AN ABSTRACT OF THE THESIS OF

Lindsey L. Thurman for the degree of Master of Science in Wildlife Science presented on June 8, 2012.

Title: Elevational Differences in UV-B Response by the Long-toed Salamander (*Ambystoma macrodactylum*)

Abstract approved:

Tiffany S. Garcia

Global amphibian declines have been attributed to numerous and often synergistic causes, such as invasive species, pathogens, and ultraviolet-B (UV-B) radiation. The effects of these stressors are context dependent and can vary with location, species, and populations. As sensitivity to UV-B has shown inconsistencies across amphibian taxa, it can be expected that variation also occurs between populations of a single species. High elevation populations of the long-toed salamander (*Ambystoma macrodactylum*) face exponentially higher UV-B radiation levels relative to low elevation populations and these levels are predicted to increase in conjunction with continued ozone depletion. We hypothesized that breeding long-toed salamander females at high elevations have modified oviposition behavior to better protect embryos from UV-B induced damage. In addition, we hypothesized that long-toed salamander embryos at high elevation would exhibit elevated photolyase activity, a photo-reactivating enzyme that repairs UV-B radiation-induced damage to DNA. We predicted that this behavioral defense strategy would be employed together with an elevated physiological response as a correlated defense response to increased levels of UV-B radiation in high elevation populations. We surveyed high and low elevation long-toed salamander breeding sites throughout Oregon to quantify oviposition site characteristics and associated UV-B profiles. We simultaneously collected embryos for quantification of photolyase activity in a bacterial transformation assay. We found significant differences in oviposition behavior across elevations, with high elevation breeding females ovipositing in deeper water and using UV-B protective refugia. Oviposition sites at low elevations, however, were most

often found in UV-B exposed microhabitats located at the surface of the water. This population difference in oviposition behavior resulted in a standardization of UV-B and temperature conditions for long-toed salamander embryos across elevation. In contrast, we found no population differentiation in photolyase activity between high and low elevation breeding sites. This indicates that behavioral selection for UV-B protected oviposition substrates may either be negating the need for increased photolyase activity in long-toed salamander embryos, or that populations lack the capacity to adapt a heightened physiological response to UV-B at high elevations. Together, these results show how trade-offs in physiology and behavior are a unique adaptation to a significant environmental stressor. Further research into the susceptibility of amphibian species to changing environmental conditions may help to demonstrate the effectiveness of correlated trait responses and plasticity in behavior, and species persistence under changing climate regimes.

Elevational Differences in UV-B Response by the Long-toed Salamander (*Ambystoma macrodactylum*)

by
Lindsey L. Thurman

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 8, 2012
Commencement June 2012

Master of Science thesis of Lindsey L. Thurman presented on June 8, 2012.

APPROVED:

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

Lindsey L. Thurman, Author

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to the community of students, faculty, and staff in the Department of Fisheries and Wildlife for continued support and encouragement. The Department of Fisheries and Wildlife, the College of Agricultural Sciences, and the OSU Graduate School have provided invaluable financial support through teaching assistantships and scholarships used for research and conferences. I was fortunate to collaborate with colleagues from a variety of departments at Oregon State, in particular Dr. John Hays' lab in the Department of Environmental and Molecular Toxicology. I would especially like to thank Peter Hoffman for his extensive instruction and time commitment to the experimental portion of my thesis, without which it would not have been possible.

Feedback provided by my committee members, Dr. Andrew Blaustein and Dr. Brian Sidlauskas, helped me to reign in my ideas and design a multi-faceted graduate project. I greatly appreciated their diversity in ideas and suggestions, which were invaluable to the development of my field surveys and data analysis. Thank you to Stephanie Gervasi, Lindsay Biga, Catherine Searle, and Paul Bradley, who provided much needed advice on mountain field sites and proper UV-B sampling methods. I would also like to thank Dr. Stan Gregory for providing me with a stream ecologists' perspective on field sampling procedures and project development. Dr. Gregory's instruction, both as a mentor and teacher, will be unforgettable.

I benefitted immensely from information provided by Robert Hoffman, Brome McCreary, and Ross Hinderer at USGS FRESC on identification and locations of long-toed salamanders. Dr. Dede Olson from the Department of Forestry at Oregon State contributed greatly to discussions on original project ideas and feasibility. In addition to folks at Oregon State, the Society for Northwestern Vertebrate Biology has been a wonderful outlet for camaraderie with fellow herpetologists throughout the Northwest and I sincerely appreciate their financial support. A special thank you to Doug DeGross and Betsy Bancroft for their advice on amphibian and UV-B sampling methods.

During my winter field season, I collaborated with Jock Beall, Wildlife Biologist at William L. Finley National Wildlife Refuge, on amphibian sampling at pond sites on the refuge. I would also like to thank Kyle Martin, the Wildlife Manager at E.E. Wilson

Management Area, for encouraging me to wear bright orange and temporarily allowing me access to the ponds during duck hunting season. Spring field work in the mountains was a unique opportunity for me to test my physical limits and I greatly respect all of my friends who joined me on these adventures. I would like to thank my undergraduate field assistant, Nikki Scott, for staying motivated and attentive! I would also like to thank Brett Hanshew, Megan Cook, Jennifer Rowe, Neil Thompson, and Dave Paoletti for hanging in there while we hiked, snow-shoed, and waded through snow and ice.

My previous employment at the Florida Museum of Natural History and my undergraduate mentor, Robert Robins, played a special role in encouraging me to pursue graduate school and my love for herpetology. I would like to thank Rob for his continued support and friendship.

A very special thank you to my advisor, Dr. Tiffany Garcia, for her encouragement and insightfulness! She has been a mentor, friend, and motivator, and I am very excited about continuing my graduate career under her supervision. My labmates, past and present – Megan Cook, Rebbecca Hill, Nick Baker, Brett Hanshew, Jennifer Rowe, Stephen Selego, Emily Nebergall, and Jenny Urbina – have been a uniquely functional group of enthusiastic scientists and friends. I could not have asked for a better lab community.

My partner in crime, Neil Thompson, has been a loving addition to my life. I am thankful for his continued support and admiration. Thank you does not seem sufficient to express my gratitude for the support given to me by my family. My aunt Linda has been a role model and confidante, and I love her for always supporting my endeavors. Last, but certainly not least, I would like to thank my mom and dad, who have fostered in me a sense of intellectual and creative curiosity. I appreciate all of you more than words can describe.

Thank you to all of my friends and family for keeping me sane.

“Climb the mountains and get their good tidings.” – John Muir

CONTRIBUTION OF AUTHORS

Tiffany S. Garcia contributed to study design and analysis in Chapter 2, and assisted in editing all aspects of the thesis. Brian Sidlauskas contributed to field study design and design of statistical analyses in Chapter 2. Peter D. Hoffman contributed to experimental design, implementation, and experimental analyses in Chapter 2.

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DEDICATION

"I went to the woods because I wished to live deliberately, to front only the essential facts of life, and see if I could not learn what it had to teach, and not, when I came to die, discover that I had not lived." – Henry David Thoreau

To all the wilderness warriors out there fighting the good fight

Elevational Differences in UV-B Response by the Long-toed Salamander

Chapter 1 – General Introduction

Freshwater ecosystems provide a substantial amount of services relative to their size and have recently been deemed biodiversity reservoirs of high conservation priority (Dudgeon *et al.*2005). In many parts of the world, these freshwater habitats produce more than 50% of the world's biomass, yet are experiencing disproportionately high extinction rates (Häder *et al.*2007). This susceptibility to species population decline may be exacerbated by environmental stressors, including contaminants, hydroperiod alteration, and changes in abiotic conditions (Beebee and Griffiths 2005; Carey and Alexander 2003).

Ozone depletion and the associated increase in harmful levels of ultraviolet radiation have aroused concern over the contribution to population declines in numerous freshwater species (Bancroft *et al.* 2007; Perin and Lean 2004). The adverse effects of solar radiation on organisms are mostly attributed to ultraviolet-B (UV-B) radiation (280-315 nm), which is absorbed in varying amounts by cellular DNA (Sinha and Häder 2002). Exposure to UV-B radiation can reduce productivity, affect reproduction and development, and increase the mutation rate in all life stages of aquatic organisms (Bancroft *et al.* 2007). The adverse effects of UV-B radiation on freshwater organisms can indirectly result in loss of species diversity, decline in ecosystem stability and alterations to trophic interactions (Häder *et al.*2007). Although freshwater organisms vary in susceptibility and tolerance to UV-B radiation, understanding the mechanisms employed by organisms to defend against UV-B damage is paramount to the conservation of vulnerable species.

The predicted long-term increase in UV-B radiation over the next century has stimulated considerable interest in the associated negative impacts to amphibian species existing in freshwater environments (Blaustein and Bancroft 2007; Stuart *et al.* 2004; Häder *et al.* 2007). Amphibians have been recognized as surrogate measures of environmental contamination, habitat quality, and population trends in other species (Beebee and Griffiths 2005). Several amphibian life history characteristics predispose

them to susceptibility to UV-B damage; amphibian skin is permeable and exposed and their eggs can readily absorb substances from the environment due to lack of a hard shell. These attributes, along with their ectothermic life history, make them especially vulnerable to environmental stressors and increases in UV-B radiation (Blaustein and Bancroft 2007).

Recent studies have shown that many amphibian species experience decreased hatching success, behavioral changes, growth impairment, deformities, or death when exposed to ambient levels of UV-B radiation (Alton *et al.*2010; Blaustein and Belden 2003). Some amphibian populations may experience these deleterious effects, and potentially population declines, from dangerous levels of UV-B before selection for adequate defense strategies occurs. While the ecological relevance of UV-B radiation as a single-factor cause for amphibian population decline has come into question (Licht 2003), recent research has highlighted the compounded impacts of UV-B with other environmental stressors (e.g. pH, contaminants, and pathogens) and pervasive biological stressors (e.g. predation and competition) on amphibian species (Alton *et al.* 2011; Searle *et al.* 2010; Bancroft *et al.* 2008; Belden *et al.* 2003; Blaustein *et al.* 2003; Blaustein *et al.* 2001; Kiesecker 2001; Hansson 2004; Hatch and Blaustein 2000). For example, UV-B radiation has been shown to interfere with predator-prey interactions by suppressing the behavioral response of larval striped marsh frogs (*Limnodynastes peronii*) to predatory cues. The physiological and behavioral disadvantages resulted in a reduction in the ability of tadpoles to plastically respond to predatory chemical cues and caused an increase in larval mortality rates (Alton *et al.* 2010).

Accumulating evidence illustrates interspecific differences in UV-B response mechanisms; however, intraspecific differences also likely exist (Belden *et al.* 2000). Different populations experience different ecological conditions such as temperature, hydroperiod, and light regimes (Belden and Blaustein 2002). Therefore, population differences in UV-B radiation response may accurately reflect disparate habitat characteristics as a result of either phenotypic plasticity or local adaptation. UV-B radiation levels are typically greater at higher altitudes, exposing high elevation populations to significantly stronger UV-B radiation selection pressure relative to populations at lower elevations (Blumthaler and Ambach 1990; Garcia *et al.* 2009).

Additionally, high altitude populations are exposed to adverse UV-B conditions during the short favorable activity period (summer), which can heighten the effect of high UV-B exposure rates (Marquis and Miaud 2008). Marquis and Miaud (2008) tested UV-B tolerance in nine populations of the common frog (*Rana temporaria*) along an altitudinal gradient in the western Alps and found that UV sensitivity (in the form of hatchability and deformity rate) decreased with increasing altitude. These results suggest local adaptations to increased UV-B at high elevations, which may provide populations with the necessary tolerance strategies and/or defense mechanisms.

The persistence of multiple biotic and abiotic stressors in aquatic systems influences habitat use and behavior in amphibian species (Bancroft *et al.* 2008). Trade-offs due to these conflicting selection pressures are important for embryonic- and larval-stage amphibians that require suitable thermal and hydroperiod conditions for growth and development (Wells 2007). Unfortunately, there is strong selection for amphibians to seek warm microhabitats to increase development rates, and these locations are often associated with relatively high levels of UV-B. At high elevations, the pressure to avoid UV-B radiation is not without consequence. For example, adult amphibians may seek oviposition locations with low UV-B transmittance, but these locations are often associated with cooler, deeper water (Palen *et al.* 2005). By attempting to reduce embryonic UV-B exposure, they are creating a conflict between thermal requirements necessary for development and the need to avoid harmful levels of UV-B radiation. Thus, significant trade-offs exist in minimizing UV-B exposure rates while maximizing growth and development trajectories.

The long-toed salamander (*Ambystoma macrodactylum*) is an excellent species with which to investigate potential population differences in UV-B radiation response mechanisms. Long-term data is not available for detecting population declines in the long-toed salamander as a result of UV-B radiation, but declines in salamander populations throughout the Northwest have been observed, largely due to trout introductions and decreased habitat quality associated with timber harvest (Jones *et al.* 2005). This species' range extends from British Columbia to the Sierra Nevada Mountains of California. In Oregon, long-toed salamanders are found in a gradient of freshwater habitats from low elevation streams within the Willamette Valley to alpine

ponds in the Cascade Mountain Range (Nussbaum *et al.* 1983). Therefore, populations experience different UV-B regimes across an elevational gradient. Previous research indicates that this species is highly sensitive to UV-B radiation relative to other species in the Pacific Northwest, yet the conservation implications of this sensitivity are largely unknown (Blaustein *et al.* 1994, Jones *et al.*2005).

Multiple field and laboratory studies have examined long-toed salamander sensitivity to various levels of UV-B radiation (Palen and Schindler 2010; Belden and Blaustein 2002; Blaustein *et al.* 2001; Belden *et al.* 2000). The general consensus is that all life stages of this species are susceptible to UV-B induced damage, resulting in increased embryonic mortality, deformity, and reduced development rates. A field experiment conducted by Blaustein *et al.* (1997) indicated that long-toed salamander embryos from the Cascade Mountains of Oregon experienced deformities and high rates of mortality when exposed to ambient levels of UV-B exposure in their natural habitat. A study of long-toed salamander eggs collected from a single population in Oregon indicated that physiological repair of UV-B damaged DNA via photolyase activity was significantly lower compared to other Pacific Northwest amphibians (Blaustein *et al.* 1994). Therefore the ability of long-toed salamanders to persist under intense UV-B radiation levels at high elevations suggests that selection for UV-B radiation tolerance and/or avoidance could be occurring in mountain populations.

We investigated the differential behavioral and physiological mechanisms for combating UV-B radiation between high and low elevation populations of the long-toed salamander. We first explored the diverse oviposition behaviors of populations from three breeding sites in the Cascade Mountain Range and three breeding sites in the Willamette Valley of Oregon. Oviposition site selection and associated UV-B radiation exposure were examined to determine if females at high elevation have modified their egg-laying behavior to protect embryos from UV-B induced damage. We also examined population differences in photolyase activity, a photoreactive enzyme that repairs UV-B induced damage to cellular DNA. Photolyase levels were compared between high and low elevation populations to determine if embryonic long-toed salamanders at high elevation have employed a relatively heightened physiological response to elevated UV-B radiation levels. Together, these two studies provide a diverse assessment of characteristics that

may allow long-toed salamanders to cope with increasing UV-B radiation and persist in stressful montane environments.

CHAPTER 2

Trade-offs in UV-B response between high and low elevation populations of the long-toed salamander (*Ambystoma macrodactylum*)

Lindsey L. Thurman, Peter D. Hoffman, and Tiffany S. Garcia

ABSTRACT

Organisms capable of maximizing trade-offs in trait response to environmental stressors may exhibit optimal strategies for defending against the effects of habitat degradation and changing environmental conditions. Species that exist over a large geographic range will likely have populations that differ in trait expression as a function of environmental gradients. Amphibian species can be highly sensitive to selection pressures that vary across an elevational gradient, such as UV-B radiation and temperature. UV-B radiation poses a significant threat to many amphibian species, with populations at high elevation sites experiencing significantly higher rates of exposure. We examined the ability of long-toed salamander (*Ambystoma macrodactylum*) populations at high and low elevations to cooperatively or alternatively employ behavioral and physiological trait response to mediate UV-B exposure. We hypothesized that long-toed salamanders in elevated UV-B environments would modify their oviposition behavior by selecting UV-B protective refugia for egg deposition. We performed a microhabitat survey to examine differences in oviposition behavior among breeding populations at high elevation (n = 3;> 1,500 m) and low elevation (n = 3;< 100 m) sites. We found significant differences in oviposition behavior across populations, with females at high elevation sites selecting oviposition substrates in UV-B protected microhabitats ($P < 0.05$). We also hypothesized that long-toed salamanders in stressful UV-B environments would exhibit a high rate of photolyase activity, a photoreactivation enzyme that repairs UV-B damage to the DNA. We collected eggs (n = 633) from each of the long-toed salamander breeding sites for analysis of photolyase activity. A bacterial transformation assay revealed no significant differences in photolyase activity between long-toed salamander populations at high and low elevations. For high elevation salamander populations, relatively low physiological repair capabilities found in embryos appear to be buffered by extensive behavioral modifications (i.e. laying eggs under UV-B protective substrates) to reduce UV-B exposure. These results indicate that UV-B may be a selective force behind population differences in oviposition site selection. This study provides valuable insight into the role of adaptive responses to pervasive environmental stressors, via the cooperation of multiple traits, in allowing sensitive species to persist in changing landscapes.

INTRODUCTION

A key issue in evolutionary ecology is how organisms optimize trait response to mediate or defend against environmental stress. This becomes particularly important in species occupying habitats that are changing as a result of environmental degradation, such as pollution, ozone depletion, and climate change. Individuals can mediate stress with cooperative or alternative trait response, utilizing a strategy that either incorporates multiple traits to defend against specific factors, or a single trait response if adequate protection is achieved (Lascoux, 1997; Endler 1995). As selection strength of stress varies over an environmental gradient, population differences in response strategies may accurately reflect these disparate habitat conditions (Caruso *et al.* 2005). In highly stressful environments, organisms may utilize a more rigorous defense strategy, employing multiple trait responses to negate potential damage (Brodie 1992; Endler 1995; Garcia *et al.* 2009). In habitats with reduced stress intensity, individuals could minimize their trait response in order to optimize energy allocation to other activities affecting fitness (e.g. growth and reproduction) (Worley *et al.* 2003).

The relative intensity of UV-B as an environmental stressor can directly influence trade-offs in trait response in sensitive species. In environments where UV-B is a significant stressor, such as at high elevations, the pressure to avoid UV-B damage while maintaining other necessary fitness components may pose a significant challenge. Organisms that exist at high elevations are exposed to amplified UV-B radiation levels because of higher maximum fluxes of incident UV radiation and reduced atmospheric attenuation due to extensive loss of stratospheric ozone (Morris *et al.* 1995). Lakes and ponds at high altitudes are generally low in vegetative cover, thus there are few sources of dissolved organic compounds (DOC) that provide protection from UV-B radiation (Schindler and Curtis 2007). In such waters, UV-B radiation can reach depths of several meters, with high UV-B exposure rates in shallower waters (Schindler and Curtis 1997). Given the extreme differences in UV-B conditions between high and low elevation sites, it is expected that populations will differ in optimal UV-B defense strategies.

Assessments of the risks posed to amphibians as a result of increasing levels of UV-B radiation have revealed disparities in sensitivity as a result of either high natural exposure to UV-B, and/or limited capacity to withstand it (Palen *et al.* 2002; Palen *et al.*

2005). In stressful UV-B environments, such as montane ponds, there exists greater selection pressure to evolve mechanisms that effectively defend against harmful UV-B radiation. Amphibians at higher elevation sites may compensate for increased UV-B radiation levels by modifying behavior to avoid prolonged UV-B exposure, such as increasing refuge use or ovipositing in UV-B protected microhabitats (Blumthaler and Ambach1990; Garcia *et al.* 2009). If behavior modification is not advantageous, amphibians may employ a heightened physiological response to counteract UV-B induced damage, such as enzymatic DNA repair (Sinha and Häder 2002). Dark body coloration, nocturnal activity patterns, and preference for deeper microhabitats can also be effective defense mechanisms to mediate UV-B exposure in aquatic environments (Belden and Blaustein 2002; Blaustein *et al.* 2004). The cooperative or alternative use of multiple trait responses will likely vary across populations as a function of exposure rates.

Appropriate UV-B defenses for amphibians will not only change with species sensitivity and stressor strength, but also with life history stage. Larval and adult amphibians can decrease UV-B exposure rates by behaviorally avoiding high UV-B microhabitats. Amphibian embryos, however, are static in location, thus breeding females must chose oviposition sites that protect developing embryos from immediate and potential stressors. Numerous working hypotheses have been employed to explain non-random oviposition site choice by oviparous species, including the maximization of maternal survival, optimization of offspring phenotype, and maintenance of natal philopatry (Refsnider and Janzen 2010; Romano *et al.* 2008; Pearl *et al.* 2007; Vredenburg 2004; Resetarits and Wilbur 1989). Maximization of embryo survival is one such hypothesis that posits females will select sites that minimize predation risk, have a low risk of desiccation, and/or maintain a microclimate suitable for embryo development (Refsnider and Janzen 2010; Rudolf and Rödel 2005). Species sensitive to UV-B radiation, or populations experiencing high UV-B exposure rates, may preferentially lay eggs in crevices, under refugia, deeper in the water column, or in water with high concentrations of dissolved organic carbon to maintain suitable UV-B exposure levels (Probst*et al.* 2009; Blaustein and Belden 2003). Palen *et al.* (2005) found that the average depth of Northwestern salamander (*Ambystoma gracile*) eggs increased with increasing

water transparency to UV-B radiation. Female marbled newts (*Triturusmarmoratus*) wrap their eggs in the leaves of aquatic vegetation; this behavior was found to effectively shield eggs from UV-B radiation and significantly decreased mortality due to UV-B induced damage (Marcos *et al*. 2001). While these various behavioral mechanisms have been effective in reducing UV-B exposure to amphibian embryos, they differ across species and even populations within species.

Amphibians can also employ physiological mechanisms to defend against and repair UV-B damage. Potential UV-B absorbing agents such as proteins, nucleic acids, and melanin exist in all amphibian species as a mechanism to reduce UV-B induced DNA damage (Sinha and Häder 2002).However, Blaustein *et al*. (1994) showed interspecific differences in ability to repair UV-B induced DNA damage As a result of UV-B radiation, lesions develop on DNA in the form of cyclobutane-pyrimidine dimers (CPDs). CPDs are typically the most abundant and highly toxic lesions present in the genome. A single CPD is sufficient to completely eliminate expression of a transcriptional unit, which can lead to misreading of the genetic code and a reduction in the functional integrity of the cell (Sinha and Häder 2002; Friedberg *et al.* 2006).

Amphibians have specialized repair proteins that scan the genome for the presence of UV-B induced DNA lesions (CPDs). Recognition of these lesions by repair enzymes is made possible by their unique structural damage to the DNA (Sinha and Häder 2002). Once a lesion is found, the protein triggers a DNA repair mechanism (Sinha and Häder 2002), such as the enzyme photolyase, which specifically binds to CPDs and reverses the damage using the energy of light. This process is known as photoreactivation, the most efficient physiological repair mechanism employed by amphibians in response to UV-B radiation (Friedberg *et al.* 2006; Friedberg *et al.* 1995; Van de Mortel *et al.* 1998). Although photolyase activity is extremely efficient and error free, the relative amount of photolyase activity among species and populations of amphibians is highly variable (Blaustein *et al.* 1994).

The physiological mechanisms that amphibians have employed to combat DNA damage have been particularly well studied in Pacific Northwest species. Blaustein *et al.* (1994) proposed the UV-Sensitivity Hypothesis, which states that the effects of ambient UV-B radiation on eggs and embryos will vary according to their natural exposure to

sunlight and their ability to repair UV-B induced damage. Photolyase activity has been quantified in five anuran and salamander species native to the Pacific Northwest (Figure 2.1; Blaustein *et al.* 1994, Blaustein *et al.* 1995; Blaustein *et al.* 1996). These studies tend to support the aforementioned hypothesis that egg and embryo survivability is, in part, dependent upon photolyase activity and relative UV exposure rates. Those species with the lowest relative photolyase activity in high UV-B environments suffered decreased hatching success and higher mortality rates relative to species with high photolyase activity.

Photolyase activity levels differ substantially among amphibian species (Blaustein *et al.* 1994), thus we expect intraspecific differences to be concordant with the relative threat of UV-B damage. For example, in high UV-B environments populations may alternatively or cooperatively employ behavioral strategies in addition to photolyase activity to produce a correlated defense strategy to UV-B radiation. We explored population differences in behavioral and physiological trait response to UV-B radiation by documenting oviposition microhabitat use and photolyase activity in long-toed salamander (*Ambystoma macrodactylum*) populations at high and low elevations. Long-toed salamanders are highly sensitive to UV-B radiation relative to other species in the Pacific Northwest (Blaustein *et al.* 1994). Embryos of long-toed salamanders from the Cascade Mountains experienced deformities and high rates of mortality when exposed to ambient levels of UV-B in the field (Blaustein *et al.* 1997) and eggs tested from a single population in Oregon revealed that long-toed salamander photolyase activity was significantly lower relative to Anuran species in the Pacific Northwest, but similar to other caudates (Figure 2.1; Blaustein *et al.* 1994). In addition, long-toed salamanders from low elevation sites were less resistant to UV-B exposure and experienced increased mortality rates relative to high elevation individuals when exposed to the same UV-B radiation levels (Belden *et al.* 2000). The ability of long-toed salamanders to persist under intense UV-B radiation levels at high elevations suggests that selection for UV-B tolerance could be occurring in mountain populations through modification of oviposition behavior and increased levels of photolyase activity relative to lower elevation populations.

The long-toed salamander exhibits diverse life history characteristics across a broad elevational gradient. Correspondingly, populations of long-toed salamanders experience differences of more than 20 $\mu W/cm^2$ of UV-B radiation (see Chapter 2 Results) between valley and mountain breeding sites (Nussbaum *et al.* 1983). In the Willamette Valley, long-toed salamander breeding, egg-laying, and embryonic development occur during the winter months when UV-B levels are lowest (Belden and Blaustein 2002). Heavy cloud cover and dense aquatic vegetation decrease UV-B exposure in these habitats even further (Nussbaum *et al.* 1983; Jones *et al.*2005; Belden and Blaustein 2002). In contrast, long-toed salamanders at high elevation will typically breed as soon as pond surfaces thaw in late spring. These Cascade Mountain water bodies are typically devoid of aquatic vegetation, there is little cloud cover, and periods of daylight are longer relative to low elevation sites (Belden and Blaustein 2002; Belden *et al.* 2000).Thus, the breeding phenology of high elevation populations results in high levels of UV-B exposure during embryonic and larval development.

We predicted that long-toed salamanders at high elevation have cooperatively employed modified oviposition behavior and increased photolyase activity to reduce embryonic damage and exposure to UV-B radiation. In high elevation populations, females have been observed depositing eggs singly on the underside of rocks, which may effectively shield them from UV-B radiation (Garcia *et al.* 2009; Jones *et al.* 2005; Belden *et al.* 2000). At low elevations, however, long-toed salamander eggs are deposited in masses of up to 100 eggs at or near the water surface attached to submerged vegetation (Jones *et al.* 2005). This use of UV-B exposed microhabitats in low elevation habitats may not deleteriously impact embryonic long-toed salamanders because of significantly lower UV-B radiation levels. However, individuals may have to employ minimal photolyase activity to repair any DNA damage that may result from oviposition in these UV-B exposed microhabitats. Due to significantly higher UV-B exposure rates at high elevations, populations may employ a cooperative trait response to UV-B radiation. This variation in trait response may be a factor in the divergence in life history strategies observed across an elevation gradient.

MATERIALS AND METHODS

Oviposition Survey – An extensive long-toed salamander breeding and microhabitat survey was conducted over two breeding seasons from May 2010 to July 2011, at three high elevation and three low elevation breeding sites. The disparity in breeding phenologies and concordant timing of sampling is a result of factors including winter precipitation events, snowmelt and/or water accumulation, and associated elevational differences in climate. Selection of breeding sites and watersheds were based on information provided by wildlife professionals from the U.S. Forest Service, U.S. Geological Survey, the Willamette Valley National Wildlife Refuge Complex, and previous surveys conducted by the USDA/CREES sponsored Conservation Easements Assessment Project. Once long-toed salamander adults and/or egg masses were encountered, repeated visits were made until newly deposited egg masses could be surveyed and collected.

To characterize the relative exposure of long-toed salamander embryos to UV-B in the natural environment, we surveyed oviposition site selection in all six field sites by measuring depth of egg clutch, number of eggs per clutch (in masses or laid singly), UV-B exposure (exposed vs. shaded), temperature (surface and subsurface), and oviposition substrate. Depth of each egg clutch was measured to the top of the shallowest egg and each oviposition site was characterized as either shaded or exposed. We considered eggs to be shaded if they were covered by an immediate barrier to light. We did not take into account shading by riparian vegetation or location relative to large objects, which may temporarily reduce UV-B exposure depending on the time of day. Oviposition substrate was qualified based on similar techniques as Adams *et al.* (2005). The primary egg substrate was characterized as silt, gravel, cobble, boulder (of various sizes), bedrock, wood, vegetation, or leaf letter. This substrate characterization was used as additional information in determining relative exposure of embryos to UV-B. For example, eggs laid atop vegetation were expected to receive different UV-B exposure rates compared to eggs laid beneath wood or boulders.

In addition, detailed UV-B radiation measurements (ambient and subsurface penetration) were taken using a handheld radiometer in clear-sky conditions at each oviposition site throughout the six breeding sites. These measurements provided a UV-B

profile of each breeding site and a record of UV-B attenuation in the water column during peak UV-B hours (1000 hours to 1500 hours). Measurements of UV-B radiation were taken at 10 cm increments from the water surface to a depth of 1 meter or until the bottom of the pond was reached. UV-B radiation levels were measured beneath the protective refugia (when present) and adjacent to the egg clutch, assuming eggs could become exposed via disturbance. These measurements provided information on the UV-B exposure rates and attenuation of UV-B in the water column for each egg clutch for effective comparisons within and among sites.

Experimental Preparation – To correlate oviposition site characteristics with photolyase activity, at least 5 (maximum of 8) long-toed salamander egg clutches were collected from each site. Clutches were collected from 3 low elevation breeding sites (LL, FL, CL) in the Willamette Valley of Oregon and 3 high elevation breeding sites (SH, EH, PH) in the Cascade Mountain Range of Oregon (Table 2.1). All eggs were staged based on Harrison (1969) Developmental Staging Table for Salamanders and collected within 48 hours after deposition, or before stage 10 to reduce the extent of environmental influence on molecular activity and condition of the embryos.

Each egg clutch was transported to Oregon State University, Corvallis, OR, in light-resistant containers. Upon arrival, a total of 633 eggs (from 37 clutches) were transferred to 1 gallon high-density polyethylene containers filled with filtered tap water that had been treated with NovAqua Plus® and Amquel Plus® (Kordon, LLC, Hayward, CA) to rid the water of contaminants and left standing for no longer than 24 hours in a 4°C refrigerator. Embryos were refrigerated to slow the meiotic process prior to enzyme analysis.

Laboratory techniques were extrapolated from Pandelova *et al.*(2005), Curtis and Hays (2007), and Matsunaga (2007). Embryos were warmed to room temperature for mechanical dissection from their jelly envelope and each clutch was combined into a separate 1 mL modified *Xenopus laevis* oocyte transcription buffer (adapted from Blaustein *et al.*1994).Using a Dounce homogenizer, the embryos were lysed and centrifuged. The exudates were recovered and centrifuged again at high speed (64,000 RPM). The supernatant was recovered and transferred to aliquots of 100-250 µL, depending on amount of extract available. Each aliquot of salamander DNA was flash-

frozen in liquid N_2 and stored at -80°C for future enzyme analysis. Eggs from low elevation populations were separated by clutch into aforementioned aliquots and kept frozen in the stable buffer solution until high elevation populations could be collected due to disparity in breeding phenologies. High elevation populations were similarly dissected, homogenized, and subdivided by clutch in buffer solutions. However, final analysis for photolyase activity was done simultaneously so as to reduce error and maximize comparability among populations.

Absolute determination of salamander photolyase activity requires calibration against irradiated DNA standards from thymine-requiring *Escherichia coli* cells, in which absolute numbers of photoproducts can be determined (Curtis and Hays 2007). To prepare plasmid DNA standards containing known levels of cyclobutane-pyrimidine dimers (CPDs; photoproduct lesions), plasmid pUC19 was extracted from the *E. coli* lysates grown in broth containing thymidine using a QIAprep® Spin Midiprep Kit (QIAGEN, Valencia, CA). The purified plasmid DNA at 200 ng/µL was then irradiated with UV-C light in a gently agitated Petri dish to doses from 50 to 200 J/m^2 at a rate of 1.6 J/m^2/s. Subsamples of irradiated plasmid DNA (200 µL) were taken every31 seconds. UV-C light is a preferred alternative because linearity between photoproduct induction and UV-C light is well known (Pandelova *et al.*2005). Only the highest fluencies (UV-C) induce enough photoproducts to be measured quantitatively. Thus, photoproducts at a lower fluency (UV-B) can be estimated by linear extrapolation.

Each irradiated DNA subsample was digested with 20 units T4 Endonuclease V (Epicentre, Madison, WI), a base excision repair pathway which recognizes CPDs and cleaves the DNA at each CPD site (Endo V-sensitive sites; ESSs), resulting in breakage of the DNA strand. Samples were electrophoresed through 1% agarose gels, and the fractions of supercoiled DNA converted to nicked DNA (corrected for preexisting nicked DNA and differential gel images) were determined from analysis of digitized gel images using ImageQuant 5.2 software (Molecular Dynamics; Figure 2.2). The average number of EndoV-sensitive sites (ESSs) per plasmid was estimated using the Poisson relationship (fraction of EndoV-resistant plasmids = e^{-x}; Figure 2.3).It is important to note that the fraction of EndoV-resistant sites in the sample irradiated at 200 J/m^2 was below the detectable limit, thus we can assume that almost the entire preexisting supercoiled DNA

was converted to nicked DNA by the T4EndoV. Based on this standard curve we can assume that the induction of CPDs is directly proportional to UV fluency. This efficiency parameter can be extrapolated to other pUC19 plasmids irradiated under identical conditions.

Experimental Design – The following procedures were conducted under gold light to ensure the prevention of unwarranted photoreactivation. long-toed salamander DNA in egg protein extracts (5, 10, 15 µL) were added to 100 ng UV-irradiated plasmid DNA (200 J/m^2) of known CPD concentration and combined with 5 µL 10x Photoreactivation Buffer (250 µL 2M Tris HCL, pH 7.4; 20 µL 500 mM EDTA; 20 µL 5M NaCl; 100 µL 1M dithiothrietol) to inhibit endonuclease degradation of the extract. Samples were replicated in quadruplicate on a 96-well microtiter plate and subdivided into UV light versus dark treatments. The UV light treatment involved incubation of salamander extracts in buffer solution under UV-B blue light for 15 minutes. The length of incubation was chosen to make the number of CPDs removed proportional to amounts of extract for the concentrations used (see Figure 2.10 Inset). The UV dark held treatment involved incubation for 15 minutes in the dark to ensure no photoreactivation occurred. This treatment was used as a control to determine the extent of degradation by endonucleases (non-light induced), which were accounted for if discovered. An additional 20 µL of the 10x Photoreactivation Buffer was added to each sample then boiled for 5 minutes and chilled on wet ice for 5 minutes.

Cyclobutane pyrimidine dimers (CPD lesions) in long-toed salamander DNA were estimated by an immuno-slot-blot technique (adapted from Curtis and Hays 2007) using a monoclonal anti-thymine dimer. Salamander DNA extracts in Photoreactivation Buffer wereadded to 0.25 mL of 1x SSPE buffer (3M NaCL, 0.23 M NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4; NaOH). Samples were vortexed and centrifuged briefly to remove debris. Aliquots of 0.5 mL DNA solution were applied to Whatman® Nytran® N+ membrane that had been prewetted and washed with 1x SSPE, using a BioRad slot-blot apparatus (Hercules, CA). Blotted DNA was washed with 0.5 mL 1x SSPE and the entire membrane fixed with 0.4N NaOH for 10 minutes. The membrane was blocked with 5% non-fat milk in 1x PBST (phosphate-buffered saline, pH 7.3, containing 0.2% Tween 20) and incubated with agitation for 60 minutes at room temperature. The DNA was washed

with 1x PBS, incubated with the monoclonal anti-thymine dimerin 1x PBS for 60 minutes. The DNA was then washed with 1x PBST, and incubated with horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Invitrogen) in 1x PBS for 60 minutes, washed with 1x PBST and incubated with ECL 2 chemiluminescent substrate (Pierce).

Detection of antibody-linked horseradish peroxidase chemiluminescent signal was conducted in a dark room by exposing the membranes onto film over a period of time (5 sec, 10 sec, 30 sec, 1 min, 2 min). Quantification of chemiluminescent signal was performed using ChemiGenius BioImaging System (Sygene, Frederick, MD). To determine concentration of solubilized proteins in salamander extracts (for quantification of enzyme activity), we conducted a Bradford protein assay. The microassay procedure for microtiter plates involves the preparation of 5 dilutions of a protein standard (bovine serum albumin; BSA), the linear range of which is 8 µg/mL to 80 µg/mL. Salamander extracts from each clutch were also assayed simultaneously. All protein solutions were combined with 40 µL Protein Assay Dye Reagent (Bio-Rad) diluted 1:4 and performed in duplicate.After incubation for 5 minutes, measurements at 595 nm wavelength were taken with a microplate reader. Comparison to a standard curve provided a relative measurement of protein concentrations per extract (Figure 2.10 Inset). Final quantification of specific photolyase activity in long-toed salamander DNA was measured in CPDs removed per µg protein per hour.

Statistical Analysis – To examine population differences in oviposition site characteristics we performed Welch's t-tests (for unequal variance) and the Student's t-test on the pooled low and high elevation sites for each variable, after assessing homogeneity in variance. Trends in UV-B attenuation with depth were analyzed using logistic regression and compared using the coefficient of determination (r^2). To examine population differences in photolyase activity, we first quantified the relative enzyme activity per clutch as the number of CPDs removed per µg protein per hour and similarly compared population differences using a Student's t-test after determining equal variance. A chi-squared test of independence was used to determine if differences in ambient and oviposition site UV-B radiation explained the observed variation in

photolyase activity among long-toed salamander egg clutches. All analyses were performed using the R statistical program (R Development Core Team, 2010).

RESULTS

Average ambient UV-B radiation levels at high elevation sites were 20.34 $\mu W/cm^2$greater than average ambient UV-B levels at low elevations during the respective long-toed salamander breeding seasons (Low: 0.69 $\mu W/cm^2 \pm 0.07$; High: 21.03 $\mu W/cm^2 \pm 0.16$; t=-118.85, df=22.73, $P<0.001$).

Oviposition site selection by adult females at our 3 high elevation breeding sites was predominantly in UV-B protected microhabitats, such as underneath rocks or buried in sediment. The deposition of eggs under refugia significantly reduced embryo exposure to harmful UV-B levels at high elevation sites, with average oviposition site UV-B radiation levels of 0.42 $\mu W/cm^2$ (SE=0.13). UV-B levels at the oviposition site at high elevations were significantly different than ambient levels, with an average reduction in UV-B of 20.28 $\mu W/cm^2$ (t =-99.27, df=34, $P<0.001$) from ambient levels. At low elevations, there was an average difference in ambient versus embryonic UV-B levels of 0.27 $\mu W/cm^2$ (t=-2.78, df=36, $P<0.05$). Thus, embryonic UV-B levels were lower than ambient levels at both the high and low elevation breeding sites.

Due to laying eggs in UV-B protected microhabitats, embryos from high elevation populations receive similar average UV-B radiation levels relative to their low elevation populations (0.75 $\mu W/cm^2$, SE = 0.08)(Figure 2.4). An F test determined that the variance in UV-B radiation levels at the site of oviposition was significantly different between high and low elevations (F=0.36, $P<0.05$), with higher variances in UV-B levels seen in high elevation populations. A Welch's t-test showed that this mean difference in UV-B radiation at the oviposition site was significantly different between high and low elevation populations ($P<0.05$, t=2.16, df=28). However, these differences are not biologically significant when considering threshold UV-B levels required to initiate biological damage in this species (Blaustein *et al.* 1996).A Welch's t-test showed that

eggs buried in sediment did not receive higher UV-B dosages relative to eggs covered beneath objects (e.g. boulders, wood, etc.) at high elevations (P >0.05, df=12).

In addition to differences in oviposition substrate, females at high elevation have modified the depth at which they lay their eggs. Average depth at which eggs were laid at high elevation was 37.84 cm (SE=7.92), compared to an average depth of 6.03 cm (SE=0.6) at low elevation. A Welch's t-test showed that this difference was significant (P<0.001, t=4.00, df=17) after determining unequal variances between populations (F=0.07, P <0.001)(Figure 2.5).

We examined UV-B attenuation (measured as percent surface UV-B) for each of the breeding sites at high and low elevation (Figures 2.6 and 2.7). One site (PH – Potholes, Mt. Bachelor) was excluded from this analysis because it was less than 15 cm deep in all areas sampled. Our measure of attenuation was calculated as percent surface UV-B remaining at 10 cm increment depths. Average UV-B radiation measurements taken at site PH showed almost complete attenuation at 10 cm, with 95.72% (SE = 0.21) of the surface UV-B attenuated at oviposition sites. As predicted by numerous models of UV-B attenuation in aquatic systems, a large proportion of the variation in UV-B radiation below the surface is explained by increases in depth (Figure 2.6 and 2.7).

While the strong correlation between UV-B attenuation and depth among high and low elevation sites were relatively indistinguishable, exponentially higher ambient UV-B levels in the mountains resulted in a greater amount of UV-B penetrating to a greater depth at high elevation sites relative to low elevation sites. For example, site EH (high elevation) and site CL (low elevation) had similar maximum depths, but UV-B penetrated to greater depths on average at the high elevation site. Site EH experienced average ambient UV-B levels of 21.22 $\mu W/cm^2$ (SE=0.29) and UV-B penetrated up to a 1-meter depth with 0.21 $\mu W/cm^2$ (SE=0.06) remaining. This is contrasted with an average ambient reading of 0.77 $\mu W/cm^2$ (SE=0.03) at low elevation site CL, which was completely attenuated at 70 cm for all UV-B measurements at this location (Figure 2.8). Given that long-toed salamanders are sensitive to UV-B radiation at all life stages, it was not surprising that oviposition site selection at site EH (high elevation) corresponded to the depths at which UV-B penetrated.

Water temperatures at the oviposition site were not significantly different between high and low elevation populations (t=-1.03, df=35, P>0.05) with an average of 3.72°C (SE=0.44) and 5.04 °C (SE=0.47) at high and low elevations, respectively. However, corresponding surface water temperatures were significantly different between populations (t= -4.38, df=27.42, P<0.001) with an average of 12.17 °C (SE=0.08) at high elevations and 4.35 °C (SE=0.45) at low elevations during long-toed salamander breeding (Figure 2.9).

Comparisons of photolyase activity between high and low elevations populations revealed no significant differences (t=1.00, df=29, P>0.05), with an average of $7.26x10^9$ CPDs removed per µg per hour (SE=$1.29x10^9$) in high elevation populations and $9.14x10^9$ CPDs removed per µg per hour (SE=$1.37x10^9$) in low elevation populations (Figure 2.10). A chi-square test of independence was performed to examine the relationship between photolyase activities and UV-B radiation at the oviposition site. The relationship between these variables was not significant (X^2=15.25, df= 12, P >0.05), therefore higher UV-B levels at the oviposition site were not associated with higher photolyase activity as was originally hypothesized. Similarly, a chi-square test revealed no relationship between ambient UV-B radiation and photolyase activity (X^2= 4.37, df= 4, P>0.05). Although UV-B conditions are significantly different between high and low elevation populations, there was no corresponding difference in photolyase activity.

DISCUSSION

Our prediction that long-toed salamanders have modified their oviposition behavior at high elevations to reduce embryonic exposure to damaging UV-B radiation was well supported. We found significant differences in microhabitat conditions such as water temperature, UV-B radiation, depth of oviposition site, and oviposition substrate. Even though ambient and surface UV-B levels were exponentially higher in the mountain sites, selection for oviposition substrates in UV-B protected microhabitats (e.g. under refugia and depths up to 1 meter) at high elevation breeding sites effectively reduced UV-B to negligible levels. This resulted in standardized UV-B conditions at the oviposition site between high and low elevations, which is a seemingly adaptive behavioral modification

for a species with high UV-B sensitivity in all life stages. Even among the various refugia selected for oviposition substrate at high elevations, UV-B levels were similar and close to zero.

Variation in available substrates across high elevation sites highlighted the different strategies employed by females when depositing eggs. Sites that were highly limited in available cover objects (such as site PH) corresponded to long-toed salamander females burying embryos in silt sediment. Our results indicate that this sediment provided similar UV-B protection relative to small boulders and rocks utilized by other high elevation populations. Thus, multiple oviposition substrates can be useful in reducing embryonic exposure to UV-B radiation at high elevations. Female salamanders are not necessarily selecting a particular substrate; instead they appear to take advantage of multiple protective substrates that limit UV-B exposure in these high elevation habitats. Oviposition behaviors utilizing UV-B protected microhabitats in high elevation populations was expected, but ovipositing females unexpectedly selected sites in deeper areas of high elevation ponds. In many instances, eggs were deposited both in deep-water microhabitats and beneath protective refugia. This coordinated defense strategy was successfully employed to reduce UV-B radiation exposure for embryos and is likely necessary when the optical properties of the water do not effectively reduce UV-B radiation to a tolerable level for this species.

Behavioral modifications to reduce UV-B exposure are also common in the larval stage of long-toed salamanders at high elevations. Belden *et al.* (2000) performed sun and shade choice tests on high elevation larval long-toed salamanders and found that larvae spent most of their time in the shade. Additionally, this study examined depth preference and larvae were most frequently observed in the deepest portion of the pond with the least amount of UV-B exposure. Behavioral avoidance strategies have similar correlates in our study, wherein adult females may have a preference for deeper, shaded locations for egg deposition substrates within the ponds at high elevation when available. It is important to note, however, that we did not examine oviposition substrate preference in this study, rather we sampled substrate use. Analysis of substrate availability at long-toed salamander breeding sites would allow us to further evaluate oviposition substrate preference in this species. It is likely that substrate preference would tend towards objects

that offer extensive UV-B protection, but they do not appear to be selective towards any particular cover object based on information gathered during our microhabitat survey.

Estimates of species sensitivity to UV-B must be considered in conjunction with assessments of UV-B exposure rates and attenuation properties to be relevant to conservation and management. The vast majority of UV-B attenuation is due to compounds in the fulvic acid fraction of dissolved organic carbon (DOC), which constitutes 40-60% of total DOC and is derived primarily from terrestrial plant decomposition (Brooks *et al.* 2005; McKnight *et al.* 1997). Light absorbance by fulvic acids can control photic zone depth and attenuation of ultraviolet light (McKnight *et al.* 1997). UV-B radiation can reach depths of several meters in high elevations waterbodies, with higher UV exposure rates in shallower waters (Schindler and Curtis 1997). At high elevations it took significantly greater depths to attenuate higher ambient UV-B levels relative to low elevation sites. Maximum attenuation was likely reached quickly at low elevation sites because there is greater input of organic matter and sub-surface UV-B levels do not significantly depart from ambient levels. However, as exemplified by one high elevation site (PH), a lack of organic matter does not completely prevent UV-B absorption in freshwater systems. Inorganic particulate matter, such as silt sediments, can infiltrate the water column and significantly reduce the transmittance of UV-B (Häder *et al.* 2007). Thus, trade-offs in organismal response to UV-B are further confounded by diverse optical properties and UV-B conditions of aquatic breeding sites.

We also predicted that photolyase activity levels would be higher in mountain populations due to exponentially higher UV-B radiation. Our results indicate that long-toed salamanders have not employed a heightened physiological response; instead we found that photolyase activity is relatively similar across populations regardless of ambient and sub-surface UV-B levels. Research conducted by Blaustein *et al.*(1994) indicated that long-toed salamanders had drastically lower photolyase activity relative to other species. We used newer methodologies to demonstrate even lower photolyase activity for this species. There are other factors that may have influenced photolyase signal in our samples. Endonucleases and exonucleases present in the salamander extracts prior to analysis can continue to breakdown the DNA if not completely removed during purification. This results in background noise and, essentially, a blurring of the signal

during later quantification. In some instances after UV-B treatments, salamander DNA showed degradation from exonucleases and endonucleases that remained after purification, so these samples were discarded during our study due to non-photolyase specific activity. Additionally, while not investigated in this species, dark excision repair (e.g. nucleotide excision repair, base excision repair) can occur in the absence of light and similarly remove thymine dimers through a multi-pathway process (Kimura *et al.* 2004). While not the main pathway for UV-specific repair, dark excision repair can act as an additional mechanism for non-specific repair of UV induced DNA damage. Dark excision repair has yet to be investigated in animal species as a mechanism for effectively removing CPD lesions, but it is an active repair process in the DNA and might be a viable strategy for highly UV-sensitive species (Kimura *et al.* 2004). We also did not measure long-toed salamander ability to repair other mutagenic photoproducts such as pyrimidine-(6-4')-pyrimidinone photoproducts. This is another multi-step pathway that is not as efficient as photolyase in removing UV-induced lesions, thus we opted to solely investigate the more efficient photolyase enzyme pathway.

Overall, it appears that long-toed salamanders can avoid UV-B damage to developing embryos by utilizing oviposition substrates with low UV-B transmittance such as shaded areas or deeper water. Cooler, deeper water, however, can reduce growth and development rates, creating a conflict between UV-B avoidance and thermal requirements as temperature is a major factor in determining amphibian hatching rates (Bancroft *et al*. 2008; Smithgill and Berven 1979; Wilbur and Collins 1973).Our results indicate that long-toed salamanders did not respond to this potential conflict by employing a physiological defense mechanism, thereby allowing individuals to develop in warmer, UV-B exposed microhabitats. Instead, relatively weak physiological repair capabilities were buffered by extensive behavioral modifications (i.e. laying eggs under UV-B protective substrates). For high elevation long-toed salamanders, the benefits of reducing UV-B exposure rates may outweigh the associated developmental costs of ovipositing in colder microhabitats. Given that this species is highly sensitive to UV-B, yet has been able to persist at high elevations, it is likely that the developmental trajectories are being satisfied regardless of the extent of UV-B avoidance. Alternatively, there is no information about the energetic costs associated with photoreactive DNA

repair in amphibian species. We can only speculate that, similar to other behaviors and metabolic pathways, there are trade-offs in the extent to which an individual can heighten the efficiency of a given trait without reducing their fitness (Worley *et al.* 2003).

We determined that long-toed salamander populations have been able to strategically modify their oviposition behavior to mitigate damaging UV-B radiation levels at high elevation. This divergence in behavior across elevations can be described as either phenotypic plasticity in response to UV-B radiation, or local adaptation to specific UV-B conditions. Analyses of population genetics and/or an experiment to test plasticity in response to UV-B radiation levels by individuals from multiple populations across an elevational gradient would be needed to determine the extent of gene flow and phenotypic plasticity in populations of the long-toed salamander. Without this information we can only speculate that the behavioral and physiological strategies employed by this species are a direct result of variability in UV-B radiation across elevations. Whether or not these strategies will promote existence into the future is critical in determining the susceptibility to decline in long-toed salamander populations. It is difficult to predict the direction a population will take in the face of both natural and rapidly mounting human-induced selection pressures. However, knowing how populations differ in stress response offers better understanding as to the overall effects of changing environmental factors.

ACKNOWLEDGEMENTS

We thank L. Ganio, K. Dugger, and J. Van Sickle for assistance with statistical analyses. We would also like to thank the Willamette Valley National Wildlife Refuge Complex and U.S. Forest Service for site access. This study was funded by scholarship stipends from The Society for Northwestern Vertebrate Biology, and the Department of Fisheries and Wildlife and OSU College of Agricultural Sciences at Oregon State University. Animals were collected under Oregon Department of Fisheries and Wildlife Scientific Taking Permit #078-10, U.S. Fish and Wildlife Service Special Use Permit #13590-11-02, E.E. Wilson Management Area Special Use Permit #1513, and Oregon State University Animal Care and Use Protocol #3978.

TABLES AND FIGURES

Table 2.1 – Long-toed salamander collection sites and respective elevations. The first letter of the Clutch I.D. represents the first initial of the site name, followed by either an L to indicate Low elevation sites, or H to indicate High elevation sites. GPS Coordinates refer to coordinates at the near center of each pond site.

Clutch I.D	**Site Name**	**Latitude**	**Longitude**	**Elevation**
LL	Lake Creek Dr. at Hinck Dr.	44.5378	-123.1377	72.24 m
FL	Finley NWR	44.4249	-123.3108	78.94 m
CL	Hwy 34 at Christmas Tree Farm	44.5629	-123.1451	66.75 m
SH	Susan's Pond	44.1116	-121.6174	1,951.33 m
EH	Eric's Pond	44.1018	-121.6162	2,024.48 m
PH	Potholes at Todd Lake	44.0333	-121.6714	1,997.05 m

Figure 2.1– Photolyase activities of 10 amphibian species from the Pacific Northwest averaged for tissues and years. Information adapted from Blaustein *et al.*1994. Each number represents the measured photolyase activity (10^{11} CPDs/µg/hr) during that study for each of the species listed. Samples were taken from various sites throughout Oregon. Photolyase activity of the long-toed salamander was measured to be much lower (0.8 10^{11} CPDs/µg/hr) than most other native Anuran species in the Pacific Northwest, but relatively similar to other Caudates.

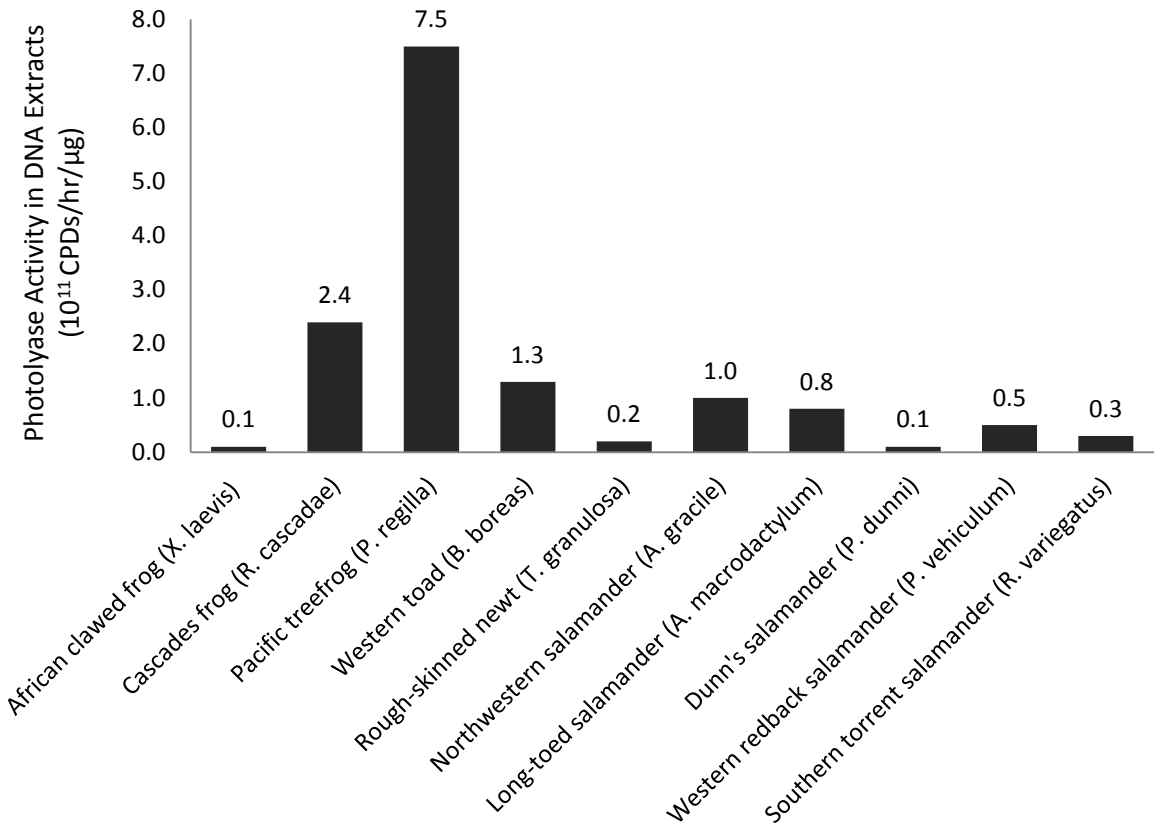


Figure 2.2 – Digitalized image of electrophoresed samples. From left to right, paired pUC19 plasmid samples irradiated at 0,50, 100, 150, and 200 J/m^2, respectively. Each pair shows the untreated (left; -) and treated (right; +) samples with T4EndoV for a visualization of *E. Coli* DNA cleavage at Endo V-sensitive sites (ESSs) caused by UV-B irradiation and subsequent CPD lesion introduction. After irradiation at 200 J/m^2, nearly the entire DNA is cleaved at lesion sites. This information is used to determine the standard curve of lesion introduction into pUC19 DNA for comparisons with salamander extracts (see Figure 2.3).

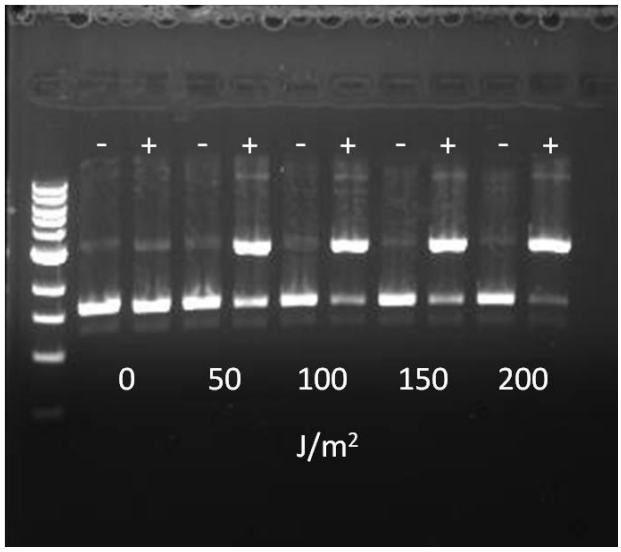


Figure 2.3 – Standard curve of the average number of Endo V-sensitive sites (ESSs) per plasmid irradiated to 0, 50, 100, 150, and 200 J/m^2. T4EndoV is a base excision repair pathway specific to cyclobutane pyrimidine dimers (UV-induced DNA lesions). This standard curve was used to assess efficiency of strand breakage at CPD sites (ESSs) in pUC19 DNA.

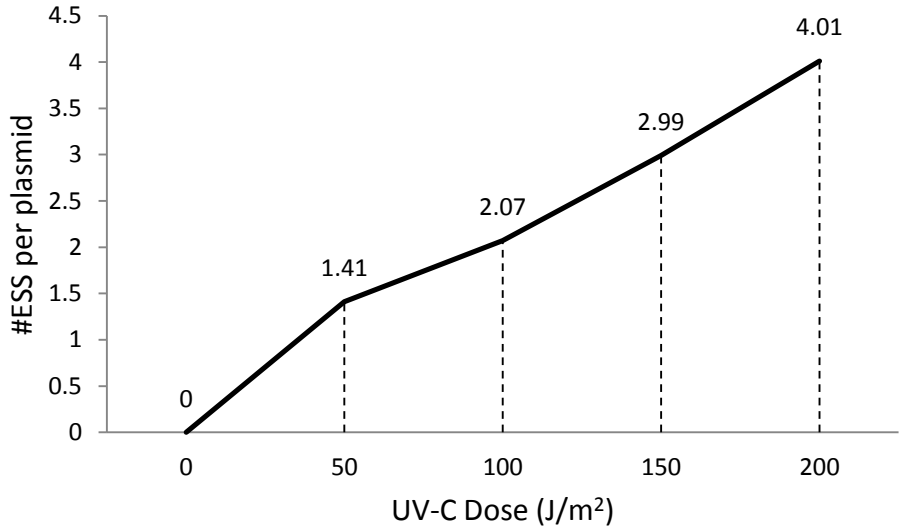


Figure 2.4 – Ambient (dark bars) and oviposition site (light bars) UV-B levels averaged for each population at high (right) and low (left) elevations. The dotted line separates the populations from high elevation from populations at low elevation. There were significant differences in ambient UV-B levels between high and low elevation breeding sites ($P < 0.001$). Within populations at high elevation, ambient and embryonic UV-B levels were also significantly different ($P < 0.01$). At low elevations, differences in ambient and embryonic UV-B levels were not biologically significant.

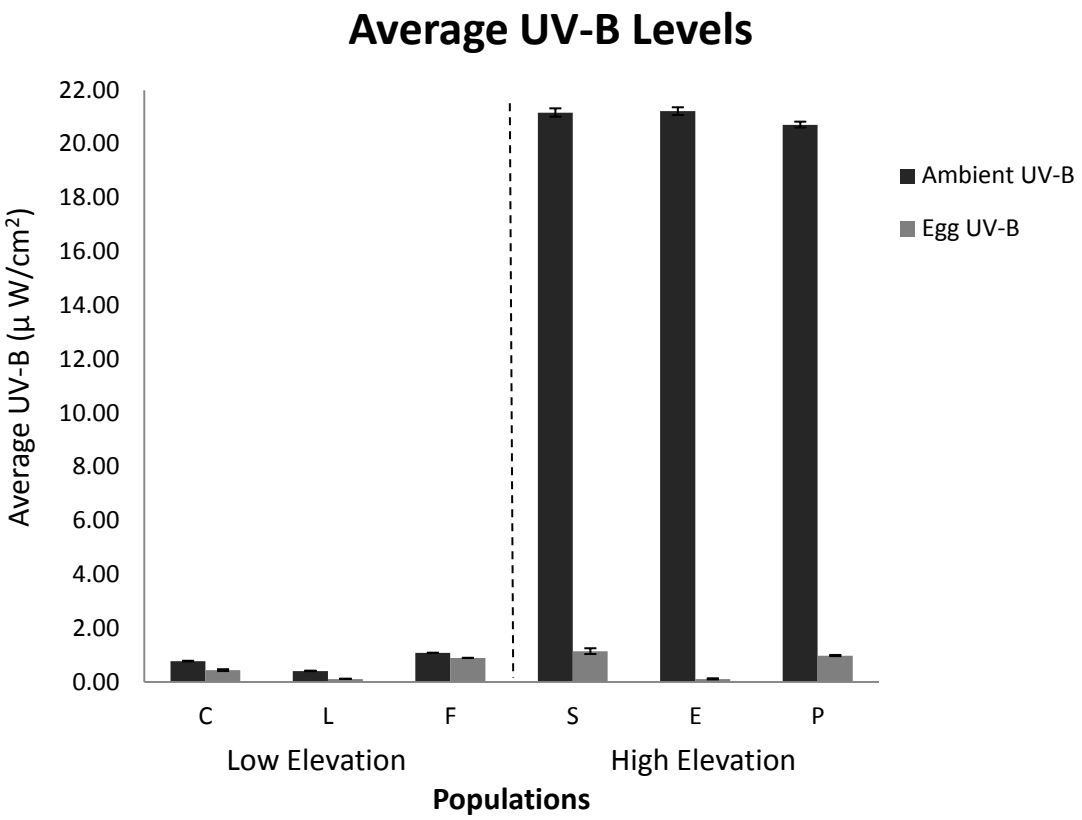


Figure 2.5 – Comparison of UV-B radiation levels averaged across populations between high (right) and low (left) elevations and the corresponding depth at which the eggs were deposited. Populations at high elevation were observed depositing eggs significantly deeper than low elevation populations, which resulted in similar UV-B levels at the oviposition site across elevations.

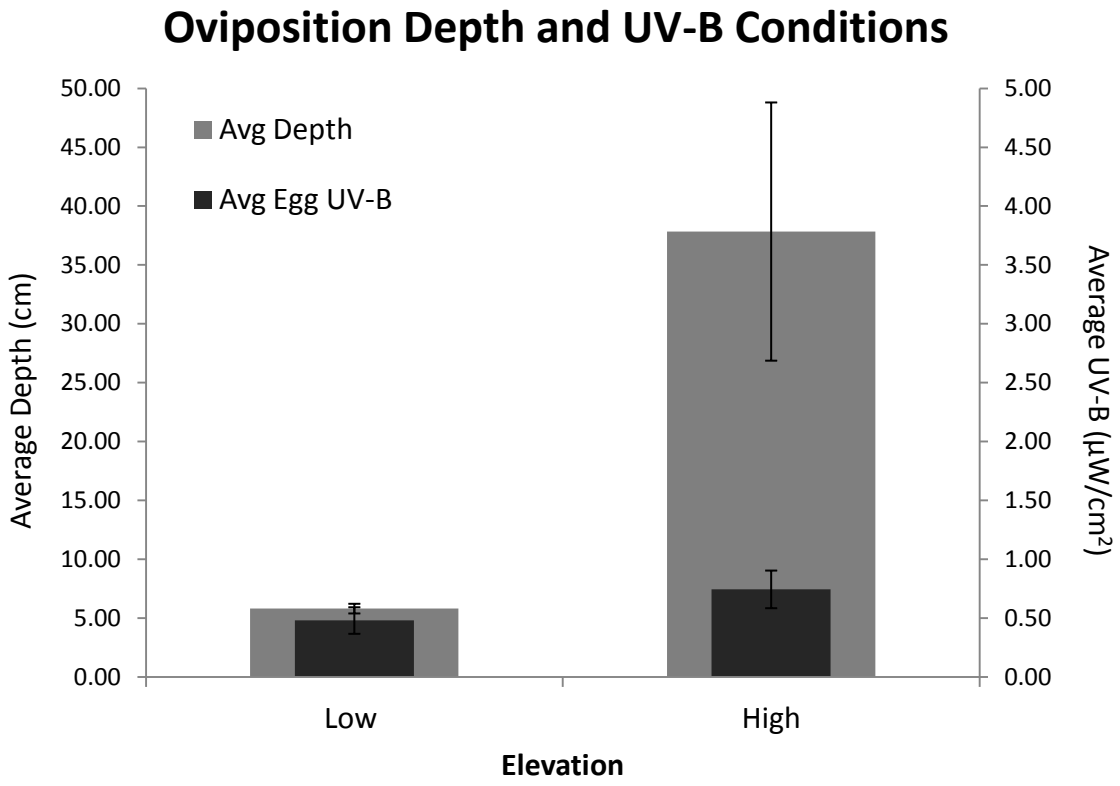


Figure 2.6 –UV-B attenuation in the water column for each breeding site (CL, LL, and FL) at low elevation. Attenuation was calculated as percent surface UV-B radiation and averaged across all locations sampled per site. The coefficient of determination (r^2) was calculated from a non-linear regression and was provided to elucidate the relationship between depth and UV-B attenuation for each site.

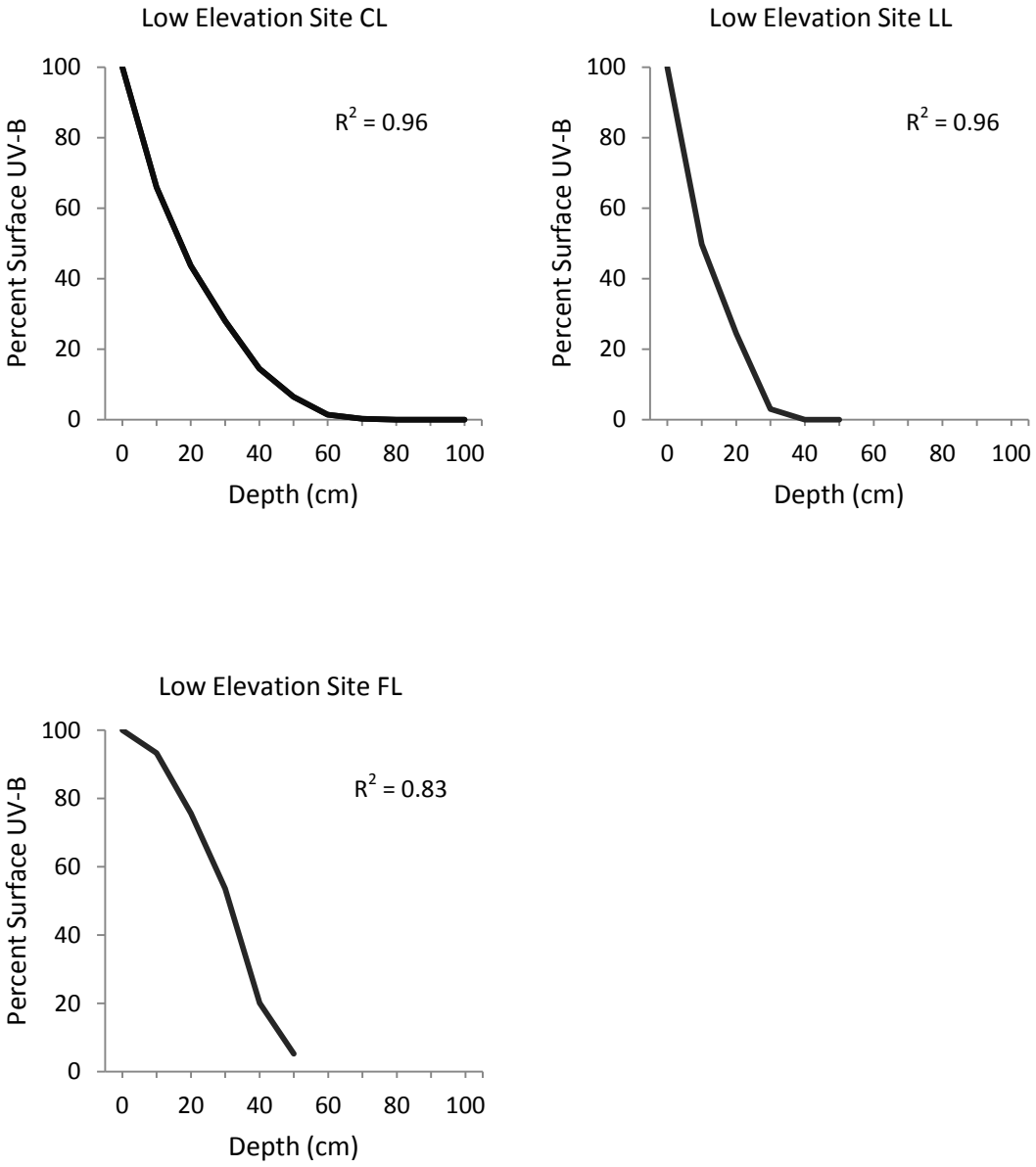


Figure 2.7 – UV-B attenuation in the water column for each breeding site (SH and EH) at high elevation. Site PH was excluded because the average water level was less than 10-15 cm and an attenuation curve was not descriptive. Attenuation was calculated as percent surface UV-B radiation and averaged across all locations sampled per site. The coefficient of determination (r^2) was calculated from a non-linear regression and was provided to elucidate the relationship between depth and UV-B attenuation for each site.

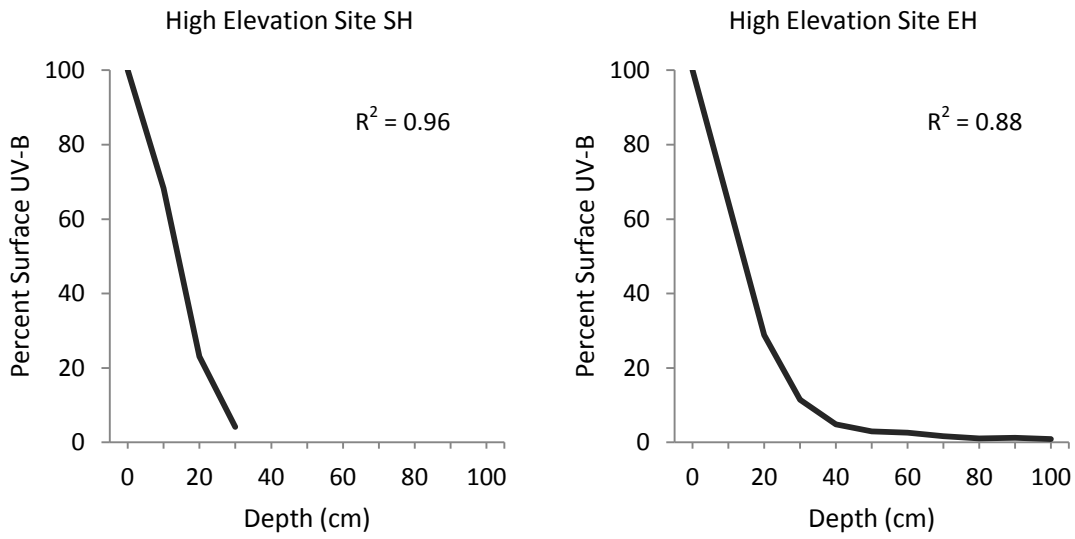


Figure 2.8 – Average decline in UV-B radiation at high elevation site EH (gray line) and low elevation site CL (black line). UV-B radiation measurements included ambient and surface UV-B, as well as UV-B penetration up to 1 meter depth at 10 cm increments. This graph is used to visualize the relative amount of UV-B that must be attenuated in the water column between high and low elevation populations, shown via non-linear regression and the correlation coefficient (r^2).

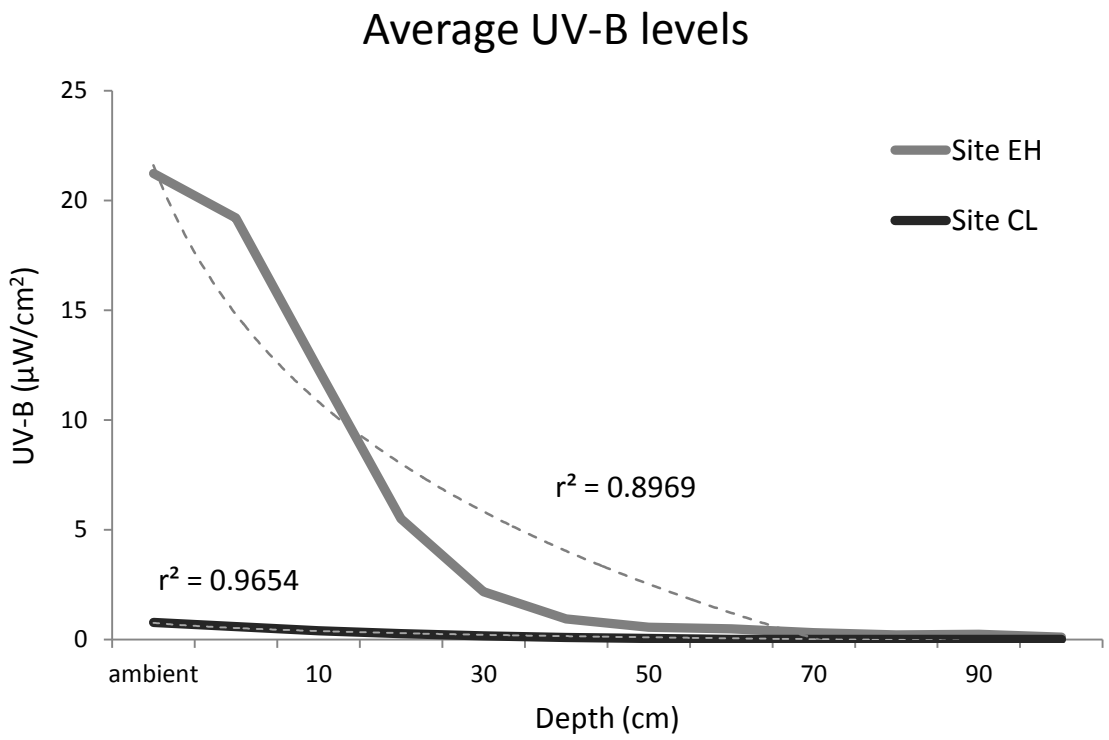


Figure 2.9 – Comparison of surface water temperature (dark) and temperature at the oviposition site (light), averaged across populations at high (right) and low (left) elevations. Surface temperatures were significantly different between high and low elevation populations, with similar water temperatures at the oviposition site across populations.

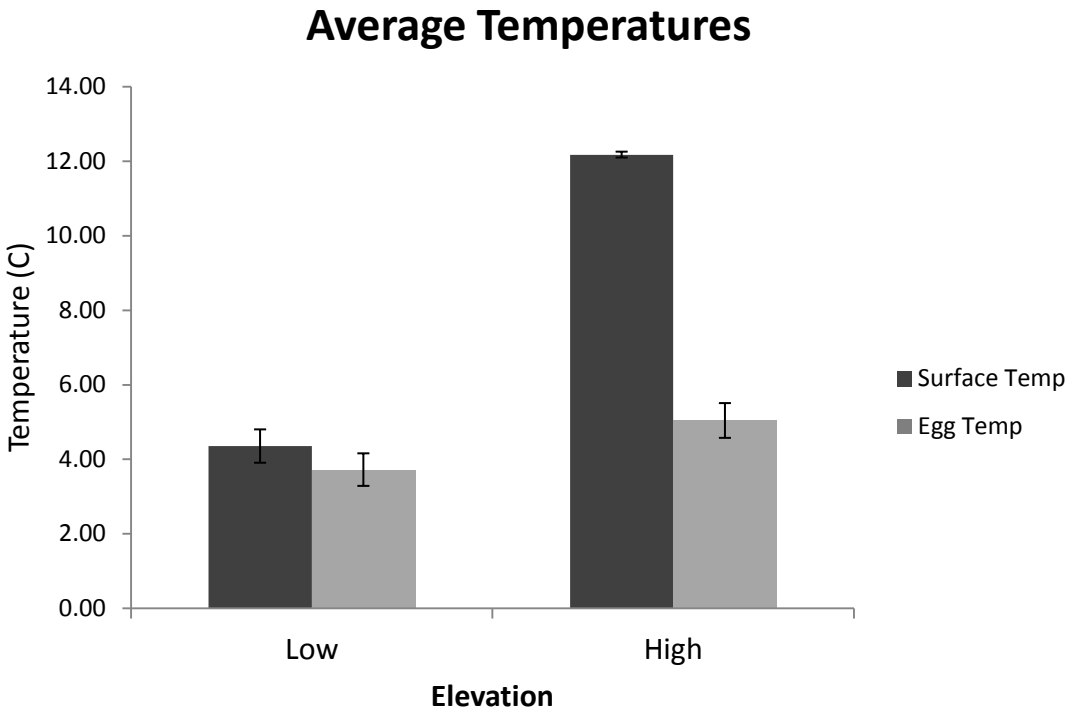
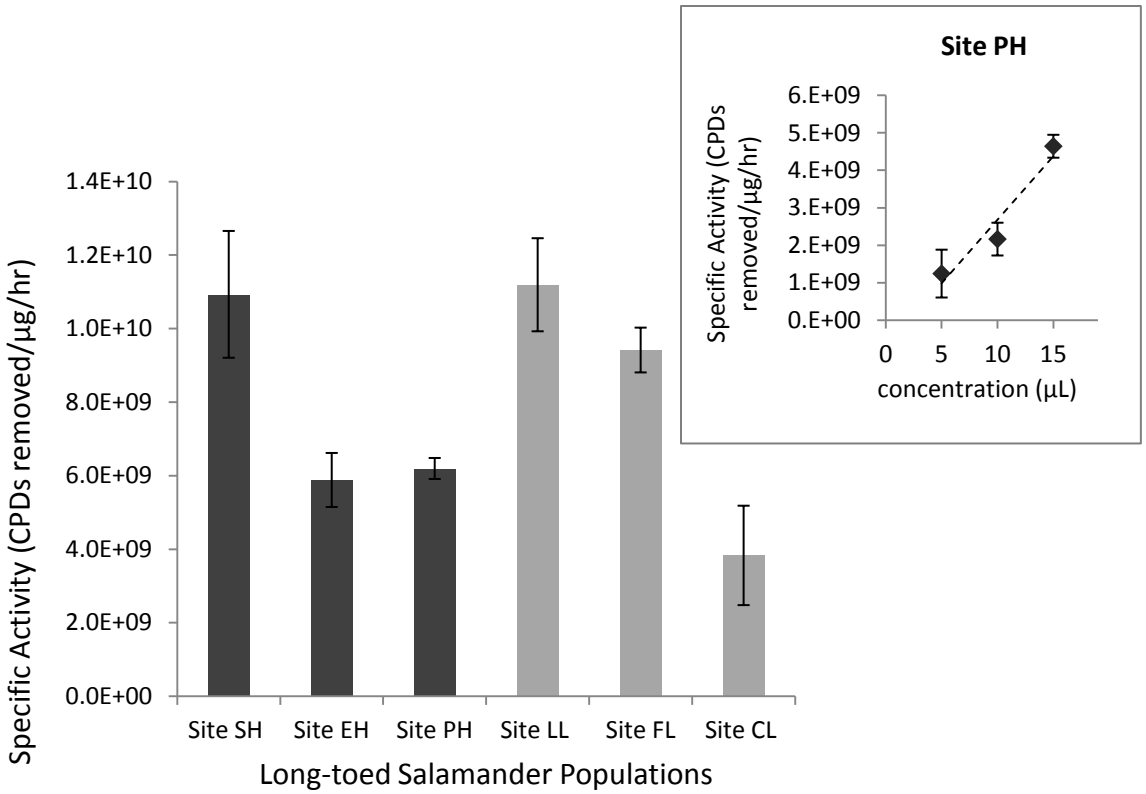


Figure 2.10 – Specific photolyase activities (rates of CPD removal per µg of protein) are averaged for all egg clutches used in simultaneous assays at extract concentrations in the linear range (see Inset for an example from site PH), all standard errors are given. The activity-concentration profiles included 100 ng irradiated DNA substrate with 5, 10, or 15 µL of salamander extract incubated for 15 minutes under UV-B light. There was no significant difference in photolyase activity between long-toed salamander populations at high and low elevation.



CHAPTER 3: Conclusions

We explored trade-offs in behavioral and physiological responses to UV-B radiation in the long-toed salamander (*Ambystoma macrodactylum*). At high elevations, exponentially higher UV-B radiation levels have resulted in a modification of life history strategies for this species. Adult female salamanders have successfully mitigated UV-B damage to their embryos at high elevations by selecting oviposition substrates that offer shading from UV-B radiation and are often at much greater depths. Lower UV-B exposure rates at low elevation have allowed females to lay eggs on a variety of vegetative substrates without need for protective cover from UV-B radiation. As a result, extensive behavioral modification in high elevation populations has standardized UV-B conditions to negligible levels for developing embryos.

The variation in life history characters exhibited by the long-toed salamander is a strategy that is suitable to the diverse habitats they occupy. UV-B radiation is a pervasive stressor that impacts these salamander populations throughout high elevation breeding sites, but is not necessarily the only factor influencing their life history. Hoffman *et al.* (2003) examined the distribution of long-toed salamanders at high elevations in Mt. Rainier National Park with respect to habitat characteristics and the presence/absence of a competitor, the Northwestern salamander (*Ambystoma gracile*). The authors found that long-toed salamanders can inhabit a diversity of ponds at high elevation, but within ponds larvae are often restricted in their microhabitat use by the presence of competitive and predatory larval *A. gracile*. Thus, complex patterns of environmental factors and biotic interactions can directly influence the distribution and persistence of long-toed salamanders at high elevation breeding sites.

While it appears that modification of oviposition site by breeding females at high elevations is adequate to reduce UV-B exposure to embryos, it is possible that other protective mechanisms are at play. Biological features inherent in the eggs, such as jelly coat, melanin, and egg thickness, are important variables that alter an embryo's *in situ* exposure to UV-B radiation (Grant and Licht 1995). These features play an important role in determining the amount of damaging UV-B that reaches the embryo (Salthe 1963; Smith *et al.* 2002). Grant and Licht (1995) investigated the capability of the jelly coat to

reduce UV-B transmission in the egg masses of 4 amphibian species from Southern Ontario, Canada, including the spotted salamander (*Ambystoma maculatum*). The authors suggested that the jelly coats, coupled with photorepair mechanisms and melanin pigmentation, afforded protection against UV-B radiation and together increased egg survivorship under various UV treatments. They found that the jelly coat surrounding spotted salamander egg masses reduced transmittance up to 77%. Similarly, Ovaska *et al.* (1997) examined jelly absorbance in Pacific chorus frogs (*Pseudacris regilla*) and Northern red-legged frogs (*Rana aurora*) and found that both species had similar patterns of absorption, but the jelly of *H. regilla* was up to two times more absorbent in the UV-B range. This difference was hypothesized to have contributed to the greater hatching success of *H. regilla* under the enhanced UV-B treatment. These studies indicate that the jelly envelope refracts light differently across amphibian taxa, and that the jelly properties exhibit unique UV-B transmission patterns.

It has been hypothesized that absorbance of the jelly at 320 nm of ultraviolet light increases as photolyase activity decreases. Therefore, at biologically relevant wavelengths, a species with less photoenzymatic repair capabilities will have jelly that absorbs more radiation in the UV-B range (Smith *et al.* 2002). We did not address the jelly absorbance hypothesis in this study, but it is likely that improved jelly absorbance in embryos at high elevation would significantly reduce UV-B penetration of the egg casing. This added defense mechanism may be another factor informing the relatively low photolyase activity in long-toed salamanders, as it appears to be an efficient UV-B protective strategy in other amphibian species (Grant and Licht 1995; Ovaska *et al.* 1997). Investigating the jelly absorbance hypothesis would have provided further insight into the potential for correlated trait responses within high elevation populations of the long-toed salamander. While there is movement towards research on paired trait responses to multiple environmental stressors (e.g. Garcia *et al.* 2009), there has yet to be studies conducted on multiple (more than 2) correlated trait responses to environmental stress in amphibian species.

Observations of long-toed salamander clutch composition offer more insight into modifications of life history characters at high elevations. Instead of laying eggs in jelly masses, all egg clutches observed at high elevation were laid singly. The direct

association of this oviposition strategy with UV-B radiation is not as apparent as the other behavioral modifications examined in our study. Numerous other explanations for differences in egg composition were researched given that there was such an obvious divergence in this particular component of long-toed salamander life history.

Multiple costs and benefits can be associated with singly laid eggs versus eggs laid in a jelly mass. Egg masses can improve thermoregulation by accumulating heat within the mass and subsequently aiding development. However, oxygen concentration is typically lower due to higher metabolically active tissue and pH can become higher towards the end of embryonic development in egg masses (Wells 2007; Surova 2002). Given that colder waters at high elevation are less likely to become hypoxic and individuals have lower metabolic demands, we do not expect that laying eggs singly is a strategy employed to combat oxygen deprivation. Long-toed salamanders at high elevation lack the thermoregulatory properties of a protective jelly mass, but this may be buffered by their selection for oviposition sites beneath cover objects and sediment. Thus, the benefits of thermoregulation may be mirrored under these abiotic substrates, which would be a useful strategy considering that temperatures during embryonic development are near 0°C and late spring freezes are frequent(personal observation).

Egg masses also offer insular eggs more protection from predation and other environmental stressors. Although long-toed salamanders are known to select breeding sites that are absent of fish predators, other amphibians and large macroinvertebrate predators may be present (Jones *et al.* 2005). By laying eggs in masses, the likelihood of losing eggs to a single predation event is much higher than eggs that are deposited singly. For long-toed salamanders at high elevation, laying eggs singly underneath cover objects is is a mechanism to reduce UV-B exposure, but could also be a strategy to hedge bets against predation by scattering the eggs in low visibility regions and risking only those that are most exposed.

Another consequence of laying eggs in masses is the increase in susceptibility to disease transmission. For example, the water mold *Saprolegnia ferax* parasitizes aquatic amphibian embryos and can increase in prevalence when embryos are aggregated in masses (Kiesecker and Blasutein 1997). The egg-laying life history strategy of long-toed salamanders at high elevation may impede the spread of *S. ferax* (and other aquatic

pathogens) by reducing density and connectivity among individuals.

Different reproductive strategies often include trade-offs in number of eggs produced and the size of each egg. The trade-off being that a female can either invest heavily in each individual to enhance its competitive ability at a cost of reduction in numbers, or she can produce large numbers of offspring with less investment in each egg. We did not find a significant difference in number of salamander eggs per clutch between high and low elevation populations during our study. Anecdotal evidence from our survey indicates that eggs at high elevation were larger, but we did not take specific measurements for comparisons. We would expect that females at high elevation would maximize individual fitness of their embryos for the purposes of producing larger, more fully developed offspring. This pattern has been seen in other amphibian species at high elevations; cold environments with low productivity require amphibian larvae to survive for a considerable period of time on nutrients derived from their egg yolk until they reach a size that allows them to feed effectively (Nussbaum and Lehtinen 2003). Long-toed salamanders at high elevation are a good example of large larval body size and long larval development periods, with many individuals over-wintering for up to three years before metamorphosing, unlike their low elevation counterparts (Hoffman *et al.* 2003).

One of the main conclusions we can draw from this discussion of life history trade-offs is that UV-B avoidance through modification of oviposition behavior may be a secondary consequence of adaptations made for other purposes (e.g. predator avoidance, reduced disease transmission). In the absence of other prevalent selection pressures, populations at high elevations may have evolved a heightened physiological response to UV-B radiation that would have reduced the need to extensively modify oviposition behavior. Regardless of which came first, long-toed salamander oviposition behavior at high elevations significantly reduces UV-B exposure rates and simultaneously offers protection from other environmental stressors.

We originally hypothesized that long-toed salamanders would cooperatively employ an added physiological defense to UV-B radiation at high elevations, but results from the bacterial transformation assay revealed this was not the case. We determined that photolyase activity is relatively consistent across populations and elevations, regardless of UV-B radiation levels. While other (less efficient) mechanisms may be

present in salamander DNA that simultaneously repair UV-B damage, we do not anticipate these would be sufficient to result in significantly higher DNA repair capabilities in high elevation populations. Additionally, it appears that photolyase levels in this species may be lower by orders of magnitude than previously measured (see Blaustein *et al.* 1994). Differences in methodologies between our studies may be revealing a wider range of photolyase activity than expected, or basic differences in accuracy of detecting photolyase signal. Given that photolyase activity in long-toed salamanders is weak relative to other amphibian species, this variation in a low range signal is somewhat irrelevant when considering the extensive behavioral modifications employed by this species to combat UV-B exposure.

Selection for trait combinations, possibly in conjunction with frequency-dependent selection, is an important factor in maintaining genetic diversity within species populations (Brodie 1992; Endler 1995). These patterns may also reflect responses to geographical variation in selective factors such as predation intensity or environmental stress. In many cases, the response to these stressors can be plastic, wherein the pattern is different depending on the relative importance of a given stressor in a specific environment. Phenotypic plasticity is an important strategy for coping with changing environments and is a well-studied mechanism in amphibian species, which often experience a high degree of variability in the temperature, hydroperiod, and photoperiod of their breeding habitats (see Springate *et al.* 2011; Gervasi and Foufopoulos 2009; Hansson 2004; Denver *et al.* 1998). In this case, seemingly distinct phenotypes may be a result of fluctuating habitat conditions that are more fine-grained relative to the overall conditions experienced by a species (Levins 1968). Alternatively, local adaptation may be an appropriate explanation for phenotypic divergence in amphibian populations experiencing different evolutionary histories, or those that are geographically isolated (Van Buskirk 2009). For this study, we can only offer initial observations of differential phenotypes, as the degree of connectivity and gene flow among high and low elevations is largely unknown in this region. Future genetic analyses are necessary to differentiate among these explanations and determine the extent of divergence among high and low elevation populations of the long-toed salamander.

As climates and environments change, amphibian conservation will become an

increasingly difficult and important issue. Changes in climate variables are occurring alongside increasing UV-B radiation at high elevations and will continue to pose new challenges for amphibian species in these environments. However, many species are able to alter life history traits and thus may exhibit optimal strategies for resisting environmental stressors. For the long-toed salamander, the persistence of high elevation populations and their capacity to adapt life history strategies concordant with dynamic physical conditions are intimately connected. UV-B will likely remain a contributing factor to amphibian population declines at high elevations, thus understanding potential variation in population responses to UV-B will be important in conservation efforts.

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