AN ABSTRACT OF THE DISSERTATION OF

Lindsay M. Biga for the degree of Doctor of Philosophy in Environmental Science presented on November 5, 2013.

Title: Effects of Pesticides on Amphibians with Special Reference to the Pyrethroid Insecticide, Cypermethrin

Abstract approved:

Andrew R. Blaustein

Pollution by pesticides is a ubiquitous concern for wildlife. The effects of pesticides are especially concerning in aquatic environments, which are particularly vulnerable as they have several exposure routes for the influx of chemicals. These effects are of particular concern as biodiversity loss reaches unprecedented rates. This includes recent declines of amphibian populations and loss of amphibian species. Pesticide exposure may contribute to some amphibian populations and extinctions. This dissertation explores the effects of pesticides on amphibians, with particular reference to the pyrethroid insecticide cypermethrin.

I first synthesized acute toxicity data of pesticides for amphibians from the literature (Chapter 2). Using 96h LC50 values for amphibian larvae exposed to insecticides, herbicides, and fungicides, I determined that amphibian species cannot be systematically classified as sensitive or tolerant to pesticides. Rather, relative acute lethal toxicities varied among species and between chemicals and types of pesticides

even within a species, indicating that no amphibian species from this analysis can represent anticipated toxic effects to all amphibians.

I next examined the differences in sensitivity to cypermethrin among three developmental stages for three anuran (frog) species (Chapter 3). I experimentally exposed embryos, recently hatched larvae and larvae with limb buds to cypermethrin in the laboratory. Exposure to cypermethrin had lethal and sublethal effects on some species and stages, but not all. The Pacific treefrog (*Pseudacris regilla*) was the most sensitive species and the early larval stage was the most sensitive for these three species. Additionally, cypermethrin exposure induced abnormal behavior in response to prodding for some species and stages. These results indicate differences in sensitivity to cypermethrin among species of amphibians.

To further understand differences in sensitivity to pesticides among amphibians, I compared how three populations of *P. regilla* responded to cypermethrin exposure (Chapter 4). I performed a time-to-death assay in the laboratory, exposing newly hatched larvae from each population to cypermethrin under identical conditions. All populations had high rates of mortality when exposed to cypermethrin compared to unexposed controls and populations varied in the time to death. Moreover, exposed individuals were smaller than unexposed controls. Interestingly, population sensitivity did not appear to be associated with proximity to agriculture, but rather with elevation gradient, indicating that an evolved tolerance to pesticide exposure is not the mechanism for the differences in sensitivity observed here.

To investigate the effects of cypermethrin on amphibians within a community context, I studied exposure in mesocosms (Chapter 5). I set up semi-natural aquatic ponds in enclosures that contained zooplankton, phytoplankton, and periphyton along with amphibian larvae. I experimentally exposed these mesocosms to cypermethrin and measured the effects on the amphibians and the other members of the community. Cypermethrin affected the entire aquatic community, even at the lowest dose, indicating the direct and indirect deleterious effects of cypermethrin on both invertebrate and vertebrate species in an aquatic community.

This dissertation provides needed data on the effects of the pyrethroid insecticide cypermethrin on amphibians and their communities. Understanding the toxicity of newer, commonly used pesticides to non-target organisms is important as conservation biologists and managers make efforts to combat future amphibian population declines which may be associated with chemicals in the environment.

Effects of Pesticides on Amphibians with Special Reference to the Pyrethroid Insecticide, Cypermethrin

by
Lindsay M. Biga

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APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Lindsay M. Biga, Author

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CHAPTER 1: GENERAL INTRODUCTION

Pollution due to pesticide use is a global concern. Tens of thousands of chemicals are regularly released into the environment with only partially understood effects (EPA 2004, Groner and Relyea 2011). Aquatic systems are particularly vulnerable to the influx of pesticides through aerial drift and run-off events. These systems provide habitat for aquatic mammals, water birds, fishes, amphibians, aquatic plants, insects, zooplankton, and phytoplankton. Though much effort has been made to understand the effects of pesticides and other pollutants on non-target organisms, a great degree of variation in effects can be observed that is not predicted by experiments with model species (Boone and James 2005). Meanwhile, pesticides have been associated with population declines in sensitive species, including amphibians (Sparling et al. 2001, Davidson 2004). Amphibians are of particular interest as they are important consumers and prey in aquatic communities (Wells 2007). Amphibians influence the habitats in which they live by affecting sediment dynamics, primary productivity, algal community composition, invertebrate grazers and predators (Blaustein et al. 2011). However, they are one of the most threatened taxa in the current biodiversity loss, with about 1910 of the 6,312 known amphibian species in danger of extinction (Stuart et al. 2005, McCallum 2007, IUCN 2012).

My dissertation examined the effects of pesticides on amphibians. In Chapter 2, I reviewed the current literature to compare the effects of insecticides, herbicides and fungicides among species of amphibians. This review offers a comprehensive view of the lethality of pesticides, including agricultural and non-agricultural chemicals, to amphibians. I compared published data of acute lethal toxicity values (specifically 96h LC50) for amphibian larvae. However, I found insufficient data on the effects of many pesticides, particularly newer classes of pesticides. Still, these results suggest that amphibian larvae cannot be systematically classified as sensitive or tolerant to pesticide exposure. Rather, relative toxicity varies among species, between chemicals and types of pesticides even within a species.

The remainder of my thesis focused on the specific effects of the pyrethroid insecticide cypermethrin (a chemical for which data on amphibian toxicity is insufficient) on amphibians under a variety of contexts. Pyrethroid insecticides are pesticides of particular concern due to their effects to non-target organisms in aquatic systems, including amphibians (Coats et al. 1989, Greulich and Pflugmacher 2003). Pyrethroids are used extensively in agricultural and non-agricultural settings as they are extremely potent neurotoxins at low concentrations. Pyrethroids, including cypermethrin, are favored over the organochlorine and organophosphate insecticides that they are replacing due to their low persistence in the environment and relatively low toxicity to mammals (Berrill et al. 1993, Moore and Waring 2001). Cypermethrin is registered for a number of uses. Agricultural uses include the treatment of insect

pests of cotton, pecans, sweet corn, lettuce, broccoli as well as pests of cattle and other livestock (EPA 2008). A wide range of non-agricultural uses including control of ants, cockroaches, fleas, and termites in indoor and outdoor structural and perimeter applications make up the majority (750,000 pounds per year) of cypermethrin use (EPA 2008).

In Chapter 3, I examined differences in species sensitivity and developmental stages of amphibians to cypermethrin in a series of laboratory experiments. Throughout their life cycle, amphibians undergo a series of developmental changes that may alter their response to chemical exposure (Berrill et al. 1994). Moreover, species differ from one another in their morphology and life history; these differences may result in differences in sensitivity to chemicals (Relyea and Hoverman 2006). Thus, exposure to cypermethrin may affect amphibians differently when comparing exposure across different developmental stages and among speices.

In Chapter 4, I investigated the effects of cypermethrin on several populations of the Pacific treefrog (*Pseudacris regilla*). While many populations of amphibians have experienced declines or extinctions, others persist (Boone and James 2005). Population level variation in sensitivity to pesticides may be due to differences in acquired tolerance from historical exposure or from adaptations to local geographic conditions (Bridges and Semlitsch 2000, Cothran et al. 2013). I experimentally exposed Pacific treefrog larvae from three populations to cypermethrin under identical

laboratory conditions. I then documented differences in growth, rates of mortality and time to death.

Finally, in Chapter 5, I utilized outdoor mesocosms to examine the effects of cypermethrin in a community context. Because cypermethrin readily partitions to sediment, vegetation and other organic matter, it has been assumed that this will reduce bioavailability to the extent that effects to aquatic organisms in natural environments will be negligible (Hill et al. 1989). To test this assumption, I experimentally examined the direct and indirect effects of cypermethrin on amphibian larvae, zooplankton, periphyton and phytoplankton in a simulated natural community.

My dissertation provides needed data on the effects of the pyrethroid insecticide cypermethrin on amphibians. Understanding the toxicity of newer, commonly used pesticides to non-target organisms is important as conservation biologists and managers make efforts to combat future amphibian population declines which may be associated with chemicals in the environment.

CHAPTER 2

Acute lethal toxicity of pesticides to amphibian larvae: A review

Lindsay M. Biga and Andrew R. Blaustein

Abstract

The release of pesticides into the environment for both agricultural and non-agricultural purposes is contributing to unprecedented rates of biodiversity loss. Non-target organisms in aquatic systems are particularly vulnerable to influxes of pesticides. Although they are not the most sensitive aquatic species, amphibians are of particular interest due to recent population declines, some of which have been associated with pesticides. However, the effects of pesticides vary among species of amphibians. We synthesized published acute lethal concentrations of pesticides to amphibian larvae to determine whether sensitivity differs systematically or randomly between species of amphibians for a variety of types and forms of pesticides. We found that amphibian species cannot simply be classified as sensitive or tolerant to insecticides, herbicides or fungicides. Rather, relative acute lethal toxicities varied among species and between chemicals and types of pesticides even within a species. Moreover, different formulations of a single active ingredient varied greatly in their toxicity among species and individual species did not demonstrate consistent sensitivity across formulations. Continued examination of the effects of pesticides on a variety of amphibian species and examination of the effects of both commercial formulations and technical grade pesticides is critical to fully understand amphibian pesticide toxicity. The accumulation of more amphibian toxicity data is critical to the conservation of sensitive amphibian species.

Introduction

In the face of unprecedented rates of biodiversity loss (Wilson 1992, Wake & Vredenburg 2008), human impact through the introduction of chemical stressors into the environment is of increasing concern (Relyea et al. 2005). While several factors clearly contribute to population declines and species loss, habitat destruction and degradation is the major underlying cause (Fahrig 2001), and the use of pesticides has paralleled habitat loss in recent history (EPA 1997). Agricultural practices account for nearly two-thirds of total pesticide expenditures, though industrial, forestry and residential uses of pesticides contribute greatly to the ubiquitous presence of these stressors in the environment (EPA 2011). Non-target organisms inhabiting freshwater ecosystems are vulnerable to the toxic effects of both agricultural and non-agricultural pesticides through numerous pathways, including direct application for pest control, overspray, aerial drift, runoff events, leaching and sediment deposition (Boone et al. 2005).

Although other freshwater taxa, particularly invertebrates, are certainly more sensitive to pesticides (Kerby et al. 2010), amphibians are of interest as they are one of the most threatened taxa in the current biodiversity crisis (Stuart 2004; IUCN 2012). Moreover, some amphibian population declines have been associated with pesticide use (Sparling et al. 2001, Davidson 2004). However, toxicological data are limited for amphibians, in large part because standardized toxicity tests with amphibian species are lacking (Hoke & Ankley 2005). Weltje and colleagues (2013) offer several

explanations for the dearth of amphibian testing: the use of surrogate species assumed to be comparable in sensitivity, ethical concerns regarding toxicity testing with vertebrates, and the absence of standard guidelines for amphibian testing. Consequently, in spite of their vulnerability to chemical contaminants and important roles in their communities (Wells 2007, Blaustein et al. 2011), risks to amphibians have not been routinely incorporated in ecological risk assessments that include assays with other freshwater aquatic species (Fort & Stover 1996a). Understanding the relative sensitivity of amphibian species to pesticide toxicity may contribute to amphibian conservation efforts worldwide to develop a specific amphibian risk assessment approach (Weltje et al. 2013).

The effects of pesticides on amphibians at environmentally relevant concentrations include mortality, altered growth and development, anatomical deformities, behavioral abnormalities, and increased susceptibility to disease (reviewed by Mann et al. 2009). However, not all species of amphibians exhibit equal effects of chemicals, as differences in life history strategies and morphology affect sensitivity (Berrill et al 1994, Rogers 1997, Yu et al. 2013). Still, most studies are limited to the effects of a single chemical on a single species (Relyea 2004). Further, a single species may be particularly sensitive to pesticides of one class, and relatively tolerant to pesticides from another (Kerby et al. 2010). This may be a result of acquired resistance to one class due to historical exposure (Cothran et al. 2013, Hua et al. 2013), or alternatively may result from traits acquired due do local geographic

conditions (Bridges & Semlitsch 2000). Additionally, pesticides are applied in a vast array of forms. Technical grade pesticides are not typically used in practice, yet are often used for toxicity testing. Though some commercial formulations vary little in terms of acute toxicity from their technical grade active ingredient (Bringolf et al. 2007), formulations often include inert ingredients, like surfactants, that affect the toxicity of these chemicals (Williamson et al. 1989, Puglis & Boone 2011). In one study, LC50 values varied by three orders of magnitude depending on the glyphosate product tested (Mann & Bidwell 1999).

Several reviews have been published on the effects of different aspects of amphibian toxicology. Some of these reviews thoroughly examine specific aspects of amphibian pesticide toxicology (e.g. ultraviolet radiation and toxic chemicals (Blaustein et al. 2003), agrochemicals (Mann et al. 2009), and pesticide mixtures (Hayes et al. 2006)). Others have compared amphibian sensitivity to other taxa (e.g. Kerby et al. 2010, Weltje et al. 2013), or conducted meta-analyses on the topic (Egea-Serrano et al. 2012, Baker et al. 2013). Our objective was to synthesize published acute lethal concentrations of pesticides to amphibian species in a comprehensive review including both agricultural and non-agricultural pesticides. Though similar comprehensive reviews have compared the effects of pesticides among amphibian species, it has been over a decade since their publication (Cowman & Mazanti 2000, Sparling 2000). An increase in amphibian toxicological testing over the past decade has greatly increased data available in the literature for such comparative purposes.

Our goal was to determine whether sensitivity differs systematically or randomly between species of amphibians for a variety of types (insecticides, herbicides and fungicides) and forms (commercial formulations and technical grade) of pesticides.

Materials and Methods

We focused on larval stages of amphibians, as the number of embryo and adult amphibian studies was inadequate; comparisons between three or more species could not be made for adults with any chemicals and could only be made for embryos with two fungicides and three herbicides. Moreover, newly hatched amphibian larvae are generally more sensitive to pesticides than embryos and advanced stages (Berrill et al. 1997). We compared data collected by a multitude of researchers using standard acute lethal toxicity studies (96h). These studies employ a dose-response curve method to estimate the concentration of a chemical required to kill 50% of the individuals exposed (this concentration is generally referred to as the LC50) and LC50s provide the best measure for comparisons for both chemicals and species (Kerby et al. 2010).

Acute toxicity data for amphibian larvae were obtained (August 2013) from the U.S. EPA ECOTOX database (http://cfpub.epa.gov/ecotox/). We initially extracted all studies on chemicals for which 96h LC50 values were available in ECOTOX for larvae of more than one amphibian species from freshwater laboratory experiments to allow for comparisons of sensitivity between species. Only data from ECOTOX that reported numerical values (as opposed to graphical representations of LC50 values)

were included. When more than one 96h LC50 value was available for the same chemical and species, all values were included. Only 96h LC50 values from the direct effects of the chemical of interest alone were included; we did not include values indicating acute lethal toxicity of exposure to multiple chemicals simultaneously or chemicals in combination with other stressors (e.g. predation, UV radiation). From all studies on chemicals obtained, we limited chemicals to those defined as fungicides, insecticides and/or herbicides by the PAN Pesticides Database (http://www.pesticideinfo.org) according to CAS number. Chemicals were included regardless of current U.S. Environmental Protection Agency restrictions on their use (e.g. DDT) as these chemicals are still in use in other countries and can persist in the environment long after their use is restricted (reviewed by Purnomo et al. 2011). After identification for inclusion, we obtained LC50 values directly from reference texts to verify that all criteria were met and to determine whether the chemical used in the experiment was of technical grade or a commercial formulation. Consequently, values were only included from peer-reviewed texts that could be obtained through the Oregon State University Libraries, its Interlibrary Loan system, or freely on the Internet.

Results

We collected and synthesized the results from 44 survival studies (references in Tables 2.2, 2.3 and 2.4) that included data from 35 amphibian species: 33 anurans

(frogs and toads) and two caudates (salamanders). Based on these data, 96h LC50 comparisons of amphibian larvae were made for 31 chemicals, of which 12 were insecticides, 14 were herbicides and 5 were fungicides (Table 2.1). Differences were seen in toxicity of commercial formulations and technical grade pesticides. Moreover, individual pesticides varied greatly in their toxicity among amphibian species (Tables 2.2, 2.3 and 2.4). Additionally, though data were not available for each chemical with all species, where comparisons could be made, species varied in their relative sensitivity to different pesticides.

Technical grade pesticides vs. commercial formulations

Technical grade insecticides generally displayed similar toxicity to commercial formulations in general (Figure 2.1). However, individual species varied in their relative sensitivity to different insecticide formulations. For example, while the Pacific treefrog, *Pseudacris regilla*, was by far the least sensitive species to the azinphos-methyl formulation Guthion, it was relatively quite sensitive to another formulation, Guthion 2S (Figure 2.1).

Unlike insecticides, technical grade herbicides and their commercial formulations did not display similar levels of toxicity (Figure 2.2). While the atrazine formulation Atrazine 4L and the paraquat formulation Gramoxone were less toxic than their technical grade active ingredient alone, all four glyphosate commercial formulations were more toxic than technical grade glyphosate (Figure 2.2). Among

species similar differences in relative toxicity of glyphosate formulations were found. Though the bell frog, *Litoria moorei*, was more sensitive to Roundup 360 than the sign-bearing froglet, *Crinia insignifera*, it was less sensitive than *C. insignifera* to technical grade glyphosate (Figure 2.2).

Insecticide toxicity

Acute lethal toxicity of insecticides ranged from 0.00021 to 55.34 mg/L (Table 2.2). Endrin was the most toxic insecticide analyzed, though its toxicity varied 1,000-fold across species assayed (Table 2.2). A commercial formulation of carbaryl was the least toxic insecticide analyzed (Table 2.2). By class, organochlorines were the most toxic insecticides, and organophosphorus (OPs) and carbamate insecticides were less toxic (Table 2.1). No single species was uniquely tolerant or sensitive to insecticide toxicity. In fact, relative sensitivities varied greatly among insecticides. For example, Fowler's toad, *Anaxyrus fowleri*, were extremely sensitive to both the azinphos-methyl formulation Guthion and technical grade malathion, yet they were among the least sensitive species to dieldrin and endrin (Table 2.2). Likewise the western chorus frog, *P. triseriata* were the most sensitive species to lindane and malathion, yet they were among the most tolerant to endrin and dieldrin. While the differences in sensitivity for *A. fowleri* correlate with chemical class, the same is not true for *P. triseiata.*

Herbicide toxicity

Acute lethal toxicity of herbicide exhibited a much smaller range than insecticide toxicity, from 0.53 mg/L to 453 mg/L (Table 2.3). Butachlor was the most toxic and DSMA was the least toxic of the herbicides analyzed (Table 2.2). Because nearly every herbicide analyzed represents a unique chemical class (except for three chemical classes which contain two herbicides), among class comparisons were not informative (Table 2.1). Several acute toxicity estimates were found for both the green frog, *Lithobates clamitans*, and the northern leopard frog, *L. pipiens*, with the glyphosate formulation Vision. Variation within these estimates for each species was more than 10-fold (Table 2.3). Further, *L. pipiens* was less sensitive than the American toad, *A. americanus*, the only other species assayed with the atrazine formulation Atrazine 4L and the alachlor formulation Alachlor EC (Figures 2.2 and 2.4). However, *L. pipiens* was the most sensitive species to the triclopyr formulation Release (Figure 2.4). Similarly, the ornate narrow-mouthed toad larvae, *Microhyla ornata*, were the most sensitive species to butachlor, yet they were the most tolerant species to zinc sulfate (Figure 2.4).

Fungicide toxicity

Acute lethal toxicity of fungicides ranged from 0.01844 mg/L to 123 mg/L (Table 2.3). Pentachlorophenol was the most toxic and sodium arsenite was the least toxic of the fungicides analyzed (Table 2.3). As only five fungicides in three chemical

classes were analyzed, comparisons between chemical classes are not possible (Table 2.1). Species exhibited more consistent relative sensitivities to fungicides than to insecticides and herbicides. For example, the Indian green frog, *Euphlyctis hexadactylus* and the Asian common toad, *Duttaphrynus melanostictus,* were relatively sensitive to each of the 3 fungicides for which they were assayed (Figure 2.5, Table 2.4). Comparably, *M. ornata* were relatively tolerant to both of the fungicides for which they were assayed (Figure 2.5).

Comparative sensitivity of Xenopus laevis across pesticides types

More acute lethal toxicity estimates (18 total) were found in our analysis for larvae of the African clawed frog, *Xenopus laevis*, than the other species (Tables 2.2, 2.3 and 2.4). *X. laevis* ranged in their sensitivity to insecticides. While they were among the most sensitive to the azinphos-methyl formulation Guthion 2S and technical grade cabaryl, they were the least sensitive species to technical grade chlorpyrifos and were moderately sensitive to dieldrin and the azinphos-methyl formulation Guthion (Figures 2.1 and 2.3). However, *X. laevis* were more consistently sensitive to herbicides and fungicides. They demonstrated relative high sensitivity to herbicides with both the glyphosate formulation Vision and the triclopyr formulation Release (Figures 2.2 and 2.4). For fungicides, *X. laevis* were moderately sensitive as demonstrated with copper sulfate and pentachlorophenol (Figure 2.5).

Discussion

Our synthesis found that amphibian larvae cannot be systematically classified as sensitive or tolerant to pesticide exposure as a group. Relative acute lethal toxicities varied among species and between chemicals and types of pesticides even within a species. Finally, different formulations of a single active ingredient varied greatly in their toxicity among species and individual species did not demonstrate consistent sensitivity across formulations.

Our synthesis of data from commercial formulations and their technical grade active ingredients indicate a great deal of variation among these pesticide formulations to amphibian species, as has been documented (Williamson et al. 1989, Mann & Bidwell 1999, Bringolf et al. 2007, Puglis & Boone 2011). The effects of inert ingredients on a formulation's toxicity are complex, in some cases increasing toxicity while decreasing it in other cases. Yet manufacturers are not required to list the inert ingredients, posing difficulty to the task of assessing toxicity of these ingredients to non-target organisms (Puglis & Boone 2011). Still, it is critical that the effects of commercial formulations as well as their active ingredients are understood to aid in our ability to predict effects of pesticides on natural populations.

Previous efforts have been made to identify particularly sensitive and tolerant amphibian species in regards to pesticide exposure. For example, Berrill and colleagues (1997) assessed the relative sensitivity of six amphibian species across eight pesticides. They determined that the northern leopard frog, L. *pipiens,* and the

American toad, *A. americanus*, are likely to be less sensitive than the green frog, *L. clamitans*, and the bullfrog, *L. catesbeiana.* While our results aligned with this finding in terms of relative sensitivity of these particular species to the insecticide dieldrin, we found the opposite to be true in terms of sensitivity to Release and Vision, the commercial formulations of the herbicides triclopyr and glyphosate respectively. We found similar inconsistencies in tolerance for insecticides and herbicides. While we found consistent relative sensitivities to fungicides, this result was only represented by comparisons between either 2 or 3 chemicals and may not hold true if additional chemicals are analyzed. The effects evoked by chemicals across a variety of classes with numerous modes of action may be too different to systematically identify species as sensitive or tolerant to pesticide exposure.

Based on its use in a common test, the frog embryo teratogenesis assay-*Xenopus* (FETAX), more data are available for *X. laevis*, the South African clawed frog, than for most other species. Consequently, some have proposed that it should be routinely used in ecological risk assesments (Hoke and Ankley 2005). However, representative species should have comparable sensitivity to the species that the represent (Weltje et al. 2013). In our analysis, we found that while *X. laevis* were consistently sensitive to herbicides and moderately sensitive to fungicides, their sensitivity to insecticides varied widely. This inconsistency may pose a challenge in using *X. laevis* as a surrogate species for toxicity testing.

Pesticides are a ubiquitous stressor in aquatic environments due to the over 5 billion pounds of active ingredient used globally each year in agricultural, residential sites, industrial sites, forestry, and other settings (EPA 2011). Moreover, frequent, substantial applications throughout the planting season coincide with rainy weather that can contribute to increased leaching and runoff of pesticides into aquatic habitats (Boone et al. 2005). This timing also coincides with the amphibian breeding season, increasing the risk of exposure of the particularly sensitive larval amphibian stage to pesticides (Blaustein & Wake 1995). Though pesticide concentrations in aquatic environments are often lower than laboratory derived LC50 values (Davidson 2004), direct lethal effects of environmentally relevant concentrations have been observed (Boone 2008). Still, as a group, amphibians are not generally included in ecological risk assessments (Fort & Stover 1996a). However, the variation in sensitivity among chemicals found in our synthesis indicates that selecting a representative model amphibian species for toxicity testing would be challenging.

This synthesis demonstrates the variation in pesticide toxicity among species and the variation within species to the effects of difference pesticides. This outcome suggests a continued need for the examination of the effects of pesticides on a variety of amphibian species. As no single species could be identified as particularly sensitive, data from multiple species are important to provide a more complete understanding of pesticide toxicity to amphibians. Additionally we argue for a need to examine the effects of both commercial formulations and technical grade pesticides. The

accumulation of more amphibian toxicity data is critical to the conservation of sensitive amphibian species. However, data continues to be limited.

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Table 2.1 Summary of pesticides, including historical and current major uses (ordered from most to least common) and modes of action, organized by chemical class.

Pesticide	Chemical	Major Use	Action
Insecticides:			
Carbamate	Carbaryl	Agriculture, rangeland, residential	Acetylcholinesterase inhibitor
Organochlorine	DDD	Public health, agriculture	Sodium channel modulator
	DDT	Public health, agriculture	Sodium channel modulator
	Dieldrin	Agriculture, industrial sites, public health	GABA-gated chloride channel antagonist
	Endosulfan	Agriculture	GABA-gated chloride channel antagonist
	Endrin	Agriculture	GABA-gated chloride channel antagonist
	Lindane	Agriculture, industrial sites, public health	GABA-gated chloride channel antagonist
	Toxaphene	Agriculture	Contact action
Organophosphorus	Azinphos-methyl	Agriculture, residential	Acetylcholinesterase inhibitor
	Chlorpyrifos	Agriculture, golf courses, industrial sites, public health	Acetylcholinesterase inhibitor

	Diazinon	Agriculture, residential	Acetylcholinesterase inhibitor
	Malathion	Agriculture, residential	Acetylcholinesterase inhibitor
Herbicides:			
Benzoic acid	Dicamba	Agriculture, golf courses, residential	Plant growth modifier
Bipyridylium	Paraquat	Agriculture, residential, forestry	Photosynthesis inhibitor
Chloroacetanilide	Alachlor	Agriculture	Mitosis & cell division inhibitor
	Butachlor	Agriculture	Mitosis & cell division inhibitor
Chlorophenoxy acid	Silvex	Agriculture, rangeland, aquatic sites	Plant growth modifier
	2,4,5-T, dimethylamine salt	Agriculture, rangeland, residential	Plant growth modifier
Chloropyridinyl	Triclopyr	Pasture & rangeland, forestry	Plant growth modifier
Inorganic	Ammonium sulfate	Agriculture	Contact action
	Zinc sulfate	Residential, industiral sites	Protein & organix acid binder

Organoarsenic	DSMA	Agriculture, golf courses, residential	Cell membrane disruptors
Phosphonoglycine	Glyphosate	Agriculture, forestry, industrial sites, residential, aquatic sites	Essential amino acid synthesis inhibitor
Pyridinecarboxylic acid	Picloram	Rangeland, forestry	Plant growth modifier
Triazine	Atrazine	Agriculture	Photosynthesis inhibitor
unclassified	Dalapon	Agriculture, residential, industrial sites	Lipid synthesis inhibitor
Fungicides:			
Chlorinated Phenol	Pentachlorophenol	Wood preservation	Oxidative phosphorylation uncoupler
Inorganic	Copper Sulfate	Agriculture	Multiple sites of action
	Mercury chloride	Agriculture	Unknown
	Sodium arsenite	Agriculture	Unknown
Organomercury	Methylmercury chloride	Agriculture	Enzyme inhibitors

Table 2.2 Lethality (96h LC50 in mg/L) of insecticides to amphibian larvae. Confidence intervals are include when provide in the text of the original source. Technical grade product (TGP) and unspecified commercial forms (CF) abbreviated.

Insecticide	Species	Compound	LC50	(95% CI)	Reference
Azinphos-methyl	*Anaxyrus fowleri*	Guthion	0.13	(0.05-0.33)	Sanders 1970
	Xenopus laevis	Guthion	2.94	(2.30-3.77)	Schuytema et al. 1995
	Pseudacris regilla	Guthion	4.14		Schuytema et al. 1995
	Xenopus laevis	Guthion 2S	0.42	(0.38-0.46)	Schuytema et al. 1995
	Pseudacris regilla	Guthion 2S	0.46	(0.39-0.55)	Schuytema et al. 1995
	Xenopus laevis	Guthion 2S	0.59	(0.43-0.80)	Schuytema et al. 1995
	Pseudacris regilla	Guthion 2S	0.84	(0.72-0.97)	Schuytema et al. 1995
	Pseudacris regilla	Guthion 2S	1.47		Nebeker et al. 1998
	Ambystoma gracile	Guthion 2S	1.67		Nebeker et al. 1998
	Ambystoma maculatum	Guthion 2S	1.9		Nebeker et al. 1998
Carbaryl	*Hoplobatrachus tigerinus*	CF	6.2		Marian et al. 1983
	Hoplobatrachus tigerinus	CF	6.3		Marian et al. 1983
	Hoplobatrachus tigerinus	CF	11.7		Marian et al. 1983
	Euphlyctis hexadactylus	CF	55.34	(42.73-69.07)	Khangarot et al. 1985c
	Rana boylii	TGP	0.58493		Kerby 2006
	Xenopus laevis	TGP	1.73	(1.31-2.16)	Zaga et al. 1998
	Hyla versicolor	TGP	2.47	(1.76-3.19)	Zaga et al. 1998
	Pseudacris regilla	TGP	3.00651		Kerby 2006
	Hoplobatrachus tigerinus	TGP	5.68		Sampath et al. 2002
	Lithobates sphenocephalus	TGP	8.4	(7.4-9.6)	Bridges et al. 2002
	Rana clamitans	TGP	11.32	(10.42-12.29)	Boone & Bridges 1999
	Anaxyrus boreas	TGP	12.3	(10.3-14.7)	EPA 1999

	Lithobates clamitans	TGP	17.36	(16.24-18.56)	Boone & Bridges 1999
	Lithobates clamitans	TGP	22.02	(20.62-23.52)	Boone & Bridges 1999
	Rhinella arenarum	TGP	24.64	(17.68-34.77)	Ferrari et al. 2004
Chlorpyrifos	*Rhinella fernandezae*	Lorsban 48E	0.151	(0.133-0.168)	Ruiz de Arcaute et al. 2012
	Rhinella fernandezae	Lorsban 48E	0.293	(0.248-0.337)	Ruiz de Arcaute et al. 2012
	Pseudacris regilla	TGP	0.122		Kerby 2006
	Rana boylii	TGP	0.205		Kerby 2006
	Rana dalmatina	TGP	5.174	(4.537-5.919)	Bernabo et al. 2011
	Xenopus laevis	TGP	14.6	(10.8-19.7)	Richards & Kendall 2002
DDD	*Anaxyrus fowleri*	TGP	0.14	(0.1-0.21)	Sanders 1970
	Pseudacris triseriata	TGP	0.40	(0.21-0.75)	Sanders 1970
DDT	*Pseudacris triseriata*	TGP	0.8	(0.5-2.3)	Sanders 1970
	Anaxyrus fowleri	TGP	1.0	(0.02-3.6)	Sanders 1970
Diazinon	*Rana boylii*	TGP	1.71455		Kerby 2006
	Pseudacris regilla	TGP	3.43354		Kerby 2006
	Rana boylii	TGP	7.488		Sparling & Fellers 2007
Dieldrin	*Pseudacris triseriata*	TGP	0.0001	(0.00003-0.00028)	Sanders 1970
	Anaxyrus fowleri	TGP	0.00015	(0.00002-0.00047)	Sanders 1970
	Lithobates catesbeiana	TGP	0.0087	(0.0078-0.0097)	Schuytema et al. 1991
	Lithobates catesbeiana	TGP	0.0303	(0.0262-0.0352)	Schuytema et al. 1991
	Xenopus laevis	TGP	0.0404	(0.0346-0.0471)	Schuytema et al. 1991

	Xenopus laevis	TGP	0.0495	(0.0333-0.0734)	Schuytema et al. 1991
	Lithobates pipiens	TGP	0.0713	(0.0713-0.0713)	Schuytema et al. 1991
Endosulfan	*Hoplobatrachus tigerinus*	TGP	0.0018	(0.0014-0.0022)	Gopal et al. 1981
	Bufo bufo	TGP	0.430		Bernabo et al. 2008
Endrin	*Euphlyctis hexadactylus*	TGP	0.00021	(0.000153-0.000285)	Khangarot et al 1985c
	Lithobates catesbeiana	TGP	0.0025		Thurston et al 1985
	Anaxyrus fowleri	TGP	0.12		Sanders 1970
	Pseudacris triseriata	TGP	0.18	(0.09-0.50)	Sanders 1970
Lindane	*Pseudacris triseriata*	TGP	2.7	(1.4-4.3)	Sanders 1970
	Euphlyctis hexadactylus	TGP	3.97	(3.35-4.48)	Khangarot et al. 1985c
	Anaxyrus fowleri	TGP	4.4	(1.8-5.6)	Sanders 1970
	Microhyla ornata	TGP	7.27	(5.640-9.364)	Pawar & Katdare 1987
Malathion	*Pseudacris triseriata*	TGP	0.2	(0.09-0.27)	Sanders 1970
	Anaxyrus fowleri	TGP	0.42	(0.09-0.98)	Sanders 1970
	Rana boylii	TGP	2.137		Sparling & Fellers 2007
	Rhinella arenarum	TGP	19.2		Venturino et al. 1992
	Pelophylax ridibundus	TGP	38	(35.11-48.25)	Sayim 2008
Toxaphene	*Anaxyrus fowleri*	TGP	0.14	(0.06-0.35)	Sanders 1970
	Pseudacris triseriata	TGP	0.50	(0.10-1.1)	Sanders 1970

Table 2.3 Lethality (96h LC50 in mg/L) of herbicides to amphibian larvae. Confidence intervals are included when provided in the text of the original source. Technical grade product (TGP) and unspecified commercial form (CF) abbreviated.

Herbicide	Species	Compound	LC50	(95% CI)	Reference
Ammonium sulfate	*Pseudacris regilla*	TGP	115.3	(93.3-142.5)	Schuytema & Nebeker 1999
	Xenopus laevis	TGP	135.0	(119.2-152.9)	Schuytema & Nebeker 1999
Alachlor	*Anaxyrus americanus*	Alachlor EC	3.3	(2.8-3.6)	Howe et al. 1998
	Lithobates pipiens	Alachlor EC	3.5	(3.1-3.8)	Howe et al. 1998
	Anaxyrus americanus	Alachlor EC	3.9	(3.7-4.2)	Howe et al. 1998
	Lithobates pipiens	Alachlor EC	11.5	(10.1-13.2)	Howe et al. 1998
Atrazine	*Anaxyrus americanus*	Atrazine 4L	10.7	(9.2-12.5)	Howe et al. 1998
	Lithobates pipiens	Atrazine 4L	14.5	(11.9-17.5)	Howe et al. 1998
	Anaxyrus americanus	Atrazine 4L	26.5	(23.0-30.5)	Howe et al. 1998
	Lithobates pipiens	Atrazine 4L	47.6	(41.4-54.8)	Howe et al. 1998
	Pseudacris regilla	TGP	1.68608		Kerby 2006
	Rana boylii	TGP	5.5169		Kerby 2006
	Rhinella arenarum	TGP	27.16	(26.01-28.35)	Brodeur et al. 2009
Butachlor	*Microhyla ornata*	CF	0.53	(0.45-0.61)	Geng et al. 2005
	Bufo gargarizans	CF	0.76	(0.47-0.97)	Yin et al. 2008
	Fejervarya multistriata	CF	1.30	(1.16-1.45)	Geng et al. 2005
	Polypedates megacephalus	CF	1.52	(1.23-1.87)	Geng et al. 2005
Dalapon	*Limnodynastes peronii*	Dowpon	2.0		Johnson 1976
	Adelotus brevis	Dowpon	4.2		Johnson 1976

Dicamba	*Limnodynastes peronii*	Banex	106		Johnson 1976
	Adelotus brevis	Banex	185		Johnson 1976
DSMA	*Limnodynastes peronii*	TGP	271		Johnson 1976
	Adelotus brevis	TGP	453		Johnson 1976
Glyphosate	*Scinax nasicus*	Glyfos	2.64	(2.19-2.84)	Lajmanovich et al. 2003
	Lithobates clamitans	Glyfos	28.6	(27.6-29.6)	Howe et al. 2004
	Litoria moorei	Roundup 360	7.6	(6.1-9.6)	Bidwell & Gorrie 1995
	Crinia insignifera	Roundup 360	39.7	(31.1-50.5)	Bidwell & Gorrie 1995
	Lithobates clamitans	Roundup Original	6.5	(6.0-7.0)	Howe et al. 2004
	Lithobates pipiens	Roundup Original	9.2		Howe et al. 2004
	Lithobates sylvaticus	Roundup Original	16.5	(15.7-17.4)	Howe et al. 2004
	Xenopus laevis	Vision	0.88	(0.84-0.92)	Edgington et al. 2004
	Lithobates pipiens	Vision	1.1	(0.96-1.14)	Edgington et al. 2004
	Lithobates clamitans	Vision	1.4	(1.2-1.7)	Edgington et al. 2004
	Anaxyrus americanus	Vision	1.7	(1.5-1.9)	Edgington et al. 2004
	Lithobates pipiens	Vision	1.8	(1.5-2.2)	Edgington et al. 2004
	Xenopus laevis	Vision	2.1	(2.0-2.7)	Edgington et al. 2004
	Anaxyrus americanus	Vision	2.9	(2.3-10.5)	Edgington et al. 2004
	Lithobates clamitans	Vision	3.5	(3.0-4.6)	Edgington et al. 2004
	Lithobates clamitans	Vision	8.71	(6.65-11.8)	Wojtaszek et al. 2004
	Lithobates pipiens	Vision	13.7	(7.90-22.9)	Wojtaszek et al. 2004
	Lithobates clamitans	Vision	14.0	(9.84-19.4)	Wojtaszek et al. 2004
	Lithobates pipiens	Vision	37.0	(30.6-46.8)	Wojtaszek et al. 2004
	Crinia insignifera	TGP	78.0	(62.9-96.6)	Bidwell & Gorrie 1995
	Litoria moorei	TGP	110.8	(95.2-128.4)	Bidwell & Gorrie 1995

Paraquat	*Limnodynastes peronii*	Gramoxone	100		Johnson 1976
	Adelotus brevis	Gramoxone	262		Johnson 1976
	Bufo gargarizans	TGP	20.1	(11.5-27.8)	Yin et al. 2008
	Anaxyrus fowleri	TGP	26.0	(11.0-43)	Sanders 1970
	Pseudacris triseriata	TGP	28.0	(21-36)	Sanders 1970
Picloram	*Adelotus brevis*	Tordon 50-D	95		Johnson 1976
	Limnodynastes peronii	Tordon 50-D	105		Johnson 1976
Silvex	*Limnodynastes peronii*	Fenoprop	22		Johnson 1976
	Rhinella marina	Fenoprop	34		Johnson 1976
	Adelotus brevis	Fenoprop	54		Johnson 1976
Triclopyr	*Lithobates pipiens*	Release	0.79	(0.75-0.93)	Edginton et al. 2003
	Lithobates pipiens	Release	0.87	(0.73-1.0)	Edginton et al. 2003
	Anaxyrus americanus	Release	0.88	(0.78-0.99)	Edginton et al. 2003
	Xenopus laevis	Release	1.0	(0.75-1.2)	Edginton et al. 2003
	Xenopus laevis	Release	1.7	(1.4-2.1)	Edginton et al. 2003
	Anaxyrus americanus	Release	2.1	(1.6-7.0)	Edginton et al. 2003
	Lithobates pipiens	Release	6.29	(4.71-9.33)	Wojtaszek et al. 2005
	Lithobates clamitans	Release	6.78	(5.52-8.86)	Wojtaszek et al. 2005
	Lithobates pipiens	Release	7.42	(5.82-10.1)	Wojtaszek et al. 2005
	Lithobates clamitans	Release	11.5	(10.1-13.2)	Edginton et al. 2003
	Lithobates clamitans	Release	18.2	(16.1-21.7)	Edginton et al. 2003
Zinc Sulfate	*Euphlyctis hexadactylus*	TGP	2.1	(1.69-3.03)	Khangarot et al. 1985a
	Duttaphrynus melanostictus	TGP	19.86	(17.68-23.90)	Khangarot & Ray 1987

	Microhyla ornata	TGP	22.41	(22.0-22.8)	Jayaprakash Rao & Madhyastha 1987
	Microhyla ornata	TGP	23.08	(22.6-23.4)	Jayaprakash Rao & Madhyastha 1987
2,4,5-T, dimethylamine	*Limnodynastes peronii*	Farmco TA-20	169		Johnson 1976
	Adelotus brevis	Farmco TA-20	200		Johnson 1976
	Rhinella marina	Farmco TA-20	340		Johnson 1976

Table 2.4 Lethality (96h LC50 in mg/L) of fungicides to amphibian larvae. Confidence intervals are included when provided in the text of the original source. Technical grade product (TGP) and unspecified commercial form (CF) abbreviated.

Fungicide	Species	Compound	LC50	(95% CI)	Reference
Copper sulfate	*Duttaphrynus melanostictus*	TGP	0.032		Khangarot & Ray 1987
	Euphlyctis hexadactylus	TGP	0.039	(0.034-0.043)	Khangarot et al. 1985a
	Epidalea calamita	TGP	0.11	(0.08-0.14)	Garcia-Munoz et al. 2009
	Anaxyrus boreas	TGP	0.12	(0.07-0.18)	EPA 1999
	Xenopus laevis	TGP	0.15	(0.10-0.20)	Fort & Stover 1996b
	Lithobates sphenocephalus	TGP	0.23	(0.21-0.25)	Bridges et al. 2002
	Xenopus laevis	TGP	0.42	(0.40-0.44)	Fort & Stover 1996b
	Xenopus laevis	TGP	1.08	(1.02-1.14)	Fort & Stover 1996b
	Microhyla ornata	TGP	5.04	(4.7-5.1)	Jayaprakash Rao & Madhyastha 1987
	Microhyla ornata	TGP	5.38	(5.0-5.8)	Jayaprakash Rao & Madhyastha 1987
Mercury chloride	*Duttaphrynus melanostictus*	TGP	0.0436	(0.0368-0.0585)	Khangarot & Ray 1987
	Euphlyctis hexadactylus	TGP	0.051	(0.033-0.053)	Khangarot et al. 1985b
	Microhyla ornata	TGP	0.1184		Ghate & Mulherkar 1980
	Duttaphrynus melanostictus	TGP	0.25	(0.219-0.285)	Paulose 1988
	Sphaerotheca rolandae	TGP	0.28	(0.250-0.320)	Paulose 1988
	Microhyla ornata	TGP	1.12	(0.9-1.3)	Jayaprakash Rao & Madhyastha 1987
	Microhyla ornata	TGP	1.43	(1.0-1.8)	Jayaprakash Rao & Madhyastha 1987

Pentachlorophenol	*Euphlyctis hexadactylus*	TGP	0.01844	(0.01476-0.02402)	Khangarot et al. 1985c
	Xenopus laevis	TGP	0.05	(0.02-0.08)	Fort & Stover 1996b
	Xenopus laevis	TGP	0.12	(0.08-0.16)	Fort & Stover 1996b
	Lithobates sphenocephalus	TGP	0.14	(0.12-0.17)	Bridges et al. 2002
	Xenopus laevis	TGP	0.35	(0.33-0.37)	Fort & Stover 1996b
	Anaxyrus boreas	TGP	0.37	(0.25-0.42)	EPA 1999
Methylmercury chloride	*Duttaphrynus melanostictus*	TGP	0.07	(0.057-0.085)	Paulose 1988
	Sphaerotheca rolandae	TGP	0.075	(0.067-0.084)	Paulose 1988
Sodium arsenite	*Limnodynastes peronii*	Arzeen	60		Johnson 1976
	Adelotus brevis	Arzeen	96		Johnson 1976
	Rhinella marina	Arzeen	123		Johnson 1976

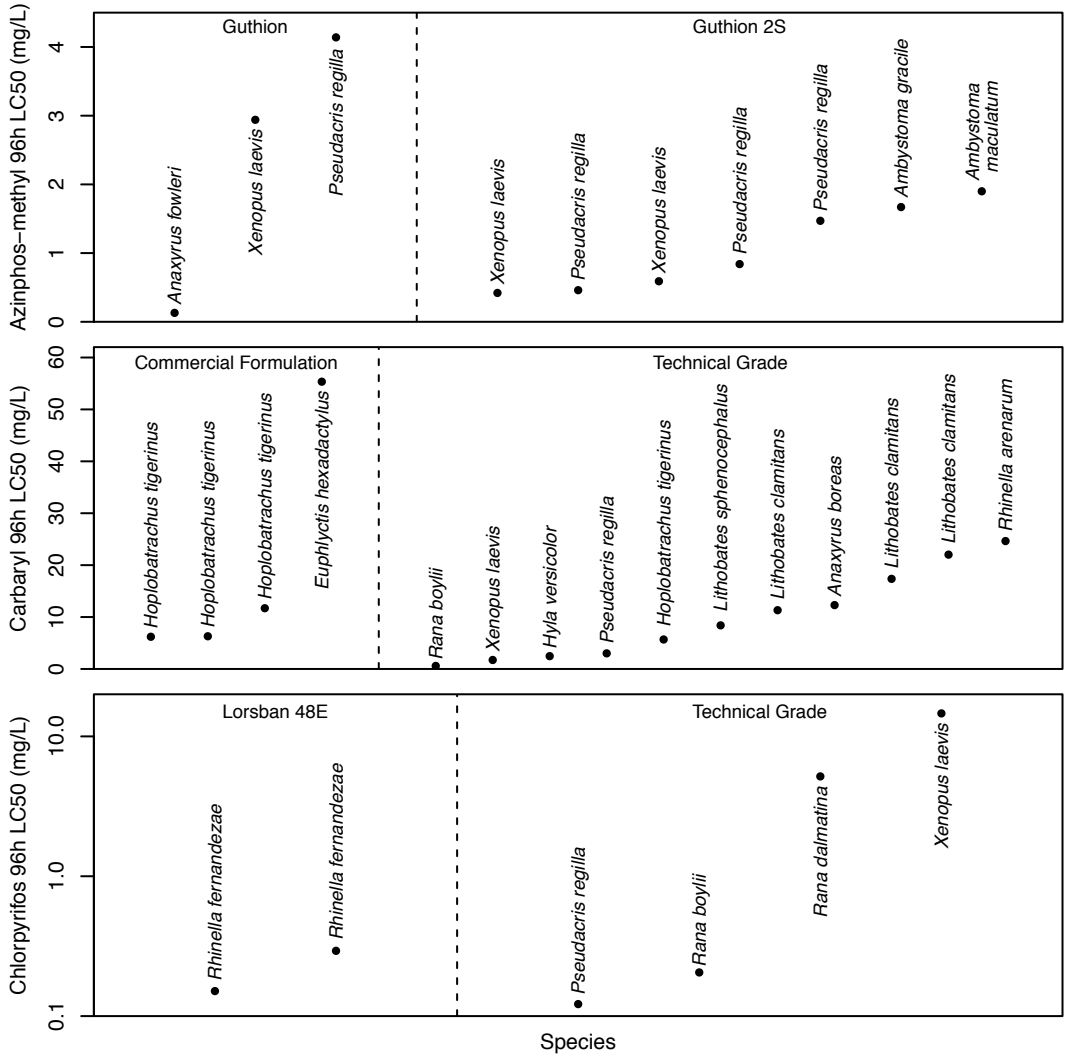

Figure 2.1 Comparative 96h median lethal concentrations (LC50) for amphibian larvae exposed to commercial formulation and/or technical grade insecticides azinphos-methyl (formulations: Guthion, Guthion 2S), carbaryl (formulation: not specified) and chlorpyrifos (formulation: Lorsban 48E).

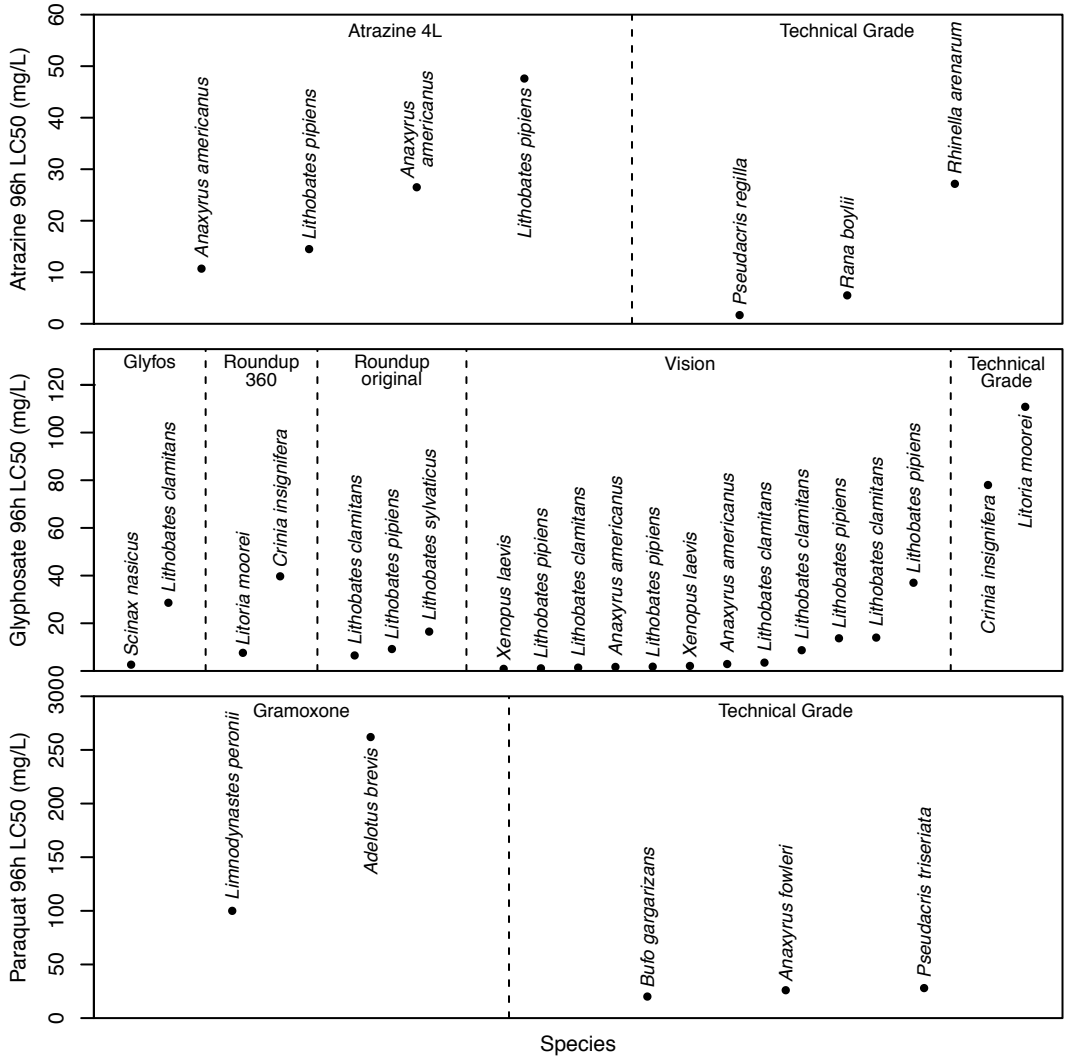

Figure 2.2 Comparative 96h median lethal concentrations (LC50) for amphibian larvae exposed to commercial formulation and technical grade herbicides atrazine (formulation: Atrazine 4L), glyphosate (formulations: Glyfos, Roundup 360, Roundup Original, Vision) and paraquat (formulation: Gramoxone).

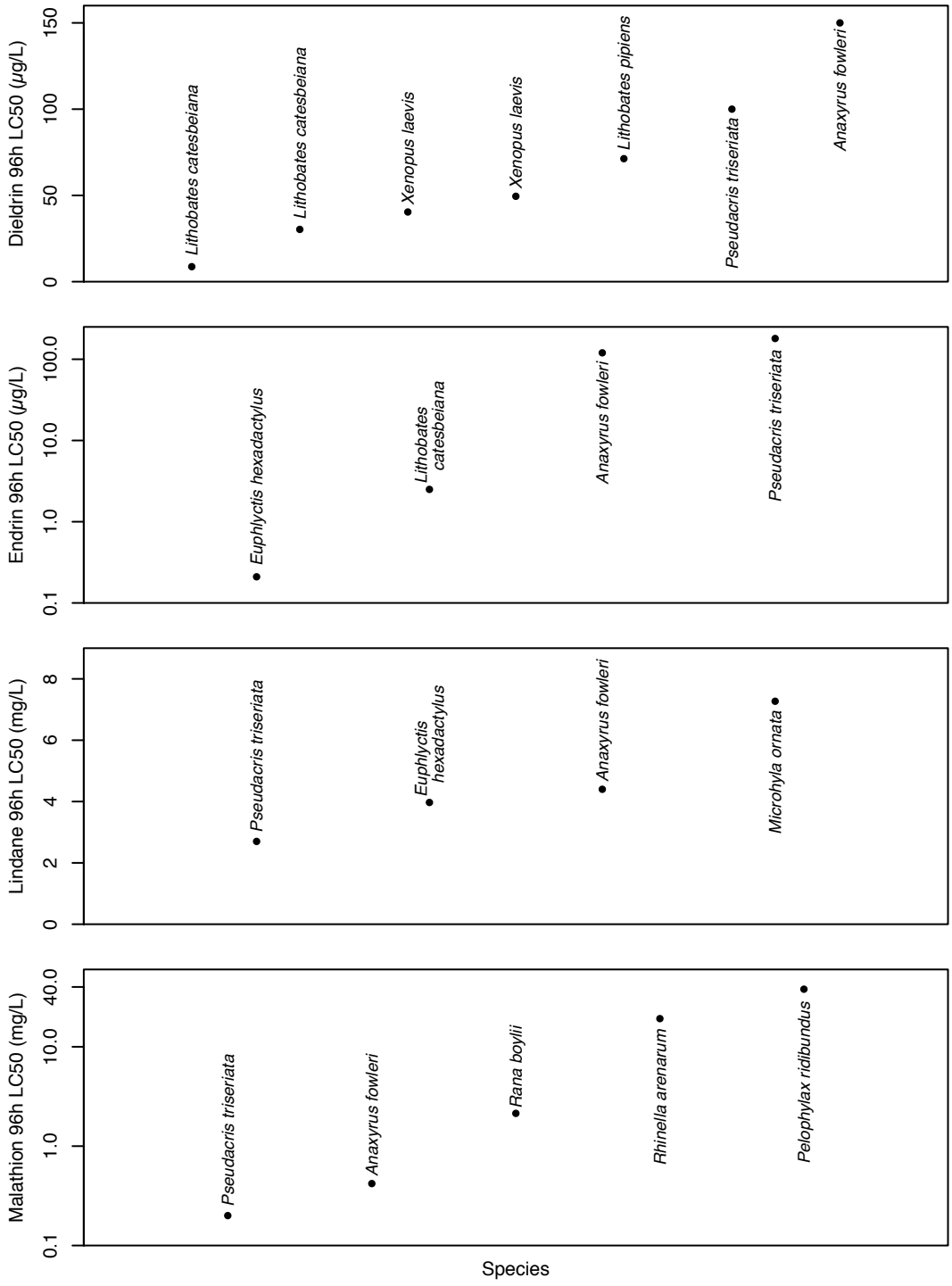

Figure 2.3 Comparative 96h median lethal concentrations (LC50) for amphibian larvae exposed to technical grade insecticides dieldrin, endrin, lindane, and malathion.

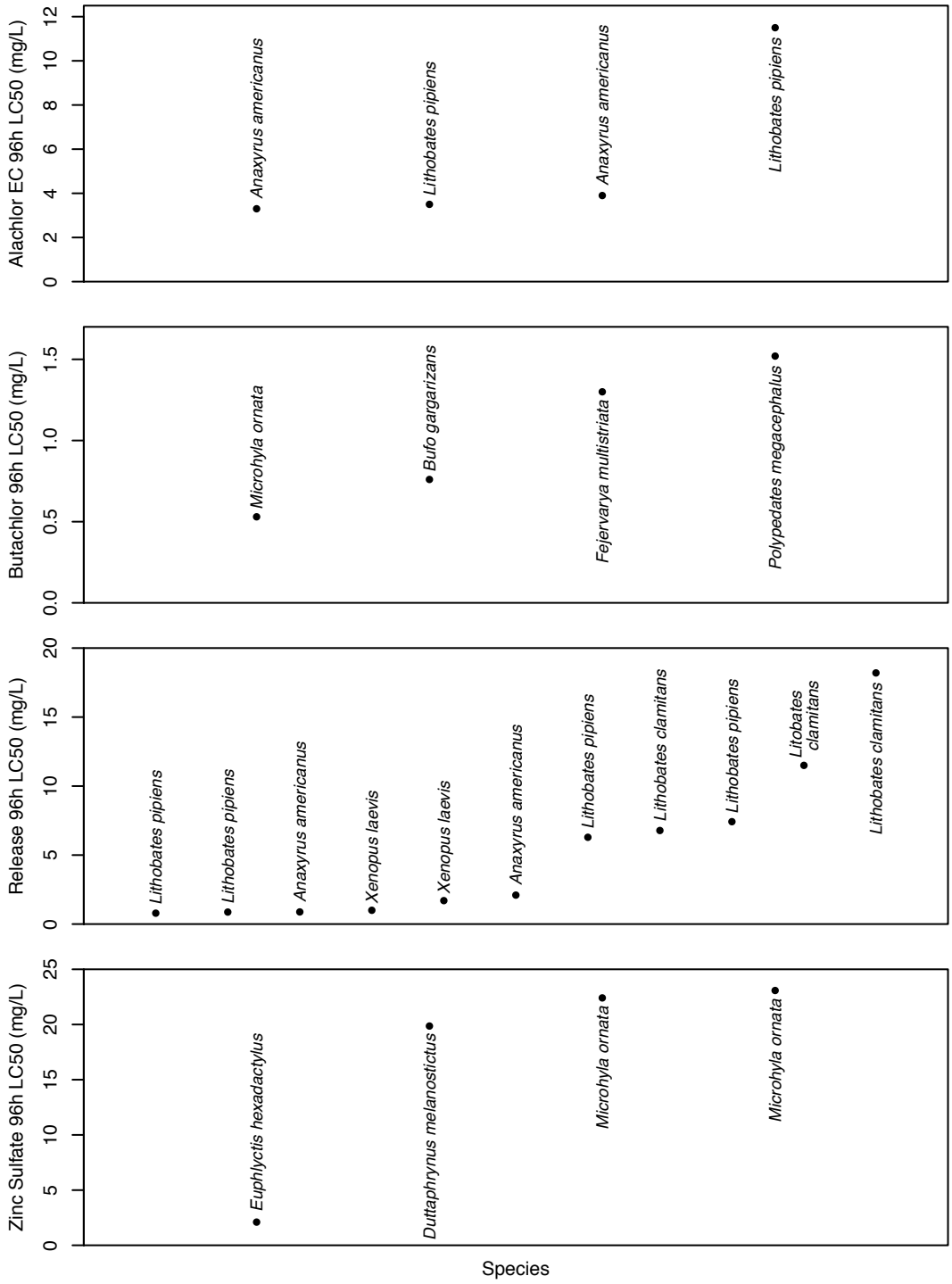

Figure 2.4 Comparative 96h median lethal concentrations (LC50) for amphibian larvae exposed to the herbicides alachlor (formulation: Alachlor EC), butachlor, triclopyr (formulation: Release), and Zinc Sulfate.

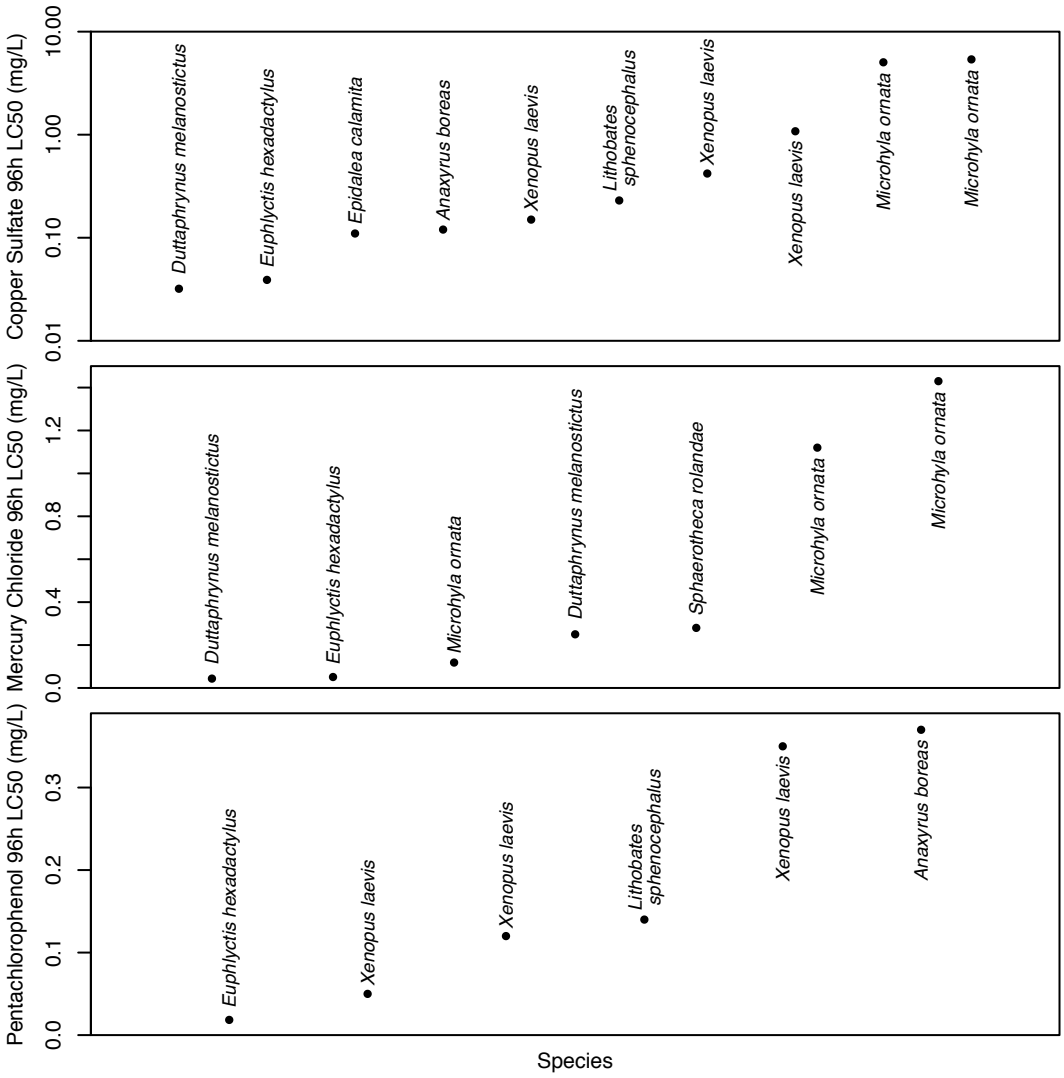

Figure 2.5. Comparative 96h median lethal concentrations (LC50) for amphibian larvae exposed to technical grade fungicides copper sulfate, mercury chloride, and pentachlorophenol.

CHAPTER 3

Variations in lethal and sublethal effects of cypermethrin among aquatic stages and species of anuran amphibians.

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Abstract

Unpredicted variation in levels of risk to organisms from xenobiotics can be observed, despite the use of model species to predict the effects of chemicals in the environment. Physiological and morphological differences between species and life stages may lead to differences in sensitivity, while seasonal and spatial variation in pesticide concentrations may affect the level of risk faced by organisms in the environment. Because anurans breed in aquatic habitats subject to contamination by run-off and spraying they are particularly vulnerable to pesticides. We exposed embryos, newly hatched larvae, and larvae with limb buds of three anuran amphibian species, *Pseudacris regilla*, *Rana cascadae* and *R. aurora*, for 48h to either 0.5 or 5.0 µg/L cypermethrin under laboratory conditions. We monitored hatching success, larval survival, and measured growth. Additionally, we assayed avoidance behavior 2wk after exposure, or 2wk after hatching for individuals exposed as embryos. Hatching and survival were not affected in animals of any species exposed as embryos. However, after exposure as embryos and as newly hatched larvae, *P. regilla* displayed behavioral abnormalities in response to prodding. Cypermethrin increased mortality in *P. regilla* exposed in both larval stages. Cypermethrin also increased mortality in larval *R. cascadae* when exposed at the early stage larvae. These results indicate variation in sensitivity to environmentally relevant concentration of cypermethrin among anuran species and life stages.

Introduction

Due to the over 5 billion pounds of active ingredient used globally each year in agricultural, residential, commercial, industrial and forest settings (EPA 2011), pesticides have become ubiquitous in the environment. Much effort has been made to understand the human health impacts and ecological effects of environmental pollutants, and pesticides in particular. Yet a great degree of variation in the effects of contaminants not predicted by dose response tests with model species can be observed in natural systems (Boone and James 2005). This may be due in part to the use of models that neglect certain ecologically relevant characteristics of different species (Stark et al. 2004, Relyea and Hoverman 2006). For example, sensitivity to chemicals may differ with life history strategies or across developmental stages (Berrill et al. 1994, Rogers 1997, Yu et al. 2013). Additionally, chemicals are not homogenous in the environment; they vary over temporal and spatial scales (Bridges and Semlitsch 2000). Thus we should expect to see variation in risk to animals exposed to environmental contaminants when spatial and temporal considerations are included. Additional work is needed to understand differences in responses to contaminants across species and stages.

Among pesticides, environmental contamination by pyrethroid insecticides is of growing concern. The restriction of the organophosphate (OP) insecticides chlorpyrifos and diazinon for residential pest control has turned users to synthetic pyrethroids as a class over the past two decades (Amweg et al. 2005, Weston et al 2005, Saha and Kaviraj 2008). Pyrethroids are favored over the OP insecticides that

they are replacing for their low persistence in the environment and relatively low toxicity to mammals (Berrill et al. 1993, Moore and Waring 2001). Pyrethroids are present in over 3,500 registered products in the U.S. alone (EPA 2012). Approximately one million pounds of active ingredient of the cyano pyrethroid insecticide cypermethrin is used in U.S. agricultural and non-agricultural settings annually (EPA 2008) and it is used extensively in other countries as well (Jergentz et al. 2005, Kim et al. 2008, Ghodageri and Pancharatna 2011). Agricultural uses include the treatment of insect pests of cotton, pecans, sweet corn, lettuce, and broccoli as well as pests of cattle and other livestock (EPA 2008). A wide range of non-agricultural uses including control of ants, cockroaches, fleas, and termites in indoor and outdoor structural and perimeter applications make up the majority (750,000 pounds annually) of cypermethrin use (EPA 2008). Although 75% of cypermethrin use is non-agricultural, due to the difficulties posed in modeling these uses, risk assessments by the EPA have only included uses on agricultural crops (EPA 2008). These assessments therefore do not include the potential effects of runoff from impervious surfaces after application in urban and industrial settings.

Pyrethroids are neurotoxins that disrupt sodium channels of nerve cells leading to repetitive firing of neurons (Soderlund and Bloomquist 1989). Despite their relative insolubility and low persistence in water, all pyrethroids are sufficiently soluble to cause adverse effects to aquatic organisms and their lipophilicity allows pyrethroids to be readily absorbed by biological membranes and tissues leading to high toxicity in non-target organisms (Oros and Werner 2005). For example, cypermethrin is

considered by the United States Environmental Protection Agency to be very highly toxic on an acute basis to marine and freshwater invertebrates and fishes, and to honeybees (EPA 2008). Moreover, evaluations in California after runoff events indicate that pyrethroids, including cypermethrin, are found at concentrations acutely toxic to invertebrates (test species *Hyalella azteca*) in urban streams (Weston et al. 2009). Despite its relatively low water solubility (4-10 µg/L), cypermethrin has been detected at levels ranging from 100–1010 µg/L in surface water (Crossland 1982, Crossland et al. 1982, Marino and Ronco 2005), and at lower levels (0.02-2.6 µg/L) in subsurface waters (Day 1989, Jergentz et al. 2005).

Understanding the toxicological effects of pesticides in animals is particularly urgent as conservation biologists are documenting population declines in birds, reptiles, amphibians and other taxa (Lawton and May 1995, Wake and Vredenburg 2008). As aquatic breeding organisms, amphibian eggs and larvae are particularly vulnerable to chemicals in their environments (Blaustein et al. 2003). Moreover, they are among the most threatened taxa in the current biodiversity loss, with approximately 1910 of 6,312 amphibian species in danger of extinction (Stuart et al. 2004, McCallum 2007, IUCN 2012) and some of these declines have been associated with pesticide use (Sparling et al. 2001, Davidson 2004). Environmentally relevant concentrations of pesticides cause adverse effects in amphibians that include altered growth and development, anatomical deformities and behavioral abnormalities, and mortality (reviewed by Mann et al. 2009). However, differences in morphology and life history among species may make amphibians differently sensitive to pollutants.

For example, breeding phenology and rate of development can influence the chance of exposure to a pesticide and exposure period.

In most taxa, the earliest life stage is considered the most sensitive, though in organisms with protective eggs like amphibians, the earliest free living (larval) stage is often more sensitive to environmental stressors (Berrill et a. 1993). Traditionally, the egg stage of aquatic animals has been considered robust, as the jelly protects them from a broad range of external disturbances (Marquis et al. 2006). However, the extent to which the jelly coat surrounding amphibian eggs protects the developing embryo from a chemical is strongly dependent on both the chemical and the species examined (Greulich and Pflugmacher 2004). Embryos are likely exposed to environmental pollutants as the jelly is filled with water shortly after being laid (Marquis et al. 2006). Moreover, uptake of waterborne contaminants has been observed in anuran eggs (Greulich and Pflugmacher 2004).

In a comparative study using three species of anuran amphibians (*Pseudacris regilla*, the Pacific treefrog; *Rana cascadae*, the Cascades frog; *R. aurora*, the northern red-legged frog), we tested the effects of cypermethrin exposure on embryos and larvae. We first assessed the effects of environmentally relevant concentrations of cypermethrin on individuals exposed as embryos, and then tested its effects on larval stages. We monitored hatching success, larval survival, and sublethal effects including growth and abnormal avoidance behaviors. We chose to assay avoidance behavior because lacking the appropriate avoidance response may reduce anti-predator and foraging success (Berrill et al. 1993). Additionally, we measured growth because

slowed growth may impair an individual's ability to metamorphose or could result in smaller size at metamorphosis (Altwegg and Reyer 2003). We made several predictions for this experiment. First, we predicted that species would differ in sensitivity to cypermethrin. We anticipated that *P. regilla* would be least sensitive as it is a generalist species that has persisted in urban and agricultural landscapes, as compared to *R. aurora* and *R. cascadae*, species with smaller ranges that have experienced population declines. Second, we predicted that sensitivity would vary depending on the timing of exposure, with newly hatched larvae exhibiting the greatest degree of sensitivity while embryos and larvae with limb buds exhibit less sensitivity. Finally, we predicted that sublethal effects of environmentally relevant concentrations of cypermethrin would impact ecologically relevant characteristics like behavior and size.

Materials and Methods

Test Species

We conducted experiments using three anuran species (*P. regilla*, *R. cascadae*, *R. aurora*) from egg masses collected within 48 hrs after they were laid in ponds in the Willamette Valley, Cascade Mountains and Coast Range of Oregon respectively during the spring and summer of 2009 and 2010. In Oregon, USA, *R. cascadae*, breed explosively in montane areas in March through July metamorphosing within 1-3 months (Jones et al. 2005). *R. aurora* breed in December through February along the coast and up to some Western mid-elevation sites with metamorphosis usually

occurring in 6-8 months (Jones et al. 2005). *P. regilla* are widespread throughout the Western US and Canada, primarily breeding in January and February (though later for higher elevation populations) and larvae typically metamorphose within 2-3 months (Jones et al. 2005).

Embryos were brought into the laboratory for rearing in 38 L tanks of aerated dechlorinated water. After hatching, larvae were fed a 3:1 mixture (by weight) of rabbit chow and fish flakes until 48h prior to initiation of the experiment. Animals were maintained in a controlled laboratory environment at 14°C, the average ambient temperature for test species, on a natural light:dark photoperiod. To examine variation in sensitivity to cypermethrin, animals were tested at three stages (Gosner 1960); embryo (stage 10-12), larvae <1wk after hatching (stage 24-25, hereafter "early larvae"), and larvae with limb buds (stage 28-30, hereafter "late larvae"). At the conclusion of the experiment, animals were anesthetized with buffered MS-222 and preserved in 95% EtOH. Due to differences in breeding phenology, species were not tested at the same time. However, all species were tested using the same methods in the same laboratory.

Cypermethrin exposure

Immediately before each experiment, fresh 100 mL stock solutions of 100 ppm cypermethrin (nominal concentration) were prepared by dissolving 99.5% standards-grade cypermethrin (ChemService) into a carrier solution of 10 mL HPLC grade acetone and 90 mL deionized water. Though analytical chemistry was not performed

on the stock solutions or exposure solutions used in the present study, cypermethrin stock solutions made with the same method in the same laboratory from an earlier experiment were analyzed at the Mississippi State Chemical Laboratory using GC/ECD. Actual concentrations of those stock solutions were 53.0, 62.1 and 67.2% of nominal concentrations. Serial dilutions (10, 1.0 and 0.10 ppm) were made from the stock solution and nominal test concentrations of 5.0 and 0.5 µg/L cypermethrin (hereafter high and low respectively) were made by adding 5 mL of the appropriate dilution to test beakers containing 1000 mL dechlorinated water. Acetone in the cypermethrin treatments did not exceed a concentration of 0.05mL/L, well below the limit of 0.1 mL/L recommended by the International Standard Organization (ISO 2012) for tests involving invertebrates. This level of acetone had no effect on amphibian embryos and larvae in our pilot experiments and was consequently not added to the controls. Exposure to cypermethrin occurred for 48h; treatments were randomly assigned to experimental units and each treatment was replication five times.

Embryo exposure

Embryos were exposed to cypermethrin for 48h in groups of ten in 1L glass beakers containing 1L of test solution. After exposure, the embryos were transferred to 1L glass beakers containing fresh dechlorinated water. They were maintained in these containers until hatching. As individuals hatched, they were transferred to 600 mL glass beakers containing 500 mL fresh dechlorinated water where they were

maintained individually as larvae for 2wk after hatching. These individuals were not fed as they maintain their yolk as a food source for some time after hatching (Stebbins and Cohen 1995).

Both hatching success and timing of hatching were determined. After hatching, we monitored mortality of larvae daily for 14d. Avoidance behavior was assessed on the final day of the experiment by prodding each larva gently on the side of the base of its tail. Behavioral abnormalities indicative of cyano-pyrethroid poisoning were recorded as present when we witnessed inactivity, twisting, trembling or weak movement over a short distance (less than 2 cm) in response to prodding (Berrill et al. 1993), while darting away (more than 2 cm) was considered a normal response.

Larval exposure

Early larvae and late larvae were exposed to cypermethrin in 1L glass beakers containing 1L of test solution for 48h. Early larvae were exposed in groups of ten but due to limited number of animals, late larvae were exposed in groups of five. After exposure, each larva was transferred individually to a 600 mL glass beakers containing 500 mL fresh dechlorinated water. Animals were maintained individually for 2wk after exposure and were fed a 3:1 mixture (by weight) of rabbit chow and fish flakes.

We monitored mortality of larvae daily during exposure and for 14d after exposure. Avoidance behavior was assessed on the final day of the experiment using the same methods as in the embryo exposure. At the conclusion of the experiment, body length and mass were measured.

Statistical analyses

We performed statistical analyses in R statistical computing environment (version 2.15.0, The R Foundation for Statistical Computing). Statistical tests were performed within species and within developmental stage. We analyzed both survival and behavior using generalized liner mixed models using a logit link function to determine the effects of cypermethrin treatments. Individuals were nested by exposure group (beaker) for all analyses to avoid pseudoreplication. To test for differences in growth, we performed multivariate ANOVA to allow quantitative partitioning of effects among experimental factors and their interactions.

Results

Embryo Exposure

There was no effect of cypermethrin exposure on survival of animals exposed as embryos for any species (Figure 3.1A, 3.1D, 3.1G). All *R. cascadae* hatched and survived for the duration of the experiment although some mortality (<10%) was seen in the two other species (Figure 3.1A, 3.1D, 3.1G). Additionally, there was no effect of cypermethrin on hatching success for any of the species ($p > 0.05$ for all species). Embryos hatched into larvae 9-13d (*P. regilla*), 12-16d (*R. aurora*) and 2-7d (*R. cascadae*) after exposure began. None of the embryos hatched prior to completion of the 48h exposure and time to hatching was not affected by exposure to cypermethrin for any of the species.

Exposure to the high treatment of cypermethrin in *P. regilla* embryos led to a 19% increase in behavioral abnormalities in response to prodding when compared to controls ($X^2 = 6.57$, $df = 2$, $p < 0.05$). Abnormalities included inactivity, twisting, trembling, or weak movement over a short distance (less than 2cm) all in response to prodding and were consistent with cyano pyrethroid poisoning. Mass and body length were not affected by either cypermethrin treatment in any species ($p > 0.05$, Table 3.1).

Larval Exposure

There was greater mortality of larvae in cypermethrin treatments than in controls (Figure 3.1B, 3.1C, 3.1E), but the effects of cypermethrin differed among species and among stage of exposure. *P. regilla* were the most sensitive to cypermethrin while *R. aurora* were the least sensitive. The high cypermethrin exposure increased mortality of *P. regilla* at the early and late larval stages (Figure 3.1B, 3.1C). In *R. cascadae*, exposure to the high level of cypermethrin increased mortality due to early larval stage exposure but not exposure in the late larval stage (Figure 3.1E). Effects of cypermethrin exposure on survival in *R. aurora* were not statistically significant. However, there was a trend towards decreased survival with cypermethrin exposure in the early larval stage (Figure 3.1H, $X^2 = 5.61$, $df = 2$, $p = 0.06$) and no effect of cypermethrin exposure on survival in the later larval stage.

Cypermethrin exposure in the low treatment led to a 7% increase in abnormal behavioral responses to prodding in *P. regilla* exposed as early larvae as compared to

controls ($X^2 = 7.19$, $df = 2$, $p < 0.05$), but did not affect behavior in the other species when exposed as larvae ($p > 0.05$). All individuals of all three species that were exposed as late larvae exhibited normal responses to prodding. Mass and body length were not affect by either the high or low treatment in any species ($p > 0.05$, Table 3.1).

Discussion

Our study demonstrates (1) that the amphibian species assayed differ in their sensitivity to cypermethrin, (2) that the degree of sensitivity varies with the life stage in which exposure occurred, and (3) cypermethrin exposure of 5.0 µg/L can lead to sublethal effects on ecologically important characteristics of these species. The effects of cypermethrin exposure, particularly at the 5.0 µg/L level, were detected in each of the three species tested and at each of the three developmental stages tested. However, these effects varied by species and life stage. For *P. regilla* exposed as embryos, sublethal effects of cypermethrin exposure were observed, while individuals of the other species appear to have been unaffected by their exposure as embryos. *P. regilla* exhibited the greatest sensitivity to cypermethrin compared to the other species as its effects were present in *P. regilla* after all three exposure time points (behavioral abnormalities for embryo exposure and mortality for both larval stages). *R. cascadae* and *R. aurora* demonstrated increased mortality at only the early larval stage, and no sublethal effects were observed in these species at any stage.

Differences in sensitivity to cypermethrin varied strongly with stage. We observed cypermethrin-induced mortality in the early and later larval stages, but not in

the embryonic stage. This increased sensitivity in the larval stages over the embryonic stages may be due to cypermethrin's action as a neurotoxin; the more developed nervous system of larval individuals may have increased their vulnerability to its effects (Berrill et al. 1993).

Although mortality was not observed in individuals exposed as eggs, sublethal effects were observed due to this exposure that were not present in older anurans. *P. regilla* exposed as embryos displayed behavioral abnormalities (such as inactivity, twisting, trembling or weak movement over a short distance in response to prodding) after hatching. The differences in tolerance to exposure in the embryonic stage may reflect the protective effects of the jelly coat surrounding anuran that others have demonstrated (Berrill et al. 1998, Pauli et al. 1999). This coat, composed of glycoproteins, mucoproteins, carbohydrates and mucopolysaccharids, differs among amphibian species in regards to the number of layers and unique molecular composition (Marquis et al. 2006). It follows that protection by the jelly coat may vary as well. Sensitivity of embryos to exposure likely varies not only by species, as seen in the present study, but also by chemical, as a chemical's ability to penetrate the jelly coat depends on the chemical's composition, as well as the morphology of the jelly coat (Greulich and Pflugmacher 2004).

Further work is needed to understand how cypermethrin might affect amphibians in the field. Others have observed effects of insecticides that appeared to detrimental to amphibians in the lab, but did not find correlating negative long-term consequences in subsequent mesocosm experiments. For instance, Relyea and Mills

(2001) documented an increase in toxicity of pesticides in the presence of predators in the lab, yet this effect has not been demonstrated in mesocosms or in the field, to our knowledge. However, a commercial formulations of permethrin, a synthetic pyrethroid insecticide with the same mode of action as cypermethrin, resulted in 98% mortality of amphibian larvae in one mesocosm experiment, indicating that direct effects of pyrethroids in aquatic systems may be severe (Boone 2008). Consequently, the sublethal effects we documented in the laboratory could have serious long-term consequences for individuals suffering similar effects in the field. Although we did not test pond water from embryo collection sites for cypermethrin, the levels tested in the present study have been observed in the environment by several others (Crossland 1982, Crossland et al. 1982, Day 1989, Jergentz et al. 2005, Marino and Ronco 2005). The behavioral effects we observed, including inactivity, twisting, trembling, or weak movement over a short distance in response to prodding, were obvious signs of cyano pyrethroid poisoning (David et al. 2012). When prodded, the initial response of anuran larvae is typically to dart away (Caldwell et al. 1980). The inability to dart away when prodded may likely to render larvae more vulnerable to predation (Berrill et al. 1993). Additionally, if a behavior is associated with foraging, these behavioral abnormalities could inhibit growth and contribute to reduced reproductive fitness. However, extrapolation to population level effect is inherently challenging, adding another layer of complexity to understanding the full impact of chemicals in the environment (Blaustein et al. 2011).

Despite being one of the most widely used pesticides, the ecological impacts of cypermethrin are not well understood (Kim et al. 2008). The results presented in the present study highlight the importance of multi-species toxicity testing and the importance of evaluating sublethal effects to better understand these impacts. We demonstrated that, at environmental relevant concentrations, cypermethrin induces behavioral abnormalities and death, but that toxicity of cypermethrin varies among amphibian species and among life stage during which exposure occurs. Cypermethrin was more toxic to *P. regilla* than *R. aurora* and *R. cascadae*. Additionally, cypermethrin toxicity was strongest when exposure occurred at the early larval stage. Our results suggest that environmentally relevant concentrations of cypermethrin are capable of causing adverse effects in anurans.

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Table 3.1 Summary of morphometric data (body length in mm and mass in g) from amphibian embryos and larvae exposed to cypermethrin treatments for 3 species. Cypermethrin exposure did not affect growth in any species at any stage ($p > 0.05$).

Stage	Control		0.5 µg/L		5.0 µg/L	
Species	Length ± SE	Mass ± SE	Length ± SE	Mass ± SE	Length ± SE	Mass ± SE
Embryo						
Pseudacris regilla	5.3 ± 0.3	0.032 ± 0.005	5.1 ± 0.2	0.028 ± 0.005	5.0 ± 0.2	0.025 ± 0.003
Rana cascadae	9.2 ± 0.1	0.094 ± 0.003	9.2 ± 0.1	0.093 ± 0.004	9.4 ± 0.2	0.112 ± 0.014
Rana aurora	8.8 ± 0.3	0.095 ± 0.007	8.8 ± 0.2	0.098 ± 0.007	8.8 ± 0.2	0.092 ± 0.006
Newly hatched larvae						
Pseudacris regilla	11.7 ± 0.2	0.269 ± 0.006	12.0 ± 0.1	0.282 ± 0.007	12.2 ± 0.2	0.289 ± 0.013
Rana cascadae	13.3 ± 0.2	0.246 ± 0.011	13.3 ± 0.2	0.241 ± 0.008	12.8 ± 0.7	0.223 ± 0.031
Rana aurora	13.1 ± 0.1	0.298 ± 0.006	12.8 ± 0.3	0.282 ± 0.014	12.6 ± 0.4	0.266 ± 0.022
Larvae with limb buds						
Pseudacris regilla	13.7 ± 0.2	0.460 ± 0.009	14.1 ± 0.4	0.457 ± 0.040	13.4 ± 0.6	0.402 ± 0.051
Rana cascadae	16.8 ± 0.3	0.537 ± 0.021	16.2 ± 0.4	0.504 ± 0.038	16.3 ± 0.2	0.515 ± 0.017
Rana aurora	15.4 ± 0.2	0.502 ± 0.031	15.6 ± 0.3	0.493 ± 0.028	15.7 ± 0.5	0.486 ± 0.043

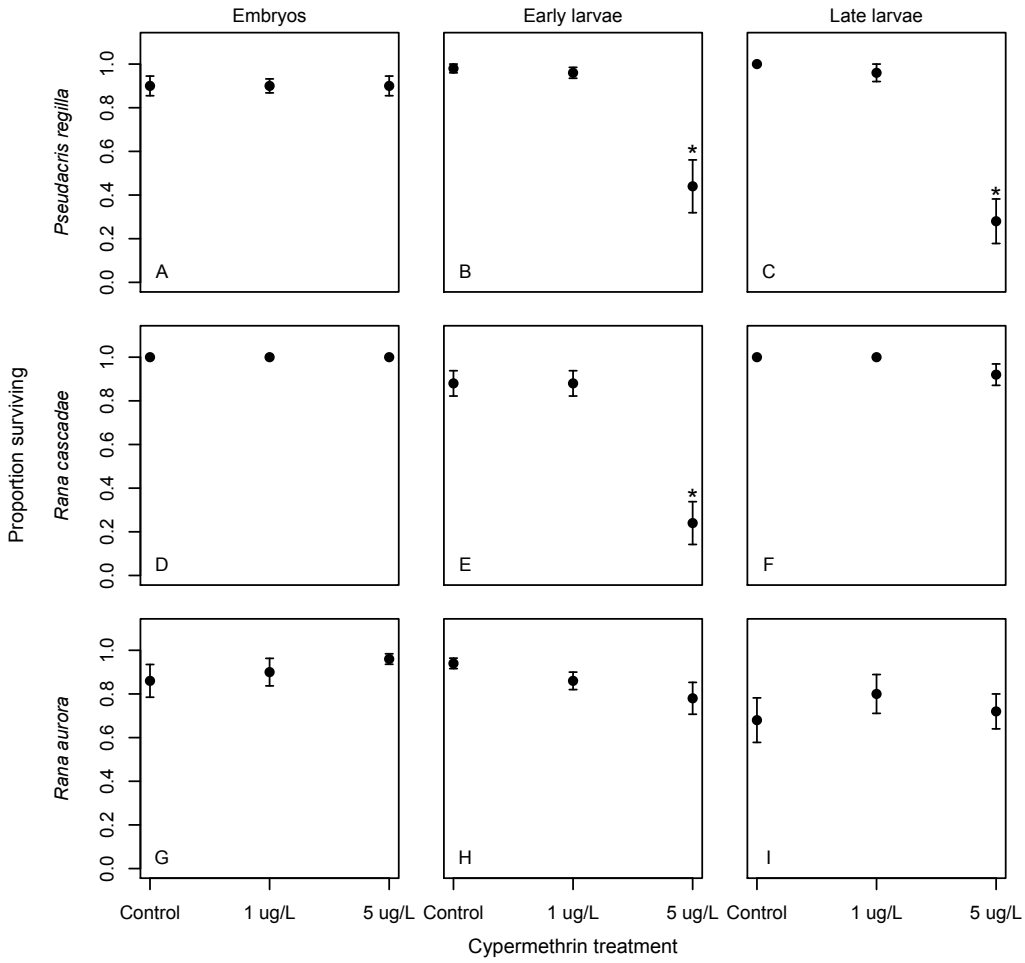

Figure 3.1 Survival of cypermethrin-exposed and control groups of amphibians of three species (top row = *P. regilla*, middle row = *R. cascadae*, bottom row = *R. aurora*) exposed at three distinct developmental time points (first column = embryos, second column = newly hatched larvae, third column = larvae with limb buds). Treatments that are significantly different (p-value < 0.05) from controls are indicated by *. Values plotted are means ± 1 SE.

CHAPTER 4

Population differences in sensitivity of Pacific treefrogs (*Pseudacris regilla*) to the pyrethroid insecticide, cypermethrin

Lindsay M. Biga and Andrew R. Blaustein

Abstract

In the face of anthropogenic change, many species are threatened with extinction. However, variations among organisms and environments have resulted in the persistence of certain populations while other populations of the same species are experiencing declines. Exposure to chemical contaminants is one factor influencing population declines. Yet chemicals in natural systems exhibit variation in effects to native populations not anticipated by toxicity tests with model species. We conducted laboratory toxicity experiments to assess the degree of population-level variation in sensitivity of a common anuran, *Pseudacris regilla* (the Pacific treefrog), to the pyrethroid insecticide cypermethrin. Cypermethrin exposure increased mortality in all groups compared to controls. We observed significant variation in time to death among the *P. regilla* populations we tested. Additionally, cypermethrin-exposed animals were significantly smaller (snout-vent length and mass) than control animals from the same population. Our results indicate that degree of sensitivity was not related to a population's proximity to urban development or agriculture, as would be expected if resistance were inherited due to historic contamination of the population's home environment. Rather, the degree of sensitivity to cypermethrin more closely aligns with differences in elevation.

Introduction

Over the past several decades, humans have dramatically altered ecosystems, leading many scientists to argue that current biodiversity loss is part of the sixth mass extinction (Wake and Vredenburg 2008). With 1,910 of 6,312 species in danger of extinction, amphibians are one of the most threatened taxonomic groups (Stuart et al. 2004, McCallum 2007, IUCN 2012). Though it is clear that many factors contribute to declines in amphibian populations (e.g. habitat destruction, disease, climate change, introduced species), variation in the effects of these factors across regions, species, life stages and populations point to the complexity and importance of ecological context in evaluating pressures on amphibian populations (Blaustein et al. 2011). Specifically, population-level variations in (1) tolerance to UV-B exposure (Belden and Blaustein 2002), (2) behavioral response to an introduced species (Kiesecker and Blaustein 1997), and (3) disease-induced rates of mortality (Tobler and Schmidt 2010) have all been established experimentally in amphibians.

A growing body of evidence links some amphibian population declines with exposure to pesticides (Sparling et al. 2001, Davidson 2004). However, a great degree of variation in sensitivity to contaminants, including pesticides, exists in nature, although this variation is not predicted by dose response tests with model species (Boone and James 2005). Variations in resistance of amphibian population to pesticides have been documented with the effects of the carbamate insecticide carbaryl

(Bridges and Semlitsch 2000, Semlitsch et al. 2000, Bridges et al. 2001), the herbicide glyphosate and the organophosphate insecticide chlorpyrifos (Cothran et al. 2013).

Population level differences in sensitivity to pesticides may be attributed to a variety of factors. Variation in sensitivity to pesticides may result from traits acquired in certain populations that result from geographic variation (i.e. altitude, climate, etc.) and confer some advantage against pesticide toxicity (Bridges and Semlitsch 2000). Alternatively, a long history of exposure may result in populations near agricultural lands that exhibit inherited tolerance. For example, Cothran et al. (2013) found that amphibians from populations closest to agriculture had the greatest tolerance to chlorpyrifos exposure, and that tolerance decreased with increasing distance from agriculture. However, inherited tolerance is less likely to be the mechanism in the case of variation in sensitivity to newer pesticides or to pesticides with modes of action novel to given area. Additionally, many pesticides have a variety of non-agricultural uses, so proximity to agricultural lands is just one of several potential exposure routes, in addition to proximity to residential and industrial development. Consequently, the sensitivity among populations to newer pesticides with novel modes of actions and a variety of agricultural and non-agricultural uses is of interest.

Pyrethroids are a newer group of pesticides with agricultural and non-agricultural uses and somewhat unique mode of action (shared only by dicofol and methoxychlor). Favored for their relatively low toxicity to mammals and low persistence in the environment (Berrill et al. 1993, Moore and Waring 2001),

pyrethroids are increasingly common insecticides present in over 3,500 registered products in the U.S. (Environmental Protection Agency 2012). For several decades, Organophosphate insecticides (OPs) have been the most commonly used insecticides. However, the emergence of data linking groundwater presence of some OPs to potentially toxic effects in humans led the EPA to restrict the residential use of diazinon and chlorpyrifos, two of the most commonly applied OPs (EPA 2012, Palmquist et al. 2012). As some principal uses of these OPs have been phased out, pyrethroid insecticides are commonly being utilized as their replacement (Oros and Werner 2005). Cypermethrin is one such pyrethroid insecticide. Agricultural uses of cypermethrin include the treatment of insect pests of cotton, pecans, sweet corn, lettuce, and broccoli as well as pests of cattle and other livestock (EPA 2008). However, the majority of cypermethrin use is for non-agricultural purposes including control of ants, cockroaches, fleas, and termites in indoor and outdoor structural and perimeter applications (EPA 2008).

Our primary objective was to determine whether population-level variation in sensitivity to cypermethrin is present in an amphibian species known to exhibit sensitivity to cypermethrin exposure. Our previous experiments on a single population of Pacific treefrogs (*Pseudacris regilla*) showed those individuals to be particularly sensitive to cypermethrin as compared to other frog species (Biga and Blaustein 2013). *P. regilla* have a wide geographic range and occupy a variety of habitats in the western United States and Canada, where they are distributed from sea level to above

3550 m (Jones et al. 2005). This broad distribution of *P. regilla* results in populations inhabiting a wide range of environments, from agricultural land and urban areas to remote wilderness. Consequently, we were able to evaluate populations from discrete geographic locations as to evaluate variation in cypermethrin sensitivity. Throughout its wide distribution, populations of *P. regilla* are faced with diverse environmental stressors, making it an ideal species for testing variations in pesticide sensitivity.

Materials and Methods

We conducted experiments on larvae from three populations of *P.* regilla. Egg masses were collected shortly after they were laid (within 48hr) in ponds from three locations in Oregon. One population was along the Oregon coast (hereafter referred to as "Coast", Lane County, elevation = 12 m), the central Willamette Valley (hereafter referred to as "Willamette Valley", Benton County, elevation = 84 m), and the Cascade Mountains (hereafter referred to as "Cascades", Linn County, elevation = 1140 m) of Oregon. The Coast population is relatively close to residential development (< 1 km). The Willamette Valley population is directly adjacent to a dairy farm and close (< 1 km) to residential development. The Cascades population is relatively remote, located in the Willamette National Forest, though it is adjacent to a state highway. The ponds from which eggs were collected were at least 60 km apart, and as such they are clearly distinct populations. Due to differences in breeding phenology among populations based on local climates, populations were not tested at

the same time. Upon initiation of an experiment, a subsample of the animals not used in the experiment was weighed (mg), measured (snout-vent length in mm), and staged (according to Gosner 1960). Although size of larvae differed slightly among populations, all tadpoles were tested at uniform stages (24-25; Gosner 1960). Additionally, all populations were tested using the same methods in in the same laboratory.

To increase the probability that our test population included a representative sample of genetic variation from their population, five egg masses from each population were used in each experiment. The five egg masses from each population were brought into the laboratory where they were reared jointly in 38 L aquaria filled with aerated dechlorinated water. Animals were maintained in a controlled laboratory environment at 21.5-23.5°C with a 13h:11h light:dark photoperiod. Experiments were initiated 2 weeks after hatching began. These individuals were not fed as they maintain their yolk as a food source for some time after hatching (Stebbins and Cohen, 1995).

We exposed cypermethrin treatment individuals to 5 µg/L cypermethrin. Immediately prior to each experiment, a fresh 100 mL stock solution of 100 mg/L cypermethrin in dechlorinated water was prepared by dissolving 99.5% standards-grade cypermethrin (ChemService, West Chester, PA, USA) into a carrier solution of 10 mL HPLC grade acetone that was subsequently added to 90 mL deionized water. Stock solutions were diluted to 10 mg/L cypermethrin. Residues were not determined.

Preliminary tests revealed no differences between dechlorinated water controls and controls containing the acetone carrier, therefore only a dechlorinated water control was used.

We performed a time-to-death assay, a surrogate for LC50 tests (which determine the concentration expected to kill 50% of the test population) to compare the relative tolerance of organisms using a smaller number of amphibian larvae (Bridges and Semlitsch 2000). For each population, time to death was measured for 50 total *P. regilla* larvae assigned randomly to either cypermethrin-exposed or control treatments (25 individuals per treatment). Individuals were exposed in 600 mL beakers filled with 500 mL dechlorinated water. Nominal test concentrations of 5 μg/L cypermethrin were achieved by adding 0.25 mL of cypermethrin dilution to 500 mL dechlorinated water. We chose this nominal concentration based on previous research which demonstrate that it is moderately lethal to *P. regilla* larvae (Biga and Blaustein 2013). Larvae were not fed during exposure. We monitored for survival at 4, 8, 12, 16, 20, 24, 36, 48, 72, 96, 120, 144, 168hr after the beginning of exposure. Mortality was defined as the absence of all movement after repeated prodding and the lack of a visible heartbeat as observed by the naked eye.

At the conclusion of the experiment, all animals were euthanized with buffered MS-222 and preserved in 95% EtOH. Animals from the Cascades population were measured (snout-vent length in mm and mass in mg) and staged (Gosner 1960) either upon mortality at the conclusion of an experiment. We were unable to collect this data

for animals from the Coast or Willamette Valley populations due to accelerated decomposition of deceased individuals.

We performed statistical analyses in R statistical computing environment (version 2.15.0, The R Foundation for Statistical Computing). We analyzed differences in survival between control and exposued groups for each population using chi square tests. To test for differences in time to death among populations, we performed multivariate ANOVA. We compared size (including both mass and SVL) of exposed and control individuals from the Cascades population using a t-test.

Results

We found that survival in the controls was high (>80%), and populations exposed to cypermethrin varied in their sensitivity (Figure 4.1). Cypermethrin treatments increased mortality in all of the populations tested as compared to controls (Cascades $X^2 = 23.53$, $p < 0.001$; Coast $X^2 = 25.76$, $p < 0.001$; Willamette Valley $X^2 = 18$, $p < 0.001$) with mortality in cypermethrin-exposed treatments ranging from 64% to 100%. Mean time to death of exposed individuals varied from approximately 4 to 6 days (Figure 4.2). Time to death significantly differed among *P. regilla* populations ($df = 2$, $F = 5.50$, $p < 0.01$). The population from the Cascades was the most tolerant whereas the population from the Coast was the most sensitive.

Cypermethrin-exposed animals from the Cascades population were smaller (mass: $t=5.38$, $df = 35.38$, $p < 0.001$; SVL: $t=5.18$, $df = 43.58$, $p < 0.001$) than those in

the control. Moreover, individuals in the cypermethrin treatment that died did not differ in size from those that survived (mass: $t = 0.10$, $df = 21.43$, $p = 0.92$; SVL: $t=-0.71$, $df = 18.48$, $p = 0.49$), indicating that size differences were a result of cypermethrin exposure and not associated with increased mortality, short life span, or an artifact of early stages of decomposition after death.

Discussion

We observed significant differences in time to death of cypermethrin-exposed populations of *P. regilla* in Oregon. This suggests that variation in chemical tolerance exists across the elevational range of this species. Recent work has provided evidence that amphibians from populations found closer to agricultural land have increased tolerance to insecticide exposure, likely an evolutionary consequence of exposure across many generations (Cothran et al. 2013, Hua et al. 2013). The insecticide evaluated here, however, is not exclusively an agrochemical. In fact, only 14% of cypermethrin uses are agricultural, with an additional 11% of use for indoor pest control and 75% for outdoor structural and perimeter application in residential and industrial areas (EPA 2008). Despite its combined urban and agricultural uses, sensitivity does not seem to be related to proximity to urban development or agriculture. The most tolerant population in the present study came from the most remote site (Cascades), while the populations found closer to urban and agricultural land (Coast and Willamette Valley) exhibited greater sensitivity to cypermethrin.

Furthermore, cypermethrin, though commonly used today, does not have a long history of extensive use as it is a newer pesticide (EPA 2008). Our results are not consistent with an evolutionary response to historical pesticide exposure as the more sensitive populations (Coast and Willamette Valley) came from areas with presumably higher levels of insecticide use. It is possible that in the case of cypermethrin toxicity in *P. regilla*, tolerance is related to a local adaptation to some other environmental stressor (i.e. climate, UV-B radiation, etc.) rather than to historical exposure to insecticides.

In our experiment, animals exposed to cypermethrin were smaller than those in control treatments, even though this experiment only lasted 7 days. If cypermethrin exposure results in significantly stunted growth on larval amphibians at the time of metamorphosis, it may have several important fitness costs for sub-lethally exposed individuals in natural populations. For example, smaller sizes at metamorphosis in anurans have been correlated with reduced juvenile survival, mating success, longer time to first reproduction and production of inferior quality eggs (Smith 1987, Howard and Kluge 1988, Altwegg and Reyer 2003).

The concentration of cypermethrin used in the present study (5 µg/L), though approaching its maximum water solubility (7.6 ppb at 25ºC; EPA 2008), is within the range of environmentally relevant concentrations observed following spray and run-off events (Marino and Ronco 2005, Weston et al. 2009). Additionally, the populations that we found to be more sensitive to cypermethrin were those more likely

to be exposed due to run off from either agricultural or residential/industrial use. Often, directly lethal concentrations are well above expected environmental concentrations (Bridges and Semlitsch 2000). Consequently observing lethality at ecologically realistic concentrations in susceptible populations is of concern for conservation.

Acknowledgements

We thank E. Hunt, P. Bradley, and C. Searle for their assistance with experiments and statistical guidance. Our thanks to T. Garcia, D. Lytle, and J. Jenkins for experimental ideas that improved the present study. Funding for this research was provided by the Oregon State University Environmental Sciences Graduate Program. This research was approved by and conducted in accordance with the regulations of the Oregon State University Institutional Animal Care and Use Committee and animals were collected according to Oregon Department of Fish and Wildlife regulations.

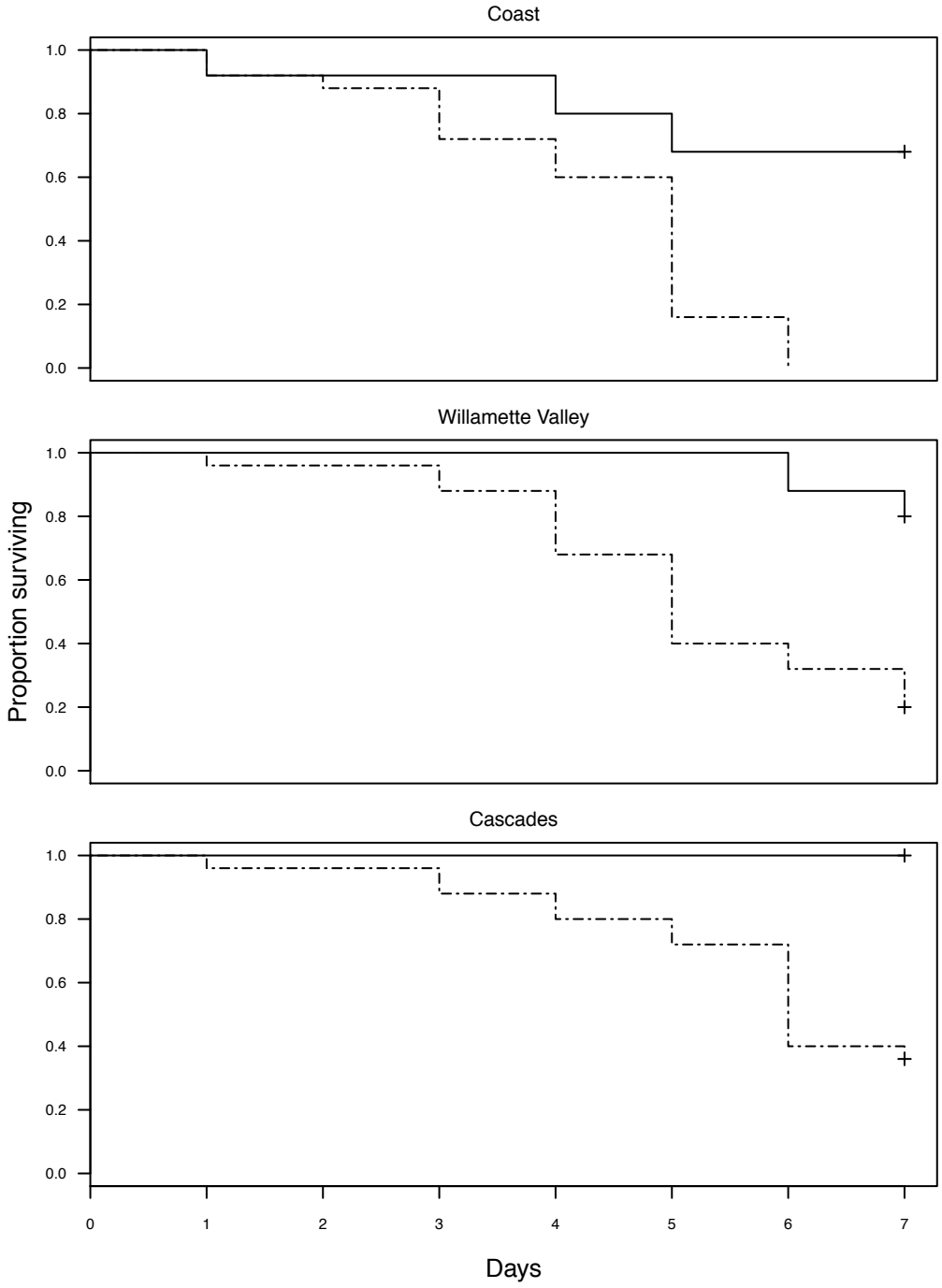

Figure 4.1 Survival of cypermethrin-exposed and control groups of *Pseudacris regilla*. Solid lines represent unexposed animals and dashed-lines represent cypermethrin-exposed animals. For each species, half of the animals were exposed to cypermethrin while the others remained unexposed. The experiments were conducted for 7 days with mortality monitored daily.

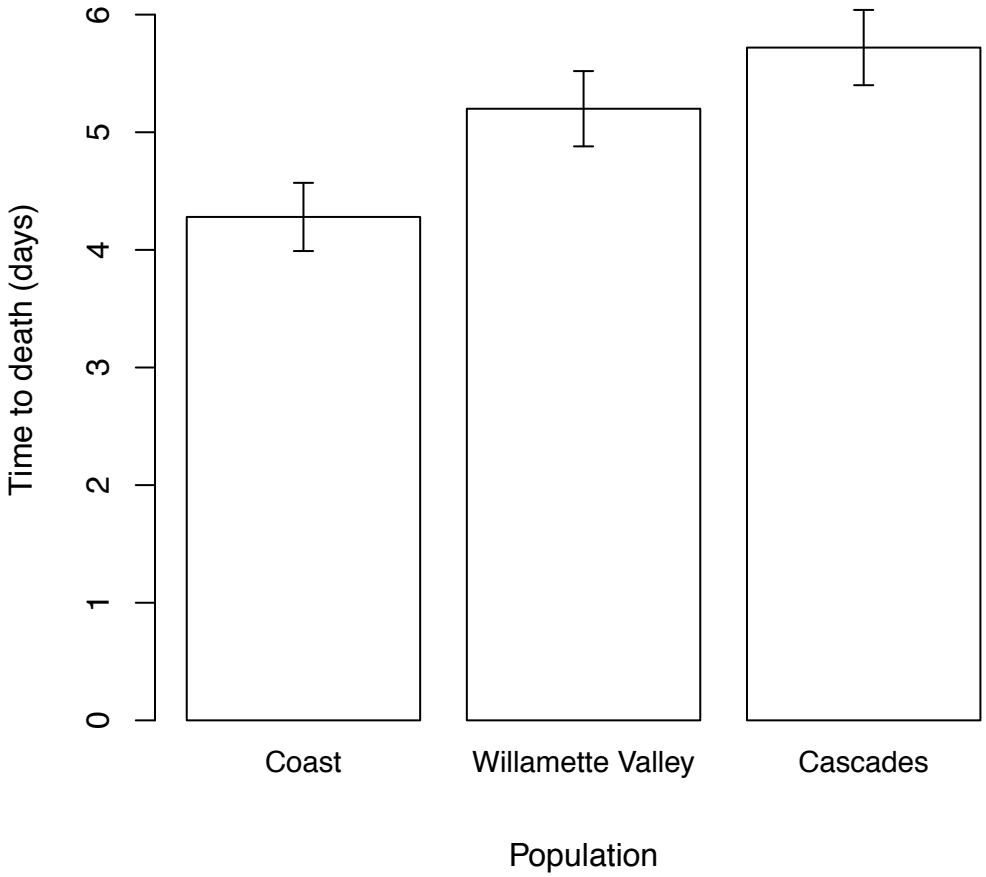

Figure 4.2 Mean number of days (+ SE) after initial exposure before death of *Pseudacris regilla* from the three populations assayed. For each species, half of the animals were exposed to cypermethrin while the others remained unexposed. The experiments were conducted for 7 days with mortality monitored daily. Time to death was significantly faster for the Coast population than for the Cascades population.

CHAPTER 5

Effects of a pyrethroid insecticide in a simulated aquatic community

Lindsay M. Biga and Andrew R. Blaustein

Abstract

Agricultural and commercial pollutants that enter lakes, ponds and streams through runoff and aerial spray can heavily impact aquatic communities in complex ways. While effects of pesticides are regularly examined in laboratory-based single species studies, the broader effects within ecosystems are less well studied. Aquatic environments provide habitat for many species whose strong interspecific interactions may lead to increased effects of pollutants through trophic relationships. We used outdoor mesocosms to simulate a natural aquatic community, including an amphibian species (*Anaxyrus boreas*), zooplankton and algae, in which we examined the effects of the synthetic pyrethroid insecticide, cypermethrin. Cypermethrin is a neurotoxic pyrethroid used for household and industrial control of ants and roaches and to control agricultural pests of cotton, fruit, and many vegetables. We examined the sensitivity of constituents of the aquatic community to varying levels (0, 1 or 5 µg/L) of cypermethrin. After exposure, we measured amphibian mortality, growth and development, as well as periphyton and chlorophyll-*a* biomass, and *Daphnia spp.* abundance. Cypermethrin affected the aquatic community, even at the lowest dose. Amphibian growth and development were inhibited and mortality increased with exposure in high treatments. Exposure to cypermethrin decreased the abundance of *Daphnia spp.* and increased the abundance of both periphyton and chlorophyll-*a* in all treatments compared to controls. The increase in periphyton and chlorophyll-*a* is likely due to the reduction of grazing pressure from zooplankton and amphibians in

the cypermethrin-exposed treatments. This experiment shows both indirect and direct deleterious effects of cypermethrin on both invertebrate and vertebrate aquatic species in an aquatic community.

Introduction

Pollution by xenobiotics is a global concern. About 80,000 registered chemicals in the United States, including about 20,000 pesticides with 675 active ingredients, are regularly released into the environment with complex and only partially understood effects (EPA 2004, Groner and Relyea 2011). While the bulk of pesticide use is concentrated in densely populated and heavily agricultural areas, residues are ubiquitous, appearing in environments as remote as the Arctic and alpine regions (reviewed in Hoferkamp et al. 2010). Aquatic systems are particularly vulnerable to the influx of pesticides through aerial drift and run-off events. These systems provide habitat for aquatic mammals, water birds fishes, amphibians, aquatic plants, insects, zooplankton, and phytoplankton. Laboratory studies have laid the foundation for understanding effects of pesticides on model species with excellent data on the acute effects of pesticides by way of LC_{50} values (the concentration required to kill 50% of the tested population). However, there is continuing debate on extrapolating laboratory results to natural situations as these experiments give an incomplete picture of the effects to individuals, population and ecosystems by overestimating, underestimating, or missing indirect effects entirely (Materna et al. 1995, Relyea et al. 2005).

The growing field of ecotoxicology has expanded upon traditional toxicological screening to encompass direct sublethal and chronic effects as well as indirect effects of pesticides on a variety of species in the laboratory and on the

residents of an ecosystem in more complex, integrated contexts (Truhaut 1977, Relyea and Hoverman 2006). Pesticides at extremely low concentrations are often directly lethal to many taxa including zooplankton (Kerby et al. 2010), a central species in many communities' food webs (Chang et al. 2005). The consequences that arise indirectly from mortality of central species can be observed throughout the food web (Fairchild et al. 1992). For example, Chang and colleagues (2005) found that exposure to the insecticide carbaryl reduced zooplankton populations, releasing rotifers from competitive pressures thus increasing their population density. In another study, Relyea and Diecks (2008) observed a trophic cascade resulting from the malathion-induced mortality of zooplankton, leading to a bloom in phytoplankton and a subsequent decline in the competing periphyton. As the food source of larval amphibians, this reduction in periphyton led to reduced growth and development in leopard frog larvae that were subsequently unable to metamorphose prior to pond drying. It is clear that low concentrations of pesticides can have complex and cascading effects.

In the face of population declines and species extinctions, we are further challenged to understand the role that pesticides play in ecological communities. Amphibians are of particular interest as they are important consumers and prey in aquatic communities that influence their environments by affecting sediment dynamics, primary productivity, algal community composition, invertebrate grazers and predators (Wells 2007, Blaustein et al. 2011). They are one of the most threatened taxa in the current

biodiversity loss, with about 1910 of the 6,312 known amphibian species in danger of extinction (Stuart et al. 2004, McCallum 2007, IUCN 2012). Moreover, some population declines of amphibians have been associated with pesticide use (Sparling et al. 2001, Davidson 2004). Unshelled eggs and semi-permeable skin make the aquatic embryos and larvae of amphibians particularly sensitive to environmental perturbations (Blaustein and Wake 1995). However, pesticide concentrations in aquatic environments are often lower than those known to cause direct lethal effects in amphibians based on laboratory tests; this disparity suggests that pesticides may be contributing to declines via sublethal and indirect effects or interactions with other factors (Davidson 2004).

Pyrethroid insecticides are a group of pesticides of particular concern due to their effects to non-target organisms in aquatic systems, including amphibians (Coats et al. 1989, Greulich and Pflugmacher 2003). Pyrethroids are used extensively in agricultural and non-agricultural settings as they are extremely potent neurotoxins at low concentrations. Cypermethrin, a cyano-pyrethroid insecticide, is used extensively in the U.S.; approximately 1 million pounds of active ingredient are used annually (Environmental Protection Agency 2008). Pyrethroids, including cypermethrin, are favored over the organochlorine and organophosphate insecticides that they are replacing for their low persistence in the environment and relatively low toxicity to mammals (Berrill et al. 1993, Moore and Waring 2001). However, their toxicity to non-target organisms is high. Cypermethrin is considered by the United States

Environmental Protection Agency to be very highly toxic on an acute basis to marine and freshwater invertebrates and fishes, and to honeybees (EPA 2008). Zooplankton display particular sensitivity to cypermethrin exposure; laboratory based LC50 values (i.e. the concentration of a chemical required to kill 50% of the individuals exposed) for *Daphnia magna* is 0.2 µg/L (Bradbury and Coats 1989).

Cypermethrin is registered for a number of uses. Agricultural uses include the treatment of insect pests of cotton, pecans, sweet corn, lettuce, broccoli as well as pests of cattle and other livestock (EPA 2008). A wide range of non-agricultural uses including control of ants, cockroaches, fleas, and termites in indoor and outdoor structural and perimeter applications make up the majority (750,000 pounds) of cypermethrin use (EPA 2008). Although 75% of cypermethrin use is non-agricultural, due to the difficulties posed in modeling these uses, risk assessments by the EPA have only included uses on agricultural crops (EPA 2008). These assessments therefore do not include the potential effects of runoff from impervious surfaces after application in urban and industrial settings. Due to their low water solubility, it has been purported that the processes of adsorption and dilution will rapidly reduce exposure and consequently negate effects of pyrethroids under field conditions (Hill 1989). Even so, evaluations in California after runoff events indicate that pyrethroids, including cypermethrin, are found at acutely toxic concentrations to invertebrates (test species *Hyalella azteca*) in urban streams (Weston et al. 2009). Moreover, cypermethrin has been detected in surface water at levels ranging from 100–1010 µg/L (Crossland 1982,

Crossland et al. 1982, Marino and Ronco 2005), and at lower levels (0.02-2.6 µg/L) in subsurface waters (Day 1989, Jergentz et al. 2005).

To our knowledge, there have been no experimental studies evaluating the effects of cypermethrin on aquatic communities using semi-natural enclosures (mesocosms) containing amphibians and few on pyrethroids in general (but see Farmer et al. 1995, Materna et al. 1995, Friberg-Jensen et al. 2003, Boone 2008). Here we investigated the effects of pyrethroid insecticides in freshwater ecosystems by examining the effects of cypermethrin on amphibians, periphyton, zooplankton and phytoplankton experimentally in mesocosms. We hypothesized that cypermethrin would have direct and indirect effects on the constituents of an aquatic communities. We made several predictions regarding community effects of cypermethrin. First, we predicted that cypermethrin exposure would be directly lethal to zooplankton. Second, we predicted that reductions in zooplankton concentration would cause algal blooms of phytoplankton. Third, we predicted that significant sublethal effects to amphibians in the form of reduced growth, development would result from exposure. Fourth, we predicted that sublethal effects to amphibians would contribute to reduced foraging efficiency thereby increasing periphyton biomass. And finally, we predicted that these effects would increase with concentration of cypermethrin exposure number of applications.

Materials and Methods

Amphibian Rearing

We collected *Anaxyrus boreas* (Western Toad, formerly *Bufo boreas*) eggs in June 2010 from Little Three Creeks Lake (Deschutes County, Oregon, USA; elevation: 2093 m). Eggs were reared in the laboratory in aerated 38 L aquaria filled with dechlorinated water at 13.4-15 °C on a photoperiod that mimics local outdoor conditions. After hatching, larvae were fed a mixture of rabbit chow and TetraMin fish food (3:1 ratio by weight). Animals were fed ad libitum, and water was changed every 2wk. Animals were not fed for 48hr prior to initiation of the experiment.

Mesocosms

The experiment was conducted in outdoor mesocosms (6 km Corvallis Benton County, Oregon, USA; elevation: 71 m) from August to October. Mesocosms were plastic containers 1.5 m in diameter filled with ~ 120 L of tap water (pH = 8) and covered with screen lids. To simulate community conditions in natural ponds, mesocosms were inoculated with zooplankton (*Daphnia spp.*), phytoplankton, periphyton collected and mixed from 10 natural ponds in Benton County, Oregon, and leaf litter (*Quercus* sp.) 10d prior to the addition of amphibian larvae to the mesocosms. In addition to creating a community with refuges for amphibians, the algae and leaf litter provided surface area for adsorption of cypermethrin, as would be present in the field. We left the mesocosms undisturbed for 10d to allow periphyton,

phytoplankton and zooplankton to establish. Twelve newly hatched, free swimming and independent feeding *A. boreas* larvae (Gosner (1960) stages 25-26) were randomly assigned and added to each of the 16 mesocosms 7d prior to initiation of the experiment. Four levels of cypermethrin (nominal concentrations in mesocosms of 1 µg/L applied once, 5 µg/L applied once, 5 µg/L applied twice, and an unexposed control) were randomly applied to mesocosm units. Doses were chosen to reflect levels of cypermethrin expected in natural waters directly after spray events (Friberg-Jensen et al. 2003). Mesocosms in the single exposure treatments were dosed with cypermethrin at the initiation of the experiment. Mesocosms in the double exposure treatments were first dosed with cypermethrin at the initiation of the experiment and again 15d later. Each treatment was replicated in 4 randomly assigned mesocosm and the experiment lasted for 35d.

Cypermethrin

Immediately before each cypermethrin exposure, a fresh 250 mL stock solution of 120 mg/L cypermethrin was prepared by dissolving cypermethrin 99.5% standards-grade cypermethrin (ChemService, West Chester, PA, USA) into a carrier solution of 250 mL HPLC grade acetone. Though analytical chemistry was not performed on the stock solutions or exposure solutions used in the present study, cypermethrin stock solutions made with the same method in the same laboratory from an earlier experiment were analyzed at the Mississippi State Chemical Laboratory using

GC/ECD. Actual concentrations of those stock solutions were 53.0, 62.1 and 67.2% of nominal concentrations. A 20% dilution was made using dechlorinated water and nominal test concentrations of 5.0 and 1.0 µg/L cypermethrin were obtained by adding 6 mL of the stock solution and dilution (respectively) to mesocosms and gently stirring the surface of the water for 10 seconds. Residues were not determined. However, exposure in mesocosms was likely much lower than nominal concentrations due to the hydrophobic (log K_{ow} = 6.6) nature of cypermethrin (Friberg-Jensen et al. 2003). The half-life for synthetic pyrethroids is typically about 1d across a variety of study designs including natural ponds, farm ponds, mesocosms and microcosms (reviewed by Hill et al. 1994). Acetone in the cypermethrin treated mesocosms did not exceed a concentration of 0.05mL/L, well below the limit of 0.1 mL/L recommended by the International Standard Organization (2012) for tests involving invertebrates. More over, this level of acetone had no effect on amphibian larvae in our pilot experiments and was consequently not added to the controls. A 6 mL dechlorinated water dose was added to control mesocosm on both treatment days and to mesocosms in the single exposure treatments on the day of the second exposure. After this addition the water was gently stirred as in the cypermethrin treatments.

Community sampling

To measure how the community was affected by treatments, data were collected on amphibians, periphyton, zooplankton and phytoplankton upon

termination of the experiment. Specifically, we quantified survival of amphibians in each mesocosm and determined developmental stage (Gosner 1960), length (snout-vent) and mass for each surviving animal. Additionally, periphyton biomass, *Daphnia spp.* concentration, and chlorophyll-*a* biomass (used to estimate phytoplankton biomass) were sampled in each mesocosm using methods similar to those described by Buck et al. (2012) which are detailed below.

We deployed periphyton samplers into each mesocosm 17d prior to the initiation of the experiment. Periphyton samplers consisted of a glass microscope slide that served as artificial substrate mounted vertically on a small Styrofoam block. At the conclusion of the experiment, the periphyton on both sides of the slide was scraped into a Petri plate using a straight-edge razor blade. These scrapings were then filtered through a 25 mm Pall A/E glass fiber filter that had been previously dried for 24h at 60 ˚C and weighed. Filters were dried again for 24h at 60˚C and reweighed to determine periphyton biomass (mg).

Zooplankton were sampled with a cylindrical water sampler (width 1.5 cm) that was dipped vertically into the water column and sealed near the bottom of the mesocosm. Three aliquots of approximately 30 mL of mesocosm water were taken - two on opposite sides of each mesocosm and one in the center - and pooled. Using this method, three pooled samples were taken from each mesocosm. The sampler was cleaned with distilled water between mesocosms to minimize dispersion of zooplankton between tanks. Water samples were sieved through 150 μm mesh

(Florida Aquatic Nurseries, Ft. Lauderdale, FL, U.S.A.), and zooplankton from each sample were preserved in 30% ethanol for later quantification. Zooplankton values are reported as concentrations per 100 mL of mesocosm water to allow for comparison with other experiments.

To estimate phytoplankton biomass, 25 mL of water from each of the three zooplankton samples per mesocosm were filtered through a Pall A/E glass fiber filter (Pall Corporation, Port Washington, NY, U.S.A.) in full shade to minimize chlorophyll breakdown. Filters were stored in 15 mL centrifuge tubes and kept on ice during sampling. Samples were then stored in the laboratory at -20˚C for 24h before chlorophyll extraction. To extract chlorophyll-*a*, centrifuge tubes were removed from the freezer, filled with 10 mL of 90% acetone, agitated for 10s, and then incubated again at -20˚C for 24h (Welschmeyer 1994). Fluorescence measurements were then taken using a Turner Designs fluorometer (model TD-700, Sunnyvale, CA, U.S.A.). The mean concentration from the three replicates of each mesocosm was used for statistical analysis.

Statistical Analysis

We analyzed data in R statistical computing environment (version 2.15.0, The R Foundation for Statistical Computing). Data from two mesocosms were excluded from the analysis. One double exposure 5 µg/L replicate was excluded as the container developed a crack and drained completely prior to sampling. Additionally one single

exposure 5 μg/L replicate was excluded as it appeared to be contaminated (as evidenced by an oily sheen visible on water's surface) and the extremely outlying results from that mesocosm were inconsistent with the remainder of the data. For amphibian larvae, effects of cypermethrin on proportion surviving and proportion reaching developmental stage 36 (Gosner 1960) were analyzed using generalized liner mixed models using a logit link function to determine the effects of cypermethrin treatments. Individuals were nested by exposure group. Both mass and length were analyzed using one-way analyses of variance (ANOVA) followed by a Tukey's HSD test. Effects of cypermethrin treatments on other members of the community (periphyton biomass, zooplankton concentration, and phytoplankton concentration) were also analyzed using ANOVA followed by Tukey's HSD test. For amphibian biometric data and community analyses, mean values per mesocosm were used as the unit of analysis. For phytoplankton concentration, we log-transformed the data to meet parametric assumptions.

Results

Exposure to cypermethrin treatments resulted in decreased amphibian survival ($X^2 = 45.31$, $p < 0.001$) with survival decreasing with increased cypermethrin concentration (Figure 5.1A). There were also several sublethal effects of cypermethrin on amphibians.

The proportion of surviving individuals reaching developmental stage 36 varied among treatments ($X^2 = 10.38$, $p < 0.05$) with inhibited development in higher cypermethrin treatments (Figure 5.1B). Only three individuals (5%) in the double high exposure treatment developed beyond Gosner stage 31 (the initial formation of a paddle-shaped foot), while 25% of individuals in the single high exposure and over 50% of individuals in the low exposure and control treatments reached stage 36 (whole foot with 5 toes formed). No amphibians metamorphosed prior to the conclusion of the experiment.

Additionally, surviving amphibians differed in both mass ($F_{3,8} = 4.87$, $p < 0.05$) and SVL ($F_{3,8} = 7.75$, $p < 0.01$) among treatments (Figure 5.1C, 5.1D). Animals exposed to the double high dose of cypermethrin were 50% lighter and 30% shorter than those in the low treatment (Tukey's HSD; mass: $p < 0.05$; SVL: $p < 0.05$) and those in the control (Tukey's HSD; mass: $p < 0.05$; SVL: $p < 0.01$).

Periphyton biomass also differed across treatments ($F_{3,10} = 4.34$, $p < 0.05$, Figure 5.2C). Compared to the controls, there was twice as much periphyton in both the low cypermethrin treatment (Tukey's HSD; $p < 0.05$) and in the single dose high cypermethrin treatment (Tukey's HSD; $p = 0.058$).

Exposure to any level of cypermethrin killed zooplankton ($F_{3,10} = 28.08$, $p < 0.001$, Figure 5.2B). There were no zooplankton found in any of our samples in mesocosms exposed to any level cypermethrin compared with an average of

approximately 79 zooplankton per 100 mL in the controls (Tukey's HSD; $p < 0.001$ for all cypermethrin treatments compared to the controls).

Phytoplankton biomass (estimated by chlorophyll-*a* concentration) also differed among treatments ($F_{3,10} = 9.14$, $p < 0.01$, Figure 5.2D). There was 16 times more phytoplankton in the low cypermethrin treatment (Tukey's HSD; $p < 0.01$) and 26 times more in the single dose high cypermethrin treatment (Tukey's HSD; $p = 0.01$) than in the control.

Discussion

Our results demonstrate the effects of a pyrethroid insecticide on a freshwater community. We quantified effects on all members of an aquatic community exposed to cypermethrin, including indirect effects through trophic interactions. Though invertebrates are the intended targets of cypermethrin, exposure impacted algal and vertebrate species. Standard LC_{50} testing on individual model organisms, though useful for establishing baseline information regarding toxicity, is unable to illustrate these complex trophic effects of pesticides (Relyea and Hoverman 2006).

Treatments differentially affected *A. boreas* larvae and effects were particularly strong in the double exposure treatment indicating that multiple doses of cypermethrin can increasingly affect amphibian larvae. We demonstrated reduced growth (mass and length) and delayed development of amphibian larvae exposed to cypermethrin. Reductions in tadpole density through contaminant-induced mortality

can lead to larger surviving tadpoles due to a decrease in competition when resources are limited (Materna 1995). However, we found that even with fewer larvae surviving, animals in higher cypermethrin treatments were smaller than those in the controls. This may indicate that cypermethrin exposure caused behavioral abnormalities that reduced foraging capabilities. Though not assayed in the present study, behavioral abnormalities are a common effect of cyano-pyrethroid exposure (Berrill et al. 1993). This indirect effect could lead to reduced size at metamorphosis which is associated with reduced juvenile survival, mating success, longer time to first reproduction and production of inferior quality eggs (Howard and Kluge 1988, Smith 1987, Altwegg and Reyer 2003). Additionally, developmental delays like those observed in the present study can be lethal to amphibian larvae which commonly occur in temporary wetlands, as pond drying can occur prior to the completion of metamorphosis (Groner and Relyea 2011).

In our mesocosms, some organic substrate was available (oak leaves and algae) for the adsorption of cypermethrin. Due to its high organic carbon (OC) partition coefficient (K_{oc} = 350,000), cypermethrin readily partitions to sediment, vegetation and other organic matter (Maul et al. 2008, Mugni et al. 2011). While pyrethroids partition more readily to sediment than to vegetation, leaf material or detritus, these other forms of organic substrate also provide surface for sorption, altering toxicity to a similar degree. For instance, Maul and colleagues (2008) found no difference in toxicity of the pyrethroid bifenthrin to the amphipod *Hyalella azteca* among systems

containing sediment versus those containing leaf material. Though it has been suggested that cypermethrin is not sufficiently bioavailable in the adsorbed state to affect amphibians (Hill 1989), we found significant effects of exposure. Multiple pathways of exposure are possible in this context including exposure of amphibians on the water's surface when they rise for air, in the water column prior to adsorption, while grazing on periphyton, or while using leaf litter as a refuge. These proposed exposure pathways are also possible routes of exposure in contaminated ecosystems. Furthermore, the aquatic communities in our mesocosms were exposed to environmentally relevant concentrations of cypermethrin (Crossland 1982, Crossland et al. 1982, Day 1989, Jergentz et al. 2005, Marino and Ronco 2005). Thus, the effects to amphibian larvae seen in the present study (mortality, reduced growth and slowed development of cypermethrin-exposed animals) are likely to occur as a result of cypermethrin exposure in the wild as well. This adds to the growing evidence that larval amphibians may be affected by pyrethroids contaminating natural water bodies (Materna et al. 1995, Boone 2008, Agostini et al. 2010, Ghodageri and Pancharatna 2011, David et al. 2012).

Cypermethrin exposure also affected all of the other members of the community that we surveyed. Exposure to any level of cypermethrin resulted in the mortality of zooplankton. These results were in line with predicted mortality based on zooplankton LC_{50} values obtained in mesocosms (Bradbury and Coats 1989, Giddings et al. 2001, Friberg-Jensen et al. 2003) and in laboratory experiments (Kim et al.

2008). This was therefore most likely a direct effect of cypermethrin's toxicity. We predicted that reduction in zooplankton populations would result in algal blooms. Indeed, increased phytoplankton (chlorophyll-*a*) concentration was observed in cypermethrin treatments. This indicates that the reduction of zooplankton populations had indirect effects on other community members. Previous research with other groups of pesticides including organophosphate and carbamate insecticides has produced similar results (Mills and Semlitsch 2004, Relyea and Diecks 2008, Groner and Relyea 2011). Phytoplankton blooms are expected to shade periphyton attached to surfaces in the water leading to a decrease in its abundance. However, we observed an increase in periphyton biomass. This trend toward increased periphyton biomass in cypermethrin treatments was likely driven by a presumable decrease in foraging pressure by fewer and smaller surviving larvae. These indirect effects of cypermethrin on an aquatic community would not have been predicted using traditional toxicological screening, which focuses on concentrations causing mortality in single species.

Collectively, these results indicate that cypermethrin, and pyrethroids as a group, are chemicals of concern for a variety of non-target taxa in aquatic environments. Pyrethroids are relevant to a wide range of aquatic habitats due to their agricultural, industrial and residential use. The concentrations used in the present study (1 - 5µg/L), while similar to some concentrations measured in the field, are probably fleeting in aquatic environments due to cypermethrin's low water solubility,

extremely high K_{oc} and short half-life (Crossland 1982, Crossland et al. 1982, Day 1989, Jergentz et al. 2005, Marino and Ronco 2005). Even so, brief exposure to these levels immediately following run-off or spray drift events can have far reaching effects as demonstrated in our single application treatments. We encourage additional mesocosm studies on the community level effects of pyrethroid insecticides as these tractable experiments contain contaminants while demonstrating their effects in a simulated community. Additionally, we encourage studies including more ecologically relevant conditions examining even lower concentrations of pyrethroids to determine a minimum concentration at which indirect effects might be observed throughout the food web.

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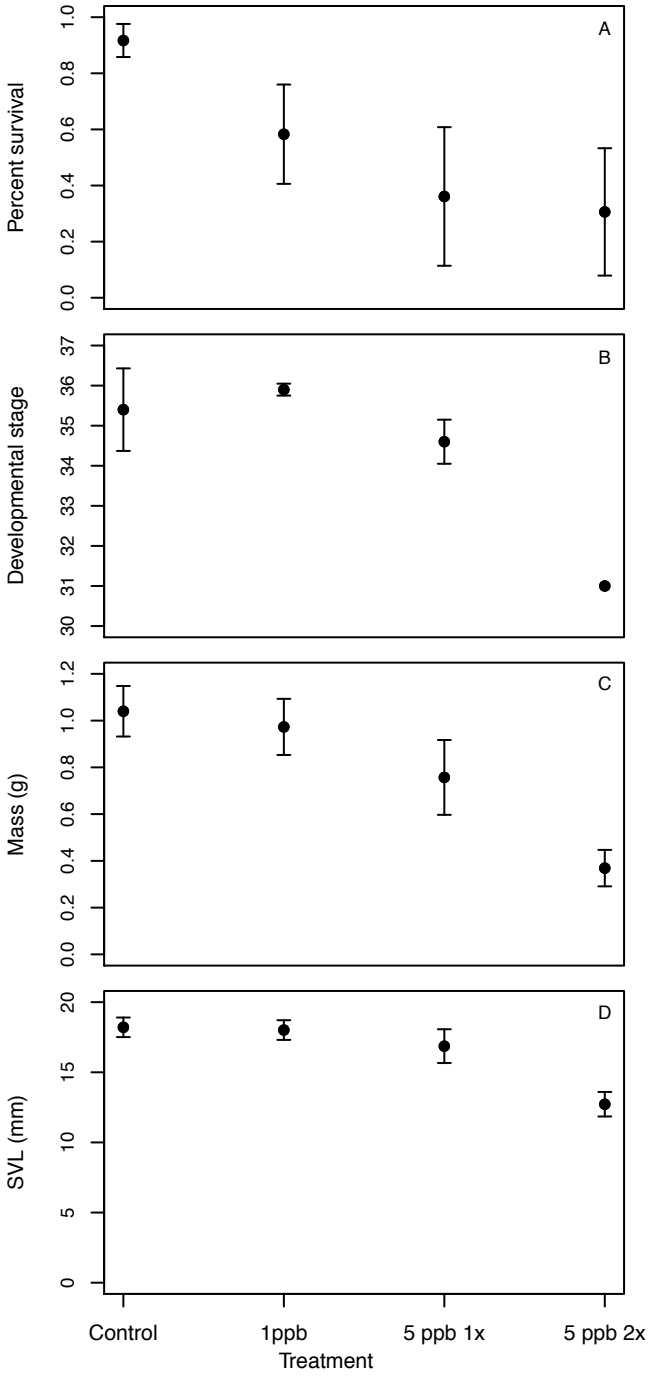

Figure 5.1 Effects of cypermethrin treatments on amphibians as A) percent survival, B) developmental stage reached (Gosner 1960), C) mass (g), and D) length (mm SVL) of amphibian larvae exposed in mesocosms (+SE). Each dot represents animals pooled by mesocosm and averaged by treatment.

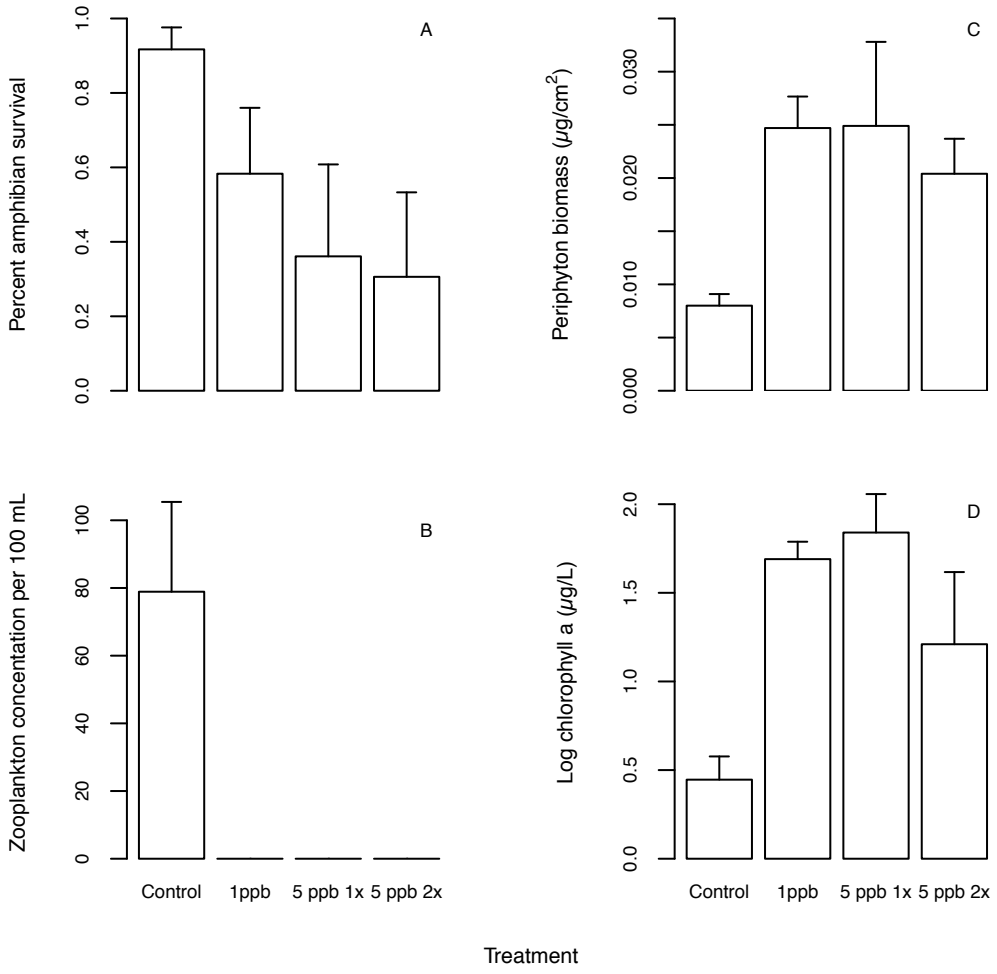

Figure 5.2 The effect of cypermethrin (+SE) on a) amphibian survival, b) zooplankton concentration, c) periphyton biomass, and d) chlorophyll-*a* concentration. Zooplankton were not detected in any of the cypermethrin-exposed treatments. Bars represent treatment averages.

CHAPTER 6: CONCLUSIONS

My dissertation explored the effects of pesticides, and in particular the pyrethroid insecticide cypermethrin, on amphibians under a variety of contexts. In the United States alone, about 20,000 pesticides with 675 active ingredients are registered for use, yet their unintended effects in the environment are not fully understood (Jones et al. 2004, Groner and Relyea 2011). Though pesticide use is most intense in agricultural and densely populated areas, wind and water currents have delivered them to even the most remote environments (Zhong et al. 2012). Pollution by pesticides is of particular concern in aquatic systems, due to their influx via run-off and aerial drift. Recent populations declines and species extinctions have made it even more pressing that we understand the role pesticides play in aquatic communities (Wake and Vredenburg 2008). Amphibians are one of the most threatened taxa associated with current biodiversity loss, and they are vulnerable to pesiticides in the aquatic environment (Stuart et al. 2005, McCallum 2007, IUCN 2012). Yet toxicological data are limited for amphibians (Hoke and Ankley 2005). My dissertation investigated the effects of pesticides, with special emphasis on the pyrethroid insecticide cypermethrin, on amphibian species, developmental stages, populations and their communities to better understand the impacts of this emerging pesticide in the aquatic environment.

In general, effects of pesticides are not consistent among species or chemicals. In Chapter 2, I reviewed the current literature on larval amphibian pesticide toxicity. No consistently sensitive or tolerant species could be identified based on my analysis

due to difference in relative sensitivity of amphibian species among chemicals and chemical types (i.e. insecticides, herbicides and fungicides). Furthermore, commercial formulations of a single active ingredient and the active ingredient alone varied greatly in toxicity among species and individual species did not demonstrate consistent sensitivity across formulations. Several classes of pesticides, especially new groups like pyrethroids, could not be included in this review due to the dearth of amphibian toxicity data for these chemicals. The recent restrictions on the uses permitted for the organophosphate insecticides chlorpyrifos and diazinon has led to a subsequent increase in usage of pyrethroid insecticides (Amweg et al. 2005, Weston et al 2005, Saha and Kaviraj 2008). Pyrethroids are extremely potent neurotoxins, yet tend not to persist in the environment and exhibit relatively low toxicity to mammals, adding further justification for their increased use (Berrill et al. 1993, Moore and Waring 2001). Cypermethrin in particular is used extensively in agricultural and non-agricultural settings for pest control (EPA 2008). Despite its relatively low toxicity to mammals, cypermethrin is considered by the United States Environmental Protection Agency to be very highly toxic on an acute basis to marine and freshwater invertebrates and fishes, and to honeybees (EPA 2008).

My experimental research illustrated differences in sensitivity to cypermethrin among amphibians. In Chapter 3 I tested the effects of cypermethrin on three amphibian species each at three distinct developmental time points. Cypermethrin exposure led to increased behavioral abnormalities and mortality for some species at

some stages. However, there was strong variation in sensitivity among both species and stages. In particular, the early larval stage and the Pacific treefrog (*Pseudacris regilla*) were found to be most sensitive. Knowing this, I experimentally exposed young larvae from three populations of *P. regilla* to cypermethrin reported in Chapter 4. Significant variation in time to death among *P. regilla* populations was observed. The results reported in Chapters 3 and 4 indicate that variation in sensitivity to pesticides exists among amphibians that may not be predicted if we rely on data from a single model species.

Although it has been hypothesized that cypermethrin's effects in natural aquatic systems will be negligible due to its low water solubility and tendency to sorb to organic material (Hill 1989), my research showed no support for this hypothesis. In the experiment reported in Chapter 5, I tested the effects of cypermethrin on amphibians in a community context using mesocosms, or semi-natural experimental ponds. Even in the presence of organic material (leaves and vegetation), cypermethrin had deleterious effects to the entire community. Amphibian growth and development were inhibited and mortality increased with exposure to cypermethrin. Moreover, cypermethrin exposure led to a decrease in the abundance of *Daphnia spp.* and an increase in the abundance of both periphyton and chlorophyll-*a* in all treatments compared to controls. Thus, even though cypermethrin is likely short lived in aquatic systems, we showed that it is capable of inducing lethal and sublethal effects in aquatic vertebrates and invertebrates. Therefore, cypermethrin exposure may

contribute to direct and indirect deleterious effect on members of aquatic ecosystems in natural settings.

Throughout my dissertation I found difference in the effects of pesticides among amphibians. This variation shows the need for more data on amphibian toxicity. Pesticide toxicity is a complex, context dependent issue. A pesticide that causes mortality in one species may have little or no effect on another at similar levels. Additionally, the effects of a pesticide in a population at one location may vary from the effects in other populations. Consequently, understanding the effects of pesticides and the factors that influence toxic effects among organisms is essential for a complete understanding of toxicity. My dissertation illustrated that toxicity may vary with species, stage, and population. As the use of pesticides continues to expand globally and new chemicals are added to the market and environment, it is increasingly important to understand the risks faced by vulnerable organisms and ecosystems. Studying pesticide toxicity across non-target species and in more natural settings is essential to influence best management and conservation practices in order to mitigate potential negative effects.

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