Effects of Chronic Fungicide and Herbicide Exposure on Survival and Development of Amphibian Larvae

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ABSTRACT

A number of factors have been suggested to explain recently observed amphibian declines, including pesticide exposure. Effects of chronic exposure to three common fungicides and herbicides on hatching success and larval survival and development of amphibians were evaluated in separate experiments. Commercial formulations of Daconil® Ultrex (chlorothalonil), Chipco® Aliette Signature (fosetyl-al) and Fore® (mancozeb) fungicides and Barricade® 65 WG (prodiamine), Roundup® Weed and Grass Killer (glyphosate) and Trimec® Classic Broadleaf (dimethylamine salt of 2,4-D, mecoprop, and dicamba) herbicides were used. Three test concentrations for each experiment were determined from preliminary LC50 tests. Wood frog (*Rana sylvatica*) and spotted salamander (*Ambystoma maculatum*) eggs were exposed to fungicides and wood frog and American toad (*Bufo americanus*) eggs were exposed to herbicides in static tests in 5-L aquaria. Hatching success of wood frogs was significantly decreased in 0.5 mg/L of Fore®. Wood frog hatchlings in Fore® concentrations of 0.5 mg/L also exhibited a significantly greater number of deformities. Hatching success of American toads was significantly decreased in Barricade® 65 WG concentrations $\geq$ 210.207 mg/L. Also, wood frog and gray treefrog (*Hyla chrysoscelis*) tadpoles were exposed to fungicides and American toad and gray treefrog tadpoles were exposed to herbicides from approximately two weeks post-hatching to metamorphosis in 60-L aquaria. In the tadpole experiments, treatments were reapplied once every two weeks with water renewal. High concentrations of Fore® (0.5 mg/L) significantly decreased survival of wood frog and gray treefrog tadpoles. High concentrations of Chipco® Aliette

Signature (28.3 mg/L) also significantly decreased survival of gray treefrog tadpoles. Metamorphosed wood frogs exposed to Fore® concentrations $\geq$ 0.1 mg/L throughout development exhibited a greater number of deformities. Survival of American toad tadpoles was significantly decreased in all concentrations of Barricade® 65 WG (8.408 – 420.415 mg/L). Survival of gray treefrog tadpoles was significantly decreased in Barricade® 65 WG concentrations $\geq$ 84.083 mg/L. Metamorphosed American toads and gray treefrogs exposed to 84.083 mg/L Barricade® 65 WG throughout development exhibited a greater number of deformities. Effects on hatching success, larval survival, and occurrence of deformities may contribute to amphibian population trends and will likely influence persistence of populations if exposure to these concentrations occur in natural environments.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Amphibian conservation has attracted global attention following accounts of declines in several species representing diverse phylogenetic groups and geographical locations [1-5]. Widespread evidence of these declines was first reported at the First World Congress of Herpetology in 1989 in Canterbury UK, although isolated cases were confirmed much earlier [2, 5]. Anthropogenic causes, such as environmental contamination and degradation, may be linked to these observations [5-7]. Moreover, some amphibian species may be sentinels whose declines indicate the presence of environmental stressors [2, 5, 8-10].

Amphibians possess several characteristics that make them potential indicators of stress on ecosystems. Many amphibians have biphasic life cycles with both aquatic and terrestrial life stages. The permeable skin of amphibians can absorb pollutants and more readily permit access to pathogens [2]. Many aquatic pollutants accumulate in sediments, and amphibians in the aquatic stage maintain close contact with the sediment either directly or through ingestion [11]. Tadpoles ingest sediment and algae and salamander larvae ingest sediment-dwelling invertebrates. Amphibians are the primary vertebrate predators on invertebrates in many freshwater and moist terrestrial environments [2]. In addition, amphibians are believed to be the most abundant vertebrate class by biomass and number in several temperate and tropical habitats [2, 12].

Habitat destruction, pathogens, over-exposure to UV light, competition with introduced exotic species, and pesticide exposure are probable factors contributing to declines of amphibian populations [6, 13-17]. Amphibians are often associated with aquatic environments in agricultural and urban areas and are vulnerable to pesticide exposure through runoff or direct application. Pesticide exposure may contribute singularly to some population declines, or be part of an additive or synergistic effect.

Pesticide studies

In natural environments, chronic or repeated exposures to acutely sublethal levels of pesticides are likely more frequent than single-event, short-term exposure to acutely lethal levels. Thus, studies that address long-term effects of exposure to low levels of pesticides can be valuable for assessing effects on natural amphibian populations. Several studies using exposure times of 96-h or less, have shown acute effects of pesticide exposure on amphibian larvae [18-24]. Several other amphibian toxicology studies have also found lethal and sublethal effects for chronic (i.e., > 96-h) exposure to pesticides, however effects vary with pesticide compound and experimental conditions. Effects on growth, neurological function, hatching success, and behavior occur from chronic exposure of amphibians to pesticides. Cooke [25] noted significant effects of field applications of the aquatic herbicides diquat and dichlobenil on growth of caged common frog (*Rana temporaria*) and common toad (*Bufo bufo*) tadpoles in natural ponds. Baker [26] exposed slimy salamanders (*Plethodon glutinosus*) and red-backed salamanders (*Plethodon cinerus*) to malathion in both laboratory and field experiments and noted significant inhibition of cholinesterase (ChE) in laboratory slimy

salamanders. However, no significant effects on ChE inhibition, survival or lipid storage were observed for either species in the field, even after 10 applications of malathion. Ingermann et al. [27] exposed long-toed salamander (*Ambystoma macrodactylum*) eggs to the estrogenic pesticide, methoxychlor, and examined the influence of this chemical on pre-hatch mortality and time-of-hatch of the embryos and post-hatch startle response of the larvae. Methoxychlor concentrations of $\geq$ 0.1 mg/L promoted early hatching of long-toed salmander eggs. Ten-day-old larvae maintained in $\geq$ 0.3 mg/L methoxychlor showed significant alterations in startle response.

Julian [28] investigated the effects of three insecticides (containing active ingredients carbaryl, chlorpyrifos, and imidacloprid) on amphibian eggs and larvae using methodology similar to that used in this study. Julian found that chronic exposure of Jefferson salamander (*Ambystoma jeffersonianum*) eggs to carbaryl insecticide at concentrations of 23–31 mg/L significantly reduced hatching success. In addition, survival rates of chorus frog (*Pseudacris triseriata*), American toad (*Bufo americanus*) and southern leopard frog (*Rana sphenocephala*) tadpoles chronically exposed from two-weeks post-hatching to metamorphosis were significantly reduced in concentrations of 0.6 – 0.7 mg/L chloropyrifos, 92 – 234 mg/L imidacloprid, and 26 – 32 mg/L carbaryl. The period of larval development of chorus frog, American toad, and leopard frog tadpoles was significantly increased in concentrations of 0.1 mg/L chloropyrifos, 18 – 47 mg/L imidacloprid, and 5 – 6 mg/L carbaryl. American toad tadpoles exhibited significantly decreased growth in concentrations of 0.1 mg/L chloropyrifos and 5 – 6 mg/L carbaryl. Southern leopard frog tadpoles exhibited decreased growth in

concentrations of 0.1 mg/L chloropyrifos, 18 – 47 mg/L imidacloprid, and 5 – 6 mg/L carbaryl. Julian's study suggests that hatching success, survival and development of amphibian species could be affected by exposure to these concentrations of pesticides in their natural habitats.

The active ingredients in test fungicide formulations used in this study were chlorothalonil, fosetyl-al, and mancozeb. The active ingredients in test herbicide formulations used in this study were glyphosate, prodiamine, and dimethylamine salt of 2-4-dichlorophenoxyacetic (2,4-D), mecocrop and dicamba. Traditionally, laboratory toxicology tests have used standard laboratory species and then extrapolated results to estimate effects on amphibians in natural environments. Sparling et al. [29] suggested that tests on resident species are more appropriate when using toxicity data to assess the potential for contaminants to cause adverse effects measured in field studies. Amphibian genera used in this study were native to the eastern United States and the particular species used for each trial depended on availability.

No current studies have investigated chronic exposure of amphibians to pesticides with active ingredients chlorothalonil, fosetyl-al, dimethylamine, or prodiamine. Harris et al. [30] exposed northern leopard frogs (*Rana pipiens*) and green frogs (*R. clamitans*) *in situ* and in the laboratory to pond water from apple orchards in southern Ontario and nonagricultural control sites. They also evaluated the toxicity of six pesticides including Dithane® DG, a fungicide containing 76-80% mancozeb, using discontinuous and continuous tests. Embryos and tadpoles exhibited poor success and survival at some orchard sites in the *in situ* assays, but differences were not consistently

associated with any single water source. Green frog 96-h LC50 values for Dithane DG contained 0.96-2.21 mg/L of mancozeb. Harris et al. also calculated LC50 values for 13-d continuous and 16-d discontinuous exposures. In discontinuous tests, tadpoles were reared in aquaria with 4-d exposure and 7.5-d nonexposure cycles. Dithane® DG LC50 concentrations for 13-d continuous and 16-d discontinuous exposures contained 0.023 and 0.20 mg/L mancozeb, respectively. Deformity frequencies were used to calculate EC50 (effective concentrations that resulted in 50% observations). The Dithane DG EC50 was 0.04 mg/L of mancozeb.

Field studies have not detected any significant effects of glyphosate spraying in the Pacific Northwest and Northwest Ontario [31, 32]. Glyphosate is the active ingredient in Roundup® herbicide. Acute toxicity studies using western Australian white-throated froglets (*Crinia insignifera*), moaning frogs (*Heleioporus eyre*), western banjo frogs (*Limnodynastes dorsalis*), and western green frogs (*Litoria moorei*) and the African clawed frog (*Xenopus laevis*) have shown that the surfactant polyoxyethyleneamine (POEA) in Roundup® formulations are sufficiently more toxic than glyphosate alone [33, 34]. Because commercial formulations can differ in toxicity from active ingredients alone, all trials in this study were done with commercial formulations. Commercial formulations, natural pond sediment, and native species were used to provide a laboratory environment that mimicked and allowed extrapolation to natural conditions.

Objectives and hypotheses

The objectives of this project were to investigate the effects of chronic exposure

to three common fungicides and three common herbicides on hatching success, and larval survival and development, of diverse amphibian taxa. I hypothesized that chronic, repeated fungicide or herbicide exposure at levels of 1 – 50% of 48-hour LC50 concentrations (the concentration lethal to 50% of test organisms) would significantly decrease survival of amphibian eggs and larvae. I also hypothesized that exposure to sublethal dosages would affect growth, time to metamorphosis, and number of gross deformities.

REFERENCES

1. Philips K. 1990. Where have all the frogs and toads gone? *Bioscience* 40:422-424.
2. Stebbins RC, Cohen NW. 1995. *A Natural History of Amphibians*. Princeton University Press, Princeton, NJ, USA.
3. Baker, B. 1999. New federal task force tackles amphibian troubles. *Bioscience* 49:366.
4. Houlahan J, Findlay CS, Schmidt B, Meyer A, Kuzmin SL. 2000. Quantative evidence for global amphibian population declines. *Nature* 404: 752-755.
5. Corn PS. 2000. Amphibian declines: Review of some current hypotheses. In Sparling DW, Linder G, and Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, USA, pp 663-696.
6. Murphy JE, Philips CA, Beasley VR. 2000. Aspects of amphibian ecology. In Sparling DW, In Sparling DW, Linder G, and Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, USA, pp 141-178.

7. Carey C, Bryant CJ. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environ Health Perspect* 103 (Suppl 4):13-17.

8. Cooke AS. Tadpoles as indicators of harmful levels of pollution in the field. *Environ Pollut* 25:123-133.

9. Vitt LJ, Caldwell JP, Wilbur HM, Smith DC. 1990. Amphibians as harbingers of decay. *Bioscience* 40:243-250.

10. Dierauf LA. 1999. Animals as environmental sentinels of human pollution. *Current topics in Veterinary Public Health.* American Veterinary Medical Association Convention, New Orleans, LA, USA, July 13, 1999.

11. Linder, G, Grillitsch B. 2000. Ecotoxicology of metals. In Sparling DW, Linder G, and Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles.* Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, USA, pp 325-459.

12. Burton TM, Likens GE. 1975. Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. *Copeia* 1975:541-546.

13. Barinaga, M. 1990. Where have all the froggies gone? *Science* 247:1033-1034.

14. Lambert, M. 1997. Effects of pesticides on amphibians and reptiles in Sub-Saharan Africa. *Rev Environ Contam Toxicol* 150:31-73.

15. Halliday, T. 1998. A declining amphibian conundrum. *Nature* 394:418-419.

16. Adams, M. 1999. Correlated factors in amphibian declines: exotic species and habitat change in western Washington. *J Wildl Manag* 63:1162-1171.

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

18. Anderson RJ, Prahlad KV. 1976. The deleterious effects of fungicides and herbicides on *Xenopus laevis* embryos. *Arch Environ Contam Toxicol* 4:312-323.

19. Judd, FW. 1977. Toxicity of monosodium methanearsonate herbicide to Couch's spadefoot toad, *Scaphiopus couchi. Herpetologica* 33:44-46.

20. Berrill M, Bertman S, McGillivray L, Kolohon M, Pauli B. 1994. Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. *Environ Toxicol Chem* 13:657-664.

21. Materna E, Rabeni CF, LaPoint TW. 1995. Effects of the synthetic pyrethroid insecticide, Esfenvalerate, on larval leopard frogs (*Rana* spp.). *Environ Toxicol Chem* 14:613-622.

22. Boone MD, Bridges CM. 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). *Environ Toxicol Chem* 18:1482-1484.

23. Bridges CM. 1999. Conservation-Effects of a pesticide on tadpole activity and predator avoidance behavior. *J Herpetol* 33:303.

24. Mann RM, Bidwell JR. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch Environ Contam Toxicol* 36:193-199.

25. Cooke AS. 1977. Effects of field applications of the herbicides Diquat and Dichlobenil on amphibians. *Environ Pollut* 12:43-50.

26. Baker K. 1985. Laboratory and field experiments on the responses by two species of woodland salamanders to malathion-treated substrates. *Arch Environ Contami Toxicol* 14:685-691.

27. Ingermann RL, Bencic DC, Eroschenko VP. 1997. Methoxychlor alters hatching and larval startle response in the salamander *Ambystoma macrodactylum*. *Bull Environ Contamin Toxicol* 59:815-821.

28. Julian SE. 2000. Effects of Chronic Pesticide Exposure on Larval Amphibians. Frostburg State University, Frostburg, MD, USA.

29. Sparling DW, Bishop CA, Linder G. 2000. The current status of amphibian and reptile ecotoxicological research. In Sparling DW, Linder G, and Bishop CA, eds, *Ecotoxicology of Amphibians and Reptiles*. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, FL, USA, pp 1-13.

30. Harris ML, Bishop CA, Struger J, Ripley B, Bogart JP. 1998. The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II Effects of pesticides and eutrophic conditions on early life stage development. *Environ Toxicol Chem* 17:1351-1368.

31. Cole EM, McComb WC, Newton M, Chambers CL, Leeming JP. 1997. Response of amphibians to clearcutting, burning, and glyphosate application in the Oregon Coast Range. *J Wildl Manag* 61:656-664.

32. Lautenschlager RA, Bell FW, Wagner RG, Reynolds PE. 1998. The Fallingsnow Ecosystem Project: Documenting the consequences of conifer release alternatives. *J For* 96:20-27.

33. Bidwell JR, Gorrie JR. 1995. Acute toxicity of herbicide to selected frog species. Final Report, Western Australian Department of Environmental Protection, Perth, Australia.

34. Perkins MJ, Boermans H, Stephenson GR. 1999. Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay – *Xenopus* (FETAX). *Environ Toxicol Chem* 19:940-945.