Turfgrass and Environmental Research Online

…Using Science to Benefit Golf

Researchers at Frostburg State University have investigated how golf courses can be designed and managed to reduce impacts on amphibian populations.

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PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 215 projects at a cost of $21 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

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Golf Course Design and Maintenance: Impacts on Amphibians

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SUMMARY

Golfing as a recreational enterprise has grown dramatically in recent years and has spawned a nation-wide proliferation of new courses. In light of this phenomenon, the industry is facing increased pressure to make development and maintenance of golf courses more sensitive to conservation issues. Concurrent with golf course expansion, one of the most alarming revelations in the conservation community in recent years has been the reports of worldwide declines in amphibian populations. Numerous investigators have observed mortality of amphibian larvae in natural populations associated with pesticide application. Increased mortality due to direct or indirect effects of pesticides in successive years may eventually result in loss of entire populations over time.

In our laboratory investigations, amphibian species exhibited reduced survival and increased time to metamorphosis with higher concentrations of many pesticides. However, there are very dramatic differences in pesticide toxicity. Many of the most toxic compounds are used during the breeding season and although there are differences among species in sensitivity, all species showed similar patterns of effect. At lower concentrations, mortality is often not the direct effect but rather we observed decreased hatching rates, slower growth rates and longer times to metamorphosis. Over many years, all of these more subtle effects can be more damaging to the persistence of amphibian populations than one large mortality event. Managers should have the data available to apply chemical treatments responsibly to reduce these hazards. That data should include information on the relative toxicity of the compounds, the persistence of those chemicals and the life stage that is most sensitive to treatments.

Some compounds appear to penetrate the jelly layers in amphibian eggs more readily than others, or are more toxic and directly impact egg hatching. Some compounds that have little detectable effect on eggs can have dramatic effects on larval growth at low concentrations.

The world-wide decline in many amphibian species has captured global attention and sparked controversy regarding the causes for the observed collapse of some populations. Some investigators have suggested that amphibian disappearances are linked to contaminates in the environment as well as the loss of wetland habitats. The creation of new wetland habitat offers an opportunity to reverse at least one of the potential causes associated with these declines.

Golf course wetlands have the potential to function as mini-preserves that can support rarer species of amphibians that have lost valuable wetland habitat elsewhere. There is an inherent danger to many of these populations if constructed wetlands are not thoughtfully developed and maintained. If species are attracted to areas of abundant water but such areas are not suitable for the maintenance of populations then the wetlands can act as population sinks further depressing the prospect of long-term survival of the species in question.

Our research focused on two major objectives. One was to design wetlands with characteristics that would create suitable breeding habitat for many rarer species of amphibians but that would restrict the establishment of aquatic predators. The second objective, and the focus of this paper, was to examine the relative toxicity of compounds commonly used in golf course maintenance to deal with turf grass pests.

Specifically we intended to: 1) develop a more complete and biologically realistic testing protocol that includes multiple species, both acute and chronic trials, multiple life history stages, multiple indicators of biological impact and an environment that provides an opportunity to detoxify or potentiate chemicals; and, 2) test the relative toxicity of several commonly used pesticides (insecticides, herbicides and fungicides). Data from such investigations would allow golf course managers to choose products less likely to endanger sensitive amphibian species and judge the timing of application to further reduce risks of impact.

The concern over amphibian declines has

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stimulated investigations on the effects of pesticides on amphibians. Application of pesticides on agricultural fields and recreational areas is heaviest in spring and summer when breeding and crucial stages of larval development occur (25). Pesticides sprayed on crops or turf accumulate in temporary pools due to surface runoff or sediment transport and may result in high concentrations in water (2, 24).

Because amphibians have skin that is highly permeable, they may be especially sensitive to environmental contamination. Their semi-aquatic lifestyle and dependence on water leaves them vulnerable to both soil and water contaminants (4). Furthermore, many larval amphibians are detritus feeders and may ingest chemicals in the substrate. Indirect effects of environmental pollution may leave amphibians more susceptible to disease, predation, and death from habitat desiccation (9, 12, 16), and may threaten the persistence of amphibian populations. Although information is available on acute toxicity of many pesticides to amphibians (19, 20, 32), the effects of low doses over longer periods of time is less well known.

The chemicals we investigated were chosen because they are commonly used on golf courses (Darin Bevard, USGA Greens Section, pers. com.) and may impact surface water in surrounding areas (30). As expected environmental concentrations and reported values of pesticides in the field are frequently well below concentrations found to be lethal in acute laboratory tests, the concentrations of pesticide we used were intended to demonstrate effects of prolonged exposure to low doses (30, 36). In addition, we added sediment to our aquaria to provide a natural route for detoxification. We anticipated, based on previous results by Berrill et al. (2), that differences in susceptibility to pesticides would exist among species tested. Thus, we selected species from different families to better represent amphibian responses.

MATERIALS AND METHODS

Prior to beginning any trials we conducted 48 hr range-finding LC_{50}s (concentration needed to kill 50% of the tadpoles in the test) to determine the relative toxicity of each pesticide we investigated. For each pesticide, 10 tadpoles were placed in each of five tanks containing different concentrations of that pesticide, and a crude LC_{50} was determined with probit analysis (11) after 48 hours. The LC_{50} was used only to estimate the three concentrations (high, medium, and low) used in the following experiments. Subsequent trials primarily used four concentrations of pesticide; a control, 0.01 X LC_{50}, 0.1 X LC_{50}, and 0.5 X LC_{50}. Because of the shape of the dose response curve in the insecticide trials, herbicide and fungicide egg hatching trials used controls, 0.1 X LC_{50}, 0.25 X LC_{50}, and 0.5 X LC_{50} concentrations. Each pesticide was evaluated in two separate experiments: an egg-hatching experiment and a larval-growth experiment.

Pesticides were applied in commercial formulations. Insecticides tested were; Sevin (41.2% carbaryl active ingredient), Dursban (22% chlorpyrifos active ingredient), and Merit (75% imidacloprid active ingredient). Fungicides tested were; Daconil Ultrex (82% chlorothalonil active ingredient), Chipco Alliette Signature (80% fosetyl-Al active ingredient), and Fore (80% mancozeb active ingredient). The herbicides tested were; Trimec Classic Broadleaf (43% dimethylamine salt active ingredient), Roundup Weed and Grass Killer (18% glyphosate active ingredient), and Barricade 65W (65% prodiamine active ingredient). Pesticide formulations were mixed with 15ml of water immediately prior to dosing.

Egg hatching trials

Eggs were either field collected (recently fertilized) or stripped from hormone-injected females ordered from a commercial supplier (Carolina Biological Supply). The egg masses from each species were divided into portions and randomly assigned to each of the dosage groups such that eggs in each treatment were represented by multiple females. All eggs were counted and added to tanks at approximately Gosner (15) stage 9, late cleavage.

Each egg mass was placed in 8 liters of dechlorinated tap water containing pesticide in one of thirty-six (72 in herbicide and fungicide trials) 15-liter aquaria. Prior to pesticide addition, 250g of sediment from an uncontaminated site where amphibians were successfully reproducing was added to each aquarium. Egg masses rested naturally upon the bottom of the aquaria such that approximately 25-50% of the egg mass was in contact with the surface of the sediment during the trial. Water temperature was 16±1 °C, and tanks received 12 hours of light per day.

Effects of three pesticides were tested simultaneously on one species in an experimental block design with four dosages (control, low, medium and high) of each pesticide and three replicates of each dosage in the insecticide trial (six replicates of each dosage in the fungicide and herbicide trials). Pesticide was not reapplied during the egg trials.

Experiments were terminated when most larvae had hatched out of the egg mass but were not yet feeding (Gosner, stage 23-24). The duration of experiments was from 9 days to 13 days depending on the species tested. Hatching rates were determined by removing the larvae and counting the number of larvae until a consensus count was reached. The larvae and remaining egg mass were preserved for later examination under a dissecting microscope. The total number and types of deformities among preserved larvae were recorded.

To examine differences among treatment groups, analysis of variance was performed on the proportion of larvae that hatched in each treatment and the proportion of hatchlings that were deformed. To estimate change in hatching success with increasing concentration of pesticide, percent eggs hatched in different treatments was examined with a binary logistic regression model. Asymptotic F tests were used to test for a significant positive or negative linear slope (33, 38).

Larval development and survival trials

In each experimental trial, ten larvae were placed in a 60-liter aquarium and maintained until metamorphosis. With forty-eight 60-liter aquaria, we were able to evaluate the effects of three pesticides simultaneously on one species. Anuran larvae were maintained on a ration of tadpole chow from a commercial supplier. Tanks were filtered through fiberglass twice per week, and all tanks received light 12 hours/day. Water temperature of all tanks was 21± 1 °C, pH was 6.9±1, and water conductivity was 0.15 mS.

The basic design of the experiment was repeated for each species. For each pesticide tested, we used four control tanks and 12 tanks containing pesticide. Four tanks contained pesticide at high concentration (0.5 X LC_{50}), and four tanks each at medium (0.1 X LC_{50}) and low (0.01 X LC_{50}) concentrations. Tanks were arranged in a randomized block design so that each concentration of each pesticide was represented in a block.

To simulate the repeated application of chemicals and subsequent runoff into wetlands (25), pesticide dosages were added at the beginning of the trial and again at two-week intervals. To prevent an increase in concentrations of persistent chemicals over time with each dosage, 75% of the water in each aquarium was removed and replaced with fresh dechlorinated water containing the same dose of pesticide. In addition, because some pesticides are absorbed by soil particles and may not be bioavailable (20), approximately 1 kg of uncontaminated sediment from a site where amphibians were successfully reproducing was added to each aquarium.

Data on tadpole survival, growth, time to metamorphosis, and development were collected during the trial. Tadpoles were observed daily for developmental abnormalities and mortality. Tadpole carcasses were removed upon discovery, and missing tadpoles were presumed dead. Wet weight of tadpoles was recorded at two weeks post-hatching (upon addition to tanks) and again at approximate Gosner (15) stage 37-39 (hind limbs with fully developed toes present). Time to metamorphosis was recorded as the number of days from first pesticide treatment to appearance of front limbs (Gosner, stage 42).

Survival of tadpoles and number of tadpoles exhibiting developmental abnormalities were examined using chi square analysis. When chi-square analysis showed that significant differences were present among treatments, subdivision of the chi square (39) was used to determine which treatments were different. Analysis of variance was used to compare average growth per tadpole and time to metamorphosis among treatments.

Water and sediment sample analysis

To validate concentrations of insecticides in each tank and to determine degradation rate, water samples were sent for analysis to the aquatic toxicology labs at The Institute of Wildlife and Environmental Toxicology (TIWET) and the Mississippi State Chemical Laboratory. Random water samples were taken three times (after the initial dosage, the second dosage, and termination of the experiment) for validation of insecticide concentrations in tanks, and intermittently during a two-week period for degradation information. Samples were taken at the end of the trial to determine the concentration of insecticide remaining in the sediment. For fungicides and herbicides water samples were sent to the University of Guelph Laboratory Services aquatic toxicology laboratory for blind analysis. Water samples were taken 24 hours after the first dosage.

RESULTS

Egg hatching trials-Insecticides

Hatching success (determined as percent eggs that hatched) of all species is expressed in (Table 1). Although fewer eggs of all species hatched in high concentrations of carbaryl, analysis of variance indicated no significant differences in percent eggs hatched from *R. pipiens*, *P. triseriata* and *B. americanus* egg masses in any treatment combinations. In *A. jeffersonianum* however, percent eggs hatching in high concentrations (1.6%) of carbaryl was significantly lower than percent eggs hatched in all other treatment groups ($F_{11,24}$=5.65, $p<0.05$).

Logistic regressions of hatching success versus pesticide concentration indicated that all four species responded similarly to increasing pesticide concentration. All species exhibited a significant decrease in hatching success with increasing dosage of carbaryl ($F_{1,39}$=26.07, $p<0.05$), however, no relationship between hatching success and dosage was evident with treatment of chlorpyrifos and imidacloprid. Relationship of hatching success to increasing dosage of carbaryl is plotted in Figure 1.

Abnormalities were observed in three regions; axial skeleton or notochord, tailblade, and gut. The most frequent malformation of the notochord or axial skeleton was a kinked tail. Gut malformations included curvature of the anal tube, gut rotation and edema. In all species, the average percent hatchlings with deformities (over all treatments) was below 12%. *Pseudacris triseriata* (11.3%) and *R. pipiens* (10.5%) exhibited more abnormal tadpoles over all treatment groups than *A. jeffersonianum* (7.0%) and *B. americanus* (3.9%). Analysis of variance performed on the total percent of hatchlings exhibiting a deformity revealed no significant differences between control and pesticide treatment groups.

Larval survival and growth-Insecticides

The LC_{50}s for three species of amphibians for the three insecticides tested is summarized in Table 2. Survival of tadpoles in all treatments is contained in Table 3. Chi square analysis indicated a significant ($p<0.05$) treatment effect on survival of all species for each pesticide (carbaryl: *Bufo americanus* χ^2_{3df}=145.37, *Rana sphenocephala* χ^2_{3df}=88.8, *Pseudacris triseriata* χ^2_{3df}=149.92, chlorpyrifos: *B. americanus* χ^2_{3df} =154.88, *R. sphenocephala* χ^2_{3df}=149.93, *P. triseriata* χ^2_{3df}=103.47, imidacloprid: *B. americanus* χ^2_{3df}=150.19, *R. sphenocephala* χ^2_{3df}=55.34, *P. triseriata* χ^2_{3df}=154.88). No tadpoles of any species survived to metamorphosis in high concentrations of carbaryl. Only 15% of *P. triseriata* tadpoles and 5% of *R. sphenocephala* tadpoles survived in high concentrations of chlorpyrifos. All *B. americanus* tadpoles in high

Insecticide	Concentration	Percent eggs hatched (standard error is in parentheses)			
		R. pipiens	*P. triseriata*	*B. americanus*	*A. jeffersonianum*
	sample size (# of aquaria in trial)	36	24	36	36
Carbaryl	control	42.50 (18.0)	85.88 (6.8)	92.33 (2.9)	65.44 (9.9)
	low	43.48 (20.5)	90.12 (0.9)	93.2 (1.3)	57.84 (10.8)
	medium	62.71 (25.4)	85.04 (4.1)	96.38 (1.3)	43.12 (3.6)
	high	17.23 (4.4)	60.09 (26.8)	54.84 (28.7)	1.61 (0.9)**
Chlorpyrifos	control	65.6 (25.6)	79.34 (2.1)	96.83 (2.1)	53.33 (5.8)
	low	54.70 (15.4)	77.64 (5.3)	98.9 (0.6)	54.99 (2.2)
	medium	41.83 (7.8)	83.34 (7.2)	97.4 (1.4)	45.73 (9.9)
	high	57.30 (7.8)	80.76 (4.0)	95.93 (1.5)	59.07 (8.4)
Imidacloprid	control	69.82 (15.9)	82.88 (4.3)	96.03 (0.6)	59.5 (5.1)
	low	48.66 (0.9)	79.59 (12.9)	98.52 (0.9)	53.03 (5.2)
	medium	47.36 (13.4)	74.36 (9.4)	95.72 (2.1)	50.2 (13.6)
	high	51.32 (5.9)	83.08 (5.0)	96.27 (1.6)	59.63 (7.6)
	** indicates significant differences				

Table 1. Effects of carbaryl, chlorpyrifos, and imidacloprid on hatching success of four species.

	Pesticide		
Species	Imidacloprid	Carbaryl	Chlorpyrifos
Rana berlandieri	184,500	51,581	1,125
Pseudacris triseriata	388,500	58,075	1,125
Bufo americanus	468,000	63,167	1,316

Table 2. Estimated LC_{50}s (ug/L) for all species.

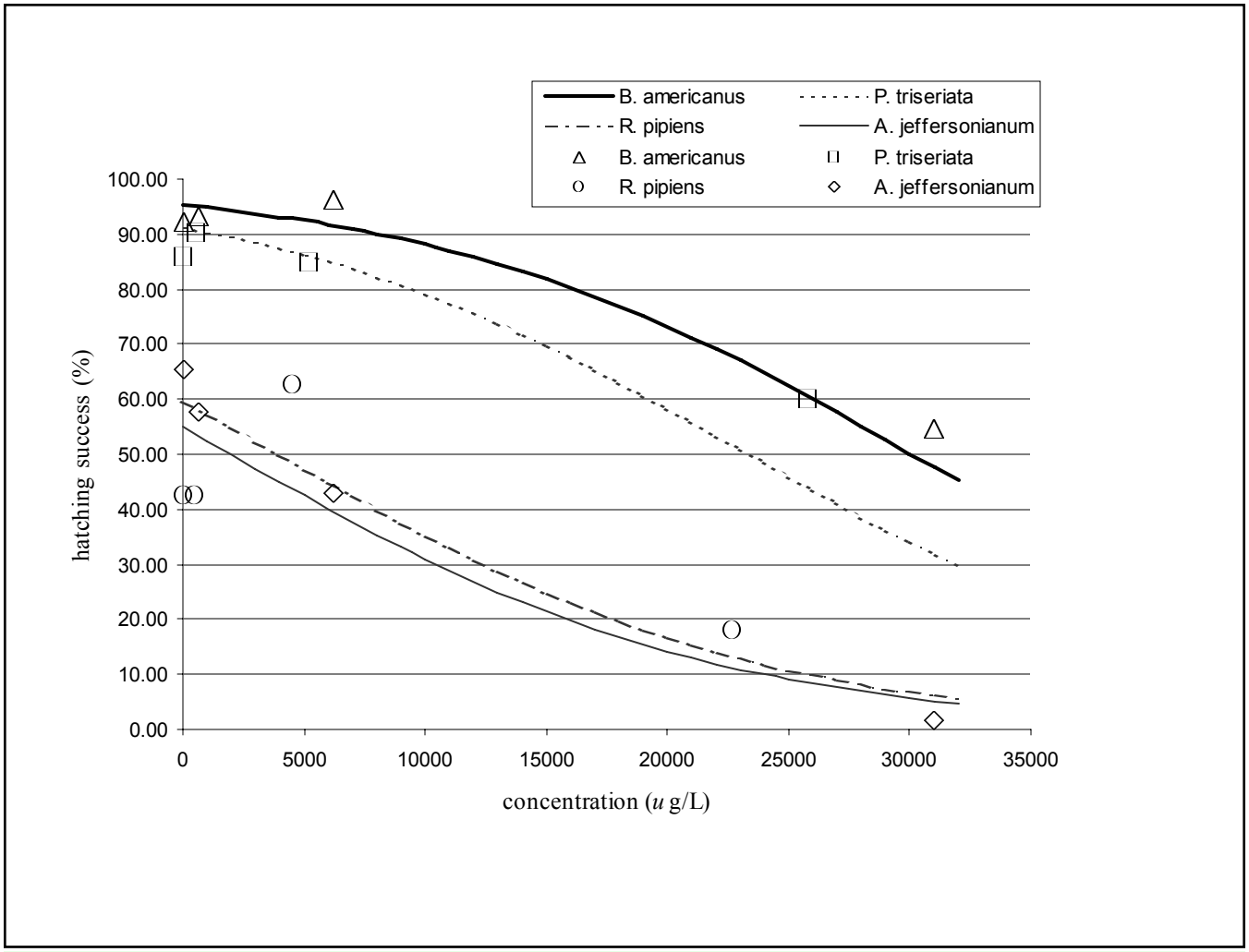

Figure 1. Relationship of hatching success to increasing dosage of carbaryl.

Pesticide	Concentration	Total surviving tadpoles		
		P. triseriata	*B. americanus*	*R. sphenocephala*
Carbaryl	control	39	39	38
	0.01xLC_{50}	40	40	30
	0.1xLC_{50}	39	38	30
	0.5xLC_{50}	0*	0*	0 *
Chlorpyrifos	control	38	40	40
	0.01xLC_{50}	40	40	36
	0.1xLC_{50}	36	39	39
	0.5xLC_{50}	6*	0*	2 *
Imidacloprid	control	40	40	37
	0.01xLC_{50}	40	40	36
	0.1xLC_{50}	39	38	37
	0.5xLC_{50}	0*	0*	14 *
* indicates significant difference from controls				

Table 3. Total number of tadpoles surviving to metamorphosis.

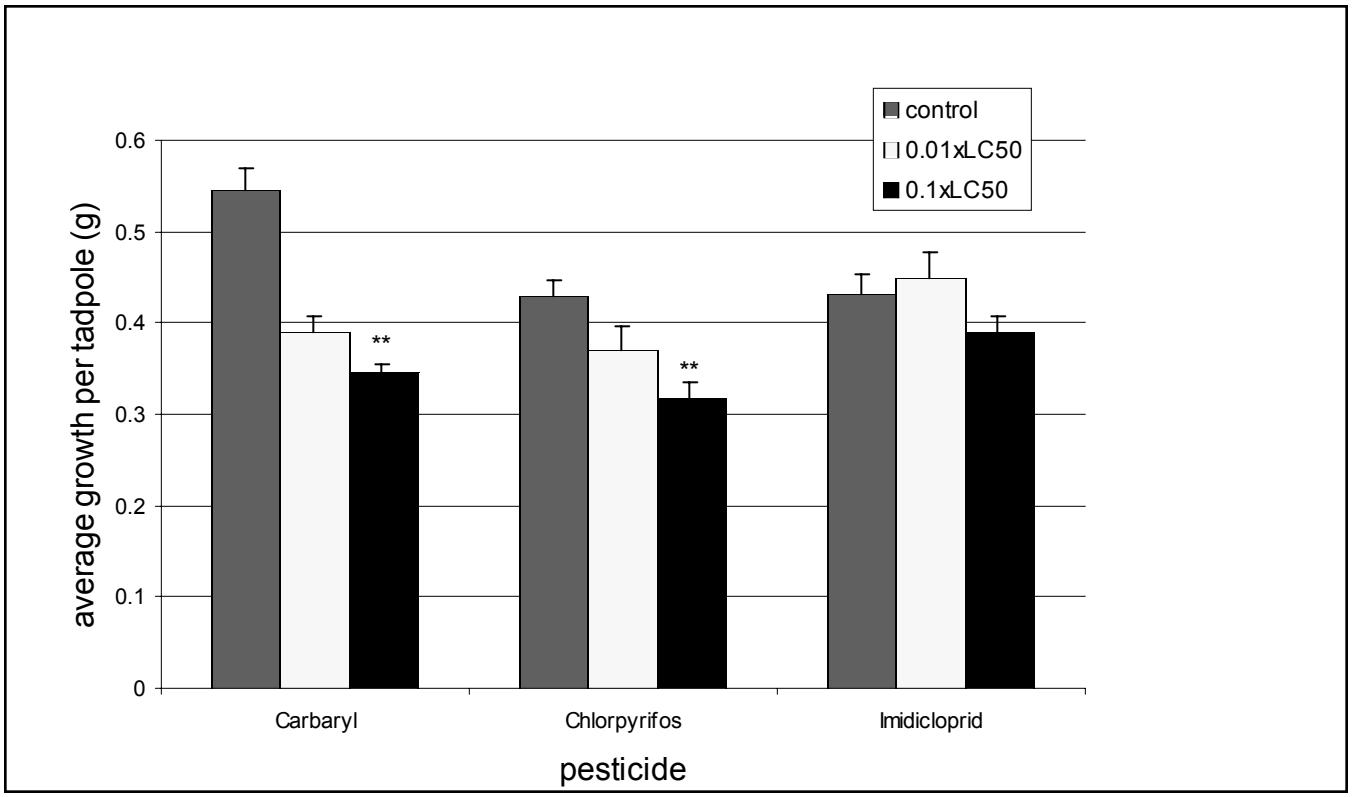

Figure 2. Average growth (g) of *B. americanus* tadpoles during pesticide treatment (** indicates significant differences from controls, error bars represent standard error).

chlorpyrifos concentrations died prior to metamorphosis. In high concentrations of imidacloprid, 35% of *R. sphenocephala* tadpoles survived to metamorphosis, however mortality of *P. triseriata* and *B. americanus* tadpoles was 100%. Average survival for all species at all other concentrations was 90% or above. Because of significant mortality at high ($0.5xLC_{50}$) concentrations, subsequent analyses were performed only on medium ($0.1xLC_{50}$), low ($0.01xLC_{50}$) concentrations, and controls.

Pseudacris triseriata tadpoles grew an average of 0.432 g from two weeks post hatching to Gosner (15) stage 37-39 , however analysis of variance revealed no significant differences in average growth between medium, low, and control treatments. Average growth of *Bufo americanus* tadpoles in control treatments was significantly higher ($F_{8,27}=5.28$, $p<0.05$) than average growth of tadpoles in medium ($0.1xLC_{50}$) concentrations of carbaryl and chlorpyrifos (Figure 2).

No growth differences in *B. americanus* tadpoles were observed between control and medium ($0.1xLC_{50}$) concentrations of imidacloprid. Growth of *R. sphenocephala* tadpoles was significantly decreased ($F_{2,24}=7.51$, $p<0.05$) by chronic exposure to $0.1xLC_{50}$ concentrations of all pesticides (Figure 3).

All pesticides had a similar effect on average days to metamorphosis of tadpoles. *Pseudacris triseriata* and *R. sphenocephala* tadpoles in medium concentrations of pesticide took an average of approximately two days longer to reach metamorphosis when compared with controls, and *B. americanus* tadpoles took an average of nearly three days longer than controls (Table 4). Analysis of variance revealed that time to metamorphosis was significantly ($p<0.05$) increased by exposure to medium concentrations of pesticide (*P. triseriata* $F_{2,24}=7.05$, *B. americanus* $F_{2,24}=8.75$, *R. sphenocephala* $F_{2,24}=16.58$).

Two *P. triseriata* tadpoles developed stunted and malformed limbs. This was observed in two tanks, both containing chlorpyrifos at a concentration of $0.5xLC_{50}$ concentration. No developmental abnormalities were observed among *B. americanus* larvae. No statistical analysis was performed on these species because of the low frequency of abnormalities.

Unlike other larval trials, a small proportion of Ranid larvae exhibited developmental abnormalities. All abnormalities observed consisted of lateral tail kinks at the base of the tail that progressed from slight to severe over several weeks. A small curvature of the spine remained in these frogs after tail resorption, but did not appear to affect movement. Low ($0.01xLC_{50}$) concentrations for all pesticides produced the greatest number of these deformities (average 13%) compared to an average of 4% for control tadpoles.

Chi square analysis did not reveal significant differences ($p>0.05$) between control tadpoles and medium or low concentrations of any pesticide. In addition, one frog in a low concentration of imidacloprid developed an extra set of hind limbs during metamorphosis.

Water and sediment sample analysis-Insecticides

Results of water sample analysis indicate that imidacloprid may be much more persistent in the water column than carbaryl or chlorpyrifos (Table 5a). After 24 hours, only about 50% of carbaryl and 29% of chlorpyrifos remained in the water, however, approximately 92% of imidacloprid was detectable. After 72 hours, carbaryl levels were undetectable and only 6% of chlorpyrifos remained, however, 25% of imidacloprid remained. After two weeks 12% of imidacloprid still remained in the water, whereas less than 1% of chlorpyrifos remained and carbaryl levels were undetectable.

Sediment samples also indicate differences in persistence of the three pesticides in the soil (Table 5b). At the termination of the experiment (3-4 weeks after the last pesticide renewal), levels of imidacloprid and chlorpyrifos were approximately equal to or greater than the concentration of pesticide added at each renewal, whereas only 1-10% of carbaryl remained in the sediment. Chlorpyrifos appeared to be most persistent with values averaging five times that added

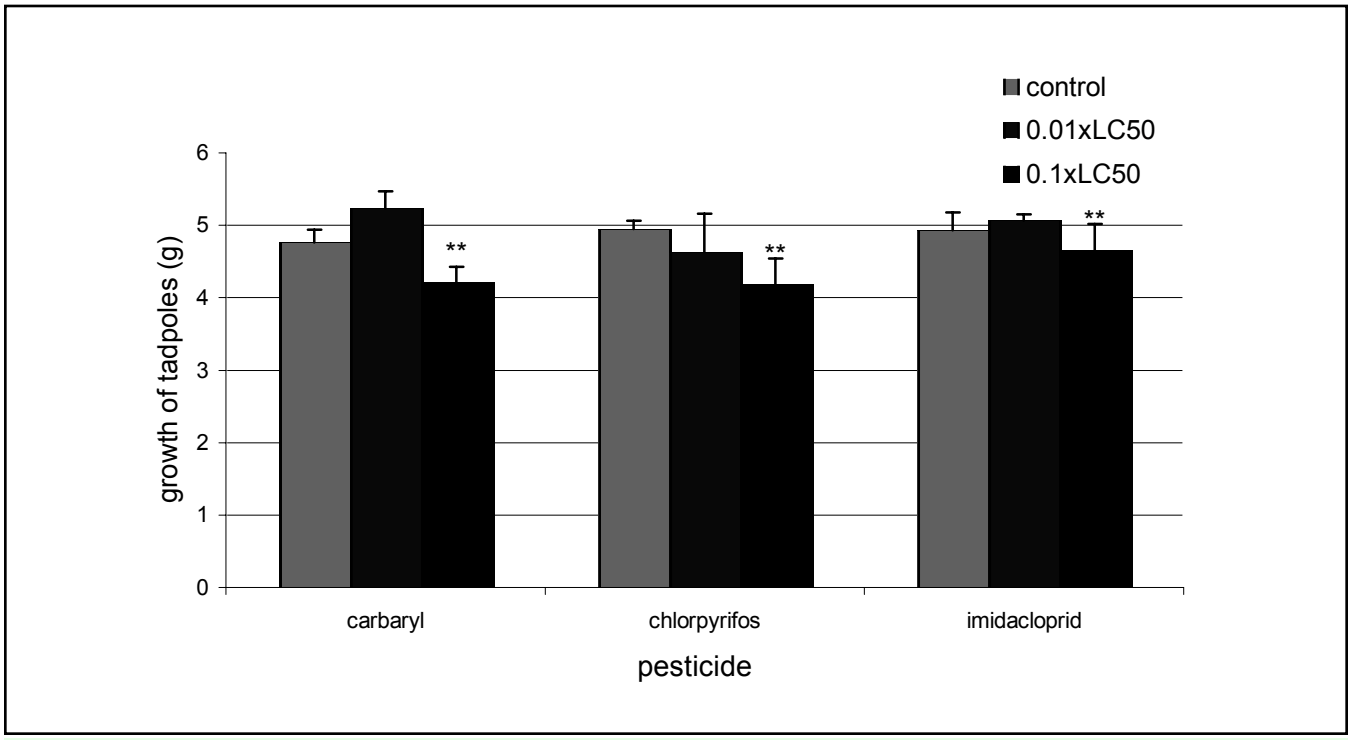

Figure 3. Average growth (g) of *R. sphenocephala* tadpoles in treatments (** indicates significant difference from controls; error bars represent standard error).

Pesticide	Concentration	Average days to metamorphosis		
		P. triseriata	*B. americanus*	*R. sphenocephala*
carbaryl	control	36.11 (0.58)	36.15 (1.08)	54.25 (1.76)
	$0.01xLC_{50}$	36.95 (0.48)	37.25 (0.97)	52.05 (1.43)
	$0.1xLC_{50}$	38.41 (1.16) *	39.89 (0.64) *	56.4 (1.24) *
chlorpyrifos	control	35.62 (0.17)	35.08 (0.75)	54.07 (1.21)
	$0.01xLC_{50}$	38.55 (0.97)	36.67 (0.95)	53.89 (2.89)
	$0.1xLC_{50}$	39.74 (1.37) *	38.65 (1.28) *	56.03 (1.83) *
imidacloprid	control	36.61 (1.11)	36.22 (0.45)	52.81 (1.42)
	$0.01xLC_{50}$	35.33 (0.74)	35.05 0.96)	52.74 (1.45)
	$0.1xLC_{50}$	37.22 (0.54) *	37.85 (0.67) *	55.24 (2.13) *

*Indicates significant difference from controls

Table 4. Average days to metamorphosis of *P. triseriata*, *B. americanus*, and *R. sphenocephala* tadpoles. Standard error is in parentheses.

Pesticide	Treatment	Initial Concentration*	Time of sampling 24hr	72hr	1week	2week
carbaryl	low	830	176[a]	nd[a]	nd[a]	nd[a]
chlorpyrifos	medium	112	24[s]	6.7[s]	1.7[s]	1.1[s]
imidacloprid	high	92,250	75,380[s]	22,560[s]	20,310[s]	11,370[s]

* Estimated from amount of pesticide added

[a] Average concentrations from 2 or 3 samples

[s] Estimated concentrations from single samples

Table 5a. Pesticide levels (ug/L) in water samples during two week time period.

Pesticide	Treatment	Initial Concentration**	Ending Concentration
chlorpyrifos	high	562	589 [a]
	medium	113	318 [a]
	low	11	133 [a]
imidacloprid	high	92,250	71,000 [s]
	medium	18,450	21,500 [a]
	low	1,845	3,500 [s]
carbaryl	high	25,777	230 [s]
	medium	5,155	98 [s]
	low	515	68 [s]

** Estimated concentration of water after each renewal

[a] Average concentrations from 2 or 3 samples

[s] Estimated concentrations from single samples

Table 5b. Concentration of pesticide (ug/L) in sediment at termination of the experiment.

at each pesticide renewal. Imidacloprid levels in sediment averaged 1.25 times the pesticide concentrations added to the water column.

PRELIMINARY RESULTS

Egg hatching trials-Fungicides

LC_{50} values (parts per billion) of commercial formulations of fungicides for *Rana utricularia* were: mancozeb, 800; fosetyl-Al, 45,280; chlorothalonil, 28.

Rana hatching success was significantly different from controls in mancozeb, low (0.1 xLC50), medium (0.25xLC_{50}), and high (0.5 x LC_{50}) concentrations (Figure 4). Ambystoma hatching success was not significantly different from controls in any test concentrations for any of the fungicides. The percentages of deformities for Rana and Ambystoma hatchlings in all mancozeb concentrations were significantly different from controls (Figure 5).

Egg hatching trial-Herbicides

LC_{50} values (parts per billion) of commercial formulations of herbicides for *Rana pipiens* were: glyphosate, 20,470; prodiamine, 840,830; dimethylamine salt, 432,000.

Rana hatching success was significantly different from controls in the dimethlylamine salt, low (0.1 xLC_{50}) and high (0.5 x LC_{50}) concentrations. *Bufo* hatching success was significantly different from controls in the glyphosate and prodiamine medium (0.25 xLC_{50}) and high concentrations, and in the dimethlylamine salt high concentration (Figure 6). The percentages of deformities for *Rana* and *Bufo* hatchlings in all test concentrations were not significantly different from controls.

Fungicide Larval Trials

Commercial formulations of three fungicides containing the active ingredients mancozeb, fosetyl-al, and chlorothalonil were used to investigate effects on *Rana sylvatica* and *Hyla chrysoscelis* larval survival and development. *Rana* survival was significantly different from controls in all mancozeb concentrations (Figure 7). *Hyla* survival was significantly different from controls in all high (0.5xLC_{50}) mancozeb and fosetyl-al concentrations. *Rana* growth was significantly lower than from controls in mancozeb high and medium (0.1 x LC_{50}) concentrations, and *Hyla* growth was significantly lower in all mancozeb concentrations. Time to metamorphosis was significantly different from controls for *Rana* in high mancozeb concentrations and for *Hyla* in mancozeb high and medium concentrations.

Herbicide Larval Trials

Commercial formulations of three herbicides containing the active ingredients glyphosate, prodiamine, and dimethylamine salt were used to investigate effects on *Bufo americanus* and *Hyla chrysoscelis* larval survival and development. *Bufo* survival was significantly different from controls in prodiamine low (0.01 xLC_{50}), medium (0.1x LC_{50}) and high (0.5 x LC_{50}) concentrations (Figure 8). *Hyla* survival was significantly different from controls in prodiamine medium (0.1x LC_{50}) and high (0.5 x LC_{50}) concentrations.

No tadpoles in prodiamine high concentrations survived to metamorphosis and prodiamine high concentration treatments were excluded from the time to metamorphosis and growth data analyses. Time to metamorphosis and growth were not significantly different from controls for prodiamine low and medium concentrations, or for any of the glyphosate or dimethylamine salt test concentrations with either *Rana* or *Hyla*.

DISCUSSION

Egg hatching trials- Insecticides

Investigations concerning the effects of pesticides on egg hatching success are imperative to understanding the impacts of pesticides on amphibians. Increased mortality of amphibian eggs due to pesticides may negatively impact breeding populations over time. Differential sensitivity of species to pesticide exposure may cause changes in species composition of breeding areas,

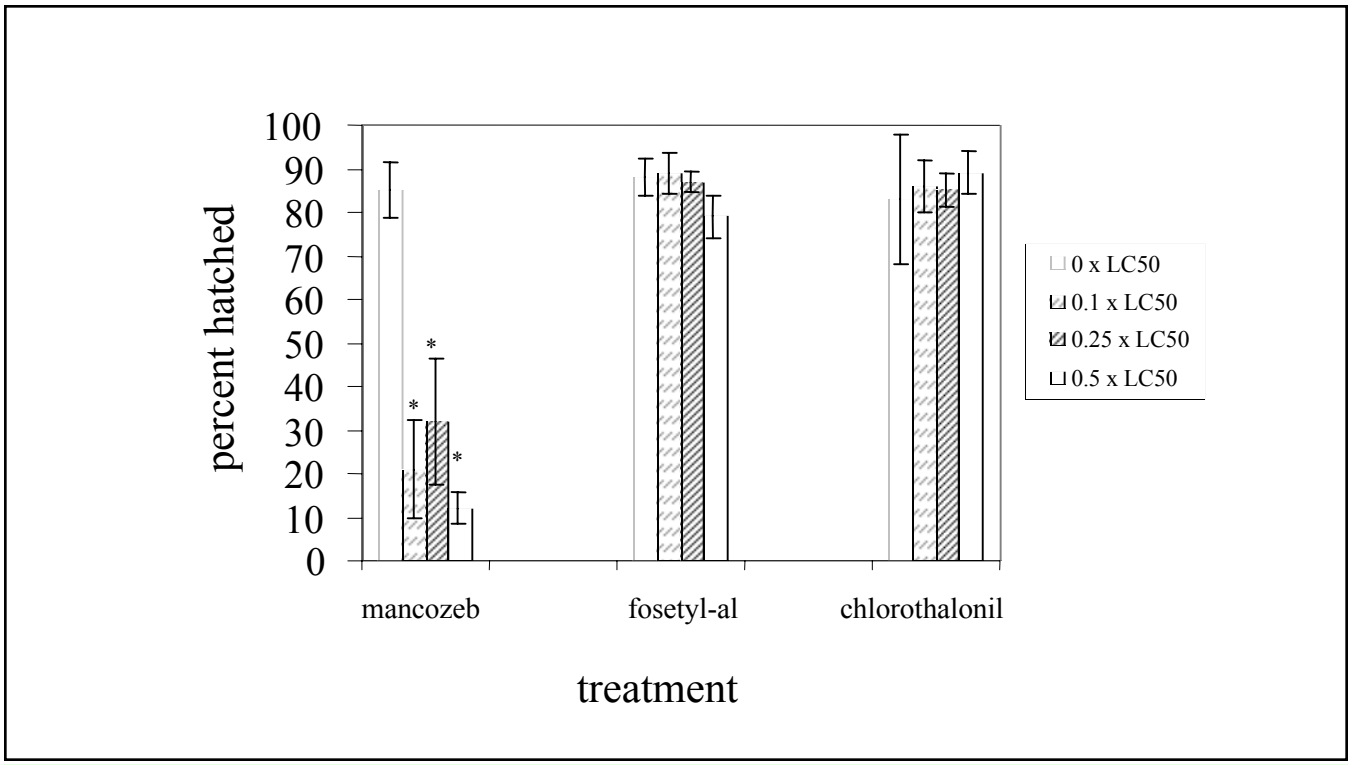

Figure 4. Average percent of *Rana* eggs hatched in all fungicide treatments.

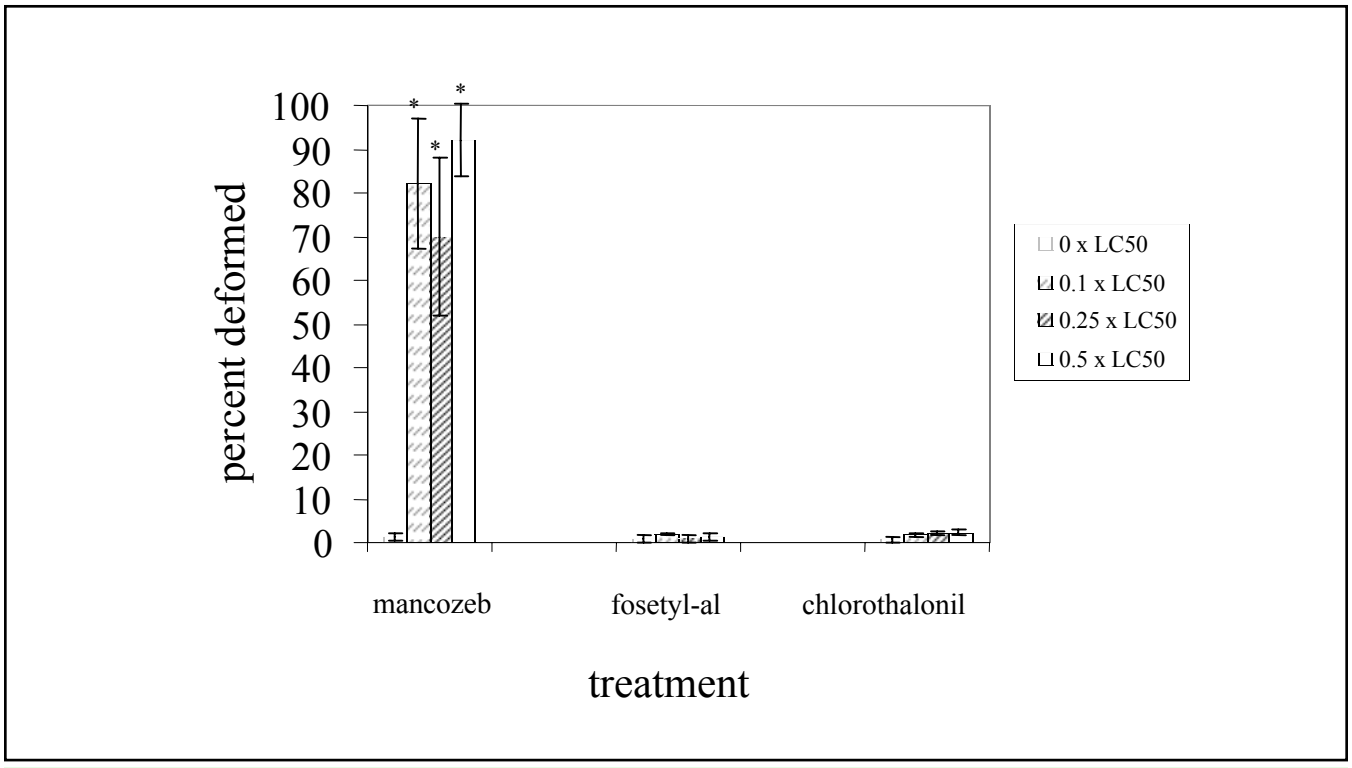

Figure 5. Average percent of *Rana* hatchlings exhibiting deformities.

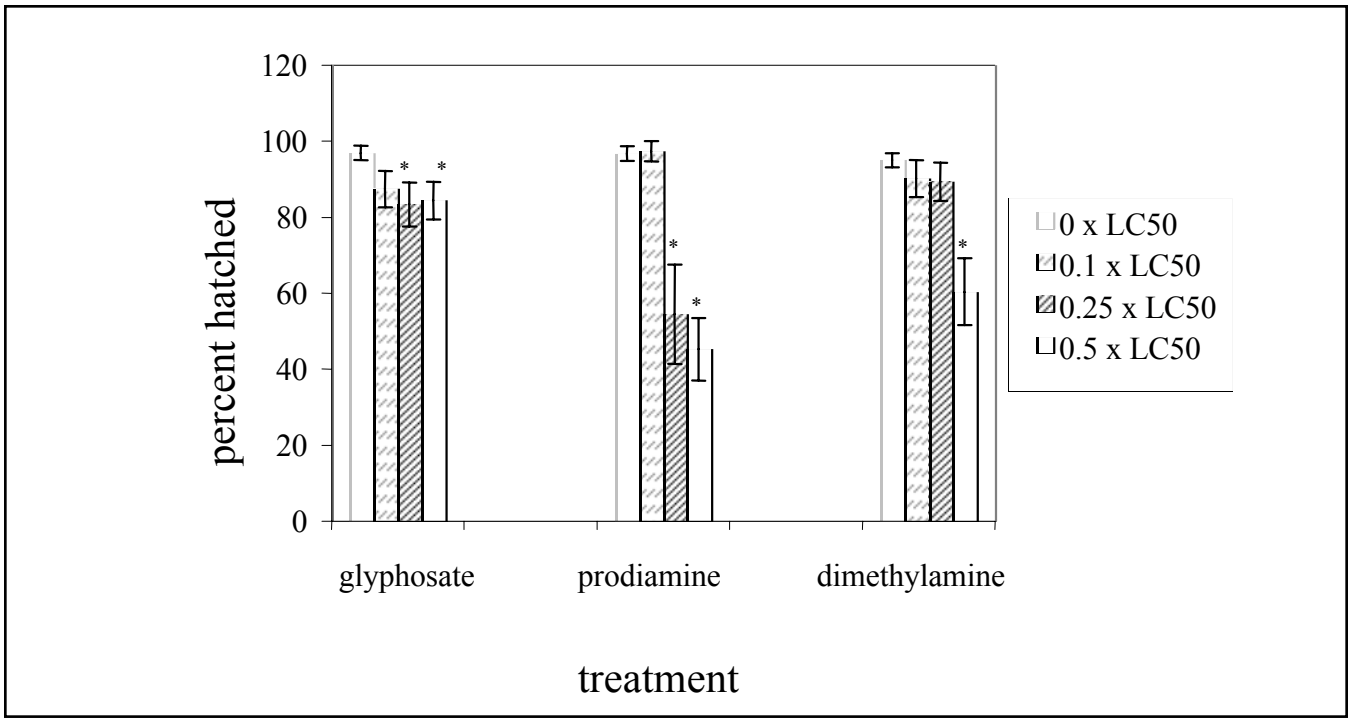

Figure 6. Average percent of *Bufo* eggs hatched in all herbicide treatments.

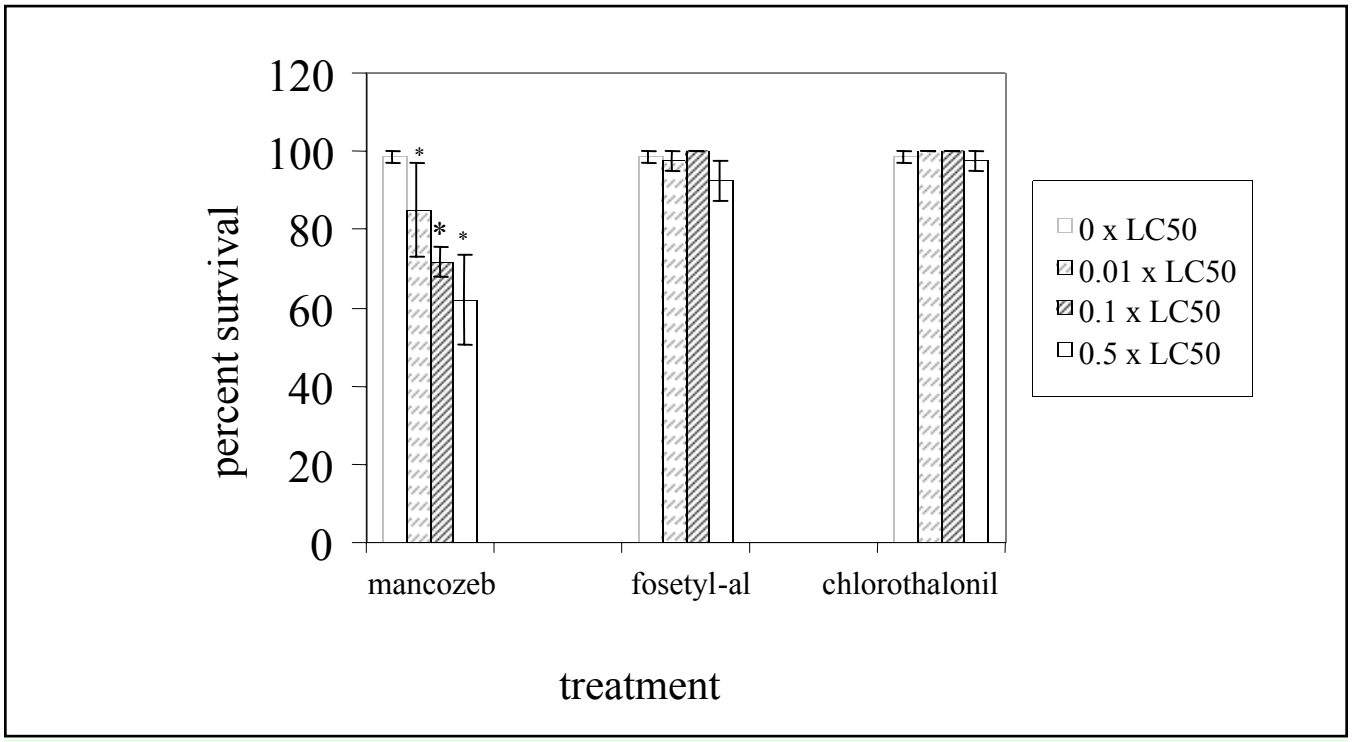

Figure 7. Percent survival of *Rana* tadpoles in fungicide treatments.

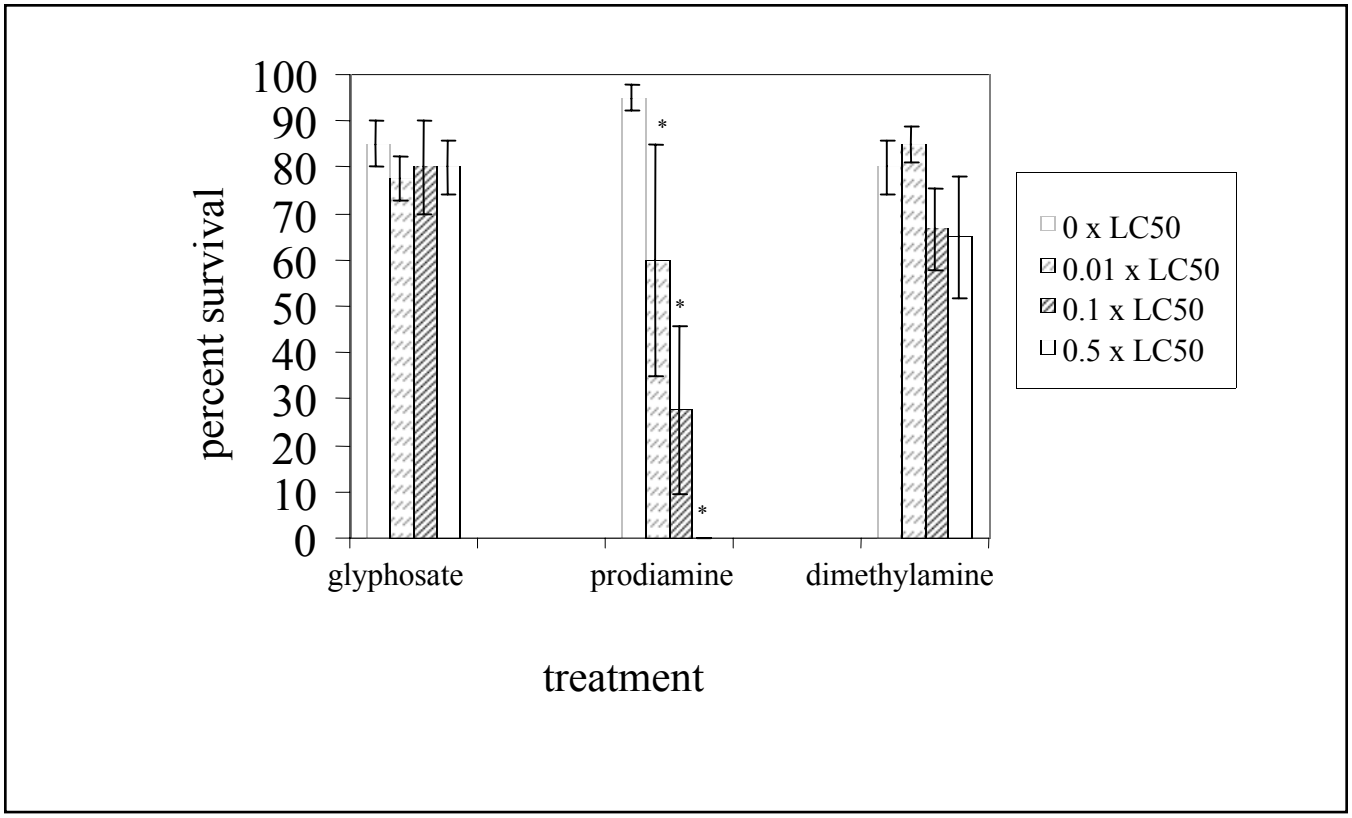

Figure 8. Percent survival of *Bufo* tadpoles in herbicide treatments.

altering interspecific interactions among larvae. This may influence growth, metamorphosis, and adult fitness of amphibians. Deformities among hatchlings may also indirectly cause pesticide-related mortality from starvation and predation. In addition, decaying eggs may foul shallow water bodies, making hatched larvae more susceptible to disease (5).

Both carbaryl (1-napthyl N-methylcarbamate, a carbamate insecticide) and chlorpyrifos ((O,O-diethylO-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, an organophosphorous insecticide) are acetylcholinesterase inhibitors and may have toxic and developmental effects at sublethal doses (22). Previous investigations involving organophosphorus and carbamate insecticides have demonstrated mortality, skeletal abnormalities, abnormal pigmentation, and edema in exposed tadpoles (1, 10, 13, 31). Imidacloprid (1-{(6-chloro-3-pyridinyl) methyl}-N-nitro-2-imidazolidinimine) is a relatively new chloronicotinyl insecticide that acts upon the nervous system (27). To our knowledge, current literature does not contain any information on the effects of imidacloprid on amphibians.

Our results suggest that species differ in sensitivity to insecticides, only *A. jeffersonianum* had hatching success significantly lower than controls. Lack of significance for *P. triseriata*, *B. americanus* and *R. pipiens* may result from high variability in response to small sample size. Small sample size and high variability decrease the power of our analysis considerably. For example, power analysis using the variance exhibited in the B. americanus trial indicates that we had a 16% chance of not detecting a significant effect if significant differences did exist (* = 0.87).

Berrill and Bertram (3) report that some species tested were consistently more sensitive to pesticides than others; *B. americanus* appeared to be most tolerant and *A. maculatum* appeared least tolerant. Of ranid frogs, *R. pipiens* was ranked more tolerant than *R. catesbeiana* and *R. clamitans*. Berrill and Bertram (3) attribute this trend of decreased tolerance to smaller egg size and earlier developmental stage at hatching. It is interesting to note that the species we found to be most sensitive (*A. jeffersonianum*) also took the longest to develop, and therefore the egg masses were exposed to the pesticide for alonger period of time.

The logistic regressions we performed indicated a significant dose-response relationship for carbaryl, and this relationship can be used to predict the amplitude of changes in hatching success as pesticide levels increase. Peterson et al. (29) estimated an expected environmental concentration of carbaryl (calculated by assuming overspray of label application rate to a 15cm deep body of water) to be approximately 3.667 mg/L.

Our results suggest that at that concentration egg masses of *A. jeffersonianum* would experience a decline in hatching success to 46.57% of eggs hatching (95% CI 42.53-50.66%), and hatching success of *R. pipiens* would decrease to 51.56% of eggs hatching (95% CI 49.53-53.57%). Due to higher hatching rates in controls, predicted declines in *B. americanus* and *P. triseriata* would not be so severe. Likewise, predicted declines in hatching success of *A. jeffersonianum* and *R. pipiens* would also be less severe if overall hatching success were greater in natural populations.

Conversely, hatching success recorded in high concentrations of chlorpyrifos and imidacloprid was greater than or almost equal to hatching success in controls of *P. triseriata*, *B. americanus*, and *A. jeffersonianum*, and our regression analysis did not suggest a dose-response relationship for these pesticides at the concentrations tested. Elliott-Feeley and Armstrong (10) found that amphibian embryos were more resistant to the organophosphate pesticide, fenitrothion, than larvae, but that the opposite pattern was observed for carbaryl. They suggested that the jelly coat covering amphibian eggs prevents penetration of some compounds to the embryo.

Our results also suggest that carbaryl may penetrate the protective jelly coat more readily than other pesticides such as organophosphates (like chlorpyrifos). Likewise, static application techniques and addition of sediment to our tanks create a natural route for detoxification, as many chemicals adhere rapidly to soil where uptake and breakdown of pesticide by microorganisms takes

place.

The high number of control tadpoles exhibiting deformities suggests that our laboratory conditions were not ideal for amphibian development. Cooke (8) stated that exposure to any environmental stress may increase incidences of abnormalities in tadpoles. We cannot rule out poor water quality as an explanation for high deformity rates since we did not measure water quality parameters.

Deformities of the notochord and spine were the most common abnormalities observed in our experiment. Most tadpoles that contained multiple deformities had a spinal abnormality in addition to other afflicted areas. Snawder and Chambers (35) observed that 60% of embryos with abnormal notochords from exposure to organophosphorous compounds also developed abnormal limbs. However, embryos that had abnormal guts or pigment alterations developed normally. The severity of many deformities we observed indicate that survival of many embryos would have been compromised once they began to feed and swim.

Larval trials- Insecticides

Our investigations demonstrate that pesticide levels not considered to be acutely toxic have deleterious effects on tadpoles exposed over longer periods of time. Although natural populations of amphibians may receive only one or two exposures to pesticides during a breeding season, most of the mortality we observed in our high concentrations took place during the two weeks following initial pesticide dosage. This suggests that even one application or runoff event exposing tadpoles to concentrations higher than the expected application rate of these pesticides could have a severe effect on survival.

Reduced growth and delayed time to metamorphosis of tadpoles may also have negative effects on population persistence. Although amphibian larvae exhibit plasticity in size at metamorphosis and length of larval period, classic studies of amphibian metamorphosis support a minimum body size needed for metamorphosis (18, 37). Tadpoles of amphibian species that breed in temporary pools need to reach this minimum body size before ponds dry up. The longer tadpoles remain in these ephemeral wetlands, the greater the competition and predation pressure (34). In addition, size of a tadpole at the time of metamorphosis affects the timing of first breeding, thus, decreased size of metamorphs could negatively impact lifelong reproductive output of adult frogs and overwintering survival (24).

Decreased activity of tadpoles has been observed in response to sublethal doses of carbaryl and is a likely cause of reduced growth in our tadpoles (6, 10). Weight increase in tadpoles is directly related to time spent feeding and rate of food intake (12). Anecdotal observations of our tanks during the first two weeks of treatment suggest less food intake in tanks with medium concentrations of pesticide compared to controls.

Although we observed only a two- to three -day difference in time to metamorphosis with higher pesticide levels, it is likely that differences in timing would be accentuated in natural populations with more environmental stress. Under favorable conditions, tadpoles may continue to grow long after minimum body size needed for metamorphosis is reached (37). Observed body size of our control tadpoles was much larger than conspecifics observed in the field, indicating our tadpoles had continued to grow long after adequate body size had been reached. However, our tadpoles in higher pesticide concentrations appeared to have lower bodymass at metamorphosis than our controls.

These observations are consistent with those of Fioramonti et al. (12) who found that a trade-off in fitness of tadpoles occurred under conditions of chemical stress (tadpoles either experienced a short larval period with lower mass at metamorphosis or long larval period with large size at metamorphosis). It is likely that our protocol provides resource rich conditions conducive to growth and tadpoles under no chemical stress delayed metamorphosis for further growth, whereas tadpoles in our higher concentrations took only a few days longer to metamorphose but at a reduced body mass.

Developmental abnormalities have been reported as a consequence of sublethal pesticide exposure by many investigators (1, 7, 8, 23, 28). Cooke et al. (8) describes lateral tail kinks as the most common deformity among older larvae, however usually tadpoles recover at front limb emergence. It is unlikely that the spinal curvatures we observed in many of our *R. sphenocephala* larvae would have any adverse effects on adult survival, although larval swimming performance may be compromised in severe cases.

The limb deformities we observed would probably affect adult survival, but occurrence was too infrequent to link them with a specific pesticide or concentration. It is possible that we saw more spinal deformities in *R. sphenocephala* because the exposure time of these larvae to the pesticide was much longer (exposure period of 52-56 days for ranid larvae compared to 32-36 days for other species). Pesticide was renewed four times in the *R. spenocephala* trials and only twice in the *P. triseriata* and *B. americanus trials.*

Water and Sediment analysis-Insecticides

The results of our sediment and water sample analysis indicate that different pesticides may primarily impact amphibians through different routes. Although imidacloprid is least toxic to amphibians, our results indicate that it remains in the water column longer than carbaryl or chlorpyrifos, and conversely that chlorpyrifos is most toxic to amphibians but leaves the water column rapidly.

Many studies on water quality do not report pesticide values higher than our lowest concentrations (14, 21, 30), however compounds that adhere rapidly to and remain in sediment (such as chlorpyrifos) have the potential to impact tadpoles through ingestion as well as absorption through the skin or gills. Giddings et al. (14) reported that chlorpyrifos was more persistent in the sediment than in the water column, and results of our sampling are consistent with their investigation. Mullins (27) reported the half-life of imidacloprid in soil as <150 days. This suggests that many amphibians would encounter imidacloprid in the sediment through the entire larval period.

SUMMARY

Insecticides

Our evaluation of three insecticides reflect dramatic differences in toxicity among the different compounds (e.g. chlorpyrifos was 200-400 times more toxic than imidacloprid for each species tested). The sublethal responses we observed are consistent with the hypothesis that exposure of some insecticides can cause decreased hatching success and increases in deformities. Even modest decreases, if persistent, can result in steady declines in numbers and eventual extirpation of impacted populations.

It is important to note that responses among amphibians we tested are not the same and some species with longer hatching times may be at greater risk to pesticides that readily penetrate the jelly coat surrounding the developing embryo. It is interesting that the only species to show a significant decline in hatching success was the only species of salamander tested. Whether this shows greater sensitivity to carbaryl or reflects differences in the architecture of the jelly coat is unclear.

The casual observation that certain amphibian species are thriving in heavily treated environments can lead to erroneous conclusions regarding long term impacts on all amphibians. Significant treatment effects were much more evident in the larval trials. Survivorship, growth and time to metamorphosis was significantly impacted by high concentrations of each insecticide. In addition, medium concentrations of both carbaryl and chlorpyrifos significantly decreased growth and time to metamorphosis. These sublethal effects would be expected to have negative effects on population persistence.

PRELIMINARY ANALYSIS

Fungicides and Herbicides

Fungicide containing the active ingredient mancozeb significantly affected hatching success of *Rana* eggs at all test concentrations, whereas *Ambystoma* hatching success was not significant-

ly affected by any of the fungicides tested. Mancozeb also resulted in a significant amount of deformities in *Rana* and *Ambystoma* hatchlings when compared to control hatchlings. These results suggest that mancozeb could affect hatching success and survival of amphibian embryos in natural environments.

All herbicides significantly affected hatching success of *Bufo* eggs at high concentrations (dimethlylamine) or high and medium concentrations (glyphosate and prodiamine), whereas *Rana* hatching success was only significantly affected by dimethlylamine at low and high concentrations. These results likely reflect differences in species sensitivity. Effects on hatching succes would likely influence population persistence in natural environments if environmental exposure was similar to test concentration values.

When tadpoles were exposed to fungicides from approximately two weeks post-hatching to metamorphosis, all concentrations of mancozeb fungicide had significant effects on survival of *Rana* larvae. High concentrations of mancozeb and fosetyl-Al had significant effects on *Hyla* larval survival. Mancozeb fungicide also had significant effects on growth and time to metamorphosis for both *Rana* and *Hyla* species. Sublethal effects on growth and time to metamorphosis would be expected to have negative effects on population persistence.

All prodiamine herbicide test concentrations significantly reduced survival of *Bufo* tadpoles. *Hyla* survival was significantly lower than controls in prodiamine medium (0.1x LC_{50}) and high (0.5 x LC_{50}) concentrations. No significant effects on growth or time to metamorphosis were noted for any of the surviving tadpoles in prodiamine treatments or tadpoles raised in glyphosate or dimethylamine salt treatments.

Pesticide Testing

Numerous investigators have observed mortality of amphibian larvae in natural populations associated with pesticide application (2, 7, 17, 26). Increased mortality due to direct or indirect effects of pesticides in successive years may eventually result in loss of entire populations over time.

In our investigations, all amphibian species exhibited reduced survival and increased time to metamorphosis with higher concentrations of pesticides. However, there are very dramatic differences in pesticide toxicity. Many of the most toxic compounds are used during the breeding season and although there are differences among species in sensitivity, all species showed similar patterns of effect.

At lower concentrations mortality is often not the direct effect but rather we observed, decreased hatching rates, slower growth rates and longer times to metamorphosis. All of these more subtle effects can, over many years, be more damaging to the persistence of amphibian populations than one large mortality event. Managers should have the data available to apply chemical treatments responsibly to reduce these hazards. That data should include information on the relative toxicity of the compounds, the persistence of those chemicals and the life stage that is most sensitive to treatments.

As we have shown with our studies, some compounds appear to penetrate the jelly layers in amphibian eggs more readily than others and directly impact egg hatching. Some compounds that have little detectable effect on eggs can have dramatic effects on larval growth at low concentrations. Hopefully, our research will encourage others to evaluate additional compounds and expand the data base available to managers.

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