Research Article

Effects of ecologically relevant doses of malathion on developing *Xenopus laevis* tadpoles

Catherine M. Webb and D. Andrew Crain

Maryville College, Maryville, Tennessee 37803

Abstract. Amphibian declines have been occurring for decades, and some of these declines are attributed to environmental contaminants. In this experiment, we examined the effects of ecologically-relevant concentrations of a common organophosphate insecticide, malathion, on developing *Xenopus laevis* tadpoles. Two-week-old *Xenopus laevis* tadpoles were divided into four experimental groups, each with a sample size of ten tadpoles: control (water), 1.0 ng/L malathion, 1.0 µg/L malathion, and 1.0 mg/L malathion. During their 30-day exposure to malathion, tadpoles in the 1.0 mg/L malathion displayed bent tails ($p<0.001$), unusual swimming behavior, and a higher mortality rate ($p<0.001$) when compared to the control group. Future experiments should examine the exact etiology of malathion-induced bent tails in *Xenopus laevis*.

Introduction

Numerous populations of amphibians have experienced declines over the last few decades. These amphibian population declines have been linked to many different causes including exposure to ultraviolet radiation (Blaustein et al., 1997), habitat destruction (Pough et al., 1998), trematode parasites (Johnson et al., 1999), environmental contaminants (Sparling et al., 2000), or a combination of these factors (e.g., Kiesecker, 2002). Of studies considering environmental contaminants, relatively few investigate the effects of contemporary-use pesticides (Sparling et al., 2000). However, recent data by Hayes et al. (2002) suggest that relatively low environmental concentrations of common modern-use pesticides can cause devastating effects to the reproductive potential and, thus, population status of frogs. Hayes et al. (2002) found that atrazine, a commonly-used herbicide, has an overall demasculinizing effect on male *Xenopus laevis* frogs, with males exhibiting decreased testosterone concentrations, smaller laryngeal openings, and some hermaphroditism. These data emphasize the potential detrimental impact of chronic low-dose pesticide exposure to amphibians.

One of the most commonly used organophosphate insecticides, malathion, has shown the potential to contribute to amphibian declines, as relatively high sublethal doses of malathion have been shown to induce abnormalities in amphibians. For instance, *Bufo woodhousi* toads externally exposed to sublethal doses (11 or 1.1 µg malathion/g toad) of malathion are more likely to

Correspondence to: Catherine Webb, Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061–0406; phone (540) 231–9183; fax (540) 231–9307; e-mail: cmwebb@vt.edu.

Bios 77(1) 1–6, 2006

contract bacterial infections, thus increasing their disease susceptibility (Taylor et al., 1999). Similarly, relatively high environmental concentrations of malathion (1 mg/L, 5 mg/L, and 10 mg/L) cause notochord defects in *Xenopus laevis* embryos (Snawder and Chambers, 1993). Such adverse effects on amphibians at relatively high dosages of malathion justify examining potential effects at lower dosages.

The half-life of malathion in water ranges from 16 hours to 21 weeks. Much of this degradation is due to hydrolysis; however, it has been suggested that high pH, photolysis, and biodegradation may also be important contributing factors to the degradation of malathion in water (Howard, 1991). Despite the fact that the half life of malathion may be relatively short, frequency of use of the insecticide may play a significant role in the persistence of malathion in aquatic environments. Indeed, chronic, low dose exposure is a current focus of environmental toxicologists (Eggen et al., 2004).

Due to the extensive use of malathion as an insecticide and the potential for malathion to alter the development of amphibians, we designed this study to evaluate the effects of ecologically-relevant concentrations of malathion on *Xenopus laevis* tadpoles. Currently, the Environmental Protection Agency sets the human drinking water equivalent level (the long-term exposure concentration that is protective of adverse effects) at 0.8 mg/L (EPA, 2004). Thus we chose to expose tadpoles to concentrations similar to and below this concentration. In this experiment we investigate the effects of chronic, low-dose malathion exposure on tadpole gross morphology, body growth, developmental stage, and tadpole activity level.

Materials and Methods

Animals and treatment groups

Eighty *Xenopus laevis* two-week-old tadpoles were purchased from Carolina Biological Supply Company (Burlington, NC). They were randomly divided into four groups of twenty tadpoles each and allowed to acclimate for six days prior to malathion exposure. Tank temperature was maintained between 18°C and 21°C from Day 0 to Day 16 (with Day 0 being the first day of treatment with malathion) and between 21°C and 24°C from Day 17 to the end of the experiment (Day 30). Lights were on for 12 hours and off for 12 hours per day. Tadpoles were raised in four plastic containers with air stones, and each tank was filled with 5 L of dechlorinated (using Wardley WaterCare Chlor Out) tap water (5 cm deep). Every third day, each tank of 20 tadpoles was fed 0.1 g pulverized Nasco Frog Brittle (Fort Atkinson, WI) after the water was changed.

The four groups of tadpoles included one control group, containing only water, and three treatment groups at concentrations of 1.0 ng/L, 1.0 μg/L, and 1.0 mg/L of malathion (Ortho Malathion 50 Plus Insect Spray Concentrate) in water. Insect spray concentrate instead of the purified malathion was chosen to mimic exposures in the environment. The malathion was added to the water after every water change (every 3 days), yielding a chronic exposure regime.

Endpoints measured

Prior to changing the water every three days, individual tadpoles were caught in a small beaker. Gross morphology and behavior were observed at each water change, and developmental stage (according to Gosner, 1960) and activity level were assessed on every fourth tadpole that was caught (a total of 5 per group). Weight was also measured on every fourth tadpole at every water change from Day 9 to Day 30; tadpoles were too small to be weighed prior to Day 9.

From Day 0 to Day 18, every fourth tadpole was observed in a watch glass for about one minute to measure its activity level. Activity levels ranged from 1 to 4, with inactive tadpoles during this time receiving a 1 and tadpoles that did not stop swimming receiving a 4. Starting on Day 21 until Day 30, activity levels were recorded using a more quantitative method. A grid with 1-inch squares was placed under each container. Prior to changing the water (but after removing air stones), five tadpoles from each group were randomly selected. Each of these individuals was then observed for two minutes while the number of lines it crossed was counted.

Statistical analysis

Statistical analyses were conducted using Microsoft Excel 2000, StatView (Version 5.0.1), and Minitab Release 14.13. Excel was used to conduct single-factor Analysis of Variance tests on measures of activity level, stage, and weight. Fisher's post-hoc tests were performed on stage and weight using StatView. Mortality rates and numbers of bent tails were compared in Minitab using binary logistic regression. For these tests, the dependent variable was whether or not the tadpole died, or whether or not it had a bent tail, and the independent variables were group and day. All p-values were analyzed at the 0.05 significance level.

Results

Most of the abnormal effects that were found in the tadpoles occurred in the highest malathion concentration (1.0 mg/L). These effects included bent tails, unusual swimming behavior, and a high mortality rate.

One effect noted in the high concentration was the bending of tadpole tails at their bases (Figure 1). This occurrence was accompanied by physical weakness (barely swimming at all) or by swimming only in the direction of the bend, either resulting in circular or corkscrew pattern swimming. The bending of tails first became apparent on Day 9 in the 1.0 mg/L group, and by Day 30, 70% of the tadpoles in this group had displayed bent tails. As indicated by logistic regression (with day being a significant variable; $p<0.001$), the 1.0 mg/L group displayed significantly more tadpoles with bent tails over time than the control group ($p<0.001$). The other two treatment groups did not display any bent tails and were not significantly different from the control group ($p=0.996$ for both groups). In all cases, the tadpoles died two to four days after the bent tail was first noted.

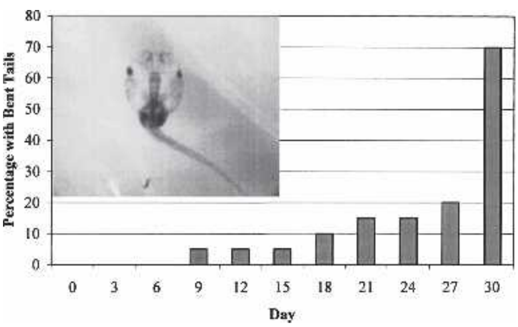

Figure 1. Percentage of *Xenopus* tadpoles that exhibited bent tails in the 1.0 mg/L group, which displayed more bent tails over time than the control group ($p<0.001$). With the exception of one tadpole in the control group at Day 30, no tadpoles in the other treatment groups exhibited bent tails. The photograph shows an example of such bent tails.

Throughout the experiment, at least several tadpoles died from each group. Figure 2 shows the numbers of live tadpoles (out of the original 20 in each group) on each day during the experiment. Logistic regression showed that the mortality rate in the high concentration (1.0 mg/L) was significantly higher than that of the control group ($p<0.001$). However, there was no significant difference between the control group and the low concentration (1.0 ng/L; $p=0.299$) or between the control group and the middle concentration (1.0 μg/L; $p=0.124$). In addition to group, day was also a significant variable in the model ($p<0.001$).

Figure 3a shows the activity levels of the tadpoles rated on a scale of 1 to 4, with 4 being the highest. The activity measured by the grid lines is shown in Figure 3b. On all days, the activity levels among the groups did not differ significantly.

The average developmental stage of tadpoles in the treatment groups did not differ at any of the sampling days ($p>0.5$ in all cases). Weights of the tadpoles did not differ significantly among the groups at any of the measurement dates (Figure 4; $p>0.05$).

Discussion

There were several major effects of malathion on tadpoles observed during the experiment. Many tadpoles in the highest concentration had their tails bent and/or swam in a disoriented manner, and this group exhibited a higher mortality rate than the other groups. There were no effects noted on tadpole weight or developmental stage.

As all organophosphates, malathion exerts its lethal action through the mechanism of acetyl-

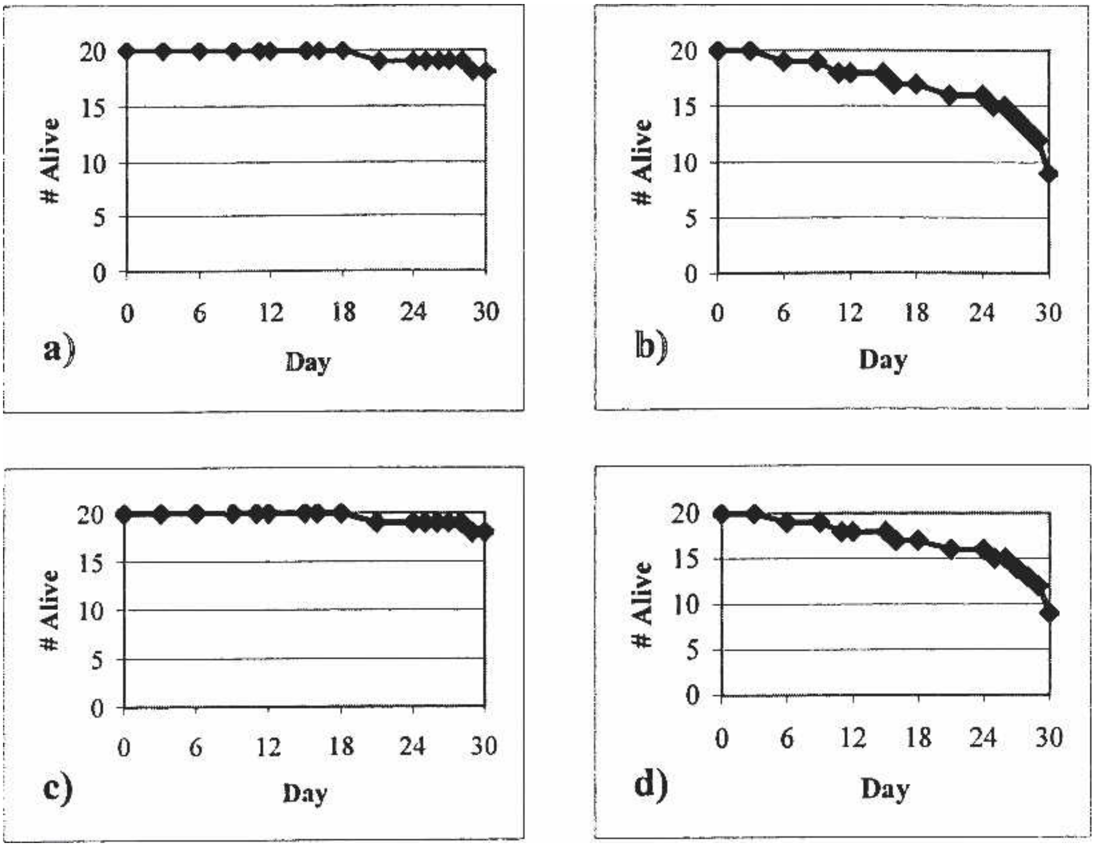

Figure 2. Numbers of surviving tadpoles throughout experiment. a) control; b) 1.0 ng/L; c) 1.0 μg/L; d) 1.0 mg/L. The 1.0 mg/L group had a higher mortality rate than the control group (p<0.001).

cholinesterase (AChE) inhibition (Ecobichon, 2001). Therefore, AChE inhibition is expected to cause abnormal muscular contraction and altered skeletal muscle function. For instance, after continuous exposure to 44 ppm malathion for 72–120 hours, *Bufo arenarum* embryos had reduced AChE activity (Caballero de Castro et al., 1991). However, the mechanism of action for sublethal

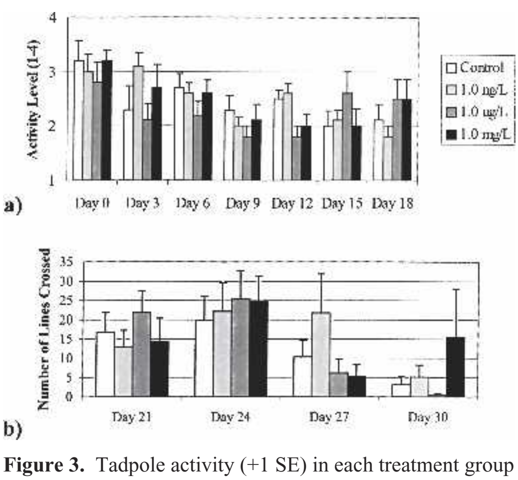

Figure 3. Tadpole activity (+1 SE) in each treatment group from Days 0–18 (a) and Days 21–30 (b).

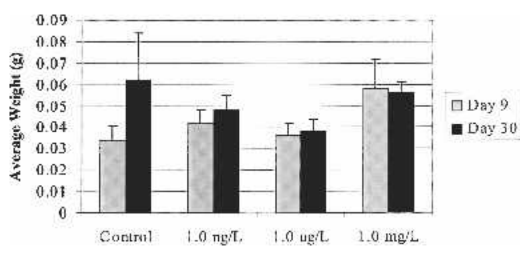

Figure 4. Average weights (+1 SE) of tadpoles at Days 9 and 30 of the experiment.

effects of lower malathion concentrations have not been characterized. Alvarez et al. (1995) found that when *Rana perezi* larvae were exposed to the organophosphate Folidol, the tadpoles displayed twisted spinal columns and tails, among other effects. In the current study, the tadpoles with bent tails in the highest concentration (1.0 mg/L) often swam in such a way that was apparently caused by the bending of their tails. For instance, many of them could turn only in the direction of the bend in the tail. Others swam upside down or swam while spinning like a corkscrew. Pawar et al. (1983) found that when *Microhyla ornata* tadpoles were exposed to 5–10 ppm malathion, they exhibited curved bodies and unusual behaviors including loss of balance, swimming in circles, and no activity unless disturbed. All of these effects were also observed in the current study. While the exact time at which tadpole tails are sensitive to malathion and other contaminants is unknown, Snawder and Chambers (1993) found that malathion induced early developmental effects causing bent notochords in *Xenopus laevis*. Indeed, malathion effects on notochords have been noted in the first four days post fertilization (Snawder and Chambers, 1990).

The occurrence of bent tails in *Xenopus laevis* tadpoles has been attributed to three causes: genetic defect (Droin et al., 1970), poor nutrition (Xenopus I, Inc., pers. com.), or environmental contaminants (e.g., Snawder and Chambers, 1993). The fact that the bent tail morphology occurred almost exclusively in the high dose malathion treatment group precludes genetics and nutrition alone as causative factors. However, it is possible that the malathion allowed expression of an otherwise masked heterozygote recessive condition or that the malathion altered foraging or digestion. Future experiments should examine the mechanism through which malathion induces the bent tail morphology.

This study found no effect of malathion on developmental progression in *Xenopus* tadpoles. In contrast, Fordham et al. (2001) found that 28 days of exposure to 1.0 mg/L malathion caused significant delays in bullfrog (*Rana catesbeiana*) development. This species difference in response to malathion is likely due to the accelerated progression of development in *X. laevis* compared to *R. catesbeiana*. Complete metamorphosis in *X. laevis* is less than 2.5 months compared to 2–3 years in *R. catesbeiana* (Carolina Biological Supply Company, 1993). The relatively fast developmental progression of *Xenopus laevis* either precluded or masked any subtle effects of malathion on developmental stage.

The malathion concentrations used in this study (1.0 ng/L, 1.0 µg/L, and 1.0 mg/L) were based on realistic environmental concentrations. Several previously recorded concentrations include 0.008–0.012 µg/L in rivers flowing into the Chesapeake Bay, up to 0.16 µg/L in an urban stream, and up to 15 µg/L in a Colorado wetland (Fordham et al., 2001). Indeed, Fordham et al. suggest that there could easily be higher concentrations in small ponds or even puddles where the water is stagnant and amphibians prefer to breed; however, concentrations of malathion are typically not measured or reported in such habitats.

Future studies should examine the influence of the timing of malathion exposure to the onset of bent tails and aberrant behavior. Tadpoles are undergoing dramatic metamorphic changes, so they may be more sensitive in some stages than in others. Indeed, exposing tadpoles from egg through metamorphosis would provide even more applicable information about the effects of malathion on amphibian development. Also, malathion concentrations are not constant in the environment, as malathion is typically sprayed several times in one area, exposing all different stages of tadpoles. Future studies should also focus on the possible influence of malathion on rates of metamorphosis in various amphibian species. In addition, malathion is rarely the only chemical found in surface water or ground water. It may be reacting with another chemical (or UV radiation) that is normally harmless; the combination may be extremely detrimental once it gets inside the organism. Future research should examine such potentially detrimental combinations.

Acknowledgements. We would like to thank Debbie Threadgill for help with obtaining supplies, Ben Cash and Kristi Kneas for help with experimental protocols, Jeff Bay for his extensive advice on statistical analyses, and the Maryville College Biology Department for funding this study.

Literature Cited

Alvarez, R., M.P. Honrubia, and M.P. Herraez. 1995. Skeletal malformations induced by the insecticides ZZ-Aphox and Folidol during larval development of *Rana perezi*. *Arch. Environ. Contam. Toxicol.* **28**:349–356.

Blaustein, A.R., J.M. Kiesecker, D.P. Chivers, and R.G. Anthony. 1997. Ambient UV-B radiation causes deformities in amphibian embryos. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 13735–13737.

Caballero de Castro, A.C., E.A. Rosenbaum, and A.M. Pechen de D'Angelo. 1991. Effect of malathion on *Bufo arenarum* Hensel development--I. Esterase inhibition and recovery. *Biochem. Pharmacol.* **41**:491–495.

Carolina Biological Supply Company. 1993. *Reptiles and amphibians: Care and culture.* Burlington, NC: Carolina Biological Supply.

Droin, A., V. Uelinger, and J. Reynaud. 1970. A recessive lethal mutation, "bt" (bent tail) in *Xenopus laevis*, Daudin. *Rev. Suisse Zool.* **77**:596–603.

Ecobichon, D.J. 2001. Toxic effects of pesticides. *In*: C.D. Klaassen, ed. *Casarett & Doull's toxicology: The basic science of poisons* (6th ed). New York: McGraw-Hill, 2001, pp. 763–811.

Eggen, R.L., R. Behra, P. Burkhardt-Holm, B. Escher, and N. Schweigert. 2004. Challenges in ecotoxicology. *Environ. Sci. Technol.* **38**(3):58A-64A.

Environmental Protection Agency. 2004. 2004 Edition of the Drinking Water Standards and Health Advisories. EPA 822-R-04–005. Office of Water, Washington, D.C.

Fordham, C.L., J.D. Tessari, H.S. Ramsdell, and T.J. Keefe. 2001. Effects of malathion on survival, growth, development, and equilibrium posture of bullfrog tadpoles (*Rana catesbeiana*). *Environ. Toxicol. Chem.* **20**:179–184.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**:183–190.

Hayes, T.B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A.A. Stuart, and A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. U.S.A.* **99**:5476–5480.

Howard, P.H. 1991. Handbook of environmental fate and exposure data for organic chemicals. Chelsea, Michigan: Lewis Publishers, pp. 473–485.

Johnson, P.T., K.B. Lunde, E.G. Ritchie, A.E. Launer. 1999. The effect of trematode infection on amphibian limb development and survivorship. *Science* **284**:802–804.

Kiesecker, J.M. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proc. Natl. Acad. Sci. U.S.A.* **99**: 9900–9904.

Pawar, K.R., H.V. Ghate, and M. Katdare. 1983. Effect of malathion on embryonic development of the frog *Microhyla ornate* (Dumeril and Bibron). *Bull. Environ. Contam. Toxicol.* **31**:170–176.

Pough, F.H., R.M. Andrews, J.E. Cadle, M.L. Crump, A.H. Savitzky, and K.D. Wells. 1998. *Herpetology*. Upper Saddle River, NJ: Prentice Hall.

Snawder, J.E., and J.E. Chambers. 1990. Critical time periods and the effect of tryptophan in malathion-induced developmental defects in *Xenopus laevis*. *Life Sci.* **46**:1635–1642.

Snawder, J.E., and J.E. Chambers. 1993. Osteolathyrogenic effects of malathion in *Xenopus* embryos. *Toxicol. Appl. Pharmacol.* **121**:210–216.

Sparling, D.W., C.A. Bishop, and G. Linder. 2000. The current status of amphibian and ecotoxicological research. *In*: D.W. Sparling, G. Linder, and C.A. Bishop, eds. *Ecotoxicology of amphibians and reptiles*. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC), 2000, pp. 1–14.

Taylor, S.K., E.S. Williams, and K.W. Mills. 1999. Effects of malathion on disease susceptibility in Woodhouse's toads. *J. Wildl. Dis.* **35**:536–541.

Received 19 August 2004; accepted 15 March 2005.