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Species- and Age-Related Differences in Susceptibility to Pesticide Exposure for Two Amphibians, *Rana pipiens*, and *Bufo americanus*

M. L. Harris,[1,*] L. Chora,[1] C. A. Bishop,[2] J. P. Bogart[1]

[1] University of Guelph, Department of Zoology, Guelph, ON, Canada N1G 2W1
[2] Canadian Wildlife Service, Environment Canada, 867 Lakeshore Road, Box 5050, Burlington, ON, Canada L7R 4A6

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Laboratory studies have a useful role to play in establishing the baseline sensitivity of various amphibian life stages and species to pesticides because other environmental stressors can be eliminated from the assessment. Further in *situ* studies may then establish their relevance to real environmental situations. Many investigators have justified using tadpoles for toxicity tests because they were described as the most sensitive life stage in some studies (Freda and McDonald 1993; Kane et al. 1993). However, this line of reasoning does not consider the relevance of each developmental stage to the timing of pesticide applications in true agricultural settings. Pesticides may be used throughout the growing season on many crops, but generally a particular formulation is only applied during an abbreviated time window of pest abundance and so will likely only directly interact with one or two amphibian morphologies. Also, published sensitivity studies may not be relevant to the exposure that a particular species of interest experiences in the natural world. A wide range in species sensitivity to pesticides has been described for amphibians (Linder et al. 1990; Schuytema et al. 1995).

In this study, we compared the responses of northern leopard frogs *(Rana pipiens)* and American toads *(Bufo americanus)* at two developmental stages to insecticide and fungicide exposure. During previous *in situ* and laboratory studies, we established that pesticides containing endosulfan, azinphos-methyl or mancozeb were applied to apple orchards in Ontario (Canada), could be detected in various environmental media in the orchards, and affected survival or growth of embryos of the green frog, *Rana clamitans,* in laboratory tests (Harris et al. 1998a, 1998b). Our objectives during this follow-up study were to 1) determine the range in sensitivity exhibited by three frog species indigenous to North America to these pesticides of concern, and 2) determine the variability in responses exhibited at two potentially sensitive developmental stages (gastrulation and metamorphic climax).

MATERIALS AND METHODS

One organochlorine insecticide (endosulfan), one organophosphate insecticide (azinphos-methyl), and one ethylenebisdithiocarbamate fungicide (mancozeb) were chosen as treatments due to their apparent liberal use in Ontario agriculture (Hunter and McGee 1994) and toxicity observed during earlier laboratory tests (Harris et al. 1998b). The formulations, Thiodan® 50WP (47% endosulfan; Agrevo Hoescht Noram Canada, Cambridge, ON), Guthion® 50WP (50% azinphos-methyl; Bayer/Mobay, Etobicoke, ON) and Dithane® DG (76-80% mancozeb; Rohm and Haas Canada, West Hill, ON) were kindly supplied by the

Present address: Lorax Environmental, 208 Spencer Road, R.D. 5 Lake Tarawera, Rotorua, New Zealand
Correspondence to: M. L. Harris

manufacturers. Formulation concentrations used for testing were 0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/L. In the following text, however, treatment concentrations were reported as the nominal concentration of active ingredient: 0.00047 to 4.7 mg endosulfan/L; 0.0005 to 5.0 mg azinphos-methyl/L; and 0.0008 to 8.0 mg mancozeb/L. Stock concentrations of each formulation were analyzed and the results described in Harris et al. (1998b). The nominal concentration ranges used for endosulfan and azinphos-methyl encompass environmental (surface water) values measured in local ponds and streams (Harris et al. 1998a, unpubl. data). Mancozeb has not been detected in local water bodies. but the range of nominal concentrations tested extends below the applied limit of detection.

One egg mass each of American toads and northern leopard frogs were collected from local ponds. Throughout this study, developmental stages were identified according to Gosner (1960). Test conditions during the first exposure at late cleavage (stage 8) were similar to those described for green frogs in Harris et al. (1998b). Ten developing embryos were placed in each of three replicate Parafilm®-covered 250-mL beakers in a temperature-(20±l°C) and photoperiod-controlled (12:12) incubation chamber. Pesticides were made up in aged (>24-hr-old) municipal tap water, and each beaker contained 150 mL solution. Solutions were two-thirds renewed at 48-hr. At 96-hr, American toads had developed to stage 17 to 19 (‘tail bud’ through to ‘heart beat’ stages), while leopard frogs had developed to stage 15 to I7 (‘rotation’ through to ‘tail bud’ stages). Thus, toads were in the process of hatching and frogs had not begun to hatch by the termination of the first 96-hr treatment.

After 96-hr, embryos were removed from treatments and placed in clean, aged tap water in shallow white enamel pans (40 x 24 x 6 cm). Pans were placed on benchtops in a room with temperature maintained at approximately 22°C. Feeding commenced at stage 22, when tadpole swimming activity became pronounced. Tadpoles were fed boiled lettuce, ad-*libitum*, until forelimb emergence from the branchial chambers (stage 42). During the interim growth period, dead individuals were removed during daily inspections and water was changed twice weekly or more frequently when faecal accumulation warranted. The embryos from each egg mass that were not used during early exposures were similarly maintained in aquaria.

Once a forelimb emerged, each individual was placed in an amber Pyrex pan (29 x 20 x 5 cm) that was placed on a slant (-20 degrees) and lined with a damp unbleached paper towel. A sufficient volume of treatment solution (pesticide or control tap water) was added to submerge the individual at the base of the slanted pan, while over half of the paper towel at the top remained moist but exposed to air. After 48-hr, individuals were transferred to clean transparent plastic boxes (13 x 18 x 4 cm) to complete metamorphosis.

Mortality was recorded daily throughout the tests, while deformities were assessed only at hatching (stage 20) and upon completion of metamorphosis (stage 46). Time-to-metamorphosis was documented for both species. but size and sex of metamorphs were only recorded for leopard frogs (the latter by identifying gonads). LC50 values were calculated, where possible, using a trimmed Spearman-Karber method (Hamilton et al. 1977). Mass (g) and snout-vent-length (cm) of leopard frog metamorphs were compared among treatments using one-way analyses of variance. The rate of deformity in a given treatment was compared to a rate of one percent using the binomial test for goodness of fit in a Poisson distribution (one-tailed, α=0.05). The sex ratio in a given treatment was compared to a 1: 1 ratio using the binomial goodness of fit test (two-tailed, α=0.05). Although tests

began with three (pseudo-) replicates of ten embryos each, equipment and space constraints prevented the maintenance of those replicates during the interim months of growth in clean water. Also, because tadpoles reached the forelimb emergence stage at varying times, the second pesticide exposure had to be conducted on individuals (e.g., individuals were the treatment unit). Therefore, rates of survival and malformation pertaining to embryos were expressed as means, but those pertaining to metamorphs were expressed as absolute values.

RESULTS AND DISCUSSION

At the concentrations tested, only mancozeb was acutely toxic to embryos (Table 1, stage G17). The 96-hr LC50 for American toad embryos was 1.4±0.3 mg/L mancozeb. The 96-hr LC50 for leopard frog embryos was 0.20±0.02 mg/L mancozeb. Previous tests showed that green frog embryos responded similarly to toad embryos, with average LC50s for the green frog ranging between 2.2 and 0.96 mg/L mancozeb (Harris et al. 1998b). Thus, leopard frogs were almost an order of magnitude more sensitive to the fungicide than toads or green frogs. Embryos of leopard frogs or American toads did not show dose-related mortality upon azinphos-methyl or endosulfan exposure of 2.3 to 2.5 mg active ingredient/L or less.

Distinctly different rates of survival were observed for metamorphs compared to embryos. Mancozeb did not elicit immediate dose-related mortality up to 8.0 mg/L at metamorphic climax (Table 1, stage G46). Sixty percent of metamorphosing leopard frogs exposed to 8.0 mg/L did die one to two weeks after test termination, suggesting that they were negatively affected at that level of exposure. Also, metamorphosing leopard frogs were unable to survive concentrations of endosulfan that were non-lethal to embryos (Table 1). All frogs exposed to ≥2.35 mg/L endosulfan died within 48-hr, while only 33% of frogs exposed to 0.47 mg/L survived. Toads at metamorphic climax did not show the same susceptibility to endosulfan. Neither species showed dose-related mortality upon exposure to azinphos-methyl (up to and including 2.5 to 5 mg/L) at metamorphosis.

These results suggest that survival rates may vary substantially with developmental stage and species for pesticide toxicity tests. This corroborates earlier acute toxicity studies completed with other amphibian species and pesticides (Sanders 1970; Linder et al. 1990). Based on recommended spray applications to Ontario apples (Harris, unpubl. data), peak concentrations of mancozeb in environmental media may be expected to coincide with early embryonic development of both leopard frogs and American toads. Endosulfan is used less frequently on apples, but when used, is applied later, around the time that both species begin transforming into juveniles. The more extensive use of endosulfan in greenhouses probably follows a similar seasonal schedule, since target pests are the same. Hence, the apparent acute sensitivities of embryos to mancozeb and of metamorphosing (leopard frog) tadpoles to endosulfan are of concern, because those stages of development are relevant to seasonal periods of most probable environmental exposure in temperate North America.

Mancozeb produced skeletal deformities at hatching in toads (Table 1, G20) at concentrations equivalent to those producing the same deformities in green frogs (Harris et al. 1998b). The majority of deformed toads were exposed to mancozeb at levels which were lethal to leopard frog embryos. While endosulfan did not produce deformities in hatched toads, hatched leopard frogs showed lateral flexure of the tail and severely impaired swimming abilities (Table 1). The observed skeletal and neural impairments disappeared within a week or two in the presence of clean water. Only mancozeb presence in the environment would usually coincide with hatching, but, given a half-life in water of less

Table 1. Effects of exposure to mancozeb or endosulfan on early development of American toads and northern leopard frogs. Treatments are described as nominal concentrations of active ingredient (in mg/L). Cumulative survivorship at the end of the first treatment (stage G17) and at forelimb emergence (stage 642) is compared to survivorship during the second treatment (stage G46). The frequency of deformities was assessed at hatching (stage G20) and upon completion of metamorphosis (stage G46). G# = Gosner (1960) developmental stage number. 'nt' = not tested; '-' = not applicable.

Test Solution	Doses[b]	American Toads					Leopard Frogs				
		% Survivorship			% Deformities[a]		% Survivorship			% Deformities[a]	
		G17	G42	G46[c]	G20	G46	G17	G42	G46[c]	G20	G46
Mancozeb											
Control	2	90	70	100	0	0	100	63	100	0	0
0.0008	2	100	40	83	0	0	100	53	100	0	0
0.008	2	100	53	88	0	0	97	50	100	0	0
0.08	2	93	73	100	14	5	93	47	100	0	0
0.8	2	87	60	100	96	0	0	0	-	0	0
4.0	2	7	3	100	100	0	0	0	-	0	0
Control	1	-	-	100	-	0	-	-	100	-	0
0.08	1	-	-	88	-	0	nt	nt	nt	nt	nt
4.0	1	-	-	88	-	0	nt	nt	nt	nt	nt
8.0	1	nt	nt	nt	nt	nt	-	-	100	-	0
Endosulfan											
Control	2	100	67	100	0	0	100	53	89	0	0
0.00047	2	80	60	94	0	0	100	50	100	0	0
0.0047	2	97	60	89	0	0	93	50	100	0	0
0.047	2	90	60	100	0	11	100	50	100	3	0
0.47	2	97	67	90	0	6	100	60	33	0	0
2.35	2	93	73	100	0	0	97	40	0	100	0
Control	1	-	-	100	-	0	-	-	100	-	0
0.047	1	-	-	100	-	0	nt	nt	nt	nt	nt
2.35	1	-	-	100	-	0	nt	nt	nt	nt	nt
4.7	1	nt	nt	nt	nt	nt	-	-	0	-	0

[a] - deformities at hatching (G20) were all skeletal in nature (lateral flexure or kinking of the tail) and leopard frogs exposed to endosulfan also displayed behaviour symptomatic of nerve-related damage (e.g., swimming in a twisting, disoriented fashion); 4 toad metamorphs (G46) showed eye deformities (3 of 4 exposed to endosulfan) - 3 were missing the right eye, while the other had one eye displaced to the top of the head; one toad with a missing eye also displayed luxation of the right front limb.

[b] - 2 = exposed at stage 8 (96-hr) and stage 42 (48-hr); 1 = exposed only at stage 42 (48-hr).

[c] - survivorship at metamorphosis was expressed as a portion of the number exposed in second test.

than one day (WHO 1988) mancozeb-induced swimming impairment would not likely affect tadpole survival unless predator densities were high. Azinphos-methyl exposure did not result in deformities at hatching in either species.

Most of the deformities that were present upon completion of metamorphosis were observed in toads (Table 1, G46, n = 4 of 310 metamorphosed toads) and all affected individuals had eye abnormalities (Fig. 1). One eye was missing completely in one toad exposed to 0.08 mg/L mancozeb, in two toads exposed to either 0.047 or 0.47 mg/L endosulfan, and in one leopard frog exposed to 0.005 mg/L azinphos-methyl. Another toad exposed to 0.047 mg/L endosulfan had two functioning eyes, but one was displaced dorsally to the top of its head (Fig. 1). One of the toads with a missing eye also had a deformed front limb (0.047 mg/L endosulfan group). All eye deformities were visible just before the second pesticide treatment. The leopard frogs that survived the second exposure to 0.47 mg/L endosulfan showed no eye deformities, but two-thirds of them suffered from nerve twitches that inhibited their ability to jump and stay upright. Control animals did not show any form of deformity at metamorphosis (n = 49). Only toads exposed to endosulfan (0.047 to 0.47 mg/L) exhibited a rate of deformity at metamorphosis that was significantly greater than the 'normal' frequency of one percent ($p = 0.007$, n = 3 of 38). Based on the typical application season in temperate North America, endosulfan could be present in the environment when American toad and leopard frog late stage tadpoles and metamorphs were present.

The predominance of deformities in American toads versus leopard frogs may only be an artifact of the study design. Leopard frogs were more acutely sensitive than toads to both mancozeb and endosulfan. Toad deformities appeared at concentrations that were lethal to leopard frogs and that were little more than an order of magnitude lower than lethal concentrations for toads, themselves. Exposure of leopard frogs within a narrow (essentially untested) sublethal range, somewhere between 0.08 and 0.8 mg/L mancozeb or between 0.047 and 2.35 mg/L endosulfan, might have also induced deformities. One concentration of endosulfan was tested within that range, but produced a 62% rate of mortality.

Upon completion of metamorphosis, leopard frogs did not differ in body mass or snout-vent length among any of the treatments ($p>0.05$). Control individuals weighed 1.6±0.1 g and had a snout-vent length of 2.5±0.05 cm. In addition, neither American toads nor leopard frogs showed differences in time-to-metamorphosis among treatments ($p>0.05$). Control-treated toads metamorphosed at 41 to 58 days, while control-treated frogs metamorphosed at 84 to 127 days.

Earlier studies with green frog embryos and tadpoles did find growth inhibition at 0.078 mg/L mancozeb and 2.5 mg/L azinphos-methyl (Harris et al. 199813); however, exposure periods were prolonged (8 to 13 d) compared to toad and leopard frog treatments. In other ten day tests with azinphos-methyl, growth inhibition of early developmental stages was observed for the Pacific treefrog *(Pseudacris regilla,* at 0.07 to 3.6 mg/L) and Ambystomatid salamanders *(A, gracile* and *A. maculatum,* at 0.03 to 0.22 mg/L) (Nebeker et al. 1998). We did not measure growth after the first exposure, so cannot directly compare our results with those cited; however, if growth differences were initially present, they disappeared during the tadpole stages of development. This brings into question the relevance of short-term growth inhibition to overall larval success. Carey and Bryant (1995) state that reduced growth may force tadpoles to overwinter when they are not physiologically equipped to do so or may place tadpoles at greater risk of predation.

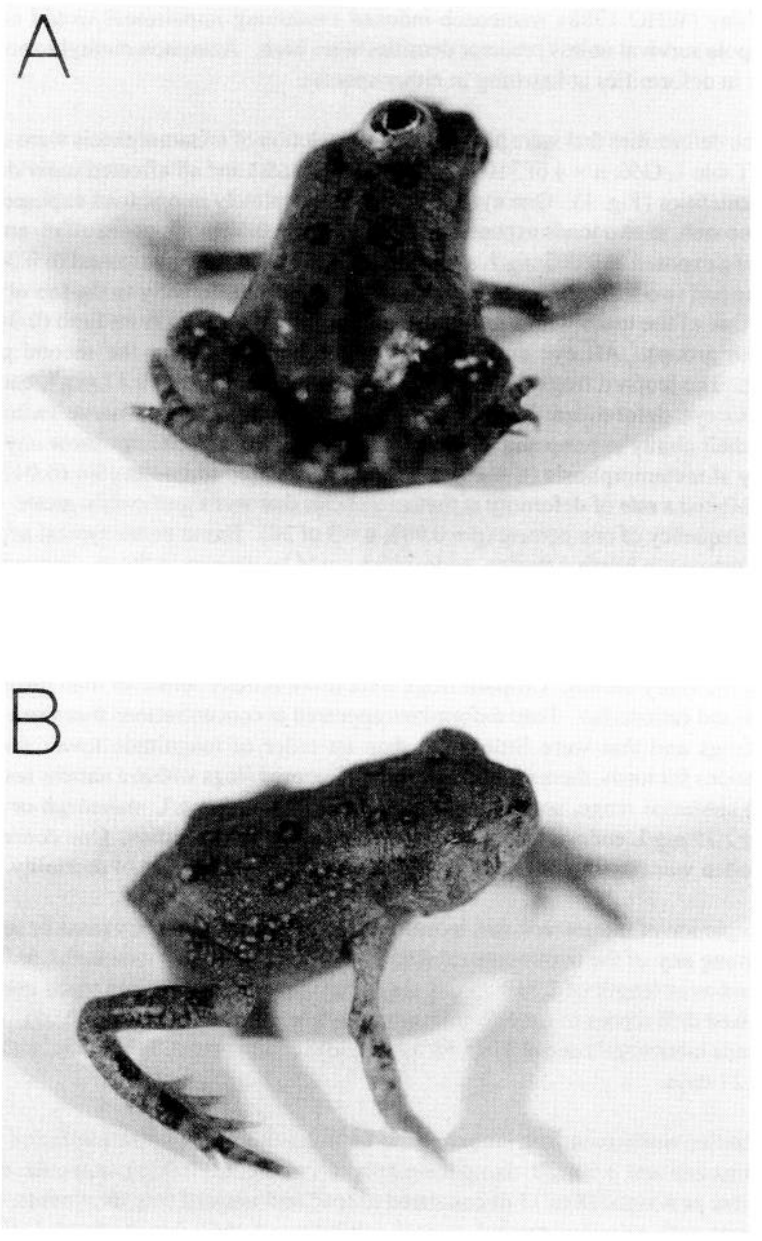

Figure 1. Eye and limb deformities expressed in American toad juveniles at metamorphosis. A) individual exposed to 0.047 mg/L endosulfan with two functional eyes, one of which was displaced to the top of its head; B) individual exposed to 0.47 mg/L endosulfan with right eye missing.

However, short-term growth has been shown to be an inaccurate reflection of metamorphic size for green frogs (Harris et al. 1998a, 1998b), presumably because it does not account for the complex interactive effects of competition and predation on overall larval growth.

Control-treated leopard frogs exhibited a 1:1 sex ratio just after metamorphosis (females 49±11%; males 51*11%, n=28). Surviving mancozeb-exposed individuals did not show an even distribution of the sexes. In the group exposed once as embryos to mancozeb, the 0.08 mg/L treated individuals (n=5) were 60% males and 40% unknown (e.g.. lacking identifiable gonads). In the group exposed twice, both as embryos and at metamorphosis, the opposite skew was apparent. Individuals exposed to 0.0008 mg/L mancozeb were 57% females and 43% unknown (n=7), while those exposed to 0.08 mg/L mancozeb were 100% females (n=7). Surviving leopard frogs exposed once (at gastrulation) to 2.35 mg/L endosulfan were also 100% females (n=5). No individuals survived a second treatment at this concentration during metamorphosis. There was no dose-response apparent in deviations from a 1:1 sex ratio in other pesticide-exposed groups. No control individuals were of unknown sex, while gonads could not be found in 21%, 4% and 2% of surviving mancozeb, azinphos-methyl and endosulfan exposed individuals, respectively.

The lack of any males in the group exposed twice to 0.08 mg/L mancozeb was a significant deviation from the expected 1:1 sex ratio ($p<0.05$). Several studies with dithiocarbamates have suggested that their degradation product, ethylenethiourea (ETU), may be responsible for the majority of toxic effects (WHO 1988). ETU produced from the degradation of mancozeb in water might have also been the cause of apparent changes in sex ratios observed here. Hayes (1997) found that treatment with thiourea produced 100% females in one frog *(Xenopus laevis)* and 100% males in another *(Hyperolius viridiflavusj*.

Interpretation of the apparent deviations from a normal 1:1 sex ratio in metamorphosed leopard frogs in our study were hindered by the small sample sizes, and further treatments with more individuals would be necessary to confirm skews. That said, the presence of pesticide-induced abnormal sex ratios is of significance because it could limit the effective size of wild breeding populations. Since male leopard frogs will mate with multiple females, an increase in the number of females may not be detrimental to the population until presence of males becomes severely restricted. However, a decline in numbers of females may produce a more rapid and extreme effect on breeding effort and success. One instance of a skewed sex ratio in wild frogs has been reported: Reeder et al. (1998) found that populations of juvenile cricket frogs were skewed in favour of males at sites contaminated with chlorinated hydrocarbons and pesticides.

In the context of local surface waters, effects were observed above maximum measured environmental concentrations of 0.54 µg/L endosulfan and 7.9 µg/L azinphos-methyl (Harris et al. 1998a, unpubl. data). Mancozeb has not been detected in local waters, but some treatment effects were expressed at nominal concentrations below the applied minimum detection limits of 10 to 50 µg/L, suggesting a need for more sensitive analyses.

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