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Source: Journal of Wildlife Diseases, 35(3) : 536-541

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-35.3.536

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Journal of Wildlife Diseases, 35(3), 1999, p. 536–541
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EFFECTS OF MALATHION ON DISEASE SUSCEPTIBILITY IN WOODHOUSE'S TOADS

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ABSTRACT: Adult male Woodhouse's toads (*Bufo woodhousi*) developed clinical disease, hepatomegaly, and died at a higher rate when externally exposed once to either a high or low sublethal dose (0.011 or 0.0011 mg malathion/g toad) of field grade malathion and challenged with a sublethal dose of *Aeromonas hydrophila* injected intraperintoneally (1.1×10^4 bacteria/g toad) when compared to toads not exposed to malathion but challenged with *A. hydrophila* ($P < 0.007$). Toads exposed to malathion (high or low dose) and challenged with *A. hydrophila* had clinical disease, hepatomegaly, and died at a higher rate [9 (90%) of 10] than toads exposed to malathion alone ($P < 0.002$). Toads exposed to the high and low doses of malathion had a 22% and 17% decrease in brain cholinesterase levels, respectively, when they were compared to nonmalathion exposed toads ($P < 0.025$, $P < 0.006$). It appears that field grade malathion applied externally to adult Woodhouse's toads may cause increased disease susceptibility when challenged with a potentially pathogenic bacteria.

Key words: *Aeromonas hydrophila*, amphibian, *Bufo woodhousi*, malathion, organophosphorus pesticide, pesticide, Woodhouse's toad.

INTRODUCTION

World wide amphibian diversity and populations numbers have been reported to be declining (Wyman, 1990; Wake, 1992; Taylor et al., 1999). Pesticides are sometimes implicated yet few studies have been conducted to determine if pesticides actually present a hazard to them (Hall and Henry, 1992). In addition, most published studies on the effects of pesticides on amphibians have been conducted on embryo and tadpole life stages (Hall and Kolbe, 1980; De Llamas et al., 1985; Rosenbaum et al., 1988; Devillers and Exbrayat, 1992; Berrill et al., 1994).

Only one study has been conducted on the effects of malathion (diethyl mercaptosuccinate, S-ester with O, O-dimethyl phophorodithioate) on amphibians in a post-metamorphic life stage. Baker (1985) examined the responses of two woodland salamander species (*Plethodon glutinosus* and *P. cinereus*) to substrates which malathion had been applied to. *Plethodon glutinosus* showed significant inhibition of cholinesterase activity after 3 days of exposure to a 5.6 kg/ha application of malathion. *Plethodon cinereus* did not show this effect, thus indicating variations in species susceptibility to malathion.

In the 1980's, malathion was applied annually to 4,486,000 ha in the United States (Smith, 1987). It is used most commonly in the control of mosquitoes, flies, household insects, animal ectoparasites, and human lice. Malathion has been element labeled and applied to fields to study its potential translocation and bioaccumulation; and small rodents, insects and birds had detectable levels 1 yr after treatment (Peterle, 1966).

Malathion is lipophilic and readily taken up through the skin, respiratory system, or gastrointestinal tract, with absorption enhanced if malathion is in the liquid form (Gunther et al., 1968). The predominant mechanism of organophosphate toxicity is inhibition of acetylcholinesterase in the nervous system causing accumulation of acetylcholine (Ecobichon, 1993). This causes hyperexcitability and multiple postsynaptic impulses generated by single presynaptic stimuli.

Minimal work has been conducted on effects of organophosphorus compounds on disease susceptibility. Dulout et al.

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(1983) demonstrated a dose-response relationship to malathion induced chromosomal aberrations in mouse bone-marrow cells. At intraperintoneally injected doses above 230 mg/kg the mice showed chromosomal abnormalities at 6 hr post-injection. Hermanowicz and Kossman (1984) observed that humans occupationally exposed to organophosphorus compounds, including malathion, have marked impairment of neutrophil chemotaxis. In addition, these workers had increased frequency of upper respiratory infections which increased with the number of years of exposure to organophosphorus compounds. Organophosphorus compounds can also affect immune function of macrophages and lymphocytes in culture (World Health Organization, 1986; Pruett, 1992).

Amphibian mortalities are often diagnosed as "red leg" which is usually attributed to the bacterium *Aeromonas hydrophila* that they are believed to have been externally exposed to from the environment (Boyer et al., 1971; Rigney et al., 1978; Nyman, 1986). The syndrome of "red leg" in amphibians appears as hyperemia of the ventral skin of the thighs and abdomen (Glorioso et al., 1974). Anorexia and dulling of the body color also frequently occur. The liver may be enlarged, lungs may appear congested, and fluid may be present in the peritoneal cavity.

Organisms within the genus *Aeromonas* are motile Gram-negative polar-flagellated rods (Nygaard et al., 1970). Aeromonads are common in aquatic environments and are found over wide ranges of salinity, conductivity, temperature, pH, and turbidity (Slotnick, 1970; Rouf and Rigney, 1971; Hazen et al., 1978). Aeromonads have several attributes which can contribute to virulence and these include production of endotoxins, hemolysins, cytotoxins, proteases, and the ability to adhere to cells (Cahill, 1990). In experimental studies to determine the pathogenicity of *A. hydrophila* in leopard frogs (*R. pipiens*) extremely high numbers of injected bacteria (1.5×10^9) were required to induce mortality (Rigney et al., 1978).

This research was conducted to determine if externally applied field grade malathion, at a sublethal exposure dose would cause increased disease susceptibility in adult Woodhouse's toads (*Bufo woodhousi*) when challenged with *A. hydrophila* injected intraperintoneally.

METHODS AND MATERIALS

Adult male free-ranging Woodhouse's toads were captured in Larimer County (Colorado, USA; 41°20′N, 105°35′W). Toads were acclimated to captivity at the Wyoming State Veterinary Laboratory (Laramie, Wyoming) for at least 2 wk prior to the start of this project. They were examined for physical condition and any toads demonstrating signs of disease or not eating were excluded from the study.

Toads were housed individually in glass terrariums with screen lids for ventilation. Terrarium substrate was peat moss initially autoclaved for 15 min at 121 C. Sterile water was provided in glass bowls that were large enough for toads to immerse themselves. Bowls were cleaned and refilled with unchlorinated well water every other day. Live crickets, obtained from a commercial cricket farm (Top Hat Cricket Farm, Kalamazoo, Michigan, USA) were fed to the toads ad libitum three times a week. Crickets were housed in the same room as the toads. The crickets were housed in glass terrariums with screen lids and fed commercial rat chow ad libitum (Purina, St. Louis, Missouri, USA). Each cricket tank contained a water dish and egg crates for shelter. The room was on a 12 hr light/12 hr dark cycle with a 15 watt full spectrum light above one end of the tank. The room temperature ranged between 20–22 C and the humidity was approximately 40%. Disposable latex gloves were worn when handling toads and were changed when moving between experimental groups.

Commercially available field grade malathion as opposed to technical grade malathion was selected for this study because this is the product applied in the environment. No carriers were added or tested because ultra low volume aerial application uses no carriers. Ventral cutaneous application of malathion was chosen to mimic a potential natural exposure in the wild.

Prior to the start of this experiment, pilot studies were conducted to determine the sublethal exposure doses of malathion for toads. This was conducted by exposing two toads to a single ventral skin exposure of malathion. If mortality occurred within 72 hr then the ex-

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TABLE 1. Results of experimental external application of a field grade malathion on disease susceptibility of Woodhouse's toads.

Group and treatment	Number with clinical disease and death/ number in group	Number with hepatomegaly/ number in group	Number isolated *A. hydrophila*/ number in group	Liver to carcass weight ratio range and mean ($\bar{x}$)
Group 1 Saline external Saline injection	0/5	0/5	0/5	0.023–0.048 (0.037)
Group 2 Malathion external (low dose)[a] Saline injection	0/5	0/5	0/5	0.027—0.066 (0.044)
Group 3 Malathion external (high dose)[b] Saline injection	2/5	2/5	0/5	0.031–0.095 (0.059)
Group 4 Saline external *A. hydrophila* injection[c]	1/5	0/5	0/5	0.032–0.056 (0.046)
Group 5 Malathion external (low dose)[a] *A. hydrophila* injection[c]	4/5[d]	4/5[d]	4/5[d]	0.046–0.098[d] (0.074)
Group 6 Malathion external (high dose)[b] *A. hydrophila* injection[c]	5/5[d]	5/5[d]	3/5[d]	0.079–0.107[d] (0.089)

[a] 0.0011 mg malathion/g toad.
[b] 0.011 mg malathion/g toad.
[c] 1.1×10^4 bacteria/g toad.
[d] Value is statistically different ($P < 0.05$) when groups 5 or 6 (separate or combined) were compared to groups 1, 2, 3, or 4.

posure dose was decreased by 25% and tried on a new group of toads. This was then done until the highest sublethal dose was determined. Malathion was found to be lethal at an exposure dose greater than 0.112 mg malathion/g toad. No morbidity or mortality occurred among five toads exposed to 0.011 mg malathion/g toad. Thus the sublethal high exposure dose of malathion was 0.011 mg malathion/g toad and the low exposure dose of malathion was set 10% of this at 0.0011 mg malathion/g toad. The highest nonlethal *A. hydrophila* dose was determined in a similar way except that exposure was through a single intraperitoneal injection and determined to be 1.1×10^4 bacteria/g toad. Injection was selected as the route of administration to deliver a known challenge dose of bacteria. This route is also the only documented proven experimental method to reproduce red leg (Rigney et al., 1978). The *A. hydrophila* culture was obtained from a Wyoming toad that died of mycotic dermatitis with secondary septicemia and identified by the same manner as described below.

Toads for the primary study were assigned randomly to one of six treatment groups of five toads each (Table 1). Group 1 toads were the control group and each received an external application of sterile saline to the ventral skin via a micro syringe (Hamilton Company, Reno, Nevada, USA). The volume administered was standardized by toad weight with sterile saline to equal the volume given to treatment toads. Toads were held until the ventral skin air dried (approximately 2 to 5 min). Prior to being returned to their aquariums, group 1 toads were injected intraperintoneally through the ventral skin surface with 0.1 ml sterile saline via 25 gauge needle.

The group 2 toads received an external application of the low exposure dose of malathion (Malathion 96.5%, Cynthion ULV, American Cyanamid Company, Wayne, New Jersey, USA) to their ventral skin via a micro syringe and an intraperitoneal injection of 0.1 ml sterile saline. The group 3 toads received an external application of the high exposure dose of malathion to their ventral skin and an intraperitoneal in-

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jection of 0.1 ml sterile saline. The group 4 toads received an external application of sterile saline to the ventral skin and an intraperitoneal injection of 1.1×10^4 bacteria/g toad of *A. hydrophila* in 0.1 ml sterile saline. Group 5 toads received an external application of the low exposure dose of malathion to their ventral skin and an intraperitoneal injection of 1.1×10^4 bacteria/g toad of *A. hydrophila* in 0.1 ml sterile saline. Group six toads received an external application of the low exposure dose of malathion to their ventral skin and an intraperitoneal injection of 1.1×10^4 bacteria/g toad of *A. hydrophila* in 0.1 ml sterile saline.

Toads were observed each day and any signs of clinical disease were recorded. Study animals were held for 30 days at which point survivors were euthanized by immersion in tricaine methane sulfonate (MS222, Sandoz Ltd., Basle, Switzerland). Toads were examined visually for gross lesions. Carcasses and livers were weighed.

Ventral abdominal skin, subcutaneous fluid, and liver were cultured for bacteria. Swabs of these tissues were placed in modified Stuart's bacterial transport medium (S/P Brand Culturette Systems, Baxter diagnostics, Deerfield, Illinois, USA). Within 1 hr swabs were plated onto Columbia agar with 5% sheep blood (Acumedia Manufacturing, Inc., Baltimore, Maryland, USA). Plates were incubated at 35 C in atmospheric air for 96 hr. Plates were examined for bacterial growth daily. Isolates were inoculated into Biolog panels (Biolog, Inc., Hayward, California, USA) for identification based on carbon utilization.

Brains were removed and individually put in 1 ml cryogenic vials (Corning, Acton, Massachusetts, USA) and frozen at −70 C. They were thawed later at 20–22 C and analyzed to detect cholinesterase activity according to the Harlin modification of the Ellman method (Harlin and Ross, 1990). Because the malathion was experimentally applied to treatment toads, a comparison of cholinesterase activity levels was made between toads that did and did not received malathion treatment.

In this prospective study it was predetermined that data would be analyzed by Fisher's exact probability tests (Minitab, State College, Pennsylvania, USA) to determine if there were statistical differences between treatment groups based on presence or absence of morbidity or mortality, presence or absence of hepatomegaly, and presence or absence of *A. hydrophila* isolation. Analysis of liver weight/carcass weight ratios and cholinesterase activity were done by paired *t*-test (Minitab). The alpha was equal to 0.05 for all statistical analyses.

RESULTS

As shown in Table 1, clinical disease, hepatomegaly, and death occurred at a higher rate when toads were exposed to a single dose of either high dose or low dose field grade malathion and challenged with *A. hydrophila* [9 (90%) of 10] when compared to toads not exposed to malathion but challenged with *A. hydrophila* [1 (20%) of 5] ($P < 0.007$). Toads in group 6 which received external exposure to the high dose of malathion and were challenged with *A. hydrophila* had clinical disease, hepatomegaly, and died at a higher rate [5 (100%) of 5] than toads in groups 1 or 2 in which none were affected ($P < 0.002$) (Table 1). Group 6 toads also were affected at higher rate than group 3 (malathion alone) [2 (40%) of 5] ($P < 0.038$) or group 4 (*A. hydrophila* alone) [1 (20%) of 5] ($P < 0.010$). However, group 4 toads did not have hepatomegaly. Statistical difference was not found when results from group 6 toads were compared to toads in group 5 [4 (80%) of 5] which were exposed to the low dose of malathion and challenged with *A. hydrophila*.

Aeromonas hydrophila was isolated from group 6 toads at a higher rate [3 (60%) of 5] than toads in groups 1, 2, 3, or 4 in which none was isolated ($P < 0.038$). *Aeromonas hydrophila* was also recovered at a higher rate from group 5 toads [4 (80%) of 5] than toads in groups 1, 2, 3 or 4 in which none were affected ($P < 0.010$). A statistical difference was not found when culture results from group 6 toads were compared to group 5 toads ($P < 0.490$).

Overall, toads exposed to high or low dose malathion and challenged with *A. hydrophila* developed clinical disease, hepatomegaly, and died at a higher rate [9 (90%) of 10] than toads exposed to high or low dose malathion and not challenged with *A. hydrophila* ($P < 0.002$). There was higher mortality in that high malathion dose groups [7 (70%) of 10] compared to toads exposed to the low dose [4 (40%) of 10] ($P < 0.178$).

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Death of affected toads in groups 3, 5, and 6 occurred between 72 to 120 hr of exposure to the pesticide and potential pathogen. The one toad in group 4 which died 432 hr post exposure did not have hepatomegaly and *A. hydrophila* was not recovered.

Liver weight/carcass weight ratios were highest in toads exposed to high or low dose of malathion and challenged with *A. hydrophila* when compared to toads that were exposed to malathion and not challenged with bacteria ($P < 0.003$). However, statistical difference was not found between groups of toads exposed to high or low dose malathion exposure and challenged with *A. hydrophila* ($P < 0.292$).

Toads not exposed to malathion had brain cholinesterase activity levels which were higher (11.33–23.89 umol/min/g, $\bar{x} = 15.17$) than malathion exposed toads (low dose 6.86–22.42 umol/min/g, $\bar{x} = 12.51$, $P < 0.006$; high dose 8.22–17.67 umol/min/g, $\bar{x} = 11.72$, $P < 0.025$). Thus, toads exposed to the high dose of malathion had a 22% decrease in brain cholinesterase activity levels and the low dose toads decreased by 17% when they were compared to non malathion exposed toads.

DISCUSSION

Disease susceptibility and mortality were increased in toads externally exposed to field grade malathion and then challenged with *A. hydrophila* injection. Statistically, both the high and low dose groups were significantly affected. Hepatomegaly was found in all toads clinically exposed to both malathion and *A. hydrophila*.

Malathion (96.5%) is registered for aerial application at a rate of 180 ml per acre (Cynthion ULV, American Cyanamid Company, Wayne, New Jersey, USA). This is approximately equal to an application of 0.002 mg malathion/square meter of land. In our study, we found that malathion was directly lethal to Woodhouse's toads when applied to their ventral skin at a single dose at or above 0.110 mg malathion/g toad. The combination of intraperitoneal injection of *A. hydrophila* following an external dose of 0.0011 or 0.011 mg malathion/g toad was lethal within 120 hr in Woodhouse's toads. In theory, in the field a toad would have to be cutaneously exposed to the equivalency of malathion that is applied once to a 2 square meter area and then be naturally challenged by a potential pathogen for increased disease susceptibility or mortality to occur. Our route of intraperitoneal injection was not a natural exposure route for the *A. hydrophila* but was done to deliver a specific dose of the organism. The aquatic terrestrial interface these animal inhabit contains a rich diversity of potentially pathogenic bacteria. This environment also contains rough substrates and it is possible that cutaneous abrasions would allow for exposure to a diversity of potential pathogenic organisms.

This study did not take into account potential increased exposure to malathion that could occur from ingestion of water and food, skin absorption of water, or multiple malathion spraying events. In addition, the effects of multiple malathion exposures on amphibians needs to be addressed. Further studies are needed to assess the actual field exposure of amphibians to malathion. In addition, studies on the amphibian immune system are needed to assess which immunological components are being adversely affected by malathion.

ACKNOWLEDGMENTS

We thank A. Boerger-Fields, D. Sanchez, V. Welch, H. Edwards, J. Russell, and S. Pistono for technical expertise. This research was supported by the United States Fish & Wildlife Service, the Wyoming Game & Fish Department, and the Department of Veterinary Sciences, University of Wyoming.

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Received for publication 6 August 1998.

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