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Tiffany Sacra Garcia University of Kentucky

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Abstract of Dissertation

Tiffany Sacra Garcia

The Graduate School

University of Kentucky

# INTERACTING COLOR AND BEHAVIOR RESPONSES TO MULTIPLE SELECTION PRESSURES IN THE SISTER SALAMANDER SPECIES *AMBYSTOMA BARBOURI* AND *AMBYSTOMA TEXANUM*.

### ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

Tiffany Sacra Garcia Lexington, Kentucky Director: Dr. Andrew Sih, Professor of Biology Lexington, Kentucky 2002

#### ABSTRACT OF DISSERTATION

# INTERACTING COLOR AND BEHAVIOR RESPONSES TO MULTIPLE SELECTION PRESSURES IN THE SISTER SALAMANDER SPECIES *AMBYSTOMA BARBOURI* AND *AMBYSTOMA TEXANUM*.

My research explores the complex strategies animals adapt to cope with multiple selection pressures. I studied the behavioral and color response of two salamander sister species, *Ambystoma barbouri* and *A. texanum*, to temperature, predation risk and ultraviolet radiation (UVR, 280-320 nm). *Ambystoma barbouri* undergo development in streams, while *A. texanum* larvae inhabit ponds. Thus, *A. barbouri* are exposed to increased habitat ephemerality, enhanced predation risk, and UVR exposure. I show how *A. barbouri* have evolved alternate coping mechanisms in response to these environmental factors, relative to *A. texanum*. In this comparison study, I've quantified the affects of these selection pressures on larval color change, refuge use and depth choice.

I found *Ambystoma barbouri* to have a significantly darker mean color than *A. texanum*. Additionally, both species significantly change color to match their background and in response to temperature. When exposed to warm temperatures, early-stage larvae of both species became lighter. Both species also changed color over ontogeny, with larvae becoming significantly lighter over development. Remarkably, *A. texanum* larvae mediated risk from predatory fish chemical cues by visually assessing the degree to which they cryptically match their background. If cryptic, *A. texanum* larvae remained on that background color rather than in refuge. *A. barbouri* larvae preferred to hide in refuge or on dark backgrounds regardless of crypticity, but quickly change color to match their new background. I found that both species darken in response to UVR. When given the choice of refuge, both species spent significantly more time in hiding when UVR was present. When given a choice of water depth, larvae preferred deep water in the presence of UVR radiation.

Adapting multiple color and behavioral responses to individual selection pressures help organisms mediate conflicting demands from multiple selection pressures. For example, when predatory fish are present, larvae should move to shallow water to avoid predation. In the presence of UVR, however, larvae should prefer deeper water. I found *A. barbouri* larvae choose deep water to avoid high UVR exposure despite the risk of predation. Evolving multiple behavioral strategies allows *A. barbouri* larvae to avoid UVR damage and mediate predation risk.

KEYWORDS: Ambystoma, color, behavior, multiple selection pressures, conflicting responses.

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The Graduate School University of Kentucky 2002

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#### Chapter 1

#### Introduction

Organisms are exposed to many environmental factors within their habitats and lifetimes. Multiple selection pressures can often create conflicts in the traits they are selecting for. However, conflicts in optimal trait response can be mediated by adapting compensatory responses (e.g., behavioral, morphological, physiological, life history) that allow organisms to cope in their environment. Evolving multiple responses to multiple selection pressures improves an individual's chance of survival in a complex environment.

Two types of traits that can be adaptive responses to multiple environmental factors are color and behavior. Color patterns can improve an individual's ability to thermoregulate, reduce predation risk and screen ultraviolet radiation (Bagnara and Hadley 1973, Endler 1988, Kollias et al 1991). Adaptive behavior can enhance fitness relative to feeding, predator avoidance, communication and reproductive needs (Krebs and Davies 1997). These two types of traits can also interact to help mediate conflicting selection pressures, or as complimentary responses to single selection pressures, (e.g. effective camouflage requires appropriate prey behavior and background color choice).

Few studies have focused on joint color and behavior responses to multiple, conflicting environmental factors. I address this issue by comparing sister species of salamanders, *Ambystoma barbouri* and *A. texanum*, which differ in mean color, behavior and habitat and thus, in selection pressures. Key selection pressures affecting these species include habitat ephemerality, predation risk and exposure to ultraviolet radiation. These environmental factors are thought to have major impacts on most amphibian populations. They are also factors that are rapidly changing due to human interference and could be contributing to amphibian population decline (Blaustein and Wake 1990, Pechmann et al. 1991).

Ephemeral habitats favor rapid development in amphibians. One way to increase metabolic and growth rates, and decrease time until metamorphosis, is to increase ambient temperature (Wilbur and Collins 1973, Voss 1993). Thus, individual fitness and population dynamics can depend on habitat temperature regimes and individual temperature preference (Carey 1978, Hutchison and Dupre 1992). Behaviors adapted to regulate body temperature (i.e.

thermal habitat choice, aggregation, refuge use) can also be complimented by thermoregulatory coloring. Color affects heat absorption and therefore, body temperature (Brattstrom 1993, Hoppe 1979, Navas 1996, Rome et al.1992).

Predation is often a major source of mortality for amphibian larvae (Werner and Anholt 1993, Sih et al. 1992). Prey commonly respond to predation risk with adaptive color and behavioral responses. Predators have been shown to alter amphibian behavior (e.g., habitat use, refuge use, activity, feeding rates (Sih 1992, Lima 1998)), and color (Kats and Van Dragt 1986, Endler 1988). In response to predatory sunfish, both *A. barbouri* and *A. texanum* decrease their activity and time spent out of refuge and thus, their feeding rate (Huang and Sih 1990, Sih et al. 1992, Sih and Kats 1994).

Ultraviolet wavelength designations [UV-A (320-400nm), UV-B (280-320nm) and UV-C (<280)] are each distinct in biological significance as well as absorption characteristics. UV-B is the most prevalent type of ultraviolet radiation (UVR) inside the Earth's atmosphere, and is regulated by the concentration of ozone in the upper stratosphere. Biological damage caused by UV-B includes immunosuppression, mutagenesis and burning of the integument (Licht and Grant 1997). Ultraviolet rays attenuate quickly out of the water column, with attenuation coefficients increasing with particulate matter concentrations and turbidity (Kirk 1983).

The influence of ultraviolet radiation on color and behavior in amphibians has not been thoroughly studied. Ultraviolet radiation has, however, been shown to be a detrimental factor in the fitness of amphibians in all life stages (Anzalone et al. 1998, Blaustein et al. 1994, 1995, 1998, Licht and Grant 1997, Ovaska et al. 1997). Because of decreasing ozone concentrations, amphibian response to increased UVR exposure is a critical question in applied evolutionary ecology (Kerr and McElroy 1993). Very little work has previously been done on how color affects UVR resistance in amphibian larvae. However, pigmentation in amphibians could be useful in either reflecting or absorbing harmful UVR wavelengths (Blaustein et al. 2001, Kollias et al 1991).

This dissertation addresses the issue of multiple responses to multiple selection pressures in the sister salamander species, *Ambystoma barbouri* and *A. texanum*. *A. texanum*, was once classified as having two forms: the pond form, which breeds in ephemeral ponds and is common in much of the eastern United States, and the stream form, confined mostly to central Kentucky ephemeral streams (Petranka 1982; Petranka et al. 1987). Evidence suggests that an *A. texanum*-

like ancestor invaded into streams and evolved into *A. barbouri* (Kraus and Petranka 1989). This move into streams resulted in exposure to novel selection pressures; in particular, increased habitat ephemerality, new visual predators and greater exposure to UVR (Petranka 1983, Petranka and Sih 1987, Sih et al. 1992, 2000, 2002). As a result, *A. barbouri* have adapted with increased activity, feeding and development rates relative to *A. texanum*, along with a difference in mean body color (Chapter 2, Petranka and Sih 1987, Maurer and Sih 1987).

Interestingly, neither *A. barbouri* nor *A. texanum* appear to exhibit effective camouflage within their respective habitats. *A. barbouri* live on light brown to yellow limestone substrate but are often dark brown to black, while the pale yellow *A. texanum* live in dark, muddy, debris-filled ponds (Storfer et al. 1999). This ineffective camouflage could be a result of conflicting selection pressures. That is, the evolution of color might be shaped by conflicting demands such as the need for rapid development, camouflage from predators, and UVR screening. The cost of ineffective color can perhaps, be mediated by compensatory behavior, such as color-dependent habitat choice and refuge use. Likewise, the ability to change color may compensate for ineffective behavioral strategies.

Examples of possible conflicts include responses to temperature and UVR. UVR is most intense in shallow, clear water. The need for increased development rates may, however, favor the use of warm, shallow areas of the stream. This conflict may be mediated by color change, background preference or refuge use. Another example of a possible conflict is the pressure for rapid development and the need for cryptic coloration. *A. barbouri* typically have a dark color in early larval stages, presumably to maintain a higher metabolic rate or as protection from UVR exposure. These larvae do not, however, cryptically match their light-colored background. *A. barbouri* inhabit streams with predatory fish, while *A. texanum* inhabit ponds with no visual predators. However, both species behaviorally respond to predators with increased refuge use and decreased activity (Huang and Sih 1990, Sih et al. 1992, Sih and Kats 1994). Selection for dark color conflicts with the cryptic needs of *A. barbouri*, but could be mediated by color-dependent habitat choice or refuge use.

In Chapter two, I studied species differences in color and color change in *A. barbouri* and *A. texanum*. Previous work has shown that amphibians can color change in response to many environmental factors, including temperature, predation risk and ultraviolet radiation. Multiple cues triggering color change may result in conflicting selection on body color. This study

focuses on species variation in mean color and physiological color change in larval *A. barbouri* and *A. texanum*. These species differ in habitat and selection pressures, with *A. barbouri* inhabiting clear, highly ephemeral streams with predatory fish, while *A. texanum* live in ponds with no visual predators and murky water, which attenuates ultraviolet radiation (UVR).

I contrasted mean color and color change on two different backgrounds (black and white) for both species, and found *A. barbouri* larvae are significantly darker than their sister species over both backgrounds. Additionally, I found both species change color to better match their background, i.e. larvae held on dark backgrounds are darker than larvae held on light backgrounds. In nature, however, neither species cryptically matches its background, with dark *A. barbouri* typically found on light colored, limestone substrate, and light *A. texanum* in muddy, dark colored ponds.

The difference in body coloration between these two species, and their conspicuousness in natural habitats despite their ability to background match, can be explained by the notion that other factors besides visual predation risk (e.g. thermoregulation and UVR screening) have major effects on the evolution of color in these species, and that the overall set of conflicting demands differ between the two habitat types. The pressure for fast development in streams could be directly, and indirectly, responsible for this difference in body color. A. barbouri larvae typically forage in the shallow, warmer areas of streams. Darker colors may increase heat absorption in larval amphibians, ultimately increasing development rates. In addition, shallow areas preferred by A. barbouri expose larvae to high levels of UVR. Previous studies have shown larvae darken when exposed to UVR, presumably because dark pigmentation absorbs short wavelengths, not allowing further penetration and damage. If dark color is a strategy against UVR damage, A. barbouri larvae may have adapted this relatively dark color to tolerate the shallow, warmer areas of the stream. These selection pressures, which influencing larval color, differ in strength between habitats and may have caused this divergence in mean coloration between sister species. The ponds larval A. texanum inhabit have higher particulate concentrations within the water column, protecting the light colored larvae from UVR, and ponds take longer to dry up relative to streams with A. barbouri, lessening the pressure to develop quickly.

In Chapter 3, I examined the effects of temperature and ontogeny on color change in *A*. *barbouri* and *A. texanum*. Temperature has been shown to affect body color in several species of amphibians. This interaction between color and temperature may also change over larval

ontogeny, perhaps due to age-related or seasonal changes in selection pressures on color. In this study, I found that early stage larvae respond to cold temperatures with a darker color relative to warm temperatures. There is also an ontogenetic effect on larval color for both species, with larvae becoming lighter with age. Older larvae show decreased plasticity in color change to temperature compared to younger stages, with no difference in color response between temperature treatments for *A. barbouri*, and a reversal in the direction of change for *A. texanum*, with cold temperatures inducing a lighter color relative to warm temperatures.

This plastic color response to temperature for young larvae, the progressive lightening of larvae over development, and the apparent loss of color plasticity to temperature over ontogeny raises interesting questions concerning environmental factors selecting for dark colored early-stage larvae and light colored late-stage larvae. At a proximate level, cold temperatures trigger the release of Melanocyte Stimulating Hormone (MSH), which causes the dispersal of pigments within the integument, and could be the mechanism by which young *A. barbouri* and *A. texanum* larvae become dark in cold temperature treatments. Cold seasonal temperatures are typical in temperate zones when *Ambystoma* eggs are hatching, and could prompt this dark color in young larvae. Seasonal warming over larval development is correlated with the ontogenetic change in color, with both species becoming lighter over time.

Ultraviolet radiation (UVR) has also been shown to cause amphibian larvae to darken (Chapter 5). Early stage larvae may be subjected to high levels of UVR because of the reduced canopy cover. Ontogenetic lightening of larvae correlates with the progression of spring, and the filling of the overhead canopy, protecting later stage larvae from harmful UVR. However, the loss of plasticity in late stage larvae, and the reversal of color change in *A. texanum*, is not explained by seasonal variation. It is possible that later in larval development, the pressure to develop quickly is not as strong, allowing other environmental factors to take precedent in selecting for body color. Predatory fish inhabit streams with *A. barbouri*, and the presence of predatory cues may initiate a background matching response. These streams are often light in color, which may explain why larvae no longer lighten in cold temperatures later in development. Predation risk may be an overriding factor in the selection of body color for late stage larvae. *A. texanum*, however, do not coexist with visual predators. Therefore, the darkening of older larvae in cold temperatures is still unexplained.

In chapter 4, I studied the effects of predation risk on color change and color-dependent

behavior in *A. barbouri* and *A. texanum*. Although many organisms show multiple responses to predation risk, relatively few studies have examined how prey integrate these multiple responses. I studied the joint expression of color and behavioral responses to predation risk in two sister species that differ in their history of exposure to predatory sunfish. *A. barbouri* inhabits streams where some pools have predatory sunfish, while *A. texanum* lives in fishless, ephemeral ponds. I examined responses to predation risk (to fish chemical cues) in three situations that differed in availability of refuge and substrate color heterogeneity, and thus availability of behavioral options for reducing risk.

With neither refuge nor variation in background color available, both species exhibited color change to better match the available background (i.e., to increase camouflage). Relative to A. texanum, A. barbouri larvae were darker in color and showed a greater range of color change to enhance camouflage. The degree of color change showed by both species, however, did not depend on predation risk. With a choice between light and dark substrates available, but no refuge, A. texanum exhibited behavioral background matching (i.e., they preferred substrates that matched their own body color), but the degree of behavioral background matching was not significantly affected by predation risk. In contrast, A. barbouri's substrate preferences did not depend on their initial body color. Instead, they responded to risk by showing a strong preference for dark substrates (i.e., they apparently associated dark substrates with safety) followed by a change to a darker body color. With refuge, and only light colored substrates available outside of refuge, both species responded to risk by increasing their use of refuge. For A. texanum, refuge use was color-dependent; lighter larvae that were well camouflaged (when out of refuge) spent less time in refuge, and should thus suffer less cost of using refuge. In contrast, A. barbouri always showed strong refuge use in response to risk, regardless of their body color. Overall, these results illustrate how environmental heterogeneity and species differences in mean color can govern the interplay of complementary and compensatory behavioral and color responses to predation risk.

In Chapter 5, I investigated color change, refuge use and depth choice responses to ultraviolet radiation in *Ambystoma barbouri* and *A. texanum*. Adaptations to avoid or cope with harmful ultraviolet radiation (UVR) have evolved in many amphibian species. Sub-lethal levels of UVR can select for simple responses in larval amphibians, such as dark pigmentation or preference for UVR protected microhabitats (i.e. under refuge or in deep water). Relatively few

studies have examined color change as a defense against UVR damage, or the interaction between color and UVR avoidance behavior. This study focused on color response to UVR, and whether avoidance behaviors like refuge use and depth choice are color dependent. I quantified these responses in two sister species of salamander larvae that differ in their history of exposure to UVR. *A. barbouri* inhabits ephemeral streams and typically reside in the shallow, clear areas of the stream, while *A. texanum* lives in muddy ponds with high particulate concentrations that can attenuate UVR.

I found that both species of larvae darken in response to UVR, and when given the choice of refuge, significantly increase the proportion of time spent in hiding. Additionally, both species used deeper microhabitats when exposed to UVR, but only *A. barbouri* larvae showed a preference for shallow waters when UVR was blocked out. Neither of these behaviors seems to be color dependent, with larvae from both species taking refuge and preferring deep water in the presence of UVR, regardless of color. Interesting behavioral trade-offs arose when larvae were confronted with conflicting selection pressures from UVR and predation risk. Since risk from predatory fish forced larvae to shallow areas, and UVR forced larvae into deeper water, the combination of the two created a conflict in optimal depth choice. Faced with this conflict, *A. barbouri* preferred deeper, risky areas over shallow water with high UVR exposure. *A. texanum* responded to predation risk with a preference for shallow water, but did not significantly alter depth in response to UVR. Neither species changed color in response to either UVR or predation risk. These UVR induced changes in behavior and color may affect larval feeding, competition and predation rates, and could thus alter aquatic community structure.

In Chapter 6, I looked at the effects of ultraviolet radiation and oviposition site on embryo survivorship in the streamside salamander, *Ambystoma barbouri*. The majority of studies on amphibian responses to ultraviolet radiation (UVR) quantify the effects of direct exposure on survivorship, while relatively few focus on the behavioral adaptations that help individuals avoid or cope with ambient UVR exposure. Protection of amphibian eggs from UVR exposure is especially critical, as UVR-induced damage in early development can significantly impact larval and adult fitness. Choice of oviposition site is a key factor in determining the amount of UVR exposure embryos will encounter during development. In this study, I examined the effects of UVR and oviposition site on egg survival in *A. barbouri*. While most *Ambystoma* are pond breeders and lay their eggs in open water attached to stems and leaves of submerged

vegetation, *A. barbouri* choose to oviposit on the undersides of rocks in ephemeral streams. This unique behavioral adaptation suggests that *A. barbouri* are exposed to selection pressures particular to streams, which are absent in ponds.

In this experiment, I found that when rocks with *A. barbouri* eggs were turned over and exposure to UVR, there was a significant decrease in overall hatching success. Additionally, in the UVR exposed treatment, there was an increase in the proportion of eggs that fell off the rock or died before hatching. This study supports the hypothesis that *A. barbouri* have evolved this cryptic ovipositing behavior in response to pressures from UVR exposure on embryonic fitness. By laying their eggs under large, submerged rocks, female *A. barbouri* adults protect their offspring from UVR damage.

#### Chapter 2

Species differences in color and color change in the sister species *Ambystoma barbouri* and *A. texanum* 

#### **Summary**

Previous work on amphibians has shown that they can color change in response to background color, temperature and ultraviolet radiation. Multiple environmental factors triggering color change may result in conflicting selection on body color. This study focuses on species variation in mean color and physiological color change in larvae of the salamander sister species Ambystoma barbouri and A. texanum. These species differ in habitat and selection pressures, with A. barbouri inhabiting clear, highly ephemeral streams with predatory fish, and A. texanum living in ponds with no visual predators and murky water, which attenuates ultraviolet radiation (UVR). I contrasted mean color and color change on two different backgrounds (black and white) for both species, and found A. barbouri larvae were significantly darker than their sister species over both backgrounds. Additionally, I found both species change color to better match their background, i.e. larvae held on dark backgrounds were darker than larvae held on light backgrounds. In nature, however, neither species cryptically matches its background, with dark A. barbouri typically found on light colored, limestone substrate, and light A. texanum in muddy, dark colored ponds. The difference in body coloration between these two species, and their conspicuousness in natural habitats despite their ability to background match, can be explained by the notion that other factors besides visual predation risk (e.g. thermoregulation and UVR screening) have major effects on the evolution of color in these species, and that the overall set of conflicting demands differ between the two habitat types.

#### Introduction

One goal of evolutionary biology is to understand how, and to what extent, species adapt to new or changing environmental conditions. Invasion by a species into a new habitat allows for exciting opportunities to study the rapid evolution of a species. By comparing closely related species that occupy different habitats, we can measure trait divergence due to changes in selection pressures and identify possible constraints affecting adaptive change (Endler 1986, Sober 1984).

Body coloration is a key adaptive trait that can influence an individual's ability to communicate, thermoregulate, and avoid predators (Endler 1988). Thus, color can be a key component of a species' adaptive response to multiple selection pressures within a given habitat. Color need not be a fixed response. Physiological color change in response to environmental factors such as temperature, predation risk and ultraviolet radiation has been documented in several amphibian and reptile taxa (Bagnara and Hadley 1973, Duellman and Trueb 1986, Endler 1988). However, conflicts in adaptive body color may arise due to multiple selection pressures; e.g. a color that enhances crypsis may conflict with the color that effectively screens ultraviolet radiation. Comparing mean color and plastic color change in closely related taxa that inhabit different environments gives us insight into how these trade-offs are resolved.

I examined larval body color in a pair of closely related sister species of salamander, *Ambystoma barbouri* and *A. texanum. Ambystoma texanum* evolved into a new species, *A. barbouri*, following a habitat shift from ponds to ephemeral streams. The ponds and streams inhabited by *A. texanum* and *A. barbouri* respectively differ in selection pressures such as predation risk, habitat ephemerality (Petranka and Sih 1987) and ultraviolet radiation intensity. Ideally, larvae should exhibit a color that improves crypticity, thermoregulation, and UV radiation screening. A significant difference in body coloration or physiological color response between these two species could represent an evolutionary divergence associated with the habitat switch. I contrasted mean color and color change for *A. barbouri* and *A. texanum* on two different background colors to gain evolutionary insight into how conflicting selection pressures in pond and stream habitats (Maurer and Sih 1996, Storfer et al. 1999) might shape the evolution of color.

#### System

Until recently, the smallmouth salamander, *Ambystoma texanum*, had been classified as having two forms: the pond form, which breeds in ephemeral ponds, and is common in much of the eastern United States, and the stream form, confined mostly to central Kentucky ephemeral streams (Petranka 1983, Petranka et al. 1987). Krause and Petranka (1989) concluded that enough life history differences existed to merit a new species. They proposed that within the last 10,000 years, some *A. texanum* colonized from ponds into ephemeral streams and evolved into the streamside salamander, *A. barbouri*. This move into streams resulted in exposure to at least three novel selection pressures for *A. barbouri* that could affect the evolution of color: increased fish predation, enhanced habitat ephemerality, and increased exposure to ultraviolet radiation (UVR) (Petranka 1983, Petranka and Sih 1987, Sih et al. 2000, 2002).

Predation can account for substantial mortality in *A. barbouri* larvae, particularly in streams with predatory sunfish (Petranka 1983, Sih et al. 1992). In contrast, *A. texanum* rarely co-occur with visual predators such as predatory fish (Petranka and Sih, 1987). Both species respond to fish chemical cues by increasing refuge use and decreasing activity; however, *A. barbouri* show stronger antipredator responses (Sih et al. 2000, 2002). Another way to reduce fish predation is via camouflage (i.e., by cryptically matching substrate color). Ponds inhabited by *A. texanum* often have turbid waters (i.e., high concentrations of suspended particulates) and dark, muddy substrates. In contrast, streams inhabited by *A. barbouri* typically have light colored substrata consisting primarily of limestone bedrock (Storfer et al. 1999). Thus, if visual predation is the dominant selective difference between the habitats, then *A. barbouri* should be lighter in color than *A. texanum*.

Other factors, however, can conflict with selection favoring lighter color for *A. barbouri* than *A. texanum*. Habitat ephemerality (i.e., early habitat drying) is a more important problem in Kentucky streams than ponds (Petranka and Sih 1987). This results in stronger selection favoring rapid development in streams than ponds. In response, *A. barbouri* larvae have evolved higher activity and feeding rates, and more rapid development than *A. texanum* (Petranka and Sih 1987, Maurer and Sih 1996). If dark coloration speeds metabolic rates by increasing heat absorption, it could facilitate activity, feeding and development rates. Dark coloration in terrestrial frogs has been shown to increase body temperature, possibly helping with digestion

and metabolic activity (Carey 1978, Hoppe 1979). However, this has never been shown for aquatic larvae. Based on this consideration, *A. barbouri* should be darker than *A. texanum*.

Color can also play an important role in screening out harmful UVR wavelengths. Both ponds and streams in Kentucky receive little shade from canopy cover until mid-late spring. However, while streams are typically clear and relatively shallow, ephemeral ponds are often murky. Thus, *A. barbouri* larvae should be more frequently exposed to high levels of potentially harmful UVR. Assuming that dark pigments can protect larvae by screening out UVR wavelengths (Kollias et al. 1991), UVR considerations also favor darker color for *A. barbouri* than *A. texanum*.

My second interest was in color change. Rapid color change has been documented in several species of amphibian larvae in response to variation in light, temperature, predation risk and background color (Duellman and Trueb 1986, Kats and van Dragt 1986, King et al 1994, Fernandez and Collins 1988). Plasticity in body color has been observed in both *A. barbouri* and *A. texanum* larvae. Both species tend to be lighter at night than in the day (Garcia and Sih, unpublished data). Here, I examined the ability of these species to change color to better match background color. I hypothesized that the both species should be lighter when held on light versus dark backgrounds. This ability to change color to better match a given background would increase crypsis. Therefore, I predict *A. barbouri*, which coexist with visual predators, will be more apt to match their background.

#### Methods

*Ambystoma barbouri* larvae were collected from streams in Raven Run Nature Preserve, Fayette County, Kentucky. I collected *A. texanum* as eggs from ponds in west central Kentucky. Both species were held in an environmental chamber with constant temperature and photoperiod (15°C, 14h light: 10h dark) and reared to middle-late larval stages (3-4 cm total length). Larvae were held in single species groups of twenty larvae and fed macroinvertebrates ad libitum.

I conducted the experiment outdoors on June 2 and 3, 1999, at the Ecological Research Facility, 10 km. northwest of the University of Kentucky campus. Weather conditions remained partly cloudy for the duration of the experiment, with water temperatures ranging from 20-24°C.

Using a 2x2 factorial design, I tested for mean color differences between larval *A*. *barbouri* and *A. texanum* on two background color treatments (black vs. white). Thirty-six ten-

liter buckets were placed in a 6x6 array in a 10x10 m grid, each sunk 8-cm into the ground to reduce temperature variation throughout the day. All buckets were randomly assigned a color and species treatment, and lined with either black or white sand and plastic sheeting. Each bucket was shaded from direct sunlight by a surrounding tree canopy and filled with eight liters of water taken from a rain-fed collecting barrel. Two larvae were placed in each bucket according to species treatment (N= 9 buckets/trt) and removed after 24 h and photographed. Negligible color change occurred in the time it took to transfer individuals from their treatment containers to the camera apparatus.

I took pictures of each larva using a 150 mm macro lens with a Nikon 90S 35 mm camera to quantify individual color at the termination of the experiment. Previous reflectance analyses on local populations of *A. barbouri* larvae showed variation primarily in brightness values (amount of black versus white), with relatively constant chroma and hue values (Storfer et al. 1999). Similar results were found when *A. texanum* larvae were tested for hue, chroma and brightness consistency (V. Rush and T.S. Garcia, unpublished data). Brightness intensities can be easily measured using black and white photographs. I took two photographs of each larva on Kodak Tmax 400 black and white film. The clearer image was then scanned and analyzed using Adobe PhotoShop 4.0 imaging software.

Using one image for each larva, I quantified brightness from three equally sized regions of the body. Measurements were taken using black vs. white pixel weights within a sizestandardized square on each side of the larval head. Dorsal coloration was quantified using the same size-standardized square at the point midway between the snout and vent on the dorsal side of each larva. Because brightness values were correlated for the three regions, I used a principal component analysis to combine the three measurements into a single measure of larval color for each image. I then ran a two-way ANOVA testing for species differences in color response to light and dark backgrounds. Species differences were assessed using a 2-tailed test. For the background effect I used a 1-tailed criteria because the a priori expectation was that larvae would be darker on darker backgrounds.

#### Results

I found *Ambystoma barbouri* larvae to be significantly darker when compared to their sister species, *A. texanum* (Figure 1). Using principal component analysis, I was able to explain

92% of the variation with the first extracted component. This principal component, representing color, was run against background color and species in a two-way ANOVA. The highly significant species effect, (p<0.0001) indicates a robust color difference between the species (Table 1). *Ambystoma barbouri* larvae were consistently darker on both backgrounds than were *A. texanum* larvae.

Background color also affected the color of individual larvae (p=.035, 1-tailed test). As predicted, both species were lighter when held for 24 hours on a lighter background than on a darker background. While figures using principal components make it difficult to ascertain degree of crypticity, I observed larvae matching their background relatively well after only a few hours on the new background. There was no significant interaction between the background color and species treatments.

#### Discussion

Although the two are closely related sister species, there was a striking difference in the mean color of *Ambystoma barbouri* versus *A. texanum* larvae. Differences in selection pressures between the pond and stream habitats may be responsible for this evolutionary change in larval coloration between species. In addition, both species of larvae exhibited short-term color change to better match their background. After 24 hours of acclimation, the color of individuals held on black backgrounds was significantly darker than the color of individuals on white backgrounds. Below, I explore several differences in selection pressures between ponds and stream that might relate to the evolution of darker color in *A. barbouri*.

When comparing the mean color of each species to the dominant substrate color within each habitat, it is apparent that neither *A. barbouri* nor *A. texanum* exhibit effective camouflage within their respective habitats. *Ambystoma barbouri* tend to be darker than their sister species, regardless of background color. This dark coloration offers relatively little crypticity on light colored limestone substrates typically found in Central Kentucky streams. The conspicuous dark coloration of *A. barbouri* larvae on their natural substrate could lead to increased mortality due to sunfish predation (Storfer and Sih 1998, Storfer et al. 1999). In contrast, ponds inhabited by *A. texanum* larvae often have dark substrates. Thus, *A. texanum* larvae also appear poorly camouflaged. However, ephemeral ponds inhabited by *A. texanum* typically contain few visual predators and therefore suffer little cost to being visually conspicuous.

The lack of cryptic coloration in larval *A. barbouri* could be the result of conflicting selection pressures within the stream habitat. A major fitness concern given *A. barbouri's* highly ephemeral habitat is the need for rapid larval development. Temperature is a major factor influencing larval development, as well as larval color and behavior (Brattstrom 1963, Voss, 1993). A color response to temperature has been found in several species of amphibian larvae (Moriya et al. 1996, Fernandez and Collins 1988, Kats and Van Dragt 1986, King et al. 1994). Cold temperatures in early spring slow down development rates, therefore imposing serious developmental constraints on young larvae (Rome et al. 1992, Petranka and Sih 1987). Selecting darker integument colors may enhance larval development rates by increasing ambient wavelength absorption (Bartlett and Gates 1967). Thermoregulatory behaviors such as aggregation in shallow, warmer waters and exposure to direct sunlight may also assist in increasing temperature, activity and development rates (Brattstrom 1962, 1963, Navas 1996).

Ultraviolet radiation is another selection pressure in both pond and stream habitats. UVR attenuation increases with turbidity, depth in the water column and increases in particulate concentrations (Kirk 1983); thus larvae in shallow, clear waters are more exposed to harmful UVR. Conversely, protection from UVR can be found in deep or murky water columns. Ponds tend to be murkier than the streams inhabited by *A. barbouri*. Therefore, *A barbouri* should be more frequently exposed to high UVR levels, particularly in early spring before the canopy fills in. One potential UVR screening strategy is to adapt a color with reflective or absorptive properties that protects larvae from harmful UVR wavelengths (Kollias 1991, Licht and Grant 1997).

I hypothesize that the darker coloration of *A. barbouri* evolved in part as an adaptation to the need for larvae to grow and develop rapidly in the spring, when temperatures are generally cold. Shallow waters are somewhat warmer, perhaps allowing higher larval activity, feeding and metabolic rates. However, shallow, clear water also exposes larvae to higher UVR levels. Darker coloration might aid in thermoregulation and in screening out UVR wavelengths. Dark color, however, has the cost of increased conspicuousness to visual predators, such as sunfish.

In addition to the difference in mean color between species, I also found that both species plastically changed color to better match their backgrounds. This daytime color change should aid in camouflage relating to visual predators (Storfer et al. 1999). Interestingly, this color change occurred even in the absence of cues from visual predators. An issue for further

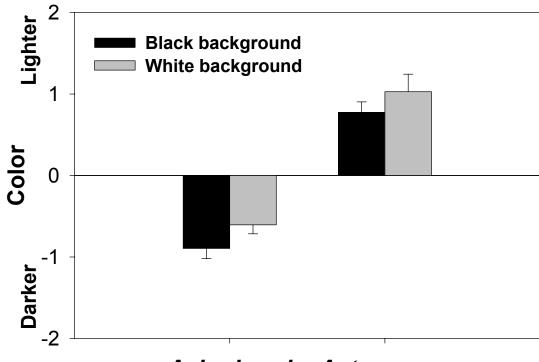
investigation is whether the degree of color change is enhanced when larvae are exposed to predatory cues (Chapter 4). I predicted that because *A. barbouri* inhabits streams with predatory fish, *A. barbouri* should show more color change to match their background relative to *A. texanum*. My results did not support this prediction. Again, it would be interesting to see of this prediction would be upheld when predator cues are present.

Adaptive color change and rapid evolution of color could be critical components affecting species persistence in a changing environment. Here, I documented both the evolution of mean color and plastic color change, but also a likely conflict between adaptive color with respect to UVR damage versus predation risk. Increases in embryo and larval mortality due to UVR exposure have been shown in several Pacific Northwest amphibian species (Blaustein et al. 1994, 1995, 1998). Predation, including exposure to novel predators, (e.g. fish, bullfrogs) is also thought to be an important contributor to the decline of come amphibian species. Amphibian evolution and plasticity is critical for understanding amphibian decline (Blaustein and Wake 1990, Pechmann et al. 1991). Studying the constraints on rapid evolution, as well as conflicting selection pressures is necessary for understanding both the evolution and ecology of amphibian persistence in the face of environmental change.

Table 1. ANOVA results for effects of background color (Black vs White) and species (*A. barbouri* and *A. texanum*) on larval color. DF = degrees of freedom, Asterisks indicate significance.

Source of Variation	SS	df	MS	F	Р
Background	1.043	1	1.043	3.421	0.035*
Species	38.54	1	38.54	126.424	0.001*
Background x Species	0.0045	1	0.0045	0.015	0.904
Error	16.157	53	0.305		

Figure 1. Principal component analysis on mean body color for larvae (*A. barbouri* and *A. texanum*) on black and white backgrounds. Shown are means and standard errors.



A. barbouri A. texanum

#### Chapter 3

Effects of temperature and ontogeny on color change in the larval salamander species *Ambystoma barbouri* and *A. texanum*.

#### **Summary**

Temperature has been shown to affect body color in several species of amphibians. This interaction between color and temperature may also change over larval ontogeny, perhaps due to age-related or seasonal changes in selection pressures on color. In this study, I quantify the effects of temperature on the color of the salamander sister species Ambystoma barbouri and A. *texanum* over larval ontogeny. I found that early stage larvae respond to cold temperatures with a darker color relative to warm temperatures. There is also an ontogenetic effect on larval color for both species, with larvae becoming lighter with age. Older larvae show decreased plasticity in color change to temperature compared to younger stages, with no difference in color response between temperature treatments for A. barbouri, and a reversal in the direction of change for A. texanum, with cold temperatures inducing a lighter color relative to warm temperatures. This plastic color response to temperature for young larvae, the progressive lightening of larvae over development, and the apparent loss of color plasticity to temperature over ontogeny raises interesting questions concerning environmental factors selecting for dark colored early-stage larvae and light colored late-stage larvae. At a proximate level, cold temperatures trigger the release of Melanocyte Stimulating Hormone (MSH), which causes the dispersal of pigments within the integument, and could be the mechanism by which young A. barbouri and A. texanum larvae becoming dark in cold temperature treatments. Cold seasonal temperatures are typical in temperate zones when *Ambystoma* eggs are hatching, and could prompt this dark color in young larvae. Seasonal warming over larval development is correlated with the ontogenetic change in color, with both species becoming lighter over time. Ultraviolet radiation (UVR) has also been shown to cause amphibian larvae to darken. Early stage larvae may be subjected to high levels of UVR because of the reduced canopy cover. Ontogenetic lightening of larvae correlates with the progression of spring, and the filling of the overhead canopy, protecting later stage larvae from harmful UVR. However, the loss of plasticity in late stage larvae, and the reversal of color

change in *A. texanum*, is not explained by seasonal variation. It is possible that later in larval development, the pressure to develop quickly is not as strong, allowing other environmental factors to take precedent in selecting for body color. When larvae are close to metamorphosis, and developmentally ahead of schedule, it is possible that other concerns can then determine mean body color. Predatory fish inhabit streams with *A. barbouri*, and the presence of predatory cues may initiate a background matching response. These streams are often light in color, which may explain why larvae no longer lighten in cold temperatures later in development. Predation risk may be an overriding factor in the selection of body color for late stage larvae. *A. texanum*, however, do not coexist with visual predators. Therefore, the darkening of older larvae in cold temperatures is still unexplained.

#### Introduction

Color change in larval amphibians occurs in response to many environmental cues, including temperature, background color, ultraviolet radiation and stress (Bagnara and Hadley 1973, Carey 1978, Hoppe 1979, Kats and Van Dragt 1986, King et al. 1994). Body color in aquatic, larval amphibians mediates thermoregulation, increases crypsis through background matching, and reduces exposure to ultraviolet radiation (UVR) by screening out harmful wavelengths with dark pigments (Chapter 5, Endler 1988). However, multiple environmental factors can exact conflicting selection pressures on color. For example, dark body color may be appropriate for screening UVR or for thermoregulation, but could increase conspicuousness to predators (Chapter 4). Color on two time scales, an immediate behavioral time scale and over larval ontogeny, may help mediate the multiple selection pressures acting on color in aquatic environments.

While temperature influences body color in amphibians, it is also a major factor in determining larval growth rates, development rates, time until metamorphosis and behavior (Smith-Gill and Berven 1979, Wilbur and Collins 1973). Behavioral responses to temperature, such as larval aggregation in warm areas and avoidance of highly variable thermal regions, illustrate the importance of temperature selection to larval fitness (Brattstrom 1962, Navas 1996). Temperature can also have indirect effects on larval amphibians. For example, in predator-prey interactions, warm temperatures can cause larvae to grow quickly, creating a size refuge from gape-limited predators (Brodie and Formanowicz 1983, Anderson et al. 2001). Temperature can also indirectly affect mortality, with warm temperatures facilitating the drying of ephemeral habitats (Laurila and Kujasalo 1999).

Color change as a function of temperature has been observed in several amphibian species. Cold temperatures trigger the dispersal of dark-colored pigment within color cells (melanophores), causing an overall darkening of the skin (Duellman and Trueb 1986). For example, *Hyla crucifer* and *H. cinerea* both darken in cold temperatures, and lighten when conditions are warmer. However, neither species effectively changes color to match light colored substrates when temperatures are cold (Kats and Van Dragt 1986, King et al. 1994). Thus, although this dark coloring in response to cold temperatures may be a good thermoregulatory defense mechanism against freezing, larvae can become less cryptic with their

background. If color change is a fixed response to temperature, they may have limited ability to modulate color in response to other environmental changes (i.e. predation risk, UVR exposure).

Although not well studied, body color in salamanders has also been shown to change over ontogeny (Fernandez and Collins 1988). Larval color change over ontogeny could be a response to seasonal variation in selection pressures. For example, if thermoregulatory concerns determine larval color, I predict that as temperatures increase from spring to summer, larvae will get lighter with age. Ultraviolet radiation is another environmental factor showing seasonal variation. If UVR exposure is responsible for a darker color in early stage larvae, seasonal growth of the UV-filtering overhead canopy may allow for lighter color in late stage larvae.

This study examines larval color response to temperature over ontogeny in two sister species of salamander. I predict a color response to temperature, and a difference in how larvae respond to temperature over ontogeny. Although closely related, these larval salamanders inhabit two distinct aquatic environments, with different degrees of predation risk, UVR exposure and ephemerality. In both habitats, for both species, selection pressures influencing color change thus vary over the course of larval development. Here, I've quantified the effects of temperature on color during early and late larval stages, and how larval color changes as a function of ontogeny.

#### System

The smallmouth salamander, *Ambystoma texanum*, was once classified as having two forms: the pond form, which breeds in ephemeral ponds and is common in much of the eastern United States, and the stream form, confined mostly to central Kentucky ephemeral streams (Petranka 1982; Petranka et al. 1987). Evidence suggests that an *A. texanum*-like ancestor invaded into streams and evolved into *A. barbouri* (Kraus and Petranka 1989). This move into streams resulted in exposure to novel selection pressures; in particular, increased habitat ephemerality, new visual predators and greater exposure to UVR (Petranka 1983, Petranka and Sih 1987, Sih et al. 1992, 2000, 2002). As a result, *A. barbouri* have adapted with increased activity, feeding and development rates relative to *A. texanum*, along with a difference in mean body color (Chapter 2, Petranka and Sih 1987, Maurer and Sih 1986).

Ambystoma barbouri larvae are significantly darker then their sister species, A. texanum, and both species respond to varying background colors with cryptic, background matching color

change (Chapter 2). The difference in mean color is presumably due to differences between habitats in selection pressures, such as UVR exposure and predation risk. Temperature, however, also affects color, thus limiting larval ability to respond to other environmental factors such as UVR and predation risk. Water temperature for both streams and ponds in late winter-early spring is relatively cold, ranging from 5-12°C. Limitations on color change due to cold temperature may only affect early stage larvae; later in development, seasonal warming of water temperature may reduce temperature constraints on color.

Ponds and streams in Kentucky receive little shade from canopy cover until middle-late spring. This exposes early stage *A. texanum* and *A. barbouri* larvae to direct sunlight and full, ambient UVR. Previous work has shown that both species get darker when exposed to ambient levels of UVR (Chapter 5). Ponds, however, offer relatively more protection from UVR than streams with murkier, deeper waters and darker substrates. Thus, the fact that early stage larvae appear to be dark in nature could be a response to either cool temperature or high UVR. In this experiment, I quantify larval color response to temperature over ontogeny to extract temperature's role in determining larval color. The degree to which temperature affects color change has potentially important impacts on larval ability to avoid predators and mediate UVR exposure.

I predict that the stream breeding species, *A. barbouri*, will respond to cold temperatures with a dark body color. Previous studies have shown *A. texanum* to be lighter than *A. barbouri* (Chapter 2). I predict that under identical temperature treatments, this relationship will continue. Thus, I expect *A. texanum* to darken in cold temperatures, but to maintain an overall lighter mean color relative to their sister species. In addition, I predict both species will lighten over larval ontogeny. Color change over larval development could be a result of seasonal increases in temperature and reductions in UVR exposure because of a growing canopy. Later stage larvae may show limited ability to plastically change color in response to temperature, although I expect the direction of color change to stay the same.

### Methods

I collected newly hatched *Ambystoma barbouri* larvae from streams in Raven Run Nature Preserve, Fayette County, Kentucky and Wildcat Creek, Anderson County, Kentucky. *Ambystoma texanum* eggs were collected from ponds in Beaver Dam, Kentucky and Livingston

County, Kentucky and reared to early larval stages to insure correct identification. Both species were held in an environmental chamber at the University of Kentucky, Lexington, Kentucky with constant temperature and photoperiod (15°C, 14h light: 10h dark). Individuals were held in single species groups of twenty larvae and fed macroinvertebrates ad libitum.

Forty early-stage *A. barbouri* larvae and 40 early-stage *A. texanum* larvae (0.1-0.2g for both species) were randomly placed into two incubation chambers (20 individuals/ per species/ per chamber) in individual 1 liter opaque Mason jars filled with 500 ml of filtered, aerated tap water. Both incubators were set at a 14h: 10h photoperiod and randomly assigned a temperature treatment, (Incubator A at 10°C, Incubator B at 20°C). After being held in their temperature treatments for 24 hours, digital images were taken of each larva using a Nikon Coolpix 950 digital camera. Negligible color change occurred during the time it took to remove larvae from the incubators and photograph them. Immediately after taking pictures, temperature treatments for both incubators were switched, (Incubator A at 20°C, Incubator B at 10°C) and larvae were held in the second temperature regime for another 24 hours. Again, digital images were taken of each larva.

Following the early stage color experiment, larvae were held individually in 17.5 cm diameter, gray colored containers with 1 liter of filtered, aerated tap water and fed macroinvertabrates ad libitum. Larvae were kept in constant 15°C temperature and fed every two days for a period of four weeks before being tested a second time.

Again, larvae were allocated randomly to temperature treatments (Incubator A at 10°C, Incubator B at 20°C). Digital images were recorded after 24 hours of exposure to these temperatures. As in the earlier experiment, the temperature in each incubator was then switched and larvae held for another 24 hours in the new temperature (Incubator A at 10°C, Incubator B at 20°C). Digital images were again taken of each larva. Each larva was treated as an individual data point, even though only two incubators were used. There is no reason to expect larvae in separate containers could influence each other's body color, as they could not see or communicate with each other. Additionally, temperature and incubator are not confounded in this experiment because both incubators experienced both temperatures.

Previous color analyses on light and dark *A. barbouri* and *A. texanum* larvae showed that larvae vary primarily in brightness values (amount of black versus white), with relatively constant chroma and hue values (Grill and Rush 1999, Storfer 2000). Using digital images of

each larva, I quantified brightness from three equal sized regions of the body. Measurements were taken using black vs. white pixel weights within a size-standardized square on each side of the larval head. Dorsal coloration was quantified using the same standardized measurement square at the point midway between the snout and vent on the dorsal side of each larva. Because brightness values were correlated for the three regions, I used a principal component analysis to combine the three measurements into a single measure of larval color for each image.

Differences between species, and effects of temperature and ontogeny on color were tested using repeated measures ANOVAs. My design included two levels of repeated measures: measurements at two temperatures for early stage larvae, and for the same two temperatures for the same larvae at a later stage. Results from a single repeated measures ANOVA using all these data are difficult to decompose and interpret. In order to examine short-term color responses to temperature I ran separate repeated measures ANOVAs for early and late stage larvae, with species as a grouping factor and temperature as the repeated measure treatment. To address ontogenetic changes in color, I ran separate repeated measures ANOVAs for 10° and 20° C, with species as the grouping factor and age as the repeated measures treatment.

### Results

# Temperature and color change

I quantified a strong difference in color between the two species (Table 2.A); *A. texanum* larvae were considerably lighter in color than its sister species *A. barbouri* (Figure 2). Temperature had a significant effect on larval color for both species in early stages of larval development. Early-stage larvae of both *A. texanum* and *A. barbouri* were lighter in the 20° C treatment than in the 10° C treatment. Although both species reacted in the same direction to warm temperatures, young *A. texanum* lightened to a greater degree than young *A. barbouri* (Table 2.B).

In late stage larvae, warm temperatures had different effects on the two species. While temperature had no significant effect on larval color in *A. barbouri*, warmer temperatures induced darker color in *A. texanum*. There also remained a striking, significant difference in color between species (Table 2.C), with older *A. texanum* being much lighter color than older *A. barbouri* over both temperature treatments (Figure 2).

Color change over Ontogeny

There was a significant difference between species in color over ontogeny for both temperatures, with *A. texanum* maintaining a lighter color relative to *A. barbouri* (Table 3.A and C, Figure 3). In general, larval color became lighter over ontogeny (Table 3.B and D). For *A. barbouri*, this was true regardless of temperature. However, for *A. texanum*, they grew much lighter over ontogeny when tested at 10°C, but showed relatively little change in color over ontogeny when tested at 20°C. Early stage *A. texanum* larvae were already light in color when tested at 20°C, and showed relatively little tendency to grow even lighter over ontogeny.

# Discussion

My study showed that early-stage *Ambystoma barbouri* and *A. texanum* larvae were lighter in the warmer temperature treatment relative to the colder temperature treatment. In addition, I found an ontogenetic change in mean body color for both *A. barbouri* and its sister species *A. texanum*. Over larval development, both species became lighter in color. However, temperature did not have an effect on the color of late-stage *A. barbouri* larvae. Furthermore, warm temperatures induced a darkening response in older *A. texanum*. While this darkening by late stage *A. texanum* goes against my predicted response, the body color of these late-stage *A. texanum* larvae in warm temperatures is still significantly lighter than most other treatments.

Temperature is one proximate mechanism controlling color in early stage larvae, with cold temperatures inducing a dark color. Cold temperatures trigger the release of Melanocyte Stimulating Hormone (MSH), which disperses dark colored pigment cells (melanophores), causing an overall darkening of the skin (Duellman and Trueb1986). Both *Ambystoma barbouri* and *A. texanum* darkened in the cold temperature treatment in the early-stages, which is consistent with the hypothesis that MSH is triggered by cold temperatures and is playing a role in *Ambystoma* larval color change.

One possible selective force explaining dark early-stage larval coloration is the need for fast development rates. Both *A. barbouri* and *A. texanum* live in ephemeral habitats. Field surveys suggest that streams inhabited by *A. barbouri* are more likely to dry than ponds used by *A. texanum* before larvae have metamorphosed into the terrestrial stage. If dark coloration speeds metabolic rates by increasing radiation absorption, it could lead to an increase in activity, feeding and development rates. Dark coloration in terrestrial frogs has been shown to increase

body temperature, possibly helping with digestion and metabolic activity (Carey 1978, Hoppe 1979). However, this has never been shown for aquatic larvae. Indeed, water has such a high specific heat capacity that it seems unlikely that color could raise individual body temperature much above ambient.

Instead, dark color might yield a thermoregulatory benefit via an indirect pathway through the effect of body color on UVR screening. Previous work on *A. barbouri* and *A. texanum* showed that both species darken in response to ambient levels of UVR (Chapter 5). Dispersal of dark pigments throughout the dermal layer is assumed to protect larvae by absorbing UVR and not allowing further penetration of the DNA damaging wavelengths. Canopy cover is lowest in the early spring when larvae are hatching. Thus, early-stage larvae receive maximum possible UVR exposure. This direct exposure could explain dark body color for young larvae of both species.

UVR screening by dark pigments can then aid in thermoregulation. Streams in particular are often considerably warmer in shallow eddies than in deeper region. Shallow areas, however, are subject to high UVR exposure. Dark color might allow larvae to use these warm, shallow zones where they can be more active, maintain high metabolic rates and thus potentially exhibit high growth and developmental rates.

Over ontogeny, larvae of both species lighten in color. This progressive lightening over time could be due to predictable changes in environmental selection pressures over the spring growing season. Water temperatures continually get warmer over the larval period. Thus, as the season progresses there should be reduction in the need for larvae to be dark as an adaptation to cool temperatures. Alternatively, selection for dark color to reduce UVR damage should also decrease seasonally due to increased canopy cover. Finally, selection for rapid development may also not be as strong in late stage larvae. These larvae appear to be able to facultatively transform. If dark color aids in rapid development, and rapid development is no longer a selective pressure for larvae able to metamorphose, then larvae may lighten as they approach transformation.

Interestingly, over larval ontogeny, individuals either stopped responding to temperature with changes in body color, or reversed the direction in which they changed color relative to earlier in larval development. While late-stage *A. barbouri* did not significantly change color between temperature treatments, *A. texanum* larvae got darker when exposed to warm

temperatures. In *A. barbouri*, this loss of a plastic response to temperature may be the result of a switch in the relative importance of environmental factors influencing color. Dark color can be beneficial for thermoregulatory and UVR screening purposes; however, in streams, dark colors (on a typically light colored substrate) make larvae highly conspicuous to visual, predatory fish (Storfer et al. 1999). Early in the season, temperature considerations appear important enough to favor a plastic color response. However, later in the season, if selection favoring a color response to temperature is relaxed, but the cost of being conspicuous remains, this might favor a decoupling of the color response to temperature in order to allow a less constrained response to risk. The explanation for why *A. texanum* reverses their color response to warm temperatures over ontogeny is unclear.

While both species lightened over larval development, in all situations tested, *A. texanum* were lighter than *A. barbouri*. This difference in color between sister species is an important indicator of differing selection pressures between habitats. *A. texanum* inhabit murky ponds with abundant refugia from UVR (i.e. under detritus or with water depth). In contrast, *A. barbouri* are usually found on shallow, bare substrates, and might remain relatively dark to protectively screen out UVR. Alternatively, *A. texanum* may have adapted this lighter mean color because of a cost associated with being dark. In the absence of forces selecting for dark coloration (i.e. UVR exposure, cold temperatures) larvae may adopt a light color. This is supported by a study showing that *A. barbouri* and *A. texanum* larvae lighten at night, presumably because of the absence of UVR and pressure from visual predators (Garcia and Sih, unpublished data). Overall, color in these two species appears to be influenced by a complex set of environmental forces. Further study is required to better understand their interacting effects.

Table 2. Repeated measures ANOVA for temperature effects (10°C and 20°C) on early and late stage larval color response (*A. barbouri* and *A. texanum*). Between subjects shows differences in color between species, and within subjects indicates color differences over ontogeny. DF = degrees of freedom, Asterisks indicate significance.

Source	dF	MS	F	Р
Early stage larvae				
A) Between Subjects				
Species	1	120.49	524.76	0.0001*
Error	44	0.23		
B) Within Subjects				
Temperature	1	11.95	61.07	0.0001*
Temperature x Species	1	5.91	30.19	0.0001*
Error	44	0.19		
Late stage larvae				
C) Between Subjects				
Species	1	66.72	94.1	0.0001*
Error	44	0.71		
D) Within Subjects				
Temperature	1	0.94	4.46	0.04*
Temperature x Species	1	3.55	16.76	0.0001*
Error	44	0.21		

Table 3. Repeated measures ANOVA for ontogenetic effects on larval color (*A. barbouri* and *A. texanum*) at two temperatures (10°C and 20°C). Between subjects shows differences in color between species, and within subjects indicates color differences over ontogeny. DF = degrees of freedom, Asterisks indicate significance.

Source	dF	MS	F	Р
Temperature = 10C				
A) Between Subjects				
Species	1	86.47	289.21	0.0001*
Error	44	0.29		
B) Within Subjects				
Time	1	148.21	385.91	0.0001*
Time x Species	1	0.57	1.48	0.231
Error	44	0.38		
Temperature = 20C				
C) Between Subjects				
Species	1	96.94	233.74	0.0001*
Error	44	0.42		
D) Within Subjects				
Time	1	60.01	241.86	0.0001*
Time x Species	1	12.68	51.12	0.0001*
Error	44	0.25		

Figure 2. Principal component analysis on mean body color for young and old *A*. *texanum* and *A. barbouri* larvae over two temperature regimes.

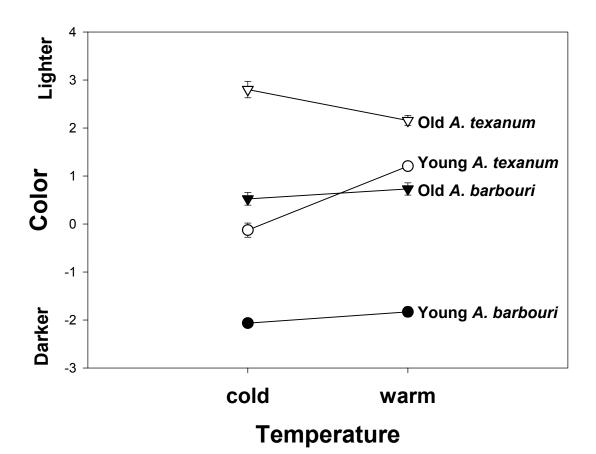
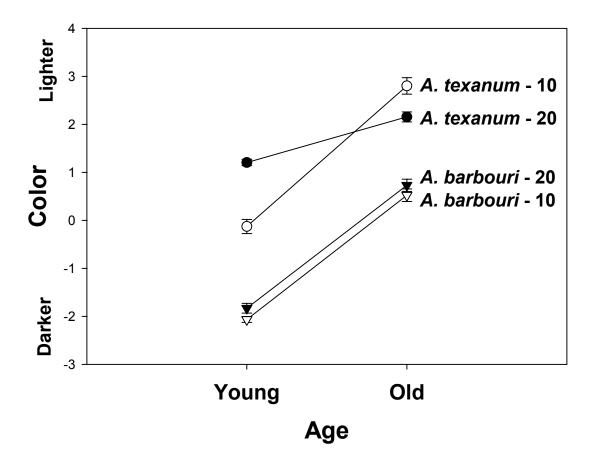


Figure 3. Principal component analysis on mean body color for young and old *A. texanum* and *A. barbouri* larvae over two temperature regimes.  $10 = 10^{\circ}$ C, and  $20 = 20^{\circ}$ C.



#### Chapter 4

Effects of predation risk on color change and color-dependent behavior in salamander sister species *Ambystoma barbouri* and *Ambystoma texanum*.

#### **Summary**

Although many organisms show multiple responses to predation risk, relatively few studies have examined how prey integrate these multiple responses. I studied the joint expression of color and behavioral responses to predation risk in two sister species of salamander larvae that differ in their history of exposure to predatory sunfish. A. barbouri inhabits streams where some pools have predatory sunfish, while A. texanum lives in fishless, ephemeral ponds. I examined responses to predation risk (to fish chemical cues) in three situations that differed in availability of refuge and substrate color heterogeneity, and thus availability of behavioral options for reducing risk. With neither refuge nor variation in background color available, both species exhibited color change to better match the available background (i.e., to increase camouflage). Relative to A. texanum, A. barbouri larvae were darker in color and showed a greater range of color change to enhance camouflage. The degree of color change showed by both species, however, did not depend on predation risk. With a choice between light and dark substrates available, but no refuge, A. texanum exhibited behavioral background matching (i.e., they preferred substrates that matched their own body color), but the degree of behavioral background matching was not significantly affected by predation risk. In contrast, A. barbouri's substrate preferences did not depend on their initial body color. Instead, they responded to risk by showing a strong preference for dark substrates (i.e., they apparently associated dark substrates with safety) followed by a change to a darker body color. With refuge, and only light colored substrates available outside of refuge, both species responded to risk by increasing their use of refuge. For A. texanum refuge use was color-dependent; lighter larvae that were well camouflaged (when out of refuge) spent less time in refuge, and should thus suffer less cost of using refuge. In contrast, A. barbouri always showed strong refuge use in response to risk, regardless of their body color. Overall, these results illustrate how environmental heterogeneity and species differences in mean color can govern the interplay of complementary and compensatory behavioral and color responses to predation risk.

### Introduction

A major issue in evolutionary ecology concerns the effects of predation risk on prey decision-making. Predators have been shown to alter prey behavior (e.g., habitat use, refuge use, activity, feeding rates (Sih 1992, Lima 1998)), morphology (Havel 1993, DeWitt et al. 1999), color (Kats and Van Dragt 1986, Endler 1988), and life history (Reznick et al. 1992, 1993, Crowl and Covich 1990, Sparkes 1996). While most studies of prey responses to predators have focused on one of these types of responses, real prey often show multiple responses (Endler 1995, DeWitt et al. 1999). In addition, the overall prey response to predators is often shaped by tradeoffs that arise from multiple, conflicting selection pressures (Sih 1987, 1992, Lima 1998).

Predation is often a major source of mortality for amphibian larvae (Sih et al. 1992, Wilbur 1972). Accordingly, larvae show various antipredator adaptations including increased refuge use, decreased activity, dispersal away from predators (Sih et al. 1992, Werner and Anholt 1993), unpalatability (Kats et al. 1988), and shifts in morphology (Van Buskirk 1998) and life history (Skelly 1992, Relyea 1999). These antipredator responses, however, can result in reduced feeding and growth rates, and ultimately, a decrease in fecundity (Lima and Dill 1990, Skelly 1992, Ball and Baker 1996). Reduced feeding, growth and developmental rates can be particularly costly to larvae that live in ephemeral habitats (e.g., shallow, temporary ponds or streams that dry up by late spring). In these shallow, temporary habitats, other selection pressures that can conflict with predator avoidance include early habitat drying (Wilbur 1972, Petranka et al. 1987), thermoregulatory needs and avoidance of damaging levels of ultraviolet radiation (Blaustein et al. 1994, 1995, 1998).

One type of larval amphibian antipredator response that has not received as much recent study is color change. Coloration is a key adaptive trait that can influence an individual's ability to communicate, thermoregulate, and protect itself from predators (Norris and Lowe 1964, Endler 1988). Color change in response to environmental factors such as predation risk, temperature, and ultraviolet radiation has been documented in several amphibian, fish and reptile taxa (Chapters 3 and 5, Duellman and Trueb 1986, Endler 1988). Color change by amphibian larvae in response to predation risk appears to increase crypticity and thus, lower predation risk (Kats and Van Dragt 1986, Endler 1995, Heinen 1994).

Color and behavior can interact as components of an overall antipredator response

(Brodie 1992). In the simplest scenario, if larvae are found on a uniform-colored substrate with no refuge, then color change to match their background (i.e., to be camouflaged) might be their primary antipredator response. To be effective, camouflage requires prey to be inactive (Wickler 1968). If, however, the substrate has a range of colors, then rather than rely on color change alone, prey can move quickly to a background that matches their current color (i.e., they can exhibit color-dependent substrate choice; Endler 1988). Over a longer period, they can use color change to further improve their background matching. Overall then, prey might show a complementary set of responses featuring behavioral background matching followed by color change to enhance camouflage, and inactivity when camouflaged.

Finally, if refuge is available, prey often chose to hide in refuge when predators are present (Sih et al. 1988, 1992). Refuge use, however, typically has a cost in reduced feeding rate (Lima 1998). Prey that are more effective at color change or behavioral background matching (i.e., that are better camouflaged and thus less susceptible to predation when outside of refuge (Storfer et al. 1999)), might have the option of spending more time feeding (as ambush predators) out of refuge. Emphasizing the converse, prey that show relatively poor color responses to risk might compensate behaviorally by using refuge; i.e., prey might show color-dependent refuge use. If behavior and color interact in any of the above ways, then a full understanding of behavior requires knowledge about prey color and vice versa. Although the above color/behavior scenarios seem reasonable, they remain relatively understudied. To my knowledge, no previous study has examined them all in a single system.

I studied the above issues in a pair of sister species that differ in color and behavioral response to predators. Larvae of the streamside salamander, *A. barbouri*, are significantly darker than their sister species, the smallmouth salamander, *A. texanum* (Chapter 2). *A. barbouri* also show stronger antipredator responses to predatory fish cues than *A. texanum* (Kats et al. 1988, Sih et al. 2000). The evolutionary divergence in color and behavior between *A. barbouri* and *A. texanum* may be, in part, due to differences in predation risk between their habitats. *A. barbouri* lives in primarily ephemeral streams that nonetheless often include permanent pools with predatory fish, while *A. texanum* lives in ephemeral ponds that rarely have fish. Here, I focus on interactions between color and behavioral responses to fish cues.

#### System

The salamander sister-species pair, *Ambystoma barbouri* and *A. texanum*, is an excellent system in which to study color and behavioral responses to predation risk. Until recently, the smallmouth salamander, *A. texanum*, had been classified as having two forms: the pond form, which breeds in ephemeral ponds, and is common in much of the eastern United States, and the stream form, confined mostly to central Kentucky ephemeral streams (Petranka et al 1987, Krause and Petranka 1989). In 1989, Krause and Petranka proposed that within the last 10,000 years, *A. texanum* colonized from ponds into ephemeral streams and evolved into the streamside salamander, *A. barbouri*.

This move into streams resulted in exposure to at least three novel selection pressures for *A. barbouri* that could affect the evolution of color and behavior: increased fish predation, enhanced habitat ephemerality, and increased exposure to ultraviolet radiation (UVR) (Petranka 1983, Petranka and Sih 1987, Sih et al. 2000). Predation accounts for a substantial amount of larval mortality in *A. barbouri*, particularly in streams with predatory sunfish (Petranka 1983, Sih et al 1992). In contrast, *A. texanum* occupy temporary ponds, and are thus rarely exposed to predatory fish (Petranka and Sih 1987). At my study sites, *A. texanum* do not appear to co-occur with any important visual predators (e.g. no fish, odonates, or large salamanders). Although *A. texanum* does not typically co-occur with fish, both *A. barbouri* and *A. texanum* larvae respond to predatory fish (and to fish chemical cues) with increased refuge use and decreased activity (Kats et al. 1988, Sih and Kats 1991 Sih et al. 1988, 2000, 2002). *A. barbouri*, however, generally show stronger responses than *A. texanum* (Sih et al. 2000). Nonetheless, in nature *A. barbouri* larvae still incur heavy predation by sunfish (Sih et al. 1992).

My primary interest here is in contrasting the joint behavioral and color responses to risk exhibited by the two species. However, other selection pressures also likely influence these traits. Both species live in ephemeral habitats. Field surveys suggest that streams inhabited by *A. barbouri* are consistently more ephemeral than the ponds used by *A. texanum* (Petranka and Sih 1987). Accordingly, the stream species has evolved higher activity, feeding, growth and developmental rates that result in a significantly shorter larval period (Petranka and Sih 1987; Maurer and Sih 1996). The streams used by *A. barbouri* are also shallow, and typically more clear than the murky ponds inhabited by *A. texanum*. Thus, the stream species likely experiences

greater selection to cope with high levels of potentially damaging UVR. Salamander larvae can respond to UVR by behavioral avoidance or by color change (Chapter 5) – the same sorts of responses shown to predators.

On average, *A. texanum* larvae are significantly lighter in color than their sister species *A. barbouri* (Chapter 2). Interestingly, this contrasts sharply with the natural substrate in their respective habitats. The ponds inhabited by *A. texanum* often have turbid waters (i.e., high concentrations of suspended particulates) and dark, muddy substrates. In contrast, streams inhabited by *A. barbouri* typically have light colored substrata consisting primarily of limestone bedrock (Storfer et al. 1999). Thus, if visual predation is the dominant selective difference between the habitats, then *A. barbouri* should be lighter in color than *A. texanum*. This, however, is not the case.

In addition to the difference in mean body coloration between species, plasticity in body color has also been observed in both species. Both species become darker when exposed to UVR (Chapter 5). Also, both species tend to be lighter at night than in the day (Garcia and Sih, unpublished data), due perhaps to the absence of UVR at night, coupled with a possible energetic cost of maintaining a dark color.

Given that these larvae exhibit both color change and behavioral responses to environmental variation, I examined interactions between these types of responses. I focused, in particular, on their joint responses to predation risk in three situations. First, I looked at larval responses to risk when neither refuge nor a choice of substrate colors was available. I predicted that larvae should change their own body color to better match the available background. Second, I provided larvae with a choice between light and dark substrates, but with no refuge available. I predicted that larvae should do behavioral background matching (i.e., prefer substrates that offer better camouflage), followed by subsequent color change to further fine tune their camouflage. Finally, I provided refuge, but only light colored substrates outside of refuge. I expected larvae to exhibit color-dependent refuge use. That is, larvae that do not match their backgrounds should compensate behaviorally by using refuges, while larvae that are better camouflaged might use refuge less. In all situations, I predicted stronger responses when prey are exposed to predator chemical cues. Because of their longer history with fish predation, *A. barbouri* have evolved a stronger response to fish cues. As a result, I predicted *A. barbouri* respond to predatory fish cues with greater color change and refuge use than *A. texanum*.

# Methods

*Ambystoma barbouri* larvae were collected from the Raven Run Nature Sanctuary, Fayette County, KY and Wildcat Creek, Anderson County, KY. *Ambystoma texanum* were collected from Jessamine and Ohio counties, KY, and raised from the egg stage to ensure correct species identification. Both species were held in a temperature and photoperiod controlled environmental chamber (20°C, 12h light: 12h dark) at the University of Kentucky in Lexington, KY. Larvae were fed macroinvertebrates and zooplankton ad libitum up until the time of the experiments.

#### Predation risk and color change

This experiment examined color change in *A. barbouri* and *A. texanum* larvae in the absence of refuge or heterogeneity in substrate color. In this situation, salamander larva should change color to better match the available substrate's color. I examined, in particular, how predator chemical cues influenced color change. Twenty-four *A. barbouri* larvae and 24 *A. texanum* larvae were tested in a  $2 \times 2 \times 2$  factorial design with species, background color and perceived predation risk (predator chemical cues) as treatments. Perceived risk was manipulated by filling 17.5 cm diameter plastic, experimental containers with 1 liter of either control freshwater or water containing predator chemical cues. Chemical cues were obtained from an 80 liter tank holding two adult bluegill sunfish (*Lepomis macrochirus*)(Sih et al. 1988).

Individual larvae were randomly distributed among containers. Half of the larvae were placed in black-bottomed containers, while the other half were placed in containers with a white bottom. All larvae were allowed to habituate for 72 hours in control freshwater. Photographs were then taken with a Nikon 90s 35 mm camera under standardized full spectrum fluorescent lighting to quantify initial color for each individual. Larvae were subsequently moved into identical 17.5 cm diameter containers, but with opposite background colors (i.e. individuals on black backgrounds were transferred to white backgrounds, and vice versa). Half of the larvae in each background color treatment were exposed to fish chemical cues, while the other half stayed in control freshwater. Photographs were taken after three hours of exposure to the new background colors and chemical cue treatments. Negligible color change occurred during the time it took to remove larvae from their treatment containers and photograph them.

Photographs were scanned into Adobe Photoshop 6.0 and analyzed for color change and degree of matching to the background. Previous color analyses on light and dark *A. barbouri* and *A. texanum* larvae showed that larvae vary primarily in brightness values (amount of black versus white), with relatively constant chroma and hue values (Grill and Rush 1999, Storfer et al. 1999). Here, I quantified brightness from three equal sized regions on each larva's body. Measurements were taken using black vs. white pixel weights within a size-standardized square on each side of the larval head. Using the same standardized measurement square, another region located midway between the snout and vent on the dorsal side of each larva was used to quantify dorsal coloration. Larger brightness values indicate a lighter, paler color relative to smaller brightness values. Because brightness values were correlated for the three regions, I used principal component analysis to combine them into a single measure of larval color for each image.

Effects of species, background color and fish chemical cues on color were analyzed using a repeated measures ANOVA. I predicted that both species should change color after their backgrounds are switched, and that the magnitude of color change should be greater when larvae are exposed to predator chemical cues.

### Predation risk and color-dependent background choice

Next, I examined larval color and behavior when provided with a choice between light and dark colored backgrounds. Sixteen *A. barbouri* larvae and 16 *A. texanum* larvae were tested in the presence versus absence of predatory fish chemical cues. Half of the larvae were habituated to a black background and the other half to a white background in individual 17.5 cm diameter containers (filled with 1 liter of control freshwater) for 72 hours. Each larva was then placed into another 17.5 cm diameter container with a substrate background split into two halves – one side black, the other side white. As above, risk was manipulated by filling each container with 1 liter of either control freshwater or water containing predator chemical cues. Chemical cues were obtained from an 80 liter tank holding two adult bluegill sunfish (*Lepomis macrochirus*)(Sih et al. 1988). Behavioral spot checks on location in the container in relation to background color were done every 15 minutes for a total of 4 hours.

Digital images were taken of each larva at the beginning and end of the experiment using a Nikon 950 Coolpix digital camera. Images were downloaded into the Photoshop 6.0 image

analysis program and brightness data taken on three standardized regions (as above) for each individual. As described above, I calculated the mean brightness value, or black vs. white pixel weight for the three standardized areas on each individual. Again, because brightness values were correlated for the three regions, I used principal component analysis to combine them into a single measure of larval color for each image.

I ran repeated measures ANOVAs with both species (and the many interaction terms) in one analysis; however, the output tables were too complicated to be easily interpreted. For more interpretable results, I ran ANOVAs separately for the two species and for the different response variables. I examined effects of initial background color (during the habituation phase) on initial color, and effects of initial background color and predator chemical cues on final color, color change and the proportion of time spent on the white background when given a choice between black and white backgrounds. My main predictions were that larvae should prefer the background color that better matches their body color at the beginning of the choice period, and that they should show stronger behavioral background choice when exposed to fish chemical cues.

#### Predation risk and color-dependent refuge use

Finally, I examined interactions between larval color and behavior when provided with refuge and a light colored substrate outside of refuge. Eighteen *A. barbouri* larvae and 18 *A. texanum* larvae were used to test whether refuge use in response to predatory fish chemical cues is color dependent. I ran a 2 x 2 factorial experiment with species and perceived predation risk as key factors. Again, risk was manipulated by filling each experimental container with 1 liter of either control freshwater or water containing predator chemical cues from two adult bluegill sunfish (*Lepomis macrochirus*).

Half of the larvae were initially held (in 17.5 cm diameter containers with 1 liter of control freshwater) on a black background, with the other half on a white background for 72 hours to generate ample variation in initial color. Digital images were then taken of each larva using a Nikon Coolpix 950 digital camera. Larvae were transferred to new 17.5 cm diameter containers, with a randomized chemical cue treatment, a white background, and a 5 cm x 7 cm piece of black corrugated plastic that larvae readily use as refuge. Spot checks recording refuge use were done at 15-minute intervals for 4 hours. Digital images were taken again at the end of

the experiment. Images were downloaded into Photoshop 6.0 and analyzed for color change over time following the same procedures as in the color-dependent background choice experiment.

As in the previous experiment, for ease of interpretation, I conducted ANOVAs separately for the two species, and for each of several variables. That is, I used ANOVAs to test for effects of previous background on initial color, and effects of previous background and predator chemical cues on time spent out of refuge and final color. Because color change during the experiment should depend on time spent out of refuge (on a white background), I also ran an ANCOVA using time spent out of refuge as the covariate.

# Results

### Predation risk and color change

Larval color change was examined using a repeated measures ANOVA to compare each individual's color before versus after 3 hours of exposure to predator chemical cue treatments (Table 4). The significant time x background interaction indicates that larvae generally responded to the switch in background colors with significant color change within a few hours (Figure 4). Larvae that were habituated to dark backgrounds became lighter when switched to a lighter background. Conversely, larvae switched from light to dark backgrounds got significantly darker to better match the dark background. Fish chemical cues, however, did not significantly influence color change; regardless of risk, both species changed color to better match their background. A significant time x species x background interaction (Table 4) indicates that *A. barbouri* showed a greater range of color change than did *A. texanum* (Compare figures 4a and 4b).

#### Predation risk and color-dependent background choice

Table 5 shows results of ANOVAs on four variables: proportion of time spent on the white background, initial color, final color, and color change. 'Initial color' addresses larval ability to match their background during the habituation period. *Ambystoma barbouri* changed color to match their substrate during the habituation period (i.e., larvae held on a black background were darker than those held on a white background; Figure 5a). In contrast, in this experiment *A. texanum* did not show significant color change to match their background (p =

0.09). All *A. texanum* were relatively light in color regardless of whether they were held on a light or dark background. Regardless of habituation conditions, *A. barbouri* larvae were generally darker than *A. texanum* larvae.

Although previous substrate experience did not influence *A. texanum* color per se, it affected their behavioral background choice. Larvae habituated to a white background spent significantly more time, when given a choice, on the light background than did larvae that previously experienced the dark background (Figure 5b). This pattern was not significantly influenced by the presence of predator chemicals. Perhaps because *A. texanum* larvae tended to prefer the background that matched their initial color, they showed no significant color change during the background choice period (Figure 5c). In contrast, *A. barbouri* background choice was not affected by their previous experience, and thus also not by their initial color (Table 5, Figure 5b). Instead, their background choice was heavily influenced by predator chemical cues. The presence of fish chemicals caused a significant increase in the tendency for these larvae to prefer dark backgrounds (Figure 5b).

Interestingly, given the options to both change color and behaviorally select a matching background, color change by *A. barbouri* depended on their previous background and on the presence of predator chemical cues (Table 5, figure 5c). In the absence of predator cues, *A. barbouri* larvae split their time roughly equally between the light and dark sides of their containers. During this period, larval color changed closer to the overall average; i.e., larvae held beforehand on dark backgrounds got lighter and larvae held beforehand on light backgrounds tended to get darker. In contrast, in the presence of predator chemicals, *A. barbouri* larvae preferred dark substrates. Larvae that were held beforehand on a dark background were already dark and showed no additional color change. Larvae held beforehand on a light background got significantly darker to better match their chosen background color (figure 5c)

### Predation risk and color-dependent refuge use

Tables 3 and 4 show results of ANOVAs and an ANCOVA on effects of previous background and predator chemicals on larval initial color, refuge use and color change during the period of exposure to control water or predator cues. During the habituation period, both species changed color to better match their background color (Table 6, Figure 6a). Again, *A. barbouri* showed a stronger magnitude of color change than *A. texanum*; i.e., at the point when larvae were

moved to predator chemical treatments, most *A. texanum* larvae were light in color, while *barbouri* varied from quite dark to relatively light (Figure 6a).

The two species, however, differed in how their refuge use and color interacted as components of an overall antipredator strategy. As expected, both *A. barbouri* and *A. texanum* increased their refuge use in response to the presence of fish chemical cues (Table 6, Figure 6b). Notably, *A. texanum*'s refuge use was color-dependent. In the presence of fish chemicals, *A. texanum* larvae that were habituated on dark backgrounds (and that were thus darker, on average) spent more than twice as much time in refuge as larvae habituated on light backgrounds. That is, larvae that matched the substrate outside of refuge remained out of refuge even under the threat of predation, while larvae that did not match the background chose to take refuge (Figure 6b). This implies that these larvae can assess their crypticity, and use refuge accordingly. In contrast, *A. barbouri* larvae showed a strong tendency to take refuge under the threat of predation regardless of the degree to which they match the background (Figure 6b).

ANCOVA showed that *A. barbouri* exhibited refuge-use dependent color change (Table 7). Their tendency to get lighter was positively related to time spent out of refuge (on a light background). Color change was particularly striking for *A. barbouri* larvae that were held beforehand on a dark substrate and then observed in water without predator chemicals (Figure 6c). These dark-colored individuals spent roughly 90% of their time out of refuge (on a light background). As a result, they became significantly lighter in color. In contrast, *A. texanum* did not exhibit significant color change during the refuge use assay period.

# Discussion

Prey often show a suite of responses to predation risk involving several types of traits (e.g., behavior, induced morphology, color change, life history shifts) and interactions among traits (Endler 1995, DeWitt et al. 1999). Relatively few studies, however, have focused on multiple responses to risk. Here, I examined interactions between color, color change and two types of behavioral response (background choice and refuge use) to predation risk. Comparisons within and between two closely related sister species showed significant differences in the way that color plasticity and color-dependent microhabitat choice interact. In particular, whether a species showed color-dependent antipredator behavior depended on the relative ability of the two species to plastically alter body color. Ultimately, the difference between the two sister species,

*A. barbouri* and *A. texanum*, in their color and behavioral responses to risk might reflect the different selection pressures found in their respective habitats. Below, I discuss multiple prey responses to predators, and constraints and conflicting demands on these multiple responses in greater detail.

### Multiple responses to predation risk

Given that prey can show multiple responses to predation risk, key issues are: how do these responses interact in terms of their effects on prey fitness? And how do prey then alter their reliance on the different types of responses in different situations? At present, there is no explicit theory to guide my understanding of this issue; however, I suggest the following framework (also see DeWitt et al. 1999).

In some situations, no single tactic works well on its own. Instead, each tactic is effective only if combined appropriately with other tactics. For example, camouflage to avoid detection depends on inactivity and vice versa. Being cryptic, but actively moving, or inactive, but non-cryptic might be largely ineffective strategies. Or, escape ability can depend on again exhibiting the correct blend of morphology and behavior. For example, Brodie (1992) showed that snake escape ability depends on having the correct combination of color pattern and escape behavior. In these scenarios, I obviously expect prey to exhibit the adaptive *complementary* responses to predators.

In other cases, even if multiple tactics or their combination could be effective at reducing predation, many of the options are ruled out by costs or constraints. For example, if prey cannot change their morphology rapidly enough to respond effectively to a new predation regime, then they might need to show *behavioral compensation* for having the 'wrong' morphology. For example, DeWitt et al. (1999) showed that snails that lack the correct size or shape to cope with crayfish predators showed stronger behavioral avoidance than snails that were better defended morphologically.

Prey use of complementary or compensatory responses to risk should depend on plasticity in prey and predator traits, and on environmental conditions. Environmental effects could involve the availability of different types of refuge for prey, or conflicting selection pressures. My experiments directly addressed effects of three environmental situations that differed in availability of refuge and substrate color variation. Differences between prey species

in their use of color change and behavior as antipredator responses in these different situations illustrates some general possibilities that might also apply to other species.

In my system, a key constraint involves species differences in average color that might then explain differences in the range of potential color change. Color change in amphibian larvae is governed by the re-distribution of pigments within pigment cells, in particular, melanophores (Duellman and Trueb 1986). Because *A. barbouri* have a high density of melanophores (relative to *A. texanum*), they have the potential to change from dark to light and vice versa. In contrast, because light colored *A. texanum* larvae have relatively few melanophores, they apparently have a limited ability to become dark. Although *A. texanum* larvae showed a significant tendency (in two out of three experiments) to match the color of their background, the degree of color change exhibited was less than for *A. barbouri*.

For *A. texanum*, their relatively limited ability to do color change required them to exhibit behavioral compensation when behavioral options were available. Rather than rely on color change, they apparently assessed how well their body color cryptically matched the background, and adjusted location (substrate choice or refuge use) accordingly. If their body color did not match the substrate, then they moved to refuge or to a cryptic background. If, however, their body color already matched the substrate, then they showed relatively little microhabitat shift. Given that antipredator behavior has concomitant costs (e.g. reduced activity and increased refuge use tend to reduce feeding rates), color-dependent antipredator responses can reduce these costs.

In contrast, *A. barbouri* larvae are darker and have highly plastic body color. In response to predatory chemical cues, larvae increased refuge use and showed a preference for dark substrates. Interestingly, these behavioral responses occurred regardless of the individual's immediate degree of crypsis; i.e. unlike *A. texanum*, *A. barbouri*'s behavioral response to predators was not color-dependent. If refuge was present, then even if an individual's body color matched the available substrate, it still took refuge. This greater reliance on using refuge when available is probably adaptive because hiding under refuge is almost certainly a more effective anti-sunfish response than relying on crypsis outside of refuge (Sih et al. 1988, 1992, 2000, pers. observation). In the absence of refuge, but with variation in substrate color available, *A. barbouri* larvae showed a strong preference for dark substrates, regardless of their own initial color. This might reflect the fact that as generally dark larvae, they cannot get light enough to be

effectively cryptic on a light colored background, or the possibility that even with perfect background matching, dark prey on a dark background might be inherently more difficult for a visual predator to locate than light prey on a light background. In any case, after exhibiting a relatively set, rapid behavioral shift to dark substrates, *A. barbouri* larvae then showed color change to enhance their background matching.

Overall, the responses exhibited by these larvae include: 1) reliance on one main tactic when one is highly effective on its own (refuge use when refuge is available); 2) complementary responses when a blend is more effective than a single response (*A. barbouri*'s tendency to prefer dark substrates and then turn darker to enhance crypsis); and 3) behavioral compensation when constraints limit one type of response (A. texanum's behavioral background matching when substrate variation in color is available). Further studies on this and other systems are needed to further develop our general understanding of prey use of multiple responses to predators.

# **Conflicting selection pressures**

Many previous studies have emphasized that behavioral responses to predators (e.g., refuge use, activity, vigilance) are shaped by conflicting demands (e.g., feeding demands, see earlier references). These conflicting demands probably also play a role in governing refuge use in my system (Sih et al. 1988, 2000, Storfer and Sih 1998). Here, I focus my discussion instead on how conflicting selection pressures in my system might affect the evolution of color. As noted above, these two sister species differ in average body color, due to a simple morphological basis (melanophore density) that probably also constrains their relative range of color change. What factors might explain the species' difference in average color? In particular, as noted earlier, neither species appears well camouflaged in their native habitats. I showed that both species can change color to better match their background. Why, then, does neither species match their mean substrate color in nature? Possible explanations involve conflicting selection pressures, such as ultraviolet radiation and habitat ephemerality, or relatively weak selection pressure from visual predators.

*Ambystoma barbouri* and *A. texanum* larvae both darken significantly in response to ambient UVR exposure (Chapter 5). The lack of a light, cryptic body coloration in *A. barbouri* could be due to selection for dark color to screen out UVR (Licht and Grant 1997, Blaustein 1998). In particular, perhaps because of habitat ephemerality and strong selection favoring high

activity and rapid development (Petranka and Sih 1987; Maurer and Sih 1996), *A. barbouri* larvae are often found in the shallower, warmer edges of stream pools where UVR exposure is likely to be particularly important. Further indirect evidence that body color might be related to UVR comes from the fact that *A. barbouri* larvae are typically darker in the day and lighter at night (Garcia and Sih, unpublished data). The lighter color at night could be due to the release from UVR pressures.

For *A. texanum*, their lack of crypsis in natural ponds might be explained by the fact that they do not often encounter predatory fish in nature. In addition, *A. texanum* can avoid harmful UVR by microhabitat choice rather than color change. UVR attenuates with increased depth and particulate concentration in the water column. Ponds inhabited by *A. texanum* tend to be deeper or murkier than streams used by *A. barbouri*, thus offering protection from UVR. The lack of selection pressure favoring dark color coupled with a presumed energetic cost of maintaining dark color (e.g., the cost of producing and maintaining pigment cells) have apparently resulted in the evolution of light color in *A. texanum*.

In sum, I suggest that the different selection pressures in streams and ponds have driven the evolution of differences in the magnitude and integration of suites of antipredator responses in these two sister species. At a simple level, because *A. barbouri* has experienced stronger selection pressure from fish, they show stronger overall responses (behavioral and color change) to fish than do *A. texanum*. The different selection pressures appear to have also driven species differences in mean color. Mean color might limit the range of possible color change. In turn, variations in the range of available color change might explain species differences in joint color and behavioral responses to risk. In *A. texanum*, limited color change can be compensated for behaviorally by color-dependent background choice or refuge use. In *A. barbouri*, larvae show strong behavioral responses to risk (microhabitat choices) regardless of color, and then change color to match the chosen substrate. Overall, my study has revealed some novel patterns of interaction between joint responses to predation risk. Further studies of multiple responses to multiple selection pressures should prove insightful. Table 4. Results for a repeated measures ANOVA on factors influencing color change for two species of salamanders (SP). Shown are within-subjects effects. Time indicates directional color change. CC indicates the effect of predator chemical cues, and background (B) is the effect of a previous background on color change. dF = degrees of freedom, Asterisks indicate significance.

Response	Effect	SS	dF	F	Р
Variable					
Color change	Time	0.000	1	0.000	0.985
	Time x CC	0.004	1	0.010	0.921
	Time x SP	0.001	1	0.004	0.953
	Time x B	16.08	1	38.74	0.000*
	Time x CC x B	0.07	1	0.172	0.681
	Time x SP x B	2.368	1	5.704	0.022*
	Time x CC x SP x B	0.216	1	0.522	0.475
	Error	15.35	37		

Response	Species	Effect	SS	dF	F	Р
Variable						
Time spent	A. texanum	Background	0.23	1	5.72	0.018*
on White						
background		CC	0.06	1	1.47	0.13
		Background x CC	< 0.001	1	0.00	0.49
		Error	0.41	10		
	A. barbouri	Background	0.002	1	0.025	0.44
		CC	0.82	1	10.26	0.003*
		Background x CC	0.02	1	0.29	0.30
		Error	1.12	14		
Initial color	A. texanum	Background	0.831	1	2.11	0.09
		Error	3.942	10		
	A. barbouri	Background	16.806	1	22.94	< 0.001*
		Error	10.258	14		
Final color	A. texanum	Background	0.799	1	2.20	0.084
		CC	0.006	1	0.02	0.452
		Background x CC	0.431	1	1.19	0.150
		Error	3.627	10		
	A. barbouri	Background	1.896	1	1.39	0.13
		CC	10.919	1	8.05	0.007*
		Background x CC	0.138	1	0.10	0.38

Table 5. ANOVA results for effects of previous background and predator chemical cues (CC) on time spent out on a white background, initial color and final color and color change for larvae of two species of salamanders. dF = degrees of freedom, Asterisks indicate significance.

	Error	18.991	14		
Color change A. texanum	Background	810.61	1	3.20	0.10
	CC	50.96	1	0.20	0.66
	Background x CC	58.13	1	0.31	0.59
	Error	2536.23	10		
A. barbouri	Background	7.413	1	9.19	0.005*
	CC	3.375	1	4.18	0.030*
	Background x CC	0.145	1	0.18	0.339
	Error	11.292	14		

Response	Species	Effect	SS	dF	F	Р
Variable						
Time spent	A. texanum	Background	0.085	1	6.22	0.014*
out of refuge		CC	0.29	1	21.15	<0.001*
		Background x CC	0.058	1	4.29	0.03*
		Error	0.16	12		
	A. barbouri	Background	0.017	1	0.20	0.33
		CC	0.84	1	10.12	0.003*
		Background x CC	0.02	1	0.24	0.314
		Error	1.24	15		
Initial color	A. texanum	Background	4.241	1	4.46	0.028*
		Error	11.409	12		
	A. barbouri	Background	9.347	1	13.41	0.001*
		Error	10.456	15		
Final color	A. texanum	Background	0.498	1	0.53	0.24
		CC	0.933	1	0.99	0.169
		Background x CC	3.555	1	3.78	0.039*
		Error	11.265	12		
	A. barbouri	Background	2.351	1	1.09	0.16
		CC	7.61	1	3.52	0.04*
		Background x CC	2.47	1	1.14	0.151
		Error	32.461	15		

Table 6. ANOVA results for effects of previous background and predator chemical cues (CC) on time spent out of refuge, initial color and final color of larvae of two species of salamanders. dF = degrees of freedom, Asterisks indicate significance.

Response	Species	Effect	SS	dF	F	Р
Variable						
Color change	e A. texanum	Background	2.998	1	2.42	0.074
		CC	0.018	1	0.01	0.454
		Background x CC	0.084	1	0.07	0.4
		Time out of refuge	1.174	1	0.95	0.16
		Error	13.61	11		
	A. barbouri	Background	3.585	1	2.74	0.06
		CC	0.355	1	0.27	0.305
		Background x CC	2.578	1	1.97	0.091
		Time out of refuge	10.912	1	8.33	0.006*
		Error	18.341	14		

Table 7. ANCOVA results for effects of initial background color, predator chemical cues (CC) and refuge use (the covariate) on color change. dF = degrees of freedom, Asterisks indicate significance.

Figure 4. Larval color for a) *A. texanum* and b) *A. barbouri* larvae before versus after being switched from either a dark background to a light one (Dark, Dark w/CC), or a light background to a dark one (Light, Light w/CC), in the absence versus presence of predator chemical cues (w/CC). Shown are means and standard errors for body color, where higher values indicate a lighter color. See the text for details on how color was quantified using principal component analysis.

Figure 4.a) A. texanum color change.

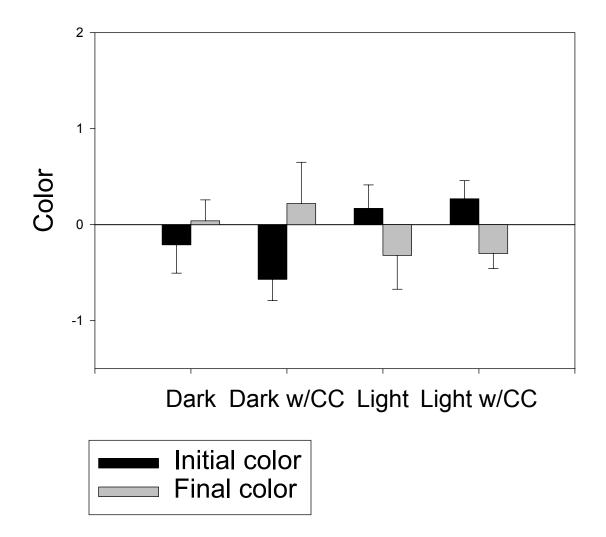


Figure 4.b) A. barbouri color change.

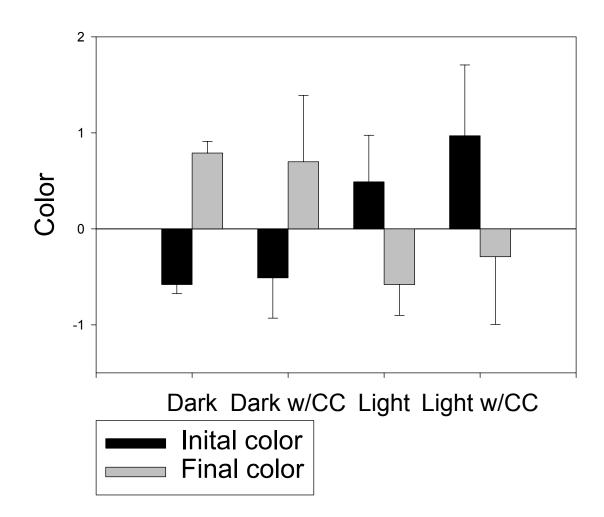


Figure 5.a) Initial body color after habituation to either a dark or light background for both *A*. *barbouri* and *A. texanum* larvae. Shown are means and standard errors for PC values (where higher values indicate a lighter color) for either *A. barbouri* (b) or *A. texanum* (t) larvae on dark or light backgrounds (Dark-b, Light-b, Dark-t, Light-t).

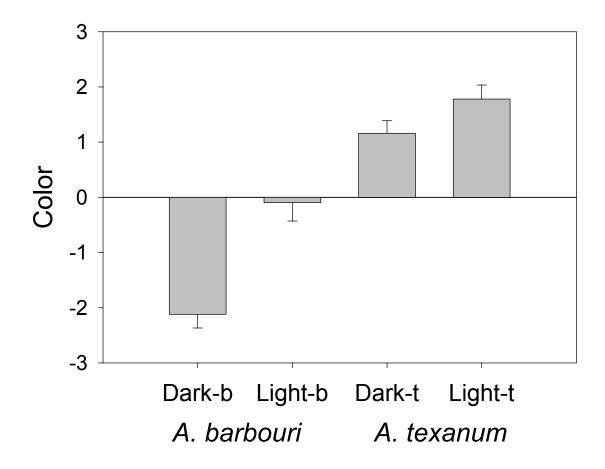


Figure 5.b) Preference for background color in *A. barbouri* (b) or *A. texanum* (t) larvae habituated to a dark or light background (Dark-b, Light-b, Dark-t, Light-t) in the presence versus absence of predator chemical cues. Shown are means and standard errors for proportion of time spent on the light background.

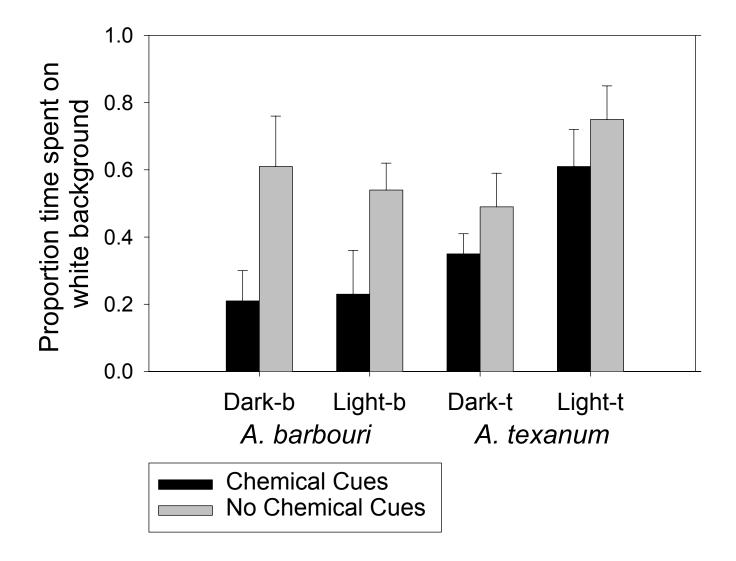


Figure 5.c) Color change in *A. barbouri* (b) and *A. texanum* (t) larvae after habituation to a dark or light background (Dark-b, Light-b, Dark-t, Light-t) and 4 hours of exposure to either control water or water with predator cues with a choice of light or dark backgrounds. Shown are means and standard errors for PC values after minus before the 4 hour predator cue test period. Positive values indicate that larvae got lighter in color.

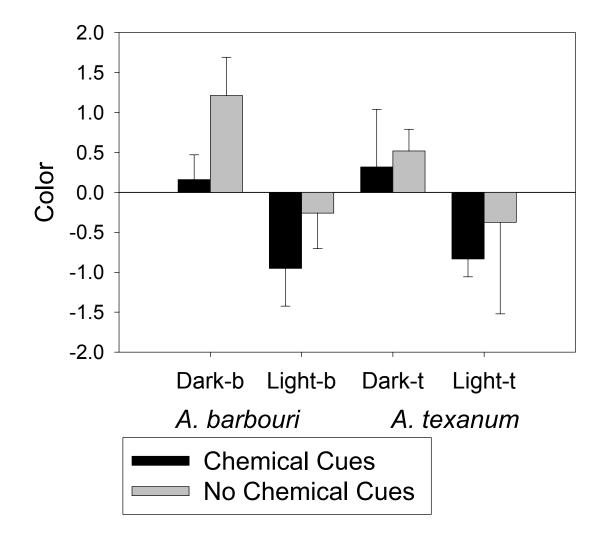


Figure 6.a) Initial body color for *A. barbouri* and *A. texanum* in the refuge use experiment, after habituation to either a dark or light background. Shown are means and standard errors for principal component values where higher values indicate a lighter color

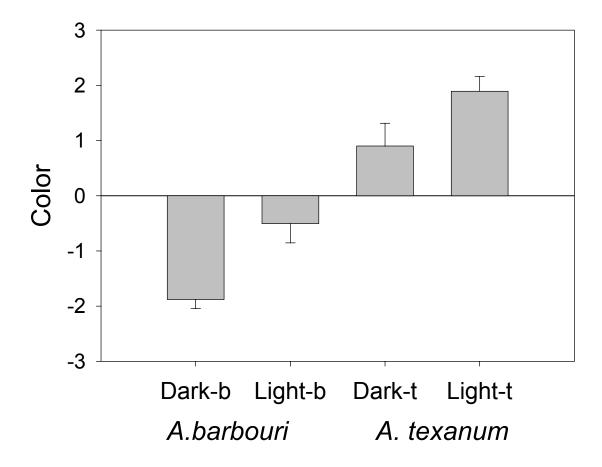


Figure 6.b) Refuge use in *A. barbouri* and *A. texanum* larvae habituated to a dark or light background (Dark-b, Light-b, Dark-t, Light-t) and then exposed to either control water, or water with predator chemical cues. Shown are means and standard errors for proportion of time spent in refuge.

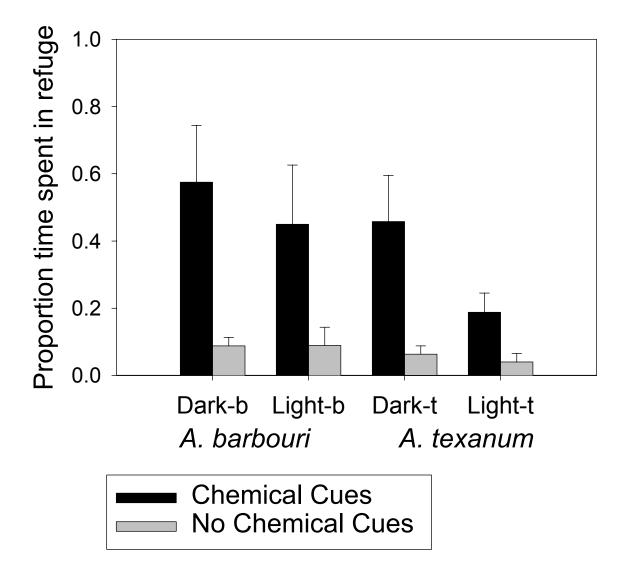
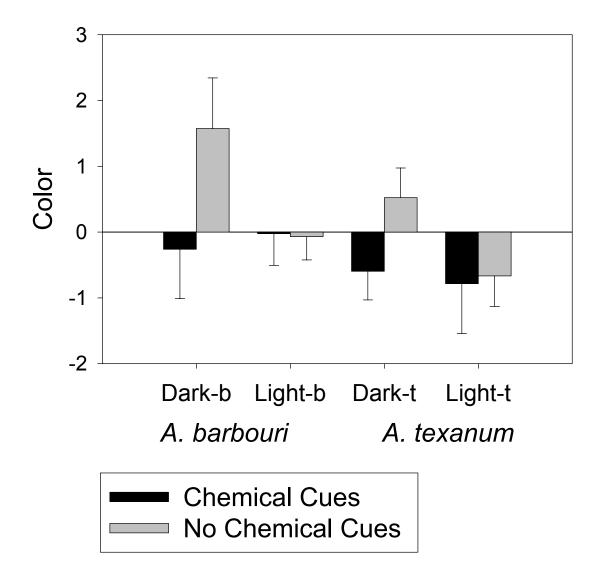


Figure 6.c) Color change in *A. barbouri* and *A. texanum* larvae after habituation to a dark or light background (Dark-b, Light-b, Dark-t, Light-t) followed by 4 hours of exposure to control water or water with predator chemical cues with refuge available. Shown are means and standard errors for PC values after minus before the 4 hour predator cue test period. Positive values indicate that larvae got lighter in color.



#### Chapter 5

Color change, refuge use and depth choice responses to ultraviolet radiation in sister species of salamander larvae, *Ambystoma barbouri* and *A. texanum*.

### **Summary**

Adaptations to avoid or cope with harmful ultraviolet radiation (UVR) have evolved in many amphibian species. Sub-lethal levels of UVR can select for simple responses in larval amphibians, such as dark pigmentation or preference for UVR protected microhabitats (i.e. under refuge or in deep water). Relatively few studies have examined color change as a defense against UVR damage, or the interaction between color and UVR avoidance behavior. This study focused on color response to UVR, and whether avoidance behaviors like refuge use and depth choice are color dependent. I quantified these responses in two sister species of salamander larvae that differ in their history of exposure to UVR. Ambystoma barbouri inhabits ephemeral streams and typically resides in the shallow, clear areas of the stream, while A. texanum lives in muddy ponds with high particulate concentrations that can attenuate UVR. I found that both species of larvae darkened in response to UVR, and when given the choice of refuge, significantly increased the proportion of time spent in hiding. Additionally, both species used deeper microhabitats when exposed to UVR, but only A. barbouri larvae showed a preference for shallow waters when UVR was blocked out. Neither of these behaviors seemed to be color dependent, with larvae from both species taking refuge and preferring deep water in the presence of UVR, regardless of their color. Interesting behavioral trade-offs arose when larvae were confronted with conflicting selection pressures from UVR and predation risk. Since risk from predatory fish forced larvae to shallow areas, and UVR forced larvae into deeper water, the combination of the two created a conflict in optimal depth choice. Faced with this conflict, A. barbouri preferred deeper, risky areas to shallow water with high UVR exposure. Ambystoma texanum responded to predation risk with a preference for shallow water, but did not significantly alter depth in response to UVR. Given the opportunity to mediate exposure to UVR and predation risk by altering depth choices, neither species changed color in response to either

UVR or predation risk. Overall, these changes in behavior and color may affect larval feeding, competition and predation rates, and could thus alter aquatic community structure.

# Introduction

Degradation of ozone levels within our stratosphere has been directly linked with increasing ultraviolet radiation (hereafter referred to as UVR (280-360 nm)) at the Earth's surface (Kerr and McElroy 1993). Biologically harmful UVR (UV-B, 280-315 nm) wavelengths have negative impacts on aquatic communities, including direct effects on survivorship in phytoplankton, zooplankton (Bothwell et al. 1994, Keller et al. 1997, Malloy et al. 1997, Villafane et al. 2001), and amphibians (Anzalone et al. 1998, Blaustein et al. 1995, 1997, Licht and Grant 1997, Ovaska et al. 1997). Adaptations to avoid or cope with harmful UVR have evolved in many aquatic species. Tunicate eggs floating on the surface of the ocean have extraembryonic cells to shield embryos from UVR wavelengths (Epel et al. 1999). *Daphnia* vertically migrate within the water column to escape UVR (Rhode et al. in press), while other freshwater zooplankton synthesize UVR absorbing compounds or accumulate them through the diet (Hairston 1976, Hansson 2000, Hebert 1990, Villafane et al 2001).

Sub-lethal levels of UV-B may select for simple avoidance behaviors in larval amphibians, such as increased activity (presumably attempting to avoid exposed sites) or preference for UVR protected microhabitats. For example, larval *Ambystoma macrodactylum* prefer shaded areas to sunny areas (Belden et al. 2000), and the newt species *Taricha granulosa* and *Taricha alpestris* show significantly more activity when exposed to UV-B (Nagl and Hofer 1997, Blaustein et al. 2000). Ultraviolet wavelengths attenuate with water depth and dissolved organic carbon (DOC) concentrations (Kirk 1986). Preferring deep microhabitats and increasing time spent in refuge may protect amphibian larvae from UV-B damage. These UVR induced behavioral changes may affect larval feeding, competition and predation rates, and could thus alter aquatic community structure.

Another potential response to UVR is color change. While color is understood in amphibians as a method of predator avoidance, communication and thermoregulation (Endler 1988), relatively few studies have examined color change as a defense against UVR damage. Color is a highly plastic trait in most amphibian larvae, with intracellular migration of melanin occurring within minutes in response to temperature change and background color (Chapters 2 and 3, Duellman and Trueb 1986, Kats and Van Dragt 1986, King et al. 1994). It has been hypothesized that skin darkening may protect amphibians from harmful UVR radiation (Cockell and Blaustein 2001). Indeed, skin darkening has been observed in several species in response to

UVR exposure, including *Hyla arborea, Hyla versicolor, Rana sylvatica* and *Xenopus laevis* (reviewed by Blaustein et al. 2001).

The relative benefits and costs of color change versus behavioral avoidance as alternative responses to UVR exposure should depend on the ecological context and associated conflicting demands. For example, even if refuge use is seemingly the most effective UVR avoidance strategy, in situations where foraging and development rates are vital to fitness, the high cost of remaining in refuge might favor foraging in the open and thus reliance on darker color to screen UVR. Alternatively, risk from predation may necessitate cryptic coloration (e.g. a light color against a sand/silt substrate), leaving larvae with only behavioral defenses against UVR. Finally, avoidance of UVR may expose larvae to abiotic stress. For example, using deeper waters to avoid UVR exposes larvae to cooler water temperatures, which tend to result in slower feeding, growth and developmental rates.

I examined the effects of UV-B on the color and behavior of two larval salamander species, *Ambystoma barbouri* and *A. texanum*, and the extent to which UVR induced behavior is color-dependent. I tested the hypotheses that exposure to sub-lethal levels of UVR should cause both species of larvae to change color, increase refuge use and increase their use of deeper microhabitats. In addition, I predicted that these responses would interact; i.e., that the degree of color change should depend on the availability of refuge or deep water, and that refuge use and use of deep microhabitats should be color-dependent. To my knowledge, this is the first study to examine interactions between behavioral and color change responses to UVR risk in any freshwater organism.

# System

Until recently, *Ambystoma texanum* was classified as having two forms: the pond form, which breeds in ephemeral ponds and is common in much of the eastern-central United States, and the stream form, confined mostly to central Kentucky ephemeral streams (Petranka 1982, Petranka et al. 1987). Several lines of evidence suggest that an *A. texanum*-like ancestor invaded into streams and evolved into *A. barbouri* (Kraus and Petranka 1989). This move into streams resulted in exposure to novel selection pressures; in particular, increased habitat ephemerality, new visual predators and greater exposure to ultraviolet radiation (Petranka 1983, Petranka and Sih 1987, Sih et al. 1992, 2000, 2002).

With regard to natural exposure to UVR, ponds and streams in Kentucky receive little shade cover in early spring, when both species breed and oviposit (February to March). Thus, eggs and early-stage larvae are exposed to a high potential risk from harmful ultraviolet wavelengths (T. Garcia pers. obs.). A canopy cover emerges in middle to late spring (April-May), partially protecting larvae in their later stages of development from UVR damage. In streams, *A. barbouri* larvae typically prefer shallow water, minimizing predation risk from deeper-dwelling fish, but increasing UVR exposure. In ponds, *Ambystoma texanum* suffer relatively less UVR exposure because of higher particulate concentrations within the water column and no threat from fish predators forcing larvae to the shallows.

Chapter 2 showed that *A. barbouri* larvae are significantly darker than their sister species, *A. texanum*, despite inhabiting streams with lighter backgrounds and visual predators. *A. texanum* larvae inhabit ponds with dark, muddy substrates, which contrasts with their light body color. This apparent lack of crypsis for both species may be the result of physiological constraints on color change or conflicting selection pressures on color. Habitat drying is an important selective force for both species, more so in streams than in ponds (Petranka and Sih 1987). In response to this increased ephemerality, *A. barbouri* larvae have evolved higher activity and feeding rates, and consequently, higher development rates relative to *A. texanum* (Petranka and Sih 1987, Sih 1992, Maurer and Sih 1996). Rapid development may also be facilitated by dark coloration, as dark colors might enhance heat absorption and result in increased larval metabolic rates (Carey 1978). Additionally, dark coloration in *A. barbouri* larvae may be a response to increased exposure to UVR in stream habitats, relative to UVR exposure in ponds inhabited by *A. texanum*.

Here, I tested for color change and color-dependent behavioral responses to harmful UVR. Do larvae plastically change color in response to UV-B radiation? Do they increase refuge use in the presence of UV-B, and is that response dependent on body color? If deeper water is available, do larvae move deeper as a UVR avoidance strategy, and is that depth choice color dependent? Do differences between species in historical selection pressures (e.g., predation risk, habitat ephemerality and UVR exposure) produce differences in their multiple responses to UVR?

To explore the question of conflicting selection pressures, I combined UVR with the risk of predation. I predicted that larvae would move to shallow water in the presence of fish, while

UVR exposure would force larvae deeper. This conflict in behavior could be mediated by skin darkening in the shallows, or by cryptic background matching in the depths. Because *A*. *barbouri* larvae can co-occur with fish (Sih et al. 1992, 2000, 2002), while *A. texanum* larvae do not, I expected *A. barbouri* to show stronger responses to fish than *A. texanum*. In particular, because *A. barbouri* are both darker and more responsive to fish (relative to *A. texanum*), I predicted that exposure to both UVR and fish cues would cause *barbouri* to use shallow water, while *texanum* would stay in deeper water (to avoid UVR) despite the presence of fish cues.

## Methods

#### Organisms

*Ambystoma barbouri* were collected as larvae from Fossil Creek (Jessamine County, Kentucky) and Raven Run Nature Preserve (Fayette County, Kentucky). To ensure correct species identification, *A. texanum* were collected as eggs from the Beaver Dam area in west central Kentucky and reared until hatching in an environmental chamber at the University of Kentucky, Lexington, KY. The larvae of both species were kept at the Ecological Research Facility, 10 km northwest of the University of Kentucky campus in Lexington, Kentucky, in an environmental chamber with constant temperature and photoperiod (15°C, 14h: 10h). Larvae were held in 15 liter gray, plastic tubs, fed *Daphnia* and macroinvertebrates *ad libitum* and reared until enough individuals met specific experimental size requirements.

## Ultraviolet radiation and color change

I tested the color response of twenty *Ambystoma barbouri* and 20 *A. texanum* larvae in a 2 x 2 factorial experiment with species and two UVR treatments (open, ambient UVR exposure and UV-B filtered exposure) as the treatment factors. Forty white, plastic containers (10 x 20 x 20 cm) with mesh sides and open tops were floated in a large rain-fed, outdoor, cattle tank (3 m diameter). A containment grid of nylon ropes effectively caged the individual floating containers within randomly assigned grid spaces (35 x 35 cm). Water levels were maintained within each container at 6 cm. Half these containers were covered with 0.3 x 22.5 x 22.5 cm clear acetate filters (Lexan Plexiglas  $^{TM}$ ), blocking 95% of the UV-B wavelengths (320-290 nm), while the other half were open to ambient light. Acetate filters were raised 10 cm above the water surface to maintain water and air flow. All containers received full sunlight (except for UVR filtering),

and filters did not affect water temperature in containers. The experiment was run on May 18, 1999.

Larvae were placed individually into randomly assigned containers and allowed to respond to treatment conditions for 24 hours. Individuals were photographed (see Color measures and statistical Analyses) the next day immediately after peak UVR hours (1100 h-1500 h). Negligible color change occurred during the time it took to remove larvae from their treatment containers and photograph them.

#### Ultraviolet radiation and refuge use

In experiment 2, I provided larvae with refuge to give them the opportunity to exhibit two responses: increased refuge use and color change. Both species were tested in a 2 x 2 x 2 factorial design with species, previous background color, and UVR as the treatment factors. Twenty-four hours prior to the experiment, twenty *Ambystoma barbouri* and 20 *A. texanum* larvae were held in the laboratory under florescent lighting conditions, in either a white or black container to test for color change to better match backgrounds. Because larvae indeed showed background matching (see Results), the variation in prior backgrounds available generated substantial variation in initial larval color. This increased my ability to detect color-dependent larval behavior when larvae were then exposed to either an open, ambient UVR or a UV-B filtered treatment. This experiment was run May 6 and 7, 2000.

Using the same set-up as described in experiment 1, 40 white plastic containers were used, each equipped with refuge in the form of a 5 x 8 cm corrugated piece of black plastic. The individuals that were habituated to either black or white backgrounds were randomly assigned a grid space and allowed to respond to UVR treatment conditions for 24 hours. Individuals were photographed twice, once before UVR exposure to record color after habituation, and again after the experiment to record color after UVR exposure. Behavioral spot checks recorded refuge use every 15 minutes during peak UVR hours (1100 h-1500 h). Larvae were considered to be in refuge if more than 50% of their body was under the piece of corrugated plastic. Analyses were done on the proportion of time (angular transformed) that larvae spent in refuge.

# Ultraviolet radiation and depth choice

I tested behavioral depth choice as a response to UVR exposure with a 2 x 2 factorial

design using a total of 50 larval *Ambystoma barbouri* and *A. texanum* over a period of five days. UVR treatments included an open UVR treatment and a UV-B blocked treatment. Half of the depth choice structures (n=10) were covered with acetate filters, while the other half were left open to ambient full sunlight. Each depth-choice apparatus was placed in one of two rain-fed cattle tanks at the Ecological Research Facility.

Each experimental enclosure consisted of a 120 cm long x 20 cm diameter, black corrugated, plastic, drainage pipe split in half longitudinally, set at a thirty-degree angle and suspended in the water column by vertical walls of fiberglass window screen mesh attached to a PVC frame. This design allowed larvae a choice of water depths ranging from the surface to a depth of 0.5 meters along a uniformly dark, textured background. One larva was placed in each enclosure (randomly assigned UVR treatments) at 1100 h, and removed at 1500 h. Behavioral spot checks recorded placement along the depth gradient every 15 minutes for the entire 4-hour period. Photographs were taken of larvae before and after the behavioral assays to quantify color change in response to UVR exposure.

With 10 enclosures, each UVR x species combination was replicated 2-3 times on each trial date. Trials were run on 5 dates (June 12-16, 2001) for a total of 50 larvae. Due to limited availability of *Ambystoma texanum* larvae, only the first 4 dates included both species. On the final day, only *A. barbouri* larvae were tested (*A. barbouri* n=30, *A. texanum* n=20).

#### Ultraviolet radiation, predation risk and depth choice

Larval *Ambystoma barbouri* and *A. texanum* were tested for behavioral depth choice and color change in response to two selection pressures: UVR and predation risk. Using a 2 x 2 x 2 factorial design, larvae of the two species were held in the depth choice apparatus used in experiment 3, in the presence or absence of predator chemical cues. Chemical cues were obtained from six bluegill sunfish (*Lepomis macrochirus*) held for 24 hours in one of the two cattle tanks containing the depth choice enclosures. The drain pipe/screen enclosures exposed larvae to chemical, and probably some visual and mechanical cues from fish, but prevented fish from having direct access to larvae.

Larvae were placed in randomly assigned experimental treatments at 1100 hr, and removed from the apparatus at 1500 hr. Behavioral spot checks recorded each larva's location along the depth gradient every 15 minutes for a total of 4 hours during peak UVR times, (1100-

1500 h). Photographs were taken of larvae before and after the behavioral assays to quantify color change in response to UVR exposure. Independent blocks of this experiment were run on four consecutive days starting on June 20, 2001.

#### **Color measures and statistical analyses**

I quantified color and color change in terms of larval brightness (i.e. amount of black versus white). Previous reflectance analysis on *Ambystoma barbouri* larvae showed variation primarily in brightness values, with relatively constant chroma and hue values (Storfer et al. 1999). Similar results were found when *A. texanum* larvae were tested for consistency of hue, chroma and brightness (Rush, unpublished data). Brightness intensities are easily measured using color, or black and white photographs. For experiments 1 and 2, pictures of each larva were taken using a Nikon 90S 35mm camera with a 150 mm macro lens to quantify individual color. Two photographs of each larva were taken on Kodak Tmax 400 black and white negative film. For experiments 3 and 4, digital images were recorded using a Nikon Coolpix 950 digital camera. In all cases, when images were taken larvae were illuminated with four surrounding 200 watt Tungsten lights. Images were scanned or downloaded into Adobe PhotoShop 6.0 imaging software for analyses.

I quantified color for each larva by measuring the amount of brightness on three regions of the body. Measurements were taken using black vs. white pixel weights within a sizestandardized square on each side of the larval head. Using this same size standardized square, color was measured on another region of the larvae, midway between the snout and vent on the dorsal side. Because brightness values were correlated for the three regions, I used principal component analysis to combine them into a single measure of larval color for each image. Hypotheses were tested by using repeated measure ANOVAs, one-way ANOVAs and paired ttests in SYSTAT 10.0.

# Results

# Ultraviolet radiation and color change

Three measures of brightness for each larva loaded strongly into PC1, with 87.9 percent of the total variance explained. Using PC1 as my measure of color, I found a strong effect of UVR exposure on larval color for both species (Table 8) as measured by principal component

analysis and two-way analysis of variance. UVR exposure caused both *A. barbouri* and *A. texanum* larvae to darken in color (Figure 7). I found no evidence of a difference between *A. barbouri* and *A. texanum* in their coloration, or in their color response to UVR exposure (species x UVR interaction). There was a tendency for *A. texanum* to become darker in response to UVR than *A. barbouri*.

### Ultraviolet radiation and refuge use

Separate ANOVA's were performed to determine the effect of UVR, species and background color on the proportion of time larvae spent in refuge, body color before and after exposure to UVB, and color change (Table 9). Three measurements of brightness for each larva loaded strongly into PC1 (color), with 95.7 percent of the total variance explained. In response to UV-B, both *A. barbouri* and *A. texanum* significantly increased their time spent in refuge (Figure 8). Although larvae changed color to better match their background color treatment (Figure 9), background color did not affect the proportion of time larvae spent in refuge (i.e. dark larvae hid just as much as light larvae). Additionally, species did not differ in the proportion of time spent in refuge.

While background color strongly affected larval color (Table 9, Figure 9), a significant difference in color between species was also detected. *A. texanum* were significantly lighter than their sister species, *A. barbouri*. I found no background x species interaction, indicating both species habituated to their background color similarly.

A one-tailed ANOVA, due to the a priori prediction that larvae would darken after exposure to UVR, showed a borderline significant color response to UVR (p=0.05). Additionally, there was a significant interaction between species and UVR, which suggests that *A. barbouri* responded to UVR with more color change than *A. texanum* (Table 9, Figure 9). Background color remained a factor affecting larval color before and after exposure to UVR treatments. Larvae held on a black background continued to be darker than larvae held on a white background (Figure 9).

Color change was calculated by subtracting larval body color after habituating to their background treatments from larval body color after exposure to UVR. There was a borderline significant interaction between UVR, species and background color (Table 9). I interpret this as

a distinct amount of color change in only one group of larvae; *A. barbouri* larvae habituated to light backgrounds and exposed to UVB became darker relative to all other groups, (Figure 9).

#### Ultraviolet radiation and depth choice

A preliminary ANOVA on results for *A. barbouri* revealed a significant, but weak UVR x tank effect (i.e., that UVR effects differed for the two cattle tanks), but no significant date or tank effects overall. Although there was a small tank x UVR effect, in order to preserve degrees of freedom for detecting treatment effects, I pooled across dates and tanks. Using principal component analysis, three measures of color for each larva loaded strongly into PC1 (color), with 83.76 percent of the total variance explained. An ANCOVA on *A. barbouri* (with two measures of color as covariates) showed that with both depth choice and color change (larval color after exposure to UVR minus larval color before exposure) as alternative responses, exposure to high UVR caused *A. barbouri* larvae to shift to deeper water (Table 10, figure 10). In addition, depth choice was not color-dependent (using either initial larval color or color change as covariates).

A significant day effect was detected for *A. texanum* larvae, which were run over a four day period. As a result, we blocked by day and ran a paired t-test (pairing values from the UVR filtered and UVR exposed treatments taken from the same tank on the same day), which showed no significant effect of UVR on depth choice (t= 0.78, d.f.= 5, P= 0.47).

#### Ultraviolet radiation, predation risk and depth choice

The effect of UVR exposure and predation risk on larval depth choice and color change for *Ambystoma barbouri* and *A. texanum* was measured by principal component analysis, an ANOVA including both species, and separate ANOVAs for each species. I conducted the separate ANOVAs for the two species in order to better examine effects of UVR and predation risk on each species (Table 11 and 12). Three measures of brightness for each larva loaded strongly into PC1, with 79.94 percent of the total variance explained. Results showed a strong species difference in depth choice in the presence of UVR and predation risk (Table 11). As predicted, both species responded to predation risk by choosing shallower areas in the presence of predator chemical cues (Table 11, Figure 11). When predator cues were absent, *A. texanum* larvae chose deeper water, regardless of UV-B filtering. *A. barbouri* larvae went to very shallow water when faced with risk and no UVR, however, when faced with conflicting demands from risk (favors going shallow) and UVR (favors going deep), they remained in the deeper water (Figure 11). A significant predation risk x UVR interaction term supports the fact that *A*. *barbouri*'s depth response to risk depended on UVR (Table 11).

Color change, however, was not affected by UVR or predation risk (Table 11). Color change was calculated by subtracting larval body color before versus after exposure to UVR and predation risk treatments. In addition, there were no significant interaction terms indicating that one group responded differently than the others.

# Discussion

I found that larval *Ambystoma barbouri* and *A. texanum* exhibit multiple responses to ultraviolet radiation. In particular, I showed that larvae from both species reacted to sub-lethal doses of harmful UVR wavelengths with increased pigmentation, refuge use and use of deep water. Their reliance on color change and different types of refuge use was context-dependent, which might reflect shifting costs and benefits of different responses. In addition, preference for a particular response may be a function of the species' evolutionary history of selection pressures.

My results showed that larval *Ambystoma barbouri* and *A. texanum* significantly darkened in response to UVR exposure (Figure 7). This suggests that UVR triggers the dispersal of dark colored melanin throughout pigment cells (melanophores), causing an overall darkening of the skin (Duellman and Trueb 1986). Increased pigmentation has been shown to be a protective response against UV-B in other organisms (Hairston 1976, Hebert 1990, Hansson 2000). By getting darker, *Ambystoma* larvae may increase the UVR absorption properties within their integument, thus not allowing further penetration of the DNA damaging wavelengths (Hansson 2000, Kollias 1991).

*A. barbouri* tend to be darker than their sister species, *A. texanum* (Figure 9). This difference in color between sister species could be an important indicator of differing selection pressures between their habitats, (i.e. habitat ephemerality and UVR exposure). *Ambystoma barbouri* larvae are heavily constrained by early habitat drying, which favors high feeding, growth and developmental rates (Petranka and Sih 1987; Maurer and Sih 1996). As a result, larvae spend much of their time foraging in UVR exposed areas. Pigmentation may be a critical defense against UV-B for rapidly developing larvae. In contrast, *Ambystoma texanum* suffer less

risk of desiccation associated with habitat ephemerality, have relatively longer larval periods (Petranka and Sih 1987; Maurer and Sih 1996), and thus less need to forage in UVR exposed areas. In addition, ponds with *A. texanum* tend to be murkier than streams with *A. barbouri*, increasing UVR attenuation. *A. texanum* 's lighter mean body color could be a result of lower exposure to harmful UVR relative to *A. barbouri*.

A relatively unexplored issue of general interest (for any species) is the interaction between multiple responses to UVR. When given the option of responding to UVR by hiding under refuge, or by changing color, *A. barbouri* showed both responses, while *A. texanum* increased the time they spent in refuge but showed no color change. While using refuge strongly reduces exposure to UVR, refuge use is costly. Increased refuge use is associated with reduced feeding rates, which can ultimately result in increased mortality for larvae in ephemeral habitats (Maurer and Sih 1996). Thus, it is necessary for larvae to spend some time out of refuge, and being darker could help to reduce UVR damage while out of refuge.

If refuge use is color-dependent, I expect the proportion of time spent in refuge to be negatively correlated with larval darkness. Given that refuge use is costly, larvae that are darker should be more willing to risk UVR exposure if, indeed, dark coloration is an effective defense against UVR damage. Here, both species hid in refuge when exposed to UVR regardless of their body color. A plausible explanation for the lack of correlation between color and refuge use is the possibility that refuge is a better strategy against high UVR than dark pigmentation. Thus, even dark colored larvae hide.

Another realistic response to UVR is preference for deeper microhabitats, as UVR wavelengths attenuate quickly with depth (Kirk 1986). *A. texanum* consistently occupied deeper waters regardless of UVR presence, while *A. barbouri* larvae chose shallow waters when UVR was blocked (Figure 11). This behavior occurred regardless of initial body color and is consistent with observations in the field. *A. texanum* larvae typically inhabit the bottoms of ponds while *A. barbouri* larvae often reside in the shallow parts of streams. This additional behavioral response to UVR allows larvae to forage, (unlike during behavioral refuge use) and may be more effective at protecting larvae from UVR damage than pigmentation. However, deeper waters are associated with colder temperatures, (T. Garcia, unpub. data) which decrease larval development rates and time until metamorphosis (Smith-Gill and Berven 1979).

In the depth choice experiment, larval color remained constant in both UVR treatments

over the course of the five-hour experiment. *A. barbouri* larvae preferred shallow water when UVR was filtered, and because UVR was filtered, no color response was detected. In the presence of UVR, larvae chose depths at which UVR was adequately attenuated, again, prompting no color response. As with refuge use, the fact that depth choice apparently took precedence over color change as a primary response to high UVR is consistent with the hypothesis that color change is not a highly effective defense against UVR damage.

The availability of multiple responses to UVR may be most important when conflicts arise from multiple selection pressures. The addition of predation risk to UVR exposed environments presents such a conflict; risk of UV-B damage forces larvae to deeper water, while predatory fish force larvae to the shallows. This conflict is probably common in nature. UVR risk invariably decreases with water depth, while risk from predatory fish is very often greater in deeper waters (Power 1987). Here, I found that in the absence of predation risk, larvae of both species tended to use deeper waters, while *A. barbouri* moved to shallower water when UVR was blocked (figure 10). In the presence of predation risk, but no UVR exposure, both species responded to predation risk by taking refuge in shallower water (Figure 11). As in previous studies, *A. barbouri* showed a stronger response to fish cues than *A. texanum* (Sih et al. 2000, 2002); however, this difference was only there in the absence of UVR (Figure 11).

Most interestingly, with both UVR and predation risk present, *A. barbouri* exhibited little or no depth response to predation risk, but instead occupied deeper, UVR protected waters. These results suggest that larvae respond more strongly to UVR risk than predation risk. It is possible that larvae exhibited other antipredator defenses, such as immobility, that would compensate for their lack of predator avoidance by depth choice; however, I did not quantify larval activity. I did, however, quantify color change, and found that neither species of larvae responded to UVR and predation risk with a change in body color. Further study of multiple responses is necessary to better understand the interaction between depth preference and multiple selection pressures.

Overall, my study addresses the important issue of how organismal responses help them to cope with high levels of UV-B, particularly in combination with other stressors. The existence of multiple responses to UV-B allows organisms, like these salamanders, to exhibit flexible, alternative defense strategies that can be adjusted to fit a range of environments with different conflicting demands. In addition, indirect effects of UVR on aquatic communities is a

growing field of interest (Bothwell et al. 1994, Cockell and Blaustein 2001, Villafane et al. 2001). Behavioral modifications in amphibians in response to UVR could affect predator-prey interactions, intra- and interspecific competition and thus overall community structure. Further experiments on effects of UVR on trait mediated indirect interactions within aquatic communities are necessary to better understand the impact of UVR on ecological patterns.

Table 8. Summary of the analysis of variance for body color in two salamander species, *A. barbouri* and *A. texanum* in response to ultraviolet radiation. Effects are SP = species, and UVR = UVR exposure. DF = degrees of freedom, Asterisks indicate significance.

Response Variable	Effect	SS	DF	F	Р
Color after UVR	UVR	9.376	1	11.751	0.001*
exposure					
	SP	0.303	1	0.380	0.541
	UVR x SP	0.597	1	0.748	0.392
	Error	28.722	36		

Table 9. Summary of ANOVAs on refuge use after exposure to UVR, body color after habituation to a black or white background, and body color after exposure to UVR in two salamander species, A*mbystoma barbouri* and *A. texanum*. DF = degrees of freedom, Asterisks indicate significance.

Response Variable	Effect	SS	DF	F	Р
Refuge use	UVR	0.661	1	14.183	0.001*
	Background color	0.044	1	0.939	0.340
	Species	0.026	1	0.56	0.460
	UV x Species	0.002	1	0.043	0.836
	Background color x	0.000	1	0.001	0.971
	Species				
	Error	1.539	33		
Color after habituation	Background color	17.278	1	10.791	0.002*
to background					
	Species	44.403	1	27.732	0.000*
	Background color x	1.226	1	0.766	0.388
	Species				
	Error	52.838	33		
Color after exposure to	UVR	2.584	1	2.787	0.05*
UVR					
	Background color	11.653	1	12.566	0.001*
	Species	52.704	1	56.833	0.000*
	UVR x Species	6.128	1	6.608	0.015*
	UVR x Background	0.968	1	1.043	0.314
	color				
	Background color x	0.001	1	0.002	0.969
	Species				
	Error	30.602	33		
Color change	UVR	1.795	1	1.706	0.101
	Background color	0.552	1	0.525	0.474

Species	0.355	1	0.338	0.565
UVR x Species	1.547	1	1.470	0.234
UVR x Background	0.195	1	0.185	0.67
color				
Background color x	1.143	1	1.086	0.305
species				
Background color x	3.389	1	3.220	0.041*
species x UVR				
Error	34.730	33	1.052	

Table 10. Results of an ANCOVA on depth choice and an ANOVA on color change in larval *Ambystoma barbouri*. Ultraviolet radiation treatments are denoted UVR. The experiment was blocked by date (8 days) and pool (2). DF = degrees of freedom, Asterisks indicate significance.

Variable	Source	SS	dF	F	Р
Depth	UVR	356.982	1	26.246	0.000*
	Color change	7.005	1	0.515	0.476
	Error	734.460	54		
Color after exposure	UVR	0.020	1	0.0015	0.904
to UVR					
	Error	76.251	55		

Table 11. Results of an ANOVA on effects of UVR and predation risk on depth choice in two salamander species, *Ambystoma barbouri* and *A. texanum*. UVR indicates the effect of ultraviolet radiation exposure, and CC is the effect of the presence of predator chemical cues on depth choice. DF = degrees of freedom, Asterisks indicate significance.

Response	Effect	SS	DF	F	Р
Variable					
Depth	Species	149.414	1	10.175	0.003*
	UVR	6.0706	1	0.457	0.505
	CC	141.995	1	9.670	0.004*
	Species x UVR	6.0108	1	0.416	0.524
	Species x CC	0.665	1	0.045	0.833
	UVR x CC	33.258	1	2.265	0.143
	Species x UVR x	13.731	1	0.935	0.342
	CC				
	Error	425.855	29	14.685	
Color	Species	0.005	1	0.003	0.956
Change					
	UVR	3.762	1	2.224	0.147
	CC	2.918	1	1.725	0.208
	Species x UVR	0.090	1	0.053	0.819
	Species x CC	0.824	1	0.487	0.495
	UVR x CC	0.951	1	0.562	0.460
	Species x UVR x	0.180	1	0.107	0.747
	CC				
	Error	45.671	27	1.692	

Table 12. Summary of ANOVAs on effects of UVR and predation risk on depth choice in two salamander species, *Ambystoma barbouri* and *A. texanum*. UVR indicates the effect of ultraviolet radiation exposure, and CC is the effect of the presence of predator chemical cues on depth choice. (DF = degrees of freedom, Asterisks indicate significance.)

A. barbouri				A. texanum					
Response	Effect	SS	dF	F	Р	SS	dF	F	Р
Variable									
depth	UVR	15.499	1	0.926	0.174	0.006	1	0.001	0.491
	CC	98.091	1	5.863	0.014*	52.492	1	4.453	0.028*
	UVR	54.298	1	3.246	0.045*	1.810	1	0.154	0.351
	x CC								
	Error	284.414	17			141.441	12		

Figure 7. Mean color values for *Ambystoma barbouri* and *Ambystoma texanum* larvae in response to UVR exposure. Color is measured by principal component 1 of a principal component analysis on brightness quantified by three larval body regions. Positive values indicate lighter larvae body color, while negative values indicate a darker color.

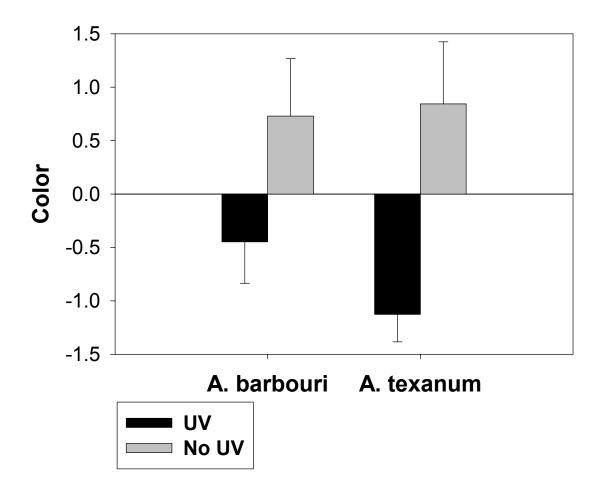


Figure 8. Proportion of time spent in refuge by larval *Ambystoma barbouri* and *A. texanum* during exposure to open (UV) and filtered UVR (No UV) treatments.

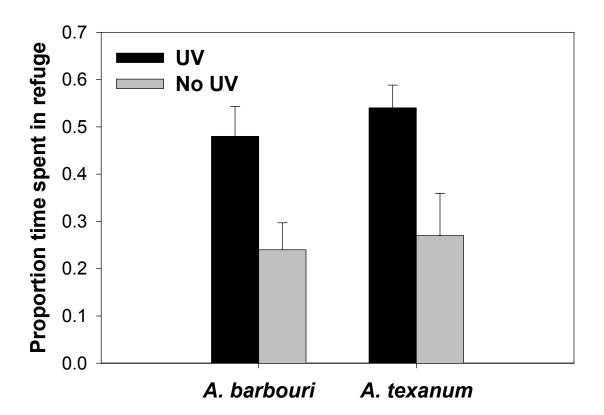


Figure 9. Color differences between species with UVR filtered, and full UVR exposure for *A*. *barbouri* and *A. texanum* with refuge available. 'Light' represents individuals habituated to white backgrounds before the experiment for twenty-four hours. 'Dark' represents individuals held on black backgrounds for twenty-four hours before the experiment.

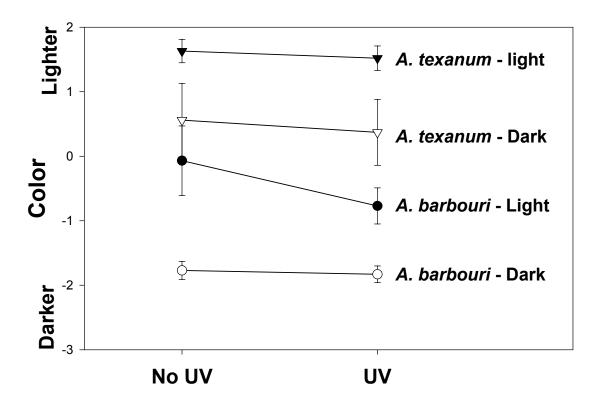


Figure 10. Depth choice for *Ambystoma barbouri* and *A. texanum* larvae due to ultraviolet radiation (UVR) treatment exposure. The y-axis represents water depth ranging from the surface (0 cm) to 50cm. A significant difference in depth choice exists for *A. barbouri* between UVR treatments.

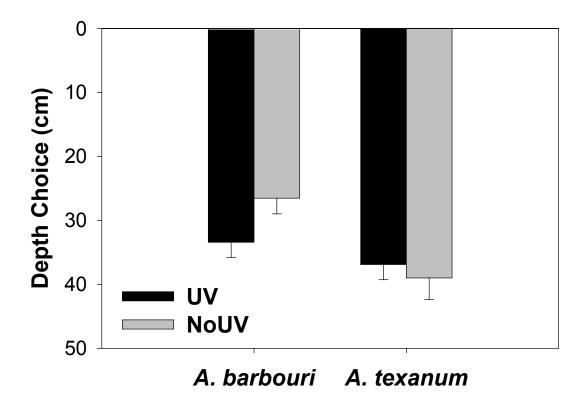
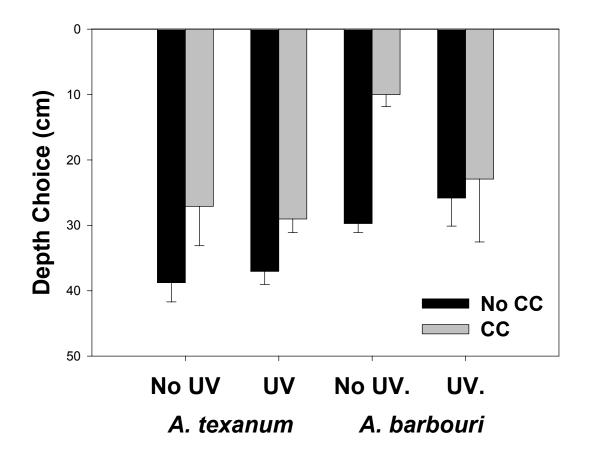


Figure 11. Depth choice for *Ambystoma barbouri* and *A. texanum* larvae due to exposure to ultraviolet radiation (UV) treatments and predator chemical cue (CC) treatments. The y-axis represents water depth ranging from the surface (0 cm) to 50cm. A significant UV x CC interaction exists for *A. barbouri*, as well as significant differences in depth choice between CC treatments for *A. texanum*.



#### Chapter 6

Effects of ultraviolet radiation and oviposition site on embryo survivorship in the streamside salamander, *Ambystoma barbouri* 

## **Summary**

Most studies on amphibian responses to ultraviolet radiation (UVR) quantify the effects of direct exposure on survivorship, while relatively few focus on behavioral adaptations that help individuals avoid or cope with potentially damaging UVR exposure. Protection of amphibian eggs from UVR exposure is especially critical, as UVR-induced damage in early development can significantly reduce larval and adult fitness. Choice of oviposition site is a key factor in determining the amount of UVR exposure embryos encounter during development. In this study, I examined the effects of UVR and oviposition site on egg survival in the streamside salamander, Ambystoma barbouri. While most Ambystoma are pond breeders and lay their eggs in open water attached to stems and leaves of submerged vegetation, A. barbouri choose to oviposit on the undersides of rocks in ephemeral streams. The laying of eggs under rocks is presumably a response to selection pressures found in streams which are absent in ponds. In this experiment, I found that when rocks with A. barbouri eggs were turned over and exposed to UVR, there was a significant decrease in overall hatching success. Additionally, in the UVR exposed treatment, there was an increase in the proportion of eggs that fell off the rock or died before hatching. This study supports the hypothesis that A. barbouri have evolved this cryptic ovipositing behavior in response to negative effects of UVR exposure on embryonic fitness. By laying their eggs under large, submerged rocks, female A. barbouri protect their offspring from UVR damage.

# Introduction

An intense debate within the fields of conservation biology and ecology focuses on the effects of ultraviolet radiation (280-360 nm) exposure on organisms under natural conditions. Most organisms are exposed to some ultraviolet radiation (hereafter referred to as UVR), but due to the degradation of atmospheric ozone, the amount of UVR reaching the Earth's surface has increased (Kerr and McElroy 1993). Several studies have shown direct negative effects of UVR on aquatic organisms, including decreased survivorship in phytoplankton, zooplankton (Bothwell et al. 1994, Keller et al. 1997, Malloy et al. 1997, Villafane et al. 2001), and amphibians (Anzalone et al. 1998, Blaustein et al. 1995, 1997, Licht and Grant 1997, Ovaska et al. 1997). While many studies quantify the direct effects of exposure to UVR on survivorship, relatively few experiments have addressed the effectiveness of adaptations that may minimize UVR-induced damage in natural settings (Epel et al 1999, Cockell and Blaustein 2001).

There are many examples of adaptations in aquatic species that help individuals avoid or cope with harmful UVR. Tunicate eggs floating on the surface of the ocean have additional embryonic cells to shield embryos from UVR wavelengths (Epel et al. 1999). *Daphnia* migrate vertically within the water column to escape UVR (Rhode et al. in press), while other freshwater zooplankton synthesize UVR absorbing compounds or accumulate them through the diet (Hairston 1976, Hansson 2000, Hebert 1990, Villafane et al 2001). In addition, several amphibian species have evolved fascinating behavioral and physiological mechanisms to decreases damage due to UVR exposure (Blaustein et al. 1998).

Many species of amphibians are sensitive to UVR, showing increased mortality and deformities when exposed to ambient levels of UVR (Anzalone et al. 1998, Blaustein et al. 1995, 1997, Langhelle et al 1999, Licht and Grant 1997, Ovaska et al. 1997). Protection of amphibian eggs from exposure is especially critical, as UVR-induced damage in early development can significantly reduce larval and adult fitness (Epel at al. 1999). One such protective mechanism is photoreactivation, or the repair of DNA damage produced by UVR exposure. Hayes et al. (1996) found that amphibian eggs vary greatly in levels of photolyase (the key DNA repair enzyme), and species with high photolyase activity are more resistant to UVR damage. Pigmentation may also act as a defense against UVR damage (Chapter 5, Cockell and Blaustein 2001, Kollias 1991). Pigmentation in the gelatinous coat enveloping amphibian eggs or dark

pigmentation in developing embryos may protect against harmful UVR wavelengths (Blaustein et al. 1998, Jablonski 1998).

Choice of oviposition site is a key factor in determining the amount of UVR exposure embryos encounter during development. UVR wavelengths attenuate quickly with water depth (Kirk 1986); thus eggs laid in deeper microhabitats are exposed to lower levels of UVR relative to eggs in shallow water (Blaustein et al. 1998). However, deeper water is typically cooler, which can negatively affect embryonic and larval development rates (Chapter 2, Duellman and Trueb 1986, Smith-Gill and Berven 1979). Oviposition sites in habitats with high particulate concentrations, or under refuge, will also decrease embryonic exposure to UVR (Blaustein et al 1998). In addition, oviposition behaviors such as egg wrapping have been shown to increase embryonic survivorship by decreasing exposure to UVR (Marco et al. 2001, Sih and Maurer 1992).

In this study, I examine the effects of UVR and oviposition site on egg survival in the streamside salamander, *Ambystoma barbouri*. Until recently, *A. barbouri* was classified as a subspecies of *A. texanum*, a pond-breeding species inhabiting much of the eastern-central United States (Petranka 1982, Petranka et al. 1987). Several lines of evidence suggest that an *A. texanum*-like ancestor invaded into streams, and evolved into what we now call *A. barbouri* (Kraus and Petranka 1989, Niedzwicki and Storfer, unpublished data). This move into streams resulted in exposure to novel selection pressures; in particular, increased habitat ephemerality, new visual predators, and greater exposure to UVR (Petranka 1983, Petranka and Sih 1987, Sih et al. 1992, 2000, 2002). Streams inhabited by *A. barbouri* are typically shallower, and have lower suspended particulate concentrations relative to ponds inhabited by *A. texanum*. However, both ponds and streams in Kentucky receive little shade cover in early spring, exposing eggs and early-stage larvae of both species to potentially harmful levels of UVR (T. Garcia, pers. obs.). This has led to the evolution of several UVR avoidance behaviors in larval *A. barbouri* and *A. texanum*, such as a preference for hiding under refuge, and the plastic ability to become darker in color when exposed to ambient levels of UVR (Chapter 5).

*Ambystoma texanum*, the pond breeding species, typically oviposits eggs in open water, attached to stems and leaves of submerged vegetation. In streams, *Ambystoma barbouri* oviposits eggs on the undersides of flat limestone rocks (Petranka 1982). This behavior of laying eggs underneath rocks suggests that an environmental factor present in streams is selecting for

cryptic oviposition. Sih and Maurer (1992) showed that when rocks harboring *A. barbouri* eggs were flipped over, survivorship of the exposed eggs was significantly less relative to eggs attached to rocks that were left unturned. In addition, embryonic developmental rates were reduced on overturned rocks. Sih and Maurer (1992) hypothesized that by ovipositing under rocks, *A. barbouri* females were protecting their eggs from either the mechanical disturbance of the stream flow, or from UVR damage.

In this study, I investigated the effect of oviposition site (under versus on top of rocks) and UV exposure on the hatching success of *A. barbouri* embryos. I predicted that when rocks with *A. barbouri* eggs were overturned, that survivorship would be significantly less for eggs that were exposed to ambient levels of UVR, relative to those on overturned rocks but with UVR filtered out. I also predicted that survivorship for egg clutches on rocks that have been overturned but have UVR filtered will be similar to survivorship for clutches that remain on the underside of rocks.

# Methods

This study was conducted in the south fork of Raven Run Creek in the Raven Run Nature Sanctuary, Fayette County, 25 km southeast of the University of Kentucky campus in Lexington, Kentucky. On March 5th, 2001, I located 22 flat, limestone rocks, each with one newly laid *Ambystoma barbouri* clutch on the underside. I recorded the total number of eggs per clutch for each rock. Eighteen of the 22 rocks were overturned, so that the eggs were exposed, although still submerged, and placed within a framed structure constructed of PVC and mesh screening. The four control rocks were manipulated in a similar way, but left unturned. Each structure was randomly assigned one of two UV-B treatments: a) a non-filtered treatment, with full exposure to UV-B, and b) a filtered treatment, with UV-B exposure reduced using Lexan Plexiglas ™(which screens 95% of UV-B wavelengths).

Experimental structures were constructed such that the 1 x 1 m Lexan filters were suspended 20 cm above the water line, and the bottom of each structure rested on the substrate, covered with mesh screening. Structures were placed out in pairs with each pair including one filtered and one unfiltered egg mass, with pairs randomly assigned to nine low flow pools along a 300 m stretch of Raven Run Creek. Each overturned rock was placed in the center of the 1 x 1 m unit to ensure that the egg clutch was shaded by the UV-B filter, regardless of the angle of the

sun. I intended to accompany each treatment pair with an unturned rock (i.e. the clutch positioned on the underside) to control for moving. However, my sample size of newly laid *A*. *barbouri* clutches was limited, resulting in only four unturned control rocks that were randomly assigned to four of the nine pools containing paired structures.

For five weeks, I gathered data every other day on the developmental stage of each clutch, and the number of eggs per clutch that had hatched, were missing, or found dead in each treatment. Unturned rocks were checked every fourth day to minimize disturbance. Eggs were considered to be dead if the embryos were milky white, and considered missing if the gelatinous coating of the egg was no longer present and the total number of eggs was less than the previous count. Missing eggs always disappeared when eggs were at an early developmental stage, far from hatching. For late stage embryos, I determined that the embryo had hatched if the gelatinous egg coating was still attached to the rock, but the embryo was missing. Decreasing water levels over the course of the experiment left some rocks above the water line. Two unturned rocks, one UVR exposed rock and three UVR filtered rocks became dry during the experiment. These clutches suffered 100% mortality due to desiccation and were excluded from the analysis.

Two-sample t-tests were run testing the treatment effects on three variables; the proportion of initial eggs that hatched, the proportion of initial eggs that were missing and the proportion that were accounted for (i.e., not missing) that were dead. Too many samples were lost to run a paired t-test, as originally planned. I applied the Dunn-Sidak adjustment to account for multiple tests. The proportion of eggs hatched, missing and dead (if not missing) were all angular transformed, and all analyses were done using transformed data.

# Results

Because sample sizes were low (N = 2) for the unturned rocks, I pooled the two treatments that had eggs that were not exposed to UVR; i.e., 'overturned, but UVR filtered' and 'unturned' rocks (hereafter, these are referred to as 'unexposed' eggs). I compared embryo fates for unexposed versus for UVR exposed rocks. Embryonic exposure to UVR resulted in a significantly lower proportion of eggs hatching, an increase in the proportion of missing eggs, and for those that were not missing, a higher proportion of dead eggs in UVR exposed clutches compared to UVR filtered clutches and clutches on unturned rocks (Figure 12).

Results from a two-sampled t test on hatching success for both treatments showed a significant UVR treatment effect on the proportion of embryos that hatched (Dunn-Sidak adjusted test; t= 2.369, d.f.= 14, one-tailed P= 0.047). The unexposed clutches had high hatching success on all rocks. In contrast, I found high variation in hatching success among the exposed rocks. While five rocks had very poor hatching success, three rocks exhibited relatively high hatching success. I assume that this variation was due to an uncontrolled factor influencing UVR levels, such as higher particulate concentrations in pools with higher hatching success. This pattern holds for all three variables.

I found a borderline significant trend for a UVR effect on the proportion of eggs missing. There was a higher proportion of missing eggs for clutches exposed to UVR relative to the unexposed treatment (Dunn-Sidak adjusted test; t= 2.181, d.f.= 14, one-tailed P= 0.067). UVR also affected mortality in exposed clutches. For eggs that were not missing, I found a significant increase in proportion dead on rocks exposed to UVR (Dunn-Sidak adjusted test; t= -2.344, d.f.= 13, one-tailed P= 0.05).

### Discussion

*Ambystoma barbouri* eggs were negatively influenced by exposure to UVR. This study shows that UVR exposure decreases the probability that *A. barbouri* eggs will hatch successfully, and increases the probability that eggs will fall off the rock or die during development. Eggs found missing were probably killed shortly after release from the protection of their gelatinous coating. I hypothesize that the integrity of the gelatinous coating was affected by UVR, causing the gelatinous matrix to weaken, allowing the egg to become detached and fall loose from the rock. Presumably, unattached, individual eggs suffer higher mortality due to mechanical disturbance in the current and exposure to other environmental stresses.

Previous studies (Sih and Maurer 1992) noted that other factors that could potentially decrease egg survivorship on overturned rocks include reduced oxygen availability to the embryo due to the thin layer of silt that can collect over the exposed egg clutches, and mechanical disturbance from exposure to water current (i.e. buffeting of the eggs by stream flow). My experimental results suggest that neither of these is a major factor in this system. With regard to silt loads, Sih and Maurer (1992) found that silted egg masses did not differ significantly in hatching rate from non-silted masses. In the present experiment, I did not observe enough silt

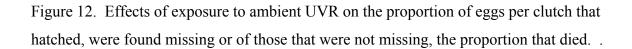
accumulation on the exposed egg clutches in this experiment to likely impede oxygen uptake. Furthermore, eggs on the underside of rocks are naturally exposed to some degree of silt without suffering high egg mortality. With regard to current flow, in the present experiment, all structures were placed in low-flow pools within the stream. Eggs on overturned rocks did not appear unduly stressed by water flow, and at no time was the current strong enough to physically remove eggs from the rocks. In addition, although eggs on overturned, but UVR-filtered rocks experienced some current flow, while eggs on unturned rocks experienced very little flow, no differences in hatching success were found between these two groups. Most importantly, eggs on overturned rocks that were protected from UVR had higher survival than eggs that were exposed to UVR, even though there were no obvious differences between these two groups in silt loads or current velocity experienced.

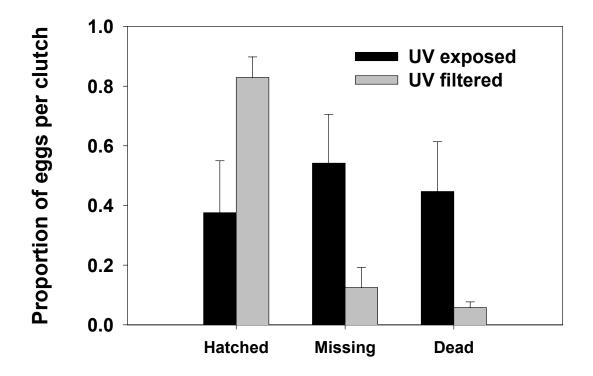
My results thus support the notion that oviposition of eggs under refuge is a beneficial behavioral adaptation to cope with increased UVR in the stream habitat. By laying their eggs under large, submerged rocks, female *A. barbouri* adults protect their offspring from UVR damage and increase overall hatching success. The absence of cryptic oviposition in the sister species *A. texanum* suggests that *A. barbouri* living in streams are subjected to selection pressures not found in ancestral ambystomatid pond habitats. Streams inhabited by *A. barbouri* are typically clearer and shallower than ponds inhabited by *A. texanum*. These clear waters expose *A. barbouri* to heavy selection pressure from UVR; thus we should see selection for other UVR avoidance strategies such as a larval preference for UVR protected areas and color change when exposed to UVR. Previous work indeed showed that *A. barbouri* exhibit a stronger shift to deep water in response to UVR relative to *A. texanum*, with larvae choosing shallow areas only when UVR has been filtered. Both *A. barbouri* and *A. texanum* prefer refuge in the presence of UVR, and become darker in color when exposed to UVR (Chapter 5).

Selection to cope with UVR stress can be enhanced by other ecological demands. Notably, *Ambystoma barbouri* larvae are heavily constrained by early habitat drying, which favors high feeding, growth and developmental rates (Petranka and Sih 1987, Maurer and Sih 1996). To facilitate faster embryonic development, female *A. barbouri* should show a preference for ovipositing in warmer areas of the stream. However, warm, shallow areas also often receive the highest levels of UVR exposure. Ovipositing under refuge in shallow portions of the stream

allows eggs to develop in the warmer parts of the stream and still be protected from UVR wavelengths.

*A. barbouri*'s tendency to prefer cryptic oviposition sites is another example of how organisms have evolved to cope with UVR exposure in nature. Avoidance of UVR by ovipositing females supports the hypothesis that ambient levels of UVR are sufficient to influence the ecology and evolution of aquatic organisms. If refuge is available, behavioral avoidance may be a highly effective mechanism for dealing with UVR. Parental care behavior such as adaptive oviposition site choice is essential in protecting egg and early larval stages from potentially damaging UVR exposure. As the global environment continues to change, and UVR becomes an increasingly important selective force, insights gained from studying how different organisms respond to UVR can help us understand relative impacts of UVR at a population or community level.





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## **PROFESSIONAL PUBLICATIONS:**

- Crowley, P. H., T. Cottrell, T. Garcia, M. Hatch, R. C. Sargent, B. Stokes and J. M.
  White. 1998. Solving the Complimentary Dilemma: Evolving strategies for simultaneous hermaphroditism. Journal of Theoretical Biology (1998) 195: 13-26.
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### **PROFESSIONAL PRESENTATIONS:**

- 2001: Ecological Society of America. Trade-offs in color and behavioral interactions: a comparison study of two larval salamander species, *Ambystoma barbouri* and *Ambystoma texanum*. Monona Terrace, Madison, Wisconson.
- 2001: Animal Behavior Society. Interacting color and behavior responses to predation risk and ultraviolet radiation in the salamander species *Ambystoma barbouri* and *Ambystoma texanum*. Oregon State University.
- 2000: Animal Behavior Society. Color and behavior response to ultraviolet radiation in the sister species *Ambystoma texanum* and *A. barbouri*. Morehouse College, Atlanta.
- 1999: Animal Behavior Society. Evolution and plasticity of color and behavior: A comparison of sister species Ambystoma texanum and Ambystoma barbouri. Bucknell University.
- 1998: Transylvania University. Invited Speaker. Amphibian Decline: examples of Applied Ecology.
- 1997: National Science Foundation. Graduate Research Training at the University of Kentucky: Promoting research and education in science and engineering, Washington, D.C.