AN ABSTRACT OF THE DISSERTATION OF

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Andrew R. Blaustein

Ultraviolet-B radiation (UV-B) has been suggested as a factor contributing to global amphibian population declines. While ambient UV-B levels damage the eggs and embryos of some amphibian species, few studies have addressed how UV-B affects other life history stages or sublethal responses. My dissertation focuses on (1) investigations of sublethal effects of UV-B exposure on growth and development in larval amphibians, (2) a preliminary examination of the hormonal stress response in larval amphibians exposed to UV-B and (3) investigations of defenses that amphibians may use to prevent UV-B damage. Delayed sublethal responses to UV-B were documented for red legged frogs (*Rana aurora*). Larvae exposed to ambient UV-B during embryonic development were smaller and less developed one month after hatching than larvae not exposed as embryos. Exposure of larval long-toed salamanders (*Ambystoma macrodactylum*) to UV-B in the laboratory resulted in sublethal effects on growth for larvae from mountain sites and in decreased survival for larvae from lower elevation. Mountain populations appear to be more resistant to the detrimental effects of UV-B exposure. However, although UV-B can have various sublethal effects on growth and development, I did not observe a hormonal stress response in larvae of four species. One potential mechanism of UV-B protection is skin darkening. Larval long-toed salamanders, Northwestern salamanders *(A. gracile)* and roughskin newts (*Taricha granulosa*) darken in response to UV-B exposure. I examined whether skin darkening had

implications for larval survival by manipulating skin color on black and white backgrounds during UV-B exposure. UV-B exposure resulted in reduced growth, regardless of background coloration, but there were no survivorship differences between the groups. In summary, my results suggest that even at current levels, UV-B may have impacts on amphibian life histories. While direct mortality in response to UV-B exposure may be observed for some species, there are many sublethal responses as well. These sublethal responses could be important for the long-term survival of amphibian populations.

Sublethal Effects of UV-B Radiation on Larval Amphibians

by

Lisa K. Belden

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Contribution of Authors

Erica L. Wildy contributed to the design and implementation of the laboratory portion of chapter two. For chapter five, Ignacio T. Moore assisted with data collection, completion of the radioimmunoassay and interpretation of results. The radioimmunoassay was performed in the laboratory of Robert T. Mason. Andrew R. Blaustein has served as my graduate advisor.

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Dedication

This dissertation is dedicated to my parents, Gwen and Jim Belden, for all their encouragement, love and support over the years, to my husband, Ignacio Moore, for being my partner in life, and to Spiros, Rya and Galgo, who put everything in perspective.

Sublethal Effects of UV-B Radiation on Larval Amphibians

Chapter 1. Introduction

Aquatic Systems and UV-B Radiation

Exposure to ultraviolet-B radiation (UV-B; 280- 320 nm) has been suggested as contributing to at least two major biodiversity crises in recent decades: bleaching events of coral reefs (e.g. Gleason and Wellington 1993, Shick et al. 1996, Lyons et al. 1998) and worldwide amphibian population declines (e.g. Blaustein et al. 1998, Alford and Richards 1999). Ultraviolet radiation has shorter wavelengths and more energy than light in the visible spectrum and is potentially damaging to biological systems. Ultraviolet radiation is subdivided into three bands: UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm). Recently, UV-B has received increasing attention from biologists because with stratospheric ozone depletion, the amount of energy in the UV-B range reaching the Earth's surface will increase (Tevini 1993, Hader et al. 1995, Hader 1997). UV-C radiation, which contains the most energy and is therefore the most damaging to biological systems, is absorbed by even small amounts of atmosphere, so that it does not reach the surface of the Earth (Molina and Molina 1986). Even with substantial ozone depletion UV-C is not expected to pose a problem for biological systems. UV-A has the least energy of the ultraviolet wavelengths and may be very important in many biological systems, potentially playing a role in cellular damage

as well as in photorepair of DNA damage caused by UV (Hearst 1995). However, UV-A is not predicted to increase with ozone depletion.

For aquatic organisms, increasing UV-B exposure can also be caused by climatic changes that alter hydrologic cycles. In some cases, increases in UV-B exposure due to climate change may be more substantial than increases due to ozone depletion (Schindler et al. 1996, Yan et al. 1996, Pienitz and Vincent 2000, Kiesecker et al. 2001). Although future increases in UV-B reaching the earth's surface may result in higher exposure for many organisms, ultraviolet light is not a new abiotic factor affecting living organisms (Sagan 1973, Caldwell 1979, Cockell 2000). Therefore, it is likely that over evolutionary time most organisms have developed defenses against potential UV light induced cellular damage.

The study of how UV-B affects aquatic systems is relatively recent. Previously, it was assumed that UV light could not penetrate far into the water column. However, it is now recognized that UV-B can penetrate into the water column to significant depths. For instance, in some marine systems, with relatively clear water, UV-B can penetrate up to 20 m (Kirk 1994). The amount of dissolved organic matter in the water column, which absorbs UV-B, strongly influences how far UV-B penetrates into the water column (Kirk 1994, Schindler et al. 1996, Yan et al. 1996). Therefore, UV-B penetration is greatest in clear marine waters, with little dissolved organic matter, and least in murky freshwater habitats, which are rich in dissolved organic matter.

The effects of UV-B exposure on aquatic organisms are varied, but there are documented negative effects on corals, aquatic insects, phytoplankton, zooplankton and fish, as well as amphibians (e.g. Shick et al. 1996, Arts and Rai 1997, Blaustein et al. 1998, Hader et al. 1998, Lyons et al. 1998, Beland et al. 1999). For instance, bleaching events in coral reefs occur when coral symbionts leave their hosts. These are likely a phenomena of warmer oceanic temperatures, possibly associated with El Niño events (Hoegh-Guldberg 1999). However, temperature alone does not explain all the variation in bleaching events and temperature may interact with increased penetration of UV radiation (Gleason and Wellington 1993, Shick et al. 1996, Hoegh-Guldberg 1999). Indeed, UV exposure has been linked to increased mortality, decreased skeletal growth and reduction of symbiont density in coral polyps (Shick et al. 1996).

Amphibian Declines and UV-B Radiation

Large losses of biodiversity, often due to habitat destruction, have been documented around the world in almost all classes of plants and animals (Lawton and May 1995, Vitousek et al. 1997). Though the exact number of species being lost is not known, it is estimated that the current rate of extinction, which is being caused by humans, is greater than any known in the last 100,000 years (Wilson 1992). In addition to actual extinction of species and a loss in the absolute numbers of organisms, these impacts have resulted in the reduction and disappearance of many organisms from their historic ranges.

As part of these losses, some amphibian species have disappeared completely (e.g. Crump et al. 1992, Richards et al. 1993), and many more appear to be undergoing population declines and range reductions (e.g. LaMarca and Reinthaler 1991, Crump et al. 1992, Fellers and Drost 1993, Richards et al. 1993, Blaustein et al. 1994a, Fisher and Shaffer 1996, Pounds et al. 1997). These amphibian population declines are global in nature (Alford and Richards 1999, Houlahan et al. 2000) and in some cases, amphibian losses appear to be more severe than losses in other taxa (Pounds et al. 1997).

Declines in amphibian populations also stand out due to the fact that many of them are occurring in areas that remain relatively undisturbed by humans, such as national parks and conservation areas. The global nature of amphibian population declines has led some scientists to suggest that they may be tied to larger global processes, such as stratospheric ozone depletion and global climate change (Blaustein et al. 1994a, Blaustein et al. 1998, Pounds et al. 1999, Kiesecker et al. 2001), perhaps in addition to more localized phenomena such as introduced species, disease, and chemical pollution (e.g. Blaustein et al. 1994b, Kiesecker and Blaustein 1995, Berger et al. 1998, Hatch and Burton 1998, Kiesecker and Blaustein 1998, Marco et al. 1999).

Increasing evidence suggests that there are likely multiple synergistic factors causing these declines, with different factors being critical at different locations. For example, red-legged frogs, *Rana aurora*, which have disappeared from much of their historic range in the Willamette Valley, Oregon, USA, are

likely being affected by habitat loss, introduced species (bullfrogs) and chemical pollution from agricultural areas (Kiesecker and Blaustein 1997, Kiesecker and Blaustein 1998, Marco et al. 1999). However, in Monteverde, Costa Rica, where a significant portion of the amphibian fauna has disappeared, climate change and disease appear to be the most important factors (Pounds et al. 1999).

Documented Effects of UV-B on Amphibians

Mortality is the most obvious direct effect that has been documented for amphibians exposed to UV-B and has been studied most intensively in the embryonic life history stage. There are also a variety of sublethal effects, including effects on growth, development and behavior of both larvae and adults. To my knowledge, no studies have addressed how UV-B exposure affects amphibian physiology, such as metabolic rates or hormonal responses. Below, I outline the major published findings regarding UV-B exposure of embryonic, larval and juvenile/adult amphibians.

Embryos

The majority of research done on amphibian sensitivity to UV-B has involved the embryonic life history stage. Most of these studies have focused on mortality as an endpoint. For species that lay their eggs in shallow water or at the surface of the water, embryos may receive relatively high doses of UV-B as behavioral avoidance is not possible. As would be expected for any potentially damaging abiotic factor, embryos of different amphibian species vary in their

sensitivity to UV-B, even within a given geographic location. Embryos of some species experience increased mortality in response to ambient exposure to UV-B, while others appear unaffected (see table in Blaustein et al. 1998, and Blaustein et al. 1999, Langhelle et al. 1999, Broomhall et al. 2000). For example, in Oregon, embryonic Cascades frogs, *Rana cascadae*, and western toads, *Bufo boreas*, are more sensitive to UV-B than Pacific treefrogs, *Hyla regilla*, at the same sites (Blaustein et al. 1994a). Differential species sensitivity of embryonic amphibians has also been documented in other regions of the world, including California (Anzalone et al. 1998), Spain (Lizana and Pedraza 1998), Sweden (Langhelle et al. 1999), and Australia (van de Mortel and Buttemer 1996, Broomhall et al. 2000).

Interactions of UV-B with other environmental factors, such as disease, can result in embryonic mortality for some species (Blaustein et al. 2001). In the Pacific Northwest, the combined presence of UV-B and the fungus, *Saprolegnia ferax*, results in higher mortality for western toad and Cascade frog embryos, than does either factor alone (Kiesecker and Blaustein 1995). This interaction is further influenced by water depth at the breeding sites, which can be affected by global climate patterns. In low water years, there is less water protecting the embryos from UV-B exposure and the result is higher mortality from *Saprolegnia* (Kiesecker et al. 2001). Some chemical contaminants may also interact synergistically with UV radiation. For example, the polycyclic aromatic hydrocarbon, fluoranthene, which is a common component of petroleum run-off

causes greater mortality of amphibian embryos when in the presence of UV radiation (Hatch and Burton 1998).

A few recent studies demonstrate that there can also be sublethal effects of UV-B exposure for embryonic amphibians. For example, embryonic exposure to UV-B can have lasting sublethal effects on growth for larvae, with exposed *Rana blairi* embryos resulting in smaller larvae (Smith et al. 2000a). This may be due to a decrease in size at hatching for UV-B exposed embryos, as was seen by Pahkala (2000) for embryos of the common frog, *Rana temporaria.*

Larvae

Fewer studies have examined the effects of UV-B on larval amphibians, although mortality has been observed for some species. For example, Nagl and Hofer (1997) found that UV-B was lethal to Alpine newt larvae, *Triturus alpestris*, in the laboratory. However, in the field high levels of dissolved organic matter in the ponds likely shield these larvae, and no UV-B induced mortality was observed. UV-B induced mortality has also been observed in larvae of another European newt, *T. cristatus*, but in the same study larvae of four other European amphibian species were not affected (Langhelle et al. 1999). Larval mortality caused by UV-B enhanced 23-30% above current ambient levels has been documented for *H. regilla* and red-legged frogs, *Rana aurora* in British Columbia (Ovaska et al. 1997, Ovaska et al. 1998).

In Oregon, adverse effects of UV, including mortality and deformities, have been demonstrated in the laboratory for larval and postmetamorphic Cascades frogs, *Rana cascadae*, and Pacific treefrogs, *Hyla regilla* (Hays et al. 1996). Developmental abnormalities were also documented in early laboratory studies of western toad larvae, *Bufo boreas*, exposed to levels of UV-B slightly above ambient levels (Worrest and Kimeldorf 1976).

A few studies have examined sublethal effects of UV-B on larval amphibians. *Xenopus laevis* larvae exposed to UV-B in the laboratory grew less with increasing doses of UV-B (Bruggeman et al. 1998). Nagl and Hofer (1997) found that UV-B exposure resulted in erratic swimming behavior for Alpine newt larvae, *Triturus alpestris,* and found that in choice experiments, this type of swimming resulted in larvae moving to areas with no UV. Behavioral avoidance of UV-B has also been observed in tadpoles of two Australian frog species, *Littoria aurea* and *L. peronii* (van de Mortel and Buttemer 1998). The effects of UV-B on antipredator behavior of larval amphibians has been examined in larval roughskin newts, *Taricha granulosa*, and Cascades frogs, *Rana cascadae* (Kats et al. 2000). *Taricha granulosa* larvae spent more time in shelter when exposed to cues from adult conspecifics, regardless of UV-B exposure. *Rana cascadae* tadpoles spent less time avoiding newts after UV-B exposure, although this difference was not statistically significant. Exposed tadpoles were significantly more active.

Juveniles and adults

The juvenile and adult amphibian life history stages are the least well studied in terms of responses to UV-B radiation. This is likely due to several factors, including the difficulty in obtaining sufficient sample sizes for experiments. To my knowledge, only a single study has investigated survival of juvenile/adult amphibians exposed to UV-B without prior exposure as embryos or larvae. Grant and Licht (1995) found that recently metamorphosed *Bufo americanus* were more resistant to high intensity UV-B exposure than were *Rana clamitans* and *R. sylvatica*, which all died when exposed. A few studies have examined newly metamorphosed individuals following larval exposure to UV-B. For example, developmental abnormalities have been seen in some species from the Pacific Northwest following metamorphosis (Worrest and Kimeldorf 1976, Hays et al. 1996), while "ecologically relevant" doses of UV-B in the laboratory did not affect time to or mass at metamorphosis for several species from Ontario, Canada (Grant and Licht 1995).

A few sublethal effects of UV-B exposure on juvenile/adult amphibians have also been documented, including effects on antipredator behavior and activity level. For example juvenile western toads, *Bufo boreas*, exposed to UV-B are less likely to avoid conspecific alarm cues than non-exposed juveniles (Kats et al. 2000) and adult roughskin newts, *Taricha granulosa*, that are exposed to UV-B are more active than non-exposed individuals (Blaustein et al. 2000). In addition, Fite et al. (1998) have documented retinal damage in adult Cascades frogs, *Rana cascadae*,

from the field that is comparable to retinal damage from leopard frogs, *Rana pipiens*, that have been experimentally light damaged in the laboratory. The implications of this are that long-term exposure to solar radiation may be damaging the eyesight of adult amphibians in the wild, which could alter their ability to catch prey and locate mates.

Potential Amphibian Defenses Against UV-B Radiation

To cope with potentially dangerous UV-B radiation animals can either prevent damage from occurring or repair damage once it occurs (Epel et al. 1999). The repair mechanisms involved in counteracting UV-B induced cellular damage to amphibian embryos have received some attention. The major damage induced to DNA by UV light is the formation of cyclobutane pyrimidine dimers, or CPDs. Formation of these dimers inhibits proper transcription and translation and therefore can result in cell death (Sancar and Tang 1993, Hearst 1995). These often lethal photoproducts can be removed by one of two known processes. The first is excision repair, which tends to be more common across taxa but can also be energetically costly if more than a single nucleotide requires repair (Sancar and Tang 1993). The second method of repair of UV-induced damage involves photoreactivation of the enzyme photolyase. Photolyase uses photons of near UV light (350-500 nm) to break the cyclobutane ring and return the pyrimidines to their

unaltered state (Hearst 1995). In the absence of light, photolyase may still facilitate repair by marking the site of damage that requires excision repair (Ozer et al. 1995).

Research on embryonic amphibians in Oregon, USA has demonstrated a strong correlation between photolyase activity and resistance to UV-B exposure (Blaustein et al. 1994a, Blaustein et al. 1996, Hays et al. 1996, Blaustein et al. 1999). A similar trend has been seen for three Australian tree frogs, although the correlation is not as strong as in the Pacific Northwest, USA (van de Mortel et al. 1998). In addition, recent research has demonstrated that photolyase production can be induced in wood frog, *Rana sylvatica*, embryos with exposure to UV-B (Smith et al. 2000b). This highlights the importance of this enzyme in protection from UV-B and implies that the individuals in high UV environments may be capable of upregulating the synthesis of photolyase.

Less research has been completed on how amphibians may prevent UV-B damage from occurring. Damage by UV-B radiation can be prevented behaviorally by spatially or temporally avoiding exposure. Thus adult amphibians that are nocturnal or live under forest debris or in closed-canopy forest effectively avoid UV-B exposure. Amphibian larvae, which are able to move in response to environmental stimuli, can also potentially avoid exposure (Nagl and Hofer 1997, van de Mortel and Buttemer 1998). In addition, females choosing to lay eggs in low UV environments, such as under logs or in deep water, limit the exposure of their eggs and embryos to UV-B. For species that lay their eggs in clear, shallow water,

the jelly matrix surrounding the eggs may prevent UV damage by absorbing damaging wavelengths of light before they reach the embryo. This hypothesis has some support, as the jellies of several species do appear to absorb wavelengths in the UV-B range (Grant and Licht 1995, Ovaska et al. 1997).

Physiological and morphological mechanisms may also limit UV-B exposure. For example, pigments in the skin, such as melanin, may provide some protection from UV-induced DNA damage (Cockell and Knowland 1999). The exact roles of various pigments in human skin that provide protection from UV damage are still being identified, as are the mechanisms involved in the process (Prota 1992). Recently, the debate has been reopened as to how various pigments respond to UV light and whether some pigments may actually contribute to the development of skin cancer (Wu 1999). However, in general, mammals with darker skin are less prone to UV-induced skin damage than those with lighter skin (Kollias et al. 1991, Barker et al. 1995). A recent hypothesis (Jablonski 1998), suggests that melanin production may protect developing amphibian embryos from neural tube defects by acting as a natural sunscreen. She suggests that melanin is a relatively inexpensive way to prevent critical metabolites, such as folate, from being degraded by UV light during development. Other evidence suggests that some amphibians may darken in response to UV-B irradiance (adult *Rana sylvatica*, Roth 1996; embryonic and larval *Hyla versicolor* and *Xenopus laevis*, Zaga 1998; larval *Hyla arborea*, Langhelle 1999). Whether this response effectively protects the individuals from UV-B damage is not known.

Heat shock proteins (HSPs), which are named for the protection they provide from temperature stress, may also play a role in protecting cells from UV-B damage (Trautinger et al. 1996, Feder and Hofmann 1999). HSPs are a large group of proteins that serve as molecular chaperones, interacting with other proteins to ensure proper structure and function of the proteins. HSPs are noted for their role in preventing the denaturation of proteins during exposure to environmental stress, especially temperature stress, but they may also be important in preventing damage from other stressors. For instance, water stress in plants and oxygen stress in brine shrimp, can result in increasing expression of HSPs (Feder and Hofmann 1999). Less is known about their role in preventing UV-B damage, but expression of at least some of these proteins is upregulated with UV-B exposure in mammals (Trautinger et al. 1996). No research has been completed on HSPs and the amphibian response to UV-B.

Thesis Organization

My dissertation has focused on the sublethal effects that UV-B exposure can have on larval amphibians, including effects on growth and development, and hormonal responses to exposure. In addition, I have examined two potential ways for larval amphibians to protect themselves from damage, namely through behavioral avoidance and the use of melanin pigmentation.

Much of the previous work investigating the effects of UV-B exposure on amphibians has been completed with embryos and has mainly examined mortality

as an endpoint. In Chapter 2, I address whether there can be sublethal effects of embryonic exposure for larval amphibians. In that study, I examined that question using embryos of the red-legged frog, *Rana aurora*, a species for which no increased mortality is observed with UV-B exposure. However, red-legged frogs breed in winter on the coast and due to low water temperatures at that time of year, embryonic development can be prolonged, thus potentially exposing embryos to UV-B for an extended period.

Blaustein (1997) found that embryonic long-toed salamanders, *Ambystoma macrodactylum*, were very sensitive to ambient UV-B exposure, with almost complete mortality of exposed embryos. However, I observed larval long-toed salamanders at the same site that were in only about 5 cm of water, fully exposed to UV-B, without obvious detrimental effects. Chapters 3, 4 and 6 represent studies I completed on larval long-toed salamander sensitivity to UV-B, including field transects to measure UV-B exposure (Chapter 3), an examination of possible UV-B avoidance behavior (Chapter 3), sublethal effects on growth and differential survivorship between populations at different elevations (Chapters 3 and 4), the effects of UV-B on food consumption (Chapter 4) and an investigation of whether skin darkening in response to UV-B might provide some protection from UV-B induced mortality (Chapter 6).

There has been a lack of research on the physiological responses of amphibians to UV-B. These types of studies could provide important information regarding the mechanisms involved in the growth, developmental and behavioral

responses that have been documented. Chapter 5 represents an initial attempt to examine a potentially important hormonal response to UV-B exposure. Vertebrates respond to stressful stimuli with increases in plasma glucocorticoid hormones. Although these hormones act to promote survival in the short-term, long-term elevation can have detrimental effects, including suppression of the immune response, decreased growth and suppression of reproduction, all of which could be important for amphibians. In Chapter 5, I investigated whether exposure to UV-B in larvae of four species of amphibians resulted in increasing circulating levels of corticosterone (the main glucocorticoid in amphibians).

Chapter 2

Exposure of Red-legged Frog Embryos to Ambient UV-B Radiation in the Field Negatively Affects Larval Growth and Development

Lisa K. Belden and Andrew R. Blaustein

Abstract

Exposure to ultraviolet-B radiation (UV-B; 280 - 320 nm) has a wide array of effects on aquatic organisms, including amphibians, and has been implicated as a possible factor contributing to global declines and range reductions in amphibian populations. Both lethal and sublethal effects of UV-B exposure have been documented for many amphibian species at various life history stages. Some species, such as red legged frogs, *Rana aurora,* appear to be resistant to current ambient levels of UV-B, at least at the embryonic and larval stages, despite the fact that they have experienced range reductions in the Willamette Valley of Oregon, USA. However, UV-B is lethal to embryonic and larval *R. aurora* at levels slightly above those currently experienced during development. Therefore, we predicted that exposure of embryos to ambient UV-B radiation would result in sublethal effects on larval growth and development. We tested this by exposing *R. aurora* embryos to ambient UV-B in the field and then raising individuals in the laboratory for one month after hatching. Larvae that were exposed to UV-B as embryos were smaller and less developed than the non-exposed individuals one month post-hatching. These types of sublethal effects of UV-B exposure indicate that current levels of UV-B could already be influencing amphibian development.

Introduction

Ultraviolet-B radiation (UV-B; 280-320 nm) as an important abiotic factor for both terrestrial and aquatic organisms has received more attention with

predictions of increasing UV-B at the Earth's surface due to stratospheric ozone depletion (Tevini 1993, Hader et al. 1995, Hader 1997). Indeed, exposure to UV-B has been suggested as contributing to at least two major biodiversity crises in recent decades: bleaching events of coral reefs (e.g. Gleason and Wellington 1993, Shick et al. 1996, Lyons et al. 1998) and worldwide amphibian population declines (e.g. Blaustein et al. 1998, Alford and Richards 1999). For amphibians, this has prompted research on the effects of UV-B exposure on embryonic and larval amphibians (Blaustein et al. 1998). As would be expected for any abiotic factor, tests on embryonic amphibians demonstrate that species vary in their sensitivity to UV-B. Even within a given geographic location, embryos of some species experience increased mortality in response to UV-B exposure, while others appear unaffected (e.g. Blaustein et al. 1994, Anzalone et al. 1998, Lizana and Pedraza 1998, Langhelle et al. 1999, Broomhall et al. 2000).

In Oregon, USA, red-legged frogs, *Rana aurora,* have disappeared over much of their historic range (see Kiesecker and Blaustein 1998), but *R. aurora* embryos and larvae do not experience increased mortality in the presence of ambient UV-B (Blaustein et al. 1996, Ovaska et al. 1997). However, Ovaska et al. (1997) observed decreased embryonic and larval survivorship at slightly enhanced UV-B levels. Mortality at enhanced levels implies that there is a specific physiological UV-B tolerance limit for this species. Even though current ambient levels are not sufficient to induce mortality, it could be energetically costly for *R. aurora* embryos exposed to UV-B to resist or repair potential cellular damage.

Because *R. aurora* egg masses are often laid at the surface of the water in direct sunlight, the embryos may receive relatively high doses of UV-B, compared to larvae or adults which may move away from sunlight. In addition, *R. aurora* embryonic development tends to be prolonged because *R. aurora* breeds in winter at the Oregon coast, when water temperatures are low. (Early *R. aurora* embryos have the lowest known temperature tolerance of the North American ranid frogs (4-21°C), Licht 1971). Thus, embryos can potentially be exposed to higher cumulative doses of UV-B than species with short times to hatching.

As UV-B is lethal to embryonic and larval *R. aurora* at levels slightly above ambient (Ovaska et al. 1997), we hypothesized that exposure of embryos to ambient levels of UV-B radiation would result in larvae that were less developed than the non-exposed individuals. These types of sublethal effects, which cross life history stages, have not been well investigated and could impact the long-term survival of many amphibian populations. We tested our hypothesis by exposing *R. aurora* embryos to ambient UV-B radiation in the field and then rearing individual tadpoles after hatching in the laboratory for one month.

Materials and Methods

In December 1999, we collected six fresh *R. aurora* egg masses from a pond 10 km S. of Waldport, Oregon. Later that day, we set up 8 containers in the laboratory with 5 eggs from each of 6 of the masses (30 eggs/container). All eggs were at Gosner stages 2-6 (Gosner 1960). The containers were left in the

laboratory overnight and the following morning were transported to outdoor mesocosms located in an open field at the Salmon Disease Laboratory of Oregon State University. Mesocosms consisted of 8 large plastic tubs (110 cm diameter, 25 cm deep) filled with well water. Within each mesocosm, we placed the eggs in a wood framed enclosure (80 cm x 80 cm x 10 cm (depth)) with mesh sides and bottom. Four of these were randomly assigned to a UV-B blocking regime (mylar filter) and the other four received an acetate filter, which allows approximately 80% of the UV-B to pass (Blaustein et al. 1994). Mylar and acetate filters were placed over the appropriate wooden enclosures and were stapled to the edges of the frame.

We measured temperatures in all 8 enclosures at noon on 5 separate days during the experiment. In addition, temperature data loggers (Hobo loggers, Onset Computer Corporation, Bourne, MA, USA) that recorded water temperature every hour for the duration of the experiment were placed in a single acetate and a single mylar enclosure. Eggs were checked for mortality and were counted every 1-2 days, and always following freezing night time temperatures. If ice was present on the enclosures, it was broken up and removed. UV-B readings were taken at the site between 1200-1300 h on 15 different days with differing weather conditions to gain an estimate of the range of exposure these larvae were receiving. All measurements of UV-B were done using a hand-held Solar Light meter with a UV-B probe (model PMA2100; Solar Light Co., Philadelphia, PA, USA).

After 6 weeks of exposure, when all embryos were nearing hatching (Gosner stages 19-21, Gosner 1960), all of them were collected and returned to the laboratory. They were set-up by mesocosm (8 groups) in plastic tubs and the total number hatched was recorded each day. All embryos had hatched (or were dead) within 5 days of being brought into the laboratory. Three days after they were all hatched, 15 individuals from each group were randomly selected and placed in individual 550 mL plastic containers filled with 350 mL of dechlorinated tap water. Containers were placed in random order on a lab bench in a 10 x 12 container grid. Every day, we removed waste products and uneaten food from all 120 containers and changed 1/2 of the water. Tadpoles were fed a 3:1 mixture of ground rabbit chow:Tetramin fish food daily, so that food was always available to them. After two weeks, all containers were completely cleaned and refilled with dechlorinated tap water. After one month, we recorded mass and developmental stage (per Gosner 1960) for all 120 individuals.

Analysis was done using MANOVA with the multivariate response of stage and mass on UV-B treatment. We used means from the original eight rearing groups in our analysis. Mean temperatures in mylar versus acetate enclosures on our five days of temperature recording were compared using a Paired t-test.

Results

Survival to hatching was high in all treatments (mylar= 93.3%, 96.7%, 100%, 100%; acetate=90%, 93.3%, 96.7%, 100%) with no difference between the

two groups (student's t-test; P = 0.388). There was no mortality during the laboratory portion of the study. However, after one month, tadpoles that were not exposed to UV-B in the field as embryos were larger and more developed than individuals that were exposed as embryos (overall MANOVA for UV-B treatment effects, p = 0.04; Table 2.1, Fig. 2.1). UV-B at the field site ranged from 0 $\mu W/cm^2$ while it was actively raining and overcast to 1.28- 1.55 $\mu W/cm^2$ on clear sunny days. There were no temperature differences between the mylar and acetate enclosures (Paired t-test; p=0.492; Fig. 2.2).

Table 2.1. Results of MANOVA for overall effects of embryonic UV-B exposure on stage and mass of *Rana aurora* tadpoles one month after hatching, and ANOVAs for each response variable.

	F	df	P
MANOVA			
constant	310175.3	2, 5	<0.001
UV treatment	6.546	2, 5	0.04
ANOVAs			
Stage	15.074	1, 6	0.008
Mass	10.352	1, 6	0.018

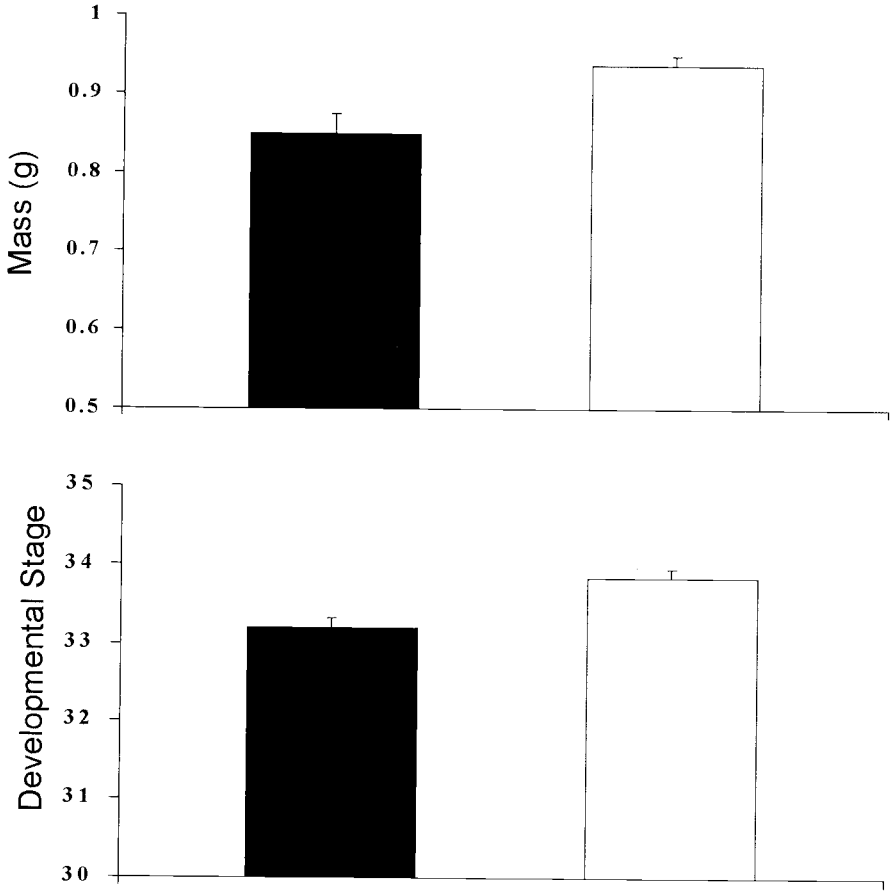

Figure 2.1. Mean (± SE) mass (g) and developmental stage (Gosner 1960) of *Rana aurora* larvae one month after embryonic UV-B exposure in the field. Black bars represent exposed larvae. White bars represent larvae that were shielded from UV-B as embryos.

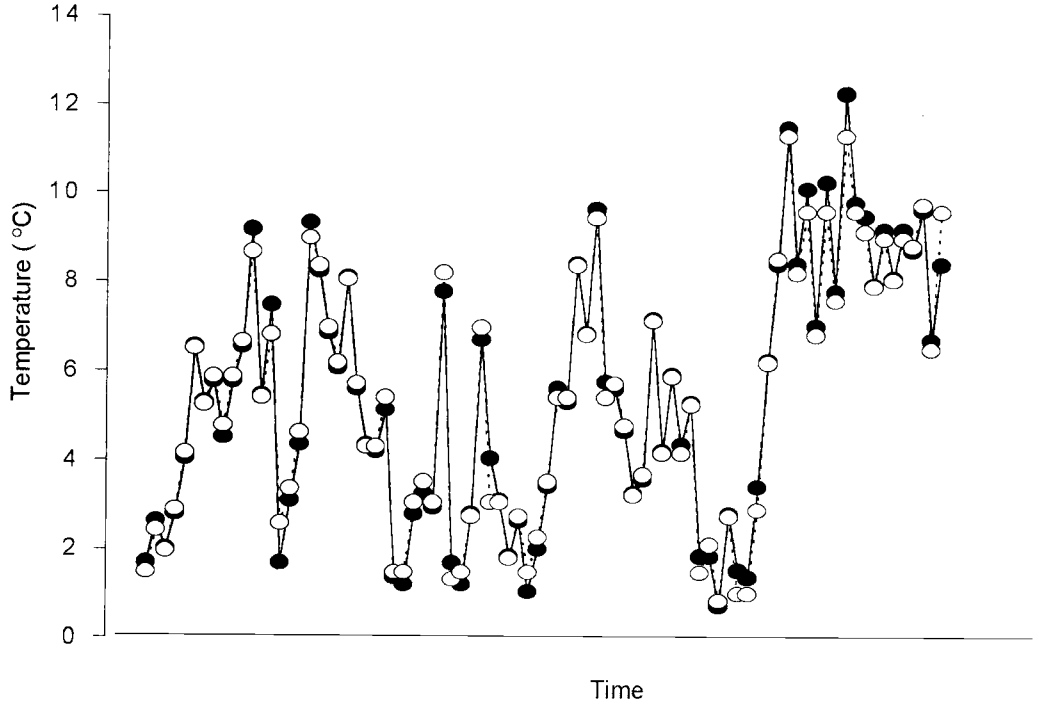

Figure 2.2. Temperatures (°C) over the course of the field exposure for a single mylar (•) and acetate (o) enclosure. Dots represent temperatures every 12 hours, starting at midnight of the first day. Statistical analysis for temperature was not performed on these data, but on means for the 4 acetate and 4 mylar enclosures taken at noon on five different days.

Discussion

Although *R. aurora* embryos have relatively high levels of the photorepair enzyme, photolyase (Blaustein et al. 1996), and do not experience greater mortality when exposed to ambient UV-B (Blaustein et al. 1996, Ovaska et al. 1997), our results suggest that there may be some energetic cost associated with UV-B exposure. In addition, our study demonstrates that UV-B exposure of embryos can

have lasting effects on the larvae, at least up to one month post-hatching. Similar results have recently been documented for plains leopard frogs, *Rana blairi* (Smith et al. 2000). Even though larvae may be able to behaviorally avoid UV-B, individuals exposed as embryos may already be at a disadvantage by the time they are able to escape from high UV-B environments. Indeed, size and rate of growth can be very important for larval anurans. Larger tadpoles may be better competitors (e.g. Travis 1980), may be more likely to attain the size threshold necessary for metamorphosis prior to pond drying (e.g. Wilbur and Collins 1973, Morey and Reznick 2000) and may be better able to avoid or ignore gape-limited predators (e.g. Puttlitz et al. 1999, Eklov 2000). In addition, larger larvae generally become larger metamorphic anurans which can have positive consequences for adult fitness (e.g. Smith 1987, Bervin 1990).

Other studies have documented growth effects on amphibians exposed to UV-B (e.g. Belden et al. 2000, Pahkala et al. 2000), but few have examined the effects of embryonic exposure on later stages. However, it is not surprising that the embryonic environment can have an influence on individuals at later life stages. This has been demonstrated for many animal groups, including mammals (e.g. Anisman et al. 1998), fish (e.g. McCormick 1998), reptiles (e.g. Shine et al. 1997) and amphibians (Watkins 2000).

In addition, various factors, such as the presence of predator cues in the environment (Sih and Moore 1993, Warkentin 1995), can alter the time to and size at hatching of embryonic amphibians. Changes in the size at hatching is likely to

have effects similar to those that we observed. It may be the case that the changes we observed were already present at hatching and were still apparent one month later in the larvae. As amphibian embryos are generally exposed directly to the environment during development and lack a protective shell, there is a good chance that many abiotic factors could have a major influence on developmental traits.

As we have demonstrated, regulating or avoiding exposure to biologically damaging UV-B radiation can be important for amphibians. This is also true for other aquatic organisms, and may become increasingly important with predictions of escalating UV-B levels at the Earth's surface due to stratospheric ozone depletion. However, in addition to ozone depletion, which will result in increases in UV-B in both terrestrial and aquatic environments, there are other factors that will alter the levels of UV-B exposure for aquatic organisms. For example, acidification of lakes and ponds results in decreased dissolved organic carbons in the water and therefore increased penetrance of UV-B in the water column (Schindler et al. 1996, Yan et al. 1996). Also, changes in hydrologic cycles that may occur with global climate change can be expected to alter water depth and availability (e.g. Schindler et al. 1996, Yan et al. 1996, Pounds et al. 1999), which could increase the UV-B exposure received by aquatic organisms. Factors such as these may be as important as ozone depletion for regulating UV-B exposure of aquatic organisms in the future (e.g. Schindler et al. 1996, Pienitz and Vincent 2000).

However, our study demonstrates that even at current levels, UV-B can influence amphibian development and is potentially already shaping life histories of aquatic organisms.

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Chapter 3

Growth, Survival and Behaviour of Larval Long-toed Salamanders (*Ambystoma macrodactylum*) Exposed to Ambient Levels of UV-B Radiation

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Abstract

Mortality is the most extreme effect of ultraviolet-B radiation (UV-B; 280-315 nm) on living organisms, but sublethal effects of UV-B may also be important. Moreover, there may be population differences in response to UV-B, but this aspect has not been well explored for animal populations. Amphibians have been a model system for studying the detrimental effects of UV-B. However, previous research on the effects of UV-B on amphibians has mainly focused on embryos. Few studies have investigated how UV-B affects larvae. We examined potential sublethal effects of UV-B on the long-toed salamander, *Ambystoma macrodactylum* from two different populations. Observational data from field transects indicated that larvae are potentially exposed to UV-B in their natural habitat. Choice tests indicated that larvae select shaded regions more often than those in the sun, but do not directly distinguish between regions with high and low UV-B. Laboratory experiments indicated a survivorship difference between individuals from low and high elevation sites. When exposed to relatively low levels of UV-B individuals from low elevation sites experienced higher mortality than controls (no UV-B). There were no differences in mortality between UV-exposed and non-exposed larvae from the high elevation population. Although mortality of UV-B exposed larvae was not significantly different from controls in the high elevation population, sublethal effects on growth were observed. Individuals from the high elevation site grew significantly less when exposed to UV-B than individuals shielded from UV-B. Our study demonstrates that larval *A. macrodactylum* are

exposed to UV-B in nature, that UV-B exposure can cause mortality as well as having sublethal effects on growth and that there are potential population differences in sensitivity to UV-B radiation.

Introduction

Increases in ultraviolet-B radiation (UV-B; 280-315 nm) have been suggested as a factor contributing to population declines and range reductions of some amphibians (Blaustein et al. 1998). Thus several recent studies have examined the sensitivity of developing amphibian embryos to UV-B radiation (reviewed by Blaustein et al. 1998). Ambient levels of UV-B radiation damage the eggs and embryos of some amphibian species, while having little apparent effect on others (e.g. Blaustein et al. 1994, Blaustein et al. 1995, Kiesecker and Blaustein 1995, Blaustein et al. 1996, van de Mortel and Buttemer 1996, Blaustein et al. 1997, Broomhall 1997, Ovaska et al. 1997, Anzalone et al. 1998, Corn 1998, Lizana and Pedraza 1998).

Few studies have examined effects of UV-B on larval amphibians. Early laboratory studies of Western toads (*Bufo boreas*) demonstrated developmental abnormalities in tadpoles exposed to levels of UV-B slightly above ambient levels (Worrest and Kimeldorf 1976). Ovaska et al., (1997) found no effects on larval survivorship in the Pacific treefrog (*Hyla regilla*) and the red-legged frog (*Rana aurora*) exposed to ambient levels of UV-B. Nagl & Hofer (1997) found mortality effects on Alpine newt (*Triturus alpestris*) larvae in the laboratory, but not in the

field. Adverse effects of UV-B, including mortality and deformities, on larval and postmetamorphic Cascades frogs (*Rana cascadae)* and Pacific treefrogs (*H. regilla*) have been experimentally demonstrated in the laboratory (Hays et al. 1996).

Although exposure to UV-B may result in reduced survivorship and developmental abnormalities that may lead to death, sublethal effects may also be important. For instance, individual growth rates may be reduced in the presence of UV-B radiation due to the cost of repairing cellular damage, producing protective pigments or behaviourally avoiding regions with sunlight and warm temperatures which also have high levels of harmful UV-B radiation. Reduced larval growth for amphibians can have direct consequences for adult fitness (Smith 1987, Semlitsch et al. 1988, Bervin 1990). Therefore, any effect that UV-B has on larval growth could impact both individual and population level processes.

Unlike embryos, larval amphibians are able to move in response to environmental stimuli. This ability allows for behavioural responses which could have drastic impacts on both immediate survival and long-term fitness. In addition, some amphibians possess ultraviolet photoreceptors in their eyes (Deutschlander and Phillips 1995) that may allow them to sense regions of high UV and alter their activity patterns accordingly. Alternatively, larvae might be able to indirectly avoid UV-B by selecting microhabitats on the basis of other factors which influence UV exposure, such as selecting deeper areas in lakes, regions in submerged vegetation, or shade.

In the Pacific Northwest (USA), the long-toed salamander, *Ambystoma macrodactylum*, appears to be one of the most UV-B sensitive species. Embryos of this species from the Cascade Mountains of Oregon experienced deformities and high rates of mortality when exposed to ambient levels of UV-B in the field (Blaustein et al. 1997), but larval sensitivity to UV-B has not been examined in this species. This study addresses three questions: 1) are larval long-toed salamanders exposed to UV-B during development in nature? (observational field transects) 2) do larval long-toed salamanders avoid UV-B exposure? (field experiment) and 3) what are the effects of UV-B exposure on larval growth? (laboratory experiment).

Materials and Methods

Study species

Long-toed salamanders are found in a wide variety of habitats in the Pacific Northwest and breed in both permanent and ephemeral bodies of water. At low elevations, breeding may begin in October, while animals at montane sites in the Cascade Mountains generally breed in May (Nussbaum et al. 1983). Eggs are laid singly or in clutches of 5-100 eggs, and are often attached to floating vegetation or laid beneath rocks (Nussbaum et al. 1983). In our growth experiments, we examined two populations of *A. macrodactylum*: *A. m. macrodactylum* from the Willamette Valley and *A.m. columbianum* from the Cascade Mountains. Most of the characteristics used to differentiate these subspecies are pigmentation patterns

and morphometric comparisons of adults; no consistent differences have been reported for the larvae of these two subspecies (Ferguson 1961). However, all of the subspecies are known to be highly variable in rates of growth and development, depending on the habitat type and altitude of the population (Kezer and Farner 1955, Ferguson 1961).

Field sites

We used a single site in the Willamette Valley and two sites in the Cascade Mountains of Oregon. The valley site was an ephemeral pond 1 km northeast of Corvallis (Benton County), OR, USA (elevation, 75 m). *Ambystoma macrodactylum* from this population were used to assess the effects of UV-B radiation on growth. The two sites in the Cascade Mountains are both ephemeral ponds 24 km south of Sisters (Deschutes County), OR, USA in the Three Creeks Basin. At Susan's pond (elevation 1939 m), we conducted microhabitat choice tests. At Eric's Pond (1 km south of Susan's pond, elevation 2005 m), we used transects to assess larval exposure to ambient UV-B radiation and collected larvae for growth studies.

Growth in the laboratory

Larval *A. macrodactylum* from two distinct populations were used to examine the effects of UV-B on growth. Experiments were conducted when eggs/larvae were available from natural oviposition sites. The first experiment

examined a population from the Willamette Valley of Oregon. Eggs were randomly collected from the valley pond in February, 1998 and returned to the laboratory. After hatching, larvae were raised in the laboratory for several months prior to being tested (mean mass (g) at testing ± SD= 0.36 ± 0.12). In August, 1998, we completed a second experiment with montane larvae collected and returned to the laboratory from Eric's Pond in the Cascade Mountains. Testing for the montane population began three days after larvae were collected (mean mass (g) at testing ± SD= 1.14 ± 0.18).

For each of the two experiments (valley and mountain populations), 28 larvae were randomly selected, weighed and placed in individual 800 ml plastic cups filled with 400 ml of dechlorinated tap water. Fourteen of these were randomly assigned to a UV-B exposed treatment (acetate filter) while the remaining 14 were shielded from UV-B (mylar filter). The mylar filter blocks 100% of UV-B radiation and the acetate filter, which serves as a control for using a filter, transmits 80% of the UV-B radiation (Blaustein et al. 1994). Filters were placed above the cups such that they never came in contact with the water in the cups or with the larvae. All 28 containers were then randomly placed under UV-B enhanced full spectrum lighting to simulate low ambient levels (3-8 $\mu W/cm^2$ at the table surface) of UV-B exposure on a 14L:10D cycle, at 13°C. We used a parallel array of lights, consisting of four UV-B lights (Q-Panel, UVB313), alternated with four fluorescent full-spectrum lights (Vita Lite), suspended 60 cm above the table surface. Levels of UV-B under mylar filters at the table surface were undetectable,

while under acetate filters exposures ranged from 1.0 to 3.5 $\mu W/cm^2$. These exposures are within the natural range experienced by larvae during development in the Cascade Mountains (see transect results below). Water changes were completed every 5-7 days and larvae were fed *Tubifex* (aquatic worms), *ad libitum*. Larvae were exposed to UV-B for four weeks, at which time they were removed from under the UV lighting and re-weighed. A four week exposure time was chosen to ensure that any measurable differences in growth due to UV-B exposure could be evaluated, without confounding changes due to metamorphosis.

Survivorship for each experiment was analysed separately using Fisher's Exact tests. Growth differences (mass) for the mountain population were analysed using ANOVA and extra sums-of-squares F-tests to compare an equal and separate means models. Not enough larvae from the valley population survived to analyse growth of individuals from that population.

Choice tests

A field experiment was conducted to determine whether individual larvae, 1) directly discriminate between regions of high and low UV-B and/or 2) indirectly discriminate between regions based on the amount of shade available. Testing was done at Susan's Pond on 21 July, 1998.

Tests were conducted on individual larvae in ten separate plastic containers (31 x 16 x 11 cm), at three consecutive time periods (11:30, 13:00, 14:30). By performing the experiment during three different times, we hoped to avoid bias due

to diel patterns in microhabitat preference. During each time period, five of the containers were randomly assigned to an acetate/mylar treatment, and the remainder received a sun/shade treatment. The five selected for the acetate/mylar treatment were covered with half mylar (UV-B blocking filter) and half acetate (UV-B transmitting filter). Pilot studies indicated that the mylar and acetate filters provided a definitive line on the bottom surface of the container between the UV-B and non UV-B exposed sides. The sun/shade containers in each time period were half covered with white plastic, which provided significant shade and reduced UV-B levels to almost zero. The other half of these containers was open to full sunlight. For both acetate/mylar and sun/shade containers, the side of the container to be exposed to UV-B, either acetate or open, was randomly selected.

Containers had mesh sides to allow for water flow and temperature buffering. They were submerged in the pond to a depth of approximately 8 cm and were floated within a wooden frame. Prior to the initial test, 35 larvae (30 for the experiment plus 5 extra) were randomly collected from the pond and placed in a 100 x 100 x 75 cm holding enclosure constructed of a wooden frame with mesh sides and bottom. This was done to ensure that individual larvae were used only once. At the beginning of each trial, ten larvae were selected from the holding enclosure and placed individually into each container. Appropriate treatment regimes were then placed over the containers. Containers were rotated in relation to the current angle of the sun such that the line between acetate/mylar and sun/shade was distinct and in the centre of the container. Larvae were then allowed

to acclimate for 10 minutes. After the acclimation time, the location of each individual in its container was recorded at 10 minute intervals for one hour. If a larva was in the centre of the container, the position of its head was used to assign location. Half-way through the testing, all containers were rotated 180° to avoid error caused by geographical orientation. At the end of the trial, temperature was recorded on each half for three randomly selected containers in each group. All ten larvae were released back into the pond and the process was repeated twice more with larvae remaining in the field enclosure.

For each individual, we summed the number of times it was located on the UV-B exposed side (acetate or open). Each set of treatments (mylar/acetate and open/shade) was analysed using a binomial test to compare the number of trials in which individuals spent more than 50% of their time on the UV-B exposed side. Temperatures were analysed using rank sum tests on data lumped across all times (3 containers x 3 times; n=9). One test compared mylar and acetate sides, and one compared sun and shade sides.

Observational field transects

To examine the levels of UV-B that *A. macrodactylum* larvae are exposed to in nature, three 10 m transects were completed on each of three days (25, 28 and 31 August, 1998) at Eric's Pond. Transects were parallel to each other and 8 m apart. On each day, 18-20 minutes was spent on each transect, so that they were all completed in 1.5 hours. The transect line was a single piece of 10 mm nylon rope,

10.2 m in length which was tied to two metal posts such that exactly 10 m remained between the posts. The posts were sunk into the pond substrate and served as endpoints and anchors for the transect. Each transect originated at the water's edge and continued for 10 m into the pond. The first transect was parallel to one edge of the pond. Transect three extended to almost the centre of the pond, and transect two was between transect one and transect three. Transects were consistently placed in the same location in the pond and were completed at the same time on each sampling day.

For each transect at each time, after the transect was established, we waited 10 minutes before sampling in case animals were disturbed during set-up. During that time, we measured ambient UV-B levels striking the surface of the water. We then walked the transect, disturbing the substrate as little as possible so that clouding of the water would not occur and animals would not be disturbed prior to measurement. We recorded UV-B exposure ($\mu W/cm^2$) of each individual larvae that was observed within 0.5 m of either side of the transect. UV-B exposure was measured using a hand held Solar Light meter (model PMA2100; Solar Light Co., Philadelphia, PA) with a UV-B probe. Measurements were recorded at the location in the water column where larvae were first observed. After UV-B measurements were completed, the maximum depth of the transect was recorded.

Results

Growth in the laboratory

For the valley population, the proportion of individuals surviving under UV-B exposed and shielded regimes was significantly different than would be expected by chance ($p < 0.001$). Only 40% of the exposed larvae survived, while 100% of the larvae that were shielded from UV-B survived (Fig. 3.1). There was no significant difference in survivorship among UV-B exposed and shielded treatments for individuals from the mountain population ($p = 1.0$). All (100%) of the mountain larvae that were shielded from UV-B survived, versus 93% in the UV-B exposed treatment.

In the mountain population, where mortality was minimal, sublethal effects on growth in UV-B exposed individuals were observed. After accounting for the initial mass of the individuals, there was a significant difference in mean final mass for UV-B exposed and non-exposed larvae ($F= 10.3$; $p=0.004$; Fig. 3.2). The mean final mass for larvae with no UV-B exposure was approximately 0.10 grams more than the mean for exposed individuals (95% confidence interval from 0.03 to 0.16 grams).

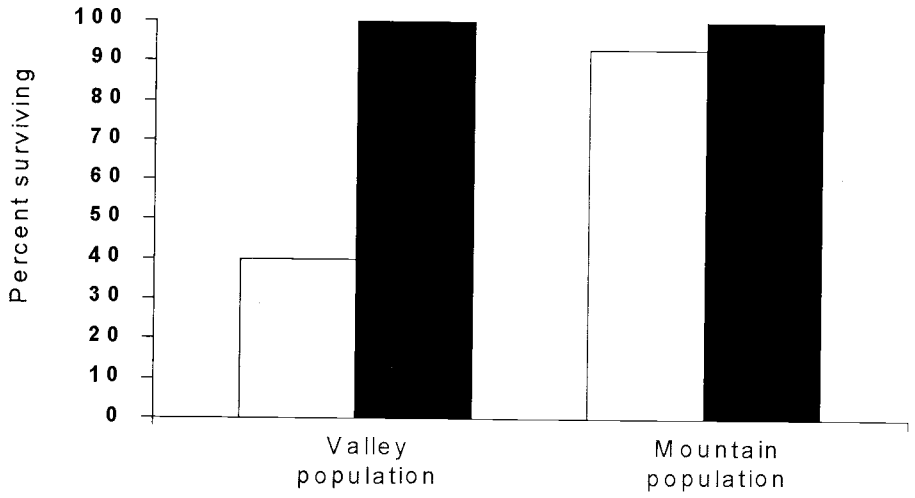

Figure 3.1. Percent of larvae from valley and mountain populations surviving after four weeks. White bars represent individuals exposed to UV-B. Black bars represent shielded individuals.

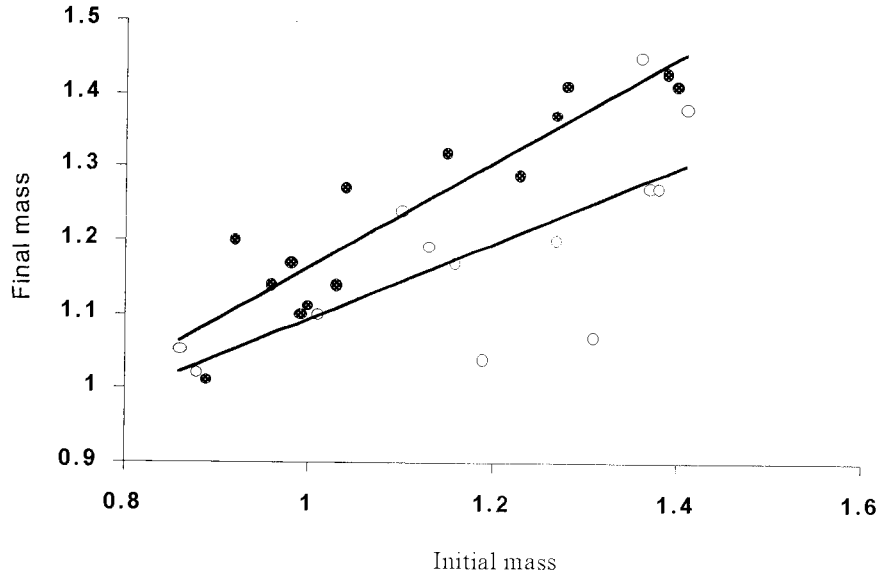

Figure 3.2. Mass (g) of larvae from the mountain population after four weeks versus initial mass (g). White dots represent individuals exposed to UV-B. Black dots represent shielded individuals.

Choice tests

When given a choice of sun or shade, larvae spent the majority of their time in the shade ($p < 0.001$; Table 3.1). The mean percent of time spent on the open (UV-B exposed) side of the container was 2.4% (Fig. 3.3). One larva in the open/shade regime died during the experiment for unknown reasons. Data for that individual were eliminated from the analysis. Individuals given a choice of acetate or mylar shields did not spend a significantly different amount of time on either side of the container ($p = 0.397$). Median water temperatures in the test containers did not vary significantly between the exposed or non-exposed sides for either set (mylar/acetate, p=0.97; sun/shade, p=0.93).

Table 3.1. Number of trials in which test larvae chose UV exposed and UV shielded sides of experimental containers

	Trials (n) where test larvae spent majority of time		
Treatment	UV-exposed	UV-shielded	P
Open/shade	0	14	<0.001
Acetate[a]/mylar[b]	7	8	0.397

[a] Transmits 80% of UV-B
[b] Blocks 100% of UV-B

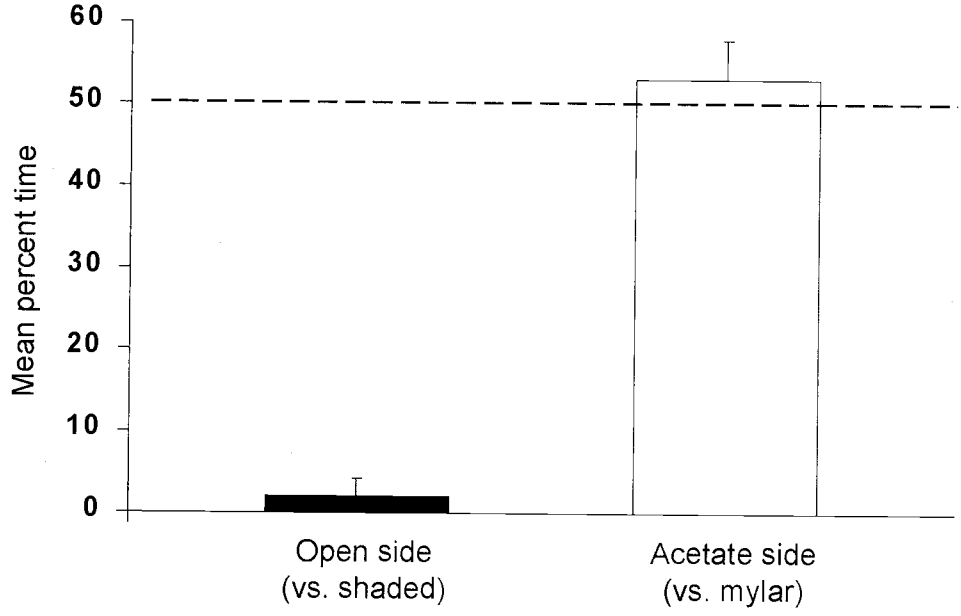

Figure 3.3. Mean (± SE) percent time larvae spent on UV exposed side of test container.

Observational field transects

Mean UV-B exposure (±SE) of individual larvae over all transects on all days was 3.4 (±0.3) $\mu W/cm^2$, while ambient levels of UV-B striking the water's surface averaged 14.7 (±0.2) $\mu W/cm^2$ (Table 3.2). Transect one was consistently the shallowest of the three transects, with a mean maximum depth of 12.7 (± 1.5) cm over the three days. This transect also consistently had the fewest larvae (N= 10, 16, 6 on the three days, respectively) and the highest UV-B exposures for individuals, with a mean of 7.9 (±0.7) $\mu W/cm^2$. In contrast, transect three, that

entered the deepest region of the pond, had a mean maximum depth of 37 (±1.7) cm over the three days. More larvae were seen along this transect (N= 23, 22, 51 on the three days, respectively) than along transect one, and mean exposure for individuals was lower, at 2.1 (± 0.3) $\mu W/cm^2$. Transect two had an intermediate mean maximum depth of 28.7 (± 1.8), and a mean exposure for individual larvae of 2.9 (±0.3) $\mu W/cm^2$.

Table 3.2 Summary of transect data from Eric's pond, Cascade Mountains, Oregon

Transect	Date	Ambient UV-B ± SE ($\mu W/cm^2$)	N	Mean exposure ± SE ($\mu W/cm^2$)	Maximum depth (cm)
1	25 August	13.9	10	8.0 ± 1.3	15
1	28 August	14.9	16	8.1 ± 1.0	13
1	31 August	14.6	6	6.8 ± 2.0	10
2	25 August	14.1	19	2.8 ± 0.6	31
2	28 August	15.0	20	3.1 ± 0.3	30
2	31 August	14.8	18	2.9 ± 0.7	25
3	25 August	14.1	23	1.4 ± 0.4	40
3	28 August	15.4	22	1.5 ± 0.5	37
3	31 August	15.3	51	2.9 ± 0.6	34
Overall	---	14.7 ± 0.2	185	3.4 ± 0.3	26.1 ± 3.7

Discussion

Although we do not know what the cumulative UV-B dose is for any single individual, larvae do appear to receive some ambient UV-B exposure during development under natural conditions. This may be especially true in ephemeral

ponds late in the summer, when drying begins to significantly reduce the water level. Prior to pond drying, larvae may be relatively protected from UV-B due to the attenuation of UV radiation in the deeper water. In many situations, larvae are therefore more likely to receive higher exposures later in development, after pond drying has commenced.

Transect data indicate that some larvae are found in sunny, UV-B exposed regions. This seems to contradict the results of the sun/shade choice tests, which indicate that larvae prefer shaded regions to those fully exposed to sun. Several factors, such as individual variation in response to environmental factors, location of food items, and predation pressure influence patterns of space use in nature. In addition to these possibilities, we suggest several other potential explanations for these findings.

In the natural habitat, temperature may be an important factor in determining microhabitat preference. Amphibian larval growth and development is largely influenced by temperature, with growth and development occurring more rapidly in warmer water (e.g., Harkey and Semlitsch 1988). In ephemeral ponds, larvae must reach a minimum size for metamorphosis prior to pond drying. Therefore spending time in warmer regions of the habitat, where growth and development will be accelerated, could be critical for survival. In high elevation montane ponds in the Cascades of Oregon, the warmest regions are generally those found in the shallower open water at the pond edges (O'Hara 1981). In the test containers used for the choice experiments, temperature did not differ significantly

between the open and shaded regions. The lack of a temperature gradient in the choice experiments could explain why larvae are seen during the day in the sunny, shallow regions of the ponds, but strongly preferred the shade in the choice test.

Another explanation is that pond drying may influence intraspecific interactions, and consequently spatial distribution, of the larvae. As the pond dries, larval density in the remaining water increases and the amount of cover available is reduced. Cover in these ponds is generally limited to rocks and small boulders and with drying, fewer rocks are left submerged, so cover availability decreases. In combination with the increasing density, a loss of cover most probably means that at least some animals will be forced into open water. Larval *A. macrodactylum* exhibit cannibalism in at least one montane population (Walls et al. 1993, Wildy et al. 1998), so in addition to the potential benefits to growth by being in warmer, shallow regions, intraspecific predation could force some larvae away from cover and into open areas.

The choice tests also indicated that larvae do not appear to discriminate between microhabitats solely on the basis of their relative UV-B exposure, as no preference was shown for mylar or acetate exposed sides of the container. This does not rule out the possibility that larvae are able to sense differences in regions of higher or lower UV. An avoidance response to levels of UV-B three times higher than summer midday exposure has been documented in several anuran larvae in the laboratory (van de Mortel and Buttemer 1998). However, our results

suggest that even if *A. macrodactylum* can sense UV, it may not be an important factor in determining microhabitat preferences.

If ambient UV-B levels continue to rise due to ozone depletion (Blumthaler and Ambrach 1990, Kerr and McElroy 1993, Zerefos et al. 1998), there may be intense selection pressure for larval *A. macrodactylum* to avoid exposure. This is especially true since our results on individual survivorship and growth in the laboratory indicate that exposure to even relatively low levels of UV-B could potentially alter individual fitness. Larval growth prior to metamorphosis is a key factor in determining adult fitness for many amphibian species (e.g. Smith 1987, Semlitsch et al. 1988, Bervin 1990). For one population of *A. macrodactylum*, we have demonstrated sublethal growth effects in the laboratory. Reduced mass in larvae exposed to UV-B could be due to the increasing physiological cost associated with repairing cellular damage caused by UV-B. With more energy allocated to repair processes, less is available for growth.

Apparent differences in survivorship between the valley and mountain populations could be due to differences in the life stage at which experimental larvae were brought into the laboratory. Individuals from the valley population were collected as embryos and raised in the lab for several months prior to being tested. Moreover, they were smaller at testing than individuals from the mountain population. Larvae from the mountain population were tested after being housed in the laboratory for less than one week. When tested they were larger than individuals from the valley population.

Sensitivity to UV-B may correlate with developmental stage and/or size. Although we do know the masses at testing, we were not able to determine the developmental stages of the larvae from the two different populations. Developmental stages of many anuran amphibian larvae have been classified through metamorphosis (e.g. Gosner 1960). However, very few species of salamanders have been staged past hatching and no developmental staging tables exist for Ambystomatid salamander larvae (see discussion in Duellman and Trueb 1986). Differences in mass are not necessarily directly correlated with developmental stage, so that larvae of different masses could be at the same point in development, and vice versa. This makes any study involving population differences in growth and development difficult.

In addition, individuals from the mountain population could have already undergone selection for UV-B resistance prior to our collection. If this occurred, we might have been testing individuals from the mountains which were survivors of this selection and were genetically more resistant to UV-B, while the valley larvae would not have undergone this selection prior to our experiments. Furthermore, the ability to cope with UV-B during late larval development may be dependent on exposure to low levels of UV-B early in development. Larvae from the valley, which were raised in the lab, did not receive any UV-B exposure prior to testing. However, UV-B levels in the Willamette Valley during the months of *A. macrodactylum* breeding (generally October - April; Nussbaum et al. 1983) are typically very low. UV-B measurements taken in January 1998 near Corvallis,

Oregon, ranged from 0.03 $\mu W/cm^2$ under cloud cover to 1.25 $\mu W/cm^2$ in bright sun (L.K.B., unpublished data). *Ambystoma macrodactylum* embryos would most likely receive little UV-B exposure in the valley.

In the mountains, ambient levels of UV-B during larval development are higher than those in the valley (Blaustein et al. 1997), and it is possible that high levels of UV-B are a significant selection pressure for the evolution of UV-B resistance. Therefore, there could be actual population differences in sensitivity to UV-B for *A. macrodactylum*. This possibility is being examined further.

Although we cannot conclude from these experiments that population survivorship differences for *A. macrodactylum* from these two populations were due to altitudinal variation in sensitivity to UV-B, the possibility does still exist. Reduced growth of the individuals from the mountain population exposed to relatively low levels indicates that UV-B could already be an important factor influencing adult fitness in this species. This could be especially true in high elevation ephemeral ponds with relatively high UV-B penetrance, like many of those in the Cascade Mountains of Oregon. Understanding how behavioural and physiological tolerance mechanisms operate in nature for larval amphibians is key to predicting the outcome of future changes in UV-B levels for these organisms.

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Chapter 4

Population Differences in Sensitivity to UV-B Radiation for Larval Long-toed Salamanders

Lisa K. Belden and Andrew R. Blaustein

Abstract

Due to ozone depletion, the intensity of ultraviolet-B radiation (UV-B; 280 - 320 nm) at the Earth's surface is increasing. UV-B penetrates some aquatic habitats to biologically significant depths and can alter life histories of aquatic organisms, including algae, zooplankton, fish and amphibians. While major species differences have been documented for UV-B sensitivity, few studies have examined differences between populations of the same species. Previous work has suggested the hypothesis that long-toed salamander larvae, *Ambystoma macrodactylum,* from valley populations (approx. 100 m elevation) were more sensitive to UV-B exposure than larvae from mountain populations (above 500 m elevation) in Oregon. To test this hypothesis in the absence of possible other confounding environmental effects, we brought early stage embryos into the laboratory from three valley populations and five mountain populations and raised them under identical conditions without UV-B for two months after hatching. Larvae from each population were then placed under UV-B lighting and growth and survivorship was recorded for three weeks. Larvae from all populations had higher mortality when exposed to UV-B than when shielded from UV-B. However, UV-B exposed individuals from low elevation populations had significantly lower survivorship than those from high elevation, suggesting an elevational difference in UV-B sensitivity. In all populations, UV-B exposed individuals were smaller than shielded individuals after one week. This reduced growth could be facilitated by a reduction in food intake of exposed individuals.

We tested for differences in food consumption in UV-B shielded and exposed larvae using a field experiment. Individuals exposed to UV-B consumed significantly fewer tadpoles than non-exposed individuals. It appears likely that there are elevational differences in UV-B sensitivity for long-toed salamanders and that reductions in growth could be due to reduced food consumption.

Introduction

Ultraviolet-B radiation (UV-B; 280-320 nm) levels at the Earth's surface are increasing with ozone depletion (Kerr and McElroy 1993, Madronich et al. 1998). Exposure to UV-B results in cellular damage (Tevini 1993, Hader 1997), which may ultimately have consequences for species interactions (Bothwell et al. 1994, Rousseaux et al. 1998). In addition, for aquatic organisms, climate change may be as critical in regulating exposure to UV-B as is ozone depletion (Schindler et al. 1996, Yan et al. 1996, Pienitz and Vincent 2000, Kiesecker et al. 2001). This is because climate change can alter water depth and water chemistry, which are important factors in determining the amount of UV-B received by aquatic organisms (Kirk 1994).

Many aquatic organisms are sensitive to UV-B exposure, including species of phytoplankton, zooplankton, aquatic invertebrates, fish and amphibians (e.g Shick et al. 1996, Blaustein et al. 1998, Hader et al. 1998, Beland et al. 1999). However, as for any abiotic factor, sensitivity to UV-B is not necessarily consistent within a given taxon, and detrimental responses in one species does not imply that

others within that taxon will respond similarly. For example, while some phytoplankton have decreased photosynthetic rates when exposed to UV-B, the specific biochemical effects vary greatly with species (Arts and Rai 1997, Hessen et al. 1997). As interspecific variation in sensitivity to UV-B radiation is well documented, we might also expect differences in sensitivity between populations of a single species. For example, UV-B may increase with increasing altitude (Blumthaler et al. 1997), so for species existing along altitudinal gradients, we might expect differences in sensitivity between high and low elevation populations. Evidence from plant research indicates that there can be altitudinal variation in response to UV-B, although there is variability between genera (Caldwell 1968, Rau and Hofmann 1996, Hubner and Ziegler 1998).

Amphibian responses to UV-B exposure have been extensively studied due to the global nature of recent amphibian population declines and range reductions. As is true for other taxa, amphibian species vary in sensitivity to UV-B. Embryos of some amphibian species die when exposed to ambient UV-B, while other species at the same sites appear unaffected (e.g. Blaustein et al. 1994, Anzalone et al. 1998, Lizana and Pedraza 1998, Broomhall et al. 2000). However, few species have been examined for population level variation in sensitivity to UV-B, and to our knowledge, no studies have examined the same species from more than two sites in the same experiment. If UV-B is a contributing factor to some amphibian population declines, then potential differences in population level response to UV-B could be important in conservation efforts.

Although their population status is not currently known, long-toed salamanders, *Ambystoma macrodactylum,* are an excellent species to use in examination of potential differences in UV-B sensitivity because they have a large range and occur from low to high elevation in the Pacific Northwest, USA (Nussbaum et al. 1983). Previous work has shown that long-toed salamander embryos are sensitive to UV-B radiation (Blaustein et al. 1997) and work with long-toed salamander larvae from two populations suggested the possibility of differences in UV-B sensitivity between populations (Belden et al. 2000). In this study, we addressed (1) whether there are differences in UV-B sensitivity for larval long-toed salamanders from valley and mountain populations and (2) whether reduced growth of UV-B exposed larvae may be facilitated by reduced food consumption of exposed individuals.

Materials and Methods

Population differences in sensitivity

To determine if there were differences in UV-B sensitivity between valley and mountain populations, we raised individuals from multiple populations in the laboratory from the early embryo stage and tested UV-B sensitivity in the laboratory eight weeks after hatching. We collected fresh eggs (Harrison stages 5-12, Harrison 1969) from eight different populations (three from the Willamette Valley (Linn and Benton counties, Oregon) and five from the Cascade Mountains

(Klickitat county, Washington; Deschutes county, Oregon) soon after oviposition (January - February for valley populations; April - June for mountain populations). The valley populations were 13 to 17 km apart and ranged from 78 to 105 m in elevation. The mountain populations were 15 - 205 km apart and ranged from 564 to 2038 m in elevation. Once in the laboratory, eggs were reared by population in plastic boxes (32 x 18 x 8 cm) filled with dechlorinated tap water (approximately 25 eggs/box and 40-50 eggs/population).

For each population, when hatching commenced, new larvae were separated from the eggs each day and maintained in the same type of boxes at a density of six individuals/box. We recorded the day of hatching for all larvae. For experiments, we used individuals that had all hatched within three days of one another. Larvae were maintained at 12.7-14.5 °C with 12L:12D flourescent lighting for eight weeks prior to testing. They were fed brine shrimp *ad libitum* three times/week, with a complete water change done the day after feeding. At eight weeks post-hatching, larvae from the population were pooled into a single tank and then placed singly into plastic petri dishes (15 cm diameter x 1.5 cm depth) filled with 1 cm of dechlorinated tap water. The total number of individuals used for each population (half went to each UV-B treatment) varied between 16 and 30 (valley A= 30; valley B= 20; valley C= 30; mountain 1= 16; mountain 2= 16; mountain 3= 24; mountain 4= 30; mountain 5= 24). Sample sizes were limited by the space available for UV-B exposure.

After transfer to the dishes, the total length of each larva was recorded to the nearest mm. Each dish was then randomly assigned to either a UV-B exposed (acetate filter) or UV-B shielded (mylar filter) treatment. Mylar blocks almost 100% of UV-B and acetate allows 80% of UV-B to pass through (Blaustein et al. 1994). All petri dishes were then randomly placed under UV-B enhanced full spectrum lighting. We used a parallel array of lights, consisting of four UV-B lights (Q-Panel, UVB313; Q-Panel Inc., Cleveland, Ohio, USA), alternated with four fluorescent full-spectrum lights (Vita Lite; Durotest Corporation, Fairfield, New Jersey, USA). These were suspended above the table to result in levels of UV-B ranging from 4 -6 $\mu W/cm^2$ in the portion of the table that we used. UV-B radiation under mylar filters ranged from 0.2 - 0.4 $\mu W/cm^2$ and under acetate ranged from 1.3 - 3.1 $\mu W/cm^2$. These levels are within the range experienced by *A. macrodactylum* larvae during development in the Cascade Mountains of Oregon (Belden et al. 2000).

Larvae were exposed to UV-B for three weeks. Survival was recorded daily during that time and length of all surviving individuals was recorded at the end of each week. During the exposure, individuals were fed approximately eight *Tubifex* worms every other day. The day after feeding, leftover food and waste was removed from all dishes and water was added so that the depth remained at 1 cm. Once a week, a complete water change was done on all the dishes.

We analyzed the mean survivorship at the end of three weeks in a two-way ANOVA, with valley vs. mountain and UV-B vs. no-UV-B as the factors. Prior to

the ANOVA, we tested data for normality and homogeneity of variance. To meet normality assumptions, survivorship data were arcsin square root transformed prior to analysis. Variance was not completely homogenous for survivorship, but ANOVA is robust to this departure from assumptions, so we continued with the analysis (Underwood 1997). We also used a two-way ANOVA, with the same factors, to examine mean growth after one week. All assumptions were met for the growth data.

Food consumption

We completed the feeding behavior trials on three days in summer 2000 at a temporary pond in the Cascade Mountains, approximately 24 km S of Sisters, Deschutes county, Oregon (elevation = 2015 m). The evening before a trial, 20 plastic containers (85 x 40 x 15 (depth) cm) were filled to a depth of 10 cm with pond water. These were placed submerged in the pond, parallel to one another and perpendicular to the pond edge. Due to variation in the bank, we alternated UV-B exposed and non-exposed treatments instead of assigning them randomly. This ensured that there would be treatments of both types on all the different slope angles of the pond margin (as in Lefcort and Blaustein 1995).

We randomly collected *A. macrodactylum* larvae from the pond and placed a single larva in each container after recording its length to the nearest 5 mm. Mean total length (± S.D.) of larvae tested was 78.2 (± 8.4) mm for non-exposed individuals and 78.8 (± 7.5) for UV-B exposed individuals. UV-B transmitting

(acetate) and blocking (mylar) filters were then placed over the containers and attached to the edges with metal clips. The following day at 1400 hours, 10 *H. regilla* tadpoles (Gosner stage 25-26, mean total length = 14 (± 3) mm) were added to each container. Tadpoles were raised in the laboratory from eggs that were collected from a site within 0.5 km of where our experiment was completed. An hour after the addition of the tadpoles to the test containers, we recorded the number of tadpoles consumed and all larvae were released back into the pond. Prior to field experiments, we tested a sample of *H. regilla* tadpoles with three of the smallest salamanders (total length = 60-65 mm) we could find to ensure that all tadpoles were of a size that could be consumed.

Temperatures were recorded in 5 mylar and 5 adjacent acetate containers at the conclusion of the experiment on each of the three days. In addition, at approximately 1430 hours on 5 days at this site, including the three experimental days, UV-B penetrance of the water column was recorded at 0, 5, 10, 15 and 20 cm depth using a hand held Solar Light meter with a UV-B probe (model PMA2100; Solar Light Co., Philadelphia, PA). We also completed these UV-B depth measurements at a site in the Willamette Valley at 1430 hours on 5 days in February, when larvae were developing, for comparison with the mountain site. We analyzed the number of tadpoles consumed by exposed and non-exposed larvae using an ANCOVA with body length as a covariate and day of the trial (1,2 or 3) and UV-B exposure (exposed or non-exposed) as factors. Individual larvae are independent samples.

Results

Population differences in sensitivity

Survival was affected by both the location (mountain or valley; $P < 0.001$) and the UV-B treatment (exposed or not exposed; $P < 0.001$). There was also a significant interaction between these two variables ($P = 0.002$), with mean survival for UV-B exposed larvae from the valley populations (7%) much lower than that for the UV-B exposed mountain larvae (74 %; fig. 4.1). Mean survival for non-exposed larvae was high for individuals from all populations (100% for mountain populations and 97% for valley populations; fig. 4.1).

Growth at one week was reduced by UV-B exposure ($P = 0.005$), but did not vary with location (mountain or valley; $P = 0.834$), nor was the interaction between the two variables ($P = 0.523$). Regardless of the population, larvae that were shielded from UV-B grew approximately twice as much, on average, during the first week than exposed individuals (fig. 4.1).

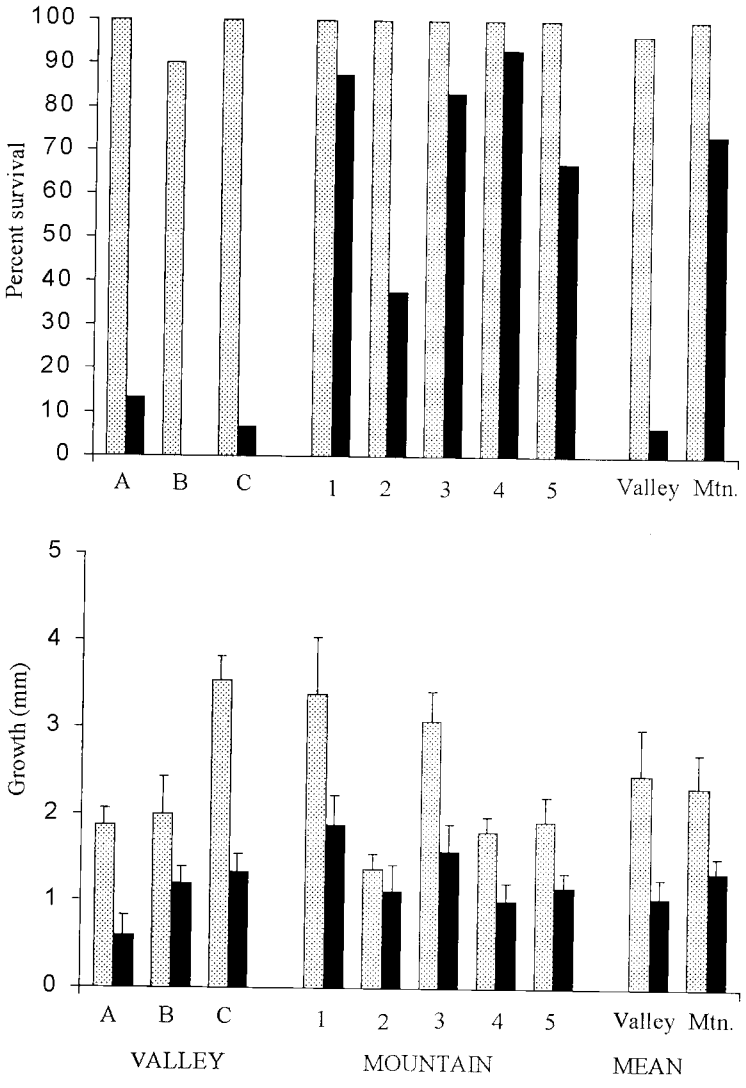

Figure 4.1. Percent survival after three weeks and growth (mm) after one week for UV-B exposed (dark bar) and non-exposed (light bar) individuals from the three valley and five mountain populations. Statistics were performed on the means for mountain and valley populations, which are also graphed here. Sample sizes are given in the text.

Food consumption

After accounting for body length and day of the trial, individuals that were exposed to UV-B ate fewer tadpoles than those not exposed to UV-B ($p = 0.004$; fig. 4.2). After accounting for UV-B treatment effects, neither body length ($p = 0.21$), day ($p = 0.17$) or a treatment by day interaction ($p=0.34$) explained a significant amount of the variation.

Mean UV-B level at the site at 1430 hours during five days (the 3 trial days plus two additional days in between trials) was 19.3 (± 1.3) $\mu W/cm^2$ at the surface of the water, and at 10 cm (the depth of water in the test containers) it was 9.4 (± 0.9) $\mu W/cm^2$ (fig. 3). In the valley, mean (± SD) UV-B at the surface of the water was 5.1 (± 0.5) $\mu W/cm^2$, and at 10 cm it was 0.8 (± 0.1) $\mu W/cm^2$ (fig. 4.3).

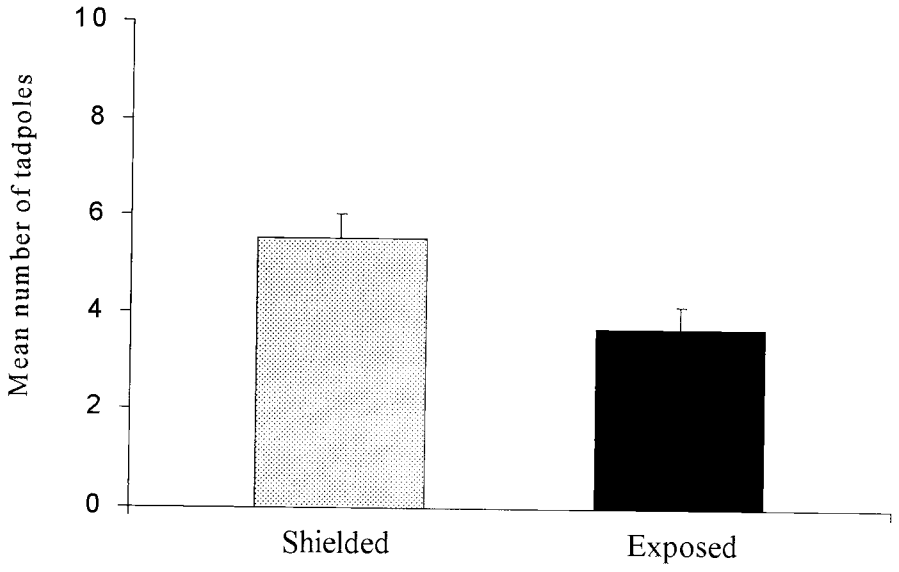

Figure 4.2. Mean number of tadpoles consumed by larvae exposed to UV-B or shielded from UV-B in the field.

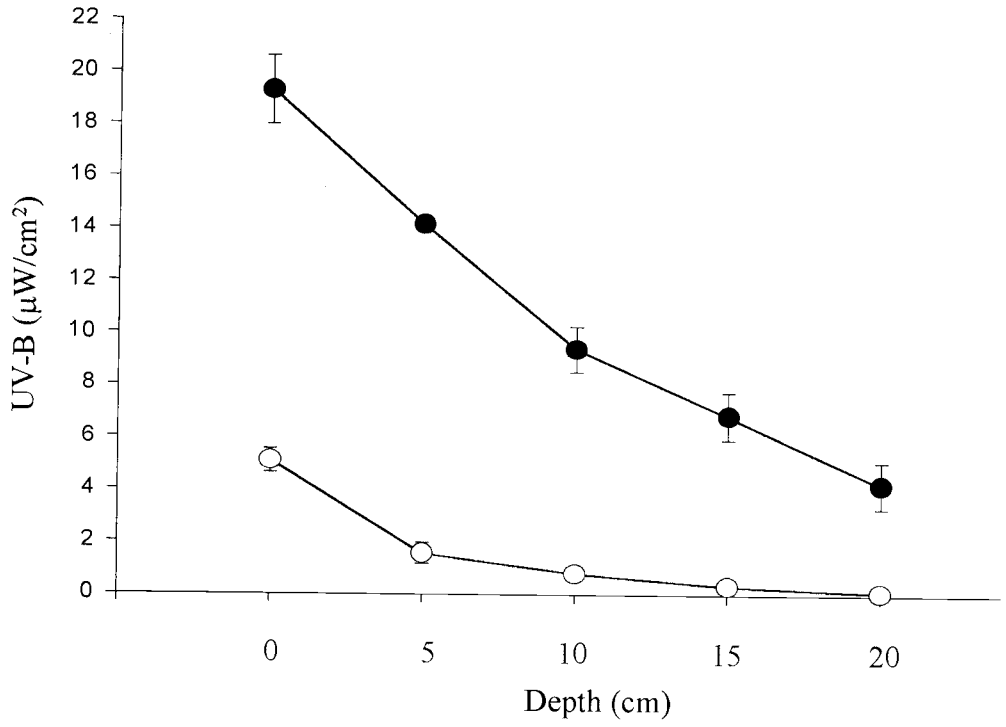

Figure 4.3. Attenuation of UV-B (μW/cm2) in the water column (0 to 20 cm depth) on five days during larval development at a mountain (●) and valley (○) site.

Discussion

To our knowledge, this study is the first to demonstrate population differences in UV-B sensitivity for a single amphibian species. Long-toed salamander larvae from valley populations experienced greater mortality when exposed to UV-B than larvae from mountain populations, although individuals from all populations experienced sublethal effects on growth after one week. We do not have genetic or dispersal data to demonstrate that the sites we sampled within the valley or within the mountains represent genetically distinct populations.

Like many amphibians, *A. macrodactylum*, is thought to have relatively low levels of dispersal, although within single mountain basins in Idaho and Montana populations appear to be very similar genetically and colonization can occur in lakes when introduced fish are removed (Funk and Dunlap 1999, Tallmon et al. 2000).

In our study, the sampling of multiple populations in the valley and mountains provides strong evidence that there are regional, and likely, elevational differences in UV-B sensitivity. In fact, the populations from the valley and mountains have previously been identified as different subspecies,with *A. m. macrodactylum* in the Willamette Valley and *A. m. columbianum* in the Cascade Mountains. This designation is based on morphometric measures and pigmentation in adults, with no consistent differences reported for the larvae (Ferguson 1961).

Blaustein et al (1994) looked at embryonic sensitivity in two populations each of Pacific treefrogs (*Hyla regilla*), Cascades frogs (*Rana cascadae*) and western toads (*Bufo boreas*) and obtained the same results within each species, with Pacific treefrogs being consistently more resistant to UV-B exposure than the other two species, regardless of population. Embryos from two populations of western spotted frogs (*Rana pretiosa*) were also found to be resistant to UV-B (Blaustein et al. 1999).

Several independent studies have examined UV-B sensitivity in the same species in different geographic regions. Three separate studies on embryonic Pacific treefrogs, *H. regilla*, completed in British Columbia (Ovaska et al. 1997),

Oregon (Blaustein et al. 1994) and California (Anzalone et al. 1998), have all reported embryonic resistance of this species to ambient UV-B radiation. Similar results have been obtained for red-legged frog (*Rana aurora*) embryos in Oregon (Blaustein et al. 1996) and British Columbia (Ovaska et al. 1997). Embryos from multiple populations of common frogs, *Rana temporaria*, also appear to be resistant to UV-B (Langhelle et al. 1999, Merilä et al. 2000, Pahkala et al. 2000), although there may be sublethal effects on growth (Pahkala et al. 2000).

Several differences in the UV environments between the valley and the mountain sites suggest that selection for UV-B tolerance could be occurring in mountain populations of salamanders. In the Willamette Valley, *A. macrodactylum* breeding and development of larvae normally occur during the winter, when UV-B levels tend to be low. Additionally, heavy cloud cover decreases exposures even further and animals are exposed to shorter daylengths during development. Many of the temporary aquatic habitats utilized in the Willamette Valley are dense with aquatic vegetation, which can further shade developing larvae. In contrast, many of the Cascade Mountain sites are devoid of vegetation and development occurs in these sites during summer months when there is little cloud cover and long periods of daylight. Overall, UV-B exposure levels at these mountain sites are likely to be much higher than those in the Willamette Valley during development of larval *A. macrodactylum*. This is supported by our field measurements of UV-B (fig. 4.3).

As none of the individuals were able to escape from exposure in our experiments and some individuals survived suggests that behavioral avoidance

alone cannot explain survival in high UV environments in nature. There appear to be physiological and/or biochemical differences regulating the variation in sensitivity. The difference in UV-B exposure during development could have resulted in selection for a number of traits providing tolerance to UV-B in the mountain populations. For example, differences in production of photolyase, the enzyme responsible for repairing a majority of UV-B induced DNA damage, may be important. There could also be differences in the production of screening compounds, such as melanin, that may effectively absorb and dissipate the energy from UV-B exposure (Jablonski 1998, Cockell and Knowland 1999).

Behavior could potentially mitigate the sublethal effects on growth that we observed. However, we have no evidence at this point that *A. macrodactylum* larvae directly avoid UV-B exposure in the field (Belden et al. 2000). In fact, field observations suggest that larvae in the mountains spend time in shallow water environments where they are exposed to UV-B radiation (Belden et al. 2000, LKB unpublished data).

Results from our field experiment indicate that the sublethal effects on growth could be caused by a decrease in food consumption. Though we did not record behavior directly in the trials, the cause of reduced consumption appeared to be reduced activity and not an inability to capture prey. We also observed this in the laboratory trials, where food was often not eaten by UV-B exposed individuals.

As part of the global amphibian population decline phenomenon, some amphibian species have disappeared completely, and many others have suffered

population and range reductions (Blaustein and Wake 1990, Alford and Richards 1999, Houlahan et al. 2000). Investigations into the causes of population declines typically focus on the variation in environmental factors at the site that could be responsible for the decline. Our study emphasizes the need to also examine the potential of within- species variation in response to environmental factors, which could result in a similar pattern of population and range reductions. Within-species variation to environmental factors, such as UV-B, may have a significant effect on the future of specific amphibian populations.

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Chapter 5

An Investigation of the Physiological Stress Response in Larvae of Four Amphibian Species Exposed to UV-B Radiation

Lisa K. Belden, Ignacio T. Moore, Robert T. Mason and Andrew R. Blaustein

Abstract

Global environmental changes, including increases in ultraviolet-B radiation (UV-B; 280-320 nm), are receiving attention from biologists interested in how these changes will alter community and ecosystem structure and function. Understanding how factors such as UV-B affect animals physiologically would greatly assist our ability to predict the outcome of future global changes. Amphibians provide a good model system for examining the physiological effects of UV-B exposure because basic studies documenting both lethal and sublethal effects have been completed on a wide array of species at many life history stages. In this study, we examined the physiological stress response, as measured by glucocorticoid hormones, for larvae of four species of amphibians (*Hyla regilla*, *Rana cascadae*, *Rana pretiosa*, *Ambystoma macrodactylum*) exposed to UV-B radiation for 7 days. Corticosterone levels between exposed and non-exposed individuals were not significantly different for any of the species. However, we did observe a stress response for *Hyla regilla* tadpoles in our experimental containers as compared to wild controls and to our knowledge this is the first presentation of baseline corticosterone levels for larvae of any of these species.

Introduction

Global environmental changes, including climate change and increasing ultraviolet-B radiation (280-320 nm), have drastic impacts on biological systems (e.g. Bothwell et al. 1994, Parmesan et al. 1999, Pounds et al. 1999, Hughes 2000).

For example, changes in moisture associated with global warming have been implicated in biodiversity losses in Monteverde, Costa Rica (Pounds et al. 1999). Although loss of moisture, increased temperature or increased exposure to UV-B may not be the direct cause of death, these types of changes are likely to physiologically stress at least some organisms within a community, making them more susceptible to factors like disease and predation, and ultimately increasing mortality risk. If changes are beyond the tolerance parameters of all the individuals in the population, and generation times are too long to allow for selection in the appropriate direction, then extinction of the population is the most probable outcome.

UV-B exposure may contribute to increasing rates of skin cancer in humans (Kollias et al. 1991, Longstreth et al. 1995), bleaching events in coral reefs (Lyons et al. 1998), and global declines in amphibian populations (Blaustein et al. 1998). In aquatic systems, exposure to UV-B can increase mortality in some species and ultimately lead to changes at the community level (e.g. Bothwell et al. 1994, Hessen et al. 1997). Because UV-B radiation reaching the Earth's surface is predicted to continue to increase due to thinning of the stratospheric ozone layer (Tevini 1993, Hader 1997, Zerefos et al. 1998), understanding the response of organisms to UV-B radiation is important. However, we know very little about the specific physiological responses of animals to UV-B exposure, which will be a necessary step if we are to predict the outcome of future changes.

Amphibians provide a good model for the investigation of such responses because UV-B exposure induces both lethal and sublethal effects in different species at varying life history stages (e.g. Blaustein et al. 1994a, Hays et al. 1996, Nagl and Hofer 1997, Anzalone et al. 1998, Fite et al. 1998, van de Mortel and Buttemer 1998, Langhelle et al. 1999, Belden et al. 2000, Blaustein et al. 2000). The majority of studies have examined direct lethal effects on amphibian embryos exposed to natural levels of UV-B in the field (Blaustein et al. 1998). These studies demonstrate variation between species in embryonic sensitivity.

Less work has been completed on larval and adult life history stages, but effects on behavior, growth and development, and survival have been documented for some species. For instance, adult roughskin newts exposed to UV-B in the laboratory increase locomotion (Blaustein et al. 2000), larval newts swim erratically when exposed (Nagl and Hofer 1997), and tadpoles of some species stop responding to predator cues after exposure (Kats et al. 2000). In addition, some larval salamanders have decreased growth when exposed to UV-B (Belden et al. 2000). Little work has been done to understand the physiological mechanism driving sublethal responses. However, at least one study has suggested that increased activity observed in response to UV-B exposure may be a physiological stress response mediated by glucocorticoid hormones (Blaustein et al. 2000). Indeed, this type of hormonal stress response has been documented for at least one fish species exposed to UV-B (Jokinen et al. 2000).

Glucocorticoid hormones are released from the adrenal cortex by activation of the hypothalamic-pituitary-adrenal axis, in response to stressful stimuli. Corticosterone, the main glucocorticoid hormone in amphibians (Idler 1972), acts to mobilize energy stores and suppress non-vital physiological processes until the stressful stimulus passes (Wingfield et al. 1998). In the short-term, this can be beneficial to the organism as immediate survival is promoted. However, long-term elevation of glucocorticoids can have deleterious effects, including decreased growth, depressed immune response and decreased reproductive output (Sapolsky 1993).

In this preliminary study, we investigated whether UV-B exposure induces a hormonal stress response in larval amphibians. We hypothesized that corticosterone levels would be higher in larvae exposed to UV-B radiation than in those shielded from UV-B. We tested this hypothesis in the larvae of four amphibian species exposed to UV-B radiation for one week. Two species, the pacific treefrog, *Hyla regilla,* and the Cascades frog, *Rana cascadae*, were tested in the field and two species, the spotted frog, *Rana pretiosa*, and the long-toed salamander, *Ambystoma macrodactylum*, were tested in the laboratory.

Materials and Methods

Documented UV-B sensitivity of study species

Previous studies have demonstrated that *H. regilla* embryos from Oregon, California and British Columbia do not experience any increase in mortality when exposed to ambient UV-B (Blaustein et al. 1994a, Ovaska et al. 1997, Anzalone et al. 1998). *Rana cascadae* embryos are susceptible to both UV-B radiation and infection by the fungus *Saprolegnia ferax* (Blaustein et al. 1994b, Kiesecker and Blaustein 1995). When these two factors are both present, egg mortality is highest. Abnormalities in metamorphic individuals that have been reared under UV in the lab have also been noted (Hays et al. 1996). For long-toed salamanders, *Ambystoma macrodactylum*, exposure to ambient levels of UV-B increased mortality and induced deformities in embryos in the Cascade Mountains of Oregon, USA (Blaustein et al. 1997). In addition, exposure to relatively low levels of UV-B in the laboratory increased mortality of *A. macrodactylum* larvae from the Willamette Valley, Oregon, USA and reduced growth for larvae from the Cascade Mountains, Oregon, USA (Belden et al. 2000). Embryos of western spotted frogs, *Rana pretiosa,* and Columbia spotted frogs, *Rana luteiventris* are resistant to ambient levels of UV-B radiation (Blaustein et al. 1999).

Field experiment of *Hyla regilla* and *Rana cascadae*

The field study of *H. regilla* and *R. cascadae* was completed at Site One (Linn County), Oregon, a natural oviposition site for both species. In April, 1998, we placed part of four *R. cascadae* egg masses in each of two field enclosures and four entire *H. regilla* clutches in each of two field enclosures. This was done to ensure that after hatching, tadpoles of each species could be readily collected for the experiments at the proper stage and with little handling time involved. Initial enclosures were constructed of a wooden frame measuring 100 x 100 x 75 cm with mesh sides and bottom that allowed for water flow. When eggs were added, several handfuls of aquatic vegetation were also added to each enclosure to provide food and cover for hatching larvae.

After all eggs hatched and tadpoles were at Gosner stage 25 (Gosner 1960), we randomly selected 24 tadpoles of each species from the enclosures and moved them into individual containers. Individual containers were 800 ml plastic cups with mesh sides and bottoms attached to 1x2 boards. Mesh sides allowed for water flow and the mesh bottom ensured that waste products did not accumulate in the containers. Twelve containers for each species were then randomly assigned a mylar filter and the remainder were covered with an acetate filter. The mylar filter blocks 100% of UV-B radiation and the acetate filter, which serves as a control for using a filter, transmits 80% of the UV-B radiation (Blaustein et al. 1994a). Natural food (phytoplankton) in each container was supplemented with ground rabbit chow at the beginning of the experiment and again on day 4. Ambient levels

of UV-B and the levels under the mylar and acetate filters were measured between 1130 am and 1230 pm at the site on days 1, 4 and 7 using a hand held Solar Light meter (model PMA2100; Solar Light Co., Philadelphia, PA) with a UV-B probe.

On day 7, all experimental animals were collected from the field for the corticosterone radioimmunoassay (RIA), with the exception of two individuals that were missing (one *R. cascadae* from the acetate group and one *H. regilla* from the mylar group). Collection involved freezing each individual within 2 minutes of capture by placing vials containing the animals in a dry ice/ethanol slurry for 60 seconds. In pilot trials in the laboratory, 60 seconds was adequate for completely solidifying larval amphibians with masses up to 0.7 grams. After freezing in the field, larvae were transferred to a cooler containing dry ice, returned to the laboratory and stored at -70°C until the RIA could be performed. In addition to experimental animals, 9 *H. regilla* (all at stage 25) were collected from the initial field enclosures and preserved for RIA. There were no *R. cascadae* remaining in the initial enclosures, so this additional control group could not be obtained for that species. The mass (g) of each individual was recorded immediately before the assay was performed. Mean mass (± SD) at collection of *H. regilla* was 0.20 (± 0.15) g, and the mean mass (± SD) of *R. cascadae* was 0.27 (± 0.13) g.

Whole body corticosterone levels for the three *H. regilla* groups (mylar, acetate and initial enclosure) were compared using an ANOVA on ranks with a post-hoc all pairwise Dunn's test. Whole body corticosterone levels between the *R. cascadae* groups were compared using a rank sum test.

Laboratory experiments of *Rana pretiosa* and *Ambystoma macrodactylum*

Parts of four *Rana pretiosa* egg clutches were collected at Gold Lake (Lane County) Oregon in the spring, 1998, and returned to the laboratory. After hatching, larvae were raised 40/ 38L tank on 14L:10D cycle at room temperature. They were fed ground rabbit chow every 2-3 days. Testing was completed when larvae were at Gosner stage 25 (Gosner 1960). Larval *A. macrodactylum* (36 individuals) were collected at Three Creeks (Deschutes County), Oregon one week prior to testing and returned to the laboratory. They were placed 6/ 38L tank, on a 14L:10D cycle at room temperature and were fed *Tubifex* (aquatic worms) every 2-3 days prior to testing.

For each of the two experiments (*Rana pretiosa* and *Ambystoma macrodactylum*), 28 larvae were randomly selected, and placed in individual 800 ml plastic cups filled with 400 ml of dechlorinated tap water. Fourteen of these were randomly assigned to a UV-B exposed treatment (acetate filter) while the remaining 14 were shielded from UV-B (mylar filter). All 28 containers were then randomly placed under UV-B enhanced full spectrum lighting to simulate low ambient levels (3-8 $\mu W/cm^2$ at the table surface) of UV-B exposure on a 14L:10D cycle, at 13°C. We used a parallel array of lights, consisting of four UV-B lights (Q-Panel, UVB313), alternated with four fluorescent full-spectrum lights (Vita Lite), suspended 60 cm above the table surface. Levels of UV-B under mylar filters at the table surface were undetectable, while under acetate filters exposures

ranged from 1.0 to 3.5 $\mu W/cm^2$. These exposures are within the natural range experienced by *A. macrodactylum* larvae in the water column during development in the Cascade Mountains (Belden et al. 2000).

Water changes were completed in all containers on day 4 of each experiment. Larval *R. pretiosa* were fed ground rabbit chow on day 1 and day 4. *Ambystoma macrodactylum* larvae were fed *Tubifex* on day 1 and day 4. On day 7 of each experiment, larvae were collected for RIA. Each individual was placed in a vial and submerged in a dry ice/ethanol slurry as in the field experiment. Larval *R. pretiosa* were submerged for 60 seconds, while larval *A. macrodactylum*, which were considerably larger, were submerged for 90 seconds to ensure complete freezing of all tissues. All samples were stored at -70°C until the RIA could be performed. Masses of individual animals were recorded at the time of RIA. The mean mass (± S.D.) of *R. pretiosa* at the time of testing was 0.11 (± 0.04) g. The mean mass of *A. macrodactylum* was 0.50 (± 0.14) g.

For both species, whole body corticosterone levels (ng/g) between exposed and non-exposed larvae were compared using rank sum tests.

Radioimmunoassay

Whole body levels of corticosterone were measured by RIA following the procedures of Moore et al. (2000) with slight modifications. Briefly, whole body homogenates were used for the assay. Each frog was weighed and homogenized with distilled water. Water was added in proportion to the mass of the frog (mass

X 10ml) with a minimum water volume of 0.5 ml and a maximum water volume of 3.0 ml. For individual recovery determination, each sample was equilibrated overnight with 2,000 cpm of tritiated corticosterone. Each sample was then triple extracted in 2 ml of diethyl ether. To break the emulsion, each sample was centrifuged at 1500 RPM for 5 min. The ether phase was then removed and dried in a warm water bath, under a stream of nitrogen gas. The extracts were then resuspended in 10% ethyl acetate in isooctane. The samples were chromatographed through individual celite columns to separate the steroid fractions and neutral lipids. The fractions were eluted using stepwise increasing proportions of ethyl acetate in isooctane. The purified eluates were dried and resuspended in buffer (phosphate buffered saline with 0.1% gelatin) for the assay.

For the assay, individual sample recoveries were determined from 50 µl of the sample while 200 µl of the sample was allocated to each of two duplicates. Serial dilutions for the standard curves were performed in triplicate. All samples, including serial dilutions and total bound, were incubated overnight with 100 µl of antibody (corticosterone antibody B21-42 from Endocrine Sciences) and 100 µl of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. The samples were resuspended in 4 ml of toluene-based scintillation fluid, incubated for 12 h and counted on a Beckman LS1800 scintillation counter. A cubic spline curve was fitted to the standard curve points and final steroid concentrations were calculated from this

curve and adjusted based on individual recoveries. Interassay and intraassay variation were 10% and 15% respectively.

Results

Field experiment of *Hyla regilla* and *Rana cascadae*

Ambient levels of UV-B at the field site on the three days of measurement ranged from 14.5 - 16.6 $\mu W/cm^2$. Values under the mylar filters ranged from 0.9 - 1.2 $\mu W/cm^2$, and under acetate ranged 10.0 - 12.1$\mu W/cm^2$. Water would attenuate this further (e.g. Schindler et al. 1996 and references therein), so that actual exposures of the larvae would be lower.

There was a significant difference between levels of corticosterone in the three groups of larval *H. regilla* (ANOVA on ranks, $H=13.67$; $P = 0.001$). Post-hoc tests indicated that the difference was between the enclosure animals and the two experimental groups. The median value for the enclosure group was 0.48 ng/g, while for the mylar and acetate groups it was 3.78 ng/g and 4.18 ng/g, respectively (fig. 1). Corticosterone levels did not differ between the mylar (-UVB) and acetate (+UVB) groups (post-hoc Dunn's test; $P > 0.05$). There was not a significant difference between corticosterone levels in the two *R. cascadae* groups either (rank sum test; $P = 0.16$). The median corticosterone level in the mylar (-UVB) group was 0.26 ng/g and in the acetate (+UVB) group the median was 0.42 ng/g (fig. 5.1).

Laboratory experiments of *Rana pretiosa* and *Ambystoma macrodactylum*

There was not a significant difference in corticosterone levels between exposed and non-exposed *R. pretiosa* larvae (rank sum test; P = 0.40; fig. 5.1). The median level for both groups was 0.03 ng/g. Corticosterone levels for exposed and non-exposed *A. macrodactylum* larvae were not significantly different either (rank sum test; P = 0.98; fig. 5.1). For exposed larvae, the median corticosterone level was 0.39 ng/g, while for non-exposed larvae it was 0.48 ng/g.

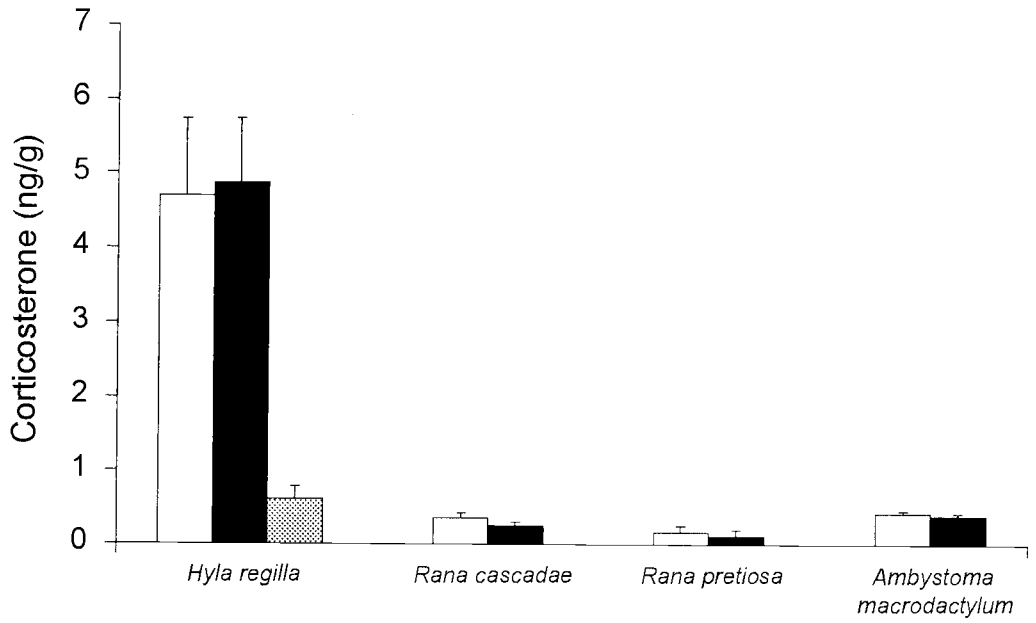

Figure 5.1. Mean (± SE) corticosterone levels (ng/g) in larvae of the four species of amphibians used in this study. White bars represent UV-B exposed larvae. Black bars represent larvae shielded from UV-B. The single gray bar represents *H. regilla* larvae from the larger initial enclosure.

Discussion

Although we did not see differences in corticosterone levels in exposed and non-exposed larvae, to our knowledge these are the first baseline corticosterone data for larvae of these four species. In addition, we have documented the occurrence of a stress response in *H. regilla* tadpoles. This is demonstrated by the elevated levels of corticosterone of individuals in the experimental containers as compared with the those individuals in the larger field enclosure. Although placement in these containers did not result in mortality, it does appear that the containers were physiologically stressful to *H. regilla* larvae. Unfortunately because of the container stress, we are unable to determine if UV-B was also a stressor for these larvae. The corticosterone levels could be maximal due to placement in the containers, so that any differences between the UV-B exposed and non-exposed treatments is undetectable.

To our knowledge, this is only the second demonstration of a stress response in a larval amphibian and the first to be documented in the field in response to experimental manipulation. Hayes (1997) has demonstrated that larval density in the laboratory can influence the endogenous levels of corticosterone in western toad tadpoles, *Bufo boreas*, at Gosner stages 29-30. Levels of corticosterone in crowded larvae (10 individuals/L) were significantly higher than levels in less crowded individuals (2 individuals/L).

There are several possible explanations for the lack of response we observed for the other three species in our experiment. We will address three of

these: 1) UV-B does not induce a physiological stress response or is not perceived as stressful, 2) there was a stress response but the timing of our experiments did not allow us to observe it or 3) the stress axis is not yet developed in these larvae.

Both glucocorticoids and thyroid hormones are critical in orchestrating the complex changes that amphibians undergo during metamorphosis (Hayes and Wu 1995). As such, many studies have examined basal levels of these hormones during that transition, and they are often reported as part of a control treatment (e.g. Krug et al. 1983, Denver 1997, Kloas et al. 1997). The larvae of at least two species of amphibian have a functional stress response during development: *Hyla regilla* (this study) and *Bufo boreas* (Hayes 1997), and a third, *Scaphiopus hammondii*, is thought to rely on environmental cues from pond drying to initiate metamorphosis via the neuroendocrine stress pathway (Denver 1995, 1997).

Perhaps the most parsimonious explanation for our negative data is that UV-B exposure at the levels and lengths of exposure that we examined does not induce a physiological stress response for *R. cascadae*, *R. pretiosa* and *A. macrodactylum*. But it could also be that UV-B exposure is not perceived as stressful or even that UV-B can not be detected by these larvae. As maximum corticosterone levels during response to stress and timing of the response varies with species and stimulus, it is also possible that we simply missed the response.

However, we find it interesting that *R. cascadae* in experimental conditions identical to those applied for *H. regilla* retained very low levels of circulating corticosterone (< 0.5 ng/g), while *H. regilla* levels in experimental containers was

elevated above 3.5 ng/g. While interspecific comparisons are difficult at best these results are surprising given that *R. cascadae* is thought to be similar to *H. regilla* in preferences for dissolved oxygen and temperature (O'Hara 1981). In addition, embryonic *R. cascadae* are much more sensitive to UV-B exposure, than are embryonic *H. regilla* (Blaustein et al. 1994a).

Another possible explanation for our results is that *R. cascadae*, *R. pretiosa* and/or *A. macrodactylum* larvae at this stage do not have a functional response to stress. If these larvae are not able to respond, they may be more susceptible to environmental stressors during this period. In addition, there is potential for stressors to actually influence the sensitivity of the developing stress axis. There is some evidence for this in mice where the extent of stressors to which neonates are exposed influences their response to stressful stimuli as adults (Anisman et al. 1998). Therefore, exposure of larval amphibians to stressful stimuli during development of the HPA axis could influence the adult stress response.

UV-B as a source of potential stress for amphibians and other organisms is a topic worthy of continued research. Levels of UV-B reaching the surface of the Earth will continue to increase with ozone depletion. Therefore, the likely scenario for most organisms is one of increasing exposure and increasing potential for UV-B induced damage to physiological systems. Exposure to UV-B has been linked to eye damage (Fite et al. 1998), cellular damage (e.g. Blaustein et al. 1994a), developmental abnormalities (e.g. Worrest and Kimeldorf 1976, Hays et al. 1996, Blaustein et al. 1997) and decreased growth (e.g. Belden et al. 2000) in amphibians,

and increased circulating glucocorticoid levels in at least one fish species (Jokinen et al. 2000). Therefore, there is good reason to believe that UV-B exposure could be physiologically stressful for amphibians. Though this study has resulted in few definitive answers regarding larval amphibians physiological response to UV-B, we feel the questions it raises concerning the response of amphibian species to an increasingly important abiotic factor are worthy of consideration and should prompt further research in this area.

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Chapter 6

UV-B Radiation Induces Skin Darkening in Three Species of Larval Salamanders

Lisa K. Belden and Andrew R. Blaustein

Abstract

Ultaviolet-B radiation (UV-B), which can cause significant cellular damage, is increasingly being recognized as an important environmental factor for animals. Interspecific differences in sensitivity to UV-B radiation are well-documented for amphibians. Yet, few studies have addressed physiological mechanisms that could be responsible for differential species survival. Skin darkening is one mechanism that may protect organisms from the detrimental effects of UV-B. The adequacy of the skin darkening response in UV-B protection has been heavily debated for mammals, but has not been explored in amphibians. However, melanin production and resultant skin darkening may protect amphibians exposed to UV-B. In this study, we examined (1) the darkening response in salamander larvae exposed to UV-B and (2) whether darker larvae have a higher survival rate than lighter larvae when exposed to UV-B. After five days of relatively low UV-B exposure in the laboratory, larval roughskin newts, *Taricha granulosa*, and Northwestern salamanders, *Ambystoma gracile*, showed a significant darkening of the skin, as compared to controls exposed to full spectrum lighting without UV-B. In addition, long-toed salamanders, *A. macrodactylum* showed the same trend for darkening, although it was not statistically significant. To investigate whether survival might be higher for darker larvae exposed to UV-B, we manipulated the skin color of *A. gracile* and *A. macrodactylum* larvae by placing them on black or white backgrounds during UV-B exposure. Larvae exposed to UV-B were smaller after three weeks, regardless of background coloration. Background coloration

effectively controlled skin color, with larvae on white consistently lighter than larvae on black backgrounds. No survival differences were observed between treatments, so it remains unclear whether skin darkening provides protection from UV-B damage. However, an understanding of how physiological tolerance mechanisms operate in response to UV-B exposure is becoming increasingly important as UV-B levels at the Earth's surface increase with ozone depletion.

Introduction

Understanding the biological effects of ultraviolet-B (UV-B; 280- 320 nm) radiation is important, as UV-B levels at the Earth's surface increase due to stratospheric ozone depletion (Kerr and McElroy 1993, Hader 1997, Zerefos et al. 1998). Varying responses of organisms to UV-B radiation have been demonstrated (e.g. Tevini 1993, Hader 1997, Cockell and Blaustein 2001). Moreover, several studies suggest that increases in ambient UV-B may have drastic biological impacts, including detrimental effects on individual organisms as well as on ecosystems (see papers in Tevini 1993, Hader et al. 1995, Hader 1997, Cockell and Blaustein 2001). Global environmental changes, including increasing UV-B exposure, have been suggested as factors contributing to current world-wide declines in amphibian populations (Blaustein and Wake 1995, Alford and Richards 1999, Kiesecker et al. 2001). Indeed, some amphibian species are extremely sensitive to even current ambient levels of UV-B (Blaustein et al. 1998).

Animals can cope with potentially dangerous UV-B radiation by preventing damage from occurring or by repairing damage once it occurs (Epel et al. 1999). Although the repair mechanisms involved in UV-B induced damage to amphibian eggs and embryos have received some attention (e.g. Blaustein et al. 1994, van de Mortel et al. 1998), little has been done to explore how amphibians may prevent damage from occurring. Behavioral avoidance of areas with high levels of UV-B is one way for larval and adult amphibians to prevent damage (Nagl and Hofer 1997, van de Mortel and Buttemer 1998, Belden et al. 2000).

Physiological and morphological mechanisms may limit UV exposure. For example, pigments in the skin, such as melanin, provide some protection from UV-induced DNA damage in mammals (Kollias et al. 1991). The exact roles of various pigments in human skin that provide protection from UV damage are still being identified, as are the mechanisms involved in the process (Prota 1992). Recently, the debate has been reopened as to how various pigments respond to UV light and whether some pigments may actually contribute to the development of skin cancer (Wu 1999). However, in general, mammals with darker skin are less prone to UV-induced skin damage than those with lighter skin (Kollias et al. 1991, Barker et al. 1995). This relationship has not been studied in amphibians.

Jablonski (1998), suggests that melanin production may protect developing amphibian embryos from neural tube defects by acting as a natural sunscreen. She suggests that melanin is a relatively inexpensive way to prevent critical metabolites, such as folate, from being degraded by UV light during development.

Other evidence suggests that some amphibians may darken in response to UV-B irradiance (adult *Rana sylvatica*, Roth et al. 1996, embryonic and larval *Hyla versicolor* and *Xenopus laevis*, Zaga et al. 1998, larval *Hyla arborea*, Langhelle et al. 1999). But to our knowledge this response has not been quantified.

Skin darkening in amphibians is controlled mainly at the level of the melanophore, which are cells in the dermis that contain melanosomes, the organelles which contain the dark melanin pigment. Darkening occurs when melanosomes are dispersed into the cytoplasm of the cell, and lightening occurs with aggregation of the melanosomes around the nucleus. In this study, we addressed whether larvae of three salamander species darken in response to UV-B exposure. In addition, to begin to address whether darkening offers a survival advantage we examined growth and survival of two of the species exposed to UV-B on either black or white backgrounds. We hypothesized that dark individuals (those on black backgrounds) would have higher survival than the lighter individuals (those on white backgrounds) when exposed to UV-B.

Materials and Methods

Study Species

We used larvae of three salamander species, roughskin newts (*Taricha granulosa)*, and Northwestern (*Ambystoma gracile)*, and long-toed (*Ambystoma macrodactylum)* salamanders to examine skin darkening in response to UV-B

exposure (experiment 1). We used *A. gracile* and *A. macrodactylum* to examine darkening effects on survival and growth (experiment 2). All three of these species are native to the Pacific Northwest, USA (Nussbaum et al. 1983).

Experiment 1: skin darkening in response to UV-B exposure

Each species was tested individually when larvae were active and could be collected from the field. All larvae were collected within 25 km of Corvallis, Benton County, OR, USA (*Taricha granulosa*, 20 km N; *Ambystoma gracile*, 25 km W; *A. macrodactylum*, 15 km E). For each species, approximately 35 larvae were collected and returned to the laboratory. These were housed 5 larvae per 38 L aquaria in dechlorinated tapwater for 14 – 18 days prior to the begininng of the experiment. They were maintained at 18 °C on a 12:12 light:dark cycle and were fed *Tubifex* worms every other day *ad libitum*. Complete water changes were done once a week. For each species, the day prior to beginning the experiment, 30 larvae were placed in individual petri dishes (15 cm diameter) filled with 1 cm of dechlorinated tap water. Length of all larvae was recorded by placing a ruler beneath the petri dish. Mean (± SD) of the tested larvae were as follows: *T. granulosa*, 28.1(± 3.5) mm; *A. gracile*, 35.0 (± 2.7) mm; *A. macrodactylum*, 47.1 (± 6.1) mm. Ten of the thirty larvae were randomly assigned to each of three treatments: 1) UV-B exposed (acetate filter), 2) UV-B shielded (mylar filter), and 3) dim light (black cover). Acetate transmits approximately 80% of UV-B and mylar blocks almost 100% (Blaustein et al. 1994). The black cover was used to

produce a low light environment. Filters were placed over the treatments such that they did not come in contact with the water.

Following assignment to treatments, larvae were transferred to the room with UV-B lighting, after the lights were off for the day. The table surface consisted of plywood. Previous trials on the plywood surface with 5 larvae of each species resulted in a melanophore index of approximately 2-3 for the larvae after 3 days. Temperature was 16 °C and lights were on a 12:12 light:dark cycle. Lighting consisted of a parallel array made up of four UV-B lights (Q-Panel, UVB313; Q-Panel Inc., Cleveland, Ohio, USA) alternated with four fluorescent full-spectrum lights (Vita Lite; Durotest Corporation, Fairfield, New Jersey, USA), suspended above the table surface to result in 3-8 $\mu W/cm^2$ of UV-B at the table surface. UV-B levels under mylar filters were undetectable. Under acetate filters, UV-B exposures ranged from 1.0 to 3.0 $\mu W/cm^2$, which is within the natural range experienced by *A. macrodactylum* larvae in the Cascade Mountains, Oregon, USA (Belden et al. 2000). Larvae were fed *Tubifex* worms *ad libitum* on day 3 of the experiment.

To quantify darkening, we staged the degree of melanosome dispersion using the five stage index created by Hogben and Slome (1931). Stage one is total aggregation of melanosomes (light skin) and stage five is total dispersion (dark skin). This index is commonly used to quantify darkening responses in amphibians (e.g. Wilson and Morgan 1979, Van Zoest et al. 1989, Rollag 1996). Melanophores were staged five times during the five day exposure (at 0, 12, 24, 36 and 120 hours). For the first two days, readings were done within the half hour after the

lights came on in the morning (0 and 24 hours) and in the half hour before lights went off at night (12 and 36 hours). The final reading was done half an hour before the lights went off at the end of day 5 (120 hours). We used this schedule to examine initial diel changes as well as the longer term response to UV-B exposure, which was our main interest. Melanophore readings were done by the same person at each time and were done blind with regard to treatment.

As we were mainly interested in the longer term responses to UV-B exposure, we analyzed the difference in the melanophore index between the three treatment groups only at the 120 hour time point using an analysis of variance (ANOVA).

Experiment 2: growth and survival

To address whether skin darkening offers a survival advantage, we exposed *A. gracile* and *A. macrodactylum* larvae to UV-B while on either a dark or light background and recorded growth and survival after three weeks. For these experiments, five *A. gracile* egg masses were collected 25 km W of Corvallis, Benton County, OR, USA, returned to the laboratory and reared until large enough to test. After hatching, larvae were randomly assigned to 38 L aquaria (5/aquaria) filled with dechlorinated tapwater. They were maintained in the laboratory at 18 °C on a 12:12 light:dark cycle, with complete water changes done once a week. Larvae were fed brine shrimp (*Artemia franciscana*) every other day until large enough to consume *Tubifex* worms, at which time they were switched to that diet.

Mean length (± SD) of the *A. gracile* at the time of testing was 26.4 (±2.3) mm. *Ambystoma macrodactylum* larvae were collected from an ephemeral pond in the Cascade Mountains, Oregon, USA approimately 25 km S of Sisters, Oregon. These were maintained in the laboratory under the same conditions as the *A. gracile* larvae for two weeks prior to testing. For *A. macrodactylum*, the mean length (± SD) at the time of testing was 32.5 (±1.6) mm.

One week prior to UV-B exposure, 28 larvae were placed in 15 cm diameter petri dishes filled with 1 cm depth dechlorinated tapwater. These were then randomly assigned to either a black or white background. Background color was used to manipulate the skin color of the larvae, as many amphibians change color in response to background coloration (Bagnara and Hadley 1973). Backgrounds were made of 20 cm^2 corrugated, plasticized black or white cardboard that were placed beneath the dishes. Larvae remained in the laboratory on these backgrounds for one week to allow them to acclimate to the appropriate background color prior to UV-B exposure. We used the same UV-B lighting regime and temperature as above. For background coloration in the UV-B room, we used white and black plastic sheeting drapped on all four sides and on the bottom of the test area for each background. The table was divided in half such that the same light tubes were used to illuminate both the black and white sides. The night before UV-B exposure began, seven larvae from each background group were randomly assigned to either UV-B exposed (acetate filter) or UV-B shielded (mylar filter) groups. This resulted in seven larvae in each of the four treatments: 1) black background with UV-B, 2)

black background without UV-B, 3) white background with UV-B and 4) white background without UV-B. Larvae were fed *Tubifex* worms every other day and complete water changes were done once a week. At the end of three weeks, the melanophore index and length was recorded for each individual. We analyzed the differences in darkening (melanophore index) using two-way ANOVAs with UV-B exposure (yes/no) and background (black/white) as the factors. Differences in length were analyzed using an ANCOVA with UV-B exposure (yes/no) and background (black/white) as factors and initial length as a covariate.

Results

Experiment 1: skin darkening in response to UV-B exposure

After five days, larval roughskin newts, *Taricha granulosa*, and Northwestern salamanders, *Ambystoma gracile*, that were exposed to UV-B were darker than the larvae exposed to full spectrum lighting without the UV-B component (overall ANOVA for *T. granulosa*, $p= 0.005$, Tukey post-hoc between acetate and mylar groups, $p =0.003$; overall ANOVA for *A. gracile*, $p= 0.015$, Tukey post-hoc between acetate and mylar groups, $p =0.03$; Figure 1). In addition, long-toed salamanders, *A. macrodactylum* showed the same trend for darkening with UV-B exposure, although it was not statistically significant (Overall ANOVA, $p=0.259$; Figure 1). In all three species, larvae in the low light treatment (black cover) were intermediate in coloration between the UV-B exposed and non-exposed individuals (Figure 1). In addition, there were strong patterns of diel

change during the first two days for both Ambystomatids (Figure 1), with melanosome dispersion (darkening) occurring over the course of each day and melanosome aggregation occuring during the night. This pattern was not as strong for *T. granulosa*.

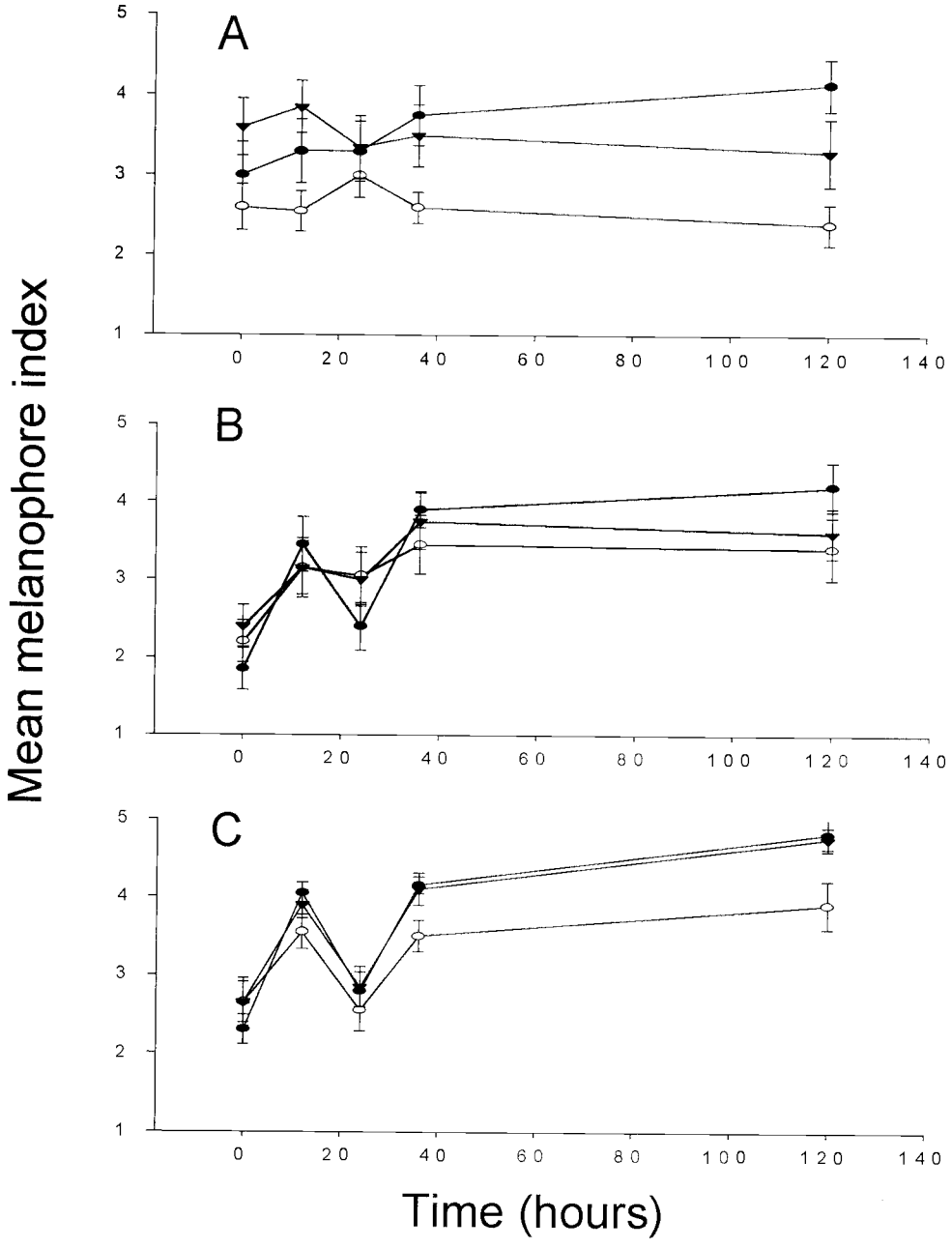

Figure 6.1. Mean melanophore index for (a) *T. granulosa*, (b) *A. macrodactylum* and (c) *A. gracile* at 0, 12, 24, 36 and 120 hours of UV-B exposure. **T** represents larvae exposed to UV-B. O represents larvae exposed to light without the UV-B component. **t** represents larvae in dim light (black cover). Melanophore index from Hogben and Slome (1931) with 1 lighter and 5 darker. Statistics were performed only on larvae at the 120 hour time point.

Experiment 2: growth and survival

In experiment 2, for both species, variation in skin color was explained best by background coloration (*A. gracile*, $p < 0.005$ for background; *A. macrodactylum*, $p < 0.005$ for background; Figure 2). Larvae in the black environment were significantly darker than larvae in the white environment. The interaction between background coloration and UV-B was non-significant for all tests, although there was a trend ($p=0.089$) for an interaction for the length of *A. gracile*. Differences in length after three weeks were best explained by UV-B exposure for both species, with intial length also explaining a significant amount of the variation (*A. gracile*, $p < 0.005$ for UV-B and $p<0.005$ for intial length; *A. macrodactylum*, $p = 0.018$ for UV-B and $p<0.005$ for intial length; Figure 2). For both species, larvae that were exposed to UV-B were smaller after three weeks. Only 3 larvae (out of 56) died during the three week exposure and these were all *A. gracile* larvae exposed to UV-B on a black backgroun

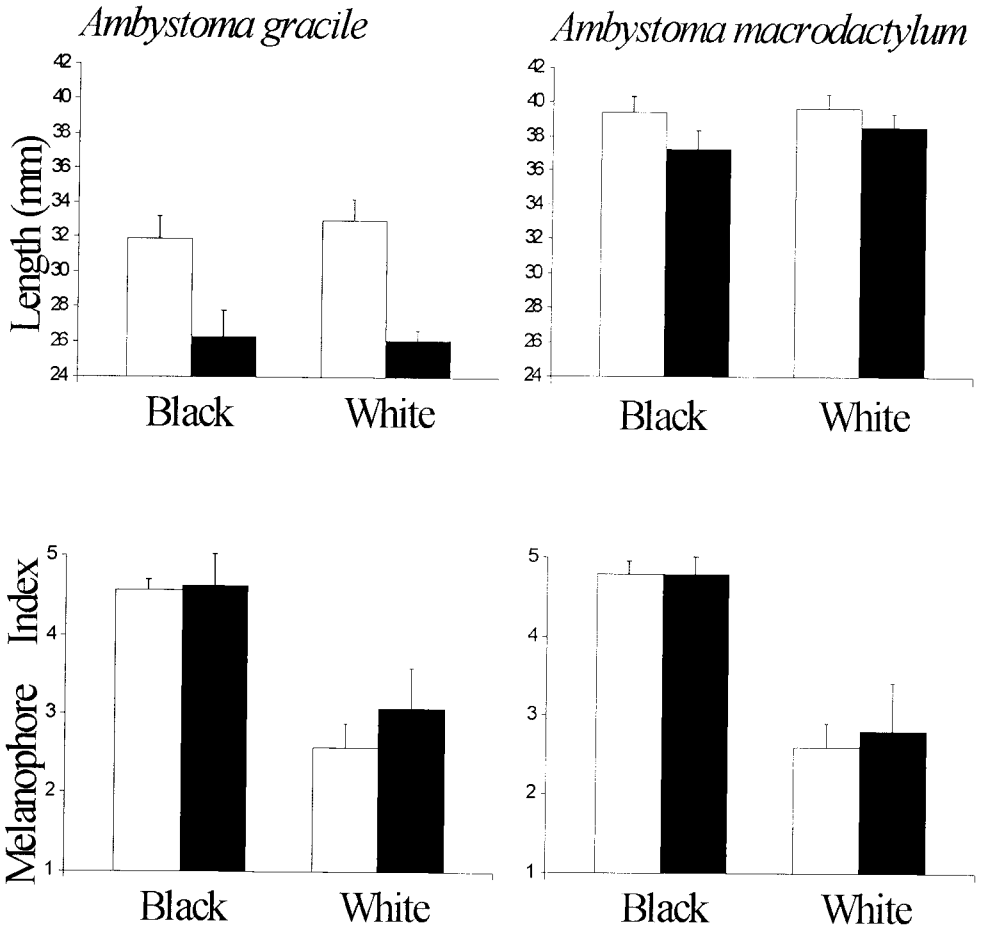

Figure 6.2. Mean length (mm) and mean melanophore index of *A. gracile* and *A. macrodactylum* on white/black backgrounds with/without UV-B after 3 weeks. Melanophore index from Hogben and Slome (1931) with 1 lighter and 5 darker. White bars represent larvae exposed to light without the UV-B component. Dark bars represent larvae exposed to UV-B.

Discussion

Color change in amphibians is controlled by a number of mechanisms, but the main endogenous regulator of the darkening response is α-MSH (α-melanocyte

stimulating hormone). This hormone is a peptide that is synthesized and released by the *pars intermedia* of the pituitary gland (Camargo et al. 1999). In response to α-MSH, melanosomes, organelles which contain the dark melanin pigment, are dispersed into the cytoplasm of the cell (melanophore), causing the skin to darken. Skin lightening occurs when the melanosomes are aggregated around the nucleus. In this study, we have demonstrated that larvae of three species of salamanders darken in response to UV-B exposure. We think it is likely that this response is controlled by α-MSH, although we have not measured α-MSH in our UV-B exposed larvae because they are too small to collect enough plasma from for the radioimmunoassay.

Melatonin, an indoleamine produced in the pineal gland, also plays an important role in color change in amphibians. Daily oscillations in color change, similar to those we saw for the two Ambystomatids in experiment 1, are thought to be controlled in large part by melatonin. Melatonin is released at night, with increased levels causing melanosome aggregation and nocturnal blanching of the skin (Bagnara 1965, Camargo et al. 1999). This role of melatonin is further supported by the fact that the daily oscillations are abolished in African clawed frogs, *Xenopus. laevis,* and bullfrogs, *Rana catesbieana,* when held under constant lighting (Camargo et al. 1999).

However, while we have demonstrated that UV-B can induce darkening on a neutral background, the results from our second experiment indicate that background color has a much stronger impact than UV-B exposure on skin color.

While individuals exposed to UV-B on white backgrounds were slightly darker than non-exposed white background individuals, this was not statistically significant.

We did not find support for our hypothesis that dark larvae would be less impacted than light larvae in terms of growth and survival. In fact, the only larvae in our second experiment that died were exposed to UV-B on dark backgrounds. One possibility for this is that larvae exposed to UV-B may be undergoing a hormonal stress response. In that case, ACTH (adrenocorticotropic hormone) would be released from the anterior pituitary. Simultaneous α-MSH release from the anterior pituitary, which results in darkening, could be a byproduct of that stress response. For some fish, MSH has been suggested to play an important role in the stress response (Wendelaar Bonga et al. 1995). However, we saw sublethal effects on growth in all UV-B exposed treatments, which might indicate stress, without concurrent skin darkening. In addition, we have measured levels of corticosterone in *A. macrodactylum* exposed to UV-B in the laboratory for seven days and have not seen signficant increases in corticosterone levels, which would be indicative of a hormonal stress response (Belden, unpublished data). The protective role of skin darkening for larval amphibians exposed to UV-B remains unclear.

We have previously observed reduced growth in *A. macrodactylum* exposed to UV-B as compared with unexposed controls (Belden et al. 2000) and we now report a similar response in *A. gracile* larvae. These types of sublethal effects can be important as larval amphibians much reach a minimum size threshold for

metamorphosis (Wilbur and Collins 1973). This is particularly critical for larval amphibians developing in temporary ponds, where they must metamorphose prior to pond drying. Larger amphibian larvae also are generally larger when they metamorphose, which can have positive consequences for adult fitness (e.g. Smith 1987, Semlitsch et al. 1988).

This study suggests that larval salamanders darken when exposed to UV-B radiation. However, it remains unclear whether skin darkening provides protection from UV-B damage for larval amphibians. Skin darkening may be a byproduct of a more general stress response. Because UV-B can reduce growth and potentially impact fitness, understanding the physiological mechanisms that may be important in regulating the effects of UV-B exposure is key to understanding the impacts that future increases in UV-B are likely to have.

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Chapter 7 General Conclusions

While many studies evoke ozone depletion as a reason to examine responses to UV-B exposure, one of the main conclusions of my research is that even at current levels, UV-B radiation is an important factor shaping the life histories of amphibians. Current UV-B levels can decrease growth and development in larval long-toed salamanders and red-legged frogs (Chapters 2, 3 and 4), can result in decreased food consumption in larval long-toed salamanders (Chapter 4), and can induce skin darkening in at least three species of larval salamanders (Chapter 6).

As current levels of UV-B can impact amphibians, it is important to consider the potential mechanisms utilized by these animals to repair or prevent damage caused by UV-B exposure. This is an especially interesting area of research, given the well-documented differences in amphibian species sensitivity to UV-B (Blaustein et al. 1998). Potential protective mechanisms include the possibility of photorepair of damaged DNA, behavioral avoidance of UV-B and the use of protective pigments. Over evolutionary time these organisms have likely developed multiple methods for coping with UV-B exposure. I examined the possibility of behavioral avoidance of UV-B in larval long-toed salamanders in the field. I found that they did not choose a low UV-B environment over a high UV-B environment per se, although they did prefer shade to sun (Chapter 3). This could mean that they are unable to detect UV-B, although at least one other

Ambystomatid salamander (*Ambystoma mexicanum*) has UV photoreceptors (Deutschlander and Phillips 1995) and a few Australian anurans may detect and avoid UV-B (van de Mortel and Buttemer 1998).

However, these larvae potentially face an interesting trade-off during development. In temporary ponds in the mountains, similar to where I completed the choice experiments, regions with the highest UV-B levels are also the warmest regions (ie, shallow water). Larvae developing in these sites must reach a minimum size for metamorphosis prior to pond drying, and they will grow fastest in the warmest water. So if they were continually avoiding UV-B, they would likely spend less time in the warmer regions of the pond, and chances of reaching metamorphosis would decrease. Larvae in the field must balance the risk of cellular damage and decreased growth associated with continual UV-B exposure, with the benefit of maximizing growth by spending time in warmer water.

In addition to behavioral avoidance, skin darkening might also prevent damage from UV-B. I found that several species of larval salamanders darken in response to UV-B exposure (Chapter 6). However, I was not able to establish that darkening prolonged survival during UV-B exposure. To the contrary, the only individuals that died during my experiment were those on black backgrounds (with dark skin) that were exposed to UV-B. For mammals, while darker skinned individuals are less prone to skin cancer than lighter skinned individuals (Barker et al. 1995), the protective role of producing melanin over the course of UV-B exposure (i.e., tanning) is unclear (Wu 1999). Salamander larvae may provide a

good model for further studies examining the UV-B induced skin darkening response in vertebrates.

Another main theme to emerge from my research is that there are a multitude of potential sublethal responses to UV-B exposure. Growth, development, behavior and physiology can all be altered by exposure to UV-B. Perhaps most interesting is that for red-legged frogs, embryonic exposure can result in smaller, less developed larvae (Chapter 2). Even though larvae may be able to behaviorally avoid UV-B or spend more time in low UV environments, individuals exposed as embryos may already be at a disadvantage by the time they hatch. Indeed, size and rate of growth can be important for larval anurans. Larger tadpoles may be better competitors (e.g. Travis 1980), be more likely to attain the size threshold necessary for metamorphosis prior to pond drying (e.g. Wilbur and Collins 1973, Morey and Reznick 2000) and be better able to avoid or ignore gape-limited predators (e.g. Puttlitz et al. 1999, Eklov 2000). In addition, larger larvae generally become larger metamorphic anurans which can have positive effects on adult fitness (e.g. Smith 1987, Bervin 1990). These types of delayed effects have not been well investigated, but could have important consequences.

In Chapter 5, I investigated whether larvae of four amphibian species would respond to UV-B exposure with a hormonal stress response. I completed these experiments to try to provide insight into the proximate mechanism of the sublethal responses to UV-B. During stressful situations, glucocorticoid hormones are released from the adrenal cortex (Wingfield et al. 1998). These hormones mobilize

energy stores and suppress non-vital physiological processes until the stress passes. In the short-term, this can be beneficial to the organism, however, long-term exposure to stressful situations can have deleterious effects, including decreased growth, depressed immune response and decreased reproductive output (Sapolsky 1993).

Growth and development can be influenced in amphibians via this stress axis (Hayes and Wu 1995a, 1995b, Denver 1997, 1998). I was interested in whether this mechanism might explain decreased growth and development observed in UV-B exposed larvae. The most important glucocorticoid hormone involved in the stress response in amphibians is corticosterone (Idler 1972). I found no differences in levels of corticosterone in UV-B exposed and shielded larvae of four species (Cascades frogs, *Rana cascadae*; Pacific treefrogs, *Hyla regilla*; long-toed salamanders, *Ambystoma macrodactylum*; spotted frogs, *Rana pretiosa*). There are several possible explanations for the lack of increased levels in UV-B exposed larvae we observed. One explanation is that UV-B does not induce a physiological stress response or is not perceived as stressful by these larvae. Another possibility is that there was a stress response but the timing of our experiments did not allow us to observe it. Though this study resulted in few definitive answers regarding larval amphibians physiological response to UV-B, the questions it raises concerning the response of amphibians to an increasingly important abiotic factor are worthy of consideration and should prompt further research in this area.

Finally, Chapters 3 and 4 highlight the necessity to consider population differences in discussions of amphibian population declines. I demonstrated population differences for long-toed salamander larvae in response to UV-B exposure. Larvae from high elevation sites are more resistant to the detrimental effects of UV-B exposure than are larvae from low elevation sites. Often we assume that spatial variation in environmental factors is the cause of specific population declines. However, there can also be differences in responses of individual populations to these factors. This could result in a pattern of amphibian population declines similar to that caused by variation in the environmental factors themselves.

The emerging view of causation for amphibian population declines is complex. Many of these declines appear to be occurring against a background of global environmental change (e.g. Pounds et al. 1999, Kiesecker et al. 2001). However, in addition to global changes, it seems likely that there are a multitude of different specific local agents at each site. For instance, red-legged frogs, *Rana aurora*, which have disappeared from much of their historic range in the Willamette Valley, Oregon, USA, are likely being affected by habitat loss, introduced species (bullfrogs) and chemical pollution from agricultural areas (Kiesecker and Blaustein 1997, Kiesecker and Blaustein 1998, Marco et al. 1999). However, for western toads, *Bufo boreas*, in the Cascade Mountains, Oregon, climate fluctuation, UV-B and disease appear to be the most important factors (Kiesecker et al. 2001).

However, despite all of our knowledge on the causes of amphibian population declines, very few management or policy changes have occurred to try and reverse these losses. Developing management strategies for declining amphibians should be one of the main objectives of future research efforts.

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