AN ABSTRACT OF THE DISSERTATION OF

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Title: MULTIPLE STRESSORS AND AMPHIBIAN POPULATION DECLINES

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Andrew R. Blaustein

In the past decade, declines in amphibian populations have captured scientific and popular interest. The causes of the declines are likely to be complex and involve interactions between several environmental stressors. Using multifactorial experiments, I investigated the combined effects of several anthropogenic stressors on developing amphibians in Oregon, USA. In laboratory experiments, I found that low levels of pH and exposure to high levels of nitrate killed larval *Rana cascadae*. Moreover, exposure to UV-B radiation and exposure to high levels of nitrate reduced larval activity level. Results suggest that in some cases, the effect of increasing nitrate level on larval activity depended on the pH level. In outdoor experiments, I investigated the combined effect of UV-B and nitrate fertilizer on two species of amphibians at both low and high elevation sites in Oregon. In *Hyla*

regilla, I found that UV-B and nitrate together had a negative effect on larval mass in the lower elevation site but adversely affected survival in the high elevation site. Nitrate increased larval mass in *Ambystoma macrodactylum*. However, in the higher elevation experiment, this effect occurred only when UV-B was blocked. Finally, using both laboratory and outdoor experiments, I investigated the combined effects of UV-B with the two commonly used pesticides, carbaryl and chlorpyrifos. I studied these effects in the larvae of three species of amphibians native to the highly agricultural Willamette Valley in Oregon: *Rana aurora*, *A. macrodactylum*, and *H. regilla*. Laboratory results for all three species revealed that a formulated pesticide product of chlorpyrifos killed larvae although the active ingredient alone did not, suggesting that some components of the pesticide formulations could be toxic to larval amphibians. In the laboratory study, there were no adverse effects caused by UV-B. However, outdoor studies indicated that ambient levels of UV-B enhance pesticide toxicity in amphibians. Both pesticides were toxic to *A. macrodactylum* in the presence of UV-B. Chlorpyrifos caused mortality in *R. aurora* in the presence of UV-B. Overall, results demonstrate the importance of considering multiple environmental stressors together in assessing amphibian population declines.

Multiple Stressors and Amphibian Population Declines

by

Audrey C. Hatch

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Major professor, representing Zoology

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Dean of the Graduate School

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.

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Audrey C. Hatch, Author

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CONTRIBUTION OF AUTHORS

Andrew R. Blaustein served as my graduate advisor and provided input in experimental design, results analysis in presentation for all components of my thesis. Lisa Belden helped with experimental design and data analysis in Chapters 4 and 5.

TABLE OF CONTENTS

TABLE OF CONTENTS, CONTINUED

TABLE OF CONTENTS, CONTINUED

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES, CONTINUED

DEDICATION

To Ian Downie.

MULTIPLE STRESSORS AND AMPHIBIAN POPULATION DECLINES

CHAPTER 1

GENERAL INTRODUCTION

AMPHIBIAN POPULATION DECLINES

Frogs, toads and salamanders are experiencing well-documented and unprecedented population declines, range reductions and extinctions (Kiesecker et al. 2001; Blaustein et al. 2001; Houlahan et al. 2000; Alford and Richards 1999; Pounds et al. 1997, 1999; Blaustein et al. 1994a,b, 1997; Pechmann 1991). Globally, there are several examples of amphibian populations declining beyond the range of natural population variability (Houlahan et al. 2000; Pounds et al. 1997, 1999). Anthropogenic contamination of the environment may be a primary reason for many population declines.

Several environmental factors have detrimental effects on amphibians and may be linked to population declines. Global climate change has been linked to range reductions and extinctions of species in Costa Rica (Pounds et al. 1999). In addition, ultraviolet radiation (UV-B) (*e.g.*, Anzalone et al. 1998, Blaustein et al.

1998, 1994a), nitrogen fertilizer runoff (*e.g.*, Rouse et al. 1999, Marco et al. 1999, Watt and Oldham 1995, Hecnar 1995), acidification (*e.g.,* Rowe et al. 1992; Warner et al. 1991; Harte and Hoffman 1989), and chemical pollutants including pesticides (*e.g.*, Howe et al. 1998, Russell et al. 1995, Materna et al. 1995; Berrill et al. 1993, 1995) and polycyclic aromatic hydrocarbons (PAHs) (Hatch and Burton 1998, Walker et al. 1998) have all been linked to detrimental effects on amphibian embryos or larvae. These effects on developing amphibians may contribute to population declines by reducing the number of individuals that reach sexual maturity. However, many amphibian population declines likely involve the combined effects of several environmental factors (*e.g.*, Kiesecker et al. 2001; Adams 1999; Alford and Richards 1999). For example, exposure to multiple pesticides can result in synergistic toxic effects (Howe et al. 1998). Low pH can enhance the toxicity of some pesticides (Fioramonti et al. 1997). Ultraviolet radiation (UV) can enhance the toxicity of some contaminants such as PAHs (Hatch and Burton 1998) or carbamate pesticides (Zaga et al. 1998) and can interact synergistically with the pathogen *Saprolegnia* (Kiesecker and Blaustein 1995) and low pH (Long et al. 1995). Given this potential for interaction between stressors, further investigation into the effects of multiple stressors on amphibians is clearly needed.

THE STUDY OF MULTIPLE STRESSORS

Introduction

Although animals are simultaneously exposed to a complex milieu of biotic and abiotic insults in nature, environmental risk assessments have traditionally consisted of studies of single stressors in controlled laboratory environments (Ferenc and Foran 2000; Foran and Ferenc 1999). When stressors are studied in combination, synergistic effects are often observed. For example, in a study along a gradient of metal pollution in Poland, food deprivation made carabid beetles more susceptible to insecticide treatment and therefore affected their distribution along the gradient (Stone et al. 2001). UV-B radiation and food shortage stress together increased the sensitivity of Antarctic amphipods to copper by several fold (Liess et al. 2001). While most studies have focused on individual animals, in some cases the effects of multiple stressors may become most apparent at higher levels of biological organization.

Effects of Stressors at the Population or Community level

At higher levels of organization such as the population or community, stress is often defined as a disorganizing process or disturbance (Newman 1995). Such disturbances have traditionally been used to help explain the distribution and abundance of organisms (*e.g.*, Paine et al. 1998; Menge and Olson 1990; Menge and Sutherland 1987; Connell and Sousa 1983; Andrewartha and Birch 1954). Examples of disturbances include fire, storms, volcanoes, disease, and biological invasion (*e.g.*, Paine et al. 1998). Both the types and number of stressful events are increasing because of human encroachment on natural habitat. In addition to altering the pattern of existing disturbances such as disease outbreaks and UV-B radiation, human activities have introduced several novel stressors such as chemical pollutants, overharvesting, global environmental change, and habitat destruction (Paine et al. 1998). Therefore, both anthropogenic factors and natural events or variables can be considered stressors.

Defining and quantifying the effects of stress on animal populations and communities is becoming increasingly important (Paine et al. 1998; Kammenga et al. 1996; Sibly and Calow 1991; Rapport et al. 1985; Odum 1985; Barrett et al. 1976; Selye 1956, 1973). Studies of the effects of stress usually focus on individual level effects. For example, Liebig's law of the minimum (defining the minimum level of a factor required to thrive) and Shelford's law of tolerance (defining the

level at which a factor becomes lethal) both concern the individual animal (*e.g.*, Newman 1995). However, individual effects can further impair higher levels of biological organization (Newman 1995, Maltby 1999). Direct effects of stressors on individuals can ultimately change the geographic distribution of animal populations and the composition of biological communities (Maltby 1999; Newman 1995; Andrewartha and Birch 1954). Along with nutrient input, environmental perturbations are one of the major factors contributing to community structure (Menge and Sutherland 1976, 1987). These two processes can directly impact predation intensity and thus affect trophic structure. In general, both predation intensity and trophic complexity are reduced in more stressful environments (Menge and Sutherland 1976, 1987; Menge and Olson 1990).

Importance of experiments

Experimentation provides a rigorous way to determine causal relationships between anthropogenic stressors and biological responses. Two concepts relating to experiments are particularly important in the study of amphibian population declines. First, the experimental venue is important when considering the effects of variables such as UV-B. Outdoor or field experiments are particularly useful in achieving natural levels of the variables of interest. This often provides for a more meaningful interpretation of results. Second, multifactorial experiments that

incorporate natural variables such as density or other important ecological factors are useful in the investigation of complex amphibian population declines. These types of studies can provide insight into how natural ecological variables might interact with anthropogenic stressors. For example, interactions between species such as competition and predation are probably influenced by many biological and abiotic factors simultaneously (Relyea and Mills 2001, Dunson and Travis 1991).

INTERACTIONS OF ABIOTIC STRESSORS

Introduction

Several studies of developing amphibians (embryos or larvae) have uncovered interactive effects between two or more environmental stressors. This work highlights the importance of considering multiple environmental factors simultaneously in studies on amphibians. Previous work (described briefly below) has focused on the effects of desiccation on other abiotic factors in the pond environment; interactions of low pH, dissolved organic carbon (DOC) and heavy metals; interactions between contaminants in the terrestrial environment and in the aquatic environment; interactions between various chemical contaminants; and interactions between UV radiation or low pH and chemical contaminants.

Desiccation

Many amphibian species use temporary freshwater pools for reproduction (Blaustein et al. 2001; Wilbur 1987, 1997; Horne and Dunson 1995a,b; Sredl and Collins 1992; Leibold and Wilbur 1992; Cortwright and Nelson 1990). Desiccation of these ponds may exacerbate the effects of environmental stressors. As ponds dry, a number of predictable changes occur that could interact with contaminants in the water. pH typically increases, and animal density increases (Wilbur 1987). In addition, water contaminants may become more concentrated as ponds dry (Rowe and Dunson 1994). Habitat desiccation also may interact with predation intensity to affect community structure (Cortwright and Nelson 1990). For example, if habitat for overwintering predatory species dries up, the community dynamics in a pond the following spring could be markedly different than if the predator were present (Cortwright and Nelson 1990). This situation highlights the importance of considering both biotic and abiotic factors in assessing effects on developing amphibians.

Interactions of low pH, DOC and heavy metals

In outdoor mesocosms, Horne and Dunson (1995a) examined interactions among low pH, metals and DOC levels in the water. DOC levels are important because they can ameliorate the effects of low pH or absorb UV rays. For both the wood frog (*Rana sylvatica*) and the spotted salamander (*Ambystoma maculatum*), at low DOC pH was the primary toxin. However, at high DOC, metal was the primary toxin. This study illustrates potential interactions between pH, DOC and metals.

Low pH increases the bioavailability of some heavy metals, making them more toxic (*e.g.*, Ferenc and Foran 2000). Horne and Dunson (1994) demonstrate an interaction among low pH, temperature, and metal toxicity in salamander larvae. Aluminum appeared to lessen the toxic effects of low pH above pH 4.5. However, at pH 4.4 or lower, aluminum did not have this effect; this may have been near the lower pH limit for salamander survival. Field surveys indicated that pH was higher in ponds that supported breeding populations of the Jefferson salamander (*Ambystoma jeffersonianum*). In a full factorial mesocosm design, Horne and Dunson (1995b) investigated the interactive effects of low pH, metals, and water hardness. Interactions were observed between pH and metals: greater wood frog survival was observed at low pH with toxic metals (aluminum, copper) than at high pH treatments with these metals (Horne and Dunson 1995b).

Tadpoles of three species of Swedish frogs (*Rana arvalis, R. temporaria* and *R. dalmatina*) had reduced survival and showed inactivity at low pH and high

aluminum concentration (Andren et al. 1988). Effects on development and activity level were enhanced by exposure to both aluminum and low pH simultaneously, when compared to the effects caused by each factor alone. In another study, survival of common frog tadpoles (*R. temporaria)* was reduced at pH 4.5 in high aluminum concentrations, and additionally, temperature-shocked embryos had reduced survival from those raised at constant temperature (Beattie et al. 1992). Low pH interacted with tannin-lignin concentrations in the water to cause mortality of spotted salamander (*Ambystoma maculatum)* embryos (Portnoy 1990). In a laboratory study, mortality of leopard frog (*Rana pipiens)* embryos was significantly greater in treatments with high levels of UV-B and low pH than in treatments with either low UV-B or neutral pH (Long et al. 1995).

Interaction of contaminants in water and soil

Contaminants in soil can influence water quality and, in turn, the resident amphibians of a pond. Sparling et al. (1995) used one hectare experimental macrocosm ponds accessible for colonization by the Northern cricket frog (*Acris crepitans*) and Gray treefrog (*Hyla versicolor*) to investigate interactions between acidification of the aquatic habitat and soil type of the terrestrial habitat. Water treatments consisted of four possibilities: (1) high aluminum; (2) low pH; (3) high aluminum and low pH; and (4) control treatments). Soil types investigated

included high metal/low organic matter clay soil or low metal/high organic matter loam soil. Soil type interacted with low pH to decrease the incidence of capturing frog tadpoles in traps. Differences among pH treatments were greater in macrocosms with clay than in macrocosms with loam soil. *Acris crepitans* abundance was lower in acidified treatments and in treatments with loam rather than clay soil. *H. versicolor* abundance was affected by acidification only in treatments with clay soils. In another study using experimental macrocosms, Sparling and Lowe (1996) found that species-specific microhabitat and soil use behavior affects ingestion of metal-contaminated sediments. Further, acidification of water reduced body concentrations of some trace metals (beryllium, magnesium and strontium). These studies illustrate the importance of considering surrounding environmental conditions and their influence on water quality. Soil type may influence the fate of contaminants in the pond.

Mixtures of chemical contaminants

The toxicological effects of chemical mixtures have been well studied (*e.g.*, Warne and Hawker 1995; Logan and Wilson 1995; Haas 1992). Many studies have documented synergistic or antagonistic effects of environmental chemicals (Newman 1995; Shirazi and Dawson 1991; Shirazi and Linder1991). Using amphibian larvae, Howe et al. (1998) studied mixtures of the commonly used

herbicides atrazine and alachlor. These herbicides are commonly applied together and are relatively stable in water. Toxicity of a mixture of equal parts of the two herbicides was greater than additive (synergistic) for leopard frog (*R. pipiens)* and American toad (*B. americanus)* larvae as well as young fry of rainbow trout (*Onchorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) at the larval life stages tested.

Ultraviolet radiation and interactions with contaminants

Several organic environmental pollutants interact with UV radiation in a synergistic manner. For example, chemicals such as polycyclic aromatic hydrocarbons (PAHs) that readily absorb UV-A (320-400 nm) are likely to be influenced by photochemical reactions (Arfsten et al. 1996, Bowling et al. 1983). PAHs are organic chemicals present in runoff from oil spills or other unburned fossil fuels (Bowling et al. 1983). Toxic effects are typically caused by phototoxic metabolites formed when PAHs are bioaccumulated (Arfsten et al. 1996). The toxicity of anthracene and the insecticide alpha-terthienyl to Northern leopard frogs (*Rana pipiens*) was enhanced by exposure to sunlight (Kagan et al. 1984). Alpha-terthienyl was not toxic in the absence of UV, or when embryos were not simultaneously exposed to UV. Similarly, anthracene was not toxic unless simultaneously exposed to UV-A (Kagan et al. 1984). Phototoxicity of several

different PAHs and of oil effluent on newt larvae (*Pleurodeles waltl*) has also been demonstrated (Fernandez and L'Haridon 1992, 1994).

Hatch and Burton (1998) demonstrated that a synergism between fluoranthene and UV reduced survival and causes embryonic deformities in three species of amphibians. In an outdoor exposure, time-to-death of spotted salamander (*Ambystoma maculatum)* and African clawed frog (*Xenopus laevis)* larvae was directly related to UV intensity and fluoranthene concentration. In a laboratory exposure, mortality of three species (*A. maculatum, Rana pipiens, and X. laevis)* occurred directly after hatching. Walker et al. (1998) found skin damage and hyperactivity in bullfrog tadpoles (*Rana catesbeiana)* as a result of exposure to fluoranthene and UV. Monson et al. (1998) found bioaccumulation of fluoranthene in *Rana pipiens* in proportion to water concentrations. Mortality was related to both UVA intensity and fluoranthene body concentration.

Some pesticides also interact with UV to become phototoxic or to degrade more rapidly. UV-B radiation alone and with carbaryl decreased the swimming activity of African clawed frog (*X. laevis)* and gray tree frog (*H. versicolor)* (Zaga et al. 1998). The authors concluded that low levels of ambient UV-B resulted in photoactivation of carbaryl.

Pesticide/pH

Pesticides such as the fungicide triphenyltin (TPT) may interact with pH (Fioramonti et al. 1997). One study investigated the combined effects of TPT with pH using a hemiclonal hybrid species in the *Rana lessonae/Rana esculenta* species complex. In this system, two separate species (*Rana lessonae* and *R. ridibunda)* mate and form a hemiclonal hybrid species (*Rana esculenta*) that is typically more tolerant of environmental stressors than the parental species (Fioramonti et al. 1997). At higher pH (8.1 vs. 6.4), *R. lessonae* had reduced survival and delayed development time in comparison to *R. esculenta.* At the lower pH, *R. lessonae* had increased growth and development time. This study demonstrates that the response to environmental stressors may depend on genotype.

ORGANIZATION OF DISSERTATION

My dissertation reports on my investigations of the effects of several multiple stressors on developing amphibians. In separate studies, I investigated the combined effects of UV-B radiation, nitrate fertilizer, and low pH; nitrate and UV-B radiation; and UV-B radiation and the pesticides atrazine, carbaryl and chlorpyrifos. Investigations focused on larval amphibians and included both lethal and sublethal endpoints.

Study System

The Pacific Northwest is relatively diverse in herpetofauna, particularly frogs, toads and salamanders (Walls et al. 1992; Nussbaum et al. 1983). My thesis focuses on several species of amphibians native to the Willamette Valley region of Oregon: the Pacific treefrog *Hyla regilla*, long-toed salamander *Ambystoma macrodactylum*, and the red-legged frog *Rana aurora*. In addition, I studied one species native to the Cascade Mountain region of Oregon: the Cascades frog *Rana cascadae*. These species vary in their conservation status and their documented sensitivity to various anthropogenic stressors. For example, red-legged frog populations appear to be declining, but are resistant to the effects of UV-B (Blaustein et al. 1996). Few data are available for long-toed salamander populations, but this species is sensitive to UV-B stress (Belden et al. 2000; Blaustein et al. 1994a). In each chapter, background for the specific species, and how this relates to their predicted sensitivity to the stressors of interest, is described.

Studies were conducted in both the Willamette Valley and the Cascade Mountains in Oregon. There are several important differences between these regions. The Willamette Valley is highly agricultural, with associated pesticide and fertilizer contamination. In contrast, amphibian breeding ponds in the Cascade

Mountain range are typically not affected by the same types of direct human impacts such as intensive agriculture or urbanization. However, amphibians breeding in roadside ponds in the Cascades may encounter road runoff with hydrocarbons, sediments or other contaminants, and native amphibians may be threatened with introduced fish. Moreover, amphibians breeding in the Cascades may be exposed to subtle types of environmental stressors. For example, UV-B intensity is usually greater in the Cascades Mountain Range compared to lower-elevation sites. Pesticides and other contaminants can be transported in the atmosphere and may accumulate to toxic levels when deposited in vegetation in mountainous regions (e.g., Aston and Seiber 1997).

Many amphibian species typically breed in small, temporary pools, where the concentrations and effects of contaminants could be exacerbated as the pond dries. Outdoor experimental ponds, or mesocosms, provide an ideal method to study the effects of abiotic and biotic factors on amphibians because they simulate the temporary pond environment. Several important studies have investigated amphibians in ecological community dynamics using mesocosms as experimental systems (*e.g.*, Wilbur 1987, 1997; Werner and Anholt 1996; Rowe and Dunson 1994; Sredl and Collins 1992; Leibold and Wilbur 1992). Mesocosms provide close approximations of natural conditions such as lighting and photoperiod, UV radiation, and temperature fluctuation, while still providing the ability to experimentally manipulate variables of interest. Mesocosm studies are useful for answering questions in both ecology and ecotoxicology, and are a useful means to

integrate ecology and ecotoxicology (Hansen and Johnson 1999, Rowe and Dunson 1994, Dunson and Travis 1991).

UV-B: Background

Levels of ultraviolet radiation (UV; especially UV-B, 280-320 nm) reaching the Earth's surface are increasing because of human-induced depletion of the ozone layer, acidification and global climate change (Middleton et al. 2001; Pienitz and Vincent 2000; Herman et al. 1996; Schindler et al. 1996; Yan et al. 1996; Kerr and McElroy 1993). These global changes increase the potential for exposure of many organisms to harmful UV-B (Cockell & Blaustein 2001, Hader et al. 1998). UV-B can cause direct mortality of amphibian embryos and larvae (*e.g.*, Broomhall et al. 2000; Lizana & Pedraza 1998; Anzalone et al. 1998; Blaustein et al. 1994a, 1995) as well as sublethal effects (Belden et al. 2000; Belden and Blaustein 2002; Blaustein et al. 2000; Kats et al. 2000; Nagl & Hofer 1997;). Both climate warming and acidification can lower dissolved organic carbon (DOC) concentrations in lakes, permitting more UV rays to penetrate the water (Schindler et al. 1996). In fact, this effect could increase UV intensity as much or more than depleting ozone levels.

Acidification: Background

Low pH in amphibian breeding ponds may result from acidic deposition, and this may decrease the survival, growth, and/or development of larval amphibians (*e.g.*, Kiesecker 1995; Rowe et al. 1992; Freda 1986). The Pacific Northwest has not experienced significant contamination from acidification (Goward and Arsenault 2000). However, montane lakes in this region, such as Waldo Lake in the Cascades Mountain Range, are highly susceptible to the effects of acid deposition because water in these lakes has a very low buffering capacity (Nelson 2000). The small temporary ponds where some amphibians breed, such as those forming from snowmelt, may have particularly low ability to buffer any changes resulting from acid deposition (Freda 1986). Furthermore, even slight acidification may be a potential problem for amphibians when considering the possibility for synergistic interactions with other environmental stressors (*e.g.*, increasing UV-B radiation; Long et al. 1995).

Nitrogenous fertilizers: Background

Fertilizer runoff from agricultural areas and game preserves likely contaminates many amphibian breeding sites, particularly temporary ponds (Boyer and Grue 1995). Nitrate contamination via fertilizer runoff and agricultural waste is

of particular concern in the highly agricultural Willamette Valley. In addition, new forest stands in the Cascades mountain range are fertilized annually by the U.S. Forest Service, and perhaps more frequently by private plantation owners (USFS, personal communication). Larval stages of amphibian species native to Oregon are sensitive to nitrite and nitrate, the breakdown products of nitrogen fertilizer contamination (Marco et al. 1999, Marco and Blaustein 1999). Other studies have demonstrated that ammonium nitrate exposure at 78 and 155 mg nitrate/L affects the growth of newts (*Triturus vulgaris*) and the behavior of newt prey (Watt and Oldham 1995). Nitrate exposure at a level of 10 mg nitrate/L significantly affected the survival of chorus frog (*Pseudacris triseriata*) and leopard frog (*Rana pipiens*) tadpoles over a 100-day exposure (Hecnar 1995). A USGS survey of Oregon watersheds reveals that nitrate levels are typically less than 5 mg nitrate/L, but that pulses of up to 20 mg nitrate/L are noted in some areas (http://water.usgs.gov/pubs/circ/circ1144/nawqa91.5.html) because of agriculture.

Pesticides (carbaryl and chlorpyrifos): Background

The effects of pesticides in amphibians are poorly understood and may differ from effects in other animals because of the unique amphibian life cycle (Sparling et al. 2001; Stebbins and Cohen 1995). For example, pesticides may be stored and mobilized at critical events such as metamorphosis, emergence from

hibernation, or egg resorption (Sparling et al. 2000; Stebbins and Cohen 1995; Russell et al. 1995). The potential for low levels of pesticides to affect amphibian reproduction (*e.g.,* Pickford and Morris 1999) or immunosuppression (*e.g.,* Gilbertson et al. 2000) are also poorly understood but of potential concern. I selected two pesticides for study: the organophosphate chlorpyrifos and the carbamate carbaryl. Both of these pesticides are frequently applied in agricultural areas, and additionally often used on private land and in forests (Barron and Woodburn 1995; Cox 1993; Racke 1993).

Chapter Objectives

Chapters 2-5 present a multifactorial assessment of stressors commonly found in agriculturally impacted areas and their effects on larval amphibians, particularly those native to the Willamette Valley, Oregon USA. In chapter 2, I investigated the combined effects of UV-B radiation, nitrate fertilizer and acidification on the survival and activity level of larval Cascades frogs (*Rana cascadae*) in the laboratory, using simulated levels of UV radiation. Factors manipulated in this experiment included UV-B (intensity of approximately 9 $\mu W/cm^2$); pH; and nitrate. These levels were similar to those larval Cascades frogs encounter in the field. UV-B levels in the mountains can be as high as 22 $\mu W/cm^2$ in the summer (pers. obs.). At larval breeding ponds, pH is typically in the range of

5.2-6.2 (pers. obs.). Nitrate levels of 5 or 20 mg/L (agricultural pulse levels) might be observed under conditions of direct runoff in the early spring, from the application of forest fertilizers.

In Chapter 3, I investigated the combined effects of UV-B radiation and nitrate fertilizer on the growth and survival of larval Pacific treefrogs (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum*). Populations of both of these species are found at both high and low elevation sites. At each of these locations, their history of exposure to UV-B may differ, affecting their sensitivity to UV-B (Belden and Blaustein 2002; Belden et al. 2000). UV-B typically is greater at the high elevation sites compared to the low elevation sites, and it is possible that high elevation populations have adapted more mechanisms to compensate for this stress (Belden and Blaustein in press). I studied ambient levels of UV radiation and nitrate for *H. regilla* in the Willamette Valley, and levels of 0, and 10 mg/L for *A. macrodactylum* in both experiments and for *H. regilla* in the Cascade Mountain experiment.

In Chapters 4-5, I assessed the combined effects of UV-B radiation and commonly used pesticides (chlorpyrifos and carbaryl) on several species of larval amphibians native to the Willamette Valley: *H. regilla, R. aurora, A. macrodactylum*, and *A. gracile*. Three-week laboratory and mesocosm exposures involved manipulating UV exposure and pesticide levels. I studied effects on larval survival, growth and activity level.

Overall, these studies investigate the potential for interactions between common environmental stressors on developing amphibians in the Willamette Valley. I hypothesized that, in some cases, the results of exposure to combined environmental stressors would result in significantly reduced survival/growth/activity level compared to exposure to single environmental stressors. These studies represent a unique cohesive effort to address the combined effects of multiple stressors on amphibians in Oregon, and have implications for the conservation of some of these declining species.

CHAPTER 2

COMBINED EFFECTS OF UV-B, NITRATE, AND LOW pH REDUCE THE SURVIVAL AND ACTIVITY LEVEL OF LARVAL CASCADES FROGS (*RANA CASCADAE*)

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ABSTRACT

We investigated interactions between low pH, high nitrate level, and ultraviolet-B (UV-B) light on the survival and activity level of larval Cascades frogs (*Rana cascadae)*. We used a fully factorial experimental design, with pH levels of 5 and 7; initial "pulse" nitrate exposure levels of 0, 5, and 20 mg/L; and UV-B present or absent. After a three-week laboratory exposure, we measured survival and activity level of the larvae. The experiment was repeated two times, in two separate years. In both years, analysis of variance (ANOVA) indicated that pH and nitrate had the greatest effect on survival, and that UV-B and nitrate had the greatest effect on activity level. Additional effects were observed in the 1998 experiment on survival. In this experiment, UV radiation and an interaction between pH and nitrate affected survival. Overall, results indicate that these important environmental factors could have detrimental effects on *R. cascadae* larvae at environmentally relevant levels.

INTRODUCTION

Amphibian populations at several locations worldwide appear to be declining (e.g., Pounds et al. 1999, Blaustein et al. 1994a, 1994b; Richards et al. 1993). Several anthropogenic factors likely contribute to these population declines,

including habitat destruction, climate change, increased intensity of ultraviolet-B radiation (UV-B; 280-315 nm), pathogens, introduced species, and chemical pollution (e.g. Pounds et al. 1999, Blaustein et al. 1997, Blaustein et al. 1994a, 1994b, 1994c). Amphibians may be particularly sensitive to these environmental impacts because of their thin permeable skin and biphasic life history, exposing them to environmental stressors in both aquatic and terrestrial environments (Blaustein et al. 1994b).

Although several environmental factors may contribute to amphibian population declines, there is probably not one single pervasive cause for the declines. In fact, developing amphibians are simultaneously exposed to a variety of stressors in nature. Moreover, detrimental effects caused by synergistic interactions have been demonstrated between several environmental stressors. For example, synergistic effects were observed between pathogens and UV (Kiesecker and Blaustein 1995), pH and UV (Long et al. 1995), and between chemical contaminants and UV (Hatch and Burton 1998, Zaga et al. 1998). In these studies, survival was reduced greatly by simultaneous exposure to stressors, in comparison to exposure to a single stressor alone.

Often, the response of developing amphibians to environmental stressors depends on other abiotic conditions or stressors that are present. For example, the effects of the fungicide triphenyltin on the growth and development of larval *Rana lessonae* and *Rana esculenta* depended on pH (Fioramonti et al. 1997). In a series of multifactorial experiments, Horne and Dunson demonstrated that some metals

ameliorate the toxicity of low pH (Horne and Dunson 1994), but at higher pH survival was lower in the presence of some nontoxic metals (Horne and Dunson 1995a). These investigators have further shown that different amphibian species respond to different combinations of environmental stressors. For example, in a study of the combined effects of metals, low pH and dissolved organic carbon (DOC) concentration, wood frogs (*Rana sylvatica*) were most negatively affected by high DOC in combination with low pH, while the salamanders *Ambystoma jeffersonianum* and *Ambystoma maculatum* were most affected by low pH in combination with high metal concentration (Horne and Dunson 1995a).

Environmental stressors may alter biological interactions with unexpected ecological consequences. For example, low dissolved oxygen alters the behavior of *Rana clamitans* tadpoles, making them more susceptible to predation by fishing spiders (*Dolomedes triton*) (Moore and Townsend 1998). Interactions between biotic and abiotic factors may differ between amphibian species. Density interacted with low pH to delay metamorphosis and decrease weight at metamorphosis for *Hyla gratiosa* but not for *Hyla femoralis* (Warner et al. 1991).

In many cases, greater toxicity or altered biological interactions are observed when animals are simultaneously exposed to more than one stressor than when animals are exposed to only a single stressor. Synergism may be attributed to a mechanism in which the toxic action of one factor is altered or enhanced by the presence of another, for example in the photoinduced toxicity of some chemical contaminants (e.g. Hatch and Burton 1999, 1998; Zaga et al. 1998). Alternatively,

interactions may result from a reduced tolerance for stressors in the presence of other stressors.

In this study, we investigated the combined effects of UV, nitrate (fertilizer runoff), and low pH on the survival and activity level of larval *Rana cascadae* (the Cascades frog), using a full factorial experimental design in the laboratory. All three factors are, currently or potentially, relevant to breeding sites of these two species in montane regions of Oregon.

The intensity of UV-B radiation that reaches the Earth's surface may be increasing because of depletion of the ozone layer (McKenzie et al. 1999, Kerr and McElroy 1993). Declines in the ozone layer have been linked to increases in biologically active UV-B in several locations worldwide, including Hawaii, Germany, Toronto Canada, Greece, New Zealand, and the South Pole (Madronich et al. 1998). In the Cascade mountain range of Oregon, ambient levels of UV-B increase egg mortality of *R. cascadae* and *Bufo boreas* (Western toad) (Blaustein et al. 1994a) and cause mortality and deformities in *Ambystoma macrodactylum* (long-toed salamander) embryos (Blaustein et al. 1997). In the Santa Monica Mountains in southern California, UV-B reduces the survival and the hatching success of embryonic *Hyla cadaverina* and *Taricha torosa* (Anzalone et al. 1998). Alpine newts (*Triturus alpestris*) in central Europe are sensitive to UV-B, exhibiting skin damage, behavioral abnormalities and mortality when exposed to UV (Nagl and Hofer 1997).

Nitrate may contaminate amphibian breeding sites as a result of runoff from agricultural use or, in the Cascades mountain range, as a result of the application of forest fertilizers. Nitrate can be toxic to some species of developing amphibians at environmentally realistic levels (Marco et al. 1999, Hecnar 1995). Several species commonly occurring in the Cascades range are sensitive to low levels of nitrate and nitrite (Marco et al. 1999). In a study of amphibians in Southern Ontario, exposure to levels of nitrate typical of agricultural areas reduced the survival of *Pseudacris triseriata* (chorus frog) and *Rana pipiens* (leopard frog) (Hecnar 1995). Acute nitrate exposure reduced the size of newt (*Triturus vulgaris*) larvae (Watt and Oldham 1995). Ammonia (NH_3), which may be associated with high nitrate input into freshwater systems from organic decomposition or animal excretion, is also toxic to amphibians at environmentally realistic levels (Jofre and Karasov 1999). Nitrite ($N\text{-}NO_2^-$), which also may be associated with nitrogen-based fertilizer application, delayed metamorphosis and altered the behavior of larval *R. cascadae*; larvae occupied shallow water more frequently than control larvae, possibly in an effort to increase their oxygen intake (Marco and Blaustein 1999).

Finally, low pH alone may impair the development of amphibians (*e.g.*, Kiesecker 1995; Rowe et al. 1992; Freda 1986). Although acidification is not an immediate threat to freshwater systems in the Pacific Northwest, even slight acidification may be a potential problem when considering the possibility for synergistic interactions with other environmental stressors (e.g. increasing UV-B radiation; Long et al. 1995). Water in the Pacific Northwest region may be at risk

for acidification because of its low buffering capacity. Moreover, small temporary ponds or ditches where some amphibians breed may have a particularly low buffering capacity (Freda 1986).

METHODS AND MATERIALS

Larvae collection and rearing

Rana cascadae larvae (Gosner stage 13-15; embryonic, neural fold developing; Gosner 1960) were collected from Parrish Pond (62 km east of Lebanon in Linn County, Oregon) upon breeding in 1998 (May 6) and 1999 (June 1). Experiments began when developing larvae were at Gosner stage 23-24 (free-swimming larvae, operculum developing, external gills disappearing; Gosner 1960) and lasted for three weeks. Larvae were at Gosner stages 30-33 (toes developing on hind limb bud; Gosner 1960) at the end of the experiment. Prior to experimental exposure, larvae were housed in 38 L aquaria with approximately 100 larvae/tank, under a 14 h light: 10 h dark photoperiod. Larvae were fed ground rabbit chow *ad libitum* during rearing and during experiments. Dechlorinated tap water was used for rearing larvae and in experimental trials.

Experimental design and water quality

UV, pH and nitrate were manipulated in a 2 X 2 X 3 factorial design, for a total of 12 treatments. Each treatment was replicated four times in 500 mL plastic cups filled with 400 mL water, containing five larvae per cup. Twenty larvae were exposed to each of the 12 treatments.

To maintain the controlled pH levels, water was changed (approx. 30%) every 48 h. Temperature, UV-B level, and pH were measured at each water change. pH was measured in the renewal water, and UV-B and temperature were measured in the constant-temperature experimental room. pH was measured using an Orion 290A pH/ISE meter with a pH electrode. UV-B was quantified at the water's surface using a UV-B meter (model 2100 PMA meter with model 2102 detector, Solar Light Co., Philadelphia, PA). UV-A was quantified after the experiment ended using the same meter and a PMA2111 detector.

To provide further information on the experimental conditions of our study, we measured nitrate, dissolved oxygen, conductivity, alkalinity and hardness levels in the water weekly. Nitrate was quantified with an Orion 290A pH/ISE meter with a nitrate electrode. Conductivity was measured using a Hanna Instruments hand-

held conductivity meter. Dissolved oxygen was calculated using the Winkler titration method (American Public Health Association 1995). Water hardness and alkalinity were measured by titration with EDTA and 0.02 N HCl, respectively (APHA 1995).

Experimental Variables

UV lighting was achieved in the laboratory using UV-B 313 light bulbs (Q Panel Inc., Cleveland, OH, USA) alternated with full spectrum Vita-Lite light bulbs (Duro-Test Corporation, Fairfield, NJ, USA). We used equal numbers of UV-B bulbs and Vita-Lite bulbs. Vita-Lite bulbs were included to ensure the presence of UV-A radiation (approximately 315-400 nm), a natural component of sunlight and essential for photorepair processes in the developing amphibians (Blaustein et al. 1994a and references therein). Two levels of UV were included in the experimental design: with UV (using acetate filters) and without UV (using Mylar® filters). The use of these filters in UV experiments has been described elsewhere (e.g., Blaustein et al. 1994a, 1997). Acetate filters transmit approximately 80% of UV-B and UV-A intensity.

We used levels of pH 5 and pH 7 in our tests. pH 7 was unmanipulated dechlorinated tap water. pH 5 was achieved by acidifying dechlorinated tap water using a ratio of 2:1 sulfuric acid (H_2SO_4) to nitric acid (HNO_3) (Kiesecker 1995).

At every water change, pH was measured and adjusted in the source water before adding water to exposure containers.

We used sodium nitrate ($NaNO_3$) to obtain three initial concentrations of nitrate: 0, 5, or 20 mg/L. Nitrate levels in the treatments were measured weekly. Although renewal water contained nitrate at the appropriate concentration, degradation of nitrate occurred over the experiment and the concentration was not maintained over the entire experiment's duration. This type of exposure regime may represent a "pulse" type of exposure similar to the type of exposure at amphibian breeding sites, where runoff of nitrate fertilizers occurs following fertilizer application and then decreases over time.

Response Variables

We assessed survival and activity level in each treatment. Dead animals were removed when observed at each water change, and overall survival was determined at the end of the experiment by counting the number of animals alive. To measure activity level of *R. cascadae*, individual larvae were placed in plastic boxes and stacked in a manner so that they could not see one another. After a 15 minute acclimation period, the observer watched animals at 30-second intervals for 10 minutes. Each animal was scored as moving or not moving, and the proportion of time spent active was calculated.

Statistical Analysis

Differences between treatment groups in the percent survival and proportion of time spent active were analyzed using analysis of variance (ANOVA) with SAS version 6.12 software. Survival was determined as the mean proportion of surviving animals per cup. Activity level was determined as the proportion of time spent active for each individual. Following these measurements, container means were used as the units for all statistical analyses. Prior to analysis, data were arc-sine-square root transformed to achieve normality.

RESULTS

Water quality

Water quality variables were within the same range in both years, with the exception of temperature and pH. Temperature in the exposure containers ranged from 14 to 17 C during the 1999 experiment and from 12 to 14 C during the 1998 experiment. pH in the pH 5 treatments varied from 4.7 to 5.3 in 1998 and from 4.8

to 5.2 in 1999. In both experiments, pH in the pH 7 treatments varied from 6.8 to 7.4. UV-B at the water's surface varied from 9-11$\mu W/cm^2$ during both years. The UV-A intensity, measured after the completion of the experiment, was 57 $\mu W/cm^2$. Alkalinity in the water ranged from 15-20 mg $CaCO_3$/L, hardness (measured as EDTA) ranged from 32-48 mg $CaCO_3$/L, and conductivity ranged from 220-248 μmhos/cm. Nitrate levels were within expected ranges based on the treatment levels. As expected, nitrate levels decreased somewhat during the three-week exposure (Table 2.1).

1998 Experiment

Both survival and activity level were reduced by the experimental variables (Table 2.3). In the low pH treatment, survival was reduced by increasing levels of nitrate. Moreover, survival was reduced by exposure to UV-B radiation. Activity level was reduced by high nitrate exposure and by UV-B exposure (Table 2.3).

Table 2.1. Measured nitrate concentrations (in units mg/L) for nominal concentrations used in nitrate/pH/UV-B experiments. nd = not detectable.

Initial:	*pH 5:*			*pH 7:*		
	0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
	0.49	4.95	20.8	0.31	5.01	19.7

One week:	*pH 5:*			*pH 7:*		
	0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:	0.24	2.91	11.8	nd	3.02	9.2
With UV:	0.47	2.98	10.0	0.32	2.76	10.2

Two weeks:	*pH 5:*			*pH 7:*		
	0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:	0.11	1.02	4.76	0.22	1.52	3.28
With UV:	nd	1.76	3.63	0.41	1.32	4.21

Three weeks:	*pH 5:*			*pH 7:*		
	0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:	0.20	0.95	2.54	0.23	0.62	2.65
With UV:	nd	0.98	3.20	0.15	0.35	1.51

Table 2.2. *Rana cascadae* survival and activity level, 1998 nitrate/pH/UV-B experiment. Mean percent survival or mean proportion of time active with standard error in parentheses.

Rana cascadae percent survival, 1998 experiment:

pH 5			pH 7		
0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:					
94% (5)	65(10)	70(10)	99(0)	94(5)	90(6)
With UV:					
90(6)	45(15)	40(8)	90(6)	90(6)	85(5)

Rana cascadae proportion of time active, 1998 experiment:

pH 5			pH 7		
0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:					
.26(.04)	.22(.05)	.21(.04)	.34(.07)	.39(.08)	.15(.05)
With UV:					
.18(.04)	.25(.08)	.14(.07)	.23(.06)	.16(.05)	.14(.03)

Table 2.3. Analysis of variance models for *Rana cascadae* survival and activity level, 1998 nitrate/pH/UV-B experiment.

Model for *Rana cascadae* survival, 1998 experiment:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
NO_3	2	1.174	0.587	10.25	<0.001
UV	1	0.429	0.429	7.51	0.009
pH	1	1.471	1.471	25.69	<0.001
UVx NO_3	2	0.005	0.003	0.05	0.952
pHxNO_3	2	0.565	0.282	4.93	0.013
pHxUV	1	0.0246	0.025	0.43	0.517
pHxUVxNO_3	2	0.060	0.030	0.53	0.595
Error	36	2.061	0.057		
Total	47	5.791			

Model for *Rana cascadae* activity level, 1998 experiment:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
NO_3	2	0.390	0.195	3.95	0.021
UV	1	0.419	0.419	8.51	0.003
pH	1	0.015	0.015	0.29	0.589
UVxNO_3	2	0.108	0.054	1.10	0.336
pHxNO_3	2	0.074	0.037	0.75	0.473
pHxUV	1	0.094	0.094	1.91	0.168
pHxUVxNO_3	2	0.219	0.109	2.22	0.111
Error	36	8.883	0.049		
Total	47	10.204			

1999 Experiment

Results from the 1999 experiment were similar to the results from the 1998 experiment. Survival was reduced by nitrate addition and by low pH (Table 2.5). Activity level was reduced by nitrate addition and by UV exposure (Table 2.5).

DISCUSSION

In both of our experiments, survival and activity level of larval *R. cascadae* were reduced by the experimental variables. The effects of pH, nitrate and UV were similar in each year: UV and pH affected survival, while UV and nitrate affected activity level. Additionally, in the 1998 experiment, the interaction term between nitrate and pH affected survival.

Table 2.4. *Rana cascadae* survival and activity level, 1999 nitrate/pH/UV-B experiment. Mean percent survival or proportion of time spent active with standard error in parentheses.

Rana cascadae percent survival, 1999 experiment:

pH 5			pH 7		
0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:					
100(0)	94(5)	90(5)	94(9)	100(0)	100(0)
With UV:					
100(0)	90(5)	75(9)	100(0)	100(0)	94(5)

Rana cascadae proportion of time active, 1999 experiment:

pH 5			pH 7		
0 mg/L	5 mg/L	20 mg/L	0 mg/L	5 mg/L	20 mg/L
Without UV:					
.65(.02)	.52(.02)	.53(.10)	.51(.02)	.53(.04)	.50(.07)
With UV:					
.45(.06)	.58(.04)	.29(.13)	.46(.05)	.45(.04)	.34(.05)

Table 2.5. Analysis of variance models for *Rana cascadae* survival and activity level, 1999 nitrate/pH/UV-B experiment.

Model for *Rana cascadae* survival, 1999 experiment:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
NO_3	2	0.229	0.115	5.01	0.012
UV	1	0.049	0.049	2.14	0.152
pH	1	0.106	0.106	4.65	0.038
$UVxNO_3$	2	0.114	0.057	2.50	0.096
$pHxNO_3$	2	0.123	0.062	2.69	0.082
pHxUV	1	0.0135	0.014	0.59	0.447
$pHxUVxNO_3$	2	0.004	0.002	0.07	0.929
Error	36	0.823	0.023		
Total	47	1.462			

Model for *Rana cascadae* activity level, 1999 experiment:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
NO_3	2	0.141	0.070	6.00	0.006
UV	1	0.145	0.145	12.39	0.001
pH	1	0.028	0.028	2.39	0.131
$UVxNO_3$	2	0.096	0.048	3.12	0.065
$pHxNO_3$	2	0.015	0.007	0.63	0.538
pHxUV	1	0.001	0.001	0.06	0.812
$pHxUVxNO_3$	2	0.048	0.024	2.04	0.145
Error	36	0.422	0.012		
Total	47	0.896			

In the current study, both nitrate and UV exposure had detrimental effects on *R. cascadae* larvae. Previous work has investigated the effects of each of these environmental agents alone on various developmental stages of *R. cascadae*. In these previous studies, *R. cascadae* was sensitive to the effects of nitrate as larvae (*e.g.*, Marco and Blaustein 1999) and to the effects of UV as embryos (Blaustein et al. 1994a). *R. cascadae* embryos are sensitive to the effects of UV-B alone in field experiments, demonstrating reduced survival and hatching success when exposed to UV-B, and relatively low levels of the repair enzyme photolyase (Blaustein et al. 1994a). *R. cascadae* larvae are sensitive to the sublethal effects of nitrite, exhibiting reduced growth and increased time to metamorphosis (Marco and Blaustein 1999). Further, *R. cascadae* larvae exhibited behavioral effects when exposed to nitrite: larvae spent more time near the water's surface than did controls, possibly in an effort to increase their oxygen intake (Marco and Blaustein 1999). In another study, other species common to the Cascades mountain range were sensitive to the effects of both nitrate and nitrite (Marco et al. 1999). Nitrite was generally more toxic than nitrate, although survival of spotted frog (*Rana pretiosa*) and salamander (*Ambystoma gracile*) larvae was reduced by exposure to nitrate greater than 10 mg/L (Marco et al. 1999).

Sublethal behavioral endpoints are useful and should be included in studies of lethal effects of stressors on developing amphibians. Behavior may be altered as a result of exposure to environmental stressors (*e.g.*, Hatch and Burton 1999, Marco and Blaustein 1999), with the potential for altered biological interactions

with species. UV-B exposure alters the orientation behavior of roughskin newts (*Taricha granulosa*) and the response to chemical cues from predators in juvenile Western toads (*Bufo boreas*) and larval *T. granulosa* and *R. cascadae* (Blaustein et al. 2000, Kats et al. 2000). Exposure to non-lethal levels of nitrate increased the feeding behavior of smooth newt (*Triturus vulgaris*) larvae, and caused newt prey (*Daphnia*) to spend more time near the top of the water column (Watt and Oldham 1995). The insecticide carbaryl reduced tadpole activity level and sprinting performance (speed and distance) (Bridges 1997). Low pH reduced the swimming activity of *Ambystoma laterale* larvae and increased the risk of predation by diving beetles to *Ambystoma maculatum* larvae (Kutka 1994).

Altered behavior may also result in altered biological interactions (Moore and Townsend 1998; Kiesecker 1995). For example, if a predator is more susceptible to low pH than its prey, the prey could potentially have enhanced survival (Kiesecker 1995). Alternatively, environmental conditions might alter the behavior of prey making them more conspicuous to predators (Moore and Townsend 1998). In our study, different experimental variables affected larval survival (a lethal response) and activity level (a sublethal response). For example, ANOVA analysis of survival in the 1998 experiment indicated that nitrate and pH contributed most to reduced survival, while nitrate and UV contributed most to reduced activity level (Tables 2.4-2.5).

The three factors that we manipulated in this study are potentially relevant to anuran breeding sites in the Cascades Range where these animals live. While

nitrate levels higher than about 5 mg/L are probably rare in breeding ponds in the Cascades, there is a potential for fertilizer runoff to enter amphibian breeding ponds. Forest stands are fertilized every few years with urea (Bill Porter, U.S. Forest Service, Sweet Home Ranger Station, personal communication). In the agricultural Willamette Valley, where fertilizer use is more prevalent, nitrate levels in runoff areas may reach as high as 20 mg/L (ACH, unpublished data). pH and UV levels used in this study are also relevant to field conditions in the Cascades. Amphibian breeding sites have been measured with water pH in the range of 4.8-5.5 (ACH, unpublished data). Also at known anuran breeding sites, UV levels of 9-25 $\mu W/cm^2$ have been recorded at the water's surface (Blaustein et al. 1997; ACH, unpublished data). The combined effect of these stressors is particularly relevant considering that low pH can increase water clarity, resulting in greater UV penetrance (Yan et al. 1996). When such potential interactions are considered, we believe that all of the environmental factors that we studied have the potential to affect developing amphibians in the aquatic environment.

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CHAPTER 3

COMBINED EFFECTS OF UV-B RADIATION AND NITRATE FERTILIZER ON LARVAL AMPHIBIANS

By Audrey C. Hatch and Andrew R. Blaustein

ABSTRACT

We studied the combined effects of ultraviolet radiation (UV-B; 280-315 nm) and nitrate fertilizer on larval amphibians (Pacific treefrogs, *Hyla regilla* and long-toed salamanders, *Ambystoma macrodactylum*) in outdoor experiments at low and high elevation sites in Oregon. Depending upon other environmental conditions, UV-B levels are usually greater in intensity at higher elevation sites compared with lower elevation sites. We found that UV-B and nitrate together reduced mass of larval Pacific treefrogs at low elevations. In the higher-elevation mountain experiment, we found that UV-B and nitrate together reduced treefrog survival. Overall, results from the two experiments with treefrogs suggest that UV-B radiation may enhance

the toxicity of nitrate. Response of long-toed salamander mass to the experimental variables could indicate a potential effect on their food base. In the lower elevation experiment, long-toed salamander mass increased with the addition of nitrate. In the higher elevation experiment, this result occurred only when UV-B was blocked. Both UV-B and nitrate are likely to impact amphibian breeding habitat, resulting in a potential for early developmental stages of amphibians to be exposed to this combination of environmental agents. The application of fertilizers in agricultural areas may coincide with the spring breeding of many amphibians. In particular, temporary ponds and ditches near agricultural areas may be subject to large amounts of runoff from fertilizer application.

INTRODUCTION

In several locations worldwide, amphibian populations appear to be declining (Blaustein et al. 2001, 1994a; Houlahan et al. 2000; Alford and Richards 1999; Pounds et al. 1999). Several factors have been linked to amphibian declines, including habitat destruction, global climate change, disease, chemical contaminants, increasing ultraviolet radiation (particularly UV-B; wavelength 280-315 nm) and nitrogenous fertilizers (Kiesecker et al. 2001; Blaustein et al. 2001, 1994a, 1994b; Marco et al. 1999, Pounds et al. 1999).

In nature, animals are exposed to a complex array of environmental insults. Therefore, interactions between two or more stressors may contribute to amphibian population declines (Blaustein et al. 2001; Kiesecker et al. 2001; Hatch and Blaustein 2000; Alford and Richards 1999; Hatch and Burton 1998). However, few studies have considered interactions of contaminants with other stressors, such as UV-B. Such studies are particularly important in light of the need to integrate toxicology with the goals of conservation biology (Hansen and Johnson 1999).

Several biological characteristics of amphibians make them particularly vulnerable to the effects of environmental contamination. For example, most amphibians have a limited geographic range and specific habitat requirements for migration, frequently making them particularly susceptible to habitat fragmentation (Blaustein et al. 1994b). The typical biphasic life history of amphibians exposes them to anthropogenic influences in both the terrestrial and aquatic environments (Blaustein et al. 1994b). Both adults and larvae have thin skin that may be highly permeable to environmental contaminants (Blaustein et al. 1994b).

The intensity of UV-B radiation reaching the Earth's surface is increasing because of anthropogenic influences including ozone depletion, acidification and climate change (Middleton et al. 2001; Pienitz and Vincent 2000; Herman et al. 1996; Yan et al. 1996). UV-B radiation penetrates aquatic habitats to biologically significant depths (Hader et al. 1998). Depending upon other environmental conditions, it is generally assumed that UV-B levels are greater in intensity at higher elevations (Blumthaler et al. 1997). Moreover, several studies have shown

that UV-B is lethal to amphibian embryos and larvae of some species (Broomhall et al. 2000; Anzalone et al. 1998; Lizana and Pedraza 1998; Blaustein et al. 1994a). In addition, UV-B may have sublethal effects on several amphibian species (Blaustein et al. 2001). For example, UV-B radiation may slow growth and development rates in amphibians (Belden and Blaustein 2002; Häkkinen et al. 2001; Belden et al. 2000; Smith et al. 2000; Blaustein et al. 1997). Sublethal effects such as altered growth may ultimately affect the interactions of species at different trophic levels (Bothwell et al. 1994) and may ultimately affect survival of adult amphibians (Bervin 1990; Semlitsch et al. 1988; Smith 1987).

The use of nitrogen fertilizers has greatly increased in recent years (Tilman 1999; Vitousek 1994). Fertilizers are applied in forests and agricultural areas. This application has several different types of impacts on amphibians (Hatch et al. 2001; Marco et al. 1999; Oldham et al. 1997). For example, nitrate may kill developing amphibians (Marco et al. 1999; Oldham et al. 1997; Hecnar 1995). Nitrate degradation products including ammonia and nitrite are also toxic to developing amphibians (Jofre and Karasov 1999; Marco and Blaustein 1999). Nitrite affects the behavior and physiology of tadpoles (Marco and Blaustein 1999). Furthermore, nutrient enrichment from nitrogen-based fertilizers may alter community dynamics by increasing the abundance of herbivores such as snails. Snails are secondary parasitic hosts for trematode parasites, and the cysts of these parasites have been linked to deformities in developing amphibians (Johnson et al. 2002, 1999).

As discussed above, several studies have documented the adverse effects of UV-B and nitrogen fertilizers alone on amphibians. In this study, we investigated the combined effects of UV-B radiation and nitrate on the growth (length and mass) and survival of developing amphibians. Field experiments in two locations at different elevations in Oregon, USA were used to evaluate these effects: the lower-elevation (approximately 10 m) agricultural Willamette Valley and the higher-elevation (approximately 1,100 m) Cascades mountain range. We studied amphibian species with populations that occur in both environments: the long toed salamander (*Ambystoma macrodactylum*) and the Pacific treefrog (*Hyla regilla*).

METHODS AND MATERIALS

Willamette Valley experiment

We tested the combined effects of ambient UV-B and nitrate on long-toed salamanders and Pacific treefrogs. In the Willamette Valley, both species breed in early spring (January-February) in roadside ditches or temporary ponds (Nussbaum et al. 1983) and often co-occur. Long-toed salamanders typically breed earlier (approximately 1-2 weeks) than Pacific treefrogs (Nussbaum et al. 1983). Therefore our experiments were not completed simultaneously. We collected egg masses

from ponds located approximately 5.5 km west of Tangent, Linn County, Oregon. We reared embryos in the laboratory until hatching (Pacific treefrogs: stage 26, Gosner 1960; long-toed salamanders: stage 46, Harrison 1969). Pacific treefrog larvae were reared in 38 L tanks filled with dechlorinated water with approximately 50 animals per tank. Half of the water was changed twice per week, and larvae were fed a mixture of ground alfalfa pellets and TetraMin® flakes *ad libitum*. Long-toed salamander larvae were reared in 4 L plastic boxes (29 x 16 cm in area, 12 cm deep) at a density of 10 larvae per container. Larvae were fed newly hatched brine shrimp *ad libitum* and half of the water was changed twice per week. All animals were maintained at room temperature (21-24°C) under a constant photoperiod of 16 h light to 8 h dark. Larvae hatched in approximately three weeks.

We used outdoor mesocosms (55 L galvanized steel cattle watering tanks lined with plastic) as experimental units. We filled tanks with well water (alkalinity 88 mg $CaCO_3$/L; hardness 102 mg $CaCO_3$/L; nitrate 2 mg/L; pH 7.2; conductivity 177 μmhos/cm; DO 11.1 mg/L at 12° C). We exposed larvae of each species separately to all possible combinations of UV-B (with, without) and nitrate (see below for specific concentrations) for three weeks and measured larval growth (length and mass). We arranged mesocosms in a randomized block design with respect to treatment. We controlled for UV-B using clear plastic filters that either transmit UV-B (acetate) or block UV-B (Mylar®; Hillcor Plastics, Baldwin Park CA). Acetate typically transmits 80% of ambient UV-B radiation and 95% of UV-A radiation, while Mylar® typically transmits 5% of ambient UV-B radiation and

30% of UV-A radiation (Blaustein et al. 1994a). We added nitrate as sodium nitrate with an initial dose at the appropriate initial concentration, to simulate a runoff event. For the Pacific treefrog experiment, we used two levels of UV-B (with, without) and three levels of nitrate (0, 5, 20 mg/L) for a total of six treatments. For each treatment we had six replicate cattle tanks with 10 larvae per replicate. For the long-toed salamander experiment, we used two levels of UV-B (with, without) and two levels of nitrate (0, 10 mg/L) for a total of four treatments. For each treatment we had four replicate cattle tanks with six larvae per replicate.

To provide food for Pacific treefrogs, we added alfalfa pellets (approx. 0.5 g) to each tank every week. To provide food for long-toed salamanders, we collected zooplankton from the original pond where animals were collected. We added approximately 100 mL of water containing zooplankton (at a density of approximately 15 zooplankton per mL) twice per week to each tank. At the completion of the three week exposure, we measured algal growth in the Pacific treefrog experiment and zooplankton abundance in the long-toed salamander experiment. We measured algal growth by placing three ceramic tiles (11 cm square) in each mesocosm and quantifying the percent cover of the tiles by algae. We measured zooplankton abundance by sieving zooplankton from tank water, preserving zooplankton in 70% ethanol, then identifying and counting the various orders of zooplankton in each tank.

We measured UV-B, nitrate, and other characteristics of water quality in at least one tank from each treatment at regular intervals. We measured UV-B levels

in the water of the tanks under the filters once per week using a model 2100 PMA meter with model 2102 UV-B detector (Solar Light Co., Philadelphia, PA). UV-A was quantified using the same meter and a PMA2111 detector. We measured nitrate levels in the water twice per week using an Orion pH/ISE nitrate probe (model 290A; Orion Research, Inc. Beverly, MA USA). Once per week we measured pH, conductivity, hardness, alkalinity and conductivity in the water of each treatment. We made these measurements to determine whether our experimental manipulations (the addition of nitrate or the blocking of UV-B) affected other aspects of water quality, potentially confounding our experiments. pH was measured using an Orion 290A pH/ISE meter with a pH electrode. Conductivity was measured using a hand-held conductivity meter (Hanna Instruments, Woonsocket, RI). Dissolved oxygen was calculated using the Winkler titration method (American Public Health Association 1995). Water hardness and alkalinity were measured by titration with EDTA and 0.02 N HCl, respectively (APHA 1995). Temperature was recorded hourly with Onset DataLoggers (Onset Computer Corporation, Bourne MA).

Cascade Mountain experiment

We tested the combined effects of ambient UV-B and nitrate on Pacific treefrogs and long-toed salamanders at a field site in the Cascade Mountains

(Parish pond; 62 km east of Lebanon in Linn County, Oregon; elevation 1022 m). In the Cascades range, both species typically breed when snowmelt fills breeding ponds in the spring (April-June) (Nussbaum et al. 1983). Both species often breed in temporary ponds that dry completely before the end of the summer. In general, UV-B levels are higher at the higher altitude Cascades mountain range sites, in comparison to sites in the lower elevation Willamette Valley (Table 3.1). Each experiment was conducted when animals were available for collection; therefore the two species were not tested simultaneously. We collected Pacific treefrog egg masses from Parish Pond, allowed eggs to hatch in the laboratory and used these larvae for the Cascades mountain experiment. We collected newly hatched long-toed salamander larvae from Susan's Pond (21 km south of Sisters in Deschutes County, Oregon; elevation approximately 1903 m) and used these larvae for the Cascades mountain experiment with this species.

We used 4 L multicolored opaque plastic buckets as experimental units. We exposed larvae to combinations of UV-B (with, without) and nitrate (0, 10 mg/L) for a total of four treatments. We used 4 replicate buckets per treatment, with 10 Pacific treefrog tadpoles per replicate or seven long-toed salamander larvae per replicate. UV-B treatments were achieved by using clear plastic filters clipped over the buckets, as described above for the Willamette Valley experiment. We added nitrate as an initial pulse of 10 mg/L. We arranged buckets in a randomized block design in the pond. We exposed animals for three weeks and then assessed effects on survival and growth (length and mass) because preliminary experiments

suggested that effects on survival might be important in the Cascades mountain experiment.

We measured UV-B and nitrate levels in the water in one bucket from each treatment under filters twice per week, and measured pH, conductivity, hardness, alkalinity and conductivity of the water once per week using the methods described above for the Willamette Valley experiment. We recorded temperature every four hours using data loggers.

Table 3.1. Ultraviolet radiation measurements at two field sites in Oregon taken during nitrate/UV-B outdoor experiments.

Site	UV-A (mW/cm^2)	UV-B ($\mu W/cm^2$)
Willamette Valley:		
Pacific treefrog experiment	1.16 - 3.68	3.88 - 7.34
Long-toed salamander experiment	2.41 - 4.11	3.18 - 9.28
Cascades Mountain range:		
Pacific treefrog experiment	2.4 - 5.03	18.7 - 21.2
Long-toed salamander experiment	2.88 - 4.43	7.77 - 15.3

Data analysis

We assessed the effects of the various nitrate and UV-B treatments on larval survival and growth (length and mass). We checked that data met the assumptions for parametric analysis (normality, homogeneity of variance) visually. Multivariate analysis of variance (MANOVA) procedures followed by univariate ANOVA F-

tests were used to analyze results, with container means used as the units of statistical analysis. Data for the proportion surviving were arcsine-square root transformed prior to analysis. For all experiments, we first tested for effects due to block before proceeding with the ANOVA. All statistical tests were completed using SAS version 6.12 for Windows.

To determine whether algal percent cover differed among treatments in the Willamette Valley experiment with Pacific treefrogs, we checked for differences in the mean percent cover between treatments using nonparametric ANOVA. To determine whether zooplankton abundance or composition differed among treatments in the Willamette Valley experiment with long-toed salamanders, we checked for differences in the relative composition between treatments using ANOVA.

RESULTS

Willamette Valley experiment: Pacific treefrog

Preliminary analysis found no effects due to block (MANOVA Wilk's lambda $F_{10,52} = 0.95$; p-value = 0.49), so we proceeded with the analysis for the

effects of experimental treatments. Mass was reduced when larvae were exposed to high levels of nitrate in the presence of UV-B (Table 3.2, Fig. 3.1).

Temperature ranged from 5 to 29 °C with a 12 °C range in any one day. There was no difference in temperature between acetate and Mylar®-covered mesocosms (paired t-test t-value 0.435; p-value 0.668; 20 df). Algal growth differed in the various treatments (Kruskal-Wallis ANOVA on ranks; $H_5 = 14.7$; p-value = 0.012). There was less algal growth in the two UV-B treatments with nitrate added and in the No UV-B treatment with high nitrate compared to the No UV-B control treatment. This suggests that either algal growth was reduced or that tadpoles consumed more algae in these treatments.

Nitrate in low treatments ranged from 5.2-6.4 mg/L in week 1; 3.3-4.0 in week 2; and 1.1-2.1 mg/L at week 3. Nitrate in high treatments ranged from 18.8-20.1 at the beginning of the experiment; 8.3 - 10.4 mg/L after the first week; 7.4-9.1 after the second week; and 2.4-3.2 mg/L at the end of the experiment. Nitrate in control treatments ranged from 0.85 - 1.89 mg/L throughout the experiment. See Table 3.1 for a summary of UV measurements for all of the experiments. We did not perform statistical tests on these measurements, but rather report the ranges measured for qualitative comparisons between experiments and to provide information on our experimental conditions. In all treatments throughout the experiment, conductivity ranged from 60 - 250 μmhos/cm; dissolved oxygen ranged from 6.3 - 13.8 mg/L; pH ranged from 7.2 - 9.1; alkalinity ranged from 16 - 114 mg $CaCO_3$/L; and hardness ranged from 22 - 116 mg $CaCO_3$/L.

Table 3.2. Multivariate analysis of variance model for *Hyla regilla* length, mass and survival in Willamette Valley nitrate/UV-B experiment

MANOVA:	Wilk's lambda	F Value	df	p-value
Nitrate		0.91	6,56	0.491
UV-B		1.75	3,28	0.179
Nitrate x UV-B		1.86	6,56	0.104

ANOVA – Length:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	2	0.261	0.130	1.16	0.327
UV-B	1	0.151	0.151	1.34	0.256
Nitrate x UV-B	2	0.066	0.033	0.30	0.746
Error	30	3.371	0.112		
Total	35	3.849			

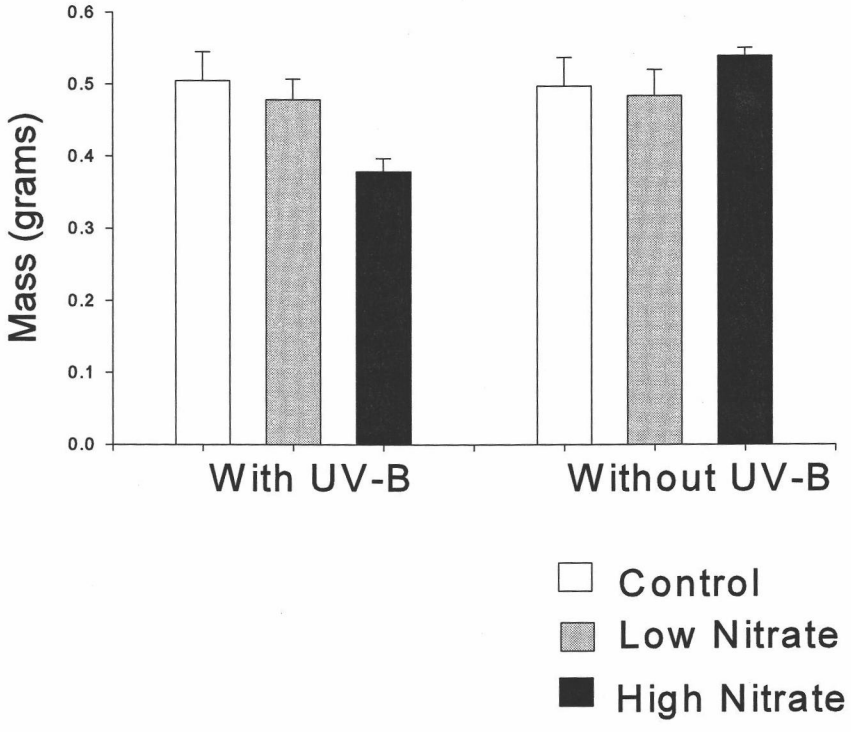
ANVOA – Mass:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	2	0.022	0.011	1.47	0.247
UV-B	1	0.024	0.023	3.21	0.084
Nitrate x UV-B	2	0.050	0.025	3.44	0.045
Error	30	0.220	0.007		
Total	35	0.315			

ANOVA – Survival:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	2	0.202	0.101	2.08	0.142
UV-B	1	0.165	0.165	3.40	0.075
Nitrate x UV-B	2	0.168	0.084	1.73	0.194
Error	30	1.452	0.048		
Total	35	1.989			

Figure 3.1. *Hyla regilla* mass in Willamette Valley nitrate/UV-B mesocosm experiment. Error bars indicate one standard error.



Willamette Valley experiment: Long-toed salamander

Preliminary analysis indicated no effects due to block (MANOVA Wilk's lambda $F_{6,18} = 0.42$; p value = 0.86). Therefore we proceeded with the analysis for treatment effects. Nitrate increased larval mass (Table 3.3; Fig. 3.2). Larval mass was marginally affected by exposure to UV-B ($p = 0.06$) and the UV-B x Nitrate interaction ($p = 0.08$) (Table 3.3; Fig. 3.2).

Temperature ranged from 8 to 32 °C with a 15 °C range in any one day. There was no difference in temperature between acetate and Mylar®-covered mesocosms (paired t-test on the highest daily temperature t-value 1.889; p-value 0.074; 20 df). There was no difference in zooplankton composition between treatments in the long-toed salamander experiment. Throughout the experiment, conductivity ranged from 88 to 150 μmhos/cm; dissolved oxygen ranged from 6.6 to 10 mg/L; pH ranged from 7.7 to 9.0; alkalinity ranged from 110 to 116 mg $CaCO_3$/L; and hardness ranged from 128 to 134 mg $CaCO_3$/L. Nitrate in nitrate treatments ranged from 9.1-10.1 at the beginning of the experiment; 6.0 to 6.8 mg/L after the first week; 4.2-6.4 after the second week; and 2.4-3.2 mg/L at the end of the experiment. Nitrate in control treatments ranged from 0.70 to 2 mg/L throughout the experiment.

Table 3.3. Multivariate analysis of variance model for *Ambystoma macrodactylum* length, mass and survival in Willamette Valley nitrate/UV-B mesocosm experiment

MANOVA:	Wilk's lambda F Value	df	p-value
Nitrate	4.39	3,10	0.033
UV-B	2.60	3,10	0.111
Nitrate x UV-B	1.51	3,10	0.272

ANOVA – Length:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.014	0.014	0.10	0.761
UV-B	1	0.001	0.001	0.00	0.959
Nitrate x UV-B	1	0.014	0.014	0.10	0.761
Error	12	1.78	0.148		
Total	15	1.809			

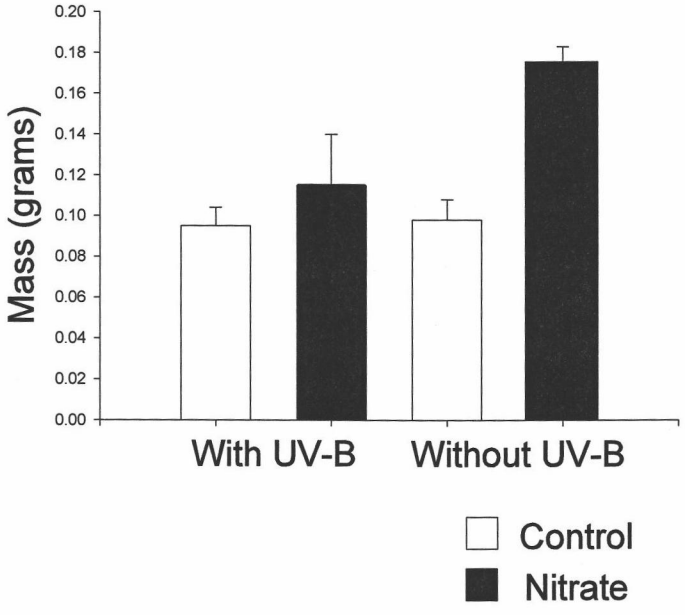
ANOVA – Mass:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.010	0.010	10.04	0.008
UV-B	1	0.004	0.004	4.24	0.062
Nitrate x UV-B	1	0.004	0.004	3.62	0.082
Error	12	0.012	0.001		
Total	15	0.029			

ANOVA – Survival:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.006	0.006	0.21	0.653
UV-B	1	0.037	0.037	1.28	0.280
Nitrate x UV-B	1	0.006	0.006	0.21	0.653
Error	12	0.346	0.029		
Total	15	0.395			

Figure 3.2. *Ambystoma macrodactylum* mass in Willamette Valley nitrate/UV-B mesocosm experiment. Error bars indicate one standard error.



Cascades Mountain experiment: Pacific treefrog

There was no block effect (MANOVA Wilk's lambda $F_{9,9,9} = 0.32$; p value = 0.95) and so we proceeded to analyze for effects due to treatment. MANOVA revealed treatment effects (Table 3.4). Although growth (length or mass) was not affected, we found treatment effects on survival (Fig. 3.3). UV-B, nitrate and their interaction were all significant in explaining the effects on survival. Survival was reduced by exposure to both UV-B and nitrate (Fig. 3.3).

Temperature was cooler over the first five days of the experiment compared to the remaining exposure time. Temperature ranged from 6 to 12 °C the first 5 days, with a 4 °C range in any one day. For the remaining 16 days of the experiment, temperature ranged from 10 to 26.5 °C, with a 12 °C range in any one day. There was no difference in temperature between acetate and Mylar®-covered enclosures (paired t-test on the highest daily temperature t-value 0.197; p-value 0.845; 20 df).

Water collected from the pond at the initiation of the experiment had less than 1 mg/L nitrate; less than 0.1mg/L ammonia; pH 6.5; conductivity 29 μmhos/cm; alkalinity 15 mg $CaCO_3$/L; and hardness 10 mg $CaCO_3$/L. Throughout the experiment conductivity ranged from 58 to 207 μmhos/cm; alkalinity ranged from 26 to 62 mg $CaCO_3$/L; hardness ranged from 14 to 50 mg $CaCO_3$/L; dissolved

oxygen ranged from 4.6 to 6.0 mg/L; and pH ranged from 6.5 to 7.8. In the nitrate treatments, nitrate levels decreased from 10 mg/L at the beginning of the experiment, to 6 mg/L after the first week, to 5 mg/L after the second week, and to 2 mg/L after the third week. There was no detectable nitrate in the control treatments.

Table 3.4. Multivariate analysis of variance model for *Hyla regilla* length, mass and survival in Cascades Mountain nitrate/UV-B outdoor experiment

MANOVA:	Wilk's lambda	F Value	df	p-value
Nitrate	17.67		3,6	0.002
UV-B	23.99		3,6	0.001
Nitrate x UV-B	21.95		3,6	0.001

ANOVA – Length:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.001	0.001	0.02	0.898
UV-B	1	0.035	0.035	0.47	0.512
Nitrate x UV-B	1	0.034	0.034	0.45	0.521
Error	8	0.606	0.076		
Total	11	0.677			

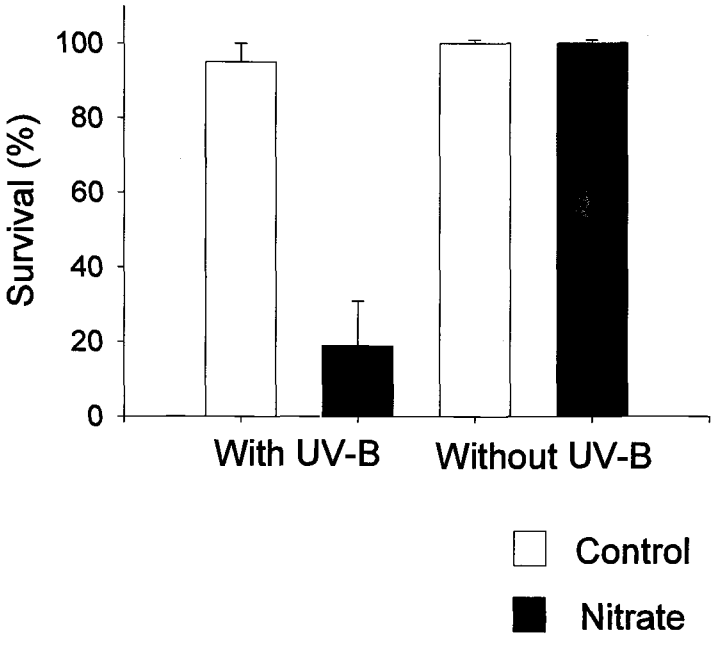
ANOVA – Mass:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.001	0.001	0.79	0.401
UV-B	1	<0.001	<0.001	0.00	0.974
Nitrate x UV-B	1	0.003	0.003	2.62	0.144
Error	8	0.008	0.001		
Total	1	0.012			

ANOVA – Survival:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.254	0.254	30.58	0.001
UV-B	1	0.219	0.219	26.40	0.001
Nitrate x UV-B	1	0.544	0.544	65.53	<0.001
Error	8	0.066	0.008		
Total	11	1.083			

Figure 3.3. *Hyla regilla* survival in Cascades mountain nitrate/UV-B outdoor experiment. Error bars indicate one standard error.



Cascades Mountain experiment: Long-toed salamander

There was no block effect (MANOVA Wilk's lambda F-statistic $_{9,17.2}$ = 1.23; p-value = 0.34) and so we proceeded to analyze for effects due to treatment. Larval mass was increased by nitrate addition when UV-B was blocked (Fig. 3.4, Table 3.5).

Temperature ranged from 7 to 33 °C, with an 18 °C range in any one day. There was no difference in temperature between acetate and Mylar®-covered enclosures (paired t-test on the highest daily temperature t-value –1.826; p-value 0.083; 20 df). Water collected from the pond at the initiation of the experiment had less than 1 mg/L nitrate; less than 0.1 mg/L ammonia; pH 7.8; conductivity 20 μmhos/cm; alkalinity 15 mg $CaCO_3$/L; and hardness 10 mg $CaCO_3$/L. Throughout the experiment conductivity ranged from 25 to 91 μmhos/cm; alkalinity ranged from 32 to 62 mg $CaCO_3$/L; hardness ranged from 20 to 62 mg $CaCO_3$/L; dissolved oxygen ranged from 5 to 8.2 mg/L; and pH ranged from 7.1 to 8.6. In the nitrate treatments, nitrate levels decreased from 10.5 mg/L at the beginning of the experiment, to 7.1 mg/L after the first week, to 4.2 mg/L after the second week, and to 2.5 mg/L after the third week. There was no detectable nitrate in the control treatments.

Table 3.5. Multivariate analysis of variance model for *Ambystoma macrodactylum* length, mass and survival in Cascades Mountain nitrate/UV-B outdoor experiment.

MANOVA:	Wilk's lambda	F Value	df	p-value
Nitrate	1.75		3,9	0.226
UV-B	0.24		3,9	0.868
Nitrate x UV-B	2.88		3,9	0.096

ANOVA – Length:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.032	0.032	2.88	0.118
UV-B	1	<0.001	<0.001	0.00	0.951
Nitrate x UV-B	1	0.011	0.011	0.98	0.343
Error	11	0.121	0.011		
Total	14	0.164			

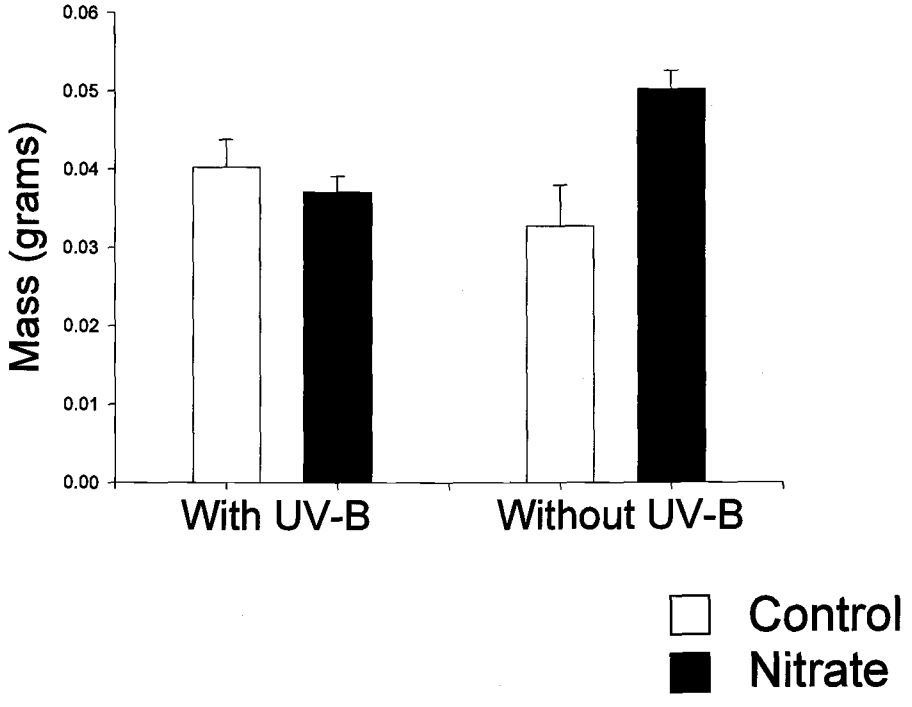
ANOVA – Mass:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.00021	<0.001	4.46	0.058
UV-B	1	0.00002	<0.001	0.50	0.494
Nitrate x UV-B	1	0.001	0.001	7.13	0.022
Error	11	0.0006	<0.001		
Total	14	0.0012			

ANOVA – Survival:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Nitrate	1	0.047	0.047	1.26	0.285
UV-B	1	0.002	0.002	0.05	0.820
Nitrate x UV-B	1	0.174	0.174	4.64	0.054
Error	11	0.413	0.037		
Total	14	0.636			

Figure 3.4. *Ambystoma macrodactylum* mass in Cascades Mountain nitrate/UV-B outdoor experiment. Error bars indicate one standard error.



DISCUSSION

Our results for Pacific treefrogs suggest that, together, nitrate and UV-B could be more toxic than either factor alone. The combination of UV-B and nitrate had a sublethal effect at the lower UV-B intensity in the lower elevation Willamette Valley, and these variables caused lethal effects in the higher UV-B intensity in the Cascades Mountain experiment. In the Willamette Valley experiment, UV-B levels are typically lower than in the Cascades range (Table 3.1). It is possible that more dramatic effects on treefrogs were observed in the Cascades mountain experiment because higher levels of UV-B radiation were present there. Alternatively, the different responses in the two habitats could also suggest a difference in sensitivity of the populations to UV-B or to nitrate. Treefrog populations in the mountains may be more sensitive to the effects of environmental agents such as fertilizers.

We do not know of a direct mechanism by which UV-B could enhance nitrate toxicity. Therefore, we suggest that our results for Pacific treefrogs are caused by reduced ability to compensate for the effects of one stressor in the presence of another. For example, in the Selyean model of stress, organisms experience increased energetic costs upon exposure to a stressor (Selye 1956). According to the Selyean general adaptation syndrome, organisms can persist in the presence of a stressor such as chemical pollution or UV-B radiation until the

stressor reaches a critical level. In our experiment, larval Pacific treefrogs may be compensating for the physiological costs of exposure to a stressor such as UV-B, and this might not become apparent until exposure to a second stressor such as nitrate pollution occurs.

Nitrate increased the mass of larval long-toed salamanders in both experiments. However, in the higher elevation Cascade Mountain experiment, this result occurred only when UV-B was blocked. The increased mass could be attributed to increased nutrients (nitrate) increasing the amount of zooplankton available as salamander food. However, if algae or zooplankton are sensitive to high levels of UV-B, they may not respond to nitrate addition by increasing their growth and this could explain the different results of salamander mass in the treatment with UV-B in the Cascade Mountain experiment.

In the Willamette Valley experiment with long-toed salamanders, zooplankton abundance and composition did not differ by treatments. However, we did find decreased abundance of algae with nitrate addition in the Pacific treefrog experiment, particularly in the presence of UV-B. Therefore, it is possible that UV-B alone or in combination with nitrate may have affected the salamander's food source. Other work has shown the potential for UV-B to impair amphibian food sources such as algae (Rogers et al. 2001), and several studies have found direct effects of UV-B on amphibian food sources. UV-B can damage bacterioplankton (Hader et al. 1998), phytoplankton (Hader et al. 1998; Hessen et al. 1997), rotifers (Vinebrooke and Leavitt 1999), and freshwater algae (Rogers et al. 2001; Arts and

Rai 1997). In other examples, UV-B can affect a particularly sensitive component of the aquatic food chain, with the potential for alterations at the community level. For example, herbivorous macroinvertebrates were more sensitive to UV-B than algae, resulting in increased algal growth in the presence of UV-B (Bothwell et al. 1994). Zooplankton fed from a UV-B treated experimental microcosm had impaired growth and survival compared to those fed from a microcosm that was not UV-B treated, although there were no direct effects of UV-B on phytoplankton, zooplankton, periphyton or macroinvertebrates (DeLange et al. 1999). Other experiments have shown that UV-B exposure reduced diatom cell size of periphyton without affecting the quality of the periphyton as food for snails (McNamara and Hill 2000).

While UV-B radiation did not directly impact amphibians in our experiments, we have shown that UV-B is important to consider in combination with other environmental agents such as nitrate. Previous work measured the activity levels of the photorepair enzyme photolyase in eggs of several species of amphibians native to the Pacific Northwest, USA (Blaustein et al. 1994a). Photolyase is important in repairing the DNA damage caused by UV-B radiation, and higher activity levels of the enzyme in eggs indicate an increased ability to repair UV-B damage (Blaustein et al. 1994a). Pacific treefrog eggs are resistant to the direct effects of UV-B, and have relatively high levels of photolyase compared to other amphibians of the Pacific Northwest (Blaustein et al. 1994a). In contrast, long-toed salamanders have relatively low levels of the enzyme photolyase

(Blaustein et al. 1994a). Field experiments indicate that ambient levels of UV-B radiation cause deformities and mortality of long-toed salamander embryos at high elevation study sites (Blaustein et al. 1997). Our experiments with Pacific treefrog larvae have indicated the potential for increased detrimental effects when both UV-B and nitrate pollution occur together.

Population differences in response to stressors such as UV-B are important to consider (Belden et al. 2000). In previous work, long-toed salamanders from lower elevation sites exhibited high mortality upon UV-B exposure, while animals from high elevation sites exhibited no significant mortality but slower growth caused by UV-B exposure (Belden et al. 2000). Our experiments at two different study sites provide further evidence of the potential differences in response to UV-B radiation between populations that have historically been exposed to different levels of UV-B. The major difference between our experiments at the two study sites was intensity of UV-B radiation (Table 3.1). Although we did not perform a statistical analysis, we did not find differences in the range of other water quality parameters measured (dissolved oxygen, pH, conductivity, hardness, alkalinity) between experimental treatments. The levels of nitrate measured degraded over time as expected, and there were no obvious differences in nitrate levels or other water quality characteristics caused by UV-B exposure.

Both UV-B and nitrate contamination are likely to exist in breeding ponds at the same time as amphibians. Furthermore, amphibians are increasingly likely to encounter both of these environmental agents because of increasing human impacts

on sensitive habitat. UV-B is increasing in intensity because of ozone depletion (Herman et al. 1996), acidification and climate change (Pienitz and Vincent 2000; Hader et al. 1998, Yan et al. 1996). Additionally, nitrate runoff is increasing with fertilizer application (Tilman 1999; Vitousek 1994). Amphibians breeding in shallow temporary ponds, particularly those impacted by nitrogenous fertilizer runoff, may frequently be exposed to both of these factors simultaneously in the field during their sensitive developmental stages. We suggest that the combined effects of environmental agents such as UV-B and nitrate must be considered when determining acceptable levels of contamination in sensitive amphibian breeding areas.

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CHAPTER 4

LARVAL AMPHIBIAN RESPONSES TO FORMULATIONS OF CARBARYL AND CHLORPYRIFOS

By Audrey C. Hatch, Lisa K. Belden, and Andrew R. Blaustein

ABSTRACT

In laboratory experiments, we investigated the effect of UV-B radiation on two commonly used pesticides, carbaryl and chlorpyrifos, in the larvae of three species of amphibians. We tested these effects in red-legged frogs (*Rana aurora*), long-toed salamanders (*Ambystoma macrodactylum*), and Pacific treefrogs (*Hyla regilla*). All of these species are commonly found in the highly agricultural Willamette Valley of Oregon. Experiments investigated the animals' response to formulated pesticide product compared to the active chemical pesticide ingredient. For all species examined, a formulated pesticide product of chlorpyrifos was more toxic than the active ingredient alone, causing complete mortality in long-toed salamanders and Pacific treefrogs. Effects of UV-B radiation on pesticide toxicity were not apparent. These results suggest that some components of the pesticide formulations, perhaps in addition to the active ingredient, were toxic to larval amphibians.

INTRODUCTION

Amphibian populations worldwide are experiencing declines in number or reductions in range (e.g., Kiesecker et al. 2001; Houlahan et al. 2000; Pounds et al. 1999). Anthropogenically enhanced climate change, disease, fertilizers, and ultraviolet radiation (UV-B; 280-320 nm) have been implicated in some instances of amphibian population declines (e.g., Kiesecker et al. 2001; Hatch et al. 2001; Davidson et al. 2001; Blaustein et al. 2000, 1998, 1994a; Alford and Richards 1999; Pounds et al. 1999; Marco et al. 1999). Additionally, pesticides have been implicated in some instances of amphibian declines near agricultural areas (Davidson et al. 2001). Adverse effects of pesticides have been demonstrated for many amphibian species (e.g., Zaga et al. 1998; Berrill et al. 1998; Berrill et al. 1993, 1995; Materna et al.1995). However, there is likely no single cause for amphibian population declines; rather, several factors likely interact at any given location (e.g., Kiesecker et al. 2001; Blaustein et al. 2001; Pounds et al. 1999). Several studies have found interactive effects when animals are exposed to more than one environmental stressor (Howe et al. 1998; Zaga et al. 1998; Hatch and Burton 1998; Horne and Dunson 1995a,b; Kiesecker and Blaustein 1995; Long et al. 1995). In this study, we examined the combined effects of UV-B radiation and pesticides in laboratory experiments.

Because of the global nature of amphibian population declines, ultraviolet radiation, increasing because of anthropogenic disturbances including stratospheric ozone depletion, acidification, and global climate change, has been investigated in a number of studies. UV-B radiation causes both lethal (Anzalone et al. 1998; Blaustein et al. 1994a) and sublethal (Belden and Blaustein 2002; Häkkinen et al. 2001; Belden and Blaustein 2001; Smith et al. 2000; Belden et al. 2000) effects on developing amphibians. Moreover, UV-B may interact with other environmental stressors such as low pH (Long et al. 1995) or pesticides (Zaga et al. 1998) to cause detrimental effects in developing amphibians. These detrimental effects may impact on the population.

Pesticide pollution may contribute to amphibian population declines, either alone or in combination with some other environmental factor. Organophosphate pesticides, such as chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate), are one of most widely used groups of pesticides (Barron and Woodburn 1995; Racke 1993). Chlorpyrifos is used in agriculture, on ornamental plants and grasses, and against household pests (Giesy et al. 1999; Barron and Woodburn 1995; Racke 1993). Chlorpyrifos and its major metabolite (3,5,6-trichloro-2-pyridinol; TCP) absorb UV radiation (at approximately 295 nm) (Racke 1993). However, the relative importance of this reaction, and any possible toxicity of chlorpyrifos photoproducts, has not been well characterized (Racke 1993). Moreover, few studies have investigated the effects of chlorpyrifos in amphibians (Giesy et al. 1999; Barron and Woodburn 1995).

Carbaryl (1-naphthyl N methylcarbamate) is a broad-spectrum pesticide used for insect control in farmland, forests, and private homes (Cox 1993). Carbaryl toxicity is enhanced by UV-B radiation, and this is likely caused by the formation of toxic chemical photo-products caused by UV-B (Zaga et al. 1998). Several studies have documented lethal and sublethal effects of carbaryl on developing amphibians (Boone and Semlitsch 2001; Relyea and Mills 2001; Bridges 1997,2000; Boone and Bridges 1999a,b; Zaga et al. 1998). Both carbaryl and chlorpyrifos may enter amphibian breeding habitats, such as ponds or wetlands, via aerial spraying of nearby fields, runoff from local application, or atmospheric deposition (Boone and Semlitsch 2001; Davidson et al. 2001; Racke 1993; Cox 1993).

Pesticides are typically applied as a commercial product formulated with several other ingredients, such as emulsifiers or solvents, in addition to the active ingredient. For most pesticides, several formulations using the same active ingredient are available (*e.g.,* chlorpyrifos; Racke 1993). In some cases, these inert ingredients may be toxic themselves. For example, components of the inert ingredients in the commercial compounds Dursban® and Lorsban®, in addition to the active ingredient (chlorpyrifos), caused inhibition of the enzyme acetylcholinesterase in frogs (Swann et al. 1996). Therefore, it is important to consider the effects of formulated products as well as the active pesticide ingredient.

In laboratory experiments, we studied the combined effects of UV-B and the pesticides carbaryl and chlorpyrifos on the larval survival of three species of amphibians native to the Pacific Northwest, USA. Specifically, we assessed the combined effects of (1) UV-B and carbaryl; and (2) UV-B and chlorpyrifos, using both the formulated product and the active ingredient of these pesticides. We examined survival of all three species after a three-week exposure.

METHODS AND MATERIALS

Animal collection and care

We examined the combined effects of ambient UV-B and pesticides on three species of amphibians native to the Willamette Valley of Oregon, USA: red-legged frogs (*Rana aurora*), long-toed salamanders (*Ambystoma macrodactylum)* and Pacific treefrogs (*Hyla regilla*). These species typically breed in early spring (January-February) in roadside ditches or temporary ponds (Nussbaum et al. 1983). Because of differences in the timing of oviposition, our experiments on the different species overlapped but were not completed simultaneously. Red-legged frog eggs (stage 15-16; Gosner 1960) were collected from a roadside pond near the coast of Oregon, USA (approximately 10 km south of Waldport in Lincoln County,

Oregon; approximately 15 m elevation). We collected Pacific treefrog (stage 15-18, Gosner 1960) and long-toed salamander (stage 20-24, Harrison 1969) egg masses from ponds located approximately 5.5 km west of Tangent in Linn County, Oregon (approximately 20 m elevation).

We reared embryos in the laboratory until hatching (three weeks under laboratory conditions). Dechlorinated tap water was used for rearing and for experiments. In the water, nitrate levels were non-detectable; dissolved oxygen ranged from 7 to 8 mg/L; pH ranged from 6.9 to 7.4; conductivity ranged from 202 to 236 μmhos/cm; alkalinity ranged from 15-20 mg $CaCO_3$/L; and hardness ranged from 26-36 mg $CaCO_3$/L.

Red-legged frog and Pacific treefrog embryos were reared in 38 L tanks filled with dechlorinated water with approximately 50 animals per tank. Half of the water was changed twice per week, and larvae were fed a mixture (approximately 50: 50) of ground alfalfa pellets and TetraMin® flakes *ad libitum*. Long-toed salamander larvae were reared in 4 L plastic boxes (29 x 16 cm in area, 12 cm deep) at a density of 10 larvae per container. Larvae were fed newly hatched brine shrimp *ad libitum* and half of the water was changed twice per week. All animals were maintained at room temperature (21-24°C) under a constant photoperiod of 16 h light to 8 h dark. Immediately prior to adding animals to experiments, we measured a random sample (n = 10) of experimental animals to determine their developmental stage, mass and length. At the beginning of experiments, red-legged frogs were at stage 25 (Gosner 1960), averaged 0.11 g (SE = 0.007 g) each, and

were approximately 1.77 cm long (SE = 0.06 cm). Long-toed salamanders were at stage 45 (Harrison 1969), averaged 0.009 g (SE = 0.002) each, and were approximately 1.04 cm long (SE = 0.051). Pacific treefrogs were at stage 25 (Gosner 1960), averaged 0.03 g (SE = 0.005 g) each, and were approximately 1.04 cm long (SE = 0.01 cm).

Experiments

For each species, we tested the effect of UV-B on carbaryl and chlorpyrifos toxicity over a three-week exposure. We tested both a formulated commercial-grade pesticide (Sevin®; active ingredient carbaryl; EPA Registration # 264-334-71004 or Kill-a-Bug®; active ingredient chlorpyrifos; EPA Registration # 7122-119-7401) and the active ingredient of the pesticide (obtained as a standard solution dissolved in methanol) diluted in water. Both formulated product and active ingredient formulations of the pesticide were diluted to the appropriate concentration (250 μg/L for carbaryl; 25 μg/L for chlorpyrifos). This resulted in a total of 10 experimental treatments: UV-B (with, without) X chemical (control, no pesticide; carbaryl active ingredient; carbaryl formulated product; chlorpyrifos active ingredient; chlorpyrifos formulated product) (Fig. 4.1). Each treatment was replicated three times in 500 mL borosilicate glass cups filled with 250 mL water. In the red-legged frog and Pacific treefrog experiments, four larvae were added per

cup; in the long-toed salamander experiment, three larvae were added per cup. We placed containers on the laboratory bench in a haphazard manner to control for any possible effects due to location.

We fed red-legged frogs and Pacific treefrogs ground alfalfa pellets (*ad libitum*) twice per week. We fed long-toed salamanders zooplankton (10 mL of water containing zooplankton at a density of approximately 15 zooplankton per mL) twice per week. We removed dead animals and waste products daily, and added water with the appropriate chemical treatment to replace any evaporate. For each of the three experiments, we measured pH, UV levels, nitrate, dissolved oxygen, conductivity, alkalinity and hardness levels in the water at the beginning and at the end of the experiment using the methods described above for the mesocosm experiment.

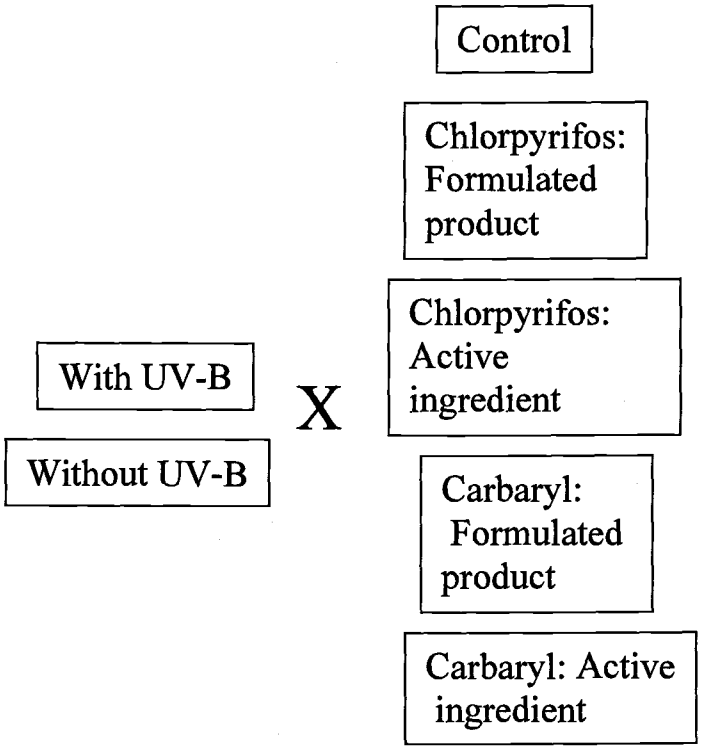
UV lighting was achieved in the laboratory using UV-B 313 light bulbs (Q Panel Inc., Cleveland, OH, USA) alternated with full spectrum Vita-Lite bulbs (Duro-Test Corporation, Fairfield, NJ, USA). We used equal numbers of UV-B bulbs and Vita-Lite bulbs. Vita-Lite bulbs were included to ensure the presence of UV-A radiation (approximately 315-400 nm), a natural component of sunlight and essential for photorepair processes in the developing amphibians (Blaustein et al. 1994a and references therein).

We controlled UV-B exposure by using clear plastic filters that either transmit UV-B (acetate) or block UV-B (Mylar®; Hillcor Plastics, Baldwin Park CA). Acetate typically transmits 80% of ambient UV-B radiation and 95% of UV-

A radiation, while Mylar® typically transmits 5% of ambient UV-B radiation and 30% of UV-A radiation (Blaustein et al. 1994a). Prior to addition of larvae, we added pesticides using the formulated product (Sevin®, active ingredient carbaryl; Kill-a-Bug®, active ingredient chlorpyrifos) with an initial dose at the appropriate concentration, to simulate a pulsed exposure from a runoff event.

We measured UV-B levels in the water using a model 2100 PMA meter with model 2102 UV-B detector (Solar Light Co., Philadelphia, PA). UV-A was quantified using the same meter and a PMA2111 detector. Nitrate was quantified with an Orion 290A pH/ISE meter with a nitrate electrode. We also measured pH, conductivity, dissolved oxygen, alkalinity and hardness in experimental water. We made these measurements to determine whether our experimental manipulations (the addition of pesticides or the blocking of UV-B) affected other aspects of water quality, potentially confounding our experiments. pH was measured using an Orion 290A pH/ISE meter with a pH electrode. Conductivity was measured using a hand-held conductivity meter (Hanna Instruments, Woonsocket, RI). Dissolved oxygen was calculated using the Winkler titration method (American Public Health Association 1995). Water hardness and alkalinity were measured by titration with EDTA and 0.02 N HCl, respectively (APHA 1995).

Figure 4.1. Schematic of experimental design for laboratory experiment with pesticides and UV-B radiation.



Chemical analysis

We measured chlorpyrifos levels from each treatment once per week using an enzyme-linked immunosorbent assay (ELISA) designed to quantify pesticides in water samples (Gaizick et al. 2001; Schraer et al. 2000; Abad et al. 1999; Rubio et

al. 1991). Carbaryl levels were not measured. The ELISA technique favorably compares with other analytical methods including gas chromatography and HPLC in terms of time and expense required to analyze samples and the accuracy and precision of the analysis (Schraer et al. 2000; Abad et al. 1999; Rubio et al. 1991).

We collected approximately 5 mL of water for chlorpyrifos analysis in borosilicate glass vials and stored the samples in the dark at 4 °C until analysis. Assay kits were obtained from Strategic Diagnostics, Inc., Newark, Delaware. Water sample analysis followed the methods recommended by the supplier and outlined in Rubio et al. (1991). Briefly, water samples were mixed with enzyme conjugate and magnetic particles, the particles were separated with a specialized magnetic rack, color reagent was added and the absorbance was read with a spectrophotometer at 450 nm. Standards were run in duplicate.

Data analysis

We visually checked that data met the assumptions for parametric analysis (normality, homogeneity of variance). One-way analysis of variance (ANOVA) was used to analyze survival, with container means used as the units of statistical analysis. Post-hoc tests (Student-Newman-Keul's; alpha = 0.05) were used to determine whether treatments differed from the No-UV-B control treatment. All statistical tests were completed using SAS version 8 for Windows.

RESULTS

Experimental conditions: All species

Temperature ranged from 14 to 16 °C. UV-A levels ranged from 0.15 to 0.17 mW/cm^2; UV-B levels ranged from 1 to 2.5 $\mu W/cm^2$. Characteristics of the dechlorinated dilution water are given above (Methods section). In water from the completion of all experiments, nitrate ranged from 0.8 to 1.2 mg/L; dissolved oxygen ranged from 7 to 8 mg/L; pH ranged from 6.9 to 8.0; conductivity ranged from 202 to 288 μmhos/cm; alkalinity ranged from 15 to 22 mg $CaCO_3$/L; and hardness ranged from 26 to 48 mg $CaCO_3$/L. Because there were no differences in the range of these water quality measurements between treatments, we did not perform statistical tests on these measurements, but rather report the ranges measured to provide information on the experimental conditions of this study.

Red-legged frog

Mortality of red-legged frogs occurred when animals were exposed to the formulated product of chlorpyrifos (Fig. 4.2). However, wide variation occurred

within treatments and this effect was not statistically significant (Table 4.1). ELISA indicated that chlorpyrifos levels in control water ranged from 0 to 1 ppb. In chlorpyrifos treatments (both chemical and formulated product), levels ranged from 20 to 30 ppb at the beginning of the experiment. Levels in the chemical treatment ranged from 8 to 22 ppb at the end of the treatment, and levels in the formulated product treatment ranged from 20 to 25 ppb at the completion of the experiment.

Table 4.1. Analysis of variance models for larval amphibian survival in laboratory pesticide/UV-B experiments

Red-legged frog:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	9	20653.333	2294.815	2.24	0.064
Error	20	20533.333	1026.667		
Total	29	41186.667			

Long-toed salamander:

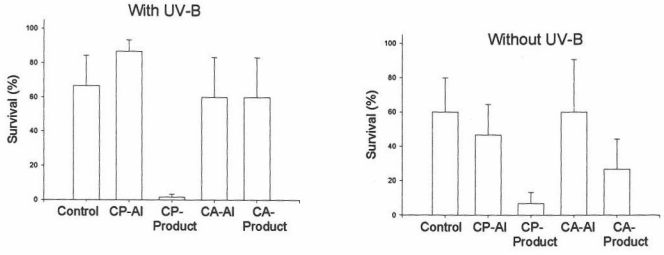
Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	9	25652.833	2850.315	5.44	0.0008
Error	20	10475.333	523.767		
Total	29	36128.167			

Pacific treefrog:

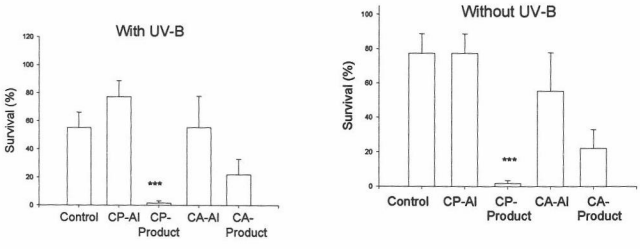
Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	9	28280.000	3142.222	5.89	0.0005
Error	20	10666.667	533.333		
Total	29	38946.667			

Figure 4.2 Survival of larval amphibians exposed to formulated products and active ingredients of the pesticides carbaryl and chlorpyrifos in laboratory experiments with and without UV-B. (a) Red-legged frog; (b) long-toed salamander; (c) Pacific treefrog. CA= carbaryl initial concentration of 250 µg/L; CP = chlorpyrifos initial concentration of 25 µg/L. Error bars represent one standard error; where no error bars were shown, there was no difference between replicates. "***" indicates treatments that are statistically significantly different from the No-UV-B control treatment (ANOVA followed by Student-Newman-Keul's test, $p < 0.05$). AI = Active ingredient of the pesticide; FP = formulated product of the pesticide.

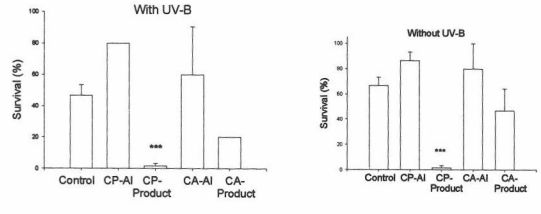
(a) Red-legged frog



(b) Long-toed salamander



(c) Pacific treefrog



Long-toed salamander

Mortality of long-toed salamander larvae was caused by the formulated product of chlorpyrifos regardless of UV-B exposure (Fig. 4.2; Table 4.1). ELISA indicated that chlorpyrifos levels in control water were not detectable. In chlorpyrifos treatments (both chemical and formulated product), levels ranged from 32 to 42 ppb at the beginning of the experiment. At the completion of the experiment, levels ranged from 22 to 27 ppb.

Pacific treefrog

Mortality of Pacific treefrog larvae was caused by the formulated product of chlorpyrifos regardless of UV-B exposure (Fig. 4.2; Table 4.1). ELISA indicated that chlorpyrifos levels in control water ranged from 0 to 1 ppb. In chlorpyrifos treatments (both chemical and formulated product), levels ranged from 22 to 38 at the beginning of the experiment. Levels in the chemical treatment ranged from 7 to 8 ppb at the end of the treatment, and levels in the formulated product treatment ranged from 8 to 11 ppb at the completion of the experiment.

DISCUSSION

Our study found that a formulated product of chlorpyrifos caused mortality in all three species tested (Table 4.1, Figure 4.2). We did not find a similar effect caused by the active ingredient alone, suggesting that in some cases toxicity may be caused by some of the inert ingredients in the formulations. Further experiments are needed to determine exactly what components in the formulations are toxic. Exposure to UV-B radiation did not affect the toxicity of the pesticides or their formulations. However, we believe it is important to examine the combined effects of stressors such as UV-B and contaminants in amphibians, because there has been little study of this important issue.

Chlorpyrifos and its major metabolite TCP absorb UV-B radiation, and exposure of chlorpyrifos to UV radiation produces several different chemical photoproducts (Racke 1993). These photoproducts have not been well characterized, and are potentially toxic (Racke 1993). In contrast to chlorpyrifos, carbaryl has been previously shown to be phototoxic to amphibian larvae in the presence of UV-B radiation (Zaga et al. 1998).

There are several possible explanations for why we did not consistently observe phototoxicity of carbaryl in our study. For example, UV-B levels could have been lower than in previous studies, or there could be other differences in UV-B exposure regime between experiments, such as the levels or doses of lighting. Alternatively, the results could reflect interspecific differences in carbaryl

sensitivity in the various amphibian species. It is also possible that the UV light levels used in the laboratory were not high enough to initiate a response in the larvae. The UV levels used in the laboratory were lower than the levels typically observed in field sites during amphibian breeding in the Willamette Valley (Table 3.1).

In addition to combined effects with UV-B, carbaryl alone has been previously shown to be toxic to amphibians. At levels similar to what animals might encounter in the field following direct application of the pesticide, carbaryl has been shown to reduce tadpole swimming and activity level (Bridges 1997). Carbaryl interacts with predator-prey dynamics (Bridges 1999a,b) and can have carry-over effects at various life history stages in amphibians (Bridges 2000). Furthermore, carbaryl can have dramatic effects on larval amphibians when they are simultaneously exposed to predator chemical cues (Relyea and Mills 2001). The many effects of carbaryl that have been documented in the amphibian ecotoxicology literature illustrate the importance of considering the role of pesticides in amphibian population declines. These effects may also occur with other pesticides commonly applied near amphibian breeding sites.

In our experiments, formulated pesticide products caused mortality while the active ingredients of the pesticides alone did not cause mortality. All three species tested responded to the formulated product of chlorpyrifos with mortality (Fig. 4.2). Ingredients used in the formulation such as emulsifiers or solvents may be toxic to aquatic species, in addition to the major active ingredients themselves.

Other work with microbial toxicity tests has also demonstrated that pesticide formulations (including their associated solvents, surfactants, etc.) can be more toxic than pesticide active ingredients (Pereira et al. 2000). However, the emulsifiers and other agents present in pesticides may not be as liable to contaminate aquatic habitat as the active ingredients of pesticides; typically, these other agents are filtered from runoff by soil or vegetation (Racke 1993). Differences between various formulations of pesticides and the active pesticide compound should be incorporated into an assessment of pesticide use on aquatic life and consider in light of the typical application procedures for a particular pesticide.

Overall, our experiments indicate the importance of considering the route of exposure and the type of chemical administered in toxicity tests. It is possible that the toxic effects we observed were caused by chemicals other than the active ingredient itself, and this issue deserves further investigation. Some possibilities for future work involve determining what components of the pesticide formulation actually reach amphibian breeding sites.

With increasing human population and agricultural expansion, the input of agricultural runoff containing pesticides is likely to increase (Tilman 1999). Therefore, the assessment of the effects of pesticides on sensitive species such as amphibians is increasingly important. In particular, studies performed in a realistic context, accounting for variables such as UV-B, are needed. Outdoor mesocosm studies may provide an ideal venue for the assessment of these effects, allowing for

natural levels of environmental variables. In a separate paper (Chapter 5), we examine the combined effects of (1) UV-B and carbaryl and (2) UV-B and chlorpyrifos in outdoor mesocosms.

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CHAPTER 5

UV-B ENHANCES PESTICIDE TOXICITY IN AMPHIBIANS

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ABSTRACT

We investigated the effect of UV-B radiation on two commonly used pesticides, carbaryl and chlorpyrifos, in larval amphibians native to the highly agricultural Willamette Valley, Oregon, USA. Outdoor mesocosm experiments investigated the effects of ambient levels of UV-B radiation on the toxicity of the pesticides in red-legged frogs (*Rana aurora*), long-toed salamanders (*Ambystoma macrodactylum*), and Pacific treefrogs (*Hyla regilla*). We found several differences in the response of the three species. Chlorpyrifos was particularly toxic to Pacific treefrogs, causing complete mortality within a few days, regardless of UV-B exposure. Carbaryl and chlorpyrifos were toxic to long-toed salamanders in the presence of UV-B radiation. Chlorpyrifos caused mortality in red-legged frogs in the presence of UV-B. Pesticide treatments did not cause mortality in long-toed salamanders or red-legged frogs without simultaneous exposure to UV-B. To our knowledge, our study is the first to document a toxic effect of the pesticide chlorpyrifos in the presence of UV-B radiation. Our results emphasize the

importance of considering UV-B radiation in the toxicity of pesticides to amphibians, and highlight the importance of species differences in responses.

INTRODUCTION

Amphibian populations worldwide are experiencing declines in number or reductions in range (*e.g.*, Houlahan et al. 2000; Pounds et al. 1999). Several agents, including climate change, disease, fertilizers, ultraviolet radiation (UV-B; 280-320 nm), and pesticides, have detrimental effects on developing amphibians and may contribute to their population declines (e.g., Hayes et al. 2002; Hatch et al. 2001; Kiesecker et al. 2001; Davidson et al. 2001; Alford and Richards 1999; Pounds et al. 1999; Marco et al. 1999; Blaustein et al. 2000, 1998, 1994a). However, animals are rarely exposed to a single stressor under natural conditions, but rather encounter a complex cocktail of stressors. Consequently, there is likely no single cause for amphibian population declines (*e.g.*, Kiesecker et al. 2001; Blaustein et al. 2001; Pounds et al. 1999).

Because of the global nature of amphibian population declines, UV-B has been investigated in a number of studies attempting to determine the factors contributing to amphibian population declines. Indeed, the intensity of UV-B reaching the Earth's surface has been increasing in both temperate and tropical regions where amphibian population declines are occurring (Middleton et al. 2001;

Herman et al. 1996; Kerr and McElroy 1993). UV-B radiation causes both lethal (Anzalone et al. 1998; Blaustein et al. 1994a) and sublethal (Belden and Blaustein 2001, 2002; Häkkinen et al. 2001; Smith et al. 2000; Belden et al. 2000) effects on developing amphibians.

Pesticides may also contribute to amphibian population declines through effects on various developmental stages (Hayes 2002; Relyea and Mills 2001; Boone and Semlitsch 2001; Sparling et al. 2000; Bridges 2000, 1999a, 1999b, 1997; Howe et al. 1998; Russell et al. 1995; Semlitsch et al. 1995; Fioramonti et al. 1997). For example, declines of red-legged frogs in California are occurring in a pattern consistent with atmospheric deposition of pesticides from the agricultural Central Valley (Davidson et al. 2001). However, most experimental studies of the effects of pesticides on amphibians are short-term, lasting only a few days.

Short-term laboratory studies may not be representative of the actual effects of pesticides on developing amphibians in a complex environment (Relyea and Mills 2001; Boone and Semlitsch 2001). A few studies have found unexpected results when the effects of pesticides were considered in a biologically realistic context. For example, in some cases the pesticide carbaryl resulted in increased tadpole growth because of its toxicity to competing zooplankton (Boone and Semlitsch 2001). Another study found that low levels of carbaryl are highly toxic to tadpoles when tadpoles are simultaneously exposed to chemical cues from potential predators (Relyea and Mills 2001). Therefore, it is important to consider the complexity of natural systems in experiments with pesticides on amphibians. One

potential approach to incorporating complexity into experiments is to use outdoor mesocosms for experimental exposures. Mesocosms allow for exposure to natural levels of light, temperature, water quality, and their variation. Furthermore, mesocosm experiments simulate the temporary pond environment where many amphibians breed and larvae develop.

With an increasing human population and associated demands on agriculture, the use of pesticides is increasing at an unprecedented rate (e.g., Tilman et al. 2001). Organophosphate pesticides, such as chlorpyrifos, parathion and malathion, are one of the most widely used groups of pesticides (Barron and Woodburn 1995; Racke 1993). Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a broad-spectrum organophosphate insecticide widely used in agriculture, on ornamental plants and grasses, and against household pests (Giesy et al. 1999; Barron and Woodburn 1995; Racke 1993).

Chlorpyrifos and its major metabolite, 3,5,6-trichloro-2-pyridinol (TCP), absorb UV radiation at approximately 295 nm (Racke 1993). However, the toxicity of chlorpyrifos photoproducts has not been well characterized (Racke 1993). The toxic chlorpyrifos oxon metabolite may be formed via photolysis when chlorpyrifos in the air is exposed to UV-B (Racke 1993). In one study of the atmospheric deposition of chlorpyrifos in the Sierra Nevada Mountain Range, levels of the toxic chlorpyrifos oxon were greater at higher elevations, and researchers concluded that this was because of photo-oxidation that occurred when chlorpyrifos molecules were transported to the higher elevations (Aston and Seiber 1997). Therefore,

animals that live at high elevations, such as some species of amphibians, may be exposed to levels of the toxic chlorpyrifos oxon that are higher than might be expected without photo-oxidation. Moreover, animals at higher altitudes may be exposed to higher doses of UV-B (Blumthaler et al. 1997; Ovaska et al. 1997).

Few studies have investigated the effects of chlorpyrifos in amphibians, and these studies indicate that chlorpyrifos toxicity in amphibians varies widely among species (Giesy et al. 1999; Barron and Woodburn 1995). Two field studies suggest that amphibians may be less sensitive to chlorpyrifos than aquatic invertebrates (Barron and Woodburn 1995) and probably respond in a manner comparable to fish (Giesy et al. 1999). However, no studies have investigated the effects of simultaneous exposure to chlorpyrifos and UV-B in amphibians.

Carbaryl (1-naphthyl N methylcarbamate) is a broad-spectrum pesticide used for insect control in farmland and private homes (Cox 1993). Several studies have documented lethal and sublethal effects of carbaryl on developing amphibians (Boone and Semlitsch 2001; Relyea and Mills 2001; Bridges 1997, 2000; Boone and Bridges 1999; Zaga et al. 1998; Marian et al. 1983). For example, larval swimming is reduced by carbaryl exposure at levels similar to what might be encountered in the field (Bridges 1997). In addition, previous work with amphibian larvae (*Xenopus laevis* and *Hyla versicolor*) indicates that carbaryl toxicity is enhanced by UV-B radiation, and this effect was attributed to the photolysis breakdown product 1-naphthol (Zaga et al. 1998). Both carbaryl and chlorpyrifos may enter amphibian breeding habitats, such as ponds or wetlands, via aerial

spraying of nearby fields, runoff from local application, or atmospheric deposition (Boone and Semlitsch 2001; Davidson et al. 2001; Racke 1993).

We studied the combined effects of UV-B radiation and the pesticides carbaryl and chlorpyrifos on the survival of the larvae of three species of amphibians native to the Pacific Northwest, USA. In outdoor mesocosm experiments, we assessed the combined effects of (1) UV-B and carbaryl; and (2) UV-B and chlorpyrifos.

METHODS AND MATERIALS

Animal collection and care

We examined the combined effects of ambient UV-B and pesticides on three species of amphibians native to the Willamette Valley of Oregon, USA: red-legged frogs (*Rana aurora*), long-toed salamanders (*Ambystoma macrodactylum)* and Pacific treefrogs (*Hyla regilla*). These species breed in early spring (January-February) in roadside ditches or temporary ponds (Nussbaum et al. 1983). Because of differences in the timing of oviposition, our experiments on the different species overlapped but were not completed simultaneously. Red-legged frog eggs (stage 15-16; Gosner 1960) were collected from a roadside pond near the coast of Oregon

(approximately 10 km south of Waldport in Lincoln County, Oregon; approx. 15 m elevation). We collected Pacific treefrog (stage 15-18, Gosner 1960) and long-toed salamander (stage 20-24, Harrison 1969) egg masses from ponds located approximately 5.5 km west of Tangent in Linn County, Oregon (approximately 20 m elevation).

We reared embryos in the laboratory in dechlorinated tap water until hatching (approximately three weeks under laboratory conditions). Red-legged frog and Pacific treefrog embryos were reared in 38 L tanks with approximately 50 animals per tank. Half of the water was changed twice per week, and larvae were fed a mixture (approximately 50: 50) of ground alfalfa pellets and TetraMin® flakes *ad libitum*. Long-toed salamander larvae were reared in 4 L plastic boxes (29 x 16 cm in area, 12 cm deep) at a density of 10 larvae per container. Larvae were fed newly hatched brine shrimp *ad libitum* and half of the water was changed twice per week. All animals were maintained at room temperature (21-24°C) under a constant photoperiod of 16 h light to 8 h dark. Immediately prior to adding animals to experiments, we measured a random sample (n = 10) of experimental animals to determine their developmental stage, mass and length. At the beginning of experiments, red-legged frogs were at stage 25 (Gosner 1960), averaged 0.11 g (SE = 0.007 g) each, and were approximately 1.77 cm long (SE = 0.06 cm). Long-toed salamanders were at stage 45 (Harrison 1969), averaged 0.009 g (SE = 0.002) each, and were approximately 1.04 cm long (SE = 0.051). Pacific treefrogs were at stage

25 (Gosner 1960), averaged 0.03 g (SE = 0.005 g) each, and were approximately 1.04 cm long (SE = 0.01 cm).

Experiments

We used outdoor mesocosms (55 L galvanized steel cattle watering tanks lined with high-density polyethylene plastic) as experimental units. We filled tanks with well water (alkalinity 88 mg $CaCO_3$/L; hardness 102 mg $CaCO_3$/L; nitrate 2 mg/L; pH 7.2; conductivity 177 μmhos/cm; DO 11.1 mg/L at 12° C). We exposed larvae of each species separately to combinations of UV-B and pesticides for three weeks and then assessed their survival.

We used two levels of UV-B (with, without) and three levels of pesticide (control, chlorpyrifos 25 μg/L, and carbaryl 250 μg/L) for a total of six treatments. For each treatment we had four replicate cattle tanks with 10 larvae per replicate. We arranged mesocosms in a randomized block design with respect to treatment. We controlled UV-B exposure by using clear plastic filters that either transmit UV-B (acetate) or block UV-B (Mylar®; Hillcor Plastics, Baldwin Park CA). Acetate typically transmits 80% of ambient UV-B radiation and 95% of UV-A radiation, while Mylar® typically transmits 5% of ambient UV-B radiation and 30% of UV-A radiation (Blaustein et al. 1994a). Prior to addition of larvae, we added pesticides using the formulated product (Sevin®, active ingredient carbaryl, EPA Registration

264-334-71004; Kill-a-Bug®, active ingredient chlorpyrifos, EPA Registration # 7122-119-7401) with an initial dose at the appropriate concentration, to simulate a pulsed exposure from a runoff event. Pesticides are typically applied as a commercial product formulated with several other ingredients, such as emulsifiers or solvents, in addition to the active ingredient. We selected to study formulated products of the pesticides of interest to simulate the conditions animals might encounter in the field upon direct application of pesticides.

To provide food for red-legged frogs and Pacific treefrogs, we added alfalfa pellets (approx. 0.5 g) to each tank every week. To provide food for long-toed salamanders, we collected zooplankton from the pond where animals were collected. We added approximately 100 mL of water containing zooplankton (at a density of approximately 15 zooplankton per mL) twice per week to each tank.

We measured UV-B, temperature, pH, conductivity, hardness, and alkalinity in the water of at least one tank from each treatment. We measured UV-B levels in the water of the tanks under the filters twice per week using a model 2100 PMA meter with model 2102 UV-B detector (Solar Light Co., Philadelphia, PA). UV-A was quantified using the same meter and a PMA2111 detector. Once per week, we measured pH, conductivity, hardness, and alkalinity in the water of at least one tank from each treatment. We made these measurements to determine whether our experimental manipulations (the addition of pesticides or the blocking of UV-B) affected other aspects of water quality, potentially confounding our experiments. pH was measured using an Orion 290A pH/ISE meter with a pH

electrode. Conductivity was measured using a hand-held conductivity meter (Hanna Instruments, Woonsocket, RI). Dissolved oxygen was calculated using the Winkler titration method (American Public Health Association 1995). Water hardness and alkalinity were measured by titration with EDTA and 0.02 N HCl, respectively (APHA 1995). Temperature in two acetate and two Mylar® covered tanks was recorded every four hours with Onset DataLoggers (Onset Computer Corporation, Bourne MA). Additionally, we measured nitrate levels in the well water at the beginning of the experiment and in water from each treatment at the completion of each experiment. Nitrate was quantified with an Orion 290A pH/ISE meter with a nitrate electrode.

Chemical analysis

We measured chlorpyrifos levels from each tank once per week using an enzyme-linked immunosorbent assay (ELISA) designed to quantify pesticides in water samples (Rubio et al. 1991, Schraer et al. 2000, Abad et al. 1999, Gaizick et al. 2001). Carbaryl levels were not measured. The ELISA technique favorably compares with other analytical methods including gas chromatography and HPLC in terms of time and expense required to analyze samples and the accuracy and precision of the analysis (Rubio et al. 1991, Schraer et al. 2000, Abad et al. 1999).

For chlorpyrifos analysis, we collected approximately 5 mL of water from each tank weekly in borosilicate glass vials and stored the samples in the dark at 4 C until analysis. Assay kits were obtained from Strategic Diagnostics, Inc., Newark, Delaware. Water sample analysis followed the methods recommended by the supplier and outlined in Rubio et al. (1991). Briefly, water samples were mixed with enzyme conjugate and magnetic particles, the particles were separated with a specialized magnetic rack, color reagent was added and the absorbance was read with a spectrophotometer at 450 nm. Standards were run in duplicate.

Data analysis

We visually checked that data met the assumptions for parametric analysis (normality, homogeneity of variance). One-way analysis of variance (ANOVA) procedures were used to analyze survival, with tank means used as the units of statistical analysis. Post-hoc tests (Student-Newman-Keul's; alpha = 0.05) were used to determine whether treatments differed from the No-UV-B control treatment. We first tested for effects due to block before proceeding with the ANOVA. Because block effects were insignificant in all cases, we proceeded to test for differences between treatments. All statistical tests were completed using SAS version 8 for Windows.

RESULTS

Experimental conditions

Nitrate in the source well water ranged from undetectable to 2.2 mg/L at the beginning of the experiments. At the completion of experiments, nitrate from water in the mesocosms ranged from 1.4 to 2.6 mg/L. In all experiments, acetate blocked approximately 10% of UV-A and approximately 12% of UV-B radiation. Mylar blocked approximately 10 % of UV-A and approximately 75% of UV-B radiation. Table 5.1 summarizes the water quality measurements from each of the experiments. We did not perform statistical tests on these measurements, but rather report the ranges measured to provide information on the experimental conditions of this study.

Table 5.1 Water quality in mesocosm pesticide/UV-B experiments

pH	Dissolved oxygen (mg/L)	Conductivity (μmhos/cm)	Alkalinity (mg $CaCO_3$/L)	Hardness (mg $CaCO_3$/L)
Red-legged frog experiment:				
6.3-7.9	7.0-8.0	291-400	90-110	110-136
Long-toed salamander experiment:				
7.6-8.8	7.2-10	239-334	96-114	104-160
Pacific treefrog experiment:				
7.2-8	7.0-8.4	256-320	102-114	114-136

Red-legged frog

Chlorpyrifos was highly toxic to red-legged frogs in the presence of UV-B (Fig. 5.1; Table 5.2). Complete mortality occurred when animals were simultaneously exposed to UV-B and chlorpyrifos. However, without UV-B, animals experienced approximately 50% mortality in response to chlorpyrifos exposure. Carbaryl exposure reduced survival, although not significantly. Throughout the red-legged frog experiment, UV-A measured at the water's surface ranged from 2.06 to 2.9 mW/cm^2; UV-B ranged from 3.18 to 5.5 $\mu W/cm^2$. Temperature in the UV-B treatments ranged from 5 to 17 °C, with a 10 °C range in any one day. Temperature in the No-UV-B treatments ranged from 5 to 19 °C, with a 10 °C range in any one day. There was no difference in temperature between the UV-B and No UV-B treatments (paired t-test using highest daily temperatures from one acetate and one Mylar® covered tank; t-value = 0.498; p-value = 0.624; 20 df). Chlorpyrifos in control tank water ranged from 0 to 1.2 ppb throughout the experiment. In the chlorpyrifos treatments, chlorpyrifos levels ranged from 32 to 39 ppb at the beginning of the experiment; 20 to 26 ppb after one week; 24 to 25 ppb after two weeks; and 9 to 11 ppb at the completion of the experiment.

Table 5.2. Analysis of variance models for larval amphibian survival in mesocosm pesticide/UV-B experiments.

Red-legged frog:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	5	239.375	47.875	4.37	0.009
Error	18	197.250	10.958		
Total	23	436.625			

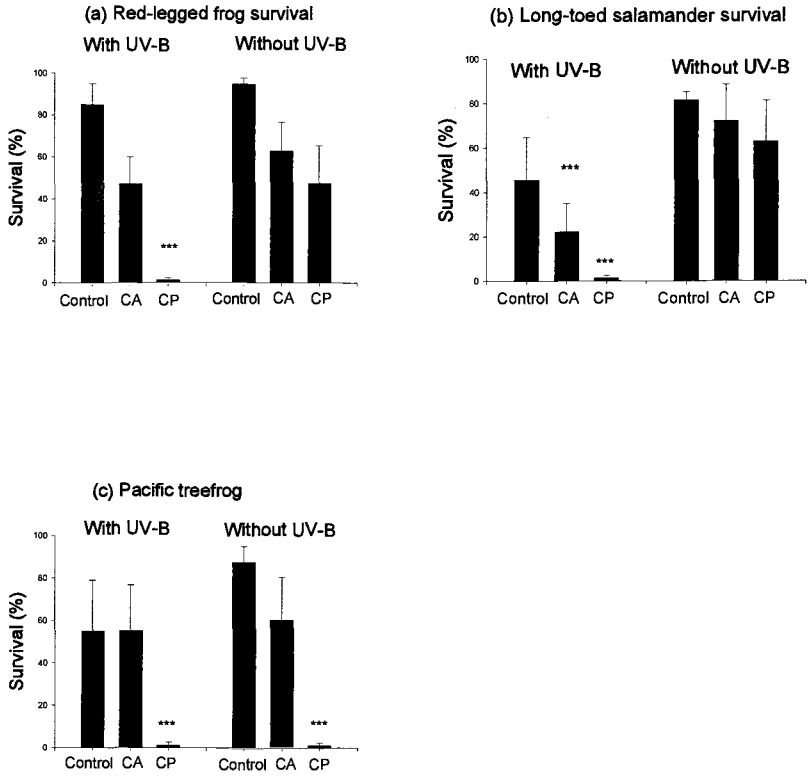
Long-toed salamander:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	5	19592.833	3918.567	5.09	0.004
Error	18	13862.500	770.139		
Total	23	33455.333			

Pacific treefrog:

Source	DF	Sum of Squares	Mean Square	F Value	p-value
Model	5	25020.833	5004.167	4.93	0.005
Error	18	18275.000	1015.278		
Total	23	43295.833			

Figure 5.1. Survival of larval amphibians exposed to formulated products of the pesticides carbaryl and chlorpyrifos in mesocosm experiments with and without UV-B. (a) Red-legged frog; (b) long-toed salamander; (c) Pacific treefrog. CA= carbaryl initial concentration of 250 μg/L; CP = chlorpyrifos initial concentration of 25 μg/L. Error bars represent one standard error. "***" indicates treatments that are statistically significantly different from the No-UV-B control treatment (ANOVA followed by Student-Newman-Keul's test, $p < 0.05$).



Long-toed salamander

Both carbaryl and chlorpyrifos caused mortality in the presence of UV-B radiation in long-toed salamanders (Fig. 5.1; Table 5.2). Animals experienced complete mortality when exposed to both chlorpyrifos and UV-B, and approximately 80% mortality when exposed to carbaryl and UV-B (Fig. 5.1). There was no significant mortality in the treatments without simultaneous exposure to UV-B radiation. Throughout the long-toed salamander experiment, UV-A ranged from 1.9 to 3.79 mW/cm^2 at the water's surface; UV-B ranged from 3.36 to 10.7 $\mu W/cm^2$. Temperature in both the UV-B and the No-UV-B treatments ranged from 4 to 26 °C, with a 12 °C range in any one day. There was no difference in temperature between the UV-B and No UV-B treatments (paired t-test using highest daily temperatures from one acetate and one Mylar® covered tank; t-value = 1.047; p-value = 0.308; 20 df). Chlorpyrifos levels in control tank water ranged from 0 to 2 ppb throughout the experiment. In chlorpyrifos treatments, levels ranged from 24-30 ppb at the beginning of the experiment; from 19-28 ppb after one week; from 5-22 ppb after two weeks; and from 5-14 ppb at the completion of the experiment.

Pacific treefrog

Complete mortality of Pacific treefrog tadpoles was caused by chlorpyrifos in both UV-B and No-UV-B treatments (Fig. 5.1; Table 5.2). Carbaryl did not cause significant mortality in Pacific treefrogs. Throughout the Pacific treefrog experiment, UV-A ranged from 2.9 to 23 mW/cm^2 at the water's surface; UV-B ranged from 5.98 to 7.2 $\mu W/cm^2$. Temperature in the UV-B treatments ranged from 7 to 27 °C, with a 12 °C range in any one day. Temperature in the No-UV-B treatments ranged from 6 to 29 °C, with a 12 °C range in any one day. There was no difference in temperature between the UV-B and No UV-B treatments (paired t-test using highest daily temperatures from one acetate and one Mylar® covered tank; t-value =-0.699; p-value = 0.493; 20 df). Chlorpyrifos levels in control tank water ranged from 0 to 5 ppb throughout the experiment. In chlorpyrifos treatments, levels ranged from 30 to 42 ppb at the beginning of the experiment; from 22 to 42 ppb after one week; from 4 to 7 ppb after two weeks; and from 2 to 7 ppb at the completion of the experiment.

DISCUSSION

Our study shows that some pesticides can cause mortality in larval amphibians when simultaneously exposed to UV-B. To our knowledge, our study is

the first to document the UV-B enhanced toxicity of the pesticide chlorpyrifos in aquatic organisms. We found that larval red-legged frogs suffered mortality when exposed to chlorpyrifos in the presence of UV-B radiation. However, this effect was not observed when animals were shielded from UV-B radiation. Similarly, larval long-toed salamanders suffered increased mortality when simultaneously exposed to chlorpyrifos and UV-B radiation, but not when shielded from UV-B.

The degradation of chlorpyrifos by UV radiation is not fully understood (Racke 1993). However, chlorpyrifos and its major metabolite TCP absorb UV-B radiation, and exposure of chlorpyrifos to UV radiation produces several different chemical photoproducts (Racke 1993). Toxicity may be caused by these photoproducts. Alternatively, it is also possible that the combined toxicity of chlorpyrifos with UV-B that we observed was simply caused by decreased resistance in the presence of multiple stressors.

In contrast to chlorpyrifos, carbaryl has been previously shown to be phototoxic to amphibian larvae (Zaga et al. 1998). In our study, this effect was apparent only for long-toed salamanders (Fig. 5.1). We observed mortality when long-toed salamander larvae were simultaneously exposed to UV-B and carbaryl, but saw no mortality when animals were exposed to carbaryl in the absence of UV-B radiation. There are several possible explanations for why we did not consistently observe phototoxicity of carbaryl in our study. For example, UV-B levels could have been lower than in previous studies, or there could be other differences in UV-B exposure regime between our experiments and those of Zaga et al. (1998), such

as the doses of UV-B received by experimental animals. Alternatively, the results could reflect interspecific differences in carbaryl sensitivity in the various amphibian species. Long-toed salamanders were particularly sensitive to the effects of UV-B alone, perhaps predisposing them to be sensitive to the combined effects of UV-B and carbaryl. Therefore, the responses of larval long-toed salamanders to the simultaneous exposure to UV-B and carbaryl could have been caused by decreased resistance in the presence of multiple stressors.

An alternative explanation for a potential interaction between UV-B and the pesticides could involve enzyme inactivation by the pesticides. Both carbaryl and chlorpyrifos inhibit the enzyme cholinesterase in vertebrates, causing nervous system damage and eventually death (Barron and Woodburn 1995; Cox 1993). In general, this effect is more readily reversible in the carbamate pesticides (such as carbaryl) in comparison to the organophosphate pesticides (such as chlorpyrifos) (Cox 1993). If there is a similar inhibitory effect on the photorepair enzyme photolyase caused by the pesticides, animals might be less able to repair the damage caused by UV-B radiation when they have been exposed to one of the pesticides. However, this idea requires additional experimentation and investigation than presented in our current study.

Our study found differences in the responses of the three species tested to pesticides and UV-B. Pacific treefrogs were particularly sensitive to chlorpyrifos, as indicated by their complete mortality within a few days of exposure (Fig. 5.1), regardless of UV-B exposure. The differences in response of each species highlight

the importance of testing a variety of species when attempting to assess the effects of environmental stressors such as pesticides.

Assuming that activity levels of the photorepair enzyme photolyase in embryos are similar to the levels in larvae, several interesting comparisons can be made between our study and previous work. The species differences found in our study correlate with species differences to UV-B alone that have been previously documented in amphibian embryos (Anzalone et al. 1998; Ovaska et al. 1997; Blaustein et al. 1994a, 1997). As embryos, Pacific treefrogs are resistant to the toxic effects of UV-B, and have relatively high activity levels of the photorepair enzyme photolyase (Blaustein et al. 1994a). Furthermore, hatching success of Pacific treefrog eggs was not affected by exposure to ambient levels of UV-B in field studies in Oregon (Blaustein et al. 1994a), California (Anzalone et al. 1998) and British Columbia, although larval survival was reduced by exposure to artificially enhanced UV-B in the British Columbia study (Ovaska et al. 1997). In our study using amphibian larvae, Pacific treefrogs were not as sensitive to UV-B radiation compared to the other two species studied (Table 5.2). In contrast to Pacific treefrogs, long-toed salamander embryos have relatively low activity levels of the enzyme photolyase, which limits their ability to repair UV-induced sublethal DNA damage (Blaustein et al. 1994a). Additionally, field exposures to ambient levels of UV-B in Oregon caused embryonic malformations in long-toed salamanders (Blaustein et al. 1997). In our study, UV-B radiation in combination with both pesticides tested caused mortality in larval long-toed salamanders.

The phototoxicity of the pesticides chlorpyrifos and carbaryl that we document in this study is of increasing importance in assessing the effects of contaminants in aquatic systems. UV-B radiation is increasing in intensity worldwide, and penetrates freshwater systems such as ponds to biologically significant depths (Middleton et al. 2001; Herman et al. 1996). These increases heighten the potential for exposure of many organisms to harmful UV-B (Cockell & Blaustein 2001). Simultaneously, with increasing human population and agricultural expansion, the input of agricultural runoff containing pesticides is likely to increase. For example, a recent State of the Environment report identifies the Willamette Valley as the region most heavily affected by agricultural practices in Oregon (http://www.econ.state.or.us/opb). This region also houses a diverse array of amphibians because of the moist, temperate climate (Nussbaum et al. 1983). Furthermore, amphibians often oviposit in roadside ditches or small ponds that are directly impacted by agricultural runoff. Therefore, the study of the combined effects of UV-B and pesticides is particularly relevant for amphibians breeding in agricultural systems such as the Willamette Valley.

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CHAPTER 6

GENERAL CONCLUSIONS

My thesis considered the combined effects of several environmental stressors on larval amphibians (Table 6.1). In some cases, results emphasize the importance of considering environmental stressors in combination. In Chapter 1, low pH and high nitrate levels caused mortality in larval Cascades frogs, while exposure to UV-B and high nitrate levels reduced their activity level. An outdoor study focusing on only nitrate and UV-B found similar effects in Pacific treefrogs (*Hyla regilla*). At lower elevations, Pacific treefrogs exhibited reduced mass when exposed to both nitrate and UV-B. However, at higher elevations, their survival was reduced by exposure to both stressors. Results from the outdoor study focusing on only nitrate and UV-B found more complex results in long-toed salamanders (*Ambystoma macrodactylum*). Larval salamander mass was enhanced by nitrate addition. However, at the higher elevation experiment with higher levels of UV-B, this effect did not occur. This result could suggest that, in the higher elevation experiment with higher UV-B levels, UV-B could have affected the salamander's food source.

Experiments with pesticides and UV-B indicate the potential for UV-B to enhance the toxicity of some pesticides. Furthermore, laboratory experiments

indicated that the formulated products of some pesticides might be more toxic than the active ingredients alone. In outdoor experiments, a formulated product of the pesticide chlorpyrifos was particularly toxic to Pacific treefrogs (*Hyla regilla*), causing complete mortality within a few days, regardless of UV-B exposure. Carbaryl and chlorpyrifos were toxic to long-toed salamanders (*Ambystoma macrodactylum*) in the presence of UV-B. Similarly, chlorpyrifos caused mortality in red-legged frogs (*Rana aurora*) in the presence of UV-B. Pesticides did not cause significant mortality in long-toed salamanders or red-legged frogs without simultaneous exposure to UV-B.

Interactive effects might be caused by decreased resistance of animals in the presence of multiple stressors, or by a specific mechanism of enhanced toxicity. In the presence of one stressor, animals may have lowered resistance to the toxicity of a subsequent stressor (*e.g.*, Paine et al. 1998; Underwood 1989; Newman 1995). We do not know of a mechanism by which UV-B might enhance the toxicity of nitrate or low pH. Therefore, increased effects in the simultaneous exposure to these stressors are likely attributed to decreased resistance. In contrast, the toxicity of pesticides in the presence of UV-B (Chapter 5) may have a mechanistic explanation. Exposure to UV-B may induce greater production of toxic metabolites (e.g., chlorpyrifos oxon; Aston and Seiber 1997). Increasingly, environmental risk assessment is focusing on the results of exposure to several stressors simultaneously (Ferenc and Foran 2000). To fully understand these assessments, it

is important to consider whether responses have a specific mechanism or simply result from the effects of multiple stressors acting together.

In some cases, the results presented in this dissertation may be useful in developing policies for the localized application of fertilizers and pesticides. When possible, the exposure of sensitive taxa such as developing amphibians to potentially harmful environmental agents should be reduced. This approach may work best when focusing on a particular geographic region, where direct cause and effect relationships can be observed. Although it may not be possible to reduce the potential for exposure to ubiquitous stressors such as UV-B, the timing of fertilizer and pesticide applications could be considered in light of the potential for amphibians to be exposed to these contaminants. For example, if there is an interactive effect between two pesticides, the timing of their application could be considered to decrease the potential for simultaneous runoff and exposure. The application of fertilizers in agricultural areas may coincide with the spring breeding of many amphibians. Furthermore, temporary ponds and ditches near agricultural areas may be subject to large amounts of runoff from fertilizer application. These regions should be included in assessments of the effects of environmental agents.

My dissertation results pose several questions for future work. The effect of UV-B on the toxicity of the pesticides carbaryl and chlorpyrifos is not fully understood. Further experiments could investigate the role of UV-B in the formation of chlorpyrifos oxon, possibly indicating whether the increased toxicity of chlorpyrifos observed in the presence of UV-B was caused by the oxon or

another metabolite. Additionally, characterization of the photoproducts of the pesticides carbaryl and chlorpyrifos, and the toxicity of these photoproducts, would be a useful avenue for future research. On a different topic, the population-level implications of detrimental effects on amphibian larvae, such as reduced survival, growth and activity level, are poorly understood. To fully understand amphibian population declines, more work is required to determine the effects of reduced larval health on the overall population.

In conclusion, the results of my thesis provide strong evidence for the importance of studying the combined effects of multiple environmental stressors in amphibians. Future work could focus on the development of a conceptual model incorporating the potential for larval amphibians to be exposed to several stressors simultaneously. To be most useful, such a model should focus on a particular geographic region (such as the Willamette Valley), accounting for the unique stressors and land use attributes of that region.

Table 6.1. Summary of dissertation results.

Chapter/Species	*Venue/Factors studied*	*Result/Conclusion*
Chapter 2:		
Rana cascadae	Laboratory	Exposure to low pH or to high nitrate caused mortality; Exposure to UV-B or to high nitrate reduced activity level
Chapter 3:		
Hyla regilla: Valley population	Field/Mesocosm UV-B and nitrate	Reduced mass in simultaneous exposure to UV-B and nitrate
Hyla regilla: Mountain population	Field/Mesocosm UV-B and nitrate	Reduced survival in simultaneous exposure to UV-B and nitrate
Ambystoma macrodactylum: Valley population	Field/Mesocosm UV-B and nitrate	Increased mass with nitrate
Ambystoma macrodactylum: Mountain population	Field/Mesocosm UV-B and nitrate	Increased mass with nitrate, shielded from UV-B
Chapter 4:		
Rana aurora	Laboratory UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by formulated product of chlorpyrifos alone
Ambystoma macrodactylum	Laboratory UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by formulated product of chlorpyrifos
Hyla regilla	Laboratory UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by formulated product of chlorpyrifos

Chapter 5:		
Rana aurora	Field/Mesocosm UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by exposure to UV-B and chlorpyrifos together
Ambystoma macrodactylum	Field/Mesocosm UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by exposure to UV-B and chlorpyrifos or by exposure to UV-B and carbaryl
Hyla regilla	Field/Mesocosm UV-B and chlorpyrifos UV-B and carbaryl	Reduced survival caused by exposure to chlorpyrifos alone

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