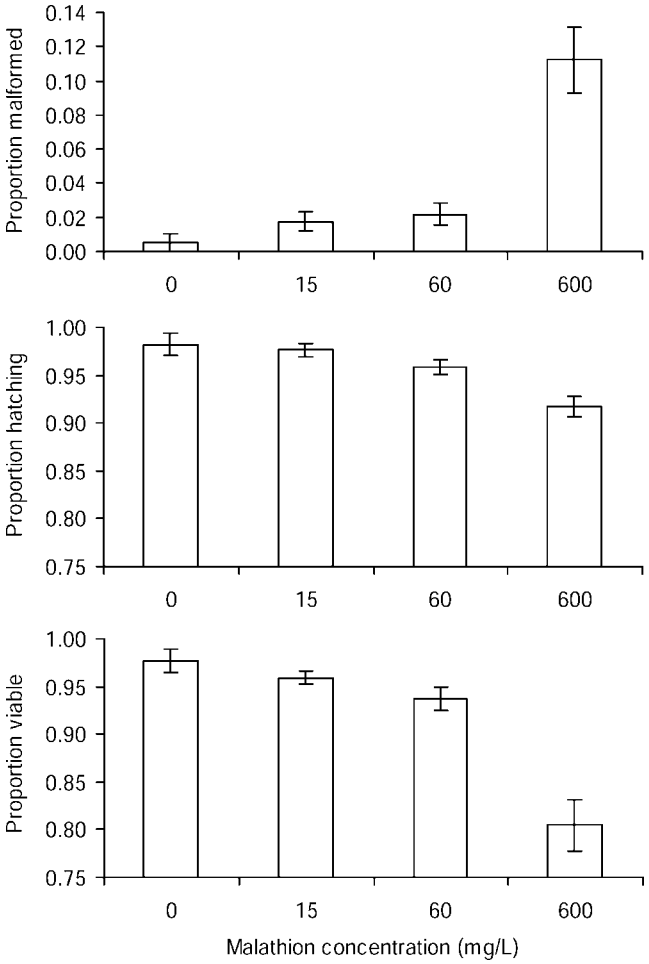

Fig. 1. Comparison of hatching success, malformation rate, and viability in *Rana palustris* tadpoles exposed to a range of malathion concentrations for 96 h. Significant effects of malathion treatment on all three variables were observed ($p < 0.003$).

duce a thin layer. Cysts were counted under ×100 magnification with a compound microscope.

Statistical analysis

Hatching success and malformation rates were not normally distributed (Shapiro–Wilks $p < 0.05$), so they were compared among treatments using Kruskal–Wallis tests. Viability was compared among pesticide concentrations using analysis of variance (ANOVA) with Tukey–Kramer pairwise comparisons. Because these three variables were not independent of each other, α was adjusted using a sequential Bonferoni procedure. The fraction of cysts, out of a potential 60, successfully encysted in each tadpole were treated as subsamples and averaged individually for each mesocosm. Encystment rates were compared among treatments using ANOVA with subsampling. The malathion treatment encystment rates were compared to the control using Dunnett's test. Tadpole size at the time of subsampling was compared among treatments using ANOVA. The relationship between tadpole mass and number of parasite cysts was examined using linear regression.

RESULTS

Malformation frequency increased and both hatching and viability decreased as malathion concentration increased (Fig. 1). Proportion hatching ($\chi^2 = 14.0$, *df* [degrees of freedom] = 3, $p = 0.003$), malformed ($\chi^2 = 15.9$, $df = 3$, $p = 0.001$), and viable ($F = 24.2$, $df = 3$, $p < 0.0001$) varied significantly among treatments. Hatching rates were high for the control and two lowest malathion treatments, ranging from 96 to 98%, but were reduced to 91.7% in the highest malathion concentration (600 μg/L). Malformation frequency was 0.7 and 2.2% in the control and 60 μg/L malathion treatments, consistent with acceptable background levels [26], but increased to 11.2% in the 600-μg/L treatment. The three malformed control tadpoles had edema, two tails, and craniofacial abnormalities with an axial flexure, respectively. The most common malformation in the lowest malathion concentration was axial flexure (Table 1). The two highest malathion concentrations were similar in their malformation profiles, both having ventralization as their most common malformation (Table 1). This distinct malformation was characterized by an underdeveloped head, reduced trunk, and abnormal notochord [27], with tails barely extending beyond the yolk sac. The proportion of viable tadpoles in the highest malathion treatment was significantly lower than the other three treatments. Viability was reduced by 17.3% in the 600-μg/L treatment compared to controls.

Table 1. Comparison of the number (and percentage of total malformations) of specific morphological abnormalities in *Rana palustris* hatchlings after a 96-h exposure to malathion. Total malformations exceeds the number of tadpoles malformed because some tadpoles had multiple malformations and are represented more than once

	Malathion treatment (μg/L)			
	0	15	60	600
Axial flexure	1 (25)	7 (70)	5 (36)	20 (35)
Craniofacial abnormality	1 (25)	1 (10)	2 (14)	7 (11)
Ventralization	0	2 (20)	7 (50)	34 (54)
Other	2 (50)	0	0	2 (3)
Total malformations	4	10	14	63
No. malformed	3	9	10	50

Neither mass ($p = 0.96$) nor length ($p = 0.96$) differed significantly among malathion treatments in the tadpoles subsampled for parasite exposure. No relationship was observed between tadpole mass ($p = 0.095$, $r^2 = 0.13$) or length ($p = 0.15$, $r^2 = 0.10$) and the number of parasites successfully encysting. The mean number of metacercarial cysts varied significantly among malathion treatments (Fig. 2; $F = 2.95$, *df*

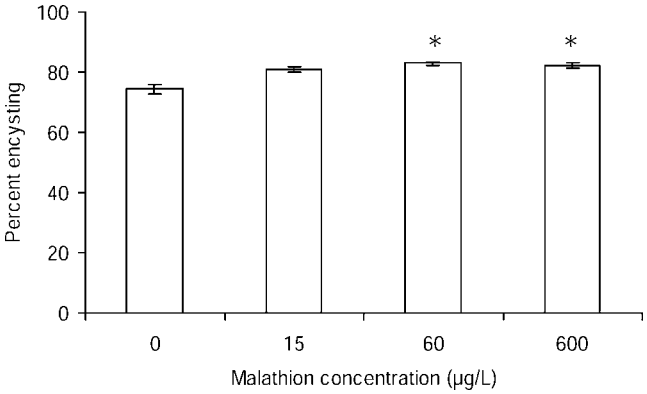

Fig. 2. Percent of *Echinostoma trivolvis* cercaria successfully encysting in *Rana palustris* tadpoles seven weeks after a 96 h of exposure to a range of malathion concentrations. Asterisks denote significant differences from the control.

= 3, 18, $p = 0.034$). Tadpoles in the two highest malathion concentrations, 60 and 600 μg/L, had 11.7 and 10.8% more cysts than tadpoles in the control treatment ($p = 0.019$ and 0.044, respectively). Although encystment in the low malathion concentration (15 μg/L) was also increased by 7% compared to controls, this difference was not significant ($p = 0.105$).

DISCUSSION

Environmentally relevant concentrations of malathion negatively affected pickerel frog hatching success, malformation frequency, and viability rates. Overall hatchling viability was decreased by 17% in our highest concentration treatment, 600 μg/L, which was the combined result of decreased hatching success and increased incidence of malformations. Ventralization, in particular, was increased in the two highest malathion treatments, accounting for over 50% of malformations. This type of malformation suggests that malathion is interfering in the development of the dorsoanterior axis [27]. Xenobiotics, including ultraviolet radiation, lithium, and retinoic acid, are known to cause ventralization [27], dorsalization [28], and posteriorization [29] in developing embryos, respectively. Although such developmental phenotypes are not listed in the malformation guide for embryo teratogenesis assays [24] or noted in any previous studies for malathion [21,22,30], they are widely utilized in developmental biology to study embryonic signaling pathways [27].

Malathion reduced hatching success to 80% in the 600-μg/L treatment, far below the 98% hatching success observed in the control treatment. Although our design precluded calculation of a 50% effective concentration (EC50), this concentration is far below the minimum concentration shown to decrease enzyme activity (4 mg/L) and the 50% lethal concentration (LC50) (19 mg/L) for *Bufo arenarum* larvae [31]. This concentration is also orders of magnitude below LC50s for embryos of *Rana tigrina* (30 mg/L) [32] and *Microhyla ornata* (20 mg/L) [33]. Pickerel frog embryos appear comparatively vulnerable to malathion, with respect to other anuran larvae, although differences in experimental conditions and exposure durations could account for some of the variation in LC50 values.

Survivors of embryonic exposures may suffer lasting consequences that would be missed by simply measuring survival and morphological parameters. We found increased susceptibility to trematode infection seven weeks after embryonic exposure to low concentrations of malathion. After a single parasite exposure, we documented an 11% increase in susceptibility to trematodes in malathion-exposed animals compared to controls. However, in nature tadpoles are repeatedly exposed to parasites. Thus, we hypothesize that a small increase in susceptibility could lead to much larger parasite burdens over time. High *E. trivolvis* burdens are known to destroy functional renal tissue in adult frogs [34], but further research is needed to determine whether latent susceptibility caused by pesticides would result in similar pathology.

We hypothesize that the most plausible explanation for increased susceptibility to infection was an altered immune response. By exposing the tadpoles to parasites in a small volume of water, we diminished their ability to behaviorally avoid cercariae, forcing them to rely primarily on physiological defenses, such as immune responses [35]. Malathion can negatively affect the immune system of amphibians [6,9], including lowering circulating eosinophilic granulocytes, the type of white blood cell known to fight parasitic infection [11]. However, because we did not measure immune function, we cannot exclude a number of other possible explanations. For example, we examined encystment at only one point in time (48 h after parasite exposure). The controls may have been capable of shedding cysts within that short time window, but the malathion-exposed tadpoles may have had a delayed rather than decreased immune response, leaving them with a higher parasite burden at the time of sampling. Alternatively, the tadpoles that died as embryos during malathion exposure may have had stronger immune systems than those that survived. Therefore, we potentially selected for less immunocompetent tadpoles in our higher pesticide treatments, compared to those in the control treatment. Clearly, additional studies that address the mechanism by which enhanced latent susceptibility occurs are needed.

The long-term and latent effects of contaminant exposure are less well known than the direct lethal and sublethal effects [12]. Chemicals that affect the immune system particularly have the potential to cause effects that do not manifest until after exposure [8]. For example, researchers that exposed chinook salmon eggs to *o,p′*-DDE (dichlorodiphenyldichloroethylene), a metabolite of DDT, found that although there was no effect on mortality, time to hatch, or gonadal development, the humoral response was significantly reduced one year after exposure [13]. Cytotoxic leukocytes from mummichog, *Fundulus heteroclitus*, in a polycyclic aromatic hydrocarbon contaminated river displayed depressed tumorolytic activity compared to those from an uncontaminated site. When moved to uncontaminated water, the polycyclic aromatic hydrocarbon–exposed mummichogs maintained their decreased tumorolytic activity for up to 28 weeks [36]. Similar long-term immunological changes would not be detected by typical short-term toxicity tests.

Latent susceptibility may lead to greater parasite transmission than when parasites and hosts are concurrently exposed to pesticides. During concurrent exposure, transmission may decrease because some pesticides reduce cercarial survival [37] and infectivity [35]. The lifetimes of many modern pesticides and free-swimming parasite stages are short compared to those of many first and second intermediate hosts. Consequently, first intermediate hosts (snails) could produce new, unaffected cercariae once pesticide exposure has ceased. The present study suggests that tadpoles (second intermediate hosts) exposed to pesticides during early development suffer increased parasite susceptibility that continues after pesticide exposure has ceased. Therefore, tadpoles exposed to pesticides early in the season (spring) could encounter newly shed parasites later in the season (summer) and ultimately accrue higher parasite burdens than when tadpoles and parasites are jointly exposed to pesticides.

Echinostoma trivolvis burdens in tadpoles from naturally infected populations can exceed those used in the present study by more than 25-fold [38] and may become an increasing problem for many amphibian species. High-nutrient conditions simulating cultural eutrophication can increase snail biomass, prevalence of snail infection, and the cercarial output of infected snails, which can result in increased tadpole infection [39]. Eutrophic habitats often have increased chemical contamination [39]; thus, understanding the interactions between pesticides and parasites could be important for conservation. Additionally, parasite hosts may increase their ranges with global climate change. Preliminary research also suggests that

many parasites proliferate more quickly in warmer waters and may benefit from longer reproductive seasons [40]. With potential for increased parasite prevalence from eutrophication and climate change, our data underscore the importance of understanding the role of pesticides in disease susceptibility and latent effects on the immune system.

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