12-1-2010

The effects of two insecticides on California anurans (Rana sierrae and Pseudacris sierra) and the implications for declining amphibian populations.

David Dimitrie
Southern Illinois University Carbondale, ddimitrie@gmail.com

THE EFFECTS OF TWO INSECTICIDES ON CALIFORNIA ANURANS (*RANA SIERRAE* AND *PSEUDACRIS SIERRA*) AND THE IMPLICATIONS FOR DECLINING AMPHIBIAN POPULATIONS

by

David A. Dimitrie

B.S., Michigan State University, 2005

A Thesis
Submitted in Partial Fulfillment of the Requirements for the
Master of Science Degree

Department of Zoology
in the Graduate School
Southern Illinois University Carbondale
December 2010

THESIS APPROVAL

THE EFFECTS OF TWO INSECTICIDES ON CALIFORNIA ANURANS (*RANA SIERRAE* AND *PSEUDACRIS SIERRA*) AND THE IMPLICATIONS FOR DECLINING AMPHIBIAN POPULATIONS

By

David A. Dimitrie

A Thesis Submitted in Partial

Fulfillment of the Requirements

for the Degree of

Master of Science

in the field of Zoology

Approved by:

Dr. Donald Sparling, Chair

Dr. Richard Halbrook

Dr. Matt Whiles

Graduate School
Southern Illinois University Carbondale
July 29, 2010

AN ABSTRACT OF THE THESIS OF

David A. Dimitrie, for the Master of Science degree in Zoology, presented on 29 July, 2010, at Southern Illinois University Carbondale.

TITLE: THE EFFECTS OF TWO INSECTICIDES ON CALIFORNIA ANURANS (*RANA SIERRAE* AND *PSEUDACRIS SIERRA*) AND THE IMPLICATIONS FOR DECLINING AMPHIBIAN POPULATIONS

MAJOR PROFESSOR: Donald Sparling

Evidence is growing that agrochemicals are playing a role in the decline of amphibians in California. An area of concern is the Sierra Nevada Mountains, where insecticides used in the Central Valley are aerially transported to amphibian habitats. I examined the effects of two of these insecticides, endosulfan and chlorpyrifos, in two experiments on anuran larvae. For the first experiment I exposed Sierra Mountain yellow-legged frog (*Rana sierrae*) larvae starting at Gosner stage 25 to each insecticide for 63 d to determine median lethal concentrations (LC_{50}) and sublethal effects on growth and development. In the second experiment Sierran treefrog (*Pseudacris regilla*) larvae were exposed to chlorpyrifos and endosulfan individually and in combination from Gosner stage 25 through metamorphosis to evaluate the interaction between these insecticides. In the first experiment the endosulfan LC_{50} was 19.8 µg/L (95% confidence interval, 15.3-52.2 µg/L) and the chlorpyrifos LC_{50} was 595 µg/L (95% confidence interval could not be determined). Endosulfan concentrations greater than 8 µg/L reduced growth but had no effect on time to metamorphosis. No larvae exposed to chlorpyrifos reached metamorphosis. All larvae exposed to greater than 737 µg/L died before the end of the experiment. Growth was reduced above 325 µg/L and cholinesterase was depressed at 737 µg/L compared to controls. In the second experiment the interactive effects of the insecticides depended on concentration and

exposure duration. Chlorpyrifos alone did not affect survival or body size after 30 d, even at concentrations greater than the previously reported LC_{50}. Survival and body size decreased with increasing endosulfan concentrations. In combination, 137 µg/L chlorpyrifos inhibited the negative effects of endosulfan on growth and survival and the positive effects of endosulfan on cholinesterase.

ACKNOWLEDGMENTS

This thesis would not have been possible without the assistance of many important individuals. My thanks are given to my graduate advisor, Donald Sparling, for his knowledge and patience in preparing me to be a better scientist. My graduate committee members Richard Halbrook and Matt Whiles have been instrumental in the development of this thesis. I also thank Gary Fellers and Patrick Kleeman of the United States Geological Survey for their assistance in collection of eggs and logistical support in preparation of the study design. I also thank them for introducing me to the herpetofuana of California and the conservation issues surrounding this important group of animals. Tricia Trimble was influential in analysis of water samples for Chapter 2. I am also thankful for the assistance of Stephanie Plautz during data collection for Chapters 1 and 2.

Special thanks are given to the United States Geological Survey's Amphibian Research and Monitoring Initiative for providing funding for this project and to the Southern Illinois University Cooperative Wildlife Research Laboratory faculty, staff, and students for guidance and overall support. I also thank the important mentors of my early biological studies career. I thank Jim Harding for instilling his passion of amphibian research in me. A tremendous thank you is given to Heather Eisthen for providing me with the foundation for sound biological research and demonstrating the importance of a strong mentor-student relationship. I am forever grateful to my family: my brother Mike; my sister Angela and brother-in-law Brian; my nephew Mason; Craig, Joyce, and Samantha Richards; and, of course, my wonderful parents Karen and Frank. Without

these people, my love and passion for the natural sciences, the outdoors, and hard work would not exist today.

Lastly, this thesis would not be possible without the love and support of my wonderful wife, Teal. You are the rock in my world. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

The decline of the world's amphibian populations has become a problem of serious concern (Blaustein and Wake 1990, Alford and Richards 1999, Houlahan et al. 2000). The International Union for Conservation of Nature (IUCN 2004) released the Global Amphibian Assessment in 2004, summarizing the results of the first globally comprehensive assessment of all described amphibian species. Of the 5,743 species described, 32.5% were listed as Vulnerable, Endangered, or Critically Endangered. Specifically, 7.4% species were listed as Critically Endangered, and at least 43% were experiencing some form of population decline (Stuart et al. 2004). Many causes have been proposed for these declines, including: habitat loss (IUCN 2004); climate change (Whitfield et al. 2007); UV-B radiation (Blaustein et al. 1994, 2003); infectious disease (Daszak et al. 1999); contaminants (Sparling et al 2001, Relyea 2005, Hayes et al. 2006); non-native predators (Kats and Ferrer 2003); and a combination of factors (Boone et al. 2007, Laurance 2008).

In North America, 21% of the 262 described amphibian species are currently threatened (Young et al. 2004). In California, 13 species and subspecies of amphibians are listed as threatened or endangered (CDFG 2010), and in the Sierra Nevada Mountains half of the 32 amphibian taxa occurring there are declining (Jennings 1996). Drost and Fellers (1996) documented serious declines in at least five of the seven anuran species that historically inhabited the Sierra Nevada Mountains in the Yosemite area. This is of particular concern because the declines have occurred in relatively undisturbed, protected areas. Introduced fish have been implicated in these declines (Bradford 1989, Knapp

2005, Davidson and Knapp 2007) as well as increased ultraviolet-B radiation (Blaustein et al. 1994, 2003) and the chytrid fungus *Batrachochytrium dendrobatidis* (Fellers et al. 2001, Rachowicz et al. 2006).

Wind-borne pesticides have also been associated with amphibian declines in California. In 2008, 73,269,299 kg of pesticide active ingredients were applied in California. Greater than 70% of this was applied in the Central Valley (CEPA 2009). Organochlorine (OC) and organophosphate (OP) insecticides from the Central Valley drift downwind into the nearby Sierra Nevada Mountains, where they are deposited through snow and rain (McConnell et al. 1998, LeNoir et al. 1999). These insecticides been measured in anuran tissue (Sparling et al. 2001, Angermann et al. 2002, Fellers et al. 2004). Additionally, cholinesterase (ChE) levels were depressed in *Pseudacris sierra* tadpoles in Sequoia National Park, directly east and downwind of the Central Valley, compared to tadpoles at sites along the Pacific coast and upwind from the Central Valley (Sparling et al. 2001). ChE activity can be used as a bioindicator of OP exposure (Melancon 2003). Davidson et al. (2001, 2002) associated anuran declines with the amount of agricultural land use in the Central Valley region and suggested that windborne agrochemicals were involved in the declines. Multiple contaminants often occur together in anuran habitats, and they can interact to affect individual fitness and aquatic community dynamics (Relyea 2004a, Hayes et al. 2006, Boone 2008). Additionally, immune defenses in anurans may be reduced after exposure to OPs, making them more vulnerable to diseases such as chytridiomycosis (Davidson et al. 2007).

Laboratory studies play a useful role in establishing baseline sensitivity of amphibians to contaminants because other environmental stressors can be controlled.

Establishing the toxicity of insecticides to amphibians can provide a better understanding of what role chemicals are playing in amphibian declines. This information can then be used to assess their ecological relevance at environmental concentrations. Past toxicity tests with native western United States anurans have included *Pseudacris sierra, Rana boylii*, and *Bufo boreas*. *P. sierra* is an abundant native anuran of the western United States and shares common larval and breeding habitats with *R. boylii, R. sierrae* and other ranid species experiencing the most severe population declines (Stebbins 2003). Thus, *P. sierra* can be used as a surrogate species for pesticide sensitivity. In addition, ranids are exposed to water during much more of their life history than *P. sierra* (e.g. larval *R. sierrae* may take ≤ 3 years to complete metamorphosis while *P. sierra* larvae take approximately 2 months; Stebbins 2003, US Geological Survey 2009). If pesticide exposure is having negative effects on *P. sierra* larvae, these effects may be magnified in ranids, especially *R. sierrae*. A heightened sensitivity to contaminants could add to the evidence of why these species are experiencing declines whereas *P. sierra* populations are still comparatively abundant.

The aim of this research was to add to the current information of pesticide impacts on anurans. Two experiments were conducted. Experiment one determined the toxicity of the insecticides endosulfan and chlorpyrifos to *R. sierrae* larvae and compared these data with past environmental measurements. Experiment two evaluated the combined toxicity of these two insecticides to *P. sierra* larvae.

CHAPTER 2

TOXICITY OF ENDOSULFAN AND CHLORPYRIFOS TO SIERRA NEVADA YELLOW-LEGGED FROG (*RANA SIERRAE)* LARVAE

INTRODUCTION

Over the past two decades biologists have accumulated evidence of a global decline of amphibians (Blaustein and Wake 1990, Alford and Richards 1999, Houlahan et al. 2000). Declines have been documented in the Americas, Europe, Australia, the Caribbean, Africa, and Asia (Semlitsch 2003). An area of particular concern is the montane regions of the western United States, including the Sierra Nevada Mountains of California, where half of the 32 amphibian taxa are declining (Jennings 1996). Drost and Fellers (1996) documented serious declines of at least five of the seven anuran species that historically inhabited the Sierra Nevada Mountains in the Yosemite area. Davidson et al. (2002) associated these declines with upwind agrochemical use in the heavily agricultural Central Valley. These chemicals are deposited downwind in the Sierra Nevada, where they have been documented in air, snow, and surface water (Zabik and Seiber 1993, McConnell et al. 1998, LeNoir et al. 1999). Concentrations of several of the most heavily used pesticides in the Central Valley have been measured in larval and adult amphibian tissues in the Sierra Nevada Mountains (Sparling et al. 2001, Angermann et al. 2002), including in the mountain yellow-legged frog (*Rana muscosa*[1]*;* Fellers et al.

[1]A 2008 classification by the Society for the Study of Amphibians and Reptiles changed the designation of *Rana muscosa (*mountain yellow-legged frog) as one 1 species to 2 species: *R. muscosa* (southern mountain yellow-legged frog) and *R. sierrae* (Sierra Nevada yellow-legged frog). Thus, most literature prior to 2008 pertaining to *R. muscosa* or the "mountain yellow-legged frog" could be in reference to either, or both, of the currently-recognized species.

2004). The Sierra Nevada yellow-legged frog (*R. sierrae)* is currently listed as a species of special concern in California, and its federal listing has been declared "warranted but excluded" due to other higher priority listing actions (CDFG 2010). The Center for Biological Diversity recently issued a petition to the California Fish and Game Commission to list all populations of the mountain yellow-legged frog (*R. sierrae* and *R. muscosa*) in California as endangered throughout their range under the California Endangered Species Act. Specifically, pesticides are implicated as a cause warranting this listing, and the petition lists researching the impacts of pesticides on mountain yellow-legged frogs as a priority for management and recovery (CBD 2010). Thus, determining the toxicity of pesticides to *R. sierrae* is an important step in the conservation of this species.

Past toxicity tests with native California anurans have included *Pseudacris regilla* (=*P. sierra*) and *R. boylii* (Sparling and Fellers et al. 2009), but no work has been conducted with *R. sierrae. R. sierrae* inhabit water throughout much more of their life history than some other native species (e.g. larval *R. sierrae* may take ≤ 3 years to complete metamorphosis while *P. sierra* larvae take 2 months; Stebbins 2003, US Geological Survey 2009). Thus, if aquatic exposure to pesticides is having negative effects on other species, these effects may be magnified in *R. sierrae*.

The objectives of this study were to determine the chronic median lethal concentrations (LC_{50}) and sublethal effects of endosulfan and chlorpyrifos to *R. sierrae* larvae, and to evaluate the ecological risk of toxicity in *R. sierrae* by comparing these toxic concentrations with past measured values of environmental concentrations in the Sierra Nevada Mountains. The insecticides endosulfan and chlorpyrifos were chosen

because of their use in the Central Valley (CEPA 2009), they have been measured in the environment (McConnell 1998) and in amphibians in the Sierra Nevada (Sparling et al. 2001), and past toxicological work with other native California amphibians have focused on these insecticides (Sparling and Fellers 2007, Sparling and Fellers 2009).

Endosulfan is an organochlorine insecticide used predominantly on alfalfa, cotton, lettuce, and tomato crops (CEPA 2009), and it has been characterized as "highly toxic" to amphibians (USEPA 1982). Endosulfan impairs neurological functioning in affected organisms by antagonizing the action of the neurotransmitter gamma-aminobutyric acid. This results in only partial repolarization of the neuron, which reduces neuronal inhibition and leads to hyper-excitation of the central nervous system (Bloomquist 1993). In 2008, 27,025 kg of active ingredient were applied in California (CEPA 2009).

Chlorpyrifos is an organophosphate (OP) insecticide that disrupts the central nervous system by inhibiting cholinesterase (ChE) activity, and it must be transformed to its oxon form to achieve maximum potency (Marrs 1993, Galloway and Handy 2003). It is commonly used to treat a variety of fruit and vegetable crops. Chlorpyrifos is the most abundantly used ChE inhibiting insecticide in California, with 612,530 kg of active ingredient applied in 2008. This comprised 27% of all ChE inhibiting insecticide use in the state that year (CEPA 2009). Previous studies have reported chlorpyrifos toxicity to California anurans to be less than that of endosulfan (e.g. Sparling and Fellers 2009). No studies have evaluated the toxicity of these two insecticides with regards to *R. sierrae*.

METHODS

Animal Husbandry

Egg masses (four masses, ca. 300 eggs total) of *R. sierrae* were collected from an unnamed pond 2.39 km south of Jeff Davis Peak, Toiyabe National Forest, California, USA (38º61.338' N, 119º89.446' W) on 2 June 2009. The eggs were placed in two bags containing ca. 4 L of original pond water and shipped in coolers with cold packs via overnight express to the Cooperative Wildlife Research Laboratory at Southern Illinois University (SIU), Carbondale, Illinois, USA. Eggs were incubated in their shipping water until they hatched and then placed in an aquarium where they were gradually assimilated to reconstituted, medium soft water (American Society for Testing and Methods 1988) over 4 d. All tadpoles were kept in the same aquarium to ensure that individuals from different egg masses were mixed prior to assignment to experimental aquaria. Vinyl gloves that had been rinsed with deionized water were worn when handling all aquaria and animals. Tadpoles were fed boiled, organic romaine lettuce and high-protein flaked fish food *ad libitum*.

Room temperature was maintained at 18-21º C and photoperiod at 12 L:12 D, both of which were checked daily. A static-renewal design was used with 100% replacement of water twice per week. Test aquaria were aerated using aquarium air pumps. Water quality parameters were measured from four randomly selected test aquaria periodically through the experiment. Dissolved oxygen (DO) and water temperatures were measured using a 550 DO Meter (YSI Incorporated, Yellow Springs, Ohio, USA), and pH was measured using a pHTestr 30 (Eutech Instruments, Oakton®, Vernon Hills, Illinois, USA). Total ammonia was measured using a LR8600 Freshwater/Saltwater Ammonia Water Test (Aquarium Pharmaceuticals, Mars Fishcare Incorporated, Chalfont, Pennsylvania, USA).

Due to the distribution of *Batrachochytrium dendrobatidis* in California (Padgett-Flohr and Hopkins 2009), I randomly sampled 15 individuals reaching Gosner stage 46 to determine if the study animals carried the fungus. Sampling followed Brem et al. (2007), and samples were sent to Pisces Molecular, Boulder, Colorado, USA for polymerase-chain reaction assays. All samples were negative for *B. dendrobatidis*. Animal husbandry and treatment were approved by the SIU Institutional Animal Care and Use Committee (protocol 08-025).

Pesticides and Treatments

Reagent-grade chlorpyrifos (99% pure, Restek, CAS 2921-88-2) and a 70:30 mixture of endosulfan I and endosulfan II (99% pure, Restek, CAS 115-29-7) were used. Pesticides were dissolved in acetone to make stock solutions and kept at -20ºC. Pesticide concentrations needed for dosing were prepared from stock solutions immediately prior to their intended use. Test concentrations were determined following a pilot test in which individual larvae were exposed to a set of concentrations ranging from 0.2 to 16 µg/L endosulfan and 25 to 800 µg/L chlorpyrifos. Each concentration was added to a separate 1 L beaker filled with 500 ml reconstituted water, and two tadpoles were placed in each beaker and monitored for behavioral effects and mortality over 96 h. Based on this screening test, geometrically arranged exposure concentrations were set as 5.75, 8, 12, 14, and 17.75 µg/L endosulfan and 325, 500, 737, 1125, and 1687 µg/L chlorpyrifos.

A negative control consisting of 1 ml of reconstituted water and a vehicle control of 1 ml of acetone were used. Each of the 10 chemical treatments and the vehicle control were represented by four replicates, and the negative control consisted of six replicates

for a total of 50 experimental aquaria. Each 8 L aquarium was filled with 7 L reconstituted water, and four randomly selected larvae at Gosner stage 25 (Gosner 1960) were placed in each tank. Individual doses of treatments for each aquarium were made by placing the amount of stock solution of each pesticide necessary to reach the desired final concentration into an amber vial and adding acetone as a vehicle for a final dose volume of 1 ml. The doses were added to each treatment aquarium at the initiation of the exposures (experimental day 0) and following each bi-weekly water change. Aquaria waters were stirred using a glass stir rod to ensure a uniform distribution of the dose in the water column.

Response variables included survivorship, days to death, body mass growth, body mass and snout-vent length (SVL) at metamorphosis, days to metamorphosis, duration of metamorphic climax (Gosner stages 42-46), and cholinesterase (ChE) activity. Each day larvae were observed for unusual behavior, morbidity and death. Dead animals were removed and weighed ($\pm$ 0.01g) using an XT Top Loading Balance (Thermo Fisher Scientific, Inc., Hampton, New Hampshire, USA), measured for SVL ($\pm$ 0.1mm) using an electronic digital caliper (Thermo Fisher Scientific, Inc., Hampton, New Hampshire, USA), staged for development, and stored in individual vials at -80ºC. Larvae from each aquarium were weighed and staged for development on day 0 and every 20 d until reaching Gosner stage 42. At Gosner stage 42, animals were measured for SVL, weighed, and placed in individual slanted 1 L Ball jars with ca. 75 ml of treatment water until completion of metamorphosis. At metamorphosis they were measured again, euthanized in tricaine methanesulfonate (MS-222), and stored in individual vials at -80ºC until analyzed for ChE activity. Due to the extended larval stage of *R. sierrae*, the study

was terminated after 63 d. Animals having reached Gosner stage 42 (i.e. moved to a slanted Ball jar) by day 63 were allowed the opportunity to complete metamorphosis. All surviving larvae not reaching Gosner stage 42 by day 63 were staged for development, weighed, measured for SVL, euthanized in MS-222, and stored in individual vials at -80ºC.

For larvae analyzed for total ChE activity, the gut coil was removed from larvae not reaching Gosner stage 46 by slightly scraping the skin from the ventral surfacing and taking out the gut. Gut coils of pre-metamorphs contain variable amounts of food and non-organismal tissue and were thus removed to normalize to the metamorphs. ChE was determined using a photometric method (Ellmann et al. 1961) modified for a 96-well microplate reader. Each sample was run in triplicate or until a coefficient of variation among three replicates was less than 5%. Mean slopes for each sample was calculated and arithmetically converted to μmol/g tissue/sec for ChE activity.

Statistical Analyses

Statistical analyses were performed using SAS 9.1 (SAS Institute Cary, North Carolina, USA). Mortality data were adjusted to control mortality before LC50 values were calculated based on Abbott's correction formula, $(p_i - p_c) / (1 - p_c)$, where p_i is the mortality proportion in treatment group i and p_c is the mortality proportion in the control group (Ostrander 1996). Abbott's correction was used because ignoring control mortality can lead to biased estimates of LC_{50} values (Hoekstra 1987). LC_{50} values were determined using the probit method when data met a goodness-of-fit based on the Pearson chi-square test statistic. For data that were statistically different from a

goodness-of-fit, a 10% trimmed Spearman-Karber (SK) method was used to calculate LC_{50} values. Where data met a goodness-of-fit, results from both methods are provided for comparison purposes. An effective concentration (EC_{50}) value based on the number of larvae exposed to endosulfan developing axial malformations was determined using the probit method following confirmation of a goodness-of-fit based on the Pearson chi-square test statistic.

The normality of response variables was tested using the Shapiro-Wilks test and by visual observations of the residual plots for normality and homogeneity of variances. Time to metamorphosis and ChE data were log transformed to normalize data and stabilize variances, and proportions of animals developing axial malformations were arcsine transformed. Survival data were still heteroscedastic following transformation, so they were ranked and analyzed using a nonparametric Kruskal-Wallis test. All other response variables met the assumptions of parametric statistics either before or after transformation. Negative controls and vehicle controls were initially compared using analysis of variance (ANOVA) to test for differences among response variables. No significant differences were observed between the two types of controls for any of the response variables ($p > 0.14$ for all comparisons), so data from both controls were pooled. Because larva in a common aquarium might be considered dependent samples, I used ANOVA to test for between-aquarium effects for response variables by examining a nested effect of aquarium within treatment. A lack of significance for the nested effect meant that an aquarium effect did not occur and individual larva could be treated as experimental units. Aquaria served as the experimental units when there was a significant aquarium effect for the response variable of SVL at metamorphosis for

animals exposed to endosulfan ($p = 0.04$). There were no inter-aquarium effects for any of the other response variables ($p > 0.23$), so individual larvae were considered experimental units for subsequent analyses of these response variables. The aquarium served as the experimental unit for body mass and SVL on day 0, 20, 40, and 60 because larvae were not individually marked, and thus mean aquarium measurements were used.

ANOVA was used to determine if treatments had an effect on time to death, time to metamorphosis, duration of metamorphic climax, and occurrence of and time to axial malformation. Analysis of covariance (ANCOVA) was used to test for an effect of insecticide concentration on final measurements at metamorphosis (i.e. body mass and SVL) with time to metamorphosis as the covariate. ANCOVA was also used to test for an effect of insecticide concentration on ChE activity with Gosner stage as the covariate.

Mean values of body mass at days 0, 20, 40, and 60 for each aquarium were used to examine growth rates. Exponential growth rates of body mass through 20, 40, and 60 d were calculated for aquaria means using the equation [ln (final mass)/ ln (mass at day 0)]. ANCOVA was used to test for an effect of insecticide concentration on growth rates with time as the covariate. For all significant statistical tests, Tukey's honestly significant difference test was used post-hoc to test for specific comparisons among insecticide concentrations. Statistical significance is based on $\alpha=0.05$.

RESULTS

Water Parameters

Dissolved oxygen ranged from 5.74 to 6.49 mg/L (mean 6.11 ± 0.17 mg/L) and water temperatures ranged from 17.9° to 20.1°C (mean 18.9° ± 0.4°C) throughout the

study. pH ranged from 6.9 to 7.2 (mean 7.1 ± 0.1). Total ammonia remained below 0.25 mg/L for the duration of the study.

Survival

Survival of control animals was 95%. A larva from an aquarium containing 5.75 µg/L endosulfan was missing on day 40 and its fate was unknown. This individual was removed from subsequent analysis (n = 15 for 5.75 µg/L). Endosulfan mortality data met the goodness-of-fit test (Pearson X^2 = 6.2, df = 3, p = 0.10) for probit analysis. The LC_{50} for the 63 d experimental period was 19.8 µg/L (95% confidence interval, 15.3-52.2 µg/L), and the SK LC_{50} was 17.2 µg/L (95% confidence interval, 14.8-20.0 µg/L). No mortality was observed in larvae exposed to 5.75 µg/L endosulfan. Mortality was significantly greater in larvae exposed to 17.75 µg/L compared to controls and 5.75 µg/L endosulfan (Kruskal-Wallis X^2 = 13.2, df = 5, p = 0.02; Figure 1). Mean time to death did not differ among treatment concentrations (Table 1). Average time to death was 43.2 d across controls and all treatments.

Chlorpyrifos mortality data did not meet the goodness-of-fit (X^2 = 11.9, df = 3, p = 0.0078) for probit analysis. The SK LC_{50} for the 63 d experimental period was 595.1 µg/L (95% confidence interval could not be determined). Mortality was significantly greater in the three highest concentrations (737, 1125, and 1687 µg/L) compared to controls and the lower concentrations (Kruskal-Wallis X^2 = 25.9, df = 5, $p < 0.0001$). Mortality in the three highest concentrations was 100% at the end of the 63 d experimental period (Figure 2); thus, a SK LC_{50} of 734.2 µg/L was calculated for mortality data after 30 d. The SK LC_{50} after 30 d was 734.2 µg/L (95% confidence

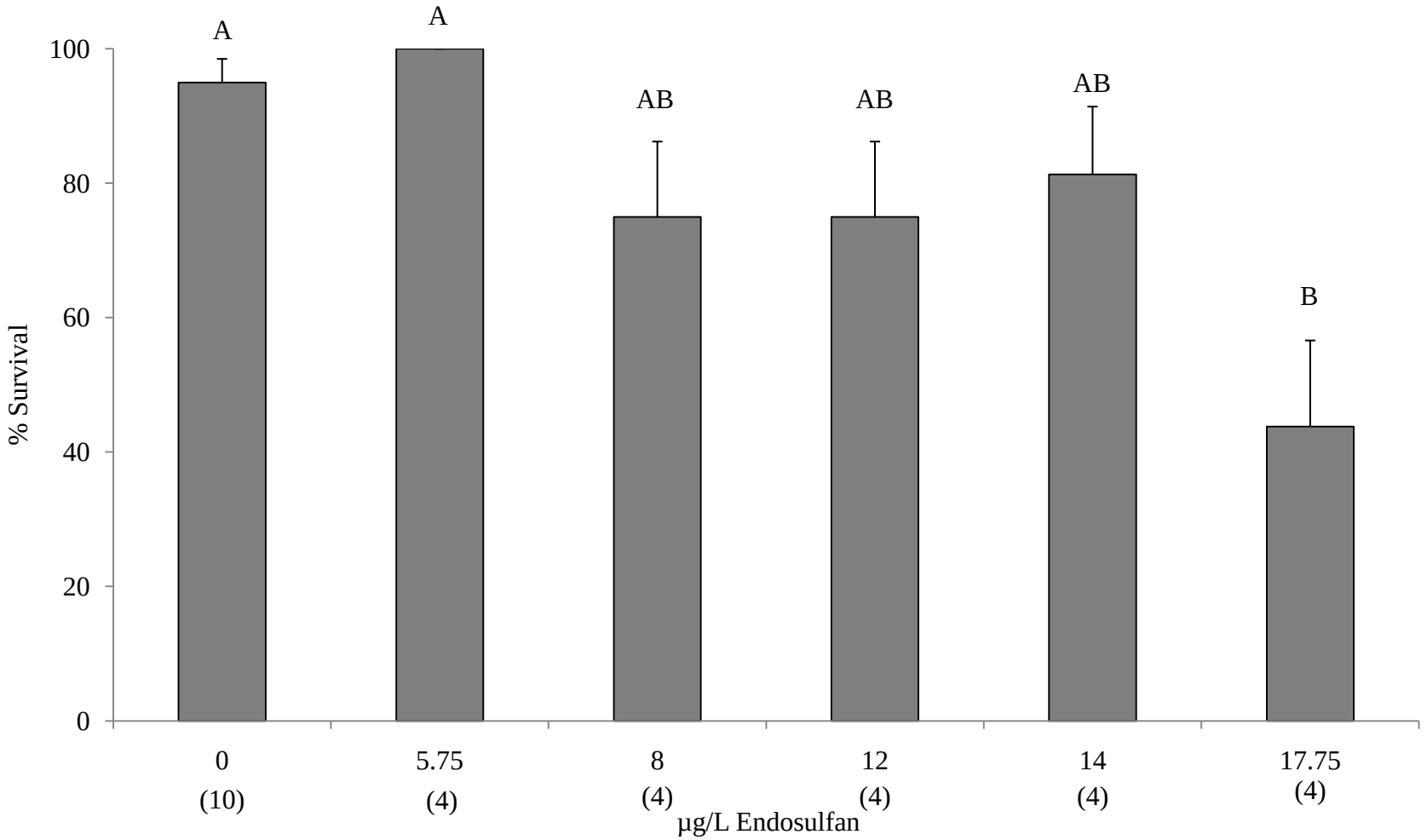

Figure 1. Mean percent survival to experimental day 63 for *Rana sierrae* larvae exposed to endosulfan. Error bars are plus one standard error. Numbers in parentheses along the horizontal axis under concentrations represent sample size (number of aquaria) per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

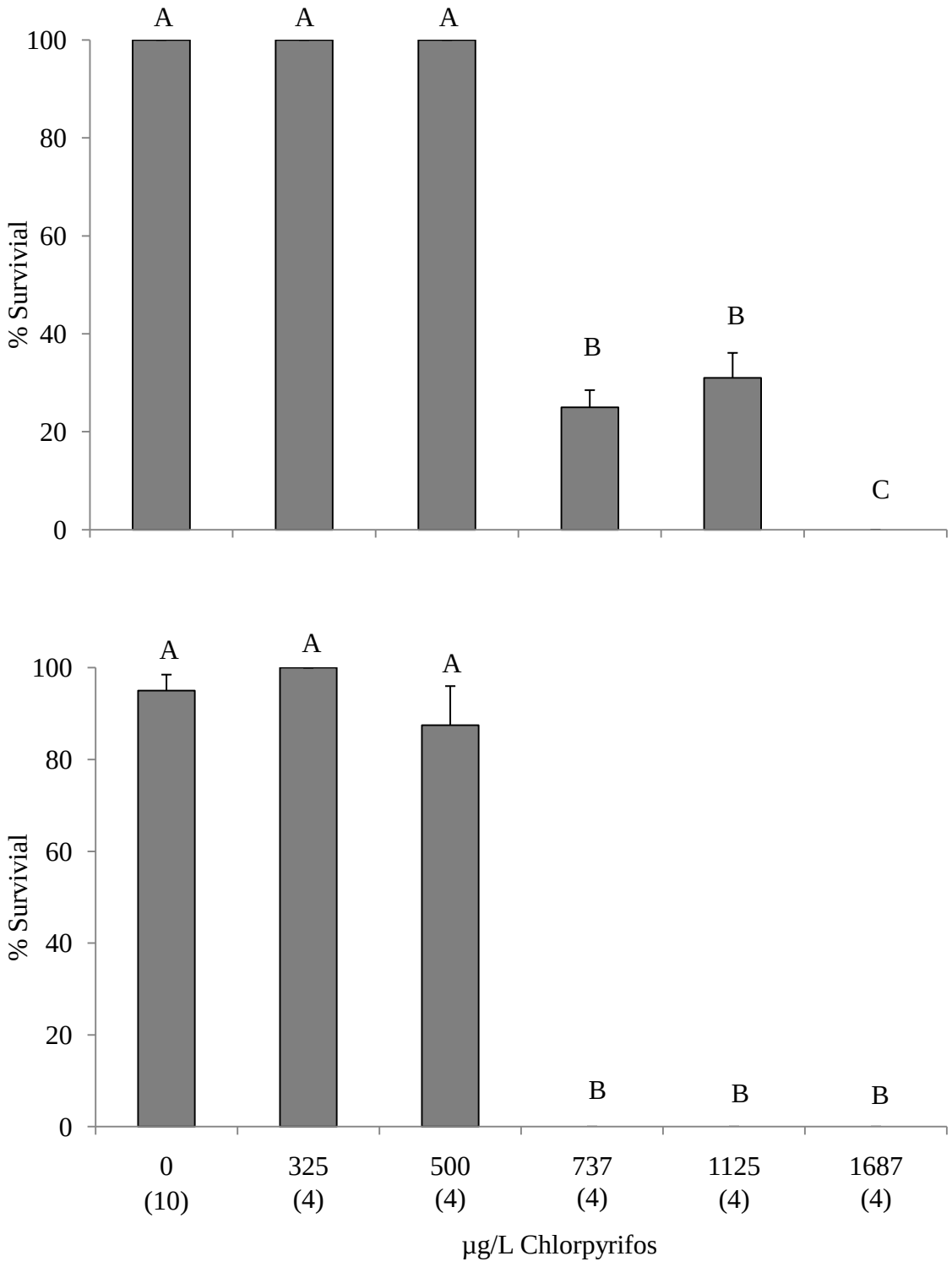

Figure 2. Mean percent survival to experimental days 30 (top) and 63 (bottom) for *Rana sierrae* larvae exposed to chlorpyrifos. Error bars are plus one standard error. Numbers in parentheses along the horizontal axis under concentrations represent sample size (number of aquaria) per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

Table 1. Summary of ANOVA results of endosulfan and chlorpyrifos on response variables for *Rana sierrae* larvae.

Response Variable	Insecticide	*df*	*F*	*p*
Time to death	Endosulfan	4,17	0.76	0.5649
	Chlorpyrifos	4,47	10.00	<0.0001
Time to metamorphosis[a]	Endosulfan	5,16	1.32	0.3050
Climax duration[a]	Endosulfan	5,16	2.13	0.1138
Malformation occurrence[a]	Endosulfan	5,24	9.72	<0.0001
Time to malformation[a]	Endosulfan	3,12	3.60	0.0462

[a]Larvae exposed to chlorpyrifos did not reach Gosner stage 42 nor did they develop axial malformations, so chlorpyrifos was not used in an ANOVA of time to metamorphosis, duration of metamorphic climax, malformation occurrence, or time to malformation.
interval, 627.5-859.1 µg/L). Survival was 100% in control larvae and larvae exposed to

325 and 500 µg/L after 30 d and 25, 31, and 0% in larvae exposed to 737, 1125, and 1687 µg/L, respectively (Kruskal-Wallis $X^2 = 27.8182$, $df = 5$, $p < 0.0001$; Figure 2). Mortality was not observed in larvae exposed to 325 µg/L chlorpyrifos over the entire 63 d experimental period. Mean time to death decreased with increasing concentration of chlorpyrifos, with larvae exposed to737 µg/L or greater dying significantly more quickly (mean 17.9 ± 2.4 d) than control larvae (mean 55.5 ± 6.6 d; Table 1; Figure 3). No larvae exposed to 737 µg/L or greater chlorpyrifos survived past day 45.

Effects on Growth and Development

The ANCOVA on body mass growth rates indicated a significant effect of endosulfan concentration, time, and their interaction (Table 2). Rates of increase for

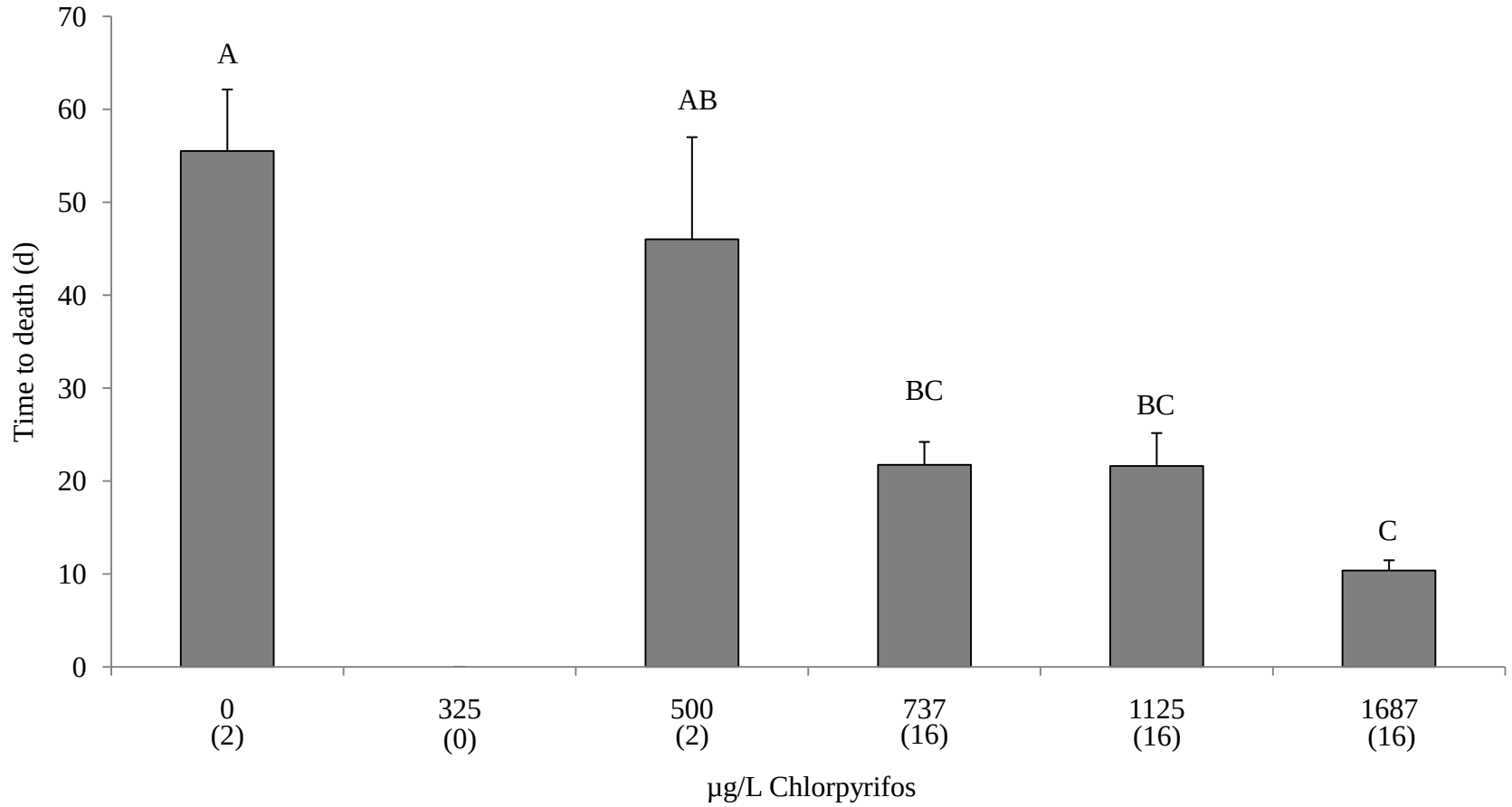

Figure 3. Mean time to death for *Rana sierrae* larvae exposed to chlorpyrifos. Error bars are plus one standard error. Numbers in parentheses along the horizontal axis under concentrations represent sample size (number of animals dead) per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

Table 2. ANCOVA results on the effects of insecticide on body mass growth rates through 20, 40, and 60 d with time as a covariate in *Rana sierrae* larvae exposed to endosulfan and chlorpyrifos.

Response Variable	Insecticide	Source	*df*	*F*	*p*
Body mass growth	Endosulfan	Concentration	5,78	13.13	<0.0001
		Time	1,78	422.34	<0.0001
		Concentration*time	5,78	4.29	0.0017
	Chlorpyrifos[a]	Concentration	4,37	3.09	0.0272
		Time	1,37	2.03	0.1625
		Concentration*time	4,37	6.91	0.0003
	Chlorpyrifos[b]	Concentration	2,48	5.44	0.0074
		Time	1,48	51.64	<0.0001
		Concentration* time	2,48	5.53	0.0069

[a]Through 20 and 40 d. 1687 µg/l chlorpyrifos was removed from analysis because no larvae in this treatment reached day 40.
[b]Through 20, 40, and 60 d. 737 µg/l, 1125 µg/l, and 1687 µg/l chlorpyrifos were removed from analysis because no larvae in these treatments reached day 60.

mass for individuals exposed to 12, 14, and 17.75 µg/L endosulfan averaged 21, 15, and 42% lower than controls, respectively (Figure 4).

Growth rates decreased with increasing concentrations of chlorpyrifos when measured through experimental days 20, 40, and 60. Initial tests were conducted on data through only 40 d because at day 60 the three highest concentrations (737, 1125, and 1687 µg/L) had experienced 100% mortality. Additionally, no animals exposed to 1687 µg/L chlorpyrifos survived to day 40; therefore, the 1687 µg/L chlorpyrifos group was excluded from all body mass analyses. Through 40 d the results of the ANCOVA on

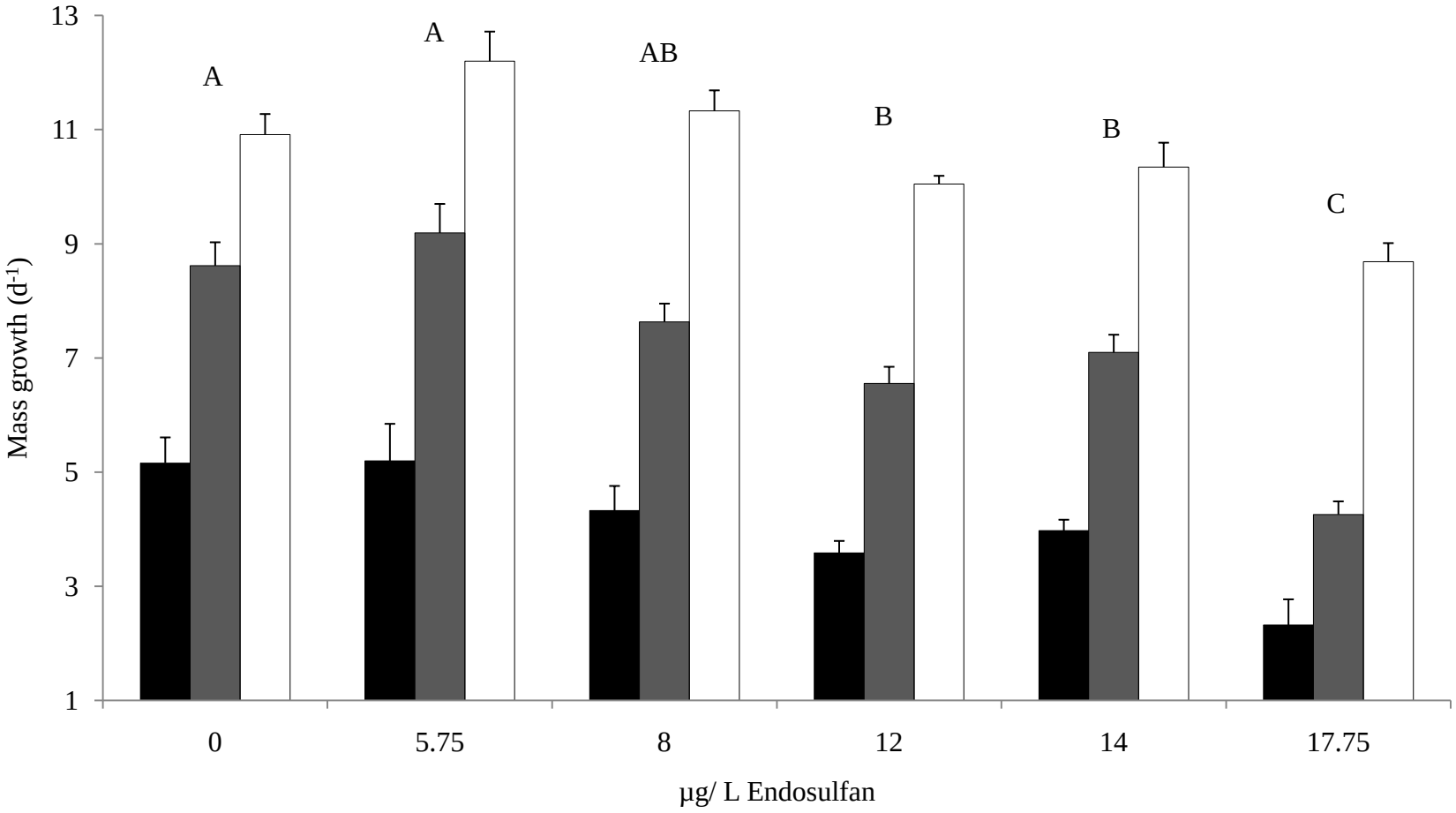

Figure 4. Mean mass growth through 20 (black bars), 40 (grey bars), and 60 (white bars) d for *Rana sierrae* larvae exposed to endosulfan. Growth was calculated by (ln current measurement / ln measurement at day 0). Error bars are ± one standard error. Groups of bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

body mass growth rates indicated a significant effect of chlorpyrifos concentration and time but not of their interaction (Table 2). Mass growth rates of individuals exposed to 325, 500, 737, and 1125 µg/L chlorpyrifos were 38, 59, 80, and 85% lower than controls, respectively (Figure 5). Through 60 d there was a significant effect of chlorpyrifos concentration, time, and the concentration-time interaction on body mass growth rates (Table 2). Growth rates of mass for individuals exposed to 325 and 500 µg/L chlorpyrifos were 41 and 64% lower than controls, respectively (Figure 6).

There was no effect of endosulfan on time to metamorphosis or on length of metamorphic climax for larvae reaching metamorphosis (Table 1). Mean time to metamorphosis was 66.9 d and metamorphic climax averaged 9.8 d across controls and all endosulfan treatment groups. No larvae exposed to chlorpyrifos reached metamorphosis by day 63. For animals reaching metamorphosis, there was a significant effect of time on body mass, but there was no effect of endosulfan concentration (Table 3). Mass at metamorphosis averaged 1.22 g across controls and all endosulfan treatment groups. Similarly, there was a significant effect of time on SVL, but there was no effect of endosulfan concentration (Table 3). SVL at metamorphosis averaged 23.1 mm across controls and all endosulfan treatment groups.

Larvae exposed to endosulfan concentrations greater than 5.75 µg/L developed axial malformations in their body just below the head (Table 1; Figure 7). The incidence of this scoliosis increased with increasing concentrations of endosulfan, with 56% of larvae exposed to 17.75 µg/L developing the malformation. Based on occurrence of axial malformations, an EC_{50} of 18.1 µg/L (95% confidence interval, 15.3-28.9 µg/L) was estimated based on probit analysis. Mean time to scoliosis decreased with increasing

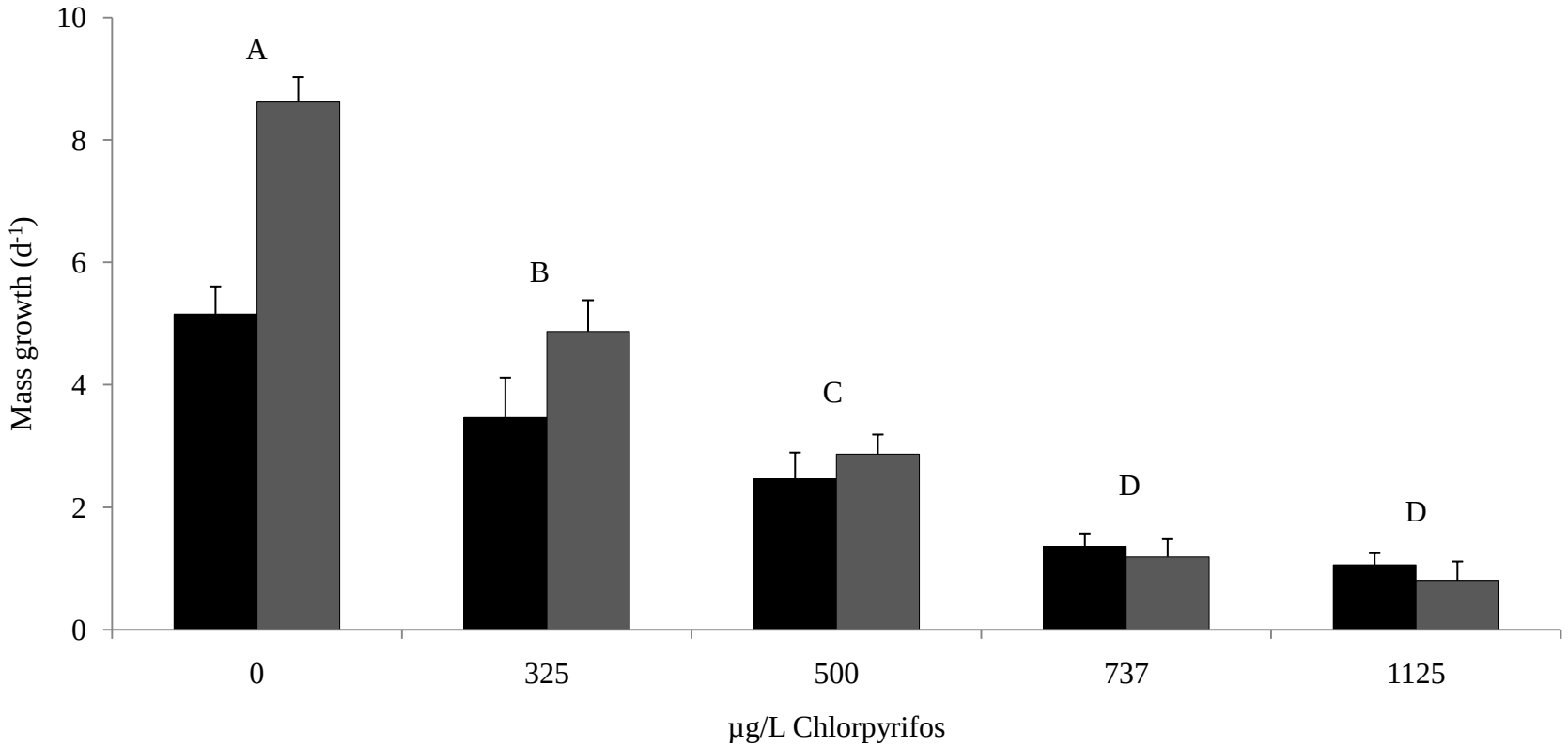

Figure 5. Mean mass growth through 20 (black bars) and 40 (grey bars) d for *Rana sierrae* larvae exposed to chlorpyrifos. Growth rates were calculated by (current measurement /measurement at day 0). Growth was calculated by (ln current measurement / ln measurement at day 0). Error bars are ± one standard error. Groups of bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

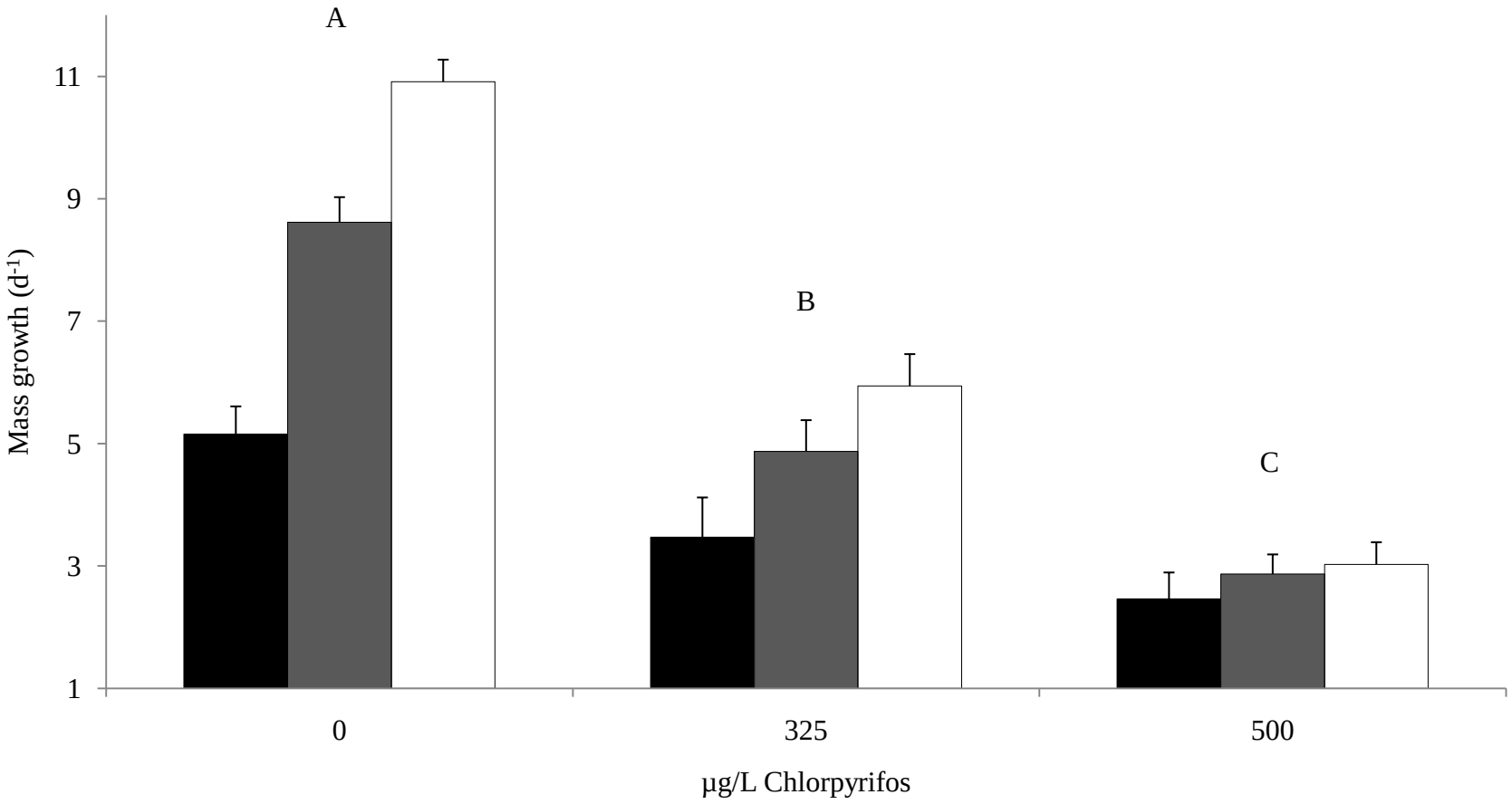

Figure 6. Mean mass growth through 20 (black bars), 40 (grey bars), and 60 (white bars) d for *Rana sierrae* larvae exposed to chlorpyrifos. Growth was calculated by (ln current measurement / ln measurement at day 0). Error bars are ± one standard error. Groups of bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

Table 3. Results of ANCOVA on the effects of insecticide on body mass and snout-vent length (SVL) at metamorphosis with time to metamorphosis as a covariate and cholinesterase (ChE) activity with Gosner stage as a covariate in *Rana sierrae* larvae exposed to endosulfan and chlorpyrifos.

Response Variable	Insecticide	Source	*df*	*F*	*p*
Body mass	Endosulfan	Concentration	5,14	2.12	0.1228
		Time	1,14	16.41	0.0012
SVL	Endosulfan	Concentration	5,11	2.87	0.0671
		Time	1,11	30.17	0.0002
ChE	Endosulfan	Concentration	5,56	0.53	0.7533
		Gosner stage	1,56	1.23	0.2714
	Chlorpyrifos	Concentration	5,52	2.67	0.0322
		Gosner stage	1,52	2.68	0.1080

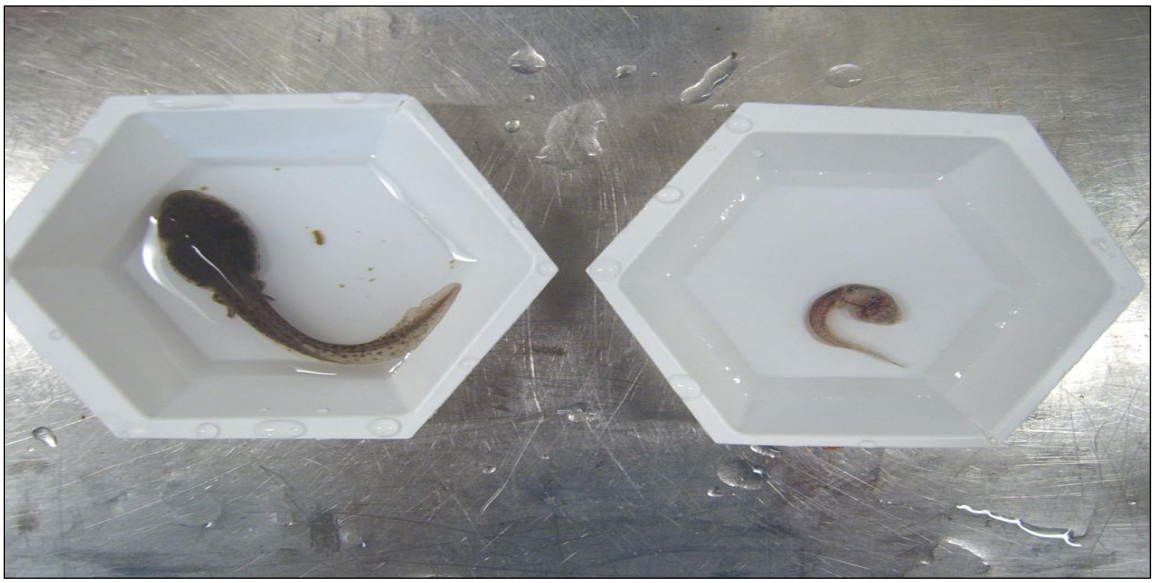

Figure 7. Dorsal view of a control larva displaying normal axial development (left) and ventral view of a larva exposed to 17.75 µg/L endosulfan displaying scoliosis (right). Photo taken at 40 d exposure.

endosulfan concentrations (Table 1; Figure 8). Animals exposed to 12 µg/L developed scoliosis after 34.5 d compared to 19.1 d for animals exposed to 17.75 µg/L. The sole larva developing scoliosis at 8 µg/L endosulfan showed signs of the malformation after only 1 d of exposure (Figure 9), suggesting a high sensitivity to endosulfan for this individual. However, this individual survived to the end of the experiment, and the malformation remained for the entire exposure period. At the end of the study the larva weighed 2.55 g and had a SVL of 24.1 mm, suggesting no effect of the scoliosis on growth.

Cholinesterase

ChE activity was significantly affected by chlorpyrifos but not by endosulfan or Gosner stage (Table 3). ChE activity was significantly greater in control animals (mean 0.45 ± 0.03 µmol/g tissue/s), compared to animals exposed to 737 µg/L (mean 0.26 ± 0.05 µmol/g tissue/s). ChE activity of animals in the 737 µg/L treatment group was 42% lower than that of controls (Figure 10).

DISCUSSION

The estimated LC_{50} of endosulfan to *R. sierrae* is 19.8 (95% confidence interval, 15.3-52.2 µg/L) and 17.2 µg/L (95% confidence interval, 14.8-20.0 µg/L) based on the probit and SK methods, respectively. All larvae exposed to 5.75 µg/L endosulfan survived the 63 d experimental period. Sparling and Fellers (2009) reported the chronic LC_{50} of endosulfan to *P. sierra* and *R. boylii* as 15.6 and 0.23 µg/L, respectively. Additionally, the authors reported that no *R. boylii* exposed to greater than 0.8 µg/L

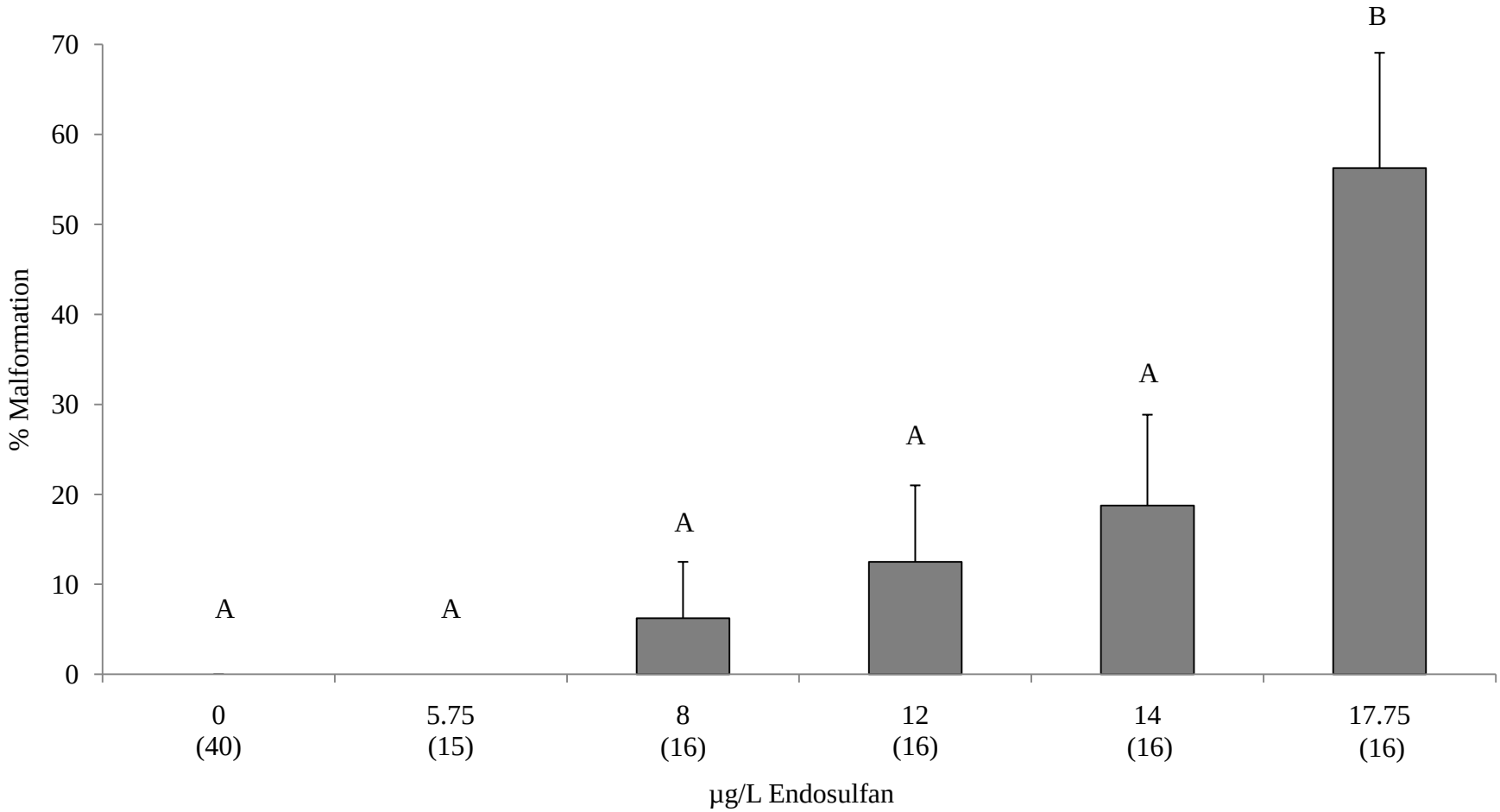

Figure 8. Mean percent of animals developing axial malformations in *Rana sierrae* larvae exposed to endosulfan. Error bars are plus one standard error. Numbers in parentheses along the horizontal axis under concentrations represent sample size (number of animals exposed) per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

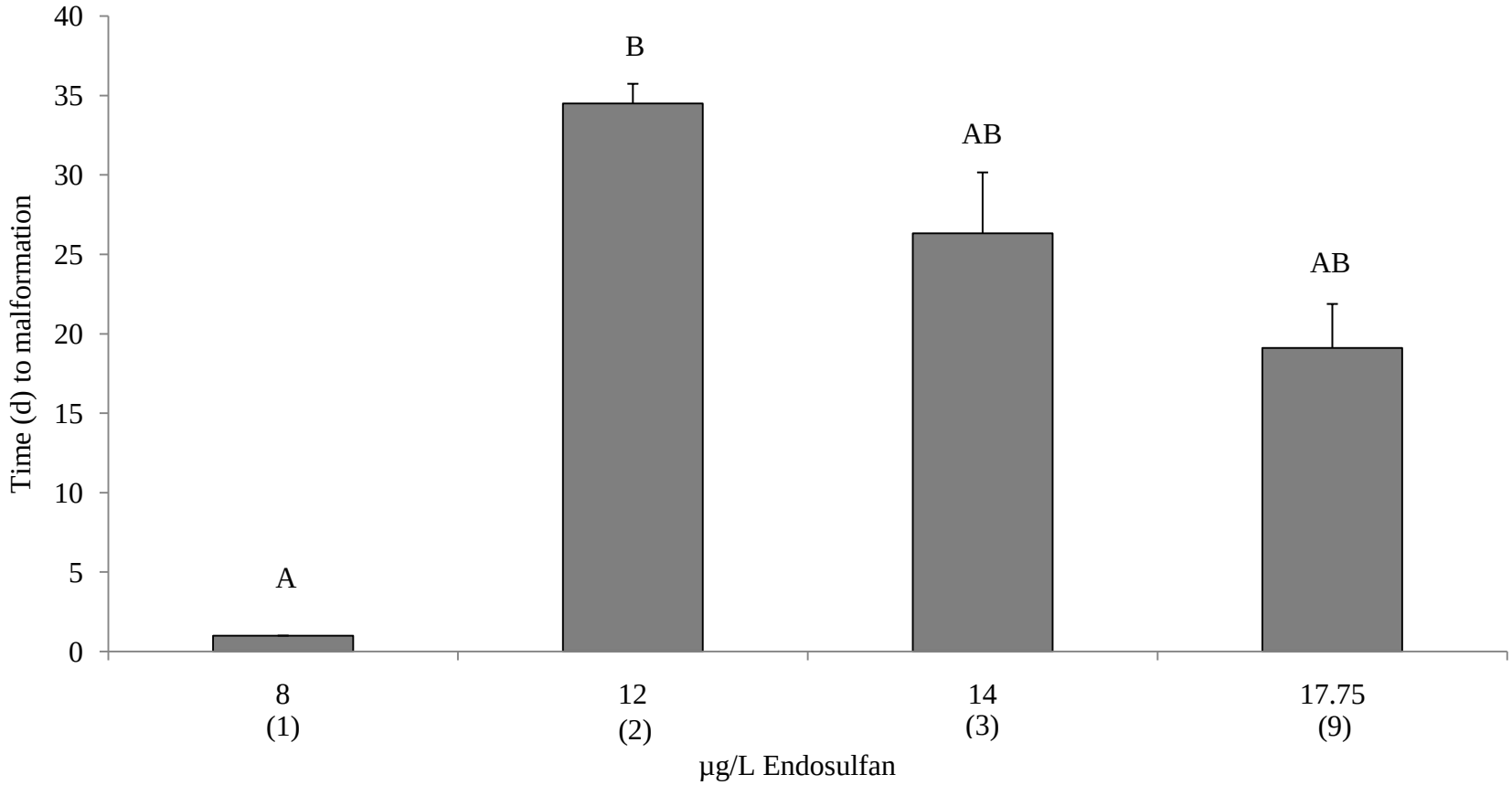

Figure 9. Mean time to development of axial malformations in *Rana sierrae* larvae exposed to endosulfan. Error bars are plus one standard error. Numbers in parentheses along the horizontal axis under concentrations represent sample size (number developing malformation out of 16) per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

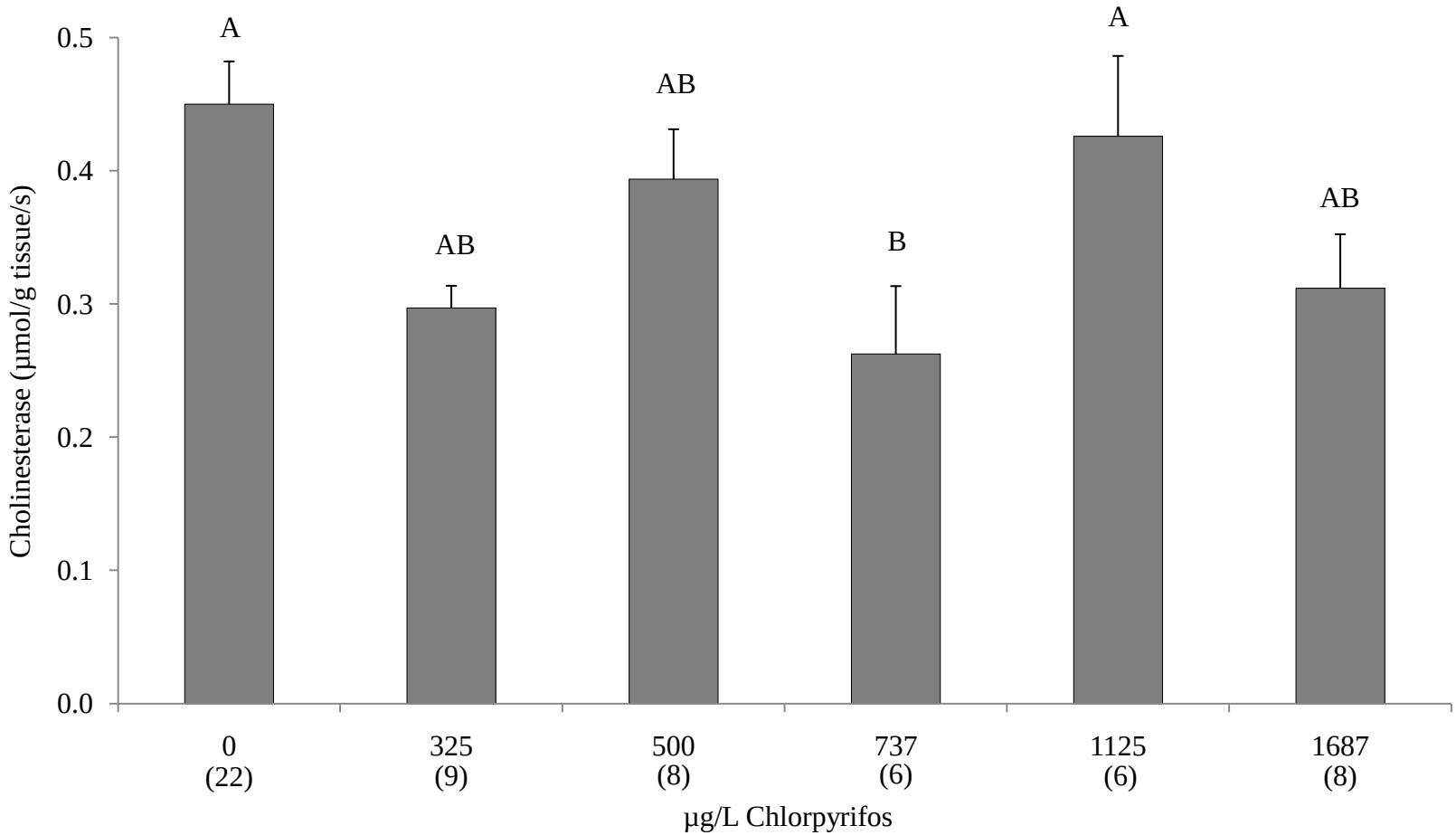

Figure 10. Mean cholinesterase (ChE) activity in *Rana sierrae* larvae exposed to chlorpyrifos. Error bars are plus one standard error. Numbers in parentheses along horizontal axis under concentrations represent sample size per concentration. Bars with the same uppercase letter cannot be distinguished at $\alpha = 0.05$.

endosulfan survived to metamorphosis. The endosulfan LC_{50} estimate from the current study with *R. sierrae*, based on examination of confidence intervals, is statistically equivalent to that of *P. sierra* while nearly two orders of magnitude higher than that of *R. boylii*. Similar to other native California anurans, endosulfan is much more toxic to *R. sierrae* larvae than is chlorpyrifos. Chlorpyrifos LC_{50} values were estimated after 30 d and 63 d. The SK LC_{50} after 30 d was 734.2 µg/L (95% confidence interval, 627.5-859.1 µg/L) and 595.1 µg/L after 63 d. The chronic LC_{50} of chlorpyrifos for *P. sierra* and *R. boylii* have been reported as 365 µg/L (confidence interval not reported) and 108.7 µg/L (95% confidence interval 0-361.3 µg/L), respectively (Sparling and Fellers 2009). Thus, the current chlorpyrifos LC_{50} estimate for *R. sierrae* is approximately twice as large as that of *P. sierra* and *R. boylii*. These results suggest using *P. sierra* as a surrogate for *R. sierrae* sensitivity to contaminants, especially endosulfan, is reasonable, as sensitivity of both species are comparable to one another. However, in other studies the exposure length for study animals differed from that of the current study. At the end of the current study only 25% (10 of 40) of control animals and 15% (12 of 79) of animals exposed to endosulfan had reached Gosner stage 42 or greater. Sparling and Fellers (2009) estimated LC_{50} values based on exposure from Gosner stage 25 through metamorphosis for both *P. sierra* and *R. boylii*. The resulting LC_{50} estimates of the current study may have been lower had the study been continued until all animals had either reached metamorphosis or died (i.e., a complete early life stage test).

Additional studies of endosulfan and chlorpyrifos toxicity have been conducted on other anuran species and have further demonstrated difference in species' sensitivities. The 96 h endosulfan LC_{50} in tiger frogs (*R. tigrina)* was estimated as 1.8 µg/L (Gopal et

al. 1981), while for the common Asian toad (*Bufo melanostictus*) it was estimated as 123 µg/L (Vardia et al. 1984). Reylea (2009) reported 10 µg/L endosulfan resulted in 84% mortality of larval northern leopard frogs (*Lithobates pipiens*) after 57 d but did not cause mortality in gray treefrogs (*Hyla versicolor*). In the same study, 10 µg/L chlorpyrifos exposure did not affect survival of either species. The 96 h chlorpyrifos LC_{50} estimates have been reported for *B. americanus* as 1 µg/L and 3,000 µg/L for *L. pipiens* (Barron and Woodburn 1995). Sparling and Fellers (2007) reported the 24 h LC_{50} of chlorpyrifos to *R. boylii* as 3,000 µg/L. In addition, chlorpyrifos is degraded into the oxon form that may be 100 times more toxic than the parental form (Sparling and Fellers 2007). The data presented in the current study corroborate the findings of chronic toxicity studies that longer exposure duration heightens the sensitivity of anurans to contaminants and results in lower estimated LC_{50} values. The estimated LC_{50} of chlorpyrifos for *R. sierrae* in the current study after 30 d was 734.2 µg/L but decreased to 595.1 µg/L after 63 d. Chronic exposures are environmentally realistic because many anurans occupy habitats that experience multiple pulses of pesticides over a longer period than several days (Relyea and Diecks 2008).

Sampling for current pesticide concentrations in the Sierra Nevada Mountains has been limited both spatially and temporally, though some general conclusions can be made in relation to the current study. Zabik and Seiber (1993) measured 10 ng/L chlorpyrifos in wintertime rain at an elevation of 2000 m in Sequoia National Park. McConnell et al. (1998) measured pesticide concentrations in winter and spring rain and snow at varying elevations in Sequoia National Park and the Lake Tahoe basin. Maximum endosulfan I and endosulfan II concentrations quantified were 6.5 and 1.4 ng/L, respectively, and the

highest concentrations were measured in Sequoia National Park directly downwind from Fresno, Kings and Tulare County, several of the heaviest pesticide using counties in the Central Valley. Endosulfan sulfate was not measured, however, so overall endosulfan concentrations were likely greater. Chlorpyrifos was measured as high as 4.9 ng/L in Sequoia National Park and 3.4 ng/L near Lake Tahoe (McConnell et al. 1998). Summertime water concentrations of endosulfan I and endosulfan II have been measured as high as 18 and 102 ng/L, respectively, and chlorpyrifos as high as 118 ng/L at an elevation greater than 2000 m in Sequoia National Park (LeNoir et al. 1999). Fellers et al. (2004) measured 4.08 ng/L total endosulfan concentration at one site in Sequoia National Park occupied by *R. muscosa*, and chlorpyrifos was detected as high as 12 ng/L at the same site. Thus, though endosulfan concentrations at some sites have been determined to be within an order of magnitude of toxic levels to *R. boylii* (Sparling and Fellers 2009), concentrations of both chlorpyrifos and endosulfan found to be toxic to *R. sierrae* in the current study are well above those that have been measured in the Sierra Nevada Mountains. More quantification of current pesticide concentrations in the Sierra Nevada, especially the northern portion of the range, should be conducted to assess this trend in specific locations that *R. sierrae* inhabit.

Direct mortality is the most extreme endpoint in toxicological responses of organisms to contaminants. However, more subtle sublethal effects can occur as a result of contaminant exposure at lower concentrations than those causing mortality (e.g. Park et al. 2001, Hayes et al. 2002). Only recently have amphibian ecotoxicology studies began examining how these sublethal effects may impact critical life history functions and generate changes at the population level. Effects on growth, development, and ChE

activity can affect larval behavior (Bridges 1997), survival in the presence of predators (Bridges 1999, Relyea 2003), and time to metamorphosis as well as future growth, reproduction, and survival in juveniles and adults following metamorphosis (Smith 1987, Semlitsch et al. 1988).

Endosulfan exposure up to 8 µg/L did not affect growth or development rates in *R. sierrae* larvae. Larvae exposed to 12 µg/L endosulfan or greater had lower growth rates than controls and larvae at lower concentrations. However, there was no effect of endosulfan on time to metamorphosis or on body mass or SVL at metamorphosis. This may be because only 13, 6, and 13% of larvae exposed to 12, 14, and 17.75 µg/L endosulfan, respectively, reached metamorphosis by the end of the experimental period compared to 25% of controls. Thus, sample sizes were small, and the individual larvae reaching metamorphosis in these concentrations were likely the least sensitive of the individuals in these treatment groups. The lack of survival for all animals exposed to 737 µg/L chlorpyrifos or greater eliminated the opportunity to examine growth and developmental rates of all concentrations of chlorpyrifos for the entire experimental period. However, the resulting analysis on data through 40 d and data on the lowest concentrations through 60 d revealed that chlorpyrifos affected growth rates throughout the study. The rate of growth of larvae exposed to 325 µg/L was less than controls, and this trend increased with increasing concentrations of the insecticide. Additionally, because of complete mortality in the highest concentrations and reduced growth and development in the lower concentrations, no animals exposed to chlorpyrifos reached metamorphosis before the completion of the experimental period. Thus, no direct conclusions can be made on the effects of chlorpyrifos on time to or body size at

metamorphosis based on the concentrations used.

In a study utilizing semipermeable membrane devices, Bridges and Little (2005) reported *P. sierra* larvae reared in water extracts from Sequoia and Kings Canyon National Park were smaller at metamorphosis and took longer to reach metamorphosis than control larvae. However, the actual compounds present in the water extracts were not determined. Findings such as these and the growth and development results of the current study are ecologically important. In addition to contaminants, other threats pose risks to anurans in the Sierra Nevada Mountains that are excluded from controlled laboratory research. These include predators, competition, climate change, and disease. Introduced trout exhibit direct predation on anuran larvae and compete with adults for resources in the Sierra Nevada (Finlay and Vredenburg 2007). Reduced growth and slower development from contaminant exposure may place larvae at a greater risk of predation. Additionally, the toxicity of the insecticides malathion and carbaryl, other ChE inhibiting insecticides, increases in several anuran species when combined with predatory stress (Relyea 2003, 2004b). The axial malformations that developed in *R. sierrae* exposed to 8 µg/L endosulfan or greater resulted in uncoordinated swimming and avoidance behavior. Similar axial malformations have been observed in *P. sierra* and *R. boylii* larvae in other laboratory tests with endosulfan (Sparling and Fellers 2009). Occurrence of such contaminant-based malformations in the natural habitat of *R. sierrae* would place them at a far greater risk of predation than normal developing larvae. The calculated EC_{50} of 18.1 µg/L endosulfan is very similar to the LC_{50} value (19.8 µg/L), indicating an effected individual would be at a high risk of mortality as well.

Delayed development and thus delayed time to metamorphosis will also place larvae at a greater risk of desiccation should their habitats dry up. Historic trout introductions in the Sierra Nevada typically occurred in deep lakes to provide the fish the ability to overwinter. Consequently, many populations of *R. sierrae* can now only occupy fishless habitats such as shallow, ephemeral water bodies that may dry in a given summer during which *R. sierrae* larvae are still developing (Matthews and Preisler 2010). Climate data for California predict a 20% decrease in average precipitation over the current century (Karl et al. 1996), thus potentially resulting in more extensive annual drying of ephemeral alpine habitats. With the already extended larval stage of *R. sierrae* in these high alpine habitats, additional delays in time to metamorphosis caused by stressors such as contaminants could further jeopardize already declining populations. Postponing metamorphosis may delay age at first reproduction and thus alter a population's demographic structure, leading to a decline in population size over time (Smith 1987). Additionally, Davidson et al. (2007) suggested that skin peptide defenses in some anurans may be reduced after exposure to OP insecticides such as chlorpyrifos and inhibit anuran immune defenses to diseases such as chytridiomycosis. Clearly, additional work on the combined effects of contaminants and other stressors in the Sierra Nevada needs to be a focal point of future research for *R. sierrae*.

Larvae exposed to chlorpyrifos experienced a reduction in ChE activity. Mean ChE activity was 5 – 42% lower in all animals exposed to chlorpyrifos than control animals, though there was no clear trend of decreasing ChE activity with increasing concentration. In a similar laboratory study, Sparling and Fellers (2009) reported a decrease in ChE activity in *R. boylii* and *P. sierra* with increasing concentration of

chlorpyrifos. Depression of ChE activity has been associated with uncoordinated behavior and a greater susceptibility to predation in anuran larvae (Bridges 1997), though studies of ChE activity in other taxa indicate a reduction of greater than 50% is necessary to elicit a negative effect at the level of the whole organism (e.g. Hill and Fleming 1982, Beauvais et al. 2000). Widder and Bidwell (2006) investigated the effects of chlorpyrifos on ChE activity and swimming speed of larval *L. sphenocephalus*. The authors reported no effect of decreased ChE on swimming speed, though the highest level of ChE inhibition measured in the greatest concentration (200 μg/L) was 43%. Thus, the maximum depression of 42% in the current study is similar to the results of Widder and Bidwell (2006). A field study conducted by Sparling et al. (2001) measured greater ChE activity of Pacific treefrog larvae from coastal California, habitat not expected to be exposed to ChE inhibiting insecticides, than that of larvae from sites in the Lake Tahoe basin and Sequoia National Park. The authors suggested ChE activity was a useful biomarker for ChE inhibiting insecticide exposure in anuran larvae. Field data on ChE activity of *R. sierrae* larvae in the Sierra Nevada Mountains are lacking, making comparisons of ChE activity in the current study to field levels difficult.

Though the results of the current study suggest that *R. sierrae* may not be as sensitive to contaminants in the Sierra Nevada as *R. boylii*, there are several limitations of this study that should be considered. *R. sierrae* inhabit high alpine (≤ 3660 m) lakes, streams, and meadows (Stebbins 2003) and thus experience a wide range of temperatures. Alpine Sierra Nevada air temperatures range from less than 0°C during winter to 25°C in July (NPS 2008). This is in contrast to the static temperature regime used in the current study. Ambient and water temperatures ranged from 18-21°C and17.9-20.1°C,

respectively. Temperature and exposure to endosulfan may have lasting effects on predator vulnerability and long-term fitness. Broomhall (2004) reared eggs of the Australian frog *Limnodynastes peronii* at two contrasting temperature regimes (14°C and 20°C) and then exposed the resulting larvae to endosulfan. Larvae from 14°C were captured faster by predators than those from 20°C. Additionally, Broomhall (2002) reported a wider temperature range (13.5-28.5°C) during tadpole exposure to endosulfan adversely affected survival and resulted in increased vulnerability to predators following exposure compared to a more stable temperature range (18-22°C). The author suggests insecticide exposure and temperature extremes may have long term impacts on fitness. Boone and Bridges (1999) reported the toxicity of carbaryl to *Lithobates clamitans* was greater at 27°C compared to 17°C. Other studies have reported the toxicity of OP insecticides to aquatic invertebrates increases up to 100-fold when temperatures increase from 10°C to 30°C (Lydy et al. 1990). Increasing temperatures increase overall uptake of OP insecticides though they may also decrease actual body burdens because of increased metabolism in the affected organism at higher temperatures (Lydy et al. 1999). Elevated metabolism would also suggest a more rapid conversion of chlorpyrifos to the more toxic chlorpyrifos oxon. Additionally, Linder et al. (2010b) pointed out "the longer the larval period, exposures to waterborne contaminants will be increased, and even relatively resistant species may express adverse effects associated with prolonged dependencies on aquatic habitats." The relatively quick development of the larvae in the current study in comparison to the 3 years they may take in natural habitat may have played a role in pesticide sensitivity observed in the current study compared to *R. boylii* exposed in similar conditions. Indeed, past toxicity tests have demonstrated that the length of

exposure to a given chemical often leads to increased sensitivity and thus lower LC_{50} estimates. Sparling and Fellers (2007) estimated the LC_{50} of chlorpyrifos to *R. boylii* as 3,000 µg/L after 24 h, but the LC_{50} decreased to 108 µg/L when the exposure period lasted from Gosner stage 25 through metamorphosis (a period of up to 100 d ; Sparling and Fellers 2009). Clearly, more relevant duration and temperature regimes should be considered to assess *R. sierrae* sensitivity to insecticides.

R. sierrae larvae occupy habitats where they will be exposed to more than one contaminant at any given time. These contaminants include chlorpyrifos, endosulfan, and other pesticides (e.g. diazinon, malathion, trifluralin; LeNoir et al. 1999) that all may be acting as stressors on developing larvae. Only recently have amphibian ecotoxicologists initiated research on interactions between multiple contaminants. Much attention has been given to the ChE inhibiting insecticide carbaryl (Boone et al. 2005, Boone 2008) and the herbicide atrazine (Hayes et al. 2006). Results pertaining to these chemicals and others have indicated contaminants may have additive (Boone and James 2003, Boone and Bridges-Britton 2006) or nonadditive (Boone et al. 2005, Hayes 2006) effects, often depending on the mode of action of the chemicals. Findings have demonstrated that, though direct lethality is important to evaluate in such studies, sublethal effects on physiological states, endocrine system disruption, and alterations to food source availability and other ecological relationships are likely occurring in nature. These effects are what ultimately may lead to population effects (Linder et al. 2010a). For example, mesocosm studies have reported the presence of more than one insecticide does not always result in stronger negative impacts than predicted by the individual effects of the insecticides present. This may be attributed to positive changes in food resources

from, for example, a fertilizer that compensates for the negative effect of an insecticide (Boone and Bridges-Britton 2006). Focusing on potential interactions among two or more insecticides to amphibians is a necessary area of continuing research. Chapter 2 of this thesis will examine the interaction between chlorpyrifos and endosulfan and its impact on California anurans.

CHAPTER 3

JOINT TOXICITY OF CHLORPYRIFOS AND ENDOSULFAN TO SIERRAN TREEFROG (*PSEUDACRIS SIERRA)* LARVAE

INTRODUCTION

Numerous causes of recent global amphibian declines have been proposed including habitat loss (IUCN 2004), climate change (Whitfield et al. 2007), infectious disease (Daszak et al. 1999), and non-native predators (Kats and Ferrer 2003, Knapp 2005, Finlay and Vredenburg 2007). There is also growing evidence that pesticides are playing a role (Sparling 2003, Relyea 2005, Hayes et al. 2006). Amphibian ecotoxicologists have made significant progress over the past decade examining this cause, though the majority of investigations have focused on individual pesticides. These single-contaminant studies, though crucial for understanding the underlying mechanisms of different classes of pesticides and their effects on amphibians, are often in contrast to exposure of amphibians to multiple pesticides in their natural habitats (e.g. LeNoir et al. 1999). Only recently has focus shifted to assessing the effects of such mixtures on amphibians (e.g. Mazanti et al. 2003, Relyea 2004, Boone and Bridges-Britton 2006, Hayes et al. 2006)

Evaluating pesticide mixtures can be difficult because pesticides may have the same mode of action and thus affect the same site of action within the organism. Conversely, pesticides may have different modes of action and affect the organism at multiple targets. These mixtures may result in additive effects, and the combined effects could be easily extrapolated to be the sum of the individual pesticides' effects. However, mixtures may also be greater than or less than additive (i.e. synergistic, potentiating, and

antagonistic) and the sum of their effects may be difficult to predict based on individual modes of action.

Recent studies have focused specifically on potential interactions between two or more classes of pesticides in anurans. Boone and Bridges-Britton (2006) reported that the presence of multiple pesticides with different modes of action did not have direct additive effects on anurans in mesocosms and thus were no more harmful than one contaminant alone. Other studies have reported that mixtures of pesticides with different modes of action have much greater effects on anurans than the respective individual pesticides (Hayes et al. 2006, Relyea 2009). Boone (2008) suggested that combinations of insecticides with the same mode of action may be more likely to have nonadditive effects than those having different modes of action. The author reported interactive effects between the cholinesterase (ChE) inhibitors carbaryl and malathion, but there were no interactions between either of the pesticides and permethrin, a sodium-channel disruptor.

In the Sierra Nevada, two pesticides are of particular interest: chlorpyrifos and endosulfan (McConnell et al. 1998). Chlorpyrifos is an organophosphate (OP) insecticide that disrupts the central nervous system by inhibiting ChE activity. In a normal functioning nervous system, acetylcholine (ACh) transmits impulses across neural synapses, and the enzyme acetylcholinesterase (AChE) breaks down the ACh and the transmission is ended. OPs bind with AChE and irreversibly inhibit it from breaking down ACh. ACh thus accumulates, leading to repeated firing of lower-order neurons (Marrs 1993, Galloway and Handy 2003). In 2008, 612,530 kg of chlorpyrifos active

ingredient were applied in California, comprising 27% of all ChE inhibiting insecticide use in California (CEPA 2009).

Endosulfan is an organochlorine (OC) insecticide that impairs neurological functioning in affected organisms by antagonizing the action of the neurotransmitter gamma-aminobutyric acid (GABA). Normal GABA activity induces the uptake of chloride ions by neurons. The blockage of this activity by endosulfan results in only partial repolarization, which reduces neuronal inhibition and leads to hyper-excitation of the central nervous system (Bloomquist 1993). Endosulfan is very toxic to amphibians (Jones et al. 2009, Relyea 2009, Sparling and Fellers 2009) and has been associated with areas of amphibian declines in California (Sparling et al. 2001). In 2008, 27,025 kg of active ingredient pesticide were applied in California (CEPA 2009). Although chlorpyrifos and endosulfan have different modes of actions, they both target the nervous system, complicating the predictive nature of their combined effects.

The objective of this study was to evaluate the interactive effects of chronic exposure of chlorpyrifos and endosulfan on survival, body size, and ChE activity of Sierran treefrog (*Pseudacris sierra)* larvae. Evaluating *P. sierra* larvae responses to both chlorpyrifos and endosulfan concurrently is important in understanding the potential interactive effects of these commonly used pesticide classes. Establishing the joint toxicity of these contaminants to amphibians can further facilitate understanding of the mechanisms of chemicals in amphibian declines both in California and in other areas where chemical stressors may be playing a role.

METHODS

Animal Husbandry

Egg masses (six masses, ca. 475 eggs) of *P. sierra* were collected from coastal ponds at Point Reyes, California, USA (38°02.850' N, 122º47.780' W and 38º03.611' N, 122º48.495' W) on 2 February 2009. This site was selected because it lies upwind of agricultural activities in the Central Valley and is not near any agricultural areas along the coast where pesticides are used in significant quantities. The eggs were placed in two bags containing ca. 4 L of original pond water and shipped in coolers with cold packs via overnight express to the Cooperative Wildlife Research Laboratory at Southern Illinois University (SIU), Carbondale, Illinois, USA. Eggs were incubated in their shipping water until they hatched and then placed in an aquarium where they were gradually assimilated to reconstituted, medium soft water (American Society for Testing and Methods 1988) over 4 d. All tadpoles were kept in the same aquarium to ensure that individuals from different egg masses were mixed prior to assignment to experimental aquaria. Vinyl gloves that had been rinsed with deionized water were worn when handling all aquaria and animals. Tadpoles were fed boiled, organic romaine lettuce and high-protein flaked fish food *ad libitum*.

Room temperature was maintained at 17-22º C and photoperiod at 12 L:12 D, both of which were checked daily. A static-renewal design was used with 100% replacement of water twice per week. Test aquaria were aerated using aquarium air pumps. Water quality parameters were measured from four randomly selected test aquaria periodically through the experiment. Dissolved oxygen (DO) and water temperatures were measured using a 550 DO Meter (YSI Incorporated, Yellow Springs,

Ohio, USA). pH was measured using a pHTestr 30 (Eutech Instruments, Oakton®, Vernon Hills, Illinois, USA). Total ammonia was measured using a LR8600 Freshwater/Saltwater Ammonia Water Test (Aquarium Pharmaceuticals, Mars Fishcare Incorporated, Chalfont, Pennsylvania, USA).

Due to the distribution of *Batrachochytrium dendrobatidis* in California (Padgett-Flohr and Hopkins 2009), I randomly sampled one individual reaching metamorphosis from each of 30 tanks to determine if the study animals carried the fungus. Sampling followed Brem et al. (2007), and samples were sent to Pisces Molecular, Boulder, Colorado, USA for polymerase-chain reaction assays. All samples were negative for *B. dendrobatidis*. Animal husbandry and treatment were approved by the SIU Institutional Animal Care and Use Committee (protocol 08-025).

Pesticides and Treatments

Reagent-grade chlorpyrifos (99% pure, Restek, CAS 2921-88-2) and a 70:30 mixture of endosulfan I and endosulfan II (99% pure, Restek, CAS 115-29-7) were used. Pesticides were dissolved in acetone to make stock solutions and kept at -20 ºC. Pesticide concentrations needed for dosing were prepared from stock solutions immediately prior to their intended use. Test concentrations consisted of three concentrations of chlorpyrifos and three concentrations of endosulfan. Based on previous findings for *P. regilla (=P. sierra*; Sparling and Fellers 2009), low, medium, and high chlorpyrifos concentrations consisted of 137, 266, and 394 μg/L, respectively, and low, medium, and high endosulfan concentrations consisted of 4.5, 7.9, and 11.3 μg/L, respectively. The experiment followed a full 4x4 factorial design with controls and the

chlorpyrifos and endosulfan concentrations. In addition to a negative control consisting of 2 ml reconstituted water, I used a vehicle control of 2 ml acetone. Chemical treatments and the vehicle control were represented by five replicates, and the negative control consisted of six replicates for a total of 86 experimental aquaria. Each 8 L test aquarium was filled with 7 L reconstituted water, and three randomly selected tadpoles at Gosner stage 25 (Gosner 1960) were placed in each aquarium. Individual doses for each aquarium were made by placing the amount of stock solution of each pesticide necessary to reach the desired final concentration into an amber vial and adding acetone as a vehicle for a final dose volume of 2 ml. The doses were added to each aquarium at the initiation of the exposures (experimental day 0) and following each bi-weekly water change. Aquaria waters were stirred using a glass stir rod to ensure a uniform distribution of the dose in the water column.

Water analyses were conducted to validate actual treatment concentrations by setting up three extra tanks each for the low/low, medium/medium, and high/high chlorpyrifos/endosulfan combinations and one negative control tank. A sample of 800 ml water was taken from each aquarium ca. 1 h after addition of the treatment. Additional samples were taken every 24 h the following 3 d to assess loss of insecticides from the aquaria. Samples were placed in a separatory funnel and a one part per million (ppm) decachlorobiphenyl/ dibromooctaflurobiphenyl surrogate was added. The samples were extracted with CH_2Cl_2 three times, and the total extract was dried with Na_2SO_4. A solvent exchange was performed with C_6H_{14}, and the samples were reduced to ca. 5 ml. The samples were further reduced to ca. 1 ml under N_2. A 10 ppm PCB 204 internal standard was added to each sample before actual concentrations were determined with

gas chromatography-mass spectrometry at the SIU Mass Spectrometry Facility. Reagent blanks and matrix spikes were subjected to the same procedures as those used on actual samples.

Response variables included survival, days to death, body mass and snout-vent length (SVL) after 10 and 30 d exposure and at metamorphosis, days to metamorphosis, duration of metamorphic climax (Gosner stages 42-46), occurrence of and time to development of axial malformations and ChE activity. Each day larvae were observed for unusual behavior, morbidity and death. Dead animals were removed and weighed (± 0.01g) using an XT Top Loading Balance (Thermo Fisher Scientific, Inc., Hampton, New Hampshire, USA), measured for SVL (± 0.1mm) using an electronic digital caliper (Thermo Fisher Scientific, Inc., Hampton, New Hampshire, USA), staged for development, and stored in individual vials at -80ºC. Larvae from each aquarium were weighed, measured for SVL, and staged for development on day 10 and 30. At Gosner stage 42, animals were measured, weighed, and placed in individual slanted 1 L Ball jars with ca. 75 ml of treatment water until completion of metamorphosis. At metamorphosis they were measured again, euthanized in tricaine methanesulfonate (MS-222), and stored in individual vials at -80ºC until analyzed for ChE activity.

Half of the individuals reaching Gosner stage 46 were analyzed for total ChE using a photometric method (Ellmann et al. 1961) modified for a 96-well microplate reader. Each sample was run in triplicate or until a coefficient of variation among three replicates was less than 5%. Mean slopes for each sample was calculated and arithmetically converted to μmol/g tissue/sec for ChE activity.

Statistical Analyses

Statistical analyses were performed using SAS 9.1 (SAS Institute Cary, North Carolina, USA). The normality of response variables was tested using the Shapiro-Wilks test and by visual observations of the residual plots for normality and homogeneity of variances. Body mass after 10 and 30 d exposure, time to death, duration of metamorphic climax, time to development of axial malformations, and SVL at metamorphosis were log transformed to normalize data and stabilize variances, and proportions of animals surviving to metamorphosis and developing axial malformations were arcsine transformed. All response variables met the assumptions of parametric statistics either before or after transformation. Negative controls and vehicle controls were initially compared using analysis of variance (ANOVA) to test for differences among response variables. No significant differences were observed for any of the response variables ($p >$ 0.07 for all comparisons), so data from both controls were pooled. Because larva in a common aquarium might be considered dependent samples, I used ANOVA to test for between-aquarium effects for response variables by examining a nested effect of aquarium within treatment. A lack of significance for the nested effect meant that an aquarium effect did not occur and individual larva could be treated as experimental units. Aquaria served as the experimental units when there were significant aquarium effects for the response variables of survival, duration of metamorphic climax, time to metamorphosis, and body mass and SVL at metamorphosis. Individual larvae were the experimental units for other response variables (time to development of axial malformations, ChE) that did not show inter-aquarium effects ($p > 0.22$). Aquaria served as experimental units for body mass and SVL on days 10 and 30 because larvae were not

individually marked, and thus mean aquarium measurements were used.

ANOVA was used to determine if treatments had an effect on all response variables except measurements at metamorphosis (i.e. body mass and SVL). I examined the effects of each insecticide alone (i.e. the concentration of the other pesticide was set at 0) using one-way ANOVA and the combined effects of both insecticides using two-way ANOVA. For measurements at metamorphosis, ANCOVA was used with time to metamorphosis as the covariate. For all significant statistical tests, Tukey's honestly significant difference test was used post-hoc to test for specific comparisons among treatment groups. Statistical significance is based on $\alpha=0.05$.

RESULTS

Water Parameters

Dissolved oxygen ranged from 5.00 to 7.50 mg/L (mean 6.46 ± 0.32 mg/L) and water temperatures ranged from 15.7° to 18.5°C (mean 17.1 ± 0.4°C) during the study. pH ranged from 7.05 to 7.22 (mean 7.13 ± 0.01). Total ammonia remained below 0.25 mg/L for the duration of the study.

Limit of quantitation was 1 µg/L for both insecticides. Measured concentrations of chlorpyrifos averaged 87%, 67%, and 66% of nominal for 134, 266, and 394 µg/L chlorpyrifos, respectively, after 1 h (Figure 11). Thus, chlorpyrifos concentrations averaged 73% of nominal. Concentrations of observed effects in the following results are reported as nominal values, though respective corrections of 73% for 137, 266, and 394 µg/L are 100, 194, and 288 µg/L, respectively. Measured concentrations of chlorpyrifos averaged 19%, 24%, and 24% of nominal concentrations after 72 h (Figure 11).

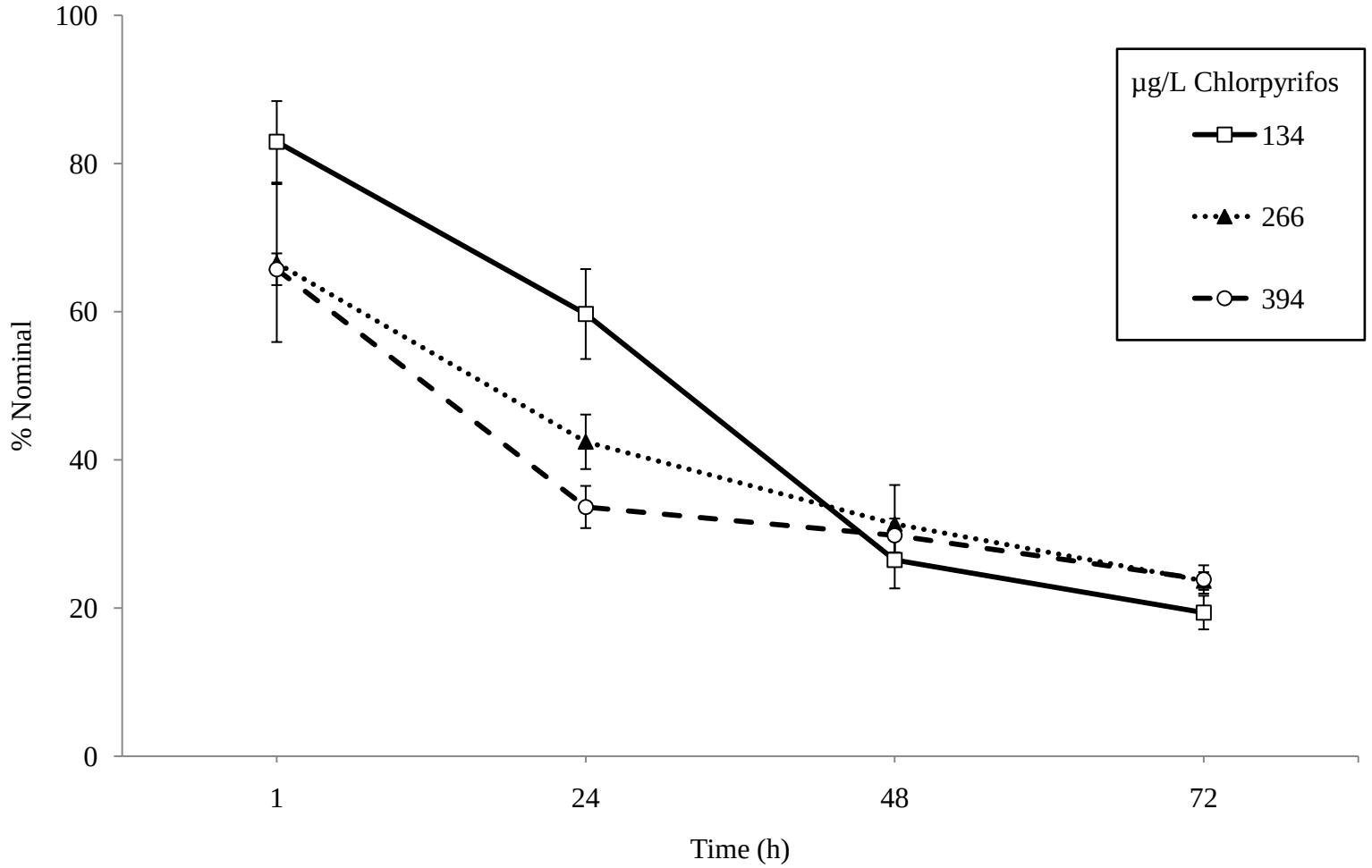

Figure 11. Mean percent of nominal chlorpyrifos concentration after 1, 24, 48, and 72 h. Error bars are ± one standard error.

Measured concentrations of endosulfan I averaged 91%, 88%, and 91% of nominal for 4.5, 7.9, and 11.3 µg/L endosulfan, respectively, after 1 h and 22%, 23%, and 21% of nominal after 72 h. Endosulfan II averaged 97%, 93%, and 96% of nominal for 4.5, 7.9, and 11.3 µg/L endosulfan, respectively, after 1 h and 27%, 29%, and 26% of nominal after 72 h (Figure 12). The following results and discussion are based on nominal 1 h concentrations of both pesticides.

Survival

Survival of control animals was 94%. Survival of larvae only exposed to chlorpyrifos exceeded 80% and was not affected by concentration (Table 4; Figure 13). Exposure to endosulfan alone resulted in decreased survival with increasing concentration of the insecticide (Table 4). Survival was 100, 67, and 33% in larvae exposed to 4.5, 7.9, and 11.3 µg/L endosulfan only, respectively (Figure 13). In the full model larval survival was also significantly affected by the interaction of chlorpyrifos and endosulfan (Table 5). In the presence of 7.9 or 11.3 µg/L endosulfan only, survival was 67 and 33%, respectively, but was 87% in larvae exposed to 7.9 or 11.3 µg/L endosulfan with 137 µg/L chlorpyrifos. All larvae exposed to 4.5 µg/L endosulfan with 137 µg/L chlorpyrifos survived, but 4.5 µg/L endosulfan with 266 or 394 µg/L chlorpyrifos decreased survival by 40 and 80%, respectively. Overall, survival decreased by 40-93% when endosulfan was combined with 266 or 394 µg/L chlorpyrifos. Similar trends were observed in survival rates after only 30 d (Figure 13). Time to death was not influenced by chlorpyrifos, endosulfan, or their interaction (Tables 4 and 5). Average time to death was 35.8 d across controls and all treatments.

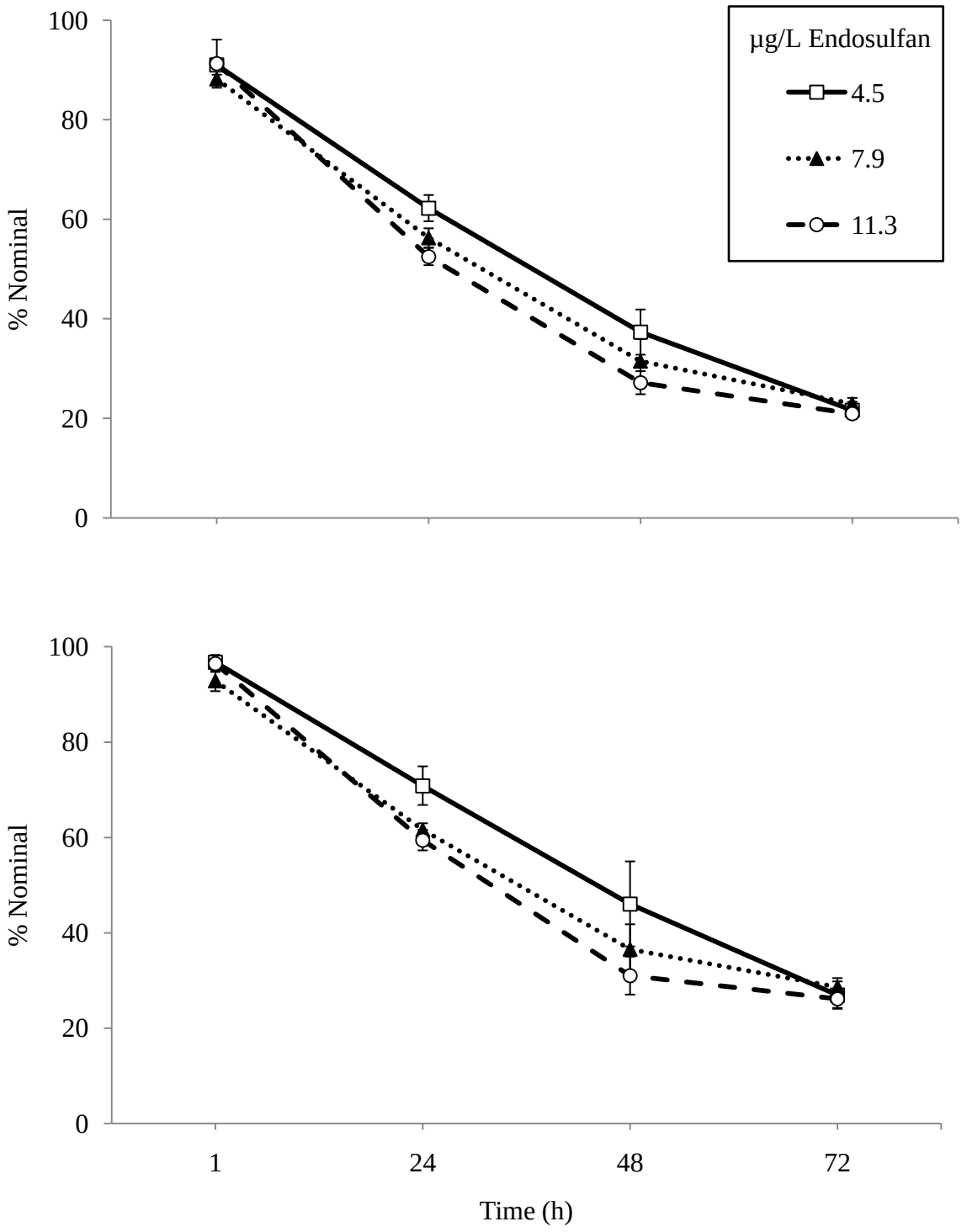

Figure 12. Mean percent of nominal endosulfan I (top) and endosulfan II (bottom) concentration after 1, 24, 48, and 72 h. Error bars are ± one standard error.

Table 4. Summary of one-way ANOVA results of the individual effects of chlorpyrifos and endosulfan on response variables for *Pseudacris sierra* larvae.

Response Variable	Treatment	*df*	*F*	*p*
Survival to metamorphosis	Chlorpyrifos	3,22	1.11	0.3680
	Endosulfan	3,22	9.19	0.0004
Time to death	Chlorpyrifos	2,3	0.18	0.8415
	Endosulfan	2,14	0.02	0.9846
Time to metamorphosis	Chlorpyrifos	3,22	6.04	0.0037
	Endosulfan	3,19	4.93	0.0107
Metamorphic climax duration	Chlorpyrifos	3,22	1.00	0.4107
	Endosulfan	3,19	2.06	0.1395
Malformation occurrence	Endosulfan	3,24	1.64	0.2059
Cholinesterase	Chlorpyrifos	3,34	1.45	0.2452
	Endosulfan	3,29	3.45	0.0294

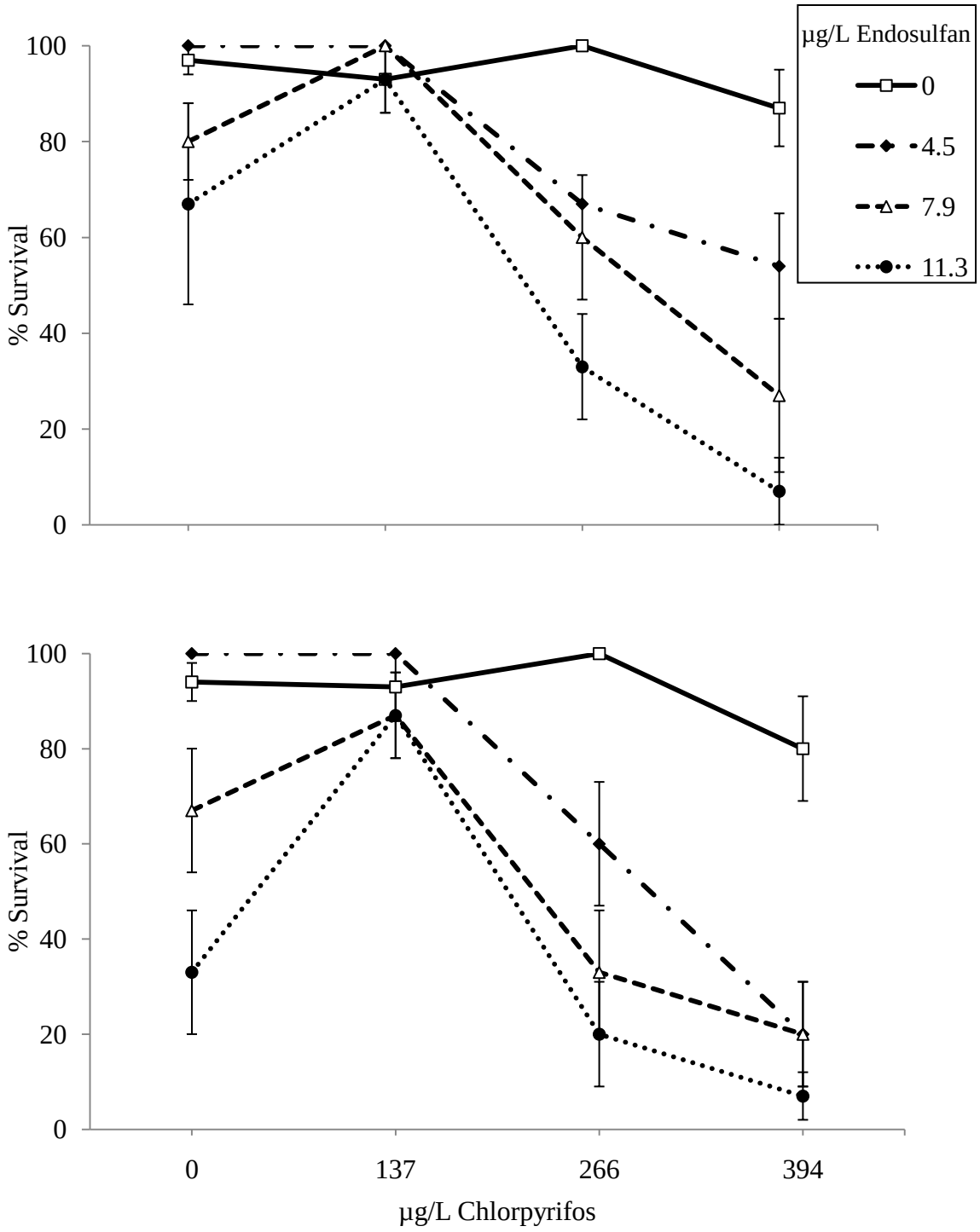

Figure 13. Mean percent survival to day 30 (top) and metamorphosis (bottom) of *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

Table 5. Summary of two-way ANOVA results of chlorpyrifos, endosulfan, and their interaction on response variables for *Pseudacris sierra* larvae.

Response Variable	Treatment	*df*	*F*	*p*
Survival to metamorphosis	Chlorpyrifos	3,70	26.80	<0.0001
	Endosulfan	3,70	25.28	<0.0001
	Chlorpyrifos* Endosulfan	9,70	3.60	0.0010
Time to death	Chlorpyrifos	3,78	1.83	0.1488
	Endosulfan	3,78	0.85	0.4688
	Chlorpyrifos* Endosulfan	6,78	1.75	0.1213
Time to metamorphosis	Chlorpyrifos	3,54	2.71	0.0538
	Endosulfan	3,54	3.24	0.0291
	Chlorpyrifos* Endosulfan	9,54	0.94	0.4967
Metamorphic climax duration	Chlorpyrifos	3,54	0.02	0.9955
	Endosulfan	3,54	1.77	0.1636
	Chlorpyrifos* Endosulfan	9,54	0.95	0.4923
Malformation occurrence	Chlorpyrifos	3,70	30.59	<0.0001
	Endosulfan	3,70	15.97	<0.0001
	Chlorpyrifos* Endosulfan	9,70	5.17	<0.0001
Time to malformation	Chlorpyrifos	3,48	1.99	0.1273
	Endosulfan	2,48	0.24	0.7882
	Chlorpyrifos* Endosulfan	2,48	0.46	0.6341
Cholinesterase	Chlorpyrifos	3,74	6.03	0.0010
	Endosulfan	3,74	3.93	0.0116
	Chlorpyrifos* Endosulfan	9,74	0.60	0.7900

Effects on Growth and Development

In the absences of endosulfan, there was a significant effect of chlorpyrifos on body mass at 10 and 30 d exposure. Similar results were observed for endosulfan in the absence of chlorpyrifos (Table 6). Control larvae were 0.14 g at 10 d exposure, and all larvae exposed to only chlorpyrifos or endosulfan were significantly smaller than controls (Figure 14). At 30 d exposure, control larvae were 0.97 g. Larvae exposed to 266 or 394 µg/L chlorpyrifos only were significantly smaller than controls, as were those exposed to only 7.9 or 11.3 µg/L endosulfan (Figure 15). Body mass at 10 and 30 d exposure was significantly affected by the chlorpyrifos-endosulfan interaction (Table 7). At 10 d exposure, chlorpyrifos and endosulfan predominantly displayed additive effects, though a slight but non-significant trend of antagonism was observed in 7.9 or 11.3 µg/L endosulfan with 137 µg/L chlorpyrifos (Figure 14). The strength of this antagonism increased at 30 d exposure. Larvae exposed to 137 µg/L chlorpyrifos with 7.9 µg/L endosulfan were more than twice as large as those exposed to 7.9 µg/L endosulfan only. A similar trend was observed in animals exposed to 137 µg/L chlorpyrifos with 11.3 µg/L endosulfan when compared to only 11.3 µg/L endosulfan, though these differences were not significant. Mean body mass of larvae exposed to 394 µg/L chlorpyrifos with 11.3µg/L endosulfan was 0.36 g and was not statistically different than control larvae, though data in the 394 µg/L chlorpyrifos and 11.3µg/L endosulfan treatment group was from only one individual larvae still alive on day 30 (Figure 15).

Similar results to body mass were observed for SVL at 10 and 30 d exposure. There were significant effects by the individual insecticides, and SVL decreased with increasing concentrations of the insecticides (Table 6). Control larvae had an average

Table 6. One-way ANOVA results of the individual effects of chlorpyrifos and endosulfan on body mass and snout-vent length (SVL) of *Pseudacris sierra* larvae after 10 and 30 d exposure.

Response Variable	Treatment	*df*	*F*	*p*
Body mass on day 10	Chlorpyrifos	3,22	93.88	<0.0001
	Endosulfan	3,22	58.11	<0.0001
SVL on day 10	Chlorpyrifos	3,22	93.76	<0.0001
	Endosulfan	3,22	64.37	<0.0001
Body mass on day 30	Chlorpyrifos	3,22	19.19	<0.0001
	Endosulfan	3,20	49.49	<0.0001
SVL on day 30	Chlorpyrifos	3,22	27.47	<0.0001
	Endosulfan	3,20	75.42	<0.0001

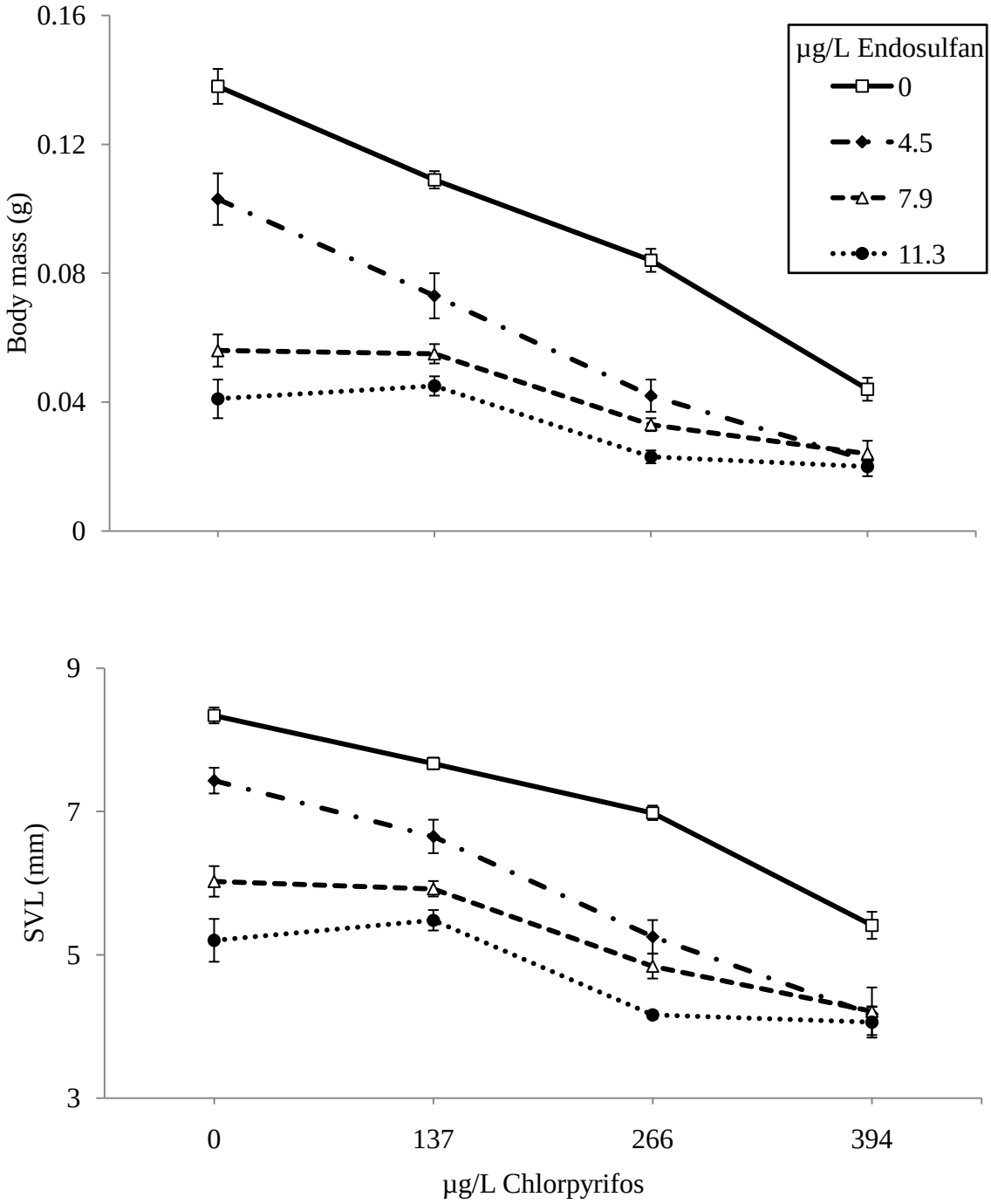

Figure 14. Body mass and snout-vent length (SVL) on day 10 of *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

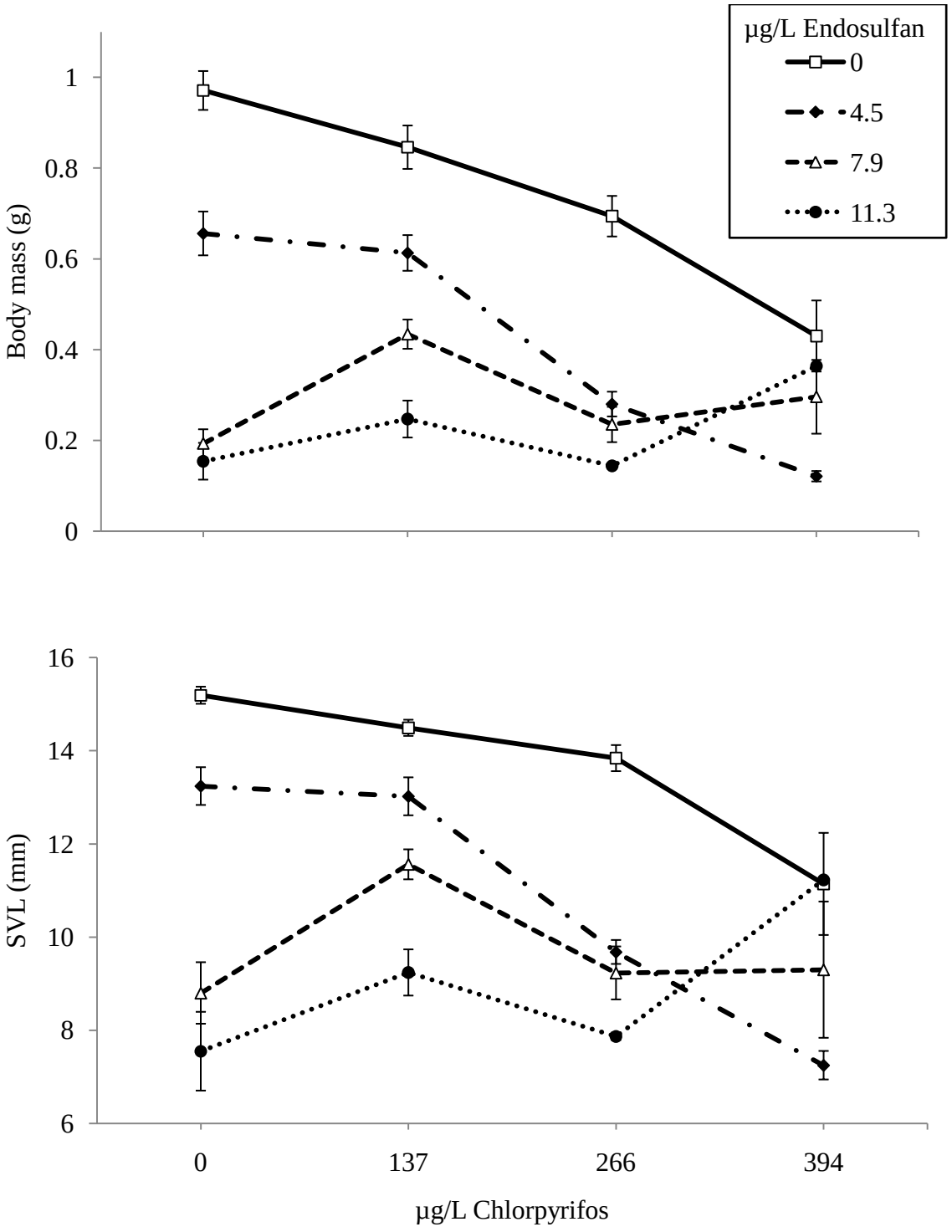

Figure 15. Body mass and snout-vent length (SVL) on day 30 of *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

Table 7. Two-way ANOVA results of the effects of chlorpyrifos, endosulfan, and their interaction on body mass and snout-vent length (SVL) of *Pseudacris sierra* larvae after 10 and 30 d exposure.

Response Variable	Treatment	*df*	*F*	*p*
Body mass on day 10	Chlorpyrifos	3,70	117.34	<0.0001
	Endosulfan	3,70	100.93	<0.0001
	Chlorpyrifos* Endosulfan	9,70	2.90	0.0057
SVL on day 10	Chlorpyrifos	3,70	128.57	<0.0001
	Endosulfan	3,70	126.93	<0.0001
	Chlorpyrifos* Endosulfan	9,70	5.26	<0.0001
Body mass on day 30	Chlorpyrifos	3,60	10.99	<0.0001
	Endosulfan	3,60	44.12	<0.0001
	Chlorpyrifos* Endosulfan	9,60	6.93	<0.0001
SVL on day 30	Chlorpyrifos	3,60	14.47	<0.0001
	Endosulfan	3,60	62.21	<0.0001
	Chlorpyrifos* Endosulfan	9,60	8.44	<0.0001

SVL of 8.3 mm at 10 d exposure, and all larvae exposed to only chlorpyrifos or endosulfan were significantly shorter than controls (Figure 14). At 30 d exposure, SVL of control larvae was 15.2 mm. Larvae exposed to 266 or 394 µg/L chlorpyrifos only were significantly shorter than controls, as were those in the 7.9 or 11.3 µg/L endosulfan

only treatment groups (Figure 15). SVL at 10 and 30 d exposure was significantly affected by the chlorpyrifos-endosulfan interaction (Table 7). At 10 d exposure, chlorpyrifos and endosulfan again predominantly displayed additive effects, though a slight but non-significant antagonism trend was observed in the 7.9 or 11.3 µg/L endosulfan with 137 µg/L chlorpyrifos groups (Figure 14). The strength of this antagonism increased by 30 d of exposure. Larvae exposed to 137 µg/L chlorpyrifos with 7.9 µg/L endosulfan were significantly larger than those exposed to7.9 µg/L endosulfan only. They were also larger than those in the 266 µg/L chlorpyrifos with 7.9 µg/L endosulfan and 394 µg/L chlorpyrifos with 7.9 µg/L endosulfan groups, though these differences were not significant. Mean SVL in larvae exposed to 394 µg/L chlorpyrifos with 11.3µg/L endosulfan was 11.2 mm and was not statistically different than control larvae (Figure 15).

Chlorpyrifos and endosulfan alone significantly affected time to metamorphosis for those animals reaching Gosner stage 46 (Table 4). Control larvae took an average of 62 d to reach metamorphosis. Larvae exposed to 394 µg/L chlorpyrifos or 11.3 µg/L endosulfan took 13 d longer than controls to reach metamorphosis (Figure 16). There was no interaction between chlorpyrifos and endosulfan on time to metamorphosis, and there were no individual effects of either insecticide or their interaction on duration of metamorphic climax (Tables 4 and 5). Mean duration of metamorphic climax was 6.5 d across controls and all treatments. For animals reaching metamorphosis, chlorpyrifos alone did not affect SVL or body mass but endosulfan did. The endosulfan-time interaction was also significant for both parameters (Table 8). Average SVL and body mass of control animals was 18.7 mm and 0.65 g, respectively. Overall, SVL and

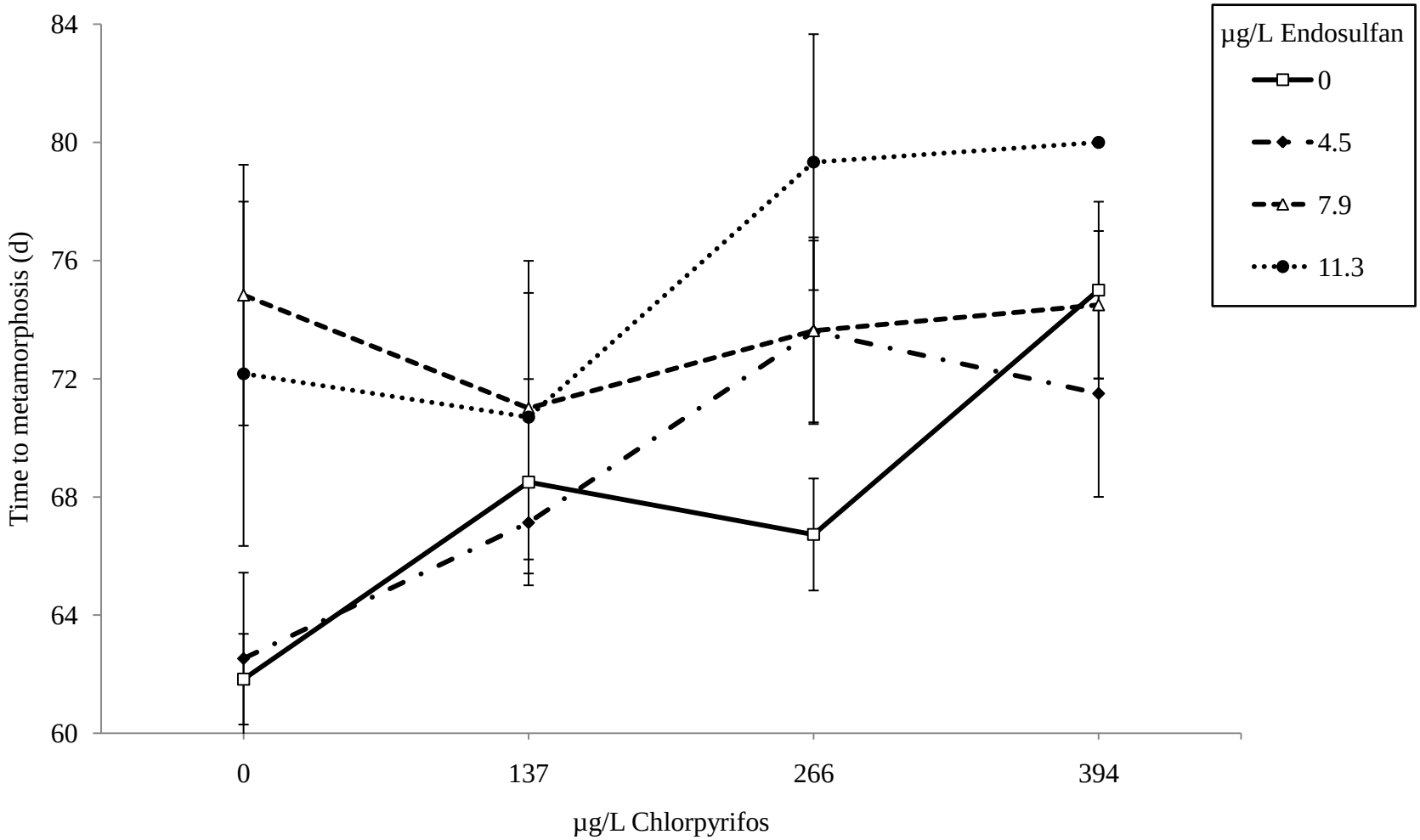

Figure 16. Time to metamorphosis for *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

Table 8. Results of ANCOVA on the individual effects of chlorpyrifos and endosulfan on snout-vent length (SVL) and body mass of *Pseudacris sierra* larvae with time to metamorphosis as a covariate.

Response Variable	Treatment	*df*	*F*	*p*
SVL	Chlorpyrifos	3,18	0.14	0.9378
	Time	1,18	8.66	0.0087
	Time* Chlorpyrifos	3,18	0.13	0.9385
	Endosulfan	3,15	4.77	0.0157
	Time	1,15	0.45	0.5139
	Time* Endosulfan	3,15	6.74	0.0043
Body mass	Chlorpyrifos	3,18	0.10	0.9602
	Time	1,18	8.46	0.0094
	Time* Chlorpyrifos	3,18	0.12	0.9480
	Endosulfan	3,15	3.76	0.0339
	Time	1,15	0.17	0.6891
	Time* Endosulfan	3,15	5.55	0.0092

body mass decreased with increasing concentrations of endosulfan. Animals exposed to 7.9 or 11.3 µg/L endosulfan only were 17 and 27% shorter and 43 and 61% smaller than controls, respectively, (Figure 17). The ANCOVA on SVL at metamorphosis with time to metamorphosis as a covariate revealed a significant effect of the chlorpyrifos and

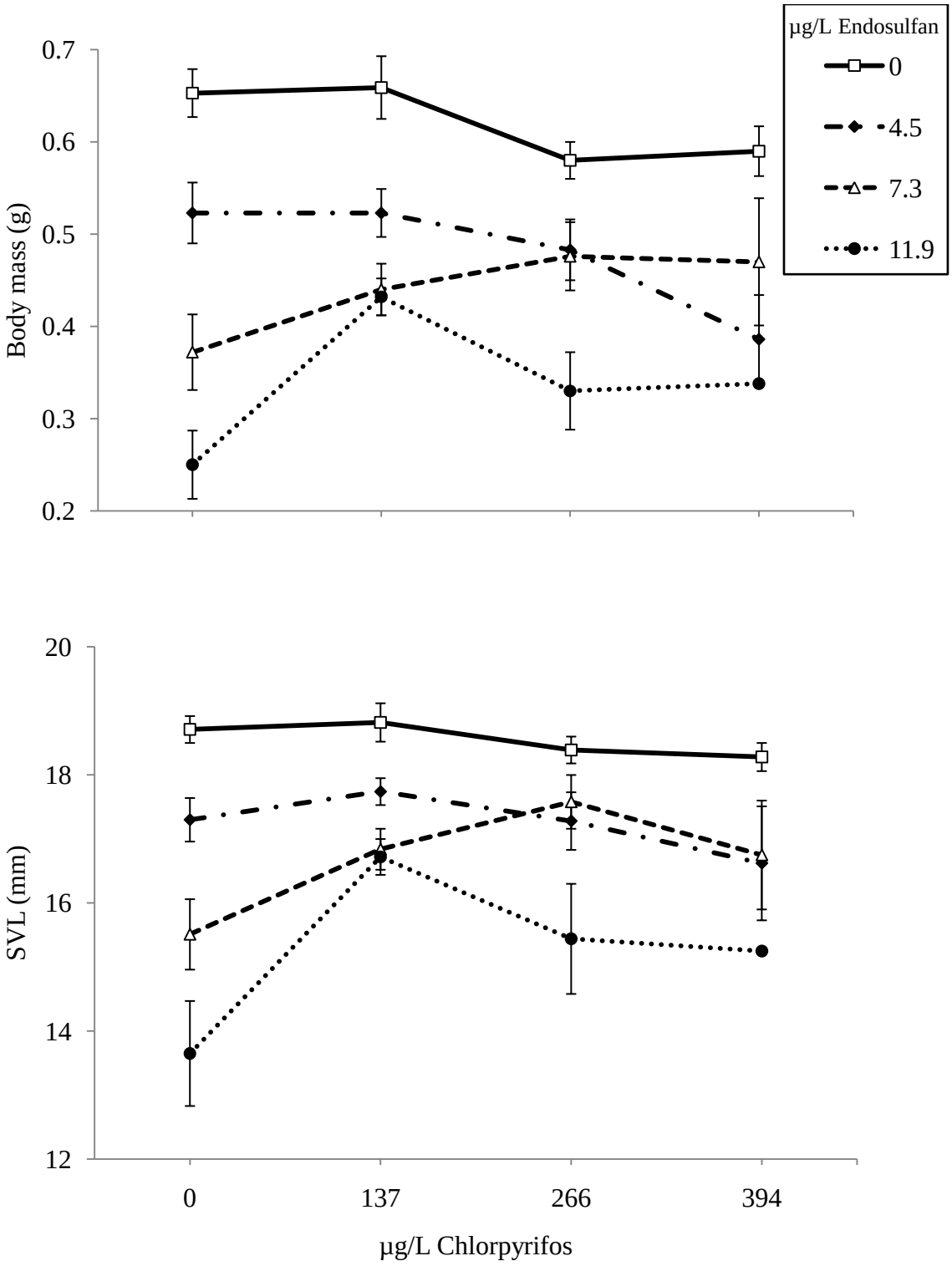

Figure 17. Body mass and snout-vent length (SVL) at metamorphosis of *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

endosulfan interaction (Table 9). SVL was greater in larvae exposed to 7.9 µg/L endosulfan with 137 or 266 µg/L chlorpyrifos than to 7.9 µg/L endosulfan alone. SVL was also greater after exposure to 11.3 µg/L endosulfan with 137 µg/L chlorpyrifos than in animals exposed to 11.3 µg/L endosulfan only. Body mass displayed similar trends, though the chlorpyrifos-endosulfan interaction was not significant.

Of those larvae exposed to either insecticide alone, development of axial malformations was rare (Table 4). Scoliosis only developed in 7% of those larvae in the 7.3 µg/L endosulfan treatment group. The interaction of chlorpyrifos and endosulfan significantly affected the development of this scoliosis (Table 5; Figure 18). Scoliosis was absent in larvae exposed to 137 µg/L chlorpyrifos and 4.5 µg/L endosulfan together but occurred in combinations of the higher concentrations and increased with increasing concentration. Scoliosis occurrence was highest (87%) in larvae exposed to 394 µg/L chlorpyrifos with 11.3 µg/L endosulfan (Figure 19). There was no effect of chlorpyrifos, endosulfan, or their interaction on time to development of scoliosis (Table 5). Mean time to development of scoliosis was 7.5 d across all treatment groups.

Cholinesterase

ChE activity was significantly affected by endosulfan alone but not by chlorpyrifos alone (Table 4). ChE activity increased with increasing concentration of endosulfan. Overall, mean ChE activity for control animals was 1.02 µmol/g tissue/s. ChE activity of animals exposed to 11.3 µg/L endosulfan was 30% greater than control animals (Figure 20). ChE activity was not affected by the chlorpyrifos and endosulfan interaction, but the main chlorpyrifos effect in the presence of endosulfan was significant

Table 9. Results of ANCOVA on the effects of chlorpyrifos, endosulfan, and their interaction on snout-vent length (SVL) and body mass of *Pseudacris sierra* larvae with time to metamorphosis as a covariate.

Response Variable	Treatment	*df*	*F*	*p*
SVL	Chlorpyrifos	3,39	1.67	0.1895
	Endosulfan	3,39	3.99	0.0142
	Chlorpyrifos* Endosulfan	8,39	2.82	0.0143
	Time	1,39	1.69	0.2017
	Time* Chlorpyrifos	3,39	1.97	0.1350
	Time* Endosulfan	3,39	5.66	0.0026
	Time* Chlorpyrifos* Endosulfan	8,39	3.18	0.0072
Body mass	Chlorpyrifos	3,39	1.40	0.2567
	Endosulfan	3,39	2.41	0.0813
	Chlorpyrifos* Endosulfan	8,39	1.54	0.1752
	Time	1,39	6.73	0.0133
	Time* Chlorpyrifos	3,39	1.35	0.2719
	Time* Endosulfan	3,39	3.69	0.0197
	Time* Chlorpyrifos* Endosulfan	8,39	1.79	0.1098

Figure 18. A control larva displaying normal axial development (left) and a larva exposed to 266 µg/L chlorpyrifos and 7.9 µg/L endosulfan displaying scoliosis (right). Photo taken at 30 d exposure.

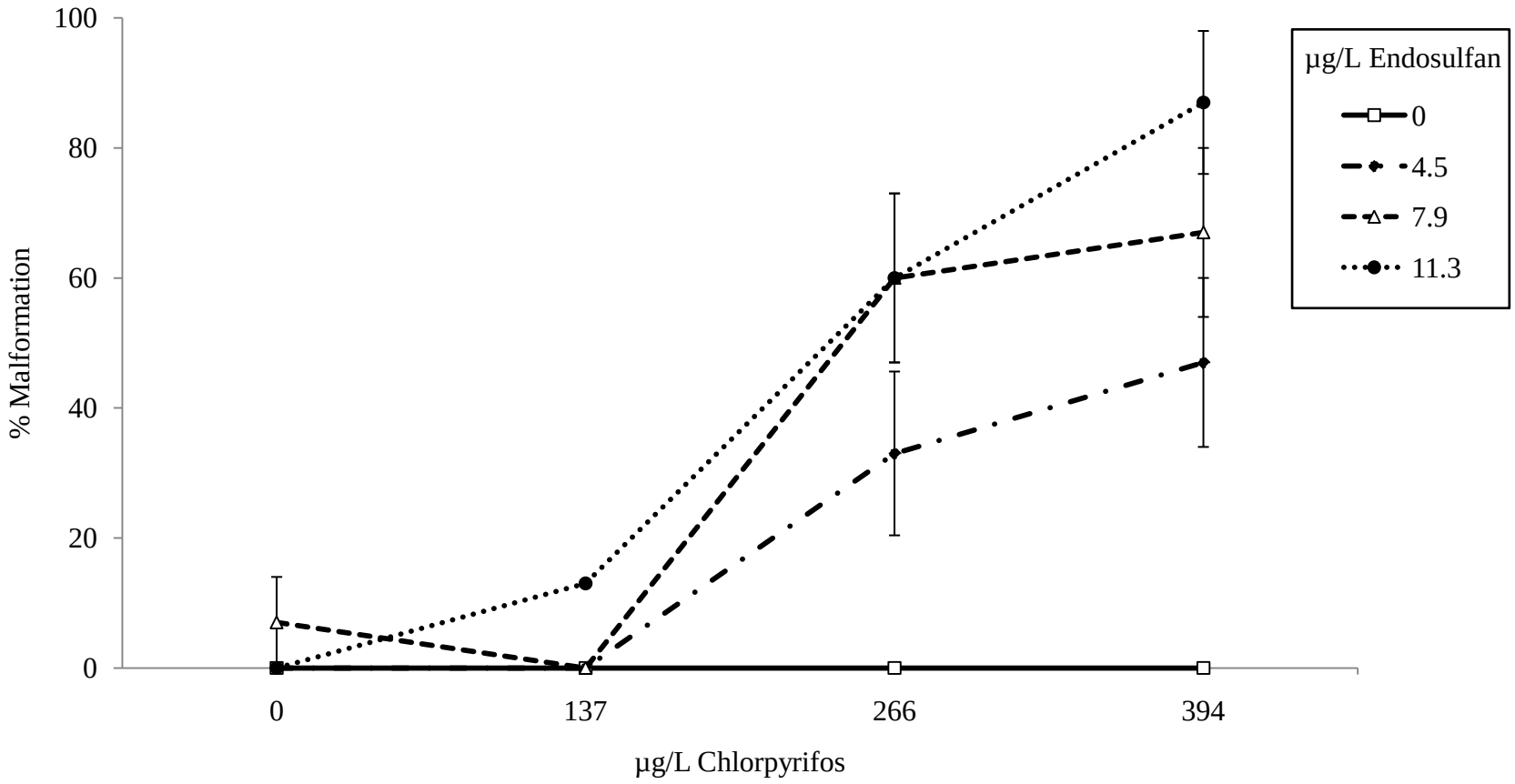

Figure 19. Mean percent of animals developing axial malformations for *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

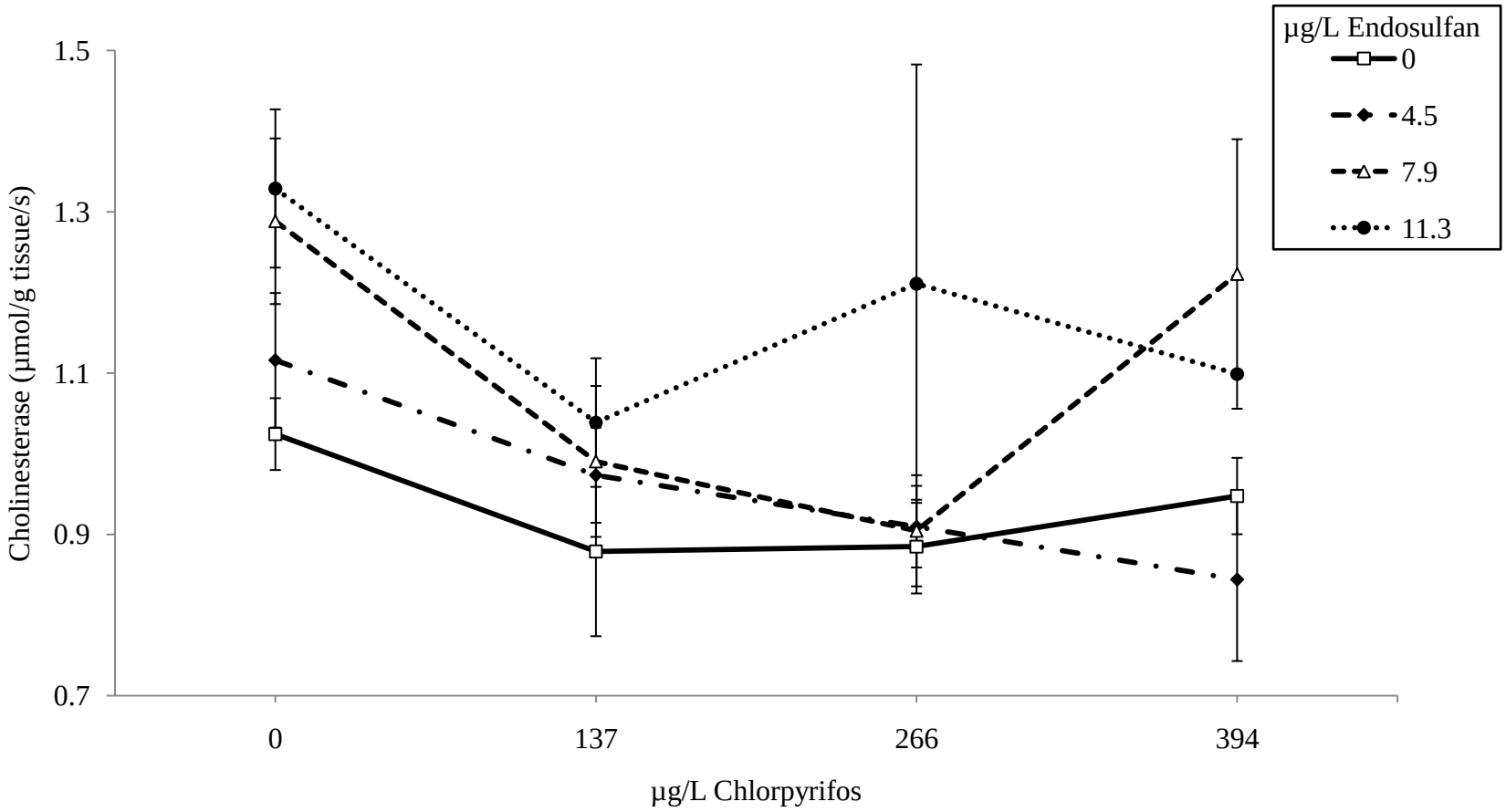

Figure 20. Mean cholinesterase for *Pseudacris sierra* larvae exposed to chlorpyrifos and endosulfan. Error bars are ± one standard error.

(Table 5). Mean ChE activity was greater in animals not exposed to chlorpyrifos (mean 1.11 µmol/g tissue/s) than in all groups exposed to chlorpyrifos. However, there was no clear trend of decreasing ChE with increasing chlorpyrifos concentrations. Mean ChE activity in animals exposed to chlorpyrifos, regardless of endosulfan concentrations, was higher in animals exposed to 394 µg/L (0.99 µmol/g tissue/s) and 134 µg/L (0.97 µmol/g tissue/s) then animals exposed to 266 µg/L (0.92 µmol/g tissue/s; Figure 20).

DISCUSSSION

Analysis of insecticides from aquaria revealed initial post-application chlorpyrifos concentrations averaged 73% of nominal. Thus, these measured concentrations should be considered along with nominal concentrations when interpreting the results of the current study. In a similar study with glass aquaria, Sparling and Fellers (2009) measured chlorpyrifos concentrations at an average of 61% of nominal concentrations within 12 h of application. Widder and Bidwell (2006) measured an initial 10 – 15% loss of chlorpyrifos from glass jar microcosms and polypropylene wading pool mesocosms, and a 90% loss following 96 h. This is consistent with the approximately 80% loss of chlorpyrifos in the current study after 72 h. Mazanti et al. (2003) measured post-application chlorpyrifos concentrations at an average of 56% of nominal in a similar laboratory aquarium study. Loss of chlorpyrifos was attributed to the aeration of the aquaria. As much as a 12% loss of bioavailable chlorpyrifos has been documented due to adsorption to glass surfaces of aquaria (Thomas and Mansingh 2002). Both of these mechanisms may have played a role in the loss of chlorpyrifos in the current study. Concentrations averaged greater than 90% of nominal for endosulfan I and endosulfan II

after initial application in the current study, suggesting agreement between the measured concentrations and the use of nominal concentrations in the results. Loss of both isomers of endosulfan was near 75 – 80% of nominal after 72 h, consistent with data following 96 h from a similar study (D. Sparling, personal communication). The dominant process for the degradation of endosulfan in water is hydrolysis (Peterson and Batley 1993).

Chlorpyrifos alone did not have a strong effect on survival. Survival was 80% even at the highest concentration of 394 µg/L. Using a similar design, Sparling and Fellers (2009) reported that survival of *P. regilla* (=*P. sierra*) larvae exposed to 200 µg/L chlorpyrifos was 67%. The authors also estimated an LC_{50} of 365 µg/L chlorpyrifos. Thus, the largest chlorpyrifos exposure in the current study was a slightly higher concentration than the estimated LC_{50}, but mortality was only 20%. This may be because measured chlorpyrifos concentrations in the current study were actually lower than the reported LC_{50} values. Endosulfan, however, resulted in a more consistent concentration-response picture. Survival of larvae exposed to 7.9 and 11.3 µg/L endosulfan only was 67 and 33%, respectively. Sparling and Fellers (2009) reported survival of larvae exposed to 3.12 and 12.5 µg/L endosulfan was 83 and 19%, respectively, and the endosulfan LC_{50} was estimated as 15.6 µg/L. Jones et al. (2009) reported an LC_{50} for *P. regilla* larvae of 21.4 µg/L after only 4 d.

The individual effects of chlorpyrifos and endosulfan on body mass and SVL after 10 and 30 d exposure also displayed an expected concentration-response similar to that reported by Sparling and Fellers (2009). Larval size decreased with increasing concentrations of both insecticides. Body mass and SVL at metamorphosis were also consistent with findings by Sparling and Fellers (2009), as chlorpyrifos did not have an

effect on either parameter of body size. Body mass and SVL decreased with increasing concentrations of endosulfan, and time to metamorphosis increased with increasing concentrations of insecticide for larvae exposed to only chlorpyrifos or endosulfan.

Exposure to chlorpyrifos, reduced tadpole ChE activity, although this reduction was not statistically significant. Mean ChE activity was 8 – 14% lower in all animals exposed to chlorpyrifos than control animals, though there was no clear trend of decreasing ChE activity with increasing concentrations. Depression of ChE activity from exposure to chlorpyrifos was not as large as predicted based on other studies with ChE inhibiting insecticides. In the current study, ChE activity was 86% of controls, and ChE depression was greatest in larvae exposed to the lowest chlorpyrifos concentration. Widder and Bidwell (2006) measured a 43% reduction in ChE in *Lithobates sphenocephalus* larvae exposed to 200 µg/L chlorpyrifos after 12 d of exposure. Sparling and Fellers (2009) reported a consistent decrease in ChE activity in *P. regilla* with increasing concentration of chlorpyrifos. In that study, animals exposed to 200 µg/L chlorpyrifos experienced an approximately 25% reduction in ChE activity compared to controls. Depression of ChE activity has been associated with uncoordinated behavior and a greater susceptibility to predation in anuran larvae (Bridges 1997).

In contrast to chlorpyrifos, ChE activity increased with increasing concentrations of endosulfan for those animals only exposed to endosulfan. Endosulfan is not a ChE inhibiting insecticide but instead reduces neuronal inhibition and leads to excitation of the central nervous system by blocking the uptake of chloride ions by neurons (Bloomquist 1993). Thus, the increase in neuron activity associated with endosulfan exposure may have led to increased activity at neural synapses, where AChE is necessary to end the

transmission of neural impulses. An increase in total AChE would then be necessary to compensate for the increase in neural activity following endosulfan exposure, and this is supported by the data in the current study of animals only exposed to endosulfan. ChE activity of animals exposed to 11.3 µg/L endosulfan was 30% greater than control animals.

The increase in ChE activity due to endosulfan exposure was unexpected based on other studies. ChE activity of larval *Rana sierrae* exposed to up to 17.75 µg/L endosulfan for 63 d was not different than that of animals not exposed to endosulfan (D. Dimitrie, unpublished data). Tu et al. (2009) measured a 30% inhibition of ChE activity in muscle of black tiger shrimp after 4 d exposure to 0.9 µg/L endosulfan. I could find no published studies on the effects of endosulfan on ChE activity in anurans or on increased ChE activity for any taxa following exposure to endosulfan. Bloomquist (2003) suggested chemicals such as OCs that target the chloride channels of neurons may additionally target ACh. If the ACh receptor for AChE was blocked by an OC such as endosulfan in the current study, it is expected that AChE would accumulate and increase with increasing concentrations of endosulfan.

Examining the combined effects of two insecticides with different modes of action in the present study demonstrates that they can have unpredictable interactions on different response variables at varying concentrations. Survival increased by as much as 54% in larvae exposed to 137 µg/L chlorpyrifos with 7.9 or 11.3 µg/L endosulfan. However, at higher concentrations of chlorpyrifos with endosulfan, survival actually decreased more than would be expected by the sum of chlorpyrifos and endosulfan alone. This likely indicates a threshold in the overall toxic load that a developing tadpole can

tolerate. The antagonism of endosulfan by chlorpyrifos was not clearly apparent at 10 d exposure, as evident by the predominantly additive effects of the two insecticides on both body mass and SVL at this time. Survival to 10 d exposure also did not show the antagonism, as survival to day 10 was ≥ 80% for all treatments except the 394 µg/L chlorpyrifos and 11.3 µg/L endosulfan group (60%). The lack of antagonism after 10 d exposure may be attributed to the period of pesticide exposure, the early developmental stage of the larvae, or a combination of both factors. By day 30, however, the trend of chlorpyrifos inhibiting the effects of 7.9 and 11.3 µg/L endosulfan was apparent. Body mass and SVL of larvae exposed to 137 µg/L chlorpyrifos with 7.9 or 11.3 µg/L endosulfan averaged 60% greater than that of larvae exposed to either of these two concentrations of endosulfan alone. This trend continued through metamorphosis, as demonstrated by body mass and SVL measurements of those animals surviving to metamorphosis. The presence of 266 or 394 µg/L chlorpyrifos also inhibited the effects of 7.9 and 11.3 µg/L endosulfan and had a positive effect on body mass and SVL at metamorphosis. This trend was apparent on day 30, though not as strongly as at metamorphosis. This is in contrast to the results on survival, where the presence of 266 or 394 µg/L chlorpyrifos with 7.9 or 11.3 µg/L endosulfan actually had a negative effect on survival compared to endosulfan alone.

Endosulfan alone increased ChE activity while chlorpyrifos had only a slight inhibitory effect on ChE activity, perhaps because chlorpyrifos had not yet been fully converted to its more toxic oxon form. In combination, however, chlorpyrifos inhibited the positive effect of endosulfan. This may be attributed to the neural effects of both insecticides. Specifically, the chlorpyrifos that was bound with the AChE could have

repressed the increase in ACh and corresponding AChE activity necessary to compensate for the excitation of the neuron by endosulfan. These physiological effects at the neural level are surprising in comparison with the results at the organism level. Animals in the 137 µg/L chlorpyrifos with 7.9 or 11.3 µg/L endosulfan treatment groups actually had a higher rate of survival than those animals only exposed to endosulfan, while these same treatment groups had a statistically significant average reduction of 22% in ChE activity compared to 7.9 or 11.3 µg/L endosulfan alone. Thus, survival rates were positively influenced even though there was a negative effect on ChE activity.

Chlorpyrifos weakly inhibited mortality and body size at the two highest concentrations of endosulfan at10 d exposure, but inhibition increased with exposure duration. Prolonged exposure to ChE inhibiting compounds has reduced ChE activity in other studies. Widder and Bidwell (2006) exposed *L. sphenocephalus* larvae to chlorpyrifos for 4 and 12 d and measured the greater reduction in ChE activity in larvae exposed for 12 d than for 4 d. Studies of other taxa have also reported an effect of exposure duration on inhibition of ChE activity by OP insecticides. Gaworecki et al. (2009) observed increased AChE inhibition in hybrid striped bass (*Morone saxatilis* × *M. chrysops*) with increasing duration of exposure to diazinon. Diazinon reduced AChE activity after 3 d exposure, and after 6 d this reduction was even greater. Longer exposure to chlorpyrifos has also increased ChE inhibition in the chironomid *Kiefferulus calligaster*. Specifically, organisms exposed for 6 d demonstrated higher levels of ChE inhibition than those of the same age exposed for 3 days (Domingues et al. 2009). ChE activity of larvae at set time points of exposure (i.e. 10 and 30 d) were not examined in the current study but were only analyzed for animals reaching Gosner stage 46. Thus,

though there was a decrease in survival with increasing concentrations of endosulfan, those animals that did survive to metamorphosis may have done so because of the positive effect endosulfan had on ChE activity. Conducting a similar study and analyzing ChE activity at specific time points could assess if trends in ChE activity correspond to results displayed for survival and body size at those same points.

The presence of both insecticides in combination induced the development of scoliosis in *P. sierra*. Development of scoliosis in larvae exposed to endosulfan alone in the current study was very low, as it also was when exposed to endosulfan and 137 µg/L chlorpyrifos. However, 266 and 394 µg/L chlorpyrifos in the presence of endosulfan induced scoliosis in 33 – 87% of larvae. Endosulfan has induced scoliosis in *P. sierra* at concentrations greater than 3 µg/L and has reached 100% occurrence at 50 µg/L (Sparling and Fellers 2009). Harris et al (2000) reported similar axial malformations in *L. pipiens* exposed to endosulfan, though at concentrations much higher (2.35 mg/L) than those in the current study. Both chlorpyrifos and endosulfan target the nervous system, and above a threshold the total insecticide load (137 µg/L chlorpyrifos and 4.5 µg/L endosulfan) may be disrupting the development of the neuromuscular system. Scoliosis developed within the first week of exposure for most larvae, suggesting sensitivity at an early Gosner stage while the neuromuscular system is still developing.

Ecologically, consideration of these malformations is important, as they cause a distinct uncoordinated swimming pattern and a weakened avoidance response. This could lead to increased vulnerability to predators and could have important implications for population dynamics. In California, introduced trout have been implicated as a factor in population declines (Finlay and Vredenburg 2007) and sublethal effects such as

developmental abnormalities from multiple contaminants may exacerbate the effects of predation. Therefore, it is important that studies examining contaminant sublethal effects such as developmental abnormalities in amphibians consider the combined effects of multiple contaminants. This may provide better estimations of the effects of contaminants on fitness when extrapolating results to effects in the field.

In California, specifically in the Sierra Nevada Mountains where amphibian species such as *R. sierrae* are experiencing precipitous declines, pesticides have been measured in amphibian habitats (Zabik and Seiber 1993, McConnell et al. 1998) and in anuran tissues (Sparling et al. 2001, Angermann et al. 2002, Fellers et al. 2004). Bridges and Little (2005) reported *P. regilla* larvae reared in water from Sequoia and Kings Canyon National Park were smaller at metamorphosis and took longer to reach metamorphosis than control larvae. The actual compounds present in the water extracts were not determined, though it is likely that the water contained mixtures of OP and OC insecticides based on previous field sampling of contaminants in Sierran habitat (e.g. McConnell et al. 1998). Water concentrations of endosulfan I and endosulfan II have been measured as high as 18 and 102 ng/L, respectively, and 118 ng/L chlorpyrifos has been measured at an elevation greater than 2000 m in Sequoia National Park. Similar concentrations have been measured at elevations as low as 118 m in the Central Valley (LeNoir et al. 1999). Thus, the concentrations of chlorpyrifos and endosulfan used in the current study with *P. sierra* are greater than what has been measured in anuran habitats. The aim of this study, however, was to examine the interactive effects of the insecticides at concentrations corresponding to previously measured effective and lethal concentrations. It would therefore also be beneficial for future research to examine the

potential for interactive effects using concentrations similar to environmental measurements to further assess the potential impacts on California anurans.

More broadly, however, this study can be used to evaluate the potential for OP and OC interactions in other locations where environmental concentrations are similar to those that have been demonstrated to exhibit lethal and sublethal effects on amphibians. For example, environmental endosulfan concentrations have been measured at 1.7 mg/L in water bodies in the vicinity of direct overhead spray events and at 0.7 mg/L up to 10 m from spray events (Ernst et al. 1991). Where direct loading of OP and OC insecticides into amphibian habitats may occur at individual concentrations considered harmful to amphibians, the potential for interactions on different indicators of fitness at the organism and sub-organism level should be considered.

The present study highlights the importance of considering concentrations used, duration, and response variables measured in studies of pesticide interactivity. In the presence of the toxic insecticide endosulfan, the less toxic chlorpyrifos reduced the negative effects on key anuran life history traits such as survival and body size at several time points and concentrations but not at others. The results indicate that contaminants with different modes of action within the nervous system can have varying interactions depending on their concentrations and on measurement endpoints being assessed.

The ability to predict the effects of mixtures can be further complicated when direct effects on individual physiology and behavior are coupled with indirect effects in a community context. Boone and Bridges-Britton (2006) suggested that multiple contaminants with different modes of action were more likely to have indirect effects on food web dynamics than direct effects on developing anuran larvae. Contaminants may

have important direct effects on individuals in a community, however, when the contaminants have similar modes of action. These effects are more likely to lead to nonadditive interactions among the contaminants (Boone 2008). Other studies have demonstrated that contaminants with different modes of actions can also have nonadditive direct effects (e.g., Boone and James 2003, Boone et al. 2005). Overall, though direct lethality is still of importance to evaluate in such studies, sublethal effects on physiological states, endocrine system disruption, and alterations to food source availability and other ecological relationships are likely occurring in nature. These effects are likely what are leading to population effects (Linder et al. 2010a). The aim of ecotoxicological studies with amphibians is shifting towards an integration of laboratory data, simulated natural mesocosms, and field studies as the effects of contaminants on amphibians are likely to be a combination of total chemical load and the interaction with the physical environment (Burkart et al. 2003). Therefore, understanding the underlying mechanisms of chemical combinations coupled with their relative importance in a community context will facilitate more accurate predictions of the effects of chemical contaminants on amphibian populations.

CHAPTER 4

CONCLUSIONS

The evidence that contaminants from the Central Valley are playing a role in the decline of Sierra Nevada amphibians is growing. Population declines have been associated with current and historical upwind agro-chemical use (Davidson et al. 2002, Davidson 2004), and these chemicals have been detected in amphibian habitat in the Sierra Nevada (McConnell et al. 1998, LeNoir et al. 1999, Fellers et al. 2004). Endosulfan and chlorpyrifos, two of the insecticides that have been detected, display similar toxicity to *Rana sierra* larvae as *Pseudacris sierra* larvae, with endosulfan being extremely more toxic than chlorpyrifos. There is no indication based on the date presented here that these insecticides demonstrate a direct threat (i.e. lethality, reduced growth) at measured environmental concentrations over a period of 63 d to developing *R. sierrae* larvae in Sierra Nevada habitat. LC_{50} estimates for endosulfan and chlorpyrifos were 19.8 and 595 µg/L, respectively, after 63 d of exposure, yet past measurements of environmental concentrations in the Sierra Nevada Mountains have been in the low ng/L range (McConnell et al. 1998, LeNoir et al. 1999, Fellers et al. 2004). These measurements, however, have been limited both spatially and temporally and do not account for seasonal fluctuations and pulses, making extrapolations to relevant environmental concentrations difficult. More quantification of current pesticide concentrations in the Sierra Nevada should be conducted to assess this in specific locations that *R. sierrae* inhabit.

Larvae survival was 100% in the presence of 5.75 µg/L endosulfan, and concentrations below 12 µg/L did not affect larval growth or development. The highest

concentrations of chlorpyrifos tested (737 – 1687 µg/L) resulted in 100% mortality, and larvae exposed to concentrations less than these demonstrated reduced growth and development compared to controls. ChE activity was reduced in larvae exposed to chlorpyrifos, but there was no trend of a greater reduction with increasing chlorpyrifos concentration. ChE activity was only significantly lower than controls for larvae exposed to 737 µg/L chlorpyrifos. All larvae were exposed for a maximum of 63 d in a static temperature regime, but *R. sierrae* may take up to three years to reach metamorphosis (Stebbins 2003). Lethal and sublethal effects may be observed at lower concentrations when exposures occur at relative time scales and when a broad temperature ranges is considered. Additionally, pesticides often occur in combinations in amphibian habitats, including in the Sierra Nevada Mountains. Thus, endosulfan and chlorpyrifos toxicity to *R. sierrae* is likely greater than presented here.

Mixtures of endosulfan and chlorpyrifos displayed varying effects on developing *P. sierra* larvae depending on the concentrations present in the mixtures, as well as the response variables examined and the time points they were examined at. Direct lethality was minimal in the presence of chlorpyrifos alone and increased with increasing concentrations of endosulfan alone. When present with 7.9 or 11.3 µg/L endosulfan, 137 µg/L chlorpyrifos had a positive effect, increasing survival by as much as 54%. However, at concentrations greater than 137 µg/L chlorpyrifos, the total insecticide load exceeded the tolerance of the larvae and increased mortality greater than the combined individual effects of each insecticide. Chlorpyrifos also inhibited the negative effects of endosulfan on body size, but this interaction was not apparent until 30 d exposure and lasted to metamorphosis. Endosulfan alone had a positive effect on ChE activity, but

chlorpyrifos ameliorated this effect. Both insecticides affect neural activity, and it appears their combined toxicity is likely causing effects at the neural sysnapse where AChE is necessary to end neural impulses. Additionally, neither insecticide alone had an effect on developmental abnormalities, though in combination the insecticides induced the development of scoliosis in larvae. This caused uncoordinated swimming and a weakened avoidance behavior in larvae which could place them at greater risk to predators such as introduced trout in Sierran lentic habitats. It is therefore important to assess the impacts of chemical combinations on developmental effects that may affect individual fitness in the field. Further examination of contaminant impacts on anuran larval development should take the effects of multiple contaminants into consideration.

Insecticide concentrations that cause direct lethal and sublethal effects on *R. sierrae* and *P. sierra* in the laboratory are above those concentrations measured in amphibian habitats in the Sierra Nevada Mountains. However, unexpected effects on the physiology of developing larvae may occur in the presence of the multiple chemical combinations. Based on the results of this study and those of others, chemical contaminants can have effects at both the individual and community level in aquatic habitats. This is likely occurring in the Sierra Nevada Mountains and consequently may be playing a significant role in alterations to amphibian populations. Contaminants such as insecticides from the Central Valley can have sublethal effects on physiological states, disrupt the endocrine system, and alter resource availability. Chemical interactions such as those in this study, coupled with complex ecological relationships, are likely occurring in nature and driving population effects. These interactions should continue to be the focus of amphibian conservation efforts in the future.

LITERATURE CITED

Alford, R. A. and S. J. Richards. 1999. Global amphibian declines: A problem in applied ecology. Annual Review of Ecology and Systematics 30:133-165.

American Society for Testing and Methods [ASTM]. 1988. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians E729-88. Pages 304-323 *in* Annual Book of ASTM Standards. ASTM, West Conshohocken, Pennsylvania, USA.

Angermann, J. E., G. M. Fellers, and F. Matsumura. 2002. Polychlorinated biphenyls and toxaphene in Pacific tree frog tadpoles (*Hyla regilla*) from the California Sierra Nevada, USA. Environmental Toxicology and Chemistry 21:2209-2215.

Barron, M. G. and K. B. Woodburn. 1995. Ecotoxicology of chlorpyrifos. Reviews of Environmental Contamination and Toxicology 144:1-93.

Beauvais, S. L., S. B. Jones, S. K. Brewer, E. E. Little. 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. Environmental Toxicology and Chemistry 19:1875-1880.

Blaustein, A. R., P. D. Hoffman, D. G. Hokit, J. M. Kiesecker, S. C. Walls, and J. B. Hays. 1994. UV repair and resistance to solar UV-B in amphibian eggs – a link to population declines? Proceedings of the National Academy of Sciences of the United States of America 91:1791-1795.

Blaustein, A. R., J. M. Romansic, J. M. Kiesecker, and A. C. Hatch. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity and Distributions 9:123-140.

Blaustein, A. R. and D. B. Wake. 1990. Declining amphibian populations: A global phenomenon? Trends in Ecology and Evolution 5:203-204.

Bloomquist, J. 1993. Toxicology, mode of action, and target site-mediated resistance to insecticides acting on chloride channels. Comparative Biochemistry and Physiology 106C:301–314.

Bloomquist, J. 2003. Chloride channels as tools for developing selective insecticides. Archives of Insect Biochemistry and Physiology 54:145-156.

Boone, M. D. 2008. Examining the single and interactive effects of three insecticides on amphibian metamorphosis. Environmental Toxicology and Chemistry 27:1561-1568.

Boone, M. D. and C. M. Bridges. 1999. The effect of temperature on the potency of

carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). Environmental Toxicology and Chemistry 18:1482-1484.

Boone, M. D. and C. M. Bridges-Britton. 2006. Examining multiple sublethal contaminants on the gray treefrog (*Hyla versicolor*): Effects of an insecticide, herbicide and fertilizer. Environmental Toxicology and Chemistry 25:3261-3265.

Boone, M. D., C. M. Bridges, J. F. Fairchild, and E. E. Little. 2005. Multiple sublethal chemicals negatively affect tadpoles of the green frog, *Rana clamitans*. Environmental Toxicology and Chemistry 24:1267-1272.

Boone, M. D. and S. M. James. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecological Applications 13:829-841.

Boone, M. D., R. D. Semlitsch, E. E. Little, and M. C. Doyle. 2007. Multiple stressors in amphibian communities: Effects of chemical contamination, bullfrogs, and fish. Ecological Applications 17:291-301.

Bradford, D. F. 1989. Allotopic distribution of native frogs and introduced fishes in high Sierra-Nevada lakes of California – implication of the negative effect of fish introductions. Copeia 3:775-778.

Brem, F., J. R. Mendelson III, and K. R. Lips. 2007. Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians, using alcohol preserved swabs. Conservation International, Arlington, Virginia, USA.

Bridges, C. M. 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Archives of Environmental Contamination and Toxicology 16:1935-1939.

Bridges, C. M. 1999. Effects of a pesticide on tadpole activity and predator avoidance behavior. Journal of Herpetology 33:303-306.

Bridges, C. M. and E. E. Little. 2005. Toxicity to amphibians of environmental extracts from natural waters in National Parks and Fish and Wildlife Refuges. Alytes 22:130-145.

Broomhall, S. 2002. The effects of endosulfan and variable water temperature on survivorpship and subsequent vulnerability to predation in *Litora citropa* tadpoles. Aquatic Toxicology 61:243-250.

Broomhall, S. 2004. Egg temperature modifies predator avoidance and the effects of the insecticide endosulfan on tadpoles of an Australian frog. Journal of Applied Ecology 41:105-113.

Burkhart, J. G, J. R. Bidwell, D. J. Fort, and S. T. Sheffield. 2003. Chemical stressors. Pages 111-128 *in* G. Linder, S. K. Krest, and D.W. Sparling, editors. Amphibian Decline: An Integrated Analysis of Multiple Stressor Effects. Society of Environmental Toxicology and Chemistry, Pensacola, Florida, USA.

California Department of Fish and Game [CDFG]. 2010. State and federally listed endangered and threatened animals of California. Sacramento, California, USA. <http://www.dfg.ca.gov/biogeodata/cnddb/pdfs/TEAnimals.pdf>. Accessed 9 Jan 2010.

California Environmental Protection Agency [CEPA]. 2009. Summary of pesticide use report data 2008, indexed by chemical. California Department of Pesticide Regulation, Sacramento, California, USA. <http://www.cdpr.ca.gov/docs/pur/pur08rep/08chem.htm>. Accessed 22 April 2010.

Center for Biological Diversity [CBD]. 2010. Petition to California Fish and Game Commission to list the mountain yellow-legged frog as endangered. San Francisco, California, USA.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735-748.

Davidson, C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. Ecological Applications 14:1892-1902.

Davidson, C., M. F. Benard, H. B. Shaffer, J. M. Parker, C. O'Leary, J. M. Conlon, and L. A. Rollins-Smith. 2007. Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. Environmental Science & Technology 41:1771-1776.

Davidson, C. and R. A. Knapp. 2007. Multiple stressors and amphibian declines: Dual impacts of pesticides and fish on yellow-legged frogs. Ecological Applications 17: 587-597.

Davidson, C., H. B. Shaffer, and M. R. Jennings. 2001. Declines of the California red-legged frog: Climate, UV-B, and pesticide hypotheses. Ecological Applications 11:464-479.

Davidson, C., H. B. Shaffer, and M. R. Jennings. 2002. Spatial tests of the pesticide drift, habitat destruction, UV-B, and climate change hypotheses for California amphibian declines. Conservation Biology 16:1588-1601.

Domingues, I., L. Guilhermino, A. Soares, A. Nogueira, and K. A. Monaghan. 2009.

Influence of exposure scenario on pesticide toxicity in the midge *Kiefferulus calligaster*. Ecotoxicology and Environmental Safety 72:450-457.

Drost, C. A. and G. M. Fellers. 1996. Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. Conservation Biology 10:414-425.

Ellmann, G.L., K. D. Courtney, V. Andres Jr., and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemistry and Pharmacology 7:88-95.

Ernst, W. R., P. Jonah, K. Doe, G. Julien, and P. Hennigar. 1991. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. Environmental Toxicology and Chemistry 10:103-114.

Fellers, G. M., D. E. Green, and J. E. Longcore. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). Copeia 4:945-953.

Fellers, G. M., L. L. McConnell, D. Pratt, and S. Datta. 2004. Pesticides in mountain yellow-legged frogs (*Rana muscosa*) from the Sierra Nevada Mountains of California, USA. Environmental Toxicology and Chemistry 23:2170-2177.

Finlay, J. C. and V. T. Vredenburg. 2007. Introduced trout sever trophic connections in watersheds: Consequences for a declining amphibian. Ecology 88: 2187-2198.

Galloway, T. and R. Handy. 2003. Immunotoxicity of organophosphorous pesticides. Ecotoxicology 12:345-363.

Gaworecki, K. M., A. P. Roberst, N. Eillis, A. D. Sowers, and S. J. Klaine. 2009. Chemical and behavioral effects of diazinon exposure in hybrid striped bass. Environmental Toxicology and Chemistry 28:105-112.

Gopal, K., R. N. Khanna, M. Anand, and G. S. D. Gupta. 1981. The acute toxicity of endosulfan to fresh-water organisms. Toxicology Letters 7:453-456.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

Harris, M. L., L. Chora, C. A. Bishop, and J. P. Bogart. 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens* and *Bufo americanus*. Bulletin of 64:263-270.

Hayes, T. B., A. Collins, M. Lee, M. Mendozza, N. Norlega, A. A. Stuart, and A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceeedings of the National Academy of Sciences of the United States of America 99:5476-5480.

Hayes, T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we understanding the impact? Environmental Health Perspectives 114:40-50.

Hill, E. F., W. J. Fleming. 1982. Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. Environmental Toxicology and Chemistry 1:27–38.

Hoekstra, J. A. 1987. Acute bioassays with control mortality. Water, Air, and Soil Pollution 35:311-317.

Houlahan, J. E., C. S. Findlays, B. R. Schmidt, A. H. Meyers, and S. L. Kuzmin. 2000. Quantitative evidence for global amphibian population declines. Nature 404:752-754.

International Union for Conservation of Nature [IUCN]. 2004. Global Amphibian Assessment. International Union for Conservation of Nature, Conservational International, NatureServe, Washington DC. USA. <http://www.globalamphibians.org>. Accessed 3 Oct 2008.

Jennings, M. R. 1996. Status of amphibians. Pages 921-944 *in* Sierra Nevada ecosystem project: Final report to congress, volume II—assessments and scientific basis for management options, report 37. University of California Davis, Davis, California, USA.

Jones, D. K, J. I. Hammond, and R. A. Relyea. 2009. Very highly toxic effects of endosulfan across nine species of tadpoles: Lag effects and family-level sensitivity. Environmental Toxicology and Chemistry 28:1939-1945.

Karl, T. R., R. W. Knight, D. R. Easterling, and R. G. Quayle. 1996. Indices of climate change for the United States. Bulletin of the American Meteorological Society 77:279-292.

Kats, L. B. and R. P. Ferrer. 2003. Alien predators and amphibian declines: Review of two decades of science and the transition to conservation. Diversity and Distributions 9:99-110.

Knapp, R. A. 2005. Effects of nonnative fish and habitat characteristics on lentic herpetofauna in Yosemite National Park, USA. Biological Conservation 121:265-279.

Laurance, W. F. 2008. Global warming and amphibian extinctions in eastern Australia. Austral Ecology 33:1-9.

LeNoir, J. S., L. L. McConnell, G. M. Fellers, T. M. Cahill, and J. N. Seiber. 1999.

Summertime transport of current-use pesticides from California's Central Valley to the Sierra Nevada Mountain range, USA. Environmental Toxicology and Chemistry 18:2715-2722.

Linder, G., C. A. Bishop, S. K. Krest, and D. Sparling. 2010a. Ecotoxicology of amphibians and reptiles – where should we be going and how do we get there? Pages 547-551 *in* D.W. Sparling, G. Linder, C. A. Bishop, and S. K. Krest, editors. Ecotoxicology of amphibians and reptiles. Society of Environmental Toxicology and Chemistry, Pensacola, Florida, USA.

Linder, G., C. M. Lehman, and J. R. Bidwell. 2010b. Ecotoxicology of amphibians and reptiles in a nutshell. Pages 69-103 *in* D.W. Sparling, G. Linder, C. A. Bishop, and S. K. Krest, editors. Ecotoxicology of amphibians and reptiles. Society of Environmental Toxicology and Chemistry, Pensacola, Florida, USA.

Lydy, M. J., T. W. Lohner, and S. W. Fisher. 1990. Influence of pH, temperature and sediment type on the toxicity, accumulation and degradation of parathion in aquatic systems. Aquatic Toxicology 17:27-44.

Lydy, M. J., J. B. Belden, and M. A. Ternes. 1999. Effects of temperature on the toxicity of m-parathion, chlorpyrifos, and pentachlorobenzene to *Chironomus tentans*. Archives of Environmental Contamination and Toxicology 37:542-547.

Marrs, T. C. 1993. Organophosphate poisoning. Pharmacology & Therapeutics 58:51-66.

Matthews, K. R. and H. K. Preisler. 2010. Site fidelity of the declining amphibian *Rana sierrae* (Sierra Nevada yellow-legged frog). Canadian Journal of Fisheries and Aquatic Sciences 67:243-255.

Mazanti, L., C. Rice, K. Bialek, D. Sparling, C. Stevenson, W. E. Johnson, P. Kangas, and J. Rheinstein. 2003. Aqueous-phase disappearance of atrazine, metolachlor, and chlorpyrifos in laboratory aquaria and outdoor macrocosms. Archives of Environmental Contamination and Toxicology 44:67-76.

Mazanti, L. E, D. W. Sparling, C. P. Rice, K. Bialek, C. Stevenson, B. M. Teels. 2003. Synergistic effects of a combined exposure to herbicides and an insecticide to *Hyla versicolor*. Pages 111-129 i*n* G. Linder, S. Krest, D. W. Sparling, E. E. Little, editors. Multiple stressor effects in relation to declining amphibian populations STP1443-EB. American Society for Testing and Materials, West Conshohocken, Pennsylvania, USA.

McConnell, L. L., J. S. LeNoir, S. Datta, and J. N. Seiber. 1998. Wet deposition of current-use pesticides in the Sierra Nevada mountain range, California, USA. Environmental Toxicology and Chemistry 17:1908-1916.

Melancon, M. J. 2003. Bioindicators of contaminant exposure and effect in aquatic and

terrestrial monitoring. Pages 257-278 *in* D. J. Hoffman, B. A. Rattner, G. A. Burton, Jr., and J. Cairns, Jr., editors. Handbook of ecotoxicology. Second edition. Lewis, Boca Raton, Florida, USA.

National Park Service [NPS]. 2008. Temperatures and precipitation: Average temperatures and precipitation for Yosemite. <http://www.nps.gov/yose/planyourvisit/climate.htm>. Accessed 13 June 2010.

Ostrander, G. K. 1996. Techniques in aquatic toxicology. CRC Press, Boca Raton, Florida, USA.

Padgett-Flohr, G. E. and R. L. Hopkins. 2009. *Batrachochytrium dendrobatidis*, a novel pathogen approaching endemism in central California. Diseases of Aquatic Organisms 83:1-9.

Park, D., S. C. Hempleman, and C. R. Propper. 2001. Endosulfan exposure disrupts pheromonal systems in the red-spotted newt: a mechanism for subtle effects of environmental chemicals. Environmental Health Perspectives 109:669-673.

Peterson, S. M. and G. E. Batley. 1993. The fate of endosulfan in aquatic ecosystems. Environmental Pollution 82:143-152.

Rachowicz, L. J., R. A. Knapp, J. A. T. Morgan, M. J. Stice, V. T. Vredenburg, J. M. Parker, C. J. Briggs. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87:1671-1683.

Relyea, R. A. 2003. Predator cues and pesticides: a double dose of danger for amphibians. Ecological Applications 13:1515-1521.

Relyea, R. A. 2004a. The growth and survival of five amphibian species exposed to combinations of pesticides. Environmental Toxicology and Chemistry 23:1737-1742.

Relyea, R. A. 2004b. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environmental Toxicology and Chemistry 23:1080-1084.

Relyea, R. A. 2005. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15:1118-1124.

Relyea, R. A. 2009. A cocktail of contaminants: How mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159:363-376.

Relyea R. A. and N. Diecks. 2008. An unforeseen chain of events: Lethal effects of pesticides on frogs at sublethal concentrations. Ecological Applications 18:1728-1742.

Semlitsch, R. D. 2003. Amphibian conservation. Smithsonian Books, Washington, D.C., USA.

Semlitsch R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69:184-192.

Smith, D. C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68:344-350.

Sparling, D. W. 2003. A review of the role of contaminants in amphibian declines. Pages 1099-1128 *in* D. J. Hoffman, B. A. Rattner, G. A. Burton Jr., J. Cairns Jr., editors. Handbook of Ecotoxicology. Lewis, Boca Raton, Florida, USA.

Sparling, D. W. and G. Fellers. 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. Environmental Pollution 147:535-539.

Sparling, D. W., and G. M. Fellers. 2009. Toxicity of two insecticides to California, USA, anurans and its relevance to declining amphibian populations. Environmental Toxicology and Chemistry. 28:1696-1703

Sparling, D. W., G. M. Fellers, and L. L. McConnell. 2001. Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry 20:1591-1595.

Stebbins, R. C. 2003. A field guide to western reptiles and amphibians. Third edition. Houghton Mifflin Company, New York, New York, USA.

Stuart, S. N., J. S. Chanson, N.A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Thomas, C. and A Mansingh. 2002. Dissipation of chlorpyrifos from tap, river, and brackish water in glass aquaria. Environmental Technology 23:1313-1323.

Tu, H. T., F. Silvestre, M. Scippo, J. Thome, N. T. Phuong, and P. Kestemont. 2009. Acetylcholinesterase activity as a biomarker of exposure to antibiotics and pesticides in the black tiger shrimp (*Penaeus monodon*). Ecotoxicology and Environmental Safety 72:1463-1470.

United States Environmental Protection Agency [USEPA]. 1982. Hexachlorohexahydromethano-2,4,3-benzodioxathiepin 3-oxide (endosulfan) pesticide registration standard. PB82-243999. National Technical Information Services, Springfield, Virginia, USA.

United States Geological Survey [USGS]. 2009. Species fact sheet: *Pseudacris sierra*. <http://nas.er.usgs.gov/queries/FactSheet.asp?speciesID=2780>. Accessed 4 January 2010.

Vardia, H. K., P. S. Rao, and V. S. Durve. 1984. Sensitivity of toad larvae to 2,4-D and endosulfan pesticides. Archives of Hydrobiology 100:395-400.

Whitfield, S. M., K. E. Bell, T. Philippi, M. Sasa, F. Bolanos, G. Chaves, J. M. Savage, and M.A. Donnelly. 2007. Amphibian and reptile declines over 35 years at La Selva, Costa Rica. Proceedings of the National Academy of Sciences of the United States of America 104:8352-8356.

Widder, P. D. and J. R. Bidwell. 2006. Cholinesterase activity and behavior in chlorpyrifos-exposed *Rana sphenocephala* tadpoles. Environmental Toxicology and Chemistry 25: 2446-2454.

Young, B. E., S. N. Stuart, J. S. Chanson, N. A. Cox, and T. M. Boucher. 2004. Disappearing jewels: the status of the new world amphibians. NatureServe, Arlington, Virginia, USA.

Zabik, J. M. and J. N. Seiber. 1993. Atmospheric transport of organophosphate pesticides from California's Great Central Valley to the Sierra Nevada Mountains. Journal of Environmental Quality 22:80-90.

VITA

Graduate School
Southern Illinois University

David A. Dimitrie Date of Birth: July 10, 1984

206 S. Gray Dr., Carbondale, Illinois 62901

42446 Ehrke Dr., Clinton Township, Michigan 48038

ddimitrie@gmail.com

Michigan State University
Bachelor of Science, Zoology, December 2005

Thesis Title:

The effects of two insecticides on California anurans (*Rana sierrae* and *Pseudacris sierra)* and the implications for declining amphibian populations

Major Professor: Donald Sparling