PESTICIDES

AND AMPHIBIAN DECLINES IN THE

SIERRA NEVADA MOUNTAINS, CALIFORNIA

A Dissertation

by

DEBORAH FAY COWMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

Co-Chairs of Committee,	Thomas E. Lacher
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	Tarla R. Peterson
	Laura L. McConnell
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ABSTRACT

Pesticides and Amphibian Declines in the Sierra Nevada Mountains, California.

(December 2005)

Deborah Fay Cowman, B.M., Coe College;

M.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Thomas E. Lacher
Dr. Donald W. Sparling

Pacific chorus frog *(Pseudacris regilla)* hatchlings were translocated and placed in cages in sites (~2,200 m elevation) located in Lassen, Yosemite, and Sequoia National Parks. DDE was found in 97% of Yosemite National Park samples, 84% in Sequoia National Park samples, and 15% of Lassen Volcanic National Park samples in 2001 and 2002. Total endosulfans were detected in 3% of Sequoia samples, 9% of Lassen samples and 24% of Yosemite samples. Both pesticides were detected in tadpoles and metamorphs raised at the three parks regardless of origin. Because the tadpoles were translocated post hatching, this finding indicates that the pesticides, particularly DDE, were accumulated at the site, instead of through deposition in the egg mass.

Liver cells from 108 newly metamorphosed frogs were examined with flow cytometry (FCM) techniques for evaluation of chromosome breakage as measured by the half-peak coefficient of variation (HPCV) of the G_1 peak. Regardless of origin,

experimental groups raised at Lassen, the reference site, had significantly less chromosomal breakage ($p=0.04$) than metamorphs raised at the other two parks. This is the first documented evidence of DNA damage in juvenile frogs in the Sierra Nevada Mountains.

Cholinesterase (ChE) was measured in tadpoles collected at 28 days and in juvenile frogs collected upon metamorphosis. In 2001, ChE activity was significantly higher in animals raised at Lassen (reference site), than at the other two parks, indicating less exposure to cholinesterase-inhibiting pesticides. This trend was not observed in 2002, although Sequoia ChE values were consistently lower than the other two parks. Temperatures were significantly different among the three parks for both years ($p<0.0001$) and lower temperatures may correlate with lower ChE levels.

Survivorship to metamorphosis, days to metamorphosis, snout-vent lengths (SVL), and malformations were evaluated. Animals raised in Sequoia had shorter SVLs, took longer to metamorphose, and had lower survivorship to metamorphosis than in the other two parks ($p<0.0001$). Effects noted in *P. regilla* may be magnified in long lived ranid species.

These findings may be important in evaluating the overall impact of aerially transported pesticides on declining frog populations in the Sierra Nevada Mountains.

I dedicate my PhD dissertation to the two most important men in my life, my father, Robert G. Murray, and my husband, Donald R. Clark.

My father was an important influence in my early years. He taught me to love Earth, and to appreciate life in its many forms. Under his guidance, my pets ranged from insects to mammals. Among other things, he taught me how to climb trees, to fish, to use tools, to love music, to ask questions, and to laugh. As a research mechanical engineer, he also instilled in me his positive "can do" spirit, his drive for excellence, and his tenacious approach to solving problems. I grew up believing he could fix anything. Sadly, he died on November 13, 2004 and so he will never read this dissertation. But without him and his loving influence, it would never have been written. Thank you, Dad, for everything.

My husband stands out as the most influential person in my later years. He taught me how to stand up for myself, how to focus my enthusiastic love of nature and wildlife, how to design and conduct a science research project, and how to write. Throughout this project, which resulted in my absence for months at a time, he kept his sense of humor and remained steadfast in his support. He helped me in the field, and afterwards would remark with a grin to any who asked, "She got me up at sunrise, worked me until dark, fed me out of tin cans, and made me sleep on the ground. And she wonders why I didn't stay longer!" And he cheerfully helped me in the lab, in spite of his assertion that "being your technician is not my idea of a fun retirement." Without my husband and his loving support, this dissertation would never have been written. Thank you, Don, for everything.

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A project of this scope would not have been possible without the help of many people. My thanks go first and foremost to my outstanding committee and project members. I am indebted to Thomas Lacher for the opportunity to be his PhD student and for his outstanding direction and support. His patient and wise counsel guided and encouraged me throughout the entire PhD process and his help in experimental design, statistics, and editing was invaluable. I am also indebted to Donald Sparling, my co-chairman and US Geological Survey supervisor, who provided the opportunity and financial support for me to pursue this project. His thoughtful leadership, excellent guidance, and well-known expertise in amphibian ecotoxicology were absolutely essential. Gary Fellers provided the Sierra Nevada amphibian decline documentation and "on the ground" mentoring in California. His vast knowledge of California amphibians, and his impressive abilities in the field, ensured the success of the field portion of this study. Laura McConnell developed analytical methods for recovery of pesticide residues and guided the difficult process of collecting and analyzing water, sediment, and tissue samples. Her outstanding analytical chemistry expertise was a key component in this project. John Bickham contributed his superior knowledge, materials, and staff for the flow cytometry methods on juvenile frog tissue, an exciting new use of this technique. This part of the study would not have been possible without his help. Tarla Peterson, taught me to think about rhetoric and its use in environmental conflicts. Her

extraordinary insights in environmental communication are invaluable when applied to controversial research involving pesticide impacts on threatened and endangered species.

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Thanks also to my parents Robert and Lois Murray for instilling in me a love of nature, a willingness to work hard, and the ability to laugh. My mother also gave me a love of education, family, and prayer. Thank you, Mom.

It isn't often that a mother can thank her daughter for helping her to obtain her PhD. My daughter, Erika Cowman, deserves a special thank you for her field and lab assistance, and for providing the inspiration for me to return to graduate school. Thank you, Erika.

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

TABLE Page

CHAPTER I

INTRODUCTION

The purpose of this dissertation is to present and interpret findings from an ecotoxicological field experiment focusing on the effects of agricultural pesticides on declining frog populations in the Sierra Nevada mountains, California. In order to address various aspects of this project appropriately, the dissertation is separated into chapters. Chapters II-VI each focus on one phase of the project and will be treated separately.

Chapter II is a review of the global decline of amphibians, amphibian population declines in the California, and supporting documentation of organophosphous insecticide effects on amphibians.

Chapter III provides site descriptions and methodology for the experiment as well as for the residue analyses. Tadpoles and metamorphs were analyzed for pesticide residues after translocation. Results for chemical residues detected in frogs is presented and interpreted here.

Chapter IV addresses DNA damage following pesticide exposures in amphibians. Methodology and results from flow cytometry experiments conducted to evaluate potential DNA damage in exposed juvenile frogs are reported and interpreted.

Format and style follows *Environmental Toxicology and Chemistry*.

Chapter V focuses on potential cholinesterase (ChE) inhibition in amphibians exposed to cholinesterase-inhibiting pesticides. Frog tissue samples from the field experiment were analyzed for ChE inhibition. Methodology and results are presented and discussed here.

And finally, chapter VI reports the results of various biological endpoints evaluated from the field experiment. Endpoints such as survivorship to metamorphosis, time to metamorphosis, size at metamorphosis, and rate of malformations are compared among the three experimental sites.

CHAPTER II

REVIEW OF SIERRA NEVADA MOUNTAIN AMPHIBIAN DECLINES AND THE ROLE OF ORGANOPHOSPHATE INSECTICIDES*

GLOBAL AMPHIBIAN DECLINES

Amphibians are an integral part of their ecosystems, affecting nutrient cycling and also serving as high-quality prey for many species [1]. In the last 15 years, scientists have accumulated evidence supporting a global decline in amphibians. As the quantitative evidence grows, it is difficult to deny the validity of this global trend [2,3]. Because amphibians have occupied Earth for 350 million years, surviving even the mass extinction of dinosaurs, their current global decline is cause for alarm.

In 1996 the International Union for Conservation of Nature (IUCN) Red List of Threatened Animals listed 156 amphibian species as extinct, critically endangered, or vulnerable to extinction [4]. Recently, the Global Amphibian Assessment (GAA) released the results of the first comprehensive and global assessment of the status of all 5,743 described species of amphibians. The data show that 1,856 amphibian species (32.5%) are now in Red List Categories of Vulnerable, Endangered, or Critically

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Endangered. Amphibians are more at risk than bird species (1,211 or 12% listed), or mammal species (1,113 or 23% listed). In addition, 427 (7.4%) of amphibian species listed are on the brink of extinction [3]. These precipitous and rapid declines are thought to be caused by one or more of the following: habitat destruction [5]; increased UV-B radiation [6–9]; climate change [10–15]; acid deposition [16,17]; bacterial, viral, and fungal infections [18–20]; introduced predators [21–25]; harvesting for food,

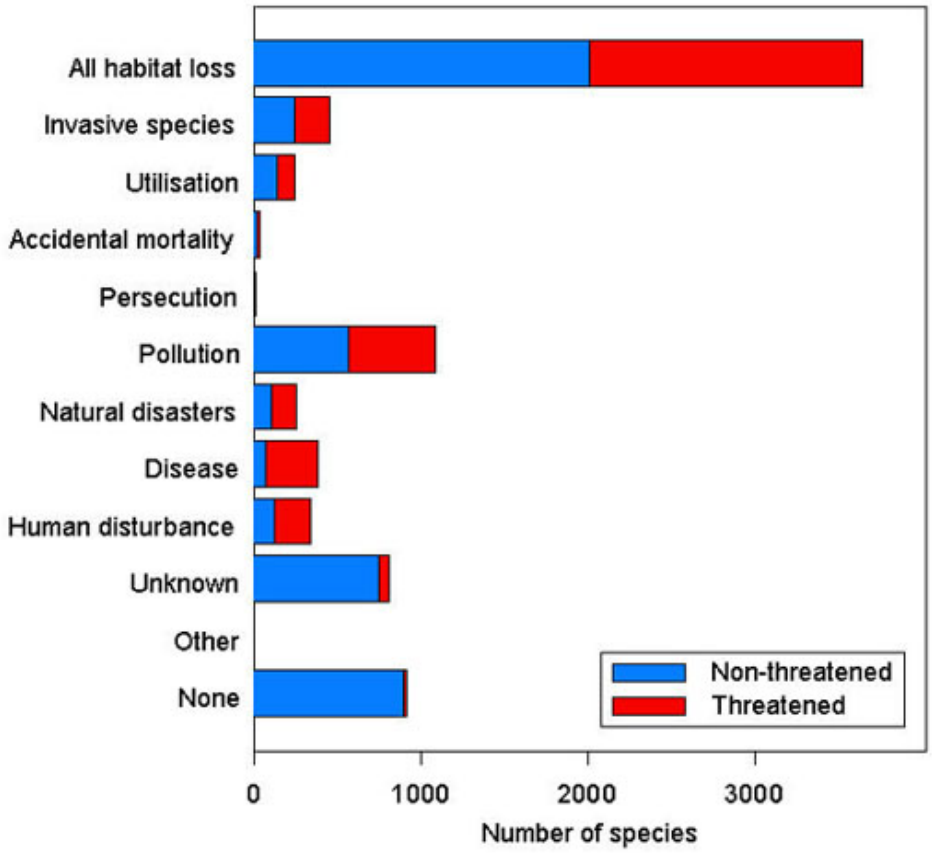

Figure 2.1. Graph depicting probable causes of amphibian declines. Source: Global Amphibian Assessment, IUCN, Conservation International, NatureServe 2004.

scientific research, and pet trade [26,27]; heavy metal, polychlorinated biphenyl (PCBs), fertilizer, and pesticide toxicities [28–38]; or a combination of two or more of these stressors [39–41].

Although Stuart et al. [3] specifically identified declines due to habitat loss and over-utilization, they acknowledge that other processes threaten 48% of rapidly declining species, with pollution second to habitat loss (Figure 2.1) [42].

CALIFORNIA DECLINES

North America has not escaped this dramatic decline [43] and one area of particular concern is the Sierra Nevada Mountains of California. Broad scale field sampling and historical analyses of museum records show an ecosystem level decline of amphibians around the Great Central Valley of California. Counties most affected are Sacramento and those of the San Joaquin Valley [44]. The collapse of a regional frog fauna, 5 of 7 species, in the Yosemite area of the California Sierra Nevada, has also been documented [45].

According to Jennings [46], all 5 native ranid species in the Sierra Nevada are in need of protection. The Cascade frog *(Rana cascadae)* [47] is declining and the northern leopard frog *(R. pipiens)* has disappeared from 99% of its range [48,49]. The California red-legged frog *(R. draytonii)* is listed as threatened [50,51]. The foothill yellow-legged frog *(R. boylii)* is in decline [45,52] and the mountain yellow-legged frog *(R. muscosa)* has disappeared from >75% of study sites where it was formerly found in California

[53–55]. In 2002, the southern-most population in California of *R. muscosa* was listed as an endangered species [56].

Various hypotheses have been presented for the decline of these species. Bradford et al. [57] suggested that predatory fish introduced into previously fish-free frog streams and ponds may have been a factor. In support of this hypothesis, Kiesecker and Blaustein [58] documented the negative effects of introduced bullfrogs *(R. catesbeiana)* and smallmouth bass *(Micropterus dolomieni)* on the growth and survival of red-legged frogs *(R. aurora)*. Other research recently published by Knapp [24], Knapp et al. [25], and Vredenburg [23] implicates introduced trout in the decline of *R. muscosa.* Periods of severe drought in California from 1987-1992 have also been offered as a possible reason for population declines of *R. muscosa* [45]. However, Drost and Fellers [45] conclude that these hypotheses do not account for widespread species declines across large geographic areas, or the probable extirpations of amphibian species in areas of relatively undisturbed habitat.

Previous studies have also shown that organophosphate (OP) pesticides from the Central Valley of California enter the Sierra Nevada ecosystem through aerial deposition in snow and rain [59–62] and that surface concentrations of certain pesticides are within an order of magnitude of the 96 hr LC_{50} for amphibians [63,64]. Datta et al. [65] documented pesticides (dichlordiphenyl dichloroethylene (p,p'-DDE), chlorothalonil and chlorpyrifos) and polychlorinated biphenyls (PCBs) in fish and Pacific chorus frog (*Pseudacris regilla*, formerly *Hyla regilla*) tadpoles from the Kaweah River Basin, CA. Angerman et al. [66] documented PCBs and toxaphene in *P. regilla* tadpoles from the

Sierra Nevada including Lassen, Yosemite, and Sequoia National Parks. Sparling et al. [64] have also found significant levels of pesticides (chlorpyrifos, diazinon, and endosulfan) in tissues of adult *P. regilla* collected in the Sierra Nevadas. In 2004, Fellers et al. [67] showed an array of pesticides in surface water and in *R. muscosa* tissues.

LeNoir et al. [62] reported that agricultural activity in California's Central Valley may be a significant source of pesticides currently being deposited in the Sierra Nevada mountain range. The authors suggest that pesticides applied to this area of intensive agriculture may be volatilized by high temperatures in the valley, transported through the atmosphere and finally deposited in cooler, higher elevations of the Sierra Nevada Mountains. Residue analyses showed that highest levels of contaminants found in surface water and dry particulate samples in the mountains were those of pesticides applied in the valley during heavy use periods in summer. Davidson et al. [68] showed an association of amphibian declines in California with the amount of upwind agricultural land use. His further research [69] demonstrated that this correlation held for declines in *R. aurora draytonii*, *R. boylii*, *R. cascadae*, and *R. muscosa*. OP and carbamate pesticides were most strongly associated with these population declines in Davidson's analysis of historical pesticide applications [69]. De Vlaming et al. [70], Hunt et al. [71], and Bailey et al. [72] also reported the presence and toxicity of chlorpyrifos and diazinon in several California agricultural and urban watersheds.

In addition to finding significant levels of pesticides in tissues of adult *P. regilla* collected in the Sierra Nevadas, Sparling et al. [64] also demonstrated that cholinesterase

activity (a bioassay reflecting OP or carbamate pesticide exposure) is significantly inhibited in tadpoles of *P. regilla* in regions where ranid frogs have experienced the worst declines. These authors hypothesize that because chorus frog tadpoles share the same habitat as larval ranids, inhibition of cholinesterase strongly suggests that carbamate or OP pesticides may be adversely affecting amphibians inhabiting these wetlands [64]. This dissertation project tested interactions between pesticide use in the Central Valley of California and declines of frog populations in the Sierra Nevada over an expanded geographic range, and with a broader, more detailed approach involving field experimentation. Because all the native true frog species (*Rana* spp.) of the Sierra Nevada are in peril, information regarding the possible role of agricultural chemicals in their decline is critical.

AMPHIBIAN PESTICIDE REVIEW

Cowman and Mazanti [33] published a review of the effects of "new generation" pesticides on amphibians. Permission has been granted by the publisher for exerts to be included here.

The effects of "new generation" pesticides (post-chlorinated hydrocarbons) such as OPs, carbamates, pyrethroids, and various herbicides and fungicides on amphibian populations are a growing concern [73-75,28,76,29,30,77,34,7]. According to Hill [78] over 100 types of carbamates and OPs alone are applied at a yearly rate of approximately 200 million-acre treatments in the United States. Although the use of cholinesterase-inhibiting insecticides has been declining in California since 1997, (Figure 2.2) total

pesticide use in the state does not appear to be decreasing. For example, in 2003 the reported pesticide use in California totaled 175 million pounds, an increase of 7.2 million pounds from 2002 [79]. When the application rates of herbicides, fungicides, and pyrethroids are also considered, along with pesticide use in the rest of the world, the potential impact becomes even greater. For example, atrazine, the most commonly used herbicide in the world, causes hermaphroditic frogs after an exposure of only ≥0.1 ppb [35,36]. Although most of these pesticides do not bioaccumulate in the manner of many chlorinated hydrocarbons (e.g. DDT's metabolite DDE) many have relatively high levels of toxicity [80–81] and may pose risks to amphibians through lethal or sublethal effects [82,28,78]. In fact, documentation of pesticide effects in other wildlife is extensive [80,83–86,78,87–89]. It is generally recognized that the biphasic life cycle of amphibians and their skin permeability may not only make them more susceptible to environmental contamination but also complicate their response patterns [90,73,74]. Differences in the diet and behavior patterns of larval and adult amphibians [91] may result in a broad range of effects caused by pesticides introduced into aquatic environments. Moreover, many agricultural pesticides are applied in a variety of chemical combinations and various authors have pointed out that few studies have evaluated the field responses of amphibians to this kind of exposure [73,92,93]. Most amphibian pesticide studies have focused on effects noted in laboratory toxicity tests involving eggs or larvae.

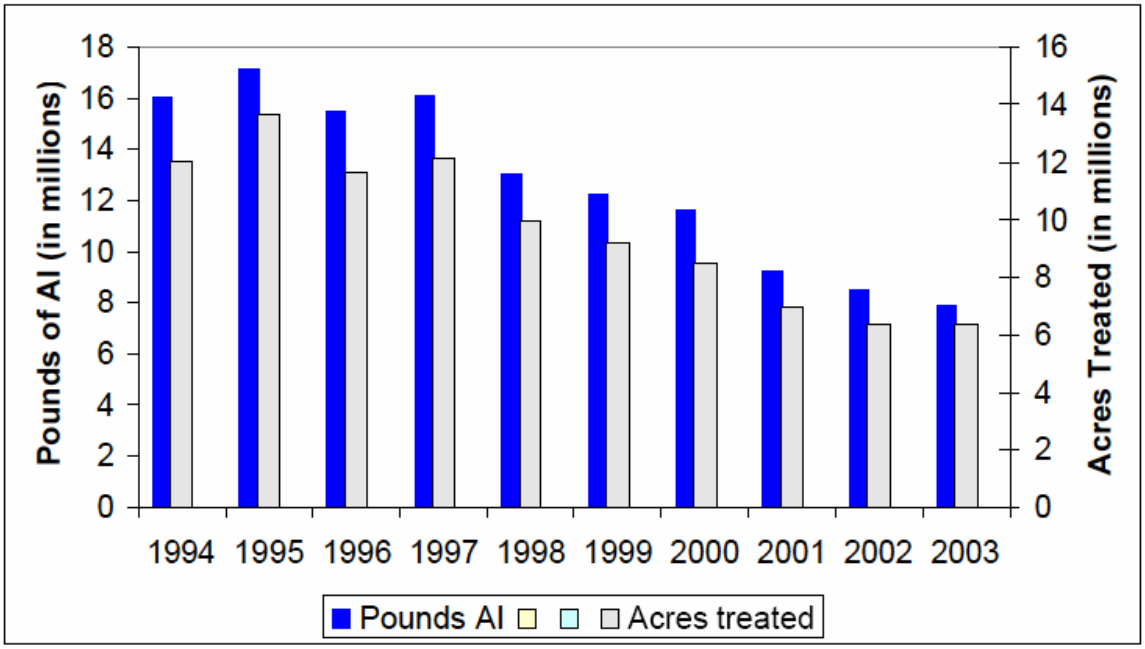

Figure 2.2. California 1994-2003 use trends of cholinesterase-inhibiting pesticides. AI = Active Ingrediant. Source: California Department of Pesticide Regulation's Pesticide Use Reports.

A summary of amphibian toxicological literature by Power et al. [82] examined toxicity tests of over 200 different contaminants and field studies of more than 50 contaminants. Their review did not focus exclusively on pesticides but did reveal an overall lack of standardized protocols or test species for amphibian contaminant studies in general [88]. Many recent investigations have used the African clawed frog *Xenopus laevis* as the test species in a bioassay called Frog Embryo Teratogenicity Assay-*Xenopus* or FETAX [94–98]. Although this bioassay is a valuable tool for determining general toxicity to amphibian eggs and larvae, and to understand developmental toxicities [99], the dependence on *X. laevis* as a test species does not address

complicated issues of species differences. In fact, *X. laevis* appears to be more tolerant to various environmental contaminants than many other amphibian species [100]. Shapira et al. [101] noted a high resistance to cholinesterase inhibitors in *X. laevis* tadpoles. Furthermore, the FETAX assay does not evaluate contaminant exposures of all life history stages in amphibians.

This section reviews publications documenting the effects of the most frequently used organophosphate insecticides in California, with particular emphasis on those used in the Central Valley, on frogs, toads, and salamanders. Studies are examined for patterns of toxicity inherent to particular species and life stages. Life stage terms used in this section are defined as follows: embryos refer to egg stage, tadpoles and larvae are used interchangeably to refer to stages after hatching but prior to complete metamorphosis, juveniles refer to metamorphosed subadults, and adults refer to sexually mature individuals [91]. Tadpole stages refer to Gosner stages [102]. Emphasis is placed on ecotoxicological studies and FETAX studies are only briefly covered. For extensive lists of LC_{50}s the reader is referred to Ecotoxicology of Chemicals to Amphibians [103].

OP Insecticides

The use of OP insecticides for pest control in agricultural crops has dramatically increased since the ban or phase-out of chlorinated hydrocarbon or organochlorine (OC) pesticides in the 1970's. While OPs are generally less persistent and bioaccumulative than OCs, most are acutely toxic to a wide variety of non-target organisms, including aquatic organisms, birds, and mammals [80,78]. OPs disrupt the central nervous system

by inhibition of cholinesterase (ChE) activity. The nervous system in animals uses acetylcholine (ACh) to transmit impulses across synapses from nerve to nerve and nerve to muscle. ChEs destroy the ACh and end the transmission. When an insecticide inhibits ChE, excessive ACh accumulates resulting in hyperactivity, muscular spasms, and eventual paralysis. Death follows due to respiratory failure [104,105,83,78]. Dermal routes of exposure to ChE- inhibiting insecticides can be particularly damaging [106,107], so animals that forage in upland crop fields (e.g. the toad *Bufo americanus)* may be vulnerable to multiple routes of exposure. The majority of published reports regarding the toxic effects of OPs on wildlife review effects on birds and fish; reptiles and amphibians are not frequently tested in spite of possible exposures in habitats receiving pesticide drift or runoff from nearby crop fields [90]. It is unknown if such scenarios result in multiple, subacute, amphibian exposures to OPs that lead to cumulative ChE inhibition and eventually death.

Galloway and Handy [108] evaluated the sublethal effects of organophosphorous pesticides on the immune systems and functions of invertebrates, fish, and higher vertebrate wildlife. Pesticides reviewed included parathion, chlorpyrifos, malathion and diazinon. While amphibians were not studied, laboratory research on fish showed immunosuppressive effects on antibody response [109]. This supports Carey and Bryant's [28] hypothesis linking immunosuppression in amphibians with contaminant exposure.

Azinphos-methyl

Azinphos-methyl (Guthion® and Guthion® 2S) is a nonsystemic, highly persistent, broad–spectrum insecticide and one of the most toxic OPs by dermal absorption, inhalation, ingestion, or eye contact [81]. Mulla [110] described a 100% mortality of adult *R. catesbeiana* at a rate of 1.8 kg ha^{-1} of azinphos-methyl field applications. A follow-up study at reduced application rates of 0.11 and 0.45 kg ha^{-1} showed no mortality of caged *Scaphiopus hammondi* and *B. boreas* adults [111]. Meyer [112] exposed *R. catesbeiana* tadpoles to 1.0 mg AI/L with no effect. The comparative toxicity of Guthion (99%) to Guthion 2S (22% active ingredient) was evaluated on the mortality and growth in *X. laevis* and *P. regilla* tadpoles [113]. Guthion caused impaired growth at 1.7 mg/L for *X. laevis* tadpoles and 9.67 mg/L for *P. regilla* tadpoles. Both species exhibited 100% mortality at 8.7 to 9.7 mg/L of Guthion and at 0.8 mg/L-1.5 mg/L Guthion 2S. Four d LC_{50}s for Guthion was 2.94 mg/L in *X. laevis* and 4.14 mg/L for *P. regilla. X. laevis* 4-d LC_{50} values for Guthion 2S were 5-7 times lower (0.42-0.59 mg/L) and those for *P. regilla* were 5-9 times lower (0.46-0.84 mg/L). No observed adverse effects levels (NOAEL) for *P. regilla* showed it to be 5 times more sensitive to Guthion 2S (0.36-0.37 mg/L) than Guthion (1.78 mg/L). NOAEL values for *X. laevis* for Guthion and Guthion 2S were 0.34 and 0.25, respectively. The LC_{50} values for *X. laevis* embryos after 4-d exposure to Guthion and Guthion 2S were found to be 2-4 times higher [114] than tadpole values. Sanders [115] and Mayer and Ellersieck [116] reported LC_{50} concentrations for *B. woodhousii fowleri* (0.109 mg/L and 0.13 mg/L, respectively). The greater toxicity of Guthion 2S compared to the technical grade

Guthion suggests that pesticide formulation increases toxicity. According to Mayer and Ellersieck [116], "inert" ingredients in water-borne pesticides may increase toxicity by as much as 2.5 orders of magnitude.

Nebeker et al. [117] determined effects of Guthion and Guthion 2S on the survival and growth of tadpoles of the treefrog *P. regilla*, and larvae of the salamanders *A. gracile*, and *A. maculatum*. Four-d LC_{50}s for *P. regilla* for Guthion and Guthion 2S were >3.6 mg/L and 1.47 mg/L, respectively. Four-d LC_{50}s for Guthion 2S for *A. gracile* and *A. maculatum* were 1.67 and 1.90 mg/L, respectively. Effects were similar to Guthion 2S in all three species with Guthion significantly more toxic, and comparable to results obtained by Schuytema et al. [113].

Chlorpyrifos

According to the United States Environmental Protection Agency (USEPA), chlorpyrifos is the most heavily used insecticide in the United States. Most of its residential use is being phased out, but agricultural use will continue [118]. USEPA classifies it as highly toxic to larval amphibians. The 24-hour LC_{50} value for *B. vulgaris formosus* tadpoles is 1 ppb [119].

In reviewing the ecotoxicology of chlorpyrifos to wildlife, Barron and Woodburn [120] noted that few laboratory or field experiments had been conducted with amphibians, and tests for chronic toxicity via dietary, water column, and sediment exposures were needed. LC_{50} values for *B. americanus* tadpoles and *R. pipiens* tadpoles were 1 μg/L and 3000 μg/L, respectively. One day and 6 d LC_{50} values for *R. tigrina*

tadpoles were 177 μg/L and 10 μg/L, respectively. Chlorpyrifos toxicity appeared to be highly species specific, and the scope of inference from the few studies available may be limited to the species tested. A field study by Moulton [121] found adverse effects including mortality in adult and larval *H. femeralis* after exposure to levels corresponding to normal field applications of chlorpyrifos. Temperature tolerance after exposure to low levels of chlorphyrifos (30-40 ppm) was reduced in *B. boreas* [122] and *P. regilla* [123].

Swann et al. [124] tested the effects of chlorpyrifos on ciliated epithelial cultures of frog palate *(R. pipiens)*. Frequency of ciliary beat decreased after exposure (24 hr EC_{50}s: Lorsban, Dursban, and pure chlorpyrifos: $2.6x10^{-8}$M, $2.1x10^{-7}$M, and $4.8X10^{-7}$M, respectively. Dilution to 10^{-5}M chlorphyrifos in either Dursban or Lorsban is the highest recommended concentration by the manufacturer). Inhibition of ciliary beating through AChE inhibition would ultimately result in cell death.

Britson and Threlkeld [125] noted positive correlations between chlorpyrifos sediment concentrations and leg malformations in *H. chrysocelis*. Calumpang et al. [126] reported 100% mortality in *B. marinus* frogs when exposed to a combination of chlorpyrifos and fenubucarb.

Gaizick et al. [127] evaluated the toxicity of chlorpyrifos to *R. pipiens* embryos and found no morphological effects or in time to hatching at normal surface water concentrations (0.1 ppb).

Malathion

Malathion is known to be highly toxic to amphibians in concentrations as small as 200 ppb [116]. Pawar et al. [128] showed mortality of *Microhyla ornata* embryos at concentrations > 10 ppm. A variety of skeletal abnormalities has also been shown in tadpoles exposed to 1-20 ppm in the embryo stage [129]. A list of peer-reviewed malathion amphibian toxicity publications can be found on the Pesticide Action Network (PAN) Pesticides Database [130].

Baker [131] conducted laboratory and field experiments on responses of the woodland salamanders, *Plethodon glutinosus* and *P. cinereus,* to malathion-treated substrates. In the laboratory 40 adult *P. cinereus* were exposed to 0, 2.24, 5.60, 8.97 kg/ha and 28 adult *P. glutinosus* were exposed to 5.60 kg/ha of malathion. In the field 10 replicate pairs of 100 m^2 study sites were divided into controls and treatment plots. The treatment plots received 10 weekly applications of malathion at 5.6 kg/ha. Sixty-seven salamanders were collected from the 20 sites and analyzed for brain ChE activity. The author also conducted behavioral experiments to determine changes in feeding, endurance, or coordination. Digestive efficiency was also studied. Brain ChE was significantly inhibited at the 5.6 kg/ha dosage for *P. glutinosus* (34%) but not for *P. cinereus. P. glutinosus* experienced reduced digestive efficiency at 5.6 kg/ha malathion, but neither species showed significant differences in feeding, endurance, or coordination following malathion exposure.

Kowsalya et al. [132] examined the myotoxic action of malathion in *R. hexadactyla.* Muscle contractions were measured on the denervated frogs after injections

of 10, 20, and 30 ppm of malathion into the gastrocnemius muscle. Contractions decreased (79%) after malathion treatments. These findings follow malathion's well-known neurotoxic action [105].

Effects of malathion on the biochemistry of *B. arenarum* embryos were evaluated by Rosenbaum et al. [133]. Enzyme activity was significantly diminished (36-, 31-, and 56-fold for acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and p-nitrophenylbutyrate, respectively) after 5 d exposure to malathion. This corroborates de Llamas et al. [134] who found inhibition of acetyl and butyryl ChE in *B. arenarum* embryos; those embryos developed in 47.3 mg/L malathion and died at 5 d. Rosenbaum et al. [133] reported that 67% of embryos died after 120 hr exposure to 44 mg/L; body curvature, tail lashing, and twisting preceded death. It was suggested that ACh and AChE play important developmental roles in undifferentiated embryos; thus, interference with this enzyme system could adversely affect morphogenesis. Cellular phospholipid content was also measured at 44 mg/L malathion, but no major changes were seen in ^{32}P labeled-phosphate incorporation. The investigators also observed a significant decrease in the 10,000 x g protein supernatant of malathion treated embryos; malathion may interfere with cellular protein synthesis mechanisms that usually control the availability of yolk platelet materials.

Malathion, dicrotophos, monocrotophos, parathion, and the metabolites malaoxon and paraoxon have been shown to be teratogenic in *X. laevis* embryos [135]. Malathion, parathion, and their metabolites, malaoxon and paraoxon were more potent teratogens than monocrotophos and dicrotophos. Specific developmental effects noted

during the first 4 days of development after malathion treatments of 1.0, 5.0, 10.0 mg/L and malaoxon treatments of 0.1, 0.5 and 1.0 mg/L included reduced size, abnormal pigmentation, abnormal gut, enlargement of atria and aorta, bent notochord, and lowered NAD+ levels [136].

Taylor et al. [137] exposed adult male Woodhouse's toads *(B. woodhousii)* to sublethal concentrations of malathion (0.001 and 0.011 mg/g) and the red leg disease bacterium *Aeromonas hydrophila*. Toads exposed to both showed 100% mortality at the high dose and 80% at the low. This was a marked increase over malathion alone (high dose=40%) or *A. hydrophila* alone (20%). Toads also showed a much higher incidence of enlarged livers (80 % -100%) when exposed to both agents versus 40% for malathion-only treatments. This suggests that malathion may increase susceptibility to *A. hydrophilia.*

Venturino et al. [138] studied the effects of exogenously applied polyamines (PAs) on malathion toxicity in the toad *B. arenarum*. Polyamines are common polycationic metabolites involved in cell division, growth, differentiation, regulation of DNA, protein synthesis, and possible AChE regulation [139]. The synergistic effects of PAs putrescine, spermidine, and spermine were tested against variable doses of malathion (0-36 mg/L) over a 144 hr period in 15-20 d-old *B. arenarum* larvae. Spermidine showed a 13-fold increase in mortality due to 12 mg/L of malathion. The authors suggested that this synergistic effect may result from stimulation of the microsomal mixed function oxidases by PAs, which in turn may accelerate production of the toxic metabolite malaoxon.

Malathion is suspected to play a role in the decline of the Wyoming toad, *B. baxteri.* Dickerson et al. [140] used caged Woodhouse's *(B. Woodhousii)* toads to evaluate the effects of malathion drift on feeding, predator avoidance, ChE activity and survival. No effects were noted, but detected levels of malathion drift observed in the study were noticeably lower than application concentrations.

Multi-chemical studies

Median tolerance limits (TL_{50}), defined as "the pesticide concentration at which half of the test animals survive during the specified time," were determined for 16 pesticides to 1 wk-old *P. triseriata* tadpoles and 18 pesticides to 4-5-wk-old *B. woodhousii fowleri* tadpoles [115]. Organophosphates included carbophenothion, Guthion, malathion, Naled®, and parathion. (Pesticide formulations were not provided in the paper). Carbophenothion was highly toxic to *P. triseriata* tadpoles (96 hr TL_{50}, 0.028 mg/L). Carbophenothion was not tested with toad tadpoles; Guthion was the most toxic chemical to *B. woodhousii fowleri* tadpoles with a 96 hr TL_{50} of 0.68 mg/L. The TL_{50} values for these species reported were not directly comparable because of the age difference in tadpoles.

Johnson [123] documented the effects of five organophosphate insecticides, temephos, fenthion, methyl parathion, chlorpyrifos, and malathion, on 3 wk old *P. regilla* tadpoles, with specific regard to thermal stress. The tadpoles were exposed to pesticide levels equivalent to common field application rates for 24 hr, and then exposed to heat; the time and heat level at which spasms occurred was recorded. All pesticide

exposed tadpoles displayed significantly lowered heat tolerance, especially when exposed to 25 and 50 ppb chlorpyrifos and 25, 50, and 100 ppb methyl parathion. Exposure to 50 ppb chlorpyrifos caused increased mortality as compared to controls. Both methyl parathion and malathion depressed tadpole activity at higher dosages. This effect was also observed previously [122] after juvenile *B. boreas* were exposed to chlorpyrifos, temephos, fenthion, methyl parathion, and methoprene.

Fulton and Chambers [141] demonstrated the toxic and teratogenic effects of phenyl saliginen cyclic phosphate (PSCP), leptophos-oxon (LPTO), tri-*o*-tolyl phosphate (TOTP) and paraoxon (PXN) in 3 species of amphibian embryos: *H. chrysoscelis*, *Gastrophryne carolinensis*, and *R. sphenecephala*. TOTP requires metabolic activation and PSCP, LPTO, and PXN are direct ChE inhibitors. PSCP caused edema in >40% of the embryos of all 3 species at concentrations as low as 0.5 ppm. Blisters and spinal deformaties occurred in 6 to 9% of all exposed embryos and >50% of surviving embryos had one or more abnormalities. Teratogenity and lethality were closely linked with PSCP (a coeffective teratogen) exposure in *H. chrysoscelis* embryos. Although LPTO did not produce abnormalities, it was toxic to *H. chrysoscelis* embryos at concentrations of 2.2 ppm. TOTP and PXM had no toxic or teratogenic effects at 10 ppm and 100 ppm, respectively. The authors suggested that use of embryos of species other than *X. laevis* and in various developmental stages may prove advantageous in the study of teratogenic effects of xenobiotics.

Lambert [142] assessed the effects of pesticide spillage caused from the bombing of a pesticide store near Hargeisa, Somalia in 1988 on reptile and amphibian species. In

addition to 4 chlorinated hydrocarbons, the primary organophosphorus insecticides spilled were fenitrothion and malathion. None of the frogs, *Tomopterna cryptotis*, put into contact with water-saturated contaminated soil survived longer than 40-45 min. Frogs were found in relative abundance in dry river-bed wells below the spillage suggesting that residues had not entered the ground water.

Ouellet et al. [143] recorded a high incidence of hindlimb deformities in wild-caught frogs, *R. clamitans*, *R. pipiens*, *B. americanus,* and *R. catesbeiana,* from 14 agricultural habitats receiving pesticide runoff in the St. Lawrence River Valley of Quebec, Canada. A variety of herbicides, fungicides, and insecticides including azinphos-methyl, oxamyl, and phorate were used on adjacent agricultural crops. Although significant differences were not found between control and pesticide-exposed habitats, 106 metamorphosing anurans out of 853 (12%, range 0 to 69%) showed severe degrees of ectromelia (absence of all or part of a limb) and ectrodactyly (absence of digit) compared to 2 out of 271 (0.7%, range 0 to 7.7%) in 12 control sites. Physical deformities, liver and kidney degeneration, or general systemic illness were found only in juveniles [144,29,30] The authors proposed that a larger number of amphibians and sites need to be evaluated for possible teratogenic effects caused by agricultural pesticides and suggested monitoring the frequency of amphibian morphological deformities as a way to evaluate agricultural impacts.

A companion study by Lowcock et al. [145] compared the incidence of abnormal DNA profile, half-peak coefficient of variation, (CV), and variation in genome size (pg DNA per haploid nucleus) through the use of flow cytometry (FCM) in green frogs from

the same agricultural areas and reference sites utilized by Ouellet et al. [143]. FCM has become a useful tool for measuring genetic damage in animals exposed to environmental mutagens [146]. Frogs from corn fields showed higher levels of abnormal DNA profiles compared to the reference sites. Elevated CV of DNA profiles were found in individuals exposed to pesticides. CV levels increased with age class in both exposed and reference populations. Visible pesticide effects in juveniles were correlated with clastogenic effects. Lowcock et al. [145] reported on 21 newly metamorphosed blue-spotted salamanders *(Ambystoma laterale)* from a ditch next to a cornfield in Cumberland Co., Nova Scotia. Hidden genotoxic effects were discovered in otherwise apparently healthy animals, leading the researchers to surmise that FCM is a useful tool for screening vulnerable populations exposed to certain environmental contaminants. This group also had a high incidence (52.3%) of hind-limb deformity [145].

The effects of six pesticides, Dithane® DG (76-80% mancozeb), a ethylenebisdithiocarbamate (EBDC) fungicide, Nova® 40W (38-42% myclobutanil), a triazole fungicide, Thiodan® 50WP (endosulfan), a chlorinated hydrocarbon insecticide, and the organophosphates Guthion® 50WP (50% zainphos-methyl), Imidian® 50WP (50% phosmet), Basudin® 500EC (diazinon), as well as the technical grade diazinon, were evaluated in *R. pipiens* and *R. clamitans* tadpoles by Harris et al. [93]. Study site pond water from 4 wetland areas within orchards in southern Ontario, Canada, and 3 nearby reference sites was used to expose embryos and larvae *in situ* and in laboratory tests for a 2 to 3 wk period coinciding with frog breeding episodes from May to July. Pesticide toxicities to *R. clamitans* were also evaluated using continuous and

discontinuous laboratory toxicity tests. Diazinon, a commonly used insecticide, was most toxic to *R. clamitans*: LC_{50} 2.8 to 5 μg/l, commercial formulation, and EC_{50} 6 to 14 μg/l, technical grade. Diazinon is known to be toxic to fish, [80,147] and its oxon is a potent enzyme inhibitor. All pesticides studied, with the exception of technical grade diazinon, are frequently applied in apple orchards. Decreased survival rates were reported for embryos and tadpoles caged in some orchard ponds, but findings were not consistent. Reduced tadpole growth was found at both reference and study sites, with the only significant factor appearing to be surface water temperature. Mortality, deformities, or delayed growth was observed at <0.01 ml/L for Basudin 500EC, technical grade diazinon, and Dithane DG in contrast to Imidan 50WP, Guthion 50WP, and Nova 40W which produced effects at higher concentrations of 5 to 10 mg/L. Although reported tests implied that ponds within apple orchards do not prevent successful development in the two species, changes in survival, deformity, and growth rates of exposed tadpoles suggested that pesticide exposure in pond water may add another environmental stressor affecting development. In addition, only short term effects were studied; cumulative frog response to mixtures of chemicals was not evaluated. Combined *in situ* and laboratory studies such as this one offer an environmentally realistic exposure for wild eggs and larvae and could be used to advantage in future studies.

Anurans studied by Bishop et al. [148] showed a negative correlation in density, development, and diversity with regards to their exposures to OPs and OCs in an agricultural watershed. Chlorpyrifos, malathion and diazinon were present in the array

of pesticides and fertilizers revealed in water analyses. Hatching success of *B. americanus* was reduced with exposure and deformity rates also increased. Deformity rates were also increased in *R. clamitans*. The findings implicate high levels of agricultural run-off with declining reproductive success in amphibians.

Harris et al. [149] exposed two developmental stages of northern leopard frogs *(R. pipiens)* and American toads *(B. americanus)* to endosulfan, an organochlorine insecticide, azinphos-methyl, an organophosphate insecticide, and mancozeb, a fungicide. Leopard frog embryos were approximately an order of magnitude more sensitive to mancozeb (96-hr LC_{50}, 0.20±0.02 mg/L) than were the toads (1.4±0.3 mg/L). Metamorphosing leopard frogs were more sensitive to endosulfan than the embryo stage. All animals exposed to 2.35 mg/L endosulfan died with 48-hrs. Dose dependant mortality did not occur with either species in regards to azinphos-methyl. Deformities were noted with all chemicals. Mancozeb exposure resulted in a skewed sex ratio (100% female). The authors conclude that survival rates post pesticide exposure can vary significantly among stages and species and that skewed sex ratios could have a negative impact on wild populations.

Relyea [150] evaluated species richness in aquatic communities containing algae and 25 species of animals post exposure (two weeks) to carbaryl (Sevin™) malathion, glyphosate (Roundup™), and 2,4-D. Species richness was reduced 30% by malathion, 22% by glyphosate, 15% by carbaryl, and not at all by 2,4-D. The insecticides reduced zooplankton and predatory insect diversity. The reduction of predatory insects correlated with an increase in tadpole survival by approximately 30%. Glyphosate eliminated two

species of tadpoles (most morality was noted in the first 24 hrs) and caused an overall 70% reduction in tadpole species richness. This study emphasizes the need to understand whether pesticide drift has detrimental effects on natural systems and contributes to declining global biodiversity.

Summary

Most organophosphate pesticide studies reviewed herein have focused on lethal toxicities and developmental effects to embryos and larvae. Effects frequently discussed include teratogenic effects and other deformities. Paralysis (partial or complete), often preceded by hyperactivity (irritability), was commonly reported and usually associated with ChE inhibition, but not exclusively. Authors frequently concluded that sublethal effects such as paralysis, reduced activity levels, and decreased swimming speed would have detrimental effects on the ability of amphibian larvae to escape predation or to forage. Reduced hatching success, decreased size at metamorphosis, physiological stress, liver and kidney degeneration, were also cited as effects of pesticide exposure. Many effects seem to be species or life stage specific [151,74] and thus these studies illustrate the difficulties in predicting pesticide toxicities to amphibians. The complex life cycle of amphibians contributes to the puzzle. Embryos, originally hypothesized to be the most susceptible life stage, have proven to be more resistant in many cases, probably due in part to their protective coating. Young tadpoles are often highly susceptible to poisoning, but actual effects vary with species, age, and environmental conditions [152]. Furthermore, selection of a frog species as a bioindicator for

environmental contamination appears to be difficult because the evidence does not support the contention that one particular species is most susceptible (or resistant) to pesticides. The reasonable conclusion to be drawn from the limited amphibian toxicology data available is that many "new generation" pesticides are highly toxic to amphibians and even low levels may cause sublethal effects that result indirectly in mortality. Extreme caution should be used in the application of these compounds to crop fields to prevent contaminated run-off or spray drift from entering nearby aquatic habitats. It appears that certain pesticides, under particular environmental conditions, may harm or extirpate individuals and even populations of amphibians. Because toxic effects may depend on many disparate factors such as temperature, organic carbon content, pesticide concentration, age of amphibians upon exposure, and other ecological factors, it is unlikely that one particular pesticide or even a class of compounds is acting in a uniformly deleterious manner across habitats and species. Rather, pesticides may work with other stressors to exert an effect. The cumulative adverse effects of multiple stressors, including pesticides, may act on susceptible amphibian species in localized environments and thereby contribute to the pattern of global amphibian decline.

CHAPTER III

PESTICIDE RESIDUES IN TRANSLOCATED TADPOLES AND METAMORPHS IN LASSEN, YOSEMITE, AND SEQUOIA NATIONAL PARKS, CALIFORNIA

SYNOPSIS

Pacific chorus frog *(Pseudacris regilla)* hatchlings were translocated (with controls in each park) and placed in cages in sites (~ 2,200 m elevation) located in Lassen, Yosemite, and Sequoia National Parks. Pesticide residues were measured in pooled samples of tadpoles from the same cages collected at 28 days and in juvenile frogs collected upon metamorphosis. *p,p'*-dichlorodiphenyldichloroethylene (DDE) was found in 97% of Yosemite samples, 84% in Sequoia samples, and 15% of Lassen samples. A chi-square test of these proportions shows Lassen (the reference site) to be significantly different ($p<0.0001$) from the other two sites in percent detections. ANOVA shows overall differences among mean DDE among parks and between age classes (<0.0001). Sheffe's post hoc test showed significant differences ($p=0.04$) between age classes (metamorphs and tadpoles) and among parks (p=<0.0001). DDE means were: Lassen metamorphs (0.183 ± 0.1 ppb); Yosemite metamorphs (2.18 ±0.24 ppb); Sequoia metamorphs (5.7 ± 0.93 ppb); Lassen tadpoles (0.066 ± 0.05 ppb); Sequoia tadpoles (2.07 ± 0.38 ppb); and Yosemite tadpoles (1.52± 0.25 ppb). There

were no differences in mean DDE levels between Sequoia and Yosemite tadpoles; nor between Yosemite metamorphs and tadpoles. Lassen DDE means for both metamorphs and tadpoles was consistently lower than the other two parks. Total endosulfans were detected in 3% of Sequoia samples, 9% of Lassen samples and 24% of Yosemite samples. Both pesticides were detected in tadpoles and metamorphs raised at the three parks regardless of origin. Because the tadpoles were translocated post hatching, this finding indicates that the pesticides, particularly DDE, were accumulated at the site, instead of through deposition in the egg mass.

INTRODUCTION

Drost and Fellers [45] and Jennings [46] documented a system wide decline of amphibians in the Sierra Nevada Mountains in California. In fact, half of the Sierra Nevada Mountain's 29 amphibian species are declining [46]. Populations of 8 of 20 salamander species, 7 of 9 frog species, and 12 of the 14 endemic species are at risk [46]. This decline correlates with historical pesticide use in the Central California Valley [69] and with the atmospheric transport of pesticides to the Sierra Nevada Mountains [59,61,62]. Cory et al. [153] documented the distribution of *p,p'*-DDE, a common metabolite of *p,p'*-DDT and frequently stored in animal tissues [154] in the mountain yellow-legged frog *(R. muscosa),* a species in marked decline and with its southern-most population currently listed as endangered [56]. Sparling et al. [64] reported DDE, endosulfan, and organophosphorus pesticide residues in *P. regilla* in these same areas, and Fellers et al. [67] also found DDE, γ-chlordane, and *trans*-nonachlor residues in *R.*

muscosa. Because amphibians are in peril worldwide, research documenting pesticide levels and potential impacts is critical. This field experiment study reports pesticide levels in *P. regilla* post translocation to potentially impacted sites.

STUDY AREAS

Lassen Volcanic National Park was chosen as a reference site because it lies north of agricultural impacts from the Central Valley. It is positioned at the southern terminus of the Cascade Mountains and at the northern tip of the Sierra Nevada Mountains. Yosemite and Sequoia National Parks were chosen as potential impacted sites because they are both downwind from areas of intense pesticide use in the Central Valley. Yosemite is located in the central Sierra Nevada Mountains and Sequoia in the southern Sierra Nevada Mountains (Figure 3.1). These three parks contain some of the largest remaining areas of late successional conifer forests at sub alpine and mid-elevations [155]. Three meadow pond sites per park were used to deploy cages of translocated *P. regilla* hatchlings (Table 3.1; Figure 3.1).

Pond meadow sites were chosen with the following characteristics: previous use by *P. regilla*; elevation (~2,200 m); lack of fish; ability to accommodate cages; and accessibility. These wetland meadow pond sites are in upper montane forests, characterized by stands of red fir (*Abies magnifica*), lodge pole pine (*Pinus contorta*), and Jeffrey pine (*Pinus jeffreyi*). There is little rainfall in the summer months in the

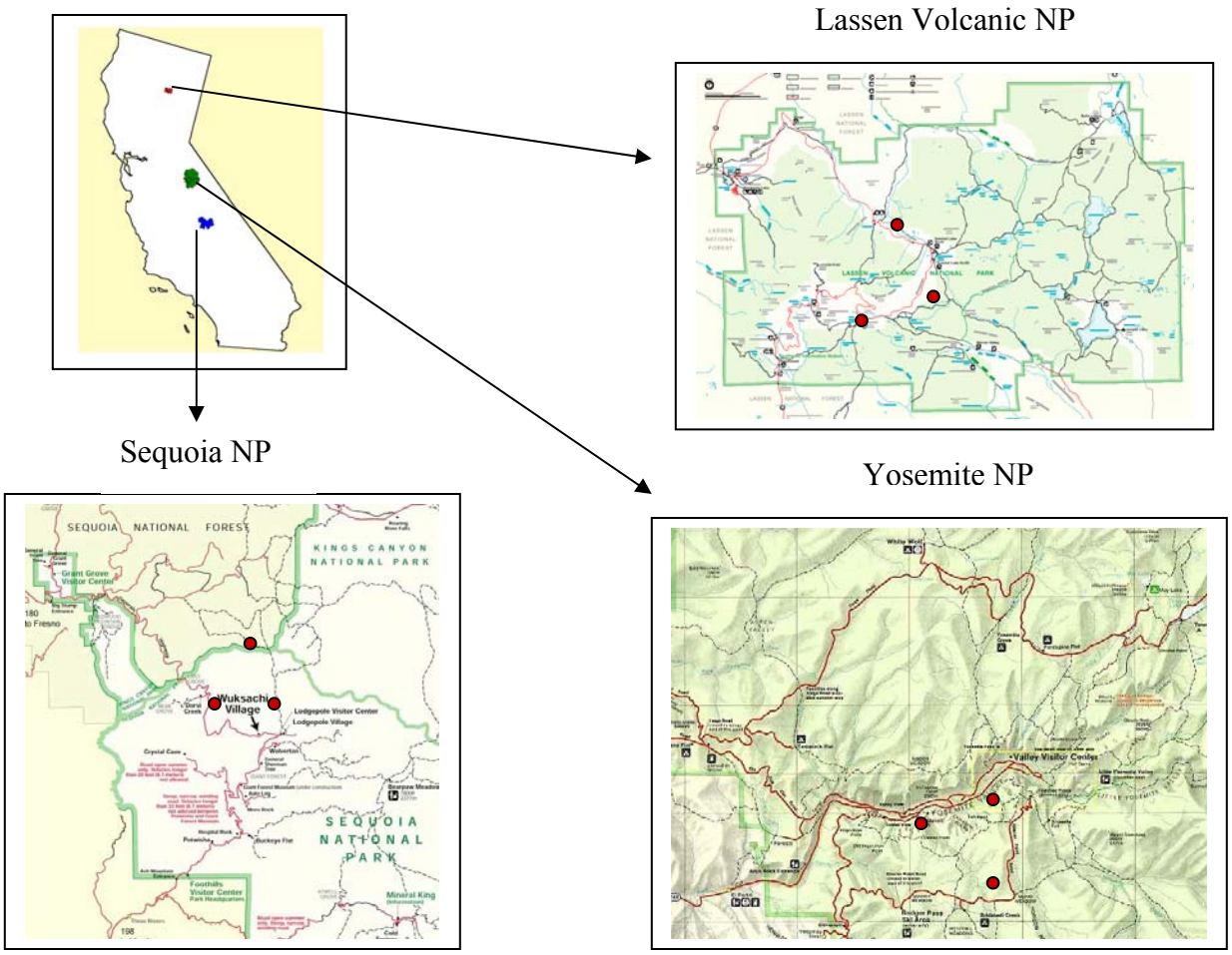

Figure 3.1 Map of California showing Lassen, Yosemite, and Sequoia National Parks. Park maps show locations of meadow ponds used to deploy translocated tadpoles. Map of California was made by Carlos Hinojosa and Amy Hays of the Land Information Systems, Texas A&M University. Parks maps are from the National Park Service, public domain.

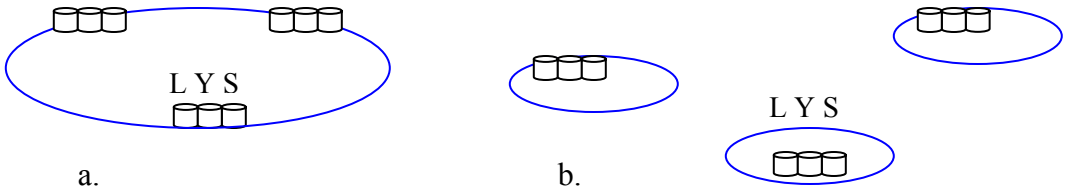

Figure 3.2. Example of cage placements. a. Three sets of 3 cages in one large pond, i.e. Long Meadow, Sequoia. b. One set of three cages in 3 small ponds in wet meadow, i.e. NE Summit Meadow, Yosemite National Parks. (L=Lassen; Y= Yosemite; S = Sequoia).

Sierras, and so most of these pond sites are fed by snow melt [156]. In Yosemite, the Tuolumne watershed in the north and the Merced watershed in the south serve most of the park and feed into the San Joaquin River basin. Sequoia has three major rivers: Kings, Kaweah and Kern. These watersheds also help to distribute winter precipitation held in the form of snow pack throughout the summer. Lassen has portions of four drainage basins including Mill, Hat, and Kings Creeks that eventually drain into the Sacramento River.

Table 3.1 Meadow pond sites in Lassen, Yosemite, and Sequoia National Parks.

Lassen	Yosemite	Sequoia
Hemlock	NE Summit Meadow	Circle Meadow
Dersch Meadow	Pothole Meadow	Huckleberry Meadow
Upper Kings Meadow	Mono Meadow	Long Meadow

METHODS

Experimental methods

The experiment was conducted June-August 2002. *P. regilla* was selected as a surrogate study species, because of its relative abundance, its wide distribution, and its ability to metamorphose in one summer compared to ~3 years for *R. muscosa.* Hatchling tadpoles were translocated from a single pond per park and care was taken to collect < 10% of total hatchlings present. Lassen hatchlings were collected from Dersch Meadow, Yosemite tadpoles from Pothole Meadow, and Sequoia tadpoles from Long Meadow. Hatchlings were placed in separate cages for each park origin and grouped in sets of threes (Lassen, Yosemite, and Sequoia) for a total of nine cages per meadow site (Figure 3.2). Cages were either placed in 3 sets of 3 cages in one large pond as in Long Meadow, Sequoia National Park, or were placed in clusters of three in small ponds in a contiguous palustrine wet meadow as in NE Summit Meadow, Yosemite National Park.

Each cage was a cylinder with bottom and removable lid constructed of rigid 75 µm white Nitex ®teflon (Sefar America Inc., Kansas City, MO) (60 cm depth x 35 cm diameter) (Figure 3.3). This field-tested design by Harris and Bogart [157] was enlarged to provide an internal cage volume for 60 tadpoles that would not cause overcrowding or possible developmental problems [158]. The cylinder was stitched together with nylon thread and plastic rings sewn to the cage sides accommodated a plastic covered metal rod that was driven into the pond bottom [157]. When necessary, cages were moved away from shore and towards the middle of the pond as ponds dried to keep them in water. The top of the cage (40cm) was attached with industrial strength

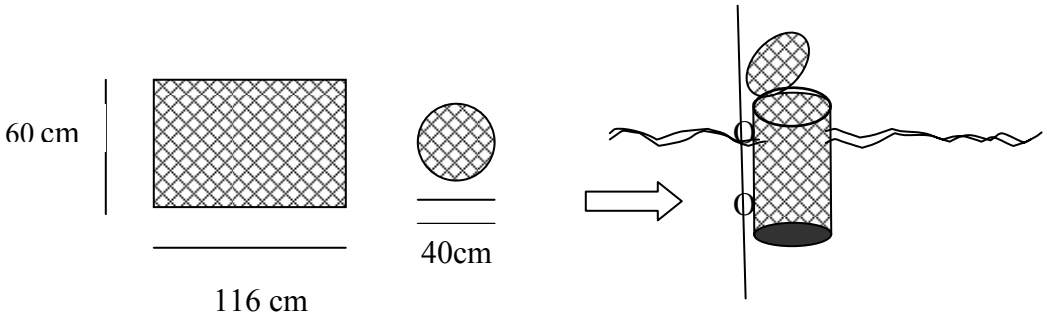

Figure 3.3 Dimensions and design of tadpole cages.

Velcro® for ease of removal. A floating platform (plastic grid side panel of a Jehmco® fry cage) was placed in each cage for metamorphosed frogs when tadpoles reached Gosner stage 42 (appearance of front limbs).

A crew of 4 technicians and the principal investigator monitored the cages daily. Boiled romaine lettuce was supplied as food. Approximately 90% of the lettuce was organic or certified pesticide-free, the other 10% was carefully washed and rinsed before boiling. Food availability was maximized by providing a continuous supply of boiled lettuce. Measurements of water pH, nitrate/nitrites, dissolved O2, ammonia, hardness, and turbidity were all within normal limits. Tadpoles were sampled at 28 days of exposure and again at metamorphosis, stage 42-46 [102].

Tissue residue methods

Tadpole and metamorph residue samples were collected from the field experiment and shipped on dry ice to Patuxent Wildlife Research Center, Laurel

Maryland, USA, where they were stored at -80° C, and then moved to -20° C storage until processing at the U.S. Department of Agriculture-Agriculture Research Station, Beltsville, Maryland, USA. Tadpole and metamorph composite samples were pooled from the same experimental cage to constitute one composite residue sample of approximately 10 g. A total of 108 composite samples (69 tadpoles; 39 metamorphs) were analyzed for pesticide residues from 2002. Samples were pulverized while immersed in liquid nitrogen with a SPEX CertiPrep 6750 freezer Mill. We used the QuEChERS dispersive solid phase extraction method [159] for sample extraction and clean up. This dispersive solid phase extraction method removed water and nontarget compounds from the samples with magnesium sulfate and sodium acetate in acetonitrile containing 1% acetic acid.

Analytes included in the study represent those most commonly found in atmospheric transport of pesticides to the Sierra Nevada Mountains [61,67] and are listed here followed by the minimum detection limit (MDL) ng/g wet wt : trifluralin (0.087); α-hexachlorocyclohexane (HCH) (0.107); diazinon (0.463); γ-HCH (0.099); heptachlor (0.143); chlorothalonil (0.00); aldrin (0.108); chlorpyrifos (0.119); fipronil (0.4); malathion (0.912); chlorpyrifos oxon (0.93); γ-chlordane (0.082); trans-nonachlor (0.112); a-chlordane (0.104); a-endosulfan (0.086); p,p'-DDE (0.114); dieldrin (0.081); cis-nonachlor (0.114); p,p'-DDD (0.114); b-endosulfan (0.099); p,p'-DDT (0.114); endosulfan sulfate (0.129); mirex (0.142); brominated diphenyl ether (BDE) 47 (0.155); BDE 99 (0.127); BDE 100 (0.142); BDE 153 (0.147); BDE 154 (0.116). Detection

limits were determined by extracting 10 g sample of shrimp spiked with a mixture of the target analytes.

Samples were analyzed in pulsed splitless mode using an Agilent 6890 gas chromatograph coupled to a 5973 inert mass spectrometer in the negative chemical ionization (NCI) selected ion-monitoring mode. Methane was the ionization gas at a pressure of 40 Pa. The persistant organic pollutants (POPs) investigated included several currently used insecticides and fungicides, as well as other banned organochlorine pesticides, five polybrominated diphenyl ethers (PBDEs). The source and quadrapole temperatures were 150° C. Gas chromatography parameters for monitored compounds were as follows: 30-m DB-17 MS capillary column (J&W Scientific Corporation, Folsom, CA), 0.25-mm inner diameter, 0.25-μm film thickness, helium carrier gas, constant flow at 1.0 ml/min; temperature program: injector port temperature 230° C, initial temperature 130° C, hold 1.0 min, 6°/min to 205° C, hold 4.5 min, 6°/min to 300, hold 6.17 min. The injection port pressure was 11.85 psi with a pulse pressure of 50.0 psi. The detector interface on each method was 300° C.

Sediment residue methods

All sediment samples were extracted and analyzed at the USDA-ARS laboratory in Beltsville, MD. The samples included in this experiment were processed along with approximately 200 sediment samples included in the larger transect experiment associated with this project.

Analytes are listed here followed by the minimum detection limit (MDL) ng/g wet wt: trifluralin (0.668); α-hexachlorocyclohexane (HCH) (0.624); diazinon (4.653); γ-HCH (0.494); heptachlor (1.064); chlorothalonil (0.16); aldrin (0.734); chlorpyrifos (0.65); fipronil (0.494); malathion (3.584); γ-chlordane (0.732); oxychlordane (0.784); Heptachlor epoxide (0.316); trans-nonachlor (0.708); a-chlordane (1.034); a-endosulfan (0.632); 4,4'-DDE (0.1.132); dieldrin (1.778); cis-nonachlor (1.312); b-endosulfan (0.308); 4,4'-DDT (0.174); endosulfan sulfate (0.56); mirex (1.348); brominated diphenyl ether (BDE) 41 (0.678); BDE 99 (0.814); BDE 100 (0.704); BDE 153 (1.76); BDE 154 (0.468). Detection limits were determined by extracting 5 g sample of sand spiked with a mixture of the target analytes.

Sediment sample collection

A Wildco model 2424 sediment corer (Wildlife Supply Company, Buffalo, NY) was used to extract 3-5 sediment cores from different areas within each sampling site. The top 2.5 cm of each sediment core was mixed in a stainless steel bowl before placing in pre-cleaned, wide-mouth amber glass jars. A chain of custody label was affixed to each jar, noting the sample ID and collection date, and stored on dry ice in the field until transport back to the lab in Pt. Reyes. All samples were wrapped in newspaper before being packed on dry ice and shipped overnight to USDA.

Sampling equipment was cleaned between sites by removing residual sediment with plastic scrub brushes and available water before immersion in a 10% bleach

solution to prevent the spread of infectious diseases. The equipment was then washed with a mild soap and tap water followed by an acetone and distilled water rinse.

Sample preparation

Frozen sediment samples were placed in the refrigerator at 4°C two days prior to sample preparation to insure complete thawing prior to processing. Each sample was mixed thoroughly with a metal spatula to make the sample as homogeneous as possible, and two 15g aliquots were removed for dry weight determination, and pesticide concentration analysis. The dry weight was determined by weighing the aliquot before and after baking at 100 ^{o}C for 24 hours.

Aliquots for pesticide determination were dried prior to extraction by using 5-10g of anhydrous magnesium sulfate ($MgSO_4$) (Fisher Scientific, Springfield, NJ) and ground into a fine powder. The dried sample was transferred to a 20-ml stainless steel extraction cell containing a cellulose filter (Dionex, Sunnyvale, CA) at the bottom of the cell. Just prior to extraction, each sample was spiked with 25 µL of diethyl-d10 as an extraction surrogate. Any remaining space in the extraction cell was filled with clean, granular sodium sulfate (Na_2SO_4). Samples were extracted in batches of 10-20 samples including one laboratory blank and one laboratory spike control using baked laboratory sand.

Method detection limits (MDLs) were determined for each analyte as described in USEPA SW-846 Test Methods for Evaluating Solid Waste [160]. MDL values were determined from analysis of at least 7 extracts from laboratory sand spiked at the lowest

point on the calibration curve for each analyte. Each compound MDL was calculated based on the standard deviation of the average mass determined multiplied by the appropriate student t-value. The limit of quantitation was defined as three times the MDL value.

Sample extraction

Samples were extracted using a Dionex Accelerated Solvent Extractor (ASE) 200 using compressed nitrogen for maintaining the pressure set point of 2000 psi, sealing the cell after loading into the oven and for purging any remaining solvent from the cell at the end of the extraction process. The ASE parameters were as follows: oven temperature, 125 EC; heat, 6 minutes; static, 5 minutes; flush, 90%; purge, 180 seconds; solvent 1: ethyl acetate 20%; solvent 2: dichloromethane (DCM) 80%; cycles, 3. After the sequence was completed, the vials containing the extract are quantitatively transferred to labeled, clean 125-mL round bottom flasks and reduced to approximately 5 mL by rotary evaporation. The extract was exchanged in hexane by adding fifteen milliliters of hexane to the flask and reduced again to approximately 5 mL.

Sample clean up

An alumina column clean up procedure was used to prepare sample extracts for analysis. Low-pressure liquid chromatography columns (11 x 250 mm, 200 mL reservoir capacity, 2 mm PFTE stopcock, Fisher Scientific, Springfield, NJ) were plugged with a small amount of glass wool, pre-cleaned with hexane through soxhlet

extraction. Alumina (80-200 mesh, Fisher Scientific, Springfield, NJ) was baked in a porcelain dish at 550°C for at least four hours. The alumina was allowed to cool to room temperature in a dessicator before measuring 4 grams into 20 mL amber vials. To each vial, 240 µL (6% by mass) of organic free water was added and vigorously shaken for approximately 10 minutes. A 4-gram aliquot of activated alumina was added to each column followed by a 1-cm thick layer of Na2SO4.

The column was pre-eluted with 25-mL of 3:1 DCM: Acetone and allowed to drip into a labeled 125-mL round bottom flask. The sample was transferred to the column using a Pasteur pipette before adding another 25 mL of the solvent mixture. After the second aliquot of 3:1 DCM Acetone had been eluted, another 15 milliliters was added. One gram of copper turnings was added to each flask prior to rotary evaporation to remove any elemental sulfur that may be present. Each sample was reduced to 2-5 mL before adding 15 mL of hexane as the exchange solvent. The extract was reduced again and transferred to calibrated 15 mL centrifuge tubes. The extract was reduced to a final volume of 1 mL using a gentle stream of nitrogen gas on the NEVAP. [161,162].

Sample analysis

Samples were analyzed Hewlett Packard 5890 gas chromatograph coupled to a 5989 mass spectrometer in the negative chemical ionization (NCI) selected ion-monitoring mode. Methane was the ionization gas at a pressure of 200 Pa. The source and quadrapole temperatures were 150^{o}C and 100^{o}C, respectively. Gas chromatography parameters for monitored compounds are as follows: 30-m DB-17 MS capillary column

(J&W Scientific Corporation, Folsom, CA), 0.25-mm inner diameter, 0.25-µm film thickness, helium carrier gas, constant flow at 1.42 ml/min; temperature program (organochlorines, organophosphates and PBDEs): injector port temperature 290^{o}C, initial temperature 130^{o}C, hold 1.0 min, 6^{o}/min to 205^{o}C, hold 4.5 min, 6^{o}/min to 300, hold 5.50 min.

PCB 204 was used as the internal standard, and a five-point calibration curve was established for each compound and instrument response was linear over the calibration standards range ($r^2 \geq 0.99$). Calibration standards, on average, ranged from 0.103 ng/µL to 0.005 ng/µL. The instrument was recalibrated every 20 to 25 sample injections. Quantification of each compound was calculated based on the area of the ion with the largest abundance. Confirmation of a particular compound in a sample was determined by the presence of at least one of the two qualifying ions in the proper ratio to the quantifying ion (± 20%). The requirement for only one qualifying ion in the proper ratio is due to the use of the NCI mode where the number of ions in the mass spectra is often dominated by one or two ions, with very small contributions from other ions.

Statistical analyses

Ratios were analyzed with a chi-square test; differences among measurable DDE levels were analyzed with analysis of variance tests (ANOVA) using StatView®, SAS Institute Inc., version 5.01. Means are presented ± 1 SE and $\alpha = 0.05$ for evaluating statistical significance. For statistical comparisons, contaminants that were detected but not quantified were calculated at one-half the quantification limit of that sample and

those not detected (ND) were treated as zeros. Samples for the ANOVA were analyzed by comparing samples that came from each park of deployment (i.e. the park where the tadpoles were raised in captivity). Lassen samples, Yosemite samples, and Sequoia samples refer to animals that were raised in each park, regardless of origin, unless otherwise stated. Animals from each meadow were combined in the analyses for each park.

RESULTS

DDE and endosulfans in tissues

Measurable levels of DDE were found in 90% of Yosemite samples, 74% of Sequoia samples, and 15% of Lassen samples. DDE detections only were in 7% of Yosemite samples, 9.6% of Sequoia samples, and 0% of Lassen samples. Total percent of samples containing either measurable or detectable levels of DDE were: 97% Yosemite; 84% Sequoia; and 15% Lassen. An overall 3x2 chi-square analysis showed a significant difference ($p<0.0001$) and three separate 2x2 chi-square analyses of the ratios of total detectable and measurable levels of DDE from the three parks revealed a significant ($p<0.0001$) difference between Lassen, the reference park, and the two other parks. Significance was calculated at $\alpha=0.0167$ according to the Bonferroni/Dunn post hoc test.

A two-way ANOVA showed overall differences in mean DDE among parks and between age classes (<0.0001) (Table 3.2, 3.3). The interaction between age and parks was also significant ($p=0.0001$) revealing that the differences between ages was

dependent upon the park where they were raised (Figure 3.4). It shows that DDE increases from tadpoles to metamorphs in all cases, but especially so in Sequoia. Sheffe's post hoc test showed significant differences (p=0.04) between age classes (metamorphs and tadpoles) and between Lassen and the other two parks (p=<0.0001) Scheffe showed a difference between Sequoia and Yosemite (p=0.014). There were no differences in mean DDE levels between Sequoia and Yosemite tadpoles; nor between Yosemite metamorphs and tadpoles. Lassen DDE means for both metamorphs and tadpoles was consistently lower than the other two parks. (Tables 3.2, 3.3, Figure 3.4). Table 3.4 gives measurable DDE concentrations in tadpoles and metamorphs from all three parks; values range from ND to 7.2 ng/g wet wt.

Table 3.2 ANOVA table for mean DDE.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Age	1	40.073	40.073	22.756	<0.0001	22.756	0.999
Park	2	163.764	81.882	46.497	<0.0001	92.994	1.000
Age*Park	2	34.924	17.462	9.916	0.0001	19.832	0.989
Residual	99	174.340	1.761				

Table 3.3 Lassen, Yosemite, and Sequoia National Parks mean DDE levels.

Park Deployed	Age Class	Mean (ppb)	n
Lassen	Metas	0.18±0.1	15
	Tadpoles	0.07±0.05	18
Yosemite	Metas	2.18±0.24	16
	Tadpoles	1.54±0.24	25
Sequoia	Metas	5.70±0.93	7
	Tadpoles	2.17±0.38	24

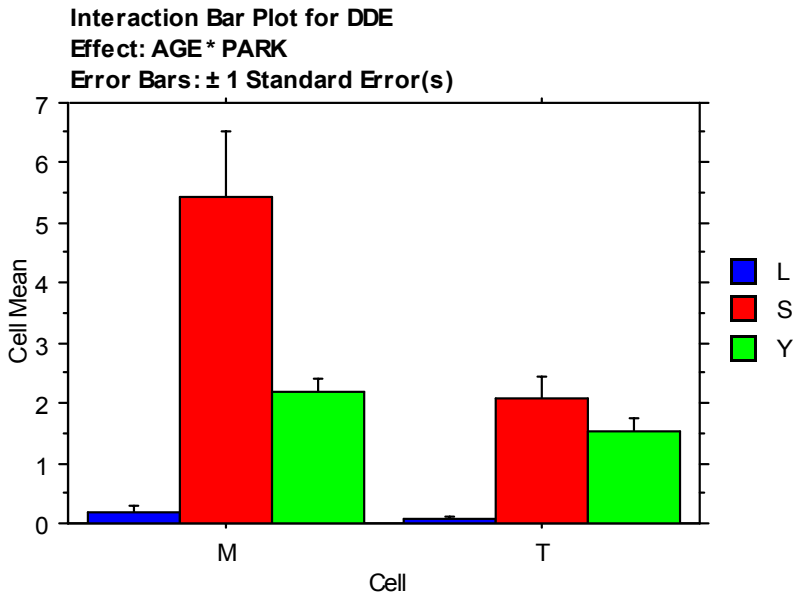

Figure 3.4. Interaction Bar Plot showing mean DDE. Values are for Lassen, Sequoia, and Yosemite National Parks for both metamorphs and tadpoles.

Total endosulfans were detected in 3% of Sequoia samples, 9% of Lassen samples and 24% of Yosemite samples. A 3x2 chi-square analysis revealed an overall significant difference (p=0.02) among parks; three 2x2 tests showed a difference between Yosemite and Sequoia % detections (p=0.0134); other comparisons were not significant. The level of signifance was reduced to $\alpha = 0.0167$ per the Bonferroni/Dunn correction.

All other chemicals

There were no detections of organophosphorous insecticides. Lassen had the following other detections: 1 sample with chlorothalonil (fungicide); 1 sample with fipronil (insecticide); 2 samples with chlordane (insecticide); 1 sample with BDE (fire-retardant). Yosemite had 3 samples with BDE detections; and Sequoia had 1 sample with dieldrin (insecticide) and 1 with BDE. Because these detections were only in a small number of samples, no statistics were calculated.

Table 3.4. Concentration of *p,p'*-DDE (ng/g wet wt) in *P. regilla* tissue collected in Lassen, Yosemite, and Sequoia National Parks, California, USA, from 2002. *Detection only; one half quantification limit entered. Samples with no detection are not shown.

Pooled Samples	Sample Size	Age class	*p,p'*-DDE ng/g wet wt
Lassen Volcanic National Park			
32L-R	n=33	T	0.61
48L-R		T	0.58
47L-RM		M	0.88

Table 3.4. Continued.

Pooled Samples	Sample Size	Age class	*p,p'*-DDE ng/g wet wt
36L-RM		M	0.77
34L-RM		M	1.10
Yosemite National Park			
43Y-R	n=41	T	2.16
37Y-R		T	5.75
30Y-R		T	1.60
53Y-R		T	1.11
50Y-R		T	0.17*
47Y-R		T	0.17*
51Y-R		T	0.78
38Y-R		T	2.07
49Y-R		T	1.67
55Y-R		T	3.04
35Y-R		T	0.64
36Y-R		T	0.99
39Y-R		T	2.90
34Y-R		T	0.67
45Y-R		T	1.22
44Y-R		T	2.54
32Y-R		T	1.97
46Y-R		T	1.02
54Y-R		T	2.07
31Y-R		T	1.36
48Y-R		T	0.17*
52Y-R		T	0.89
42Y-R		T	2.21
41Y-R		T	0.83
50Y-RM		M	2.05
32Y-RM		M	1.10
47Y-RM		M	2.78
48Y-RM		M	1.19
51Y-RM		M	2.07
41Y-RM		M	2.83
42Y-RM		M	1.60
43Y-RM		M	0.87
38Y-RM		M	3.44
32Y-RM		M	1.04
38Y-RM		M	2.93

Table 3.4. Continued.

Pooled Samples	Sample Size	Age class	*p,p'*-DDE ng/g wet wt
39Y-RM		M	1.46
31Y-RM		M	2.11
44Y-RM		M	1.96
39Y-RM		M	3.24
34Y-RM		M	4.19
Sequoia National Park			
39S-R	n=31	T	4.86
46S-R		T	1.26
47S-R		T	2.52
52S-R		T	5.25
31S-R		T	1.42
53S-R		T	2.63
36S-RM		M	1.07
50S-R		T	2.68
55S-R		T	0.17*
38S-RM		M	3.37
32S-RM		M	7.20
52S-R		T	5.25
31S-R		T	1.42
53S-R		T	2.63
32S-RM		M	7.20
32S-R		T	2.13
56S-R		T	6.61
49S-R		T	2.81
34S-RM		M	7.19
51S-RM		M	6.85
54S-R		T	0.92
54S-RM		M	7.02
51S-R		T	0.17*
43S-R		T	1.38
40S-R		T	4.13
37S-R		T	1.37
35S-R		T	2.09

Sediment samples

In 2002, one sediment sample was collected from each of the nine experimental meadows, and of these, three were from each park. Lassen National Park samples were from Dersch, Kings, and Hemlock sites and the only detection was that of endosulfan in the Hemlock sample. Yosemite samples were from Pothole, NE Summit, and Mono meadows and only the NE Summit meadow sample detected endosulfan. Sequoia samples were from Long, Circle, and Huckleberry meadows, with detections of endosulfan found in the Long and Huckleberry samples. Because these detections were only in a small number of samples, no statistics were calculated. However, the detection of endosulfan in the sediments is consistent with its detection in the tissues.

DISCUSSION

The mechanisms by which pesticides enter the air through volatilization or application drift and their subsequent atmospheric transport as small particles, aerosols, or vapors from California's Central Valley to the western slopes of the Sierra Nevada Mountains, have been reported in detail [59,61,163,62]. Because agricultural pesticides are heavily applied at all times of the year in this region, they are available for year round transport [61,62]. Several studies have documented the pattern of increasing pesticide concentrations on a north to south gradient in the Sierra Nevada Mountains [153,164,62,64]. Cory et al. [153] reported mean levels of DDE in 142 adult *R. muscosa* of 3.19 ppm (μg/g) and 3.46 ppm in the central and southern Sierra Nevada Mountains, respectively. These samples were collected throughout the Sierra Nevada Mountains in

1966-1968 when this frog was still relatively abundant. He also reported particularly high mean concentrations of DDE in *R. muscosa* in the Yosemite-Sonora area (5.38 ppm). This higher mean was attributed to applications of DDT in the Tuolumne Meadows and Stanislaus National Forest areas for the control of lodgepole needle miner *(Coleotechnites milleri)* in 1953 and 1956 and may explain the greater percentage of detections and measurable amounts of DDE in this study's Yosemite samples.

Sparling et al. [64] found measurable concentrations of DDTs (4,4'-DDE, 4,4'-DDT, and 2,4'-DDT) primarily in adult *P. regilla* to differ significantly ($p=0.04$) among Sequoia, Yosemite, and Tahoe sites with the maximal concentration at 38.7 ppb at Sequoia. His report of widespread DDTs with greater concentrations in the south fits the north south gradient previously described. Fellers et al. [67] documented DDE concentrations in adult *R. muscosa* of 46±20 ng/g (ppb) at Tablelands, Sequoia National Park and 17 ± 8 ng/g at Sixty Lakes, Kings Canyon National Park. This was one to two orders of magnitude higher than other organochlorine residues found in these areas but considerably less than the levels found by Cory et al. in 1970 [153]. Datta et al. [65] reported average levels of DDE of 9 ng/g for *H. regilla* (*H. regilla* is now *P. regilla,* per Silva [165] tadpoles from Sycamore Creek, Sequoia National Park (610 m elevation), and 258 ng/g from an agricultural area at Davis, California. Russell et al. [166] documented levels of DDE in green frogs *(R. clamitans)* of 3.54 ppb in Long Point and 5.84 ppb in Hillman Marsh, Southern Ontario, Canada.

Persistent organochlorine compounds can be found in biota in remote pristine polar locations at high altitude [167]. Blais et al. [168] suggested that the phenomenon

of cold condensation leads to higher concentrations of these compounds at altitudes. He reported a 10- to 100-fold increase in organochlorine concentrations in snow between 770 and 3,100 m altitude in the mountains of western Canada [168]. These findings support the pattern of organochlorine depositions in the Sierra Nevada Mountains. Angermann et al. [66] documented levels of two organochlorine compounds: polychlorinated biphenyls (PCBs) (244 -1.6 ng/g wet wt) and toxaphene (15.6-1.5 ng/g) in *P. regilla* tadpoles in the Sierra Nevada Mountains. Ohyama et al. [169] also reported DDE levels in trout at high altitudes over wide areas in the Sierra Nevada, further supporting atmospheric transport of these compounds.

This study is unique in its reporting of DDE levels because it involves the only field experimental translocation of hatchling tadpoles in the Sierra Nevada Mountains and their subsequent adjacent development in each of the three locations, Lassen, Yosemite, and Sequoia National Parks. DDE, a metabolite of DDT, is highly lipophilic and in birds is passed into the egg and developing embryo [170,171]. However, this translocation experiment clearly shows that regardless of origin, frogs raised in Lassen had lower levels of DDE than frogs deployed at Yosemite or Sequoia. Thus, concentrations of DDE measured in tadpoles and metamorphs primarily came from their environment as opposed to being deposited in the egg mass by the maternal frog. It is well documented that water is the primary source of organochlorines in aquatic organisms [172]. This suggests that Yosemite and Sequoia are still impacted from the heavy use of DDT on cotton in the Central Valley (especially Fresno, Madera, Tulare and Kings Counties) in California prior to its ban in 1972 [153]. Datta et al.'s [65]

report of no detectable levels of DDE in 8 *P. regilla* egg masses collected from Upper Meadow in Lassen Volcanic National Park further supports our finding.

DDT and its metabolites are known endocrine disruptors [173,174]. Furthermore, Datta et al. [65] postulated that the porous and vascular skin of frogs (especially frogs such as *R. muscosa* that spend much of their time in and around bodies of water) would be particularly vulnerable to the dermal absorption of lipophilic compounds such as DDT and its metabolites. Moreover, recent levels of DDT in the Great Lakes area have been implicated in decreased fertility, egg survival, and thyroid hormone levels in fish [175]. Thyroid hormones play a major role in amphibian metamorphosis [176,177] and in metabolic adjustments for overwintering in cold climates [178]. Thyroid hormones in amphibians are inhibited by some xenobiotics [179,180].

In vertebrates, the thyroid is also implicated in the control of resting metabolic rates. An increase in metabolic rates post DDE exposure has been noted in shrews and bats [181]. This is particularly significant during hibernation when premature energy depletion coupled with the release of DDE from stored fats may result in mortality [181]. Linzey et al. [182] implicated DDE (0.027-1.626 ppm) in liver abnormalities indicative of altered metabolism and immunosuppression in the marine toad (*B. marinus*). Bradford et al. [183] attributed incidents of mortality in *R. muscosa* during hibernation in lakes and ponds to oxygen depletion. It is unknown whether DDE exposure could result in metabolic changes in hibernating frogs in water at low temperatures that would

negatively affect oxygen consumption, hypoxia tolerance, or liver glycogen, which provides most winter fuel needs of aquatic frogs [178].

Angermann et al. [66] also postulated that suppression of the immune system in frogs in cold climates [184] might increase the risk of exposure to chlorinated compounds. The toxicity of PCBs to *R. pipiens* (LC_{50} of 3.5 ug/L) was established by Birge [185] and research by Cook [186] highlighted sublethal and toxic effects of DDT in *R. temporaria*. Gilbertson et al. [187] reported immunosuppression in northern leopard frog *(R. pipiens)* field populations after exposure to DDT. Russell et al. [188] reported an association between levels of DDT, DDE, dichlorodiphenyldichloroethane (DDD), and dieldrin in southern Ontario spring peepers *(P. crucifer)* and local documented amphibian extinctions. And finally, Reeder et al. [38] recently documented historic endocrine disruption and intersexuality in Illinois cricket frogs *(Acris crepitans)* correlated with DDT and PCB exposures. Their research suggests that organochlorine-induced endocrine disruption contributed to *A. crepitans* population declines in Illinois.

Despite the banning of DDT in the United States in 1972, it is still used in many parts of the world and continues to be aerially transported to North America [189]. USEPA reported 32 countries currently using DDT for disease vector control under provisions in the Stockholm Convention [190]. Temperate zone mountain regions with high rates of precipitation may be especially at risk for the accumulation of organochlorine compounds [168]. It is possible that DDT, a persistent, endocrine disrupting, bioaccumulative, and aerially transported compound may pose a threat to high elevation amphibian populations.

In the Sierra Nevada Mountains, Bradford [53] documented mass mortality and extinction in a high elevation population of *R. muscosa* associated with the bacterium *Aeromonas hydrophila.* Carey et al. [191] noted that 2 museum specimens of the Yosemite toad *(Bufo carnorus)* collected during a Sierra Nevada Mountains mass mortality event in the 1970's contained chytrid fungi *(Batrachochytrium dendrobatidis)*. Among other hypotheses, they postulate that contaminant exposures may inhibit amphibian immune systems and make them more susceptible to disease. Many populations of high elevation amphibians in Central and South America are currently experiencing dramatic declines attributed to chytrid fungi [192,193]. Mass mortality events are frequently at high elevations and in cooler regions, where they result in 50-100% mortality of the populations [53,194–197]. Further study is needed to assess the role of aerially transported pesticides in these amphibian disease events.

Endosulfan, an organochlorine highly toxic to amphibians, [82,198,199] is also a known endocrine disruptor. Yosemite had a higher level of % detections of endosulfan in the samples than the other two parks. Because endocrine disruption may be increased by additive effects of chemicals [200], it is noteworthy that both endosulfan and DDE were detected in Yosemite samples. DDE has also been shown to have additive and synergistic effects on toxicity in birds when combined with other pesticides [201].

Much remains to be learned about effects of pesticides on amphibian populations in the Sierra Nevada Mountains. Our choice of *P. regilla* for a surrogate species was a logical choice based on its abundance and range, and a conservative one as we suspect it is less susceptible than *R. muscosa* to pesticide effects. The life history strategy of *R.*

muscosa (approximately 3 years to metamorphosis; longer life span; greater body size; longer time to sexual maturity; and more time spent in the water as an adult) vs. *P. regilla* (about 2-3 mo to metamorphosis; shorter life span; smaller body size; less time to sexual maturity, and less time in the water as an adult) may place *R. muscosa* at greater risk for accumulation of these compounds. Unless solutions are discovered and implemented quickly, this species and other declining amphibian populations in the Sierra Nevada Mountains may not recover.

CHAPTER IV

FLOW CYTOMETRIC ASSESSMENT OF GENOTOXIC EFFECTS OF PESTICIDE EXPOSURE IN PACIFIC CHORUS FROGS IN THE SIERRA NEVADA MOUNTAINS, CALIFORNIA

SYNOPSIS

Previous studies have shown that pesticides from the Central Valley of CA enter the Sierra Nevada ecosystem through aerial deposition in snow and rain, and that surface concentrations of certain pesticides are within an order of magnitude of the 96 hr LC_{50} of amphibians [61,64]. *Pseudacris regilla* hatchlings were translocated (with controls in each park) and placed in cages in sites located in Lassen Volcanic, Yosemite, and Sequoia National Parks. Liver cells from 108 newly metamorphosed frogs were examined with flow cytometry (FCM) techniques for evaluation of chromosomal breakage as measured by the half-peak coefficient of variation of the G_1 cell population (HPCV). Regardless of origin, experimental groups raised at Lassen, the reference site, had significantly less variation in DNA content ($p=0.04$, Fisher's PLSD test) than metamorphs raised at the other two parks. Mean HPCV values for the three parks are: Lassen (4.97±0.31); Yosemite (5.75±0.19); Sequoia (5.73±0.23). This is the first documented evidence of DNA damage in juvenile frogs in the Sierra Nevada Mountains.

This finding may be important in evaluating the overall impact of aerially transported pesticides on declining frog populations in the Sierra Nevada Mountains.

INTRODUCTION

Over the past decade, scientists have documented amphibian declines and population crashes in at least 16 countries on 6 continents [3]. It is now widely accepted that we are experiencing a global decline of amphibians [2,3]. Amphibians represent a significant part of vertebrate biomass and nutrient cycling in many ecosystems [1] and so these precipitous declines are cause for grave concern.

Many of these declines are occurring in mountain regions at high elevations [53,194,54,195–197]. One region of particular concern in North America is the Sierra Nevada Mountains of California. Broad scale field sampling compared with historical analyses of museum records shows an ecosystem level decline of amphibians around the Central Valley of California. Counties most affected are Sacramento and those of the San Joaquin Valley [44]. The collapse of a regional frog fauna, 5 of 7 species, in the Yosemite area of the California Sierra Nevada, has also been documented [45].

According to Jennings [46], all 5 native ranid species in the Sierra Nevada are in need of protection. The Cascade frog (*Rana cascadae*) is declining [47] and the northern leopard frog (*R. pipiens*) has disappeared from 99% of its range [48,49]. The California red-legged frog (*R. draytonii*) is listed as threatened [50,51]. The foothill yellow-legged frog (*R. boylii*) is in decline [45,52] and the mountain yellow-legged frog (*R. muscosa*)

has disappeared from >75% of study sites where it was formerly found in California [53–55]. In 2002, the southern-most population in California of *R. muscosa* was listed as an endangered species [56].

These declines correlate with historical pesticide use in the Central California Valley [69] and with the atmospheric transport of pesticides to the Sierra Nevada Mountains [59,62,61]. The global use of pesticides has increased in the last decade and in 1989 was estimated to be at ~6.3 billion pounds [202] with ~2.1 billion pounds used in the United States [202]. The 2003 United States estimate was ~5 billion pounds [203] and global use has risen to ~13.2 [204]. California has followed this global trend and in 2003 reported the sale of 570 million pounds and the application of 175 million pounds of active ingredient [79].

Pesticides have been reported in frog tissues collected in the Sierra Nevada Mountains [153,65,64,66,67]. Relatively little research has been done in general on amphibian ecotoxicology; a recent survey revealed only 2.7% of all vertebrate toxicological studies included amphibians [32] whereas amphibians make up approximately 10% of vertebrate species. Even less amphibian research has been done in the area of genotoxic effects [205,145,206]. Various chemical contaminants, including pesticides, have been implicated in genotoxicity [207,206] and a correlation between elevated HPCVs and contaminant exposures (both radiation and chemical) has been established [208,207].

Several studies have documented DNA alterations in amphibians post contaminant exposure through the use of the comet and/or micronucleus assay [209–

214]. A variety of studies have successfully used flow cytometry to evaluate effects in other wildlife vertebrates [146,215–221]. Vinogradov [222] and Lizana et al. [223] used flow cytometry to establish genome size in unexposed amphibians. Only a few studies have used flow cytometry to look at chromosomal breakage and potential genotoxicity in amphibians [145,224].

Flow cytometry is a relatively recent and rapid cytological technique that quantifies cellular DNA content [208] and makes it possible to measure contaminant effects on the vertebrate genome [222,206]. This is the first flow cytometric investigation of genotoxic effects of agricultural pesticides on newly metamorphosed frog tissues from a field translocation experiment in the Sierra Nevada Mountains.

STUDY AREAS

Lassen Volcanic National Park was chosen as a reference site because it lies north of agricultural impacts from the Central Valley. It is positioned at the southern terminus of the Cascade Mountains and at the northern tip of the Sierra Nevada Mountains. Yosemite National Park and Sequoia National Park were chosen as potential impacted sites because they are both downwind from areas of intense pesticide use in the Central Valley. Yosemite is located in the central Sierra Nevadas and Sequoia in the southern Sierra Nevadas (Figure 4.1). These three parks contain some of the largest remaining areas of late successional conifer forests at sub alpine and mid-elevations [155]. Three meadow pond sites per park were used to deploy cages of translocated *P. regilla* hatchlings (Table 4.1; Figure 4.1).

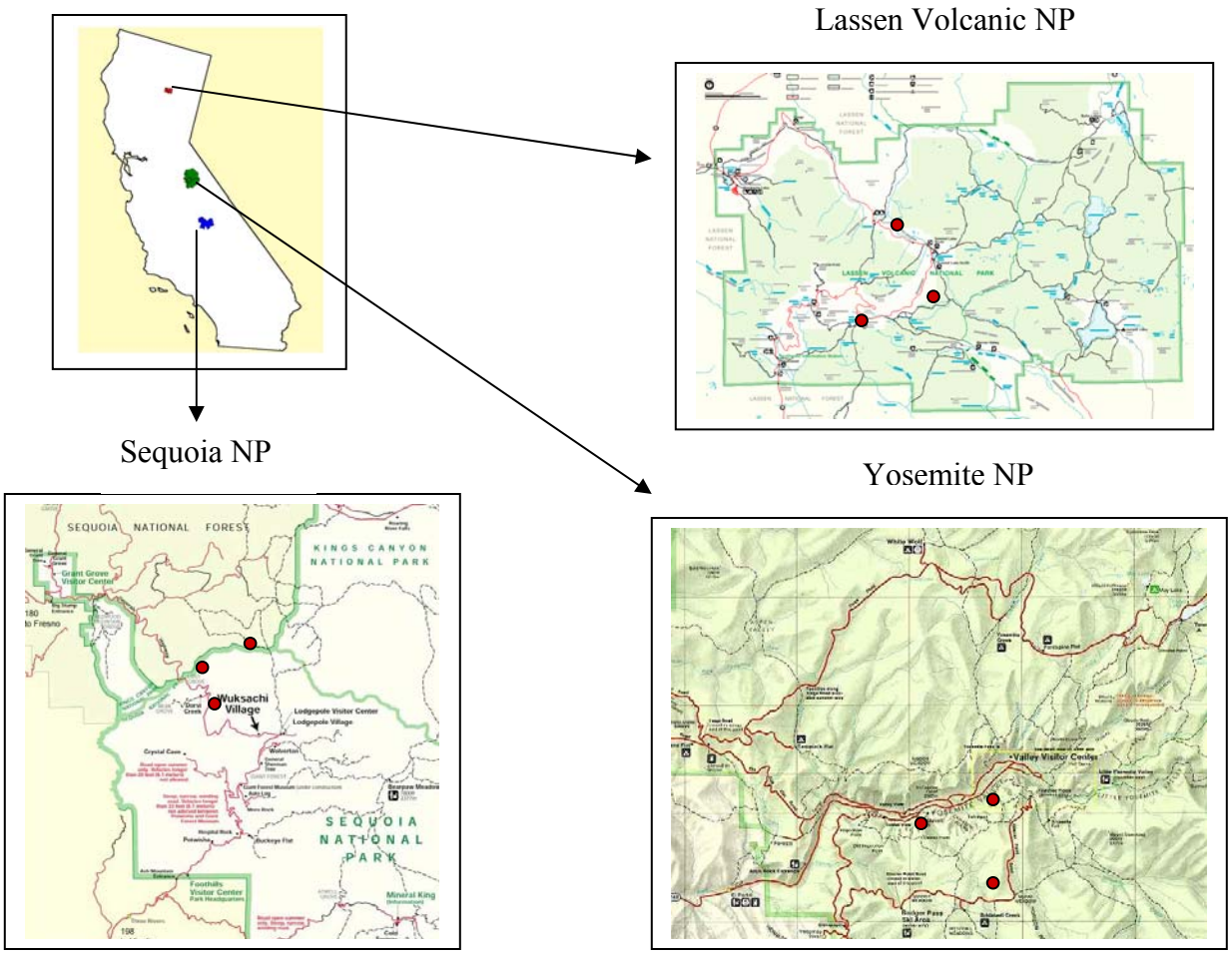

Figure 4.1 California map showing Lassen Volcanic, Yosemite, and Sequoia National Parks. Park maps show locations of meadow ponds used to deploy translocated tadpoles. Map of California was made by Carlos Hinojosa and Amy Hays of the Land Information Systems, Texas A&M University. Parks maps are from the National Park Service, public domain.

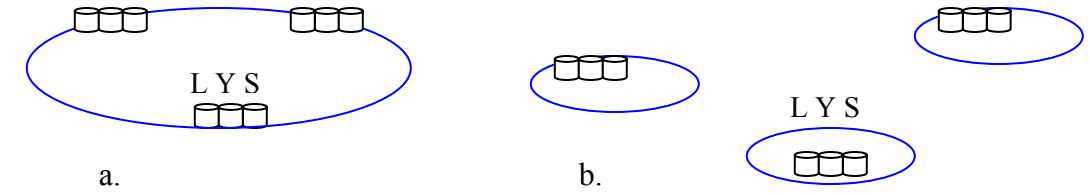

Figure 4.2. Cage placements. a. Three sets of 3 cages in one large pond, i.e. Long Meadow, Sequoia. b. One set of three cages in 3 small ponds in wet meadow, i.e. NE Summit Meadow, Yosemite National Park. (L=Lassen; Y= Yosemite; S = Sequoia).

Pond meadow sites were chosen with the following characteristics: previous use by *P. regilla*; elevation (~2,200 m); lack of fish; ability to accommodate cages; and accessibility. These wetland meadow pond sites are in upper montane

Table 4.1 Experimental pond sites in Lassen, Yosemite, and Sequoia National Parks.

Lassen	Yosemite	Sequoia
Hemlock	NE Summit Meadow	N Circle Meadow
Dersch Meadow	Pothole Meadow	Huckleberry Meadow
Upper Kings Meadow	Mono Meadow	S Circle Meadow

forests, characterized by stands of red fir (*Abies magnifica*), lodgepole pine (*Pinus contorta*), and Jeffrey pine (*Pinus jeffreyi*). There is little rainfall in the summer months in the Sierras, and so most of these pond sites are fed by snow melt [156]. In Yosemite,

the Tuolumne watershed in the north and the Merced watershed in the south serve most of the park and feed into the San Joaquin River basin. Sequoia has three major rivers: Kings, Kaweah and Kern. These watersheds also help to distribute winter precipitation held in the form of snow pack throughout the summer. Lassen has portions of four drainage basins including Mill, Hat, and Kings Creeks that eventually drain into the Sacramento River.

METHODS

Experimental methods

The experiment was conducted June-August 2001. *P. regilla* was selected as a surrogate study species, because of its relative abundance, its wide distribution, and its ability to metamorphose in one summer compared to ~3 years for *R. muscosa.*

Hatchling tadpoles were translocated from a single pond per park and care was taken to collect < 10% of total hatchlings present. Lassen hatchlings were collected from Dersch Meadow, Yosemite tadpoles from Dana Meadow, and Sequoia tadpoles from Table Meadow. Hatchlings were placed in separate cages for each park origin and grouped in sets of threes (Lassen, Yosemite, and Sequoia) for a total of nine cages per meadow site (Figure 4.2). Cages were either placed in 3 sets of 3 cages in one large pond as in Long Meadow, Sequoia National Park, or were placed in clusters of three in small ponds in a contiguous palustrine wet meadow as in NE Summit Meadow, Yosemite National Park.

Each cage was a cylinder with bottom and removable lid constructed of rigid 75 µm white Nitex ®teflon (Sefar America Inc., Kansas City, MO) (60 cm depth x 35 cm diameter) (Figure 4.3). This field-tested design by Harris and Bogart [157] was

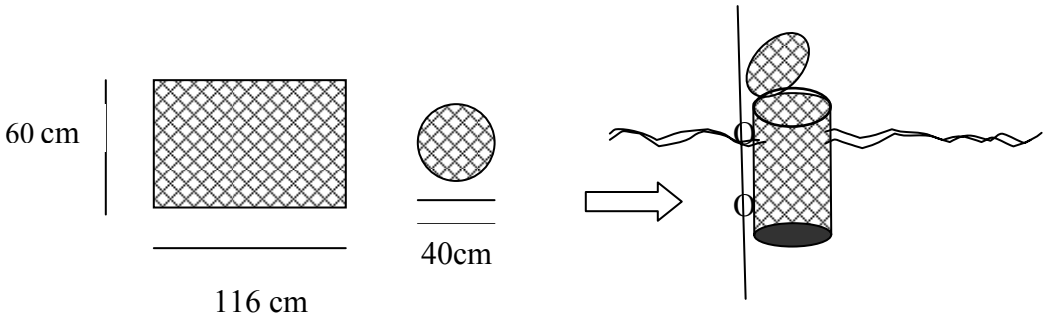

Figure 4.3 Tadpole cage dimensions and design.

enlarged to provide an internal cage volume for 60 tadpoles that would not cause overcrowding or possible developmental problems [158]. The cylinder was stitched together with nylon thread and plastic rings sewn to the cage sides accommodated a plastic covered metal rod that was driven into the pond bottom [159]. When necessary, cages were moved away from shore and towards the middle of the pond as ponds dried to keep them in water. The top of the cage (40cm) was attached with industrial strength Velcro® for ease of removal. A floating platform (plastic grid side panel of a Jehmco® fry cage) was placed in each cage for metamorphosed frogs when tadpoles reached Gosner stage 42 (appearance of front limbs).

A crew of 4 technicians and the principal investigator monitored the cages daily. Boiled romaine lettuce was supplied as food. Approximately 90% of the lettuce was organic or certified pesticide-free, the other 10% was carefully washed and rinsed before boiling. Food availability was maximized by providing a continuous supply of boiled lettuce. Measurements of water pH, nitrate/nitrites, dissolved O2, ammonia, hardness, and turbidity were all within normal limits. Juvenile frogs were collected upon metamorphosis, stage 42-46 [102].

Flow cytometry methods

Newly metamorphosed frogs were collected from the field experiment and shipped on dry ice to Patuxent Wildlife Research Center, Laurel Maryland, USA, where they were stored at -80° C, and then moved to -80° C storage until processing at the Brazos Field Station, Texas A&M University, College Station, Texas, USA.

Various frog tissues were tested for suitability and optimal results were obtained from frog livers. Previously developed protocols were used to measure DNA content in cells [208,215]. In order to avoid bias, samples were randomized prior to the experiment, and the sample group identifications were unknown to the cytometer operator. All samples for this experiment were run on the same day in order to reduce variation caused by cytometer drift. Livers were dissected from slightly thawed specimens. Nuclear suspensions were obtained by adding 15 µl of trypsin/detergent solution for digestion [225] and later by physical disruption by douncing. Ten minutes after the digestion solution was introduced to the sample, a trypsin inhibitor and RNAse was added [225].

The solution was filtered through 30 μm nylon mesh and dounced for 10 seconds, after which propidium iodide (PI) was added to intercalate between the bases [226] and to stain the DNA. Samples were covered with foil, place on ice, and allowed to stain for 15 minutes.

A Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA) was used in the generation of DNA flow histograms. Cells were illuminated with a Coherent laser at 514 nm at 500 mW of power in order to measure red fluorescent emissions of propidium iodide-stained nuclei, which is proportional to DNA content [215]. Gating of cells was established on forward and side scatter and the ratio of peak to integrated fluorescence. Light scatter parameters indicate levels of cytoplasm remaining attached to the nucleus. Stability of the instrument is ensured by time during analyses. Cells with high levels of side scatter were not included in the analysis in order to minimize sample preparation variation. Any samples showing evidence of drift were reanalyzed. The cytometer computer program determines HPCV, mean, standard deviation, and number of cells for region determined by the operator. Only samples that measured ten thousand nuclei and that satisfied all gating parameters were included in the statistical analyses. Intercellular variation in DNA content was reported as the half peak percent coefficient of variation (HPCV) of the gated G_1 cell population. Fluorescent microspheres and chicken red blood cells are used as standards to check the alignment and focus of the system.

Statistical analyses

Mean HPCVs among parks were analyzed by analysis of variance (ANOVA) using StatView®, SAS Institute Inc., version 5.01. Means are presented ± 1 SE and $\alpha = 0.05$ for evaluating statistical significance. Data were plotted and appeared normally distributed and so actual values were used to compute means. Separate analyses were conducted evaluating total samples for each park by origin of tadpoles and also for total samples of tadpoles deployed at each park regardless of origin. Lassen samples, Yosemite samples, and Sequoia samples refer to animals that were raised in each park, regardless of origin, unless otherwise stated. Animals from each meadow were combined in the analyses for each park.

RESULTS

Comparisons were made among the three National Parks based on park of origin and park of deployment. Data were plotted and appeared normally distributed and so parametric ANOVA was used to compare the groups. There was a significant difference among mean HPCV values by park of deployment only based upon ANOVA ($p=0.04$; DF=2, 105; F-value = 3.28 (Tables 4.2, 4.3; Figure 4.4). Two separate *a posteriori* tests were performed; Fisher's PLSD fixes only the comparison wide error rate ($p = 0.05$) and is a liberal test, whereas Scheffé's test is conservative and fixes the experiment wide error rate at $p = 0.05$. Both were used given the marginal level of significance of the overall ANOVA so that a clearer understanding of the likely cause of the overall significance could be determined.

Fisher's PLSD test was significant for differences between Lassen and the other two parks (Table 4.4). Scheffe's post-hoc test was not significant for comparisons among the means for any of the three parks (Table 4.5), though p-values were only marginally non-significant. It is clear from the inspection of these results that the significance in the overall ANOVA was related to the low mean values for HPCV at Lassen.

Table 4.2 ANOVA table for mean HPCV.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	13.485	6.743	3.275	0.0417	6.549	0.605
Residual	105	216.207	2.059				

Table 4.3. Lassen Volcanic, Yosemite and Sequoia National Park mean HPCV levels. M=metamorph.

Park	Age Class	Mean HPCV	n
Lassen	M	(4.97±0.31) [a]	31
Yosemite	M	(5.75±0.19)[b]	38
Sequoia	M	(5.73±0.23) [b]	39

[a,b] Means followed by the same letter were not significantly different.

Table 4.4. Fisher's PLSD for juvenile *P. regilla* HPCV means by parks deployed.

Parks Deployed	Mean Diff.	Critical Diff	P-Value
L,S	-0.79	0.69	0.02
L,Y	-0.77	0.69	0.03
S,Y	-0.03	0.65	0.94

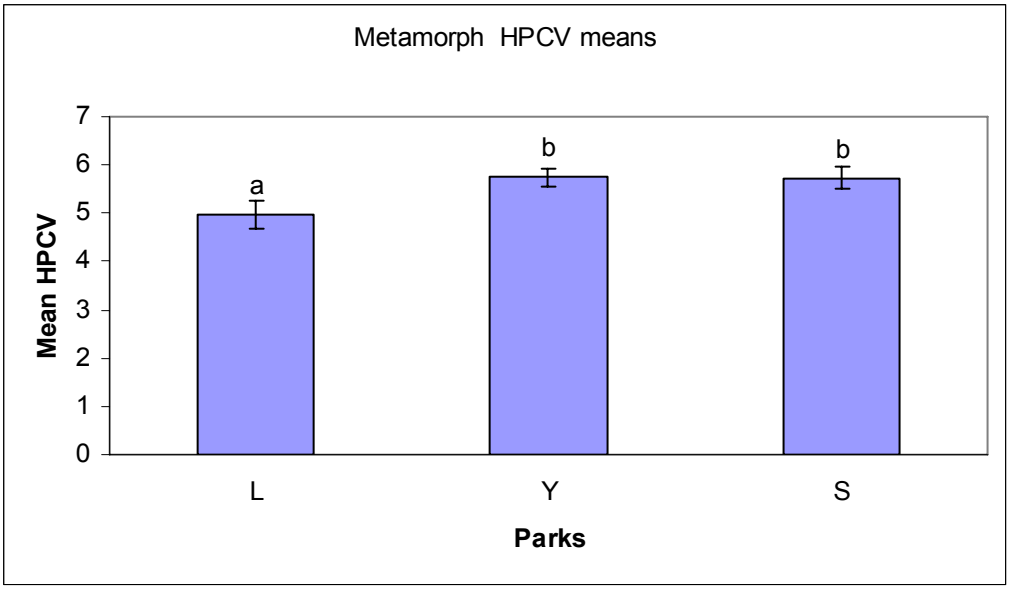

Figure 4.4. Graph of juvenile *P. regilla* HPCV means.

Table 4.5. Scheffe for juvenile *P. regilla* HPCV means by parks deployed.

Parks Deployed	Mean Diff.	Critical Diff	P-Value
L,S	-0.79	0.86	0.08
L,Y	-0.77	0.86	0.09
S,Y	-0.03	0.81	1.0

DISCUSSION

DNA strand breakage is a cellular response to a genotoxic agent and usually occurs within hours or days of exposure [206]. It has also been documented that HPCV values correlate with age class (adult animals show greater HPCV than juveniles) [146,145]. Lowcock et al. [145] compared HPCV between juvenile and adult green frogs *(R.clamitans)* exposed to a variety of pesticides on potato and corn crops versus a control site. They found increased strand breakage among both age classes in corn crop areas where carbofuran, atrazine, glyphosate, and butylate were used. Adult frogs also showed higher HPCV values in the potato crop areas. Juvenile frogs with deformities showed higher HPCV values than normal frogs [145].

Matson [224] showed genetic damage based on flow cytometry and a micronucleus assay in marsh frogs *(R. ridibunda)* in Sumgayit, Azerbaijan. Frogs were sampled post-exposure to a variety of environmental contaminants: petrochemical, persistent organic pollutants (POPs) (including pesticides) and heavy metals [224].

Pesticide residues in frog tissues in Lassen, Yosemite and Sequoia National Parks have been documented by Sparling et al. [64] and Cowman et al. (Chapter III), and

in snow, sediment, and water by McConnell et al. [61]and (McConnell et al. unpublished data). Maximal levels of pesticides found in tissues included: chlorpyrifos (190 ppb, Yosemite), diazinon (190 ppb Yosemite), endosulfans (21.9 ppb), DDTs (38.7 ppb, Sequoia), and α- and γ-hexachlorocyclohexane (HCH) (1.57 ppb, Yosemite) [64]. Levels at Lassen were low or often zero in comparison [64] (McConnell et al. unpublished data) and support our hypothesis that this park is less impacted from aerially transported pesticides than the other two. Our findings of increased HPCV values in Yosemite and Sequoia compared to those of Lassen also supports our hypothesis that frogs at Yosemite and Sequoia National Parks have levels of DNA strand breakage correlated with increased pesticide exposures.

Of the pesticides found in frog tissues, chlorpyrifos, diazinon, endosulfan, and DDE, all are known genotoxic agents [227–230]. Chlorpryrifos and diazinon (to a lesser degree) have both been identified as neuroteratogens in the inhibition of DNA synthesis [227]. DDE has been found to cause genetic alterations in vertebrates in a variety of studies [228] and has been implicated in cytotoxicity in amphibians [229]. Endosulfan is reported to be hepatotoxic in mammals and induces DNA strand breaks and increased micronuclei [230,231]. However, not all of these compounds have been directly linked to DNA strand breakage in vertebrates.

The implications of DNA strand breakages are two fold. Somatic cells constitute most of the cells in a vertebrate animal and DNA damage to these cells may be inconsequential if the cells die or are repaired [207,232]. However, affected somatic cells may also lead to mutations. This could result in increased cancer risk, decreased

immune system responses, or reduced reproductive fitness [206,207]. Hence somatic cell damage could affect the health and integrity of a particular organism [206]. When damage occurs to germ cells, the resultant effects could be far more serious for the population as a whole. Germ cell damage may become fixed in the gene pool, transmitted to future generations, and potentially affect genetic diversity, mutation loads, and ultimately reproductive fitness of the population [232].

Amphibian populations in the Sierra Nevada Mountains are in decline, and we have evidence of genotoxic pesticide residues in frog tissues in this area [153,64,67]. Furthermore, our study shows greater DNA damage in *P. regilla* metamorphs at Yosemite and Sequoia National Parks versus our reference site, Lassen Volcanic National Park. This finding may be important in evaluating the overall effects that aerially transported pesticides are having on amphibian population declines.

This is only the first step in determining whether genotoxicity is playing a definitive role in the declines. More direct documentation of genetic effects in amphibians post-pesticide exposure is needed before we can establish a link between genetic damage (i.e. DNA strand breakage) and amphibian population declines in the Sierra Nevada Mountains.

CHAPTER V

CHOLINESTERASE INHIBITION IN METAMORPHS AND TADPOLES IN THE SIERRA NEVADA MOUNTAINS, CALIFORNIA

SYNOPSIS

Declines of native ranid frog populations in the Sierra Nevada Mountains may be linked to aerial deposition of pesticides originating from the Central Valley of CA. *Pseudacris regilla* (Pacific chorus frog) hatchings were translocated (with controls in each park) and placed in cages in sites (~ 2,200 m elevation) located in Lassen, Yosemite, and Sequoia National Parks. Cholinesterase (ChE) was measured in tadpoles collected at 28 days and in juvenile frogs collected upon metamorphosis. Animals were staged according to Gosner; then grouped in the following categories: (PL) prelimb (24-26); (LB) limbbud (27-34); (EHL) early hind limb (35-36); (MHL) middle hind limb (37-39); (LHL) late hind limb (40-41); (Meta) metamorph (42-46).

For the 2001 field experiment, ANOVA showed overall differences ($p<0.0001$; DF=5, 722; F=286) among ChE means by stage for all parks; Scheffe's post-hoc test showed differences ($p<0.0001$) between Metas and all other groups; between LHL and EHL($p=0.02$) and LHL and LB ($p<0.002$); there were no differences among MHL, EHL, LB, and PL. ANOVA showed overall differences ($p<0.0001$; DF=2, 271; F= 47.6)

among ChE Meta means (Table 3.4; Figure 4.5) by park deployed; Scheffe's test showed differences ($p<0.0001$) between Lassen and the other two parks and a difference ($p=0.002$) between Yosemite and Sequoia (Table 3.5). Tadpoles PL-MHL had an overall ANOVA significance of ChE means (Table 3.6) by park deployed ($p<0.0001$; DF=2, 400; F= 24) and Scheffe's post-hoc test revealed significant differences among all three parks. Park deployment was important, but park origin was insignificant.

For the 2002 field experiment, ANOVA showed overall differences ($p<0.0001$; DF=3, 655; F=733.8) among ChE means by stage; Scheffe's test showed differences ($p<0.0001$) for metas and all other groups; between MHL and LB ($p=0.01$); there were no differences between MHL and EHL. Based on these values, Metas and LB were analyzed as separate groups; MHL and EHL were analyzed as one group. ANOVA showed no overall differences ($p=0.26$; DF=2, 250; F= 1.35) among Meta ChE means by park deployed; Scheffe showed no differences among the three parks. No differences were detected in the overall ANOVA ($p=0.14$; DF=2, 297; F-value=2) for LB ChE (Lassen 0.6; ± 0.02; Sequoia 0.61± 0.02; Yosemite 0.65 ± 0.02 μmol/min/gr); Scheffe also revealed no differences. The overall ANOVA for EHL-MHL showed a significant difference ($p=0.04$; DF=2,103; F=3.23) (Table 4.2); but Scheffe only showed a difference between Sequoia and Yosemite ($p=0.04$).

Temperatures were significantly different among the three parks for both years ($p<0.0001$) and may play a role in ChE levels. ChE inhibition can be an indicator of organophosphate insecticide exposure. Effects noted in *P. regilla* may be magnified in long lived ranid species.

INTRODUCTION

Approximately 5 billion pounds of all pesticide groups (including conventional, specialty biocides, wood preservatives, and choline/hypochlorites) were used in the United States in 2001, an increase from 2.1 billion pounds in 1989 [202,203]. Although the world estimate given by USEPA for 2001 was for conventional pesticides only (~5 billion pounds), because the United States represents close to one third of the overall world market, the total global use of all pesticide groups can be estimated at ~13.2 billion pounds [204]. California has followed this global trend and in 2003 reported the sale of 570 million pounds and the application of 175 million pounds of active ingredient [79].

Organophosphate pesticide use

According to Hill [233], organophosphate insecticides total more than one third of registered pesticides in the world, and most are used in agriculture or for mosquito control. Many water soluble organophosphorous compounds are acutely toxic and are accessible for long periods in water [233].

Although the use of cholinesterase-inhibiting insecticides has been declining in California since 1997, [79] the use of these chemicals peaked in the early 1990's (Figure 5.1) and coincided with reports of amphibian declines in California

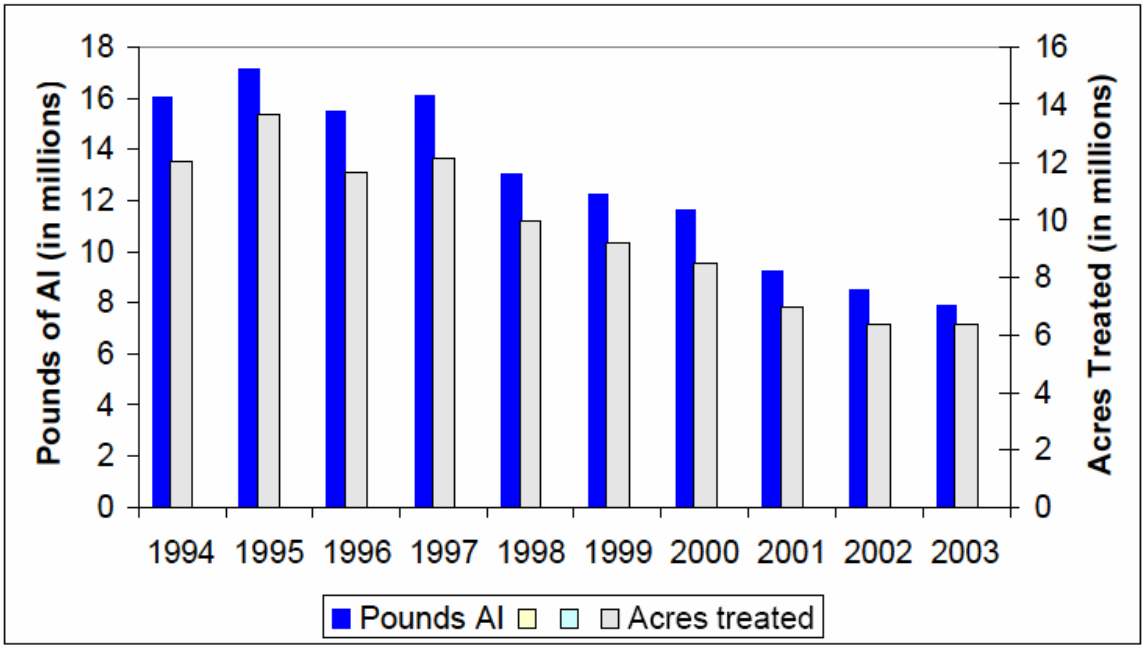

Figure 5.1. Use trends of cholinesterase-inhibiting pesticides, California 1994-2003. Source: California Department of Pesticide Regulation's Pesticide Use Reports.

[45,46]. Acute toxicities of cholinesterase-inhibiting pesticides to amphibians have been established [116,82,103] as has a suite of sub-lethal effects including paralysis, malformations, effects of predator/prey interactions, decreased activity and growth rates, DNA damage, and immunosupression [33,234].

The atmospheric transport of cholinesterase-inhibiting pesticides is also well documented [59–62]. LeNoir et al. [62] reported that agricultural activity in California's Central Valley may be a significant source of pesticides currently being deposited in the Sierra Nevada Mountain range. The authors suggest that pesticides applied to this area of intensive agriculture may be volatilized by high temperatures in the valley, transported through the atmosphere and finally deposited in cooler, higher elevations of the Sierra

Nevada Mountains. Residue analyses showed that highest levels of contaminants found in surface water and dry particulate samples in the Mountains were those of pesticides applied in the valley during heavy use periods in summer. Davidson et al. [68] showed an association of amphibian declines in California with the amount of upwind agricultural land use. His further research [69] demonstrated that this correlation held for declines in *R. aurora draytonii*, *R. boylii*, *R. cascadae*, and *R. muscosa*. OP and carbamate pesticides were most strongly associated with these population declines in Davidson's analysis of historical pesticide applications [69]. De Vlaming et al. [70], Hunt et al. [71], and Bailey et al. [72] also reported the presence and toxicity of chlorpyrifos and diazinon in several California agricultural and urban watersheds.

McConnell et al. [61] reported levels of malathion (0.045-6 ng/L), chlorpyrifos (1.1-13 ng/L) and diazinon (0.57-14 ng/L) at an elevation of 1,920 m in Sequoia National Park. Their study also revealed these same chemicals at 2,200 m in Lake Tahoe: malathion (0.46-18 ng/L); diazinon (0.057-7ng/L); and chlorpyrifos (0.30-3.4 ng/L).

Datta et al. [65] documented pesticides (dichlordiphenyl dichloroethylene (p,p'-DDE), chlorothalonil and chlorpyrifos) and polychlorinated biphenyls (PCBs) in fish and Pacific chorus frog (*P. regilla)* tadpoles from the Kaweah River Basin, CA.

Sparling et al. [64] reported levels of chlorpyrifos and diazinon in frog tissues in Yosemite and Sequoia National Parks. Maximal levels of pesticides found in tissues included chlorpyrifos (190 ppb, Yosemite), and diazinon (190 ppb Yosemite). Levels at Lassen were low or often zero in comparison [64].

Chlorpyrifos, diazinon, and malathion are continuously applied throughout the year in California and in counties adjacent to Yosemite and Sequoia National Park [79] (Figure 5.2 and 5.3).

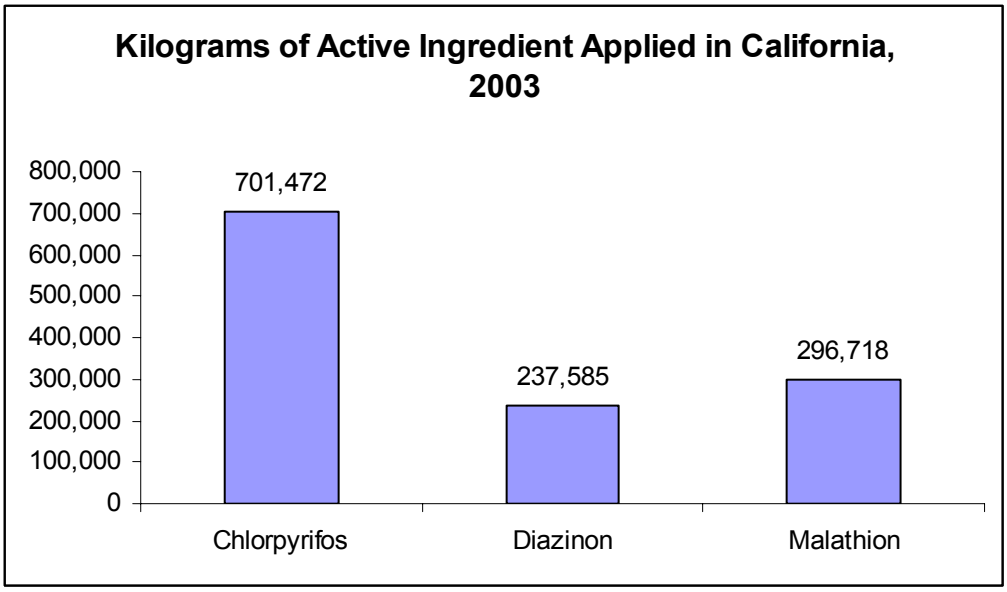

Figure 5.2. Total kilograms of chlorpyrifos, diazinon, and malathion applied in California, 2003. Source: California Department of Pesticide Regulation.

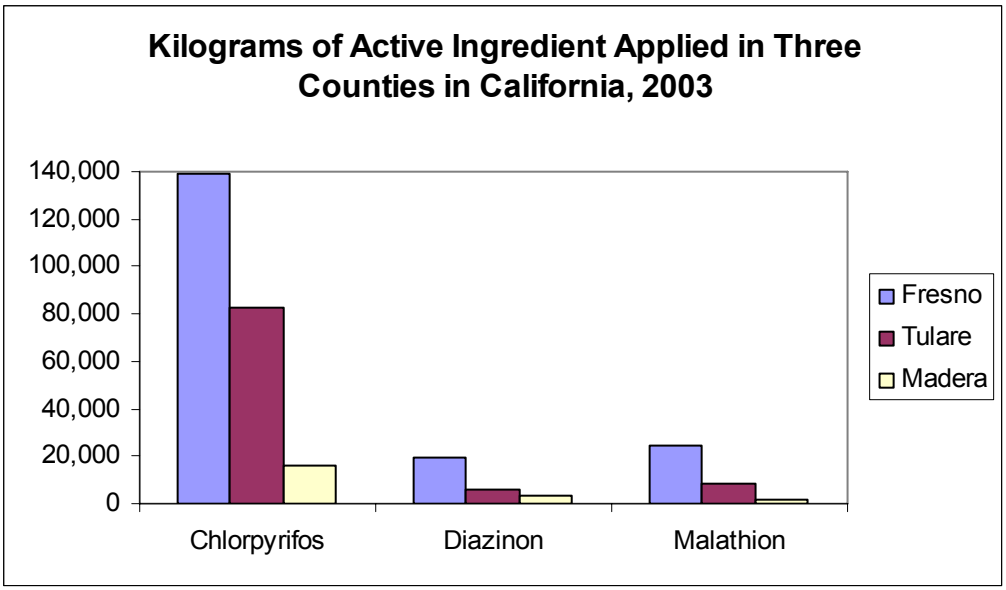

Figure 5.3. Kilograms of chlorpyrifos, diazinon, and malathion. Applications in Fresno, Tulare, and Madera counties, California, 2003. Source: California Department of Pesticide Regulation. Fresno is 55 miles west of Sequoia National Park and Sequoia lies in the eastern part of Tulare County. Part of Yosemite National Park is in Madera County, and Fresno is 65 miles south of Yosemite.

According to the United States Environmental Protection Agency (USEPA), chlorpyrifos is the most heavily used insecticide in the United States. Most of its residential use is being phased out, but agricultural use will continue [118]. USEPA classifies it as highly toxic to larval amphibians. The 24-hour LC_{50} value for *B. vulgaris formosus* tadpoles is 1 ppb [119].

In addition to its acute toxicity to amphibians, chlorpyrifos has also been implicated in amphibian leg malformations [125] and decreased low temperature tolerance [122,123]. In mammals, chlorpyrifos has been implicated in inhibition of DNA synthesis [227] and immunotoxicity [108].

Malathion is highly toxic to amphibians in concentrations as small as 200 ppb [116]. It is associated with the following sublethal effects: delayed development [235]; enhanced toxicity when combined with predatory stress [236]; amphibian hindlimb deformities [40]; immunotoxicity in fish [108]; immunosuppression in amphibians [137,187]; and mutations in T-lymphocytes in humans [237].

The residential use of diazinon was phased out in 2004 but 70% of its agricultural use is still permitted [203]. It is acutely toxic to fish [238], causes mortality in green frog tadpoles at 5.9 ppb [93], and is also associated with deformities and growth inhibition in ranid embryos and tadpoles [93].

Cholinesterase inhibition

Cholinesterase inhibition is a well established biomarker for the detection of organophosphorous insecticides [239,240] and has been used in amphibian toxicological research [106,107,33,64,234]. Organophosphates phosphorylate ChEs which results in the accumulation of acetylcholine at receptor sites, ultimately causing death from respiratory failure [233].

Although acutely toxic, the relative inability of organophosphorous compounds to bioaccumulate in vertebrates often makes it difficult to detect their residues in animal tissues. The cholinesterase inhibition biomarker allows for the detection of the effect of the pesticide even when residues are not present [239]. Cholinesterase activity levels may also be affected by various factors such as the health of the animal, seasonal variation, cold or heat stress, and nutritional status [239,234].

In addition to finding significant levels of pesticides in tissues of adult *P. regilla* collected in the Sierra Nevadas, Sparling et al. [64] also demonstrated that cholinesterase activity (a bioassay reflecting OP or carbamate pesticide exposure) is significantly inhibited in tadpoles of *P. regilla* in regions where ranid frogs have experienced the worst declines. These authors hypothesize that because chorus frog tadpoles share the same habitat as larval ranids, inhibition of cholinesterase strongly suggests that carbamate or OP pesticides may be adversely affecting amphibians inhabiting these wetlands [64]. This project examined cholinesterase levels among tadpoles and metamorphs translocated as hatchlings and raised in Lassen Volcanic, Yosemite, and Sequoia National Parks. Because all the native true frog species (*Rana* spp.) of the Sierra Nevada are in peril, information regarding the possible role of agricultural chemicals in their decline is critical.

STUDY AREAS

Lassen Volcanic National Park was chosen as a reference site because it lies north of agricultural impacts from the Central Valley. It is positioned at the southern terminus of the Cascade Mountains and at the northern tip of the Sierra Nevada Mountains. Yosemite and Sequoia National Parks were chosen as potential impacted sites because they are both downwind from areas of intense pesticide use in the Central Valley. Yosemite National Park is located in the central Sierra Nevadas and Sequoia National Park in the southern Sierra Nevadas (Figure 5.4). These three parks contain some of the largest remaining areas of late successional conifer forests at sub alpine and

mid-elevations [155]. Three meadow pond sites per park were used to deploy cages of translocated *P. regilla* hatchlings (Table 5.1; Figure 5.4).

Pond meadow sites were chosen with the following characteristics: previous use by *P. regilla*; elevation (~2,200 m); lack of fish; ability to accommodate cages; and accessibility. These wetland meadow pond sites are in upper montane forests,

Table 5.1 Lassen, Yosemite, and Sequoia National Parks meadow pond sites.

Lassen	Yosemite	Sequoia
Hemlock	NE Summit Meadow	Circle Meadow
Dersch Meadow	Pothole Meadow	Huckleberry Meadow
Upper Kings Meadow	Mono Meadow	Long Meadow

characterized by stands of red fir (*Abies magnifica*), lodge pole pine (*Pinus contorta*), and Jeffrey pine (*Pinus jeffreyi*). There is little rainfall in the summer months in the Sierras, and so most of these pond sites are fed by snow melt [156]. In Yosemite, the Tuolumne watershed in the north and the Merced watershed in the south serve most of the park and feed into the San Joaquin River basin. Sequoia has three major rivers: Kings, Kaweah and Kern. These watersheds also help to distribute winter precipitation held in the form of snow pack throughout the summer. Lassen has portions of four drainage basins including Mill, Hat, and Kings Creeks that eventually drain into the Sacramento River.

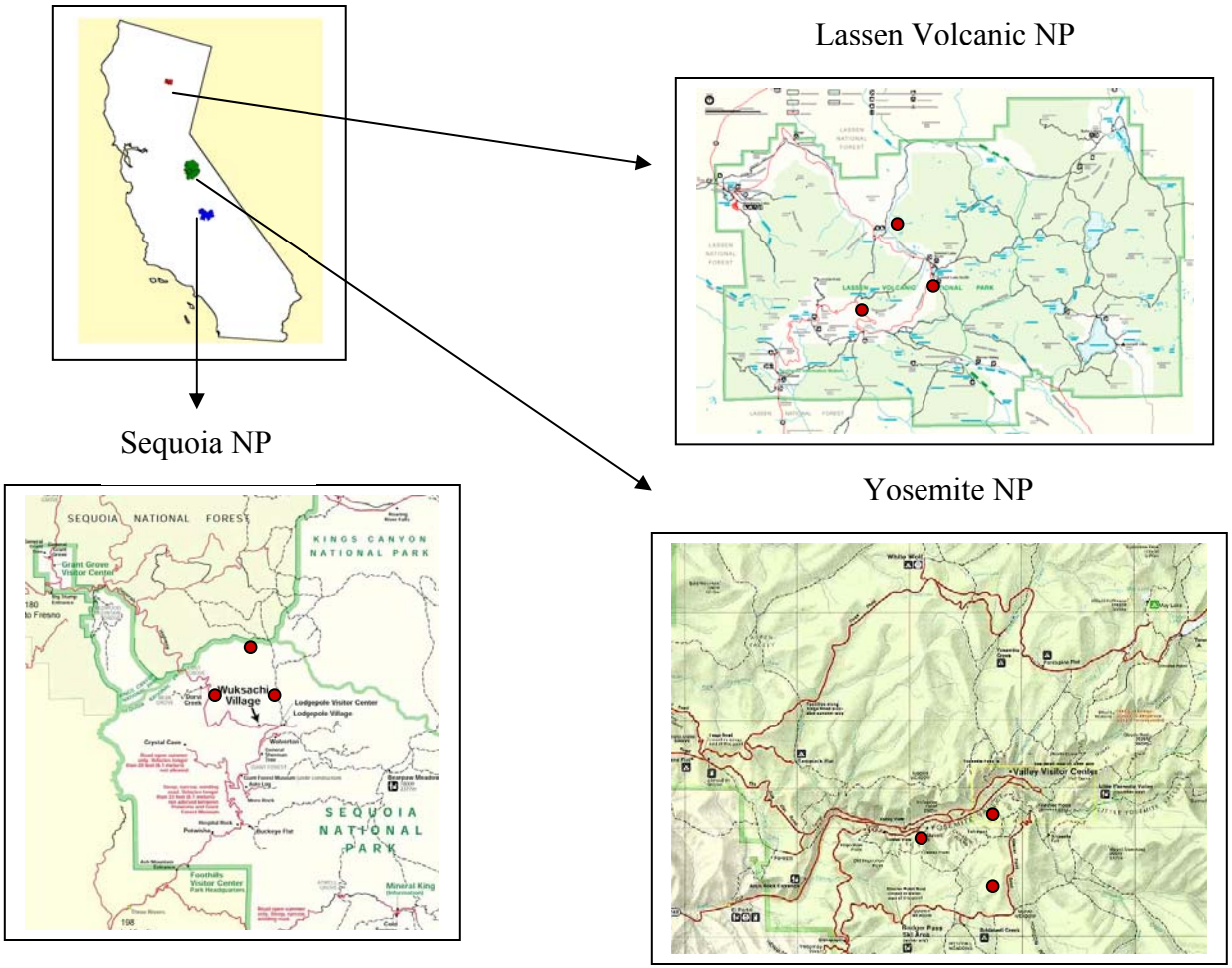

Figure 5.4. Lassen Volcanic, Yosemite, and Sequoia National Parks. Park maps show locations of meadow ponds used to deploy translocated tadpoles. Map of California was made by Carlos Hinojosa and Amy Hays of the Land Information Systems, Texas A&M University. Parks maps are from the National Park Service, public domain.

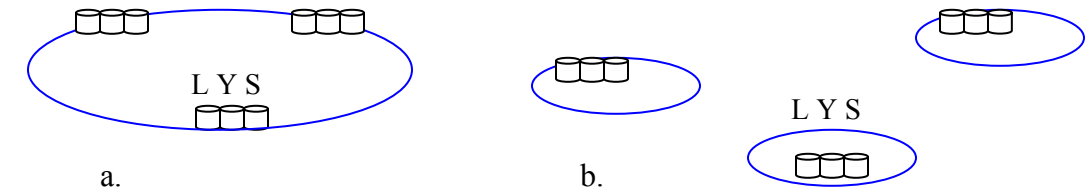

Figure 5.5. Placements of cages. a. Three sets of 3 cages in one large pond, i.e. Long Meadow, Sequoia. b. One set of three cages in 3 small ponds in wet meadow, i.e. NE Summit Meadow, Yosemite National Parks. (L=Lassen; Y= Yosemite; S = Sequoia).

METHODS

Experimental methods

Two field experiments were conducted: June-August 2001 and June-August 2002. *P. regilla* was selected as a surrogate study species, because of its relative abundance, its wide distribution, and its ability to metamorphose in one summer compared to ~3 years for *R. muscosa.*

Hatchling tadpoles were translocated from a single pond per park and care was taken to collect < 10% of total hatchlings present. Lassen hatchlings were collected from Dersch Meadow, Yosemite tadpoles from Pothole Meadow, and Sequoia tadpoles from Long Meadow. Hatchlings were placed in separate cages for each park origin and grouped in sets of threes (Lassen, Yosemite, and Sequoia) for a total of nine cages per meadow site (Figure 5.5). Cages were either placed in 3 sets of 3 cages in one large pond as in Long Meadow, Sequoia National Park, or were placed in small ponds in a contiguous palustrine wet meadow as in NE Summit Meadow, Yosemite National Park.

Each cage was a cylinder with bottom and removable lid constructed of rigid 75 µm white Nitex ®teflon (Sefar America Inc., Kansas City, MO) (60 cm depth x 35 cm diameter) (Figure 5.6). This field-tested design by Harris and Bogart [157] was enlarged to provide an internal cage volume for 60 tadpoles that would not cause overcrowding or possible developmental problems [158]. The cylinder was stitched together with nylon thread and plastic rings sewn to the cage sides accommodated a plastic covered metal rod that was driven into the pond bottom [157]. When necessary, cages were moved away from shore and towards the middle of the pond as ponds dried to keep them in water. The top of the cage (40cm) was attached with industrial strength Velcro® for ease of removal. A floating platform (plastic grid side panel of a Jehmco® fry cage) was placed in each cage for metamorphosed frogs when tadpoles reached Gosner stage 42 (appearance of front limbs).

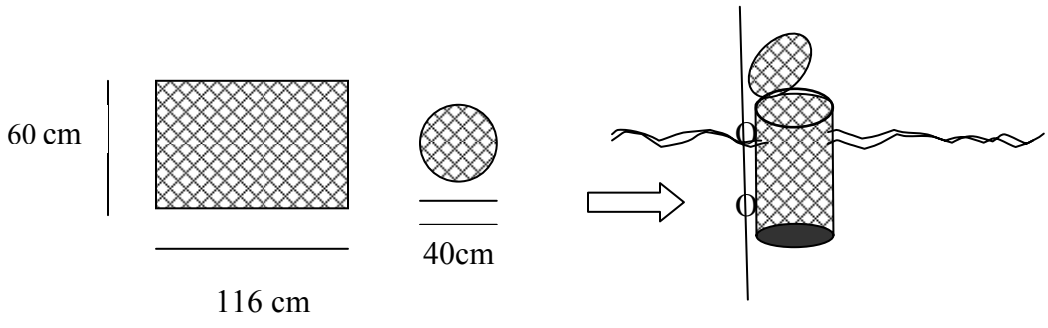

Figure 5.6. Design and dimensions of tadpole cages.

A crew of 4 technicians and the principal investigator monitored the cages daily. Boiled romaine lettuce was supplied as food. Approximately 90% of the lettuce was organic or certified pesticide-free, the other 10% was carefully washed and rinsed before boiling. Food availability was maximized by providing a continuous supply of boiled lettuce. Measurements of water pH, nitrate/nitrites, dissolved O2, ammonia, hardness, and turbidity were all within normal limits. Tadpoles were sampled at 28 days of exposure and again at metamorphosis, stage 42-46 [102].

Cholinesterase methods

Sparling et al. [64] reported a mean difference in ChE for tadpoles at various stages of development. Therefore, all tadpoles were staged (Gosner 1960) before ChE analyses and placed in the following categories: (PL) prelimb (Gosner 24-26); (LB) limbbud (Gosner 27-34); (EHL) early hind limb (Gosner 35-36); (MHL) middle hind limb (Gosner 37-39); (LHL) late hind limb (Gosner 40-41); (Meta) metamorph (Gosner 42-46). Five tadpoles from each cage were randomly selected by stage and snout-vent length (SVL) and 5 metamorphs were also randomly selected from each cage (numbers permitting) for ChE analyses.

Tadpole and metamorph ChE samples were collected from the field experiment, frozen in liquid nitrogen, and shipped on dry ice to Patuxent Wildlife Research Center, Laurel, Maryland, USA, where they were stored at -80° C until processing at the Patuxent Wildlife Research Center Laboratory in Beltsville, Maryland, USA. Tadpoles were partially thawed for removal of gut coils, homogenized in Tris buffer (whole bodies

minus gut coil) and then centrifuged. The supernatant, free of suspended particles, was measured for cholinesterase activity with a SpectraMax® spectrophotometer per Ellman [241] with modifications for a multi-plate reader per Hooper [242]. All samples were run in triplicate or until the coefficient of variation was less than 5% for each run. Values are expressed in μmol/min/g.

Statistical analyses

Mean ChE among parks for tadpoles and metamorphs were analyzed with analysis of variance tests (ANOVA) using StatView®, SAS Institute Inc., version 5.01. Means are presented ± 1 SE and $\alpha = 0.05$ for evaluating statistical significance. Values did not deviate from a normal distribution and so actual values were used to compute means. Separate analyses were conducted evaluating total samples for each park by origin of tadpoles and also for total samples of tadpoles deployed at each park regardless of origin. Lassen samples, Yosemite samples, and Sequoia samples refer to animals that were raised in each park, regardless of origin, unless otherwise stated. Animals from each meadow were combined in the analyses for each park.

RESULTS

2001 Field experiment

For the 2001 field experiment, ANOVA showed overall differences ($p<0.0001$; DF=5, 722; F=286) among ChE means (Tables 5.2, 5.3; Figure 5.7); Scheffe's post-hoc test showed differences for Metas and all other groups; between LHL and EHL; and LHL

and LB; there were no differences among MHL, EHL, LB, and PL. Based on these values, Metas and LHL were analyzed as separate groups, and MHL, EHL, LB and PL were analyzed as one group.

Table 5.2 ANOVA table of ChE means by stage for total samples, 2001.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Stage	5	125.895	25.179	286.006	0.0001	1430.031	1.000
Residual	722	63.562	0.088				

Table 5.3. ChE by stage for total samples.

STAGE	N	Mean (µmol/min/gr)	Standard Error
PL	25	0.501	0.022
LB	90	0.491	0.014
EHL	198	0.549	0.009
MHL	79	0.625	0.017
LHL	62	0.710	0.018
META	274	1.421	0.027

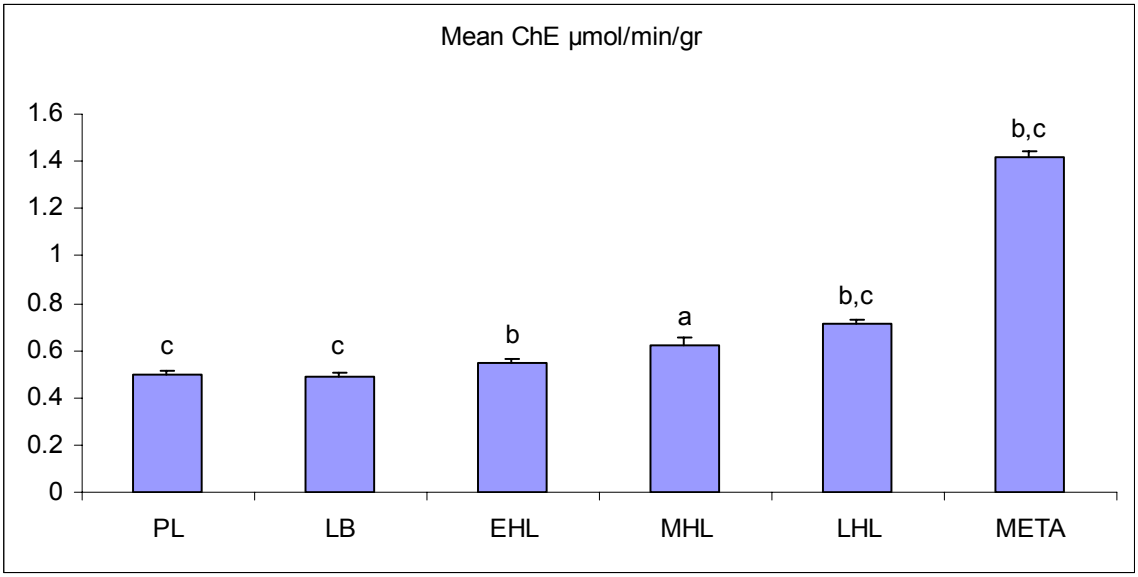

Figure 5.7. ChE means by stage for total samples. Lowercase letters indicate differences among means.

One-way ANOVA showed overall differences ($p<0.0001$; DF=2, 271; F= 47.6) among ChE Meta means (Tables 5.4, 5.5; Figure 5.8) by park deployed; Scheffe's test showed differences between Lassen and the other two parks and a difference between Yosemite and Sequoia. ChE values for Metas raised at Lassen National Park (reference site) were significantly higher ($p<.0001$) than values for Metas raised at Sequoia and Yosemite National Parks regardless of origin (Figure 5.8).

Table 5.4. ANOVA table of Meta ChE means by park deployed, 2001.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	14.414	7.207	47.572	<0.0001	95.144	1.000
Residual	271	41.057	0.152				

Table 5.5. Meta ChE means by park deployed.

Park	Stage	N	Mean (µmol/min/gr)	Standard Error
L	Meta	187	1.57	0.03
Y	Meta	64	1.2	0.04
S	Meta	23	0.86	0.04

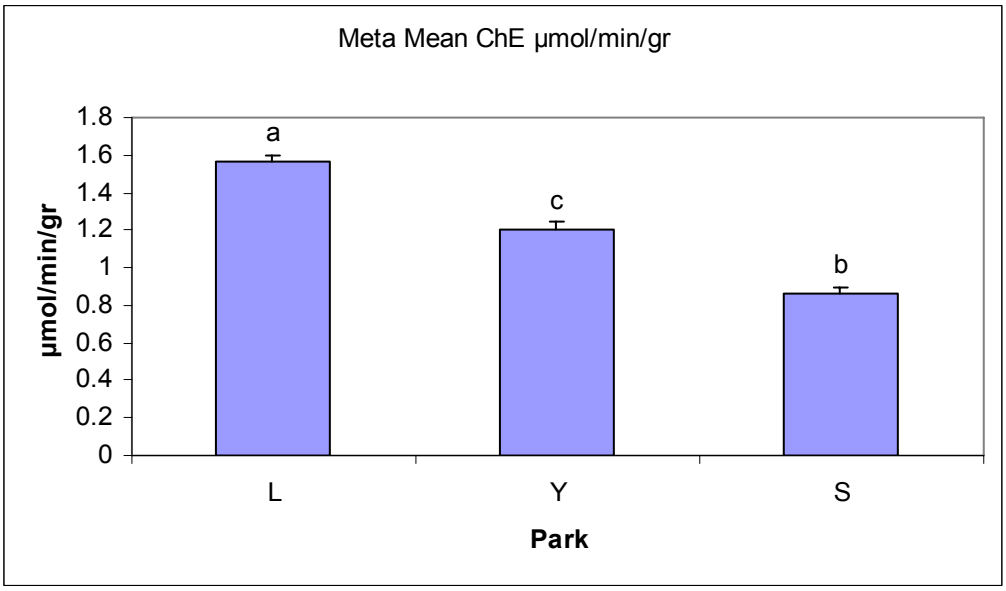

Figure 5.8. Meta ChE means by park deployed. Lowercase letters indicate differences among means.

There was no overall difference in the ANOVA for LHL ChE (Figure 5.9). Tadpoles PL-MHL had an overall ANOVA significance of ChE means (Tables 5.6, 5.7) by park deployed ($p<0.0001$; DF=2, 400; F= 24) and Scheffe's post-hoc test revealed significant differences among all three parks (Figure 5.10).

Table 5.6. ANOVA table of PL-MHL ChE means by park deployed.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	0.815	0.408	24.044	<0.0001	48.088	1.000
Residual	388	6.579	0.017				

Table 5.7. PL-MHL ChE means by park deployed.

Park	Stage	N	Mean	Standard Error
L	Meta	197	0.588	0.009
Y	Meta	103	0.479	0.12
S	Meta	91	0.55	0.14

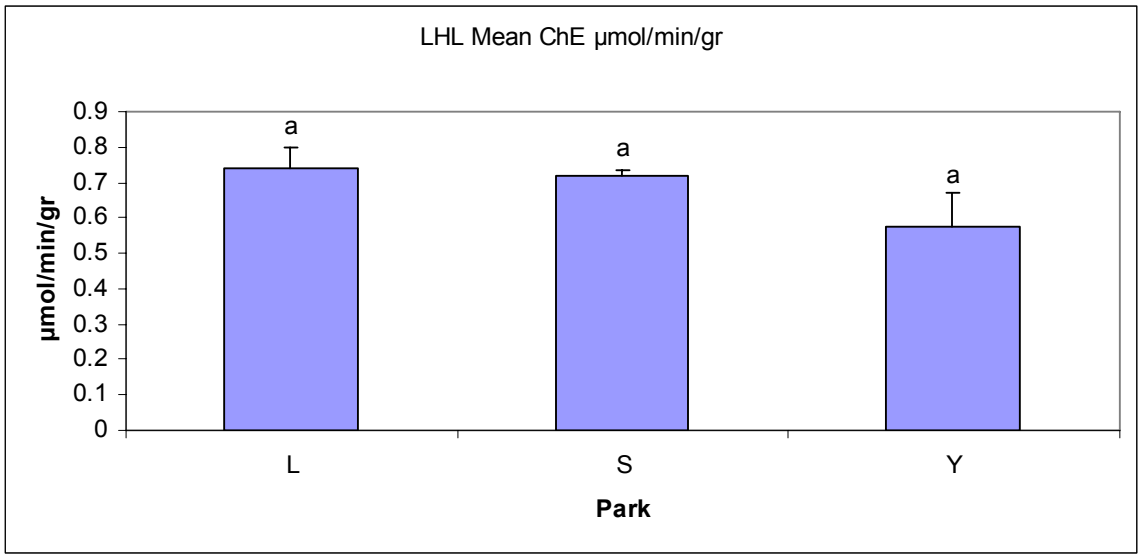

Figure 5.9. LHL mean ChE by park deployed. Lowercase letteres indicate differences among means.

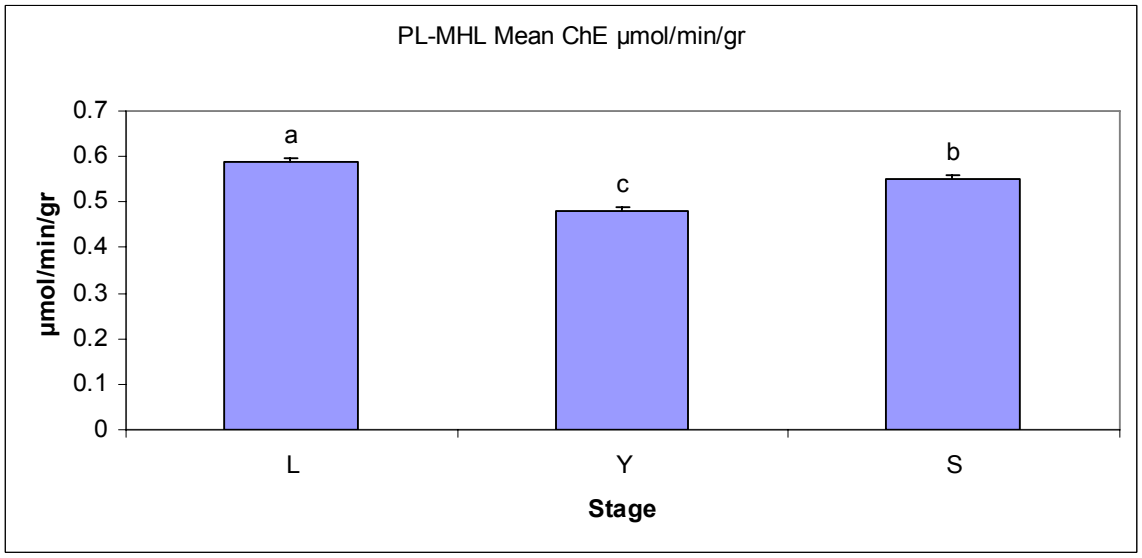

Figure 5.10. PL-MHL ChE means by park deployed. Lowercase letters indicate differences among means.

In summary, the means for all of the groups from the 2001 field experiment indicate that animals raised at Lassen, regardless of origin had significantly higher ChE means, and less exposure to ChE inhibitors than animals raised at the other two parks.

2002 Field experiment

For the 2002 field experiment, ANOVA showed overall differences ($p<0.0001$; DF=3, 655; F=733.8) among ChE means by stage (Tables 5.8, 5.9; Figure 5.11); Scheffe's test showed differences for Metas and all other groups; between MHL and LB there were no differences between MHL and EHL. Based on these values, Metas and LB were analyzed as separate groups; MHL and EHL were analyzed as one group. No LHL tadpoles were analyzed.

Table 5.8 ANOVA table of total ChE means by stage, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	3	205.244	68.415	733.752	<0.0001	2201.256	1.000
Residual	655	61.072	0.093				

Table 5.9. Total ChE means by stage.

STAGE	N	Mean	Standard Error
EHL	56	0.69	0.02
LB	300	0.61	0.01
META	253	1.79	0.03
MHL	50	0.77	0.02

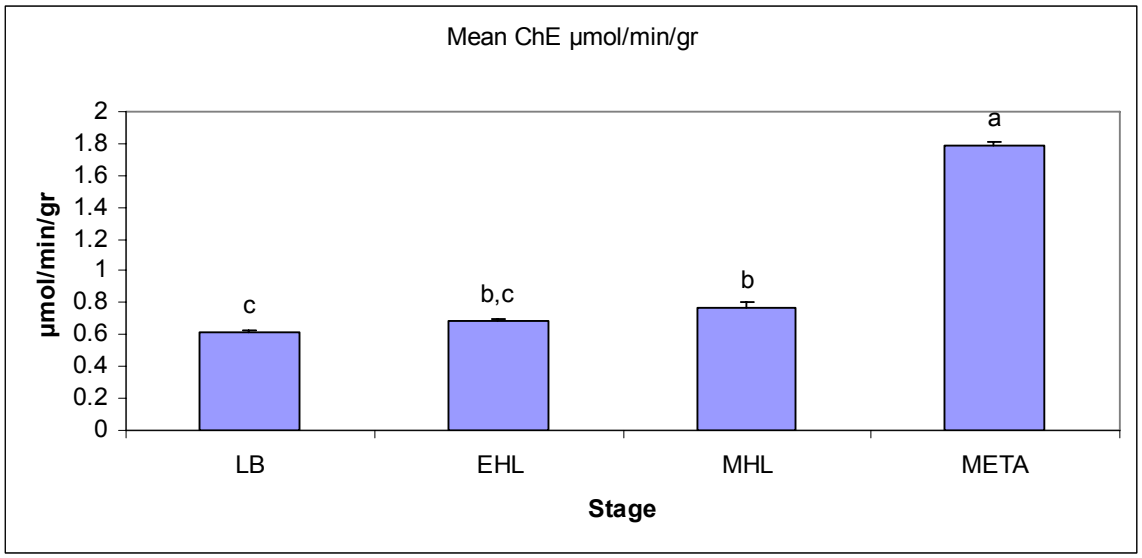

Figure 5.11. Total ChE means by stage. Lowercase letters indicate differences among means.

ANOVA showed no overall differences (p=0.26; DF=2, 250; F= 1.35) among Meta ChE means (Tables 5.10, 5.11).

Table 5.10. ANOVA table of Meta ChE means by park deployed, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	0.554	0.277	1.347	0.2619	2.694	0.278
Residual	250	51.458	0.206				

Table 5.11. 2002 Meta ChE means by park deployed.

Park	Stage	N	Mean	Standard Error
L	Meta	104	1.78	0.05
Y	Meta	90	1.87	0.06
S	Meta	59	1.74	0.04

No differences were detected in the overall ANOVA (p=0.14; DF=2, 297; F-value=2) for LB ChE (Lassen 0.60 ± 0.02; Sequoia 0.61± 0.02; Yosemite 0.65 ± 0.02); The overall ANOVA for EHL-MHL showed a significant difference (p=0.04; DF=2,103; F=3.23) (Tables 5.12, 5.13); Scheffe showed a difference between Sequoia and Yosemite (Table 5.14; Figure 5.12).

Table 5.12 ANOVA table of EHL-MHL mean ChE by park deployed, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	0.111	0.056	3.226	0.0438	6.451	0.597
Residual	103	1.778	0.017				

Table 5.13. 2002 EHL-MHL ChE means by park deployed.

Park	Stage	N	Mean	Standard Error
L	EHL-MHL	30	0.73	0.22
Y	EHL-MHL	45	0.76	0.18
S	EHL-MHL	31	0.68	0.27

Table 5.14. Scheffe's post-hoc test for EHL-MHL mean ChE by park deployed.

Parks	Mean Diff.	Critical Diff	P-Value
L,S	0.05	0.08	0.35
L,Y	-0.03	0.08	0.65
S,Y	-0.08	0.08	0.04*

*Indicates statistical significance

In summary, the ChE means for both Metas and tadpoles in the 2002 field experiment were not significantly different among parks by deployment, except for slight differences between Yosemite and Sequoia EHL-MHL. Lassen, our reference site, did not show the same significantly higher pattern for ChE that it presented in 2001.

Water temperatures

Daily water temperatures for each cage were collected in 2001. The means of these daily temperatures for each cage were compared among park of deployment. An overall analysis of variance showed statistical significance among the means ($p<0.0001$; DF=2, 78; F-value=71.6) (Tables 5.15, 5.16). Scheffe's post hoc test showed significance among mean water temperatures at all three parks (Figure 5.12).

Table 5.15. 2001 ANOVA table of water means by park deployed.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	642.056	321.028	71.567	<0.0001	143.134	1.000
Residual	78	349.884	4.486				

Table 5.16. 2001 water temperature means by park.

Park	N	Mean	Standard Error
L	27	22.1	0.23
Y	27	18.3	0.65
S	27	15.2	0.17

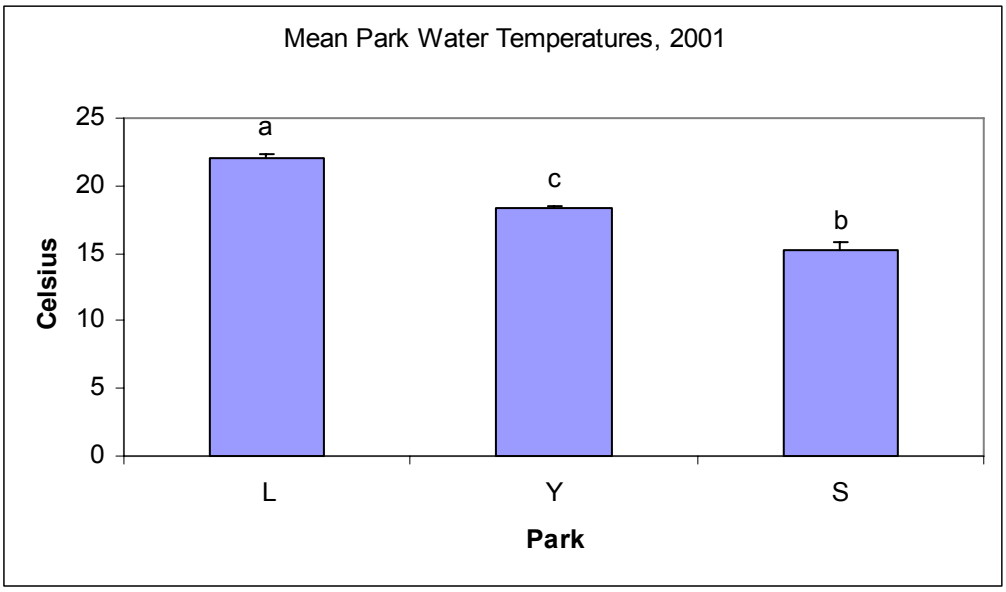

Figure. 5.12. Water temperature means by park, 2001. Lowercase letters indicate differences among means.

Minimum and maximum water temperatures were collected daily for each meadow pond in 2002 and ANOVA was used to compare means among parks. The overall ANOVA was not significant for minimum temperatures (Tables 5.17, 5.18). However, maximum water temperatures were significantly different among the three

parks in 2002 (Tables 5.19, 5.20; Figure 5.13). Overall ANOVA was significant ($p<0.0001$, DF= 2, 549; F-value=135), and Sheffe's post-hoc test was also significant among all three parks (Figure 5.13).

Table 5.17 ANOVA table of minimum water temperature means by park, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	229.192	114.596	2.337	0.0976	4.673	0.461
Residual	549	26925.550	49.045				

Table 5.18. 2002 Minimum water temperature means by park.

Park	N	Mean	Standard Error
L	166	14.1	0.39
S	200	12.6	0.72
Y	186	12.7	0.23

Table 5.19. ANOVA table of maximum water temperature means by park.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	2741.518	1370.759	135.122	<0.0001	270.244	1.000
Residual	549	5569.393	10.145				

Table 5.20. 2002 Maximum water temperature means by park.

Park	N	Mean	Standard Error
L	166	21.4	0.35
S	200	19.3	0.17
Y	186	24.6	0.19

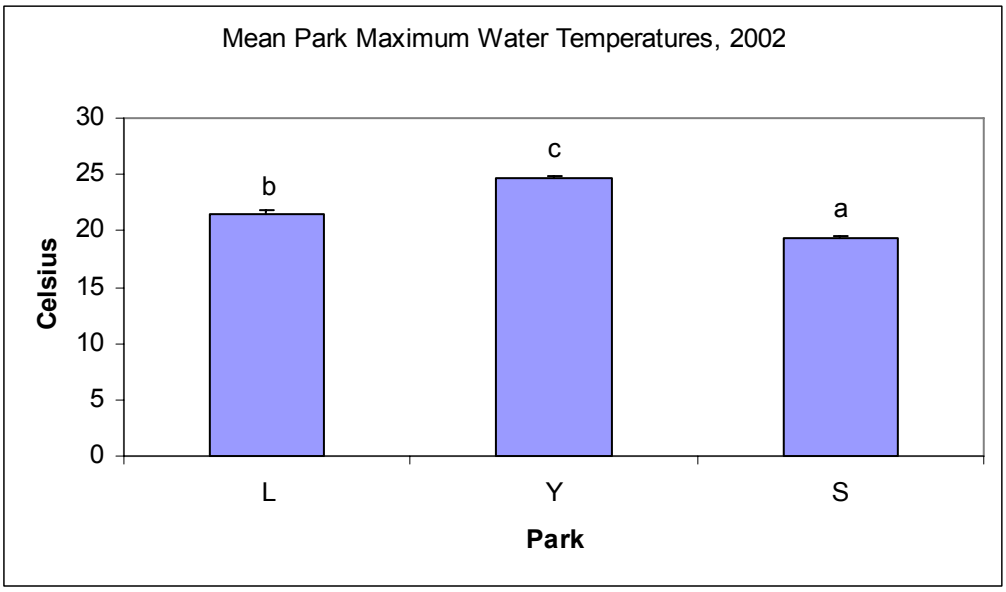

Figure 5.13. Maximum mean water temperatures by park, 2002. Lowercase letters indicate differences among means.

DISCUSSION

The mean cholinesterase activities determined in *P. regilla* in the 2001 field experiment, correlate with other data we have regarding known pesticide residues in frog tissues [64], atmospheric deposition [61], DNA strand breakage (Chapter IV), and

historic pesticide use and frog declines [69]. This data supports our hypothesis that cholinesterase-inhibiting pesticides are reaching the parks through atmospheric deposition and possibly contributing to frog population declines.

However, the 2002 field experiment did not reveal the same pattern. Animals raised at Lassen in 2002 may have been low temperature stressed due to an unusually large and late winter storm that year [243]. Our entry into our study sites at Lassen was delayed because of road closures due to snow hazards. Approximately 4 cages of the first group of tadpoles translocated to our Hemlock site in Lassen died within days due to extreme low temperature shock and had to be replaced. The minimum temperatures recorded the first week at this site ranged from 0.1-5.1°C and the maximum temperatures were 2.6-9.9°C. These temperatures fall outside the range recorded our first year (9-32°C) for developing tadpoles in the Sierra Nevadas. The remaining animals may have suffered cold stress that resulted in decreased ChE levels [244].

Temperature has recently been shown by [245] to be a significant factor effecting acetylcholinesterase (AChE) levels in *P. regilla*. They reported that animals raised at 8°C had significantly lower AChE levels ($p<0.001$) than animals raised at 19°C. The ponds in our study did not remain at a constant temperature as in [245] but fluctuated daily, and at times above and below this range, so it is unclear if this finding is environmentally relevant. However, there appears to be a correlation between the temperatures and the ChE levels reported in our study. The 2001 data showed that Lassen animals had significantly higher ChE values and the mean water temperatures were also significantly higher that year at Lassen than at the other parks. The same

relationships held true for 2002: Yosemite had significantly higher maximum water temperatures and although it did not have significantly higher ChE means, it did present the highest ChE mean for Metas (1.84±0.06 μmol/min/gr), EHL-MHLs (0.75±0.18 μmol/min/gr), and LBs (0.64±0.02 μmol/min/gr) of the three parks.

It is also possible that levels of pesticides fluctuate from year to year based on weather conditions. We did not detect organophosphate residues in *P. regilla* tadpoles or metamorphs in either year of field experiments (Chapter III) but low concentrations of these residues (ppb) are not easy to detect in animal tissues.

It is also difficult to establish causality between cholinesterase-inhibiting pesticides in the field in the Sierra Nevada Mountains and amphibian declines. The cumulative effects of organochlorine pesticides and cholinesterase-inhibiting pesticides may have had their largest impacts over two decades ago, initiating a steady decline that went unnoticed for years. Sierra Nevada amphibian declines may have been instigated by a variety of pesticide-induced factors, including reduced populations from acute toxicity mortality, increased disease susceptibility (e.g chytrid and red-leg disease), reduced health or reproductive fitness (e.g. genetic mutations), or impaired reproduction due to endocrine disruption. Although our data contributes to the "weight of evidence" towards solving this puzzle, more research is needed to answer these questions.

CHAPTER VI

SURVIVORSHIP, GROWTH, AND MALFORMATIONS IN PACIFIC CHORUS FROGS IN THE SIERRA NEVADA MOUNTAINS, CALIFORNIA

SYNOPSIS

Previous studies have shown that pesticides from the Central Valley of CA enter the Sierra Nevada ecosystem through aerial deposition in snow and rain, and that surface concentrations of certain pesticides are within an order of magnitude of the 96 hr LC_{50} of amphibians [61,64]. *Pseudacris regilla* hatchlings were translocated (with controls in each park) and placed in cages in sites (~ 2,200 m elevation) located in Lassen Volcanic, Yosemite, and Sequoia National Parks.

Survivorship to metamorphosis, days to metamorphosis, snout-vent lengths (SVL), and malformations were evaluated. Both 2001 and 2002 field experiments showed lower SVLs in tadpoles and metamorphs raised in Sequoia than in the other two parks ($p<0.0001$). Animals raised at Sequoia also took significantly longer to metamorphose ($p<0.0001$). This trend also held for survivorship to metamorphosis. In 2001, 71% (591/836) of Lassen tadpoles, 45% (244/295) of Yosemite tadpoles, and 8 % (68/842) of Sequoia tadpoles reached metamorphosis. In 2002, 53% (541/1018) of Lassen tadpoles, 57% (489/374) of Yosemite tadpoles, and 25 % (275/809) of Sequoia

tadpoles reached metamorphosis. A hind limb deformity was noted both years. In 2001, 6% (42/675) of Lassen animals, 25% (70/275) of Yosemite animals, and 7% (24/337) of Sequoia animals showed bilateral brachydactyly, a shortening of the proximal hind limb joint. In 2002, 20% (114/452) of Lassen animals, 14% (71/507) of Yosemite animals, and 7% (43/561) showed this hind limb deformity.

Time to metamorphosis, size at metamorphosis, survivorship to metamorphosis, and malformations have all been shown to be altered or caused by pesticide exposures. Because amphibians are in serious decline in the Sierra Nevada Mountains, information regarding the possible effects of agricultural chemicals is critical.

INTRODUCTION

Recently, the Global Amphibian Assessment (GAA) released the results of the first comprehensive and global assessment of the status of all 5,743 described species of amphibians. The data show that 1,856 amphibian species (32.5%) are now in Red List Categories of Vulnerable, Endangered, or Critically Endangered [3]. Although Stuart et al. [3] specifically identified declines due to habitat loss and over-utilization, they acknowledged that other processes threaten 48% of rapidly declining species, with pollution second to habitat loss [42].

North America has not escaped this dramatic decline [43], and one area of particular concern is the Sierra Nevada Mountains of California. Broad scale field sampling compared with historical analyses of museum records shows an ecosystem level decline of amphibians around the Great Central Valley of California. Counties most

affected are Sacramento and those of the San Joaquin Valley [44]. The collapse, 5 of 7 species, of the regional frog fauna, in the Yosemite area of the California Sierra Nevada, has also been documented [45].

According to Jennings [46] all 5 native ranid species in the Sierra Nevada are in need of protection. The Cascade frog *(Rana cascadae)* [47] is declining and the northern leopard frog *(R. pipiens)* has disappeared from 99% of its range [48,49]. The California red-legged frog *(R. draytonii)* is listed as threatened [50,51]. The foothill yellow-legged frog *(R. boylii)* is in decline [45,52] and the mountain yellow-legged frog *(R. muscosa)* has disappeared from > 75% of study sites where it was formerly found in California [53–55]. In 2002, the southern-most population in California of *R. muscosa* was listed as an endangered species [56].

Previous studies have also shown that organophosphate (OP) pesticides from the Central Valley of California enter the Sierra Nevada ecosystem through aerial deposition in snow and rain [59–62] and that surface concentrations of certain pesticides are within an order of magnitude of the 96 hr LC_{50} for amphibians. Datta et al. [65] documented pesticides (dichlordiphenyl dichloroethylene (p,p'-DDE), chlorothalonil and chlorpyrifos) and polychlorinated biphenyls (PCBs) in fish and Pacific chorus frog (*Pseudacris regilla,* formerly *Hyla regilla)* [165] tadpoles from the Kaweah River Basin, CA. Angerman et al. [66] documented PCBs and toxaphene in *P. regilla* tadpoles from the Sierra Nevada including Lassen, Yosemite, and Sequoia National Parks. Sparling et al. [64] have also found significant levels of pesticides (chlorpyrifos, diazinon, and endosulfan) in tissues

of adult *P. regilla* collected in the Sierra Nevadas. In 2004, Fellers et al. [67] showed an array of pesticides in surface water and in *R. muscosa* tissues.

Davidson et al. [68] showed an association of amphibian declines in California with the amount of upwind agricultural land use. His further research [69] demonstrated that this correlation held for declines in *R. aurora draytonii, R. boylii, R. cascadae*, and *R. muscosa*. OP and carbamate pesticides were most strongly associated with these population declines in Davidson's analysis of historical pesticide applications [69].

This field experiment examined differences in survivorship to metamorphosis, time to metamorphosis, growth, and rate of malformations in *P. regilla* translocated and raised to metamorphosis in Lassen Volcanic (reference site), Yosemite, and Sequoia National Parks. Because amphibian declines in the Sierra Nevada Mountains may result in species extinctions, information regarding possible pesticide impacts to amphibian populations in these areas is critical.

STUDY AREAS

Lassen Volcanic National Park was chosen as a reference site because it lies north of agricultural impacts from the Central Valley. It is positioned at the southern terminus of the Cascade Mountains and at the northern tip of the Sierra Nevada Mountains. Yosemite and Sequoia National Parks were chosen as potential impacted sites because they are both downwind from areas of intense pesticide use in the Central Valley. Yosemite is located in the central Sierra Nevadas and Sequoia in the southern Sierra Nevadas (Figure 6.1). These three parks contain some of the largest remaining areas of

late successional conifer forests at sub alpine and mid-elevations [155]. Three meadow pond sites per park were used to deploy cages of translocated *P. regilla* hatchlings (Table 6.1; Figure 6.1).

Pond meadow sites were chosen with the following characteristics: previous use by *P. regilla*; elevation (~2,200 m); lack of fish; ability to accommodate cages; and accessibility. These wetland meadow pond sites are in upper montane forests, characterized by stands of red fir (*Abies magnifica*), lodge pole pine (*Pinus contorta*), and Jeffrey pine (*Pinus jeffreyi*). There is little rainfall in the summer months in the Sierras, and so most of these pond sites are fed by snow melt [156]. In Yosemite, the Tuolumne watershed in the north and the Merced watershed in the south serve most of

Table 6.1. Pond sites in Lassen, Yosemite, and Sequoia National Parks.

Lassen	Yosemite	Sequoia
Hemlock	NE Summit Meadow	Circle Meadow
Dersch Meadow	Pothole Meadow	Huckleberry Meadow
Upper Kings Meadow	Mono Meadow	Long Meadow

the park and feed into the San Joaquin River basin. Sequoia National Park has three major rivers: Kings, Kaweah and Kern. These watersheds also help to distribute winter precipitation held in the form of snow pack throughout the summer. Lassen Volcanic.

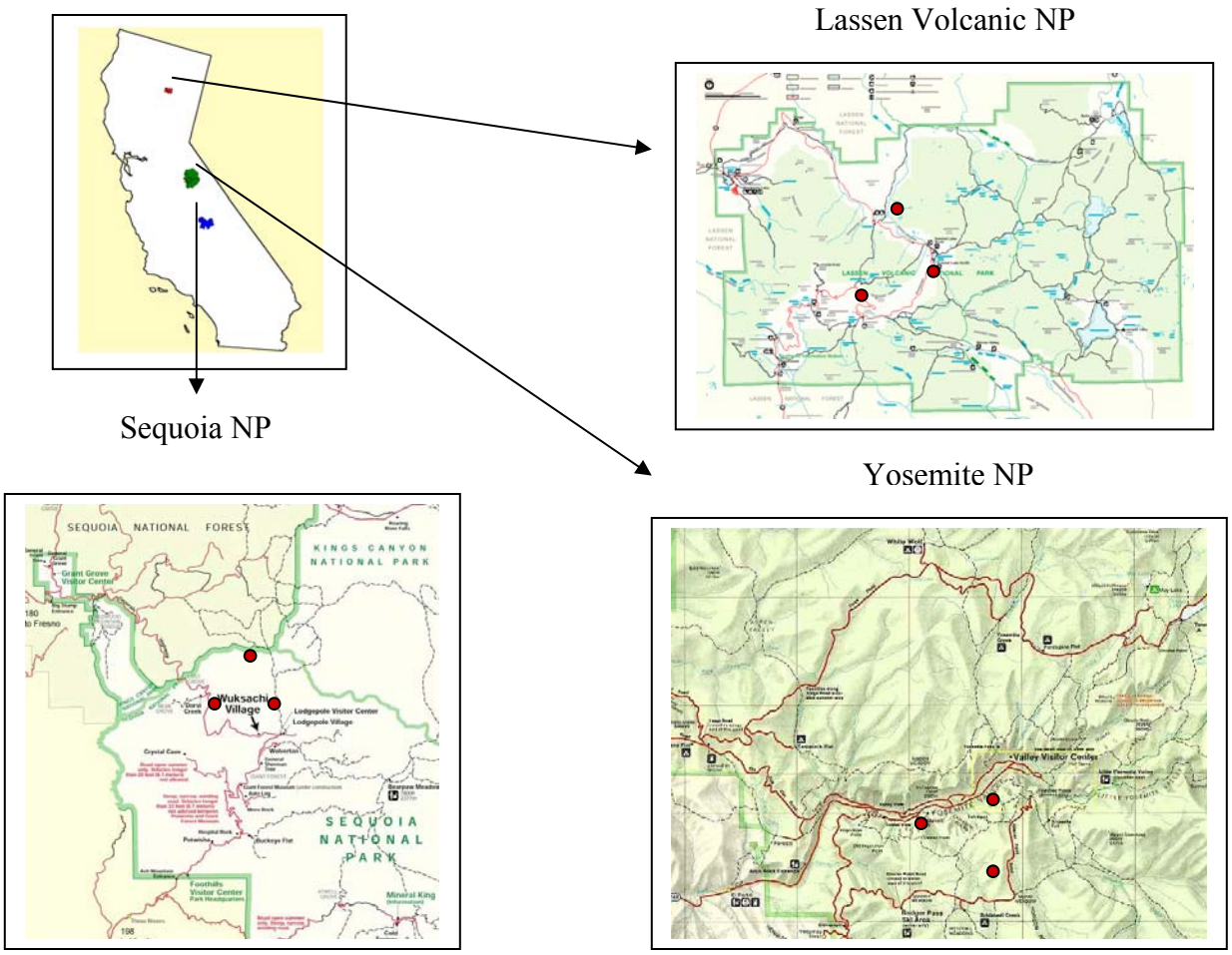

Figure 6.1. Map of California showing meadow pond sites in Lassen Volcanic, Yosemite, and Sequoia National Parks. Map of California was made by Carlos Hinojosa and Amy Hays of the Land Information Systems, Texas A&M University. Parks maps are from the National Park Service, public domain.

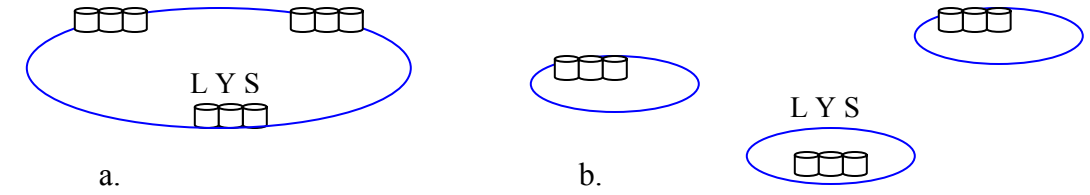

Figure 6.2. Diagram showing cage placements. a. Three sets of 3 cages in one large pond, i.e. Long Meadow, Sequoia. b. One set of three cages in 3 small ponds in wet meadow, i.e. NE Summit Meadow, Yosemite National Park. (L=Lassen; Y= Yosemite; S = Sequoia).

National Park has portions of four drainage basins including Mill, Hat, and Kings Creeks that eventually drain into the Sacramento River.

METHODS

Experimental methods

Two field experiments were conducted: June-August 2001 and June-August 2002. *P. regilla* was selected as a surrogate study species, because of its relative abundance, its wide distribution, and its ability to metamorphose in one summer compared to ~3 years for *R. muscosa.*

Hatchling tadpoles were translocated from a single pond per park and care was taken to collect < 10% of total hatchlings present. Lassen hatchlings were collected from Dersch Meadow, Yosemite tadpoles from Pothole Meadow, and Sequoia tadpoles from Long Meadow. Hatchlings were placed in separate cages for each park origin and

grouped in sets of threes (Lassen, Yosemite, and Sequoia) for a total of nine cages per meadow site (Figure 6.2). Cages were either placed in 3 sets of 3 cages in one large pond as in Long Meadow, Sequoia National Park, or were placed in small ponds in clusters of three in a contiguous palustrine wet meadow as in NE Summit Meadow, Yosemite National Park.

Each cage was a cylinder with bottom and removable lid constructed of rigid 75 µm white Nitex ®teflon (Sefar America Inc., Kansas City, MO) (60 cm depth x 35 cm diameter) (Figure 6.3). This field-tested design by Harris and Bogart [157] was enlarged to provide an internal cage volume for 60 tadpoles that would not cause overcrowding or possible developmental problems [158]. The cylinder was stitched together with nylon thread and plastic rings sewn to the cage sides accommodated a plastic covered metal rod that was driven into the pond bottom [157]. When necessary, cages were moved away from shore and towards the middle of the pond as ponds dried to keep them in water. The top of the cage (40cm) was attached with industrial strength Velcro® for ease of removal. A floating platform (plastic grid side panel of a Jehmco® fry cage) was placed in each cage for metamorphosed frogs when tadpoles reached Gosner stage 42 (appearance of front limbs).

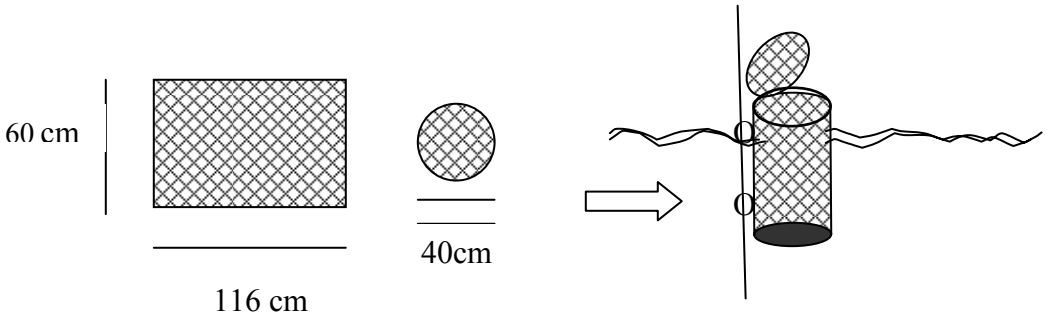

Figure 6.3. Cage dimensions and design.

A crew of 4 technicians and the principal investigator monitored the cages daily. Boiled romaine lettuce was supplied as food. Approximately 90% of the lettuce was organic or certified pesticide-free, the other 10% was carefully washed and rinsed before boiling. Food availability was maximized by providing a continuous supply of boiled lettuce. Measurements of water pH, nitrate/nitrites, dissolved O2, ammonia, hardness, and turbidity were all within normal limits. Tadpoles were sampled at 28 days of exposure and again at metamorphosis, stage 42-46 [102].

Sampling methods

Tadpoles were counted at 28 days and up to 20 tadpoles were collected from each cage. Only half of the tadpoles were sampled in cages that held less than 40 animals. Metamorphs were collected when they reached Gosner stage 42-46. Animals

were immediately transported in plastic fish bags in coolers back to the field research camp and processed.

In 2001, field processing included length measurements, examination for deformities, and the separation of metamorphs from tadpoles. In 2002, animals were measured, weighed, examined for deformities, and staged according to Gosner [102]; then grouped in the following categories: (PL) prelimb (24-26); (LB) limbbud (27-34); (EHL) early hind limb (35-36); (MHL) middle hind limb (37-39); (LHL) late hind limb (40-41); (Meta) metamorph (42-46). Animals were frozen in individual cyrovials in liquid nitrogen dewers for subsequent analyses and shipped on dry ice to Patuxent Wildlife Research Center, USGS, Laurel, Maryland, USA.

Statistical analyses

Mean water temperatures, days to metamorphosis, and snout-vent lengths (SVL) among parks for tadpoles and metamorphs were analyzed with analysis of variance tests (ANOVA) using StatView®, SAS Institute Inc., version 5.01. Chi-square analyses were used to evaluate differences among parks for percent survivorship to metamorphosis and percent malformations. Means are presented ± 1 SE and $\alpha = 0.05$ for evaluating statistical significance. Values did not deviate from a normal distribution and so actual values were used to compute means. Analyses were conducted evaluating total samples for each park by origin of tadpoles and also for total samples of tadpoles deployed at each park regardless of origin. Lassen samples, Yosemite samples, and Sequoia samples

refer to animals that were raised in each park, regardless of origin, unless otherwise stated. Animals from each meadow were combined in the analyses for each park.

Percent survivorship to metamorphosis by park was determined by using the ratio of the total number of metamorphs sampled from that park to the number of animals remaining in a total park's cages after the 28-d tadpoles were sampled.

RESULTS

2001 Field experiment

Snout-vent lengths

A two-way ANOVA showed overall significance ($p<0.0001$) among mean tadpole SVL among parks and between park origin and deployment (Tables 6.2, 6.3). The interaction between origin and deployment was also significant ($p=0.0001$) revealing that the differences between tadpole SVLs by park origin was dependent upon the park where they were raised (Figure 6.4). Sheffe's post hoc test showed significant differences between Lassen and the other two parks by park origin and between Sequoia and the other two parks by park of deployment (Tables 6.4, 6.5).

Table 6.2 ANOVA table of tadpole SVL means by park deployed and park origin for total samples, 2001.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	1457.129	728.565	44.707	<0.0001	89.414	1.000
Park Origin	2	352.477	176.238	10.815	<0.0001	21.629	0.996
Park Deployed*Park Origin	4	692.824	173.206	10.629	< 0.0001	42.514	1.000
Residual	1692	27573.441	16.296				

Table 6.3. Tadpole SVL means by park deployed and park origin for total samples, 2001.

Park Origin	Park Deployed	N	Mean (µmol/min/gr)	Standard Error
L	L	237	15.3	0.13
S	L	218	15.5	0.12
Y	L	113	16.6	0.17
L	Y	194	14.4	0.22
S	Y	155	16.8	0.15
Y	Y	87	16.6	0.33
L	S	325	14.1	0.45
S	S	239	14.4	0.12
Y	S	133	13	0.19

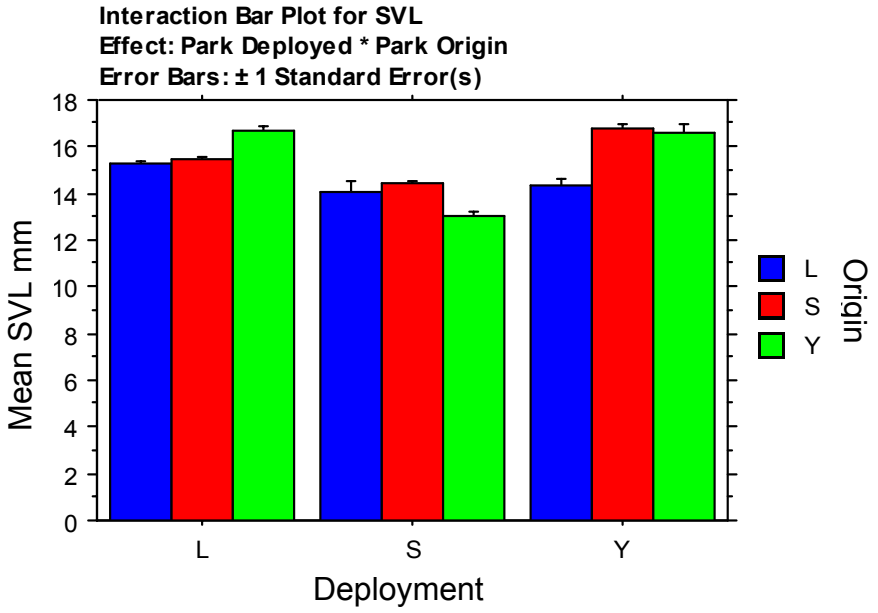

Figure 6.4. Interaction graph for tadpole SVLs, 2001. (Legend refers to origin of tadpoles; x axis indicates park deployed.)

Table 6.4. Scheffe for tadpole SVL by park origin, 2001.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	14.1, 15.2	-0.85	0.54	0.0006*
L,Y	14.1, 15.2	-0.67	0.65	0.04*
S,Y	15.2, 15.2	0.18	0.67	0.8

*Indicates statistical significance (Means represent total animals by origin. Lassen origin means: 15.3, 14.4, 14.1 mm; Yosemite origin means: 16.6, 16.6, 13 mm; and Sequoia origin means: 15.5, 14.4, 16.6 mm.)

Table 6.5. Scheffe for tadpole SVL by park deployment, 2001.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	15.6, 13.9	1.63	0.56	<0.0001*
L,Y	15.6, 15.6	-0.04	0.63	0.99
S,Y	13.9, 15.6	-1.66	0.6	<0.0001*

*Indicates statistical significance (Means represent total animals by origin. L, Y, and S. Lassen deployment means: 15.3, 16.6, 15.5 mm; Yosemite deployment means: 14.4,16.6, 16.8 mm; and Sequoia deployment means: 14.1, 13, 14.4 mm.)

A two-way ANOVA showed an overall significance ($p<0.0001$) among mean Meta SVL among parks and between park origin and deployment (Tables 6.6, 6.7). The interaction between origin and deployment was not significant ($p=0.14$) (Figure 6.5). Sheffe's post-hoc test showed significant differences between Yosemite and the other two parks by park origin and between Yosemite and the other two parks by park of deployment (Tables 6.8, 6.9).

Table 6.6. ANOVA table of Meta SVL means by park deployed and park origin for total samples, 2001.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	212.748	106.374	30.278	<0.0001	60.555	1.000
Park Origin	2	200.748	106.374	28.570	<0.0001	57.140	1.000
Park Deployed*Park Origin	4	24.568	6.142	1.748	0.1373	6.993	0.529
Residual	848	2979.257	3.513				

Table 6.7. Meta SVL means by park deployed and park origin for total samples, 2001.

Park Origin	Park Deployed	N	Mean	Standard Error
L	L	266	15.0	0.13
S	L	220	15.0	0.12
Y	L	97	17.2	0.18
L	Y	105	16.4	0.18
S	Y	57	16.2	0.16
Y	Y	47	17.7	0.21
L	S	14	14.7	0.37
S	S	33	14.6	0.25
Y	S	18	15.9	0.36

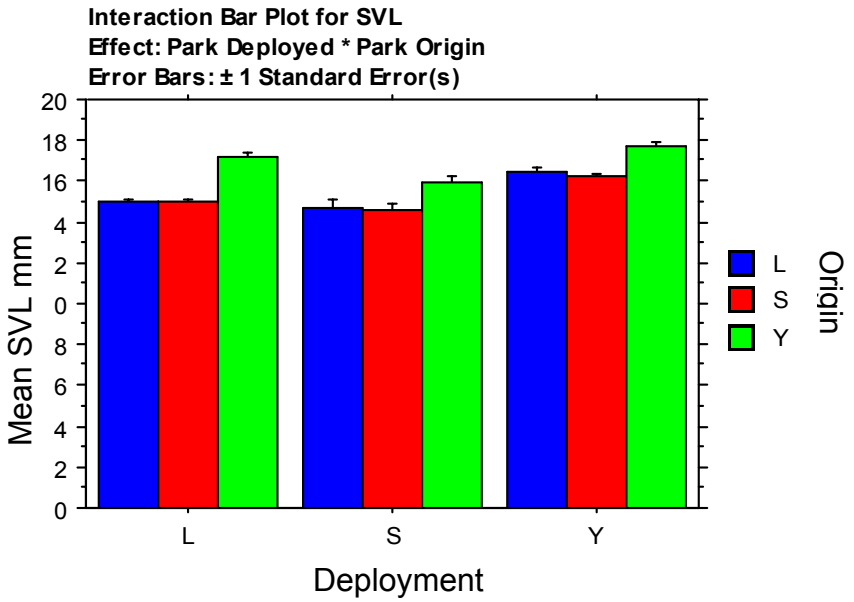

Figure 6.5. Interaction graph for Meta SVLs, 2001. (Legend refers to origin of tadpoles; x axis indicates park deployed.)

Table 6.8. Scheffe for Meta SVL by park origin, 2001.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	15.4, 15.3	.237	0.35	0.26
L,Y	15.4, 17.2	-1.81	0.43	<0.0001*
S,Y	15.3, 17.2	-2.05	0.45	<0.0001*

*Indicates statistical significance (Means represent total animals by origin. L, Y, and S. Lassen origin means: 15, 16.4, 14.7 mm; Yosemite origin means: 17.2, 17.7, 15.9 mm; and Sequoia origin means: 15, 16.2, 14.6 mm.)

Table 6.9. Scheffe for Meta SVL by park deployment, 2001.

Parks	Mean (mm)	Mean Diff.	Critical Diff	P-Value
L,S	15.4, 15	0.36	0.6	0.34
L,Y	15.4, 16.6	-1.35	0.37	<0.0001*
S,Y	15, 16.6	-1.71	0.65	<0.0001*

*Indicates statistical significance (Means represent total animals by origin. L, Y, and S. Lassen deployment means: 15, 17.2, 15 mm; Yosemite deployment means: 16.4, 17.7, 16.2 mm; and Sequoia deployment means: 14.7, 15.9, 14.6 mm.)

Time (days) to metamorphosis

A two-way ANOVA showed an overall significance ($p<0.0001$) among mean time (in days) to metamorphosis among parks and between park origin and deployment (Tables 6.10, 6.11). The interaction between origin and deployment was significant ($p=0.003$) indicating that differences among mean time to metamorphosis by park origin may be dependent upon the park where they were raised (Figure 6.6). Sheffe's post-hoc test showed significant differences among means time to metamorphosis for all three parks for both origin and deployment (Tables 6.12, 6.13). Overall values ranged from 25 to 73 days; Lassen had the shortest mean time to metamorphosis regardless of origin.

Table 6.10. ANOVA table of mean time (days) to metamorphosis by park deployed and park origin for total samples, 2001.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	41254.881	20627.440	481.490	<0.0001	962.980	1.000
Park Origin	2	5639.740	2819.870	65.822	<0.0001	131.644	1.000
Park Deployed*Park Origin	4	692.879	173.220	4.043	0.0030	16.173	0.923
Residual	863	36971.654	42.841				

Table 6.11. Mean time (days) to metamorphosis by park deployed and park origin for total samples, 2001.

Park Origin	Park Deployed	N	Mean (d)	Standard Error
L	L	267	42.8	0.34
S	L	223	35.6	0.4
Y	L	99	42.1	0.57
L	Y	108	53.5	0.66
S	Y	61	45.5	1.29
Y	Y	48	56.8	1.45
L	S	14	68	0.83
S	S	34	59.5	0.95
Y	S	18	63.9	1.63

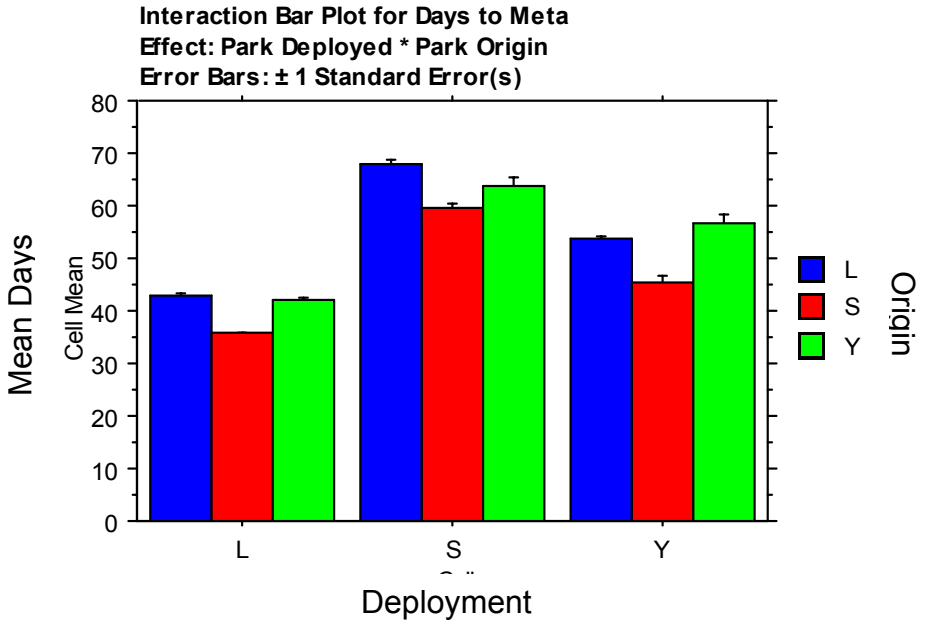

Figure 6.6. Interaction graph for mean time (days) to metamorphosis. (Legend refers to origin of tadpoles; x axis indicates park deployed.)

Table 6.12. Scheffe for time (days) to Meta by park origin, 2001.

Parks	Means (d)	Mean Diff.	Critical Diff	P-Value
L,S	46.7, 40.1	6.6	1.21	<0.0001*
L,Y	46.7, 48.8	-2.05	1.49	0.004*
S,Y	40.1, 48.8	-8.654	1.54	<0.0001*

*Indicates statistical significance (Means represent total animals by origin. Lassen origin means: 42.8, 53.5, 68 d; Yosemite origin means: 42.1, 56.8, 63.9 d; and Sequoia origin means: 35.6, 45.5, 59.5 d.)

Table 6.13. Scheffe for time (days) to Meta by park deployed, 2001.

Parks	Means (d)	Mean Diff.	Critical Diff	P-Value
L,S	40, 62.5	-22.56	2.08	<0.0001*
L,Y	40, 52	-12.06	1.27	<0.0001*
S,Y	62.5, 5	10.49	2.26	<0.0001*

*Indicates statistical significance (Means represent total animals by deployment. Lassen deployment means: 42.8, 42.1, 35.6 d; Yosemite deployment means: 53.5, 56.8, 45.5 d; and Sequoia deployment means: 68, 63.9, 59.5 d.)

Survivorship to metamorphosis

In 2001, 71% (591/836) of Lassen tadpoles, 45% (244/295) of Yosemite tadpoles, and 8 % (68/842) of Sequoia tadpoles reached metamorphosis. Three separate 2x2 chi-square analyses of the ratios of survivorship to metamorphosis from the three parks revealed a significant ($p<0.0001$) difference in these ratios among all three parks. Significance was calculated at $\alpha=0.0167$ according to the Bonferroni/Dunn post hoc test.

Bilateral hind limb deformity

A bilateral hind limb deformity (brachydactyly), a truncation of the proximal hindlimb joint, was observed in late hind limb tadpoles and metamorphs (Figure 6.7).

Figure 6.7. Photographs of late hind limb tad and metamorph with brachydactyly. The white line indicates the truncation of the joint.

The rate of deformities per park deployed was calculated by using the ratio of the number animals with the deformity (both late stage tadpoles and metamorphs) to the total number of animals reaching metamorphosis or surviving as undeveloped tads at the end of the experiment. In 2001, 6% (42/675) of Lassen animals, 25% (70/275) of Yosemite animals, and 7% (24/337) of Sequoia animals showed this hind limb deformity.

Three separate 2x2 chi-square analyses of the ratios of hind limb deformity from the three parks revealed a significant ($p<0.0001$) difference in these ratios between Yosemite and the other two parks. Significance was calculated at $\alpha=0.0167$ according to the Bonferroni/Dunn post hoc test.

2002 Field experiment

Snout-vent lengths

A two-way ANOVA showed an overall significance ($p<0.0001$) among mean tadpole SVL among parks and between park origin and deployment (Tables 6.14, 6.15). The interaction between origin and deployment was also significant ($p=0.0001$) revealing that the differences between tadpole SVLs by park origin was dependent upon the park where they were raised (Figure 6.8). Sheffe's post hoc test showed significant differences between Lassen and the other two parks by park origin and among all parks by deployment (Tables 6.16, 6.17).

Table 6.14. ANOVA table of mean tadpole SVL by park deployed and park origin for total samples, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	2049.579	1024.789	161.725	<0.0001	323.449	1.000
Park Origin	2	284.640	142.320	22.460	<0.0001	44.920	1.000
Park Deployed*Park Origin	4	603.820	150.955	23.823	<0.0001	95.291	1.000
Residual	1836	11634.049	6.337				

Table 6.15. Mean tad SVL by park deployed and park origin for total samples, 2002.

Park Origin	Park Deployed	N	Mean	Standard Error
L	L	167	15.1	0.15
S	L	165	14.7	0.21
Y	L	176	12.8	0.17
L	Y	139	15.6	0.20
S	Y	184	14.1	0.18
Y	Y	180	15.7	0.15
L	S	323	13	0.14
S	S	300	12.6	0.16
Y	S	211	12.2	0.21

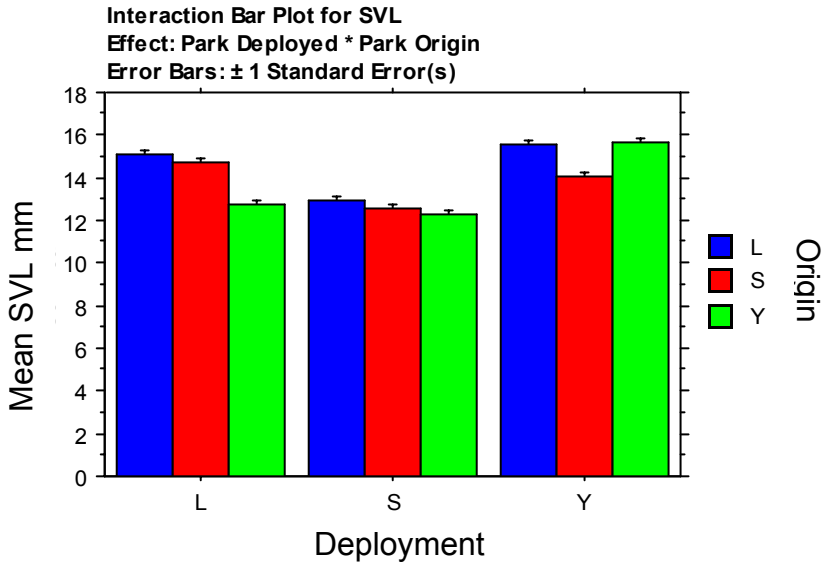

Figure 6.8. Interaction graph for mean tadpole SVLs, 2002. (Legend refers to origin of tadpoles; x axis indicates park deployed.)

Table 6.16. Scheffe for mean tadpole SVLs by park origin, 2002.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	14.1, 13.6	0.55	0.35	0.0005*
L,Y	14.1, 13.5	0.59	0.36	0.0003*
S,Y	13.6, 13.5	0.03	0.35	0.98

*Indicates statistical significance (Means represent total animals by origin. Lassen origin means: 15.1, 15.6, 13 mm; Yosemite origin means: 12.8, 12.2, 15.7 mm; and Sequoia origin means: 14.7, 12.6, 14.1 mm.)

Table 6.17. Scheffe for mean tadpole SVLs by park deployed, 2002.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	14.1 12.6	1.49	0.35	<0.0001*
L,Y	14.1, 15.1	-0.92	0.39	<0.0001*
S,Y	12.6, 15.1	-2.41	0.35	<0.0001*

*Indicates statistical significance (Means represent total animals by deployment. Lassen deployment means: 15.1, 14.7, 12.8 mm; Yosemite deployment means: 15.6, 14.1, 15.7 mm; and Sequoia deployment means: 13, 12.6, 12.2 mm.)

A two-way ANOVA showed an overall significance ($p<0.0001$) among mean Meta SVL among parks and between park origin and deployment (Tables 6.18, 6.19). The interaction between origin and deployment was not significant ($p=0.14$) (Figure 6.9). Sheffe's post-hoc test showed significant differences between Yosemite and the other two parks by park origin and between Sequoia and the other two parks by deployment (Tables 6.20, 6.21).

Table 6.18 ANOVA table of mean Meta SVL by park deployed and park origin for total samples, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	661.422	330.711	106.081	<0.0001	212.161	1.000
Park Origin	2	70.084	35.042	11.240	<0.0001	22.481	0.997
Park Deployed*Park Origin	4	55.789	13.947	4.474	0.0014	17.895	0.951
Residual	1403	4373.914	3.118				

Table 6.19. Mean Meta SVL by park deployed and park origin for total samples, 2002.

Park Origin	Park Deployed	N	Mean	Standard Error
L	L	248	16.3	0.13
S	L	159	17.2	0.16
Y	L	204	17	0.13
L	Y	124	16.2	0.15
S	Y	151	16.1	0.13
Y	Y	251	16.8	0.1
L	S	58	14.7	0.2
S	S	129	14.8	0.15
Y	S	88	15.2	0.16

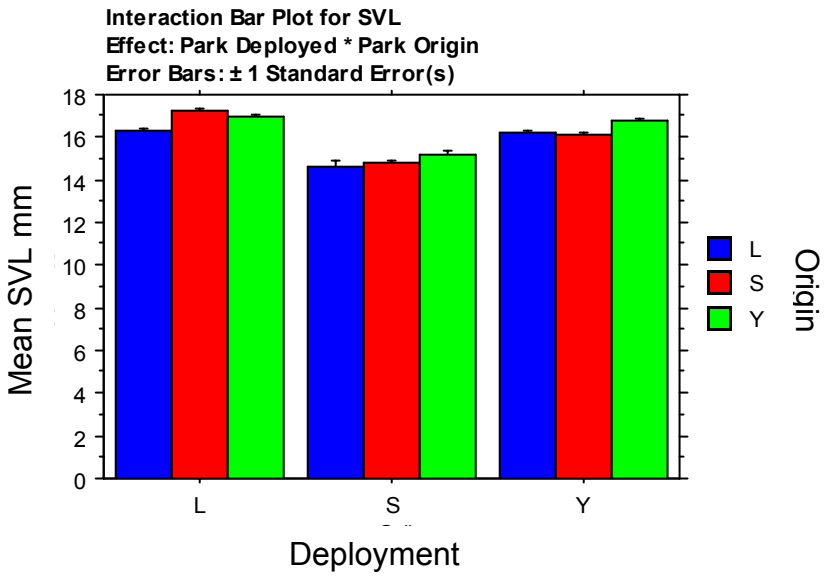

Figure 6.9. Interaction graph for mean Meta SVLs, 2002. (x axis indicates park deployed.)

Table 6.20. Scheffe for mean Meta SVLs by park origin, 2002.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	16.1, 16.1	-0.07	0.35	0.86
L,Y	16.1, 16.6	-0.56	0.36	<0.0001*
S,Y	16.1, 16.6	-0.5	0.35	<0.0001*

*Indicates statistical significance (Means represent total animals by origin. Lassen origin means: 16.3, 14.7, 16.2 mm; Yosemite origin means: 17, 15.2, 16.8 mm; and Sequoia origin means: 17.2, 14.8, 16.1 mm.)

Table 6.21. Scheffe for mean Meta SVLs by park deployed, 2002.

Parks	Means (mm)	Mean Diff.	Critical Diff	P-Value
L,S	16.8, 14.9	1.87	0.32	<0.0001*
L,Y	16.8, 16.5	0.31	0.26	0.014*
S,Y	14.9, 16.5	-1.56	0.32	<0.0001*

*Indicates statistical significance (Means represent total animals by deployment. Lassen deployment means: 16.3, 17.2, 17 mm; Yosemite deployment means: 16.2, 16.1, 16.8 mm; and Sequoia deployment means: 14.7, 14.8, 15.2 mm.)

Time (days) to metamorphosis

A two-way ANOVA showed an overall significance ($p<0.0001$) among mean time (days) to metamorphosis among parks and between park origin and deployment (Tables 6.22, 6.23). The interaction between origin and deployment was significant ($p<0.0001$) indicating that differences among mean time to metamorphosis by park origin was dependent upon the park where they were raised (Figure 6.10). Sheffe's post-hoc test showed significant differences among means days to metamorphosis for all three parks for deployment; Lassen was different between the other two parks by origin. (Tables 6.24, 6.25). Lassen had the shortest mean time to metamorphosis regardless of origin. Overall values ranged from 27 to 88 days.

Table 6.22. ANOVA table of mean time (days) to metamorphosis by park deployed and park origin for total samples, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Park Deployed	2	32444.167	16222.083	221.326	<0.0001	442.653	1.000
Park Origin	2	13046.201	6523.101	88.998	<0.0001	177.996	1.000
Park Deployed*Park Origin	4	4998.113	1249.528	17.048	0.0014	68.192	1.000
Residual	1403	102832.672	73.295				

Table 6.23. Mean time (days) to metamorphosis by park deployed and park origin for total samples, 2002.

Park Origin	Park Deployed	N	Mean (d)	Standard Error
L	L	248	48.5	0.4
S	L	159	51.4	0.8
Y	L	204	56	0.44
L	Y	124	58.7	0.63
S	Y	151	58.7	0.86
Y	Y	251	53.6	0.58
L	S	58	58.5	1.08
S	S	129	68.8	1
Y	S	88	68.5	0.77

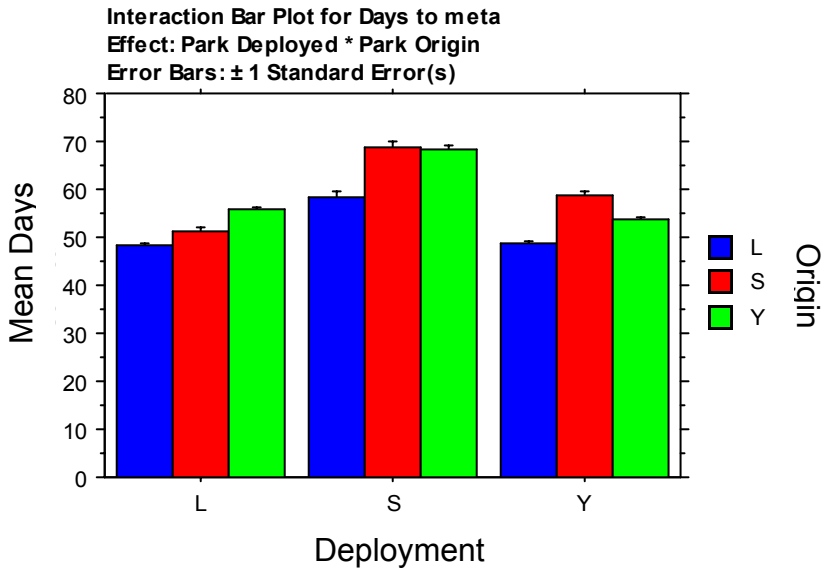

Figure 6.10. Interaction graph for time (days) to metamorphosis. (x axis indicates park deployed.)

Table 6.24. Scheffe for mean time (days) to Meta by park origin, 2002.

Parks	Means (d)	Mean Diff.	Critical Diff	P-Value
L,S	52.8, 59	-9.08	1.14	<0.0001*
L,Y	52.8, 56.9	-7.0	1.09	<0.0001*
S,Y	59, 56.9	2.09	1.08	0.0002*

*Indicates statistical significance (Means represent total animals by origin. Lassen origin means: 48.5, 58.5, 58.7 d; Yosemite origin means: 56, 68.5, 53.6 d; and Sequoia origin means: 51.4, 68.8, 58.7 d.)

Table 6.25. Scheffe for mean time (days) to Meta by park deployed, 2002.

Parks	Means (d)	Mean Diff.	Critical Diff	P-Value
L,S	51.8, 66.5	-14.8	1.22	<0.0001*
L,Y	51.8, 56.3	-2.18	1.0	<0.0001*
S,Y	66.5, 56.3	12.61	1.25	<0.0001*

*Indicates statistical significance (Means represent total animals by deployment. Lassen deployment means: 48.5, 51.4, 56 d; Yosemite deployment means: 58.7, 58.7, 53.6 d; and Sequoia deployment means: 58.5, 68.8, 68.5 d.)

Survivorship to metamorphosis

In 2002, 53% (541/1018) of Lassen tadpoles, 57% (489/374) of Yosemite tadpoles, and 25 % (275/809) of Sequoia tadpoles reached metamorphosis. Three separate 2x2 chi-square analyses of the ratios of survivorship to metamorphosis from the three parks revealed a significant ($p<0.0001$) difference in these ratios between Sequoia and the other two parks. Significance was calculated at $\alpha=0.0167$ according to the Bonferroni/Dunn post hoc test.

Bilateral hind limb deformity

The rate of deformities per park deployed was calculated by using the ratio of the number animals with the deformity (both late stage tadpoles and metamorphs) to the total number of animals reaching metamorphosis or surviving as undeveloped tads at the end of the experiment. In 2002, 20% (114/452) of Lassen animals, 14% (71/507) of Yosemite animals, and 7% of Sequoia animals (43/561) showed this hind limb deformity.

Three separate 2x2 chi-square analyses of the ratios of hind limb deformity from the three parks revealed significant (L,S $p<0.0001$; L,Y $p=0.008$; Y,S $p=0.0002$) differences in these ratios among all three parks. Significance was calculated at $\alpha=0.0167$ according to the Bonferroni/Dunn post hoc test.

Water temperatures

Daily water temperatures for each cage were collected in 2001. The means of these daily temperatures for each cage were compared among parks of deployment. An overall analysis of variance showed statistical significance among the means ($p<0.0001$; DF=2, 87; F-value=89) (Tables 6.26, 6.27). Scheffe's post hoc test shows significance among mean water temperatures at all three parks (Figure 6.11).

Table 6.26. ANOVA table of water means by park deployed.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	642.056	321.028	71.567	<0.0001	143.134	1.000
Residual	78	349.884	4.486				

Table 6.27. Water temperature means by park, 2001.

Park	N	Mean	Standard Error
L	27	22.1	0.23
S	27	15.2	0.17
Y	27	18.3	0.65

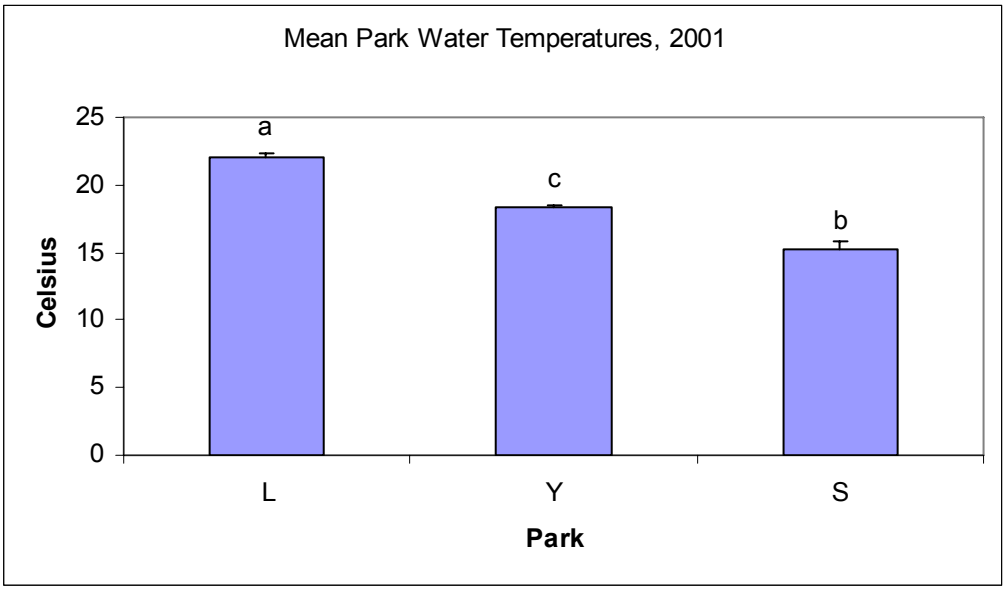

Figure. 6.11. Mean water temperature by park, 2001. Lowercase letters indicate differences among means.

Minimum and maximum water temperatures were collected daily for each meadow pond in 2002 and ANOVA was used to compare means among parks. The overall ANOVA was not significant for minimum temperatures (Tables 6.28, 6.29). However, maximum water temperatures were significantly different among the three

parks in 2002 (Tables 6.30, 6.31; Figure 6.12). Overall ANOVA was significant ($p<0.0001$, DF= 2, 549; F-value=135), and Sheffe's post-hoc test was also significant among all three parks (Figure 6.12).

Table 6.28. ANOVA table of minimum water temperature means by park, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	229.192	114.596	2.337	0.0976	4.673	0.461
Residual	549	26925.550	49.045				

Table 6.29. Minimum mean water temperature by park, 2002.

Park	N	Mean	Standard Error
L	166	14.1	0.39
S	200	12.6	0.72
Y	186	12.7	0.23

Table 6.30. ANOVA table of maximum water temperature means by park, 2002.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Deployed	2	2741.518	1370.759	135.122	<0.0001	270.244	1.000
Residual	549	5569.393	10.145				

Table 6.31. Maximum mean water temperature by park, 2002.

Park	N	Mean	Standard Error
L	166	21.4	0.35
S	200	19.3	0.17
Y	186	24.6	0.19

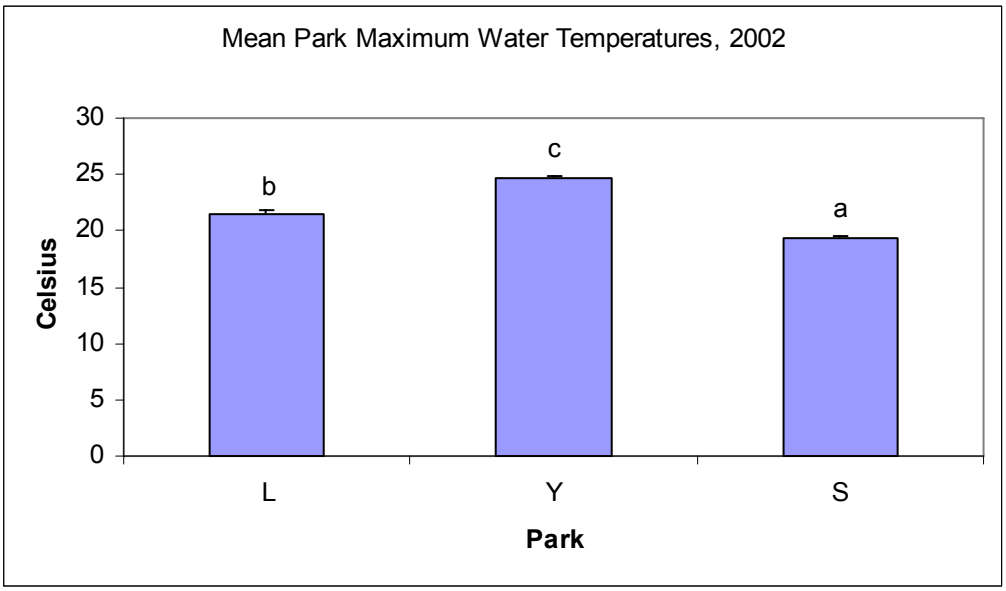

Figure 6.12. Mean maximum water temperatures by park, 2002.

DISCUSSION

Snout-vent lengths

Tadpole mean SVLs in 2001 were significantly lower in animals raised in Sequoia and were significantly lower in animals originating from Lassen. Tadpoles

originating from Lassen declined in SVLs when moved, tadpoles originating from Yosemite declined/or stayed the same in SVLs when moved and tadpoles originating from Sequoia increased in SVLs when moved. In 2002, tadpoles deployed in Sequoia again showed the lowest means for SVLs, however, Lassen origin tadpoles showed significantly higher means. Lassen origin SVLs were smaller when moved to Sequoia; Sequoia origin SVLs were always larger when moved; and Yosemite origin SVLS were smaller when moved in all cases. Yosemite SVLs seem to be largest by deployment when pooled across origin; Lassen SVLs seem to be largest when pooled across deployment.

Meta mean SVLs in 2001 showed Yosemite metamorphs by origin and deployment to be significantly larger than those of Lassen or Sequoia. Metamorphs of Lassen origin declined in SVL in Sequoia and increased in Yosemite; metamorphs of Sequoia origin increased when moved; and metamorphs of Yosemite origin declined in Sequoia and increased in Lassen. While there was no interaction effect for both years for Metas, Yosemite origin animals were again larger in 2002, and Sequoia Metas continued to be smaller. In 2002, Lassen origin metamorphs were smaller when moved to Sequoia (than when deployed at the other two parks); Sequoia origin metamorphs were always bigger when moved to the other two parks (than when raised at Sequoia); and Yosemite origin metamorphs were smaller when moved to Sequoia (than when raised at the other two parks). Animals deployed in Lassen were always the largest of the three parks, and animals deployed in Sequoia were always the smallest of the three parks.

Time to metamorphosis

In 2001, tadpoles raised at Lassen reached metamorphosis more quickly than tadpoles raised at the other two parks. This factor could be influenced by temperature, in that Lassen also had the highest mean water temperature (22.1±0.23 °C) of the three parks in 2001. In 2002, however, Yosemite had the highest mean water temperature (24.6±0.19 °C) and showed the largest animals, but still had a longer mean time to metamorphosis than did animals raised at Lassen. Tadpoles raised at Sequoia took the longest time to reach metamorphosis in 2001 (62.5±0.8 d) and 2002 (66.5±0.63 d) and also had the lowest mean water temperatures for both years (2001, 15.2±0.17 °C; 2002, minimum 12.6±0.72 °C, maximum 19.3±0.17 °C). In 2001, Lassen origin animals' time to metamorphosis increased when deployed in Sequoia or Yosemite; Sequoia origin animals' time to metamorphosis decreased when deployed in Lassen or Yosemite; and Yosemite origin animals' time to metamorphosis increased in Sequoia, and declined in Lassen. This same trend held for 2002, with the exception that Yosemite origin animals' time to metamorphosis stayed the same in Lassen.

Larval growth history is an important factor affecting metamorphosis [246]. Time to metamorphosis and size at metamorphosis may be altered by pesticide exposure [247,248,235,249] and have also been correlated with adult survival and reproductive success [250,251,252].

Survivorship to metamorphosis

In 2001, survival from day 28 to metamorphosis at Lassen (reference site) was significantly different from the other two parks; many more animals survived in Lassen compared to the other two parks. While this correlates with known pesticide exposures, we did not see the same pattern in 2002. In that year survival at Lassen did not differ from that at Yosemite but both were significantly different than Sequoia.

It is possible we are seeing the effects of low water temperatures early in the season in Lassen in 2002. Animals raised at Lassen in 2002 may have been low temperature stressed due to an unusually large and late winter storm that year [243]. Our entry into our study sites at Lassen was delayed because of road closures due to snow hazards. Approximately 4 cages of the first group of tadpoles translocated to our Hemlock site in Lassen died within days due to extreme low temperature shock and had to be replaced. The minimum temperatures recorded the first week at this site ranged from 0.1-5.1°C and the maximum temperatures were 2.6-9.9°C. These temperatures fell outside the range recorded our first year (9-32°C) in this area. The remaining animals may have suffered cold stress that resulted in decreased health and survival [184].

Bilateral hind limb deformity

The hind limb deformity observed in 2001 was most pronounced in animals raised in Yosemite. However, in 2002, Lassen had the greatest rate of deformity among the three parks. Because this deformity is bilateral, it appears to be developmental in origin and does not fit the typical profile of a deformity caused by trematodes [253–255].

Amphibian limb deformities have been associated with agricultural pesticides [41,256,182,257,143]. In several cases the link has been shown to be related to suppressed immune function and a subsequent increase in parasitic loads, resulting in limb deformities. However, Bridges [248] also showed developmental limb malformations in *R. sphenocephala* exposed in the laboratory to carbaryl.

Our research assistant in Yosemite did observe free-swimming late hind limb to early stage metamorphs with this deformity. However, a field survey of free-ranging newly metamorphosed animals at all three parks did not reveal any deformed animals, although the techniques used for capture (sweep nets combined with visual hand captures) probably pre-selected for active animals. Frogs with this deformity were observed to have a severely limited range of motion in their hind limbs. The limbs were usually locked in an awkward position extending at right angles from the body. Their ability to hop was extremely limited and they often drowned in our cages because they could not crawl or hop onto the platform provided for newly metamorphosing animals. Based on the average size ranges of metamorphs at the three parks, this problem becomes apparent when the proximal hind limb joint is less than 5 mm in length.

While it is difficult to postulate the cause of this deformity, it is certain that it causes severe problems for the animal. It is probable that we were able to observe this phenomenon because our cages protected these animals from predation.

Summary

Although it is difficult to tease out clear pattern, overall, animals at Sequoia did not fare as well in survivorship to metamorphosis, time to metamorphosis, and size at metamorphosis. The deformity results do not fit this pattern, however, this may be related to the fact that fewer Sequoia animals developed to the stage where a deformity could be observed. This trend for Sequoia may be related to temperature differences, which were also lowest at this park, or they may also be related to pesticide impacts. Sequoia is the southern-most park of the three and is adjacent to and downwind of intensive agriculture.

When combined with historical pesticide use and amphibian declines [69], current pesticide use [79], pesticide residues in *P. regilla* [64] (Chapter III), DNA damage (Chapter IV), and ChE inhibition [64] (Chapter V), the argument for pesticide impacts becomes even more compelling. Further statistical studies are needed to better understand other possible relationships among possible pesticide effects and amphibians at these three parks.

CHAPTER VII

CONCLUSIONS

Amphibian declines in the Sierra Nevada Mountains are dramatic and will probably result in species extinctions if probable causes and solutions are not discovered quickly. My research in this area is based on the documentation of aerial transport of agricultural chemicals into Yosemite and Sequoia National Parks. Most of the ranid species in these parks are in decline; therefore I chose a surrogate species, the Pacific chorus frog *(Pseudacris regilla)* for my translocation experiments. The field experiment design allowed me to examine differences in various biological endpoints affected by pesticide exposure.

Most ecotoxicological studies are forensic or epidemiological in nature. It is difficult to show causality, especially with pesticides that do not readily bioaccumulate in animal tissues. Although I did not find organophosphate residues in frog tissues, the presence of DDE in tadpoles and metamorphs raised in Yosemite and Sequoia regardless of origin indicates the animals obtained most of this chemical through the water. Even though it is no longer being applied in this area, frogs are still able to accumulate it in their tissues in a matter of weeks. While adult chorus frogs do not spend large amounts of time in water, ranid frogs such as the declining mountain yellow-legged frog often take up to three years to metamorphose, and spend significant time in the water as adults.

Variation in cellular DNA content, which indicates chormosomal damage, was also significantly higher in frogs raised in Yosemite and Sequoia regardless of origin. Although this measurement cannot prove causality, it adds to our weight of evidence. It is noteworthy that this was discovered in newly metamorphosed chorus frogs. Longer lived ranid frogs may accumulate genetic damage resulting in mutations, possible disease, or decreased reproductive fitness. Over time, such damage could lead to loss of genetic variability and ultimately to species extinctions.

Cholinesterase (ChE) inhibition is another biomarker of pesticide exposure and can be a powerful tool when combined with other indicators. In 2001, the reference site produced the hypothesized result. Frogs raised at Lassen had higher levels of ChE activity when compared to animals raised at Yosemite and Sequoia. However, the second year the ChE levels for animals raised at Lassen were not significantly different from those raised at Yosemite, even though Sequoia levels were still low. Pesticide levels may fluctuate from year to year due to prevailing winds, snow melt, or other weather conditions. Frogs raised at Lassen in 2002 may have also been severely cold stressed at the beginning of the season, and not able to recover fully.

Other researchers have shown that time to metamorphosis, size at metamorphosis and survivorship to metamorphosis may be altered by pesticide exposure, and affect subsequent reproductive fitness and survival. These endpoints are now being used as reliable indicators to assess sublethal effects of pesticides. I saw a similar trend in these measurements; animals raised at Sequoia had significantly longer time to metamorphosis, a lower rate of animals reaching metamorphosis, and smaller size at

metamorphosis (Table 7.1). Sequoia National Park is also adjacent and downwind of intensive agriculture where thousands of pounds of pesticides are applied annually.

Table 7.1. Weight of evidence table.

Evidence	Lassen	Yosemite	Sequoia
Pesticide Residues—DDE	**15%**	**97%**	**84%**
Residues—Endosulfan	**9%**	**24%**	**3%**
DDE—Metas (ppb)	**0.18**	**2.18**	**5.70**
DDE—Tadpoles (ppb)	**0.07**	**1.54**	**2.17**
Cellular DNA Content	**4.97**	**5.75**	**5.73**
ChE—Metas 2001	**high**	**medium**	**low**
ChE—Metas 2002	**ns**	**ns**	**ns**
SVL—Tadpoles 2001	**medium**	**biggest**	**smallest**
SVL—Metas 2001	**medium**	**biggest**	**smallest**
SVL—Tadpoles 2002	**medium**	**biggest**	**smallest**
SVL—Metas 2002	**biggest**	**medium**	**smallest**
Survivorship 2001	**71%**	**45%**	**8%**
Survivorship 2002	**53%**	**57%**	**25%**
Metamorphosis 2001	**fastest**	**medium**	**slowest**
Metamorphosis 2002	**fastest**	**medium**	**slowest**
Malformations 2001	**6%**	**25%**	**7%**
Malformations 2002	**20%**	**14%**	**7%**
Best outcome	**10**	**4**	**2**
Worst outcome	**1**	**4**	**11**

Table 7.1 compiles the weight of evidence and summary of results from this study. The best to worst outcomes follow the documented concentration gradient of pesticides reaching the three parks. Lassen has the best outcome and also the least amount of pesticides documented in water, sediment, and tissues. Yosemite is in the moderate range, and Sequoia National Park, which has the closest proximity to intensive agriculture, has the worst outcome.

All these findings are pieces of the large and complex puzzle we are trying to solve. When added together with historical pesticide-use data that correlates with amphibian declines in California; with current pesticide use upwind from these areas; with documented atmospheric transport of pesticides to the Sierra Nevada Mountains; with pesticide residues found in frog tissues in these areas by other researchers; and with the dramatic declines of amphibians; the pieces begin to fit together. As the evidence grows, it becomes increasingly probable that pesticides are having or have had detrimental effects on Sierra Nevada amphibian populations.

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