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UV-B Induced Skin Darkening in Larval Salamanders Does Not Prevent Sublethal Effects of Exposure on Growth

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Interspecific differences in sensitivity to ultraviolet-B radiation (UV-B; 280–315 nm) are well documented for amphibians. However, few studies have addressed physiological mechanisms underlying differential species survival to such exposure. One potential mechanism that might protect amphibians from damaging UV-B involves melanin production and resultant skin darkening. In this study, we examined (1) the darkening response in salamander larvae exposed to UV-B and (2) whether darker larvae have a higher survival rate than lighter larvae when exposed to UV-B. After five days of relatively low UV-B exposure in the laboratory, larval roughskin newts, *Taricha granulosa*, and Northwestern salamanders, *Ambystoma gracile*, showed a significant darkening of the skin, as compared to controls exposed to full-spectrum lighting without UV-B. In addition, long-toed salamanders, *Ambystoma macrodactylum*, showed the same trend for darkening, although it was not statistically significant. To investigate whether survival might be higher for darker larvae exposed to UV-B, we manipulated the skin color of *A. gracile* and *A. macrodactylum* larvae by placing them on black or white backgrounds during UV-B exposure. Larvae exposed to UV-B were smaller after three weeks, regardless of background coloration. Background coloration effectively controlled skin color, with larvae on white backgrounds consistently lighter than larvae on black backgrounds. No survival differences were observed between treatments; thus, it remains unclear whether skin darkening provides protection from UV-B damage.

UNDERSTANDING the biological effects of ultraviolet-B (UV-B; 280–315 nm) radiation is important, as UV-B levels at the Earth's surface increase due to stratospheric ozone depletion (Kerr and McElroy, 1993; Hader, 1997; Zerefos et al., 1998). Varying responses of organisms to UV-B radiation have been demonstrated (e.g., Tevini, 1993; Hader, 1997; Cockell and Blaustein, 2001). Moreover, several studies suggest that increases in ambient UV-B may have drastic biological impacts, including detrimental effects on individual organisms as well as on ecosystems (see papers in Tevini, 1993; Hader, 1997; Cockell and Blaustein, 2001). Global environmental changes, including increasing UV-B exposure, have been suggested as factors contributing to current worldwide declines in amphibian populations (Blaustein and Wake, 1995; Alford and Richards, 1999; Kiesecker et al., 2001). Indeed, some amphibian species are extremely sensitive to even current ambient levels of UV-B radiation (Blaustein et al., 1998).

Animals can cope with potentially dangerous UV-B radiation by preventing damage from occurring or by repairing damage once it occurs (Epel et al., 1999). Although the mechanisms involved in repairing UV-B-induced damage to amphibian eggs and embryos have received some attention (e.g., Blaustein et al., 1994; van de Mortel et al., 1998), there is little information on how amphibians prevent damage from occurring.

Behavioral avoidance of areas with high levels of UV-B is one way for larval and adult amphibians to prevent damage (Nagl and Hofer, 1997; van de Mortel and Buttemer, 1998; Belden et al., 2000). In addition, behavioral decisions by adults regarding oviposition locations can limit exposure of embryos to UV-B (e.g., Marco et al., 2001). Physiological and morphological mechanisms may also limit UV exposure. A recent study by Hofer and Mokri (2000) suggests a role for a specific UV-B absorbing substance isolated from the skin of *Rana temporaria* tadpoles in preventing UV-B damage. In addition, pigments in the skin, such as melanin, provide some protection from UV-induced DNA damage in mammals (Kollias et al., 1991). The exact roles of various pigments in human skin that provide protection from UV damage are still being identified, as are the mechanisms involved in the process (Prota, 1992). Although the matter is still open for some debate (Wu, 1999), in general, mammals with darker skin are less prone to UV-induced skin damage than those with lighter skin (Kollias et al., 1991; Barker et al., 1995). This relationship has not been well studied in amphibians.

Jablonski (1998) suggests that melanin production may protect developing amphibian em-

bryos from neural tube defects by acting as a natural sunscreen. She suggests that melanin is a relatively inexpensive way to prevent critical metabolites, such as folate, from being degraded by UV light during development. Other evidence suggests that UV-B irradiance may induce skin darkening in some amphibians (embryonic and larval *Hyla versicolor* and *Xenopus laevis*, Zaga et al., 1998; larval *Hyla arborea*, Langhelle et al., 1999).

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

Materials and Methods

Study species.—We used larvae of three salamander species, roughskin newts (*Taricha granulosa)* and Northwestern (*Ambystoma gracile)* and longtoed (*Ambystoma macrodactylum)* salamanders, to examine skin darkening in response to UV-B exposure (experiment 1). We used *A. gracile* and *A. macrodactylum* to examine darkening effects on survival and growth (experiment 2). All three species are native to the Pacific Northwest, USA (Nussbaum et al., 1983). We were most interested in UV-B-induced darkening in larval *A. macrodactylum*, because we have previously documented sublethal effects of UV-B exposure in that species (Belden et al., 2000). However, both *A. gracile* and *A. macrodactylum* embryos are sensitive to UV-B exposure (Blaustein et al., 1995, 1997); thus, we included both of them in these experiments. In addition, as larval *T. granulosa* develop in many of the same sites as *A. macrodactylum* and *A. gracile*, we were also interested in their response.

Experiment 1: Skin darkening in response to UV-B exposure.—Each species was tested individually when larvae were active and could be collected from the field. All larvae were collected within 25 km of Corvallis, Benton County, Oregon (*T. granulosa*, 20 km north; *A. gracile*, 25 km west; *A. macrodactylum*, 15 km east). For each species, approximately 35 larvae were collected and returned to the laboratory. These were housed five larvae per 38-liter aquarium in dechlorinated tapwater for 14–18 days prior to the beginning of the experiment. They were maintained at 18 C on a 12:12 h light:dark cycle and were fed *Tubifex* worms ad libitum every other day. Complete water changes were done once a week. For each species, on the day prior to beginning the experiment, 30 larvae were placed in individual petri dishes (15 cm diameter) filled with 1 cm of dechlorinated tap water. Length of all larvae was recorded by placing a ruler beneath the petri dish. Means (± SD) of the tested larvae were as follows: *T. granulosa*, 28.1(± 3.5) mm; *A. gracile*, 35.0 (± 2.7) mm; *A. macrodactylum*, 47.1 (± 6.1) mm. Ten of the 30 larvae were randomly assigned to each of three treatments: (1) UV-B exposed (acetate filter); (2) UV-B shielded (Mylar filter); and (3) dim light (black cover). Acetate transmits approximately 80% of UV-B and mylar blocks almost 100% (Blaustein et al., 1994). The black cover was used to produce a low light environment. Filters were placed over the treatments such that they did not come in contact with the water.

Following assignment to treatments, larvae were transferred to the room with UV-B lighting, after the lights were off for the day. The table surface consisted of plywood. Previous trials on the plywood surface with five larvae of each species resulted in a melanophore index (see description below; Hogben and Slome, 1931) of approximately 2–3 for the larvae after three days. Temperature was 16 C, and lights were on a 12:12 h light:dark cycle. Lighting consisted of a parallel array made up of four UV-B lights (Q-Panel, UVB313; Q-Panel, Inc., Cleveland, Ohio) alternated with four fluorescent full-spectrum lights (Vita Lite; Durotest Corp., Fairfield, NJ), suspended above the table surface to result in 3–8 $\mu W/cm^2$ of UV-B at the table surface. UV-B levels under Mylar filters were undetectable. Under acetate filters, UV-B exposures ranged from 1.0 to 3.0 $\mu W/cm^2$, which is within the natural range experienced by *A. macrodactylum* larvae developing in the Cascade Mountains, Oregon, during the summer (Belden et al., 2000). Larvae were fed *Tubifex* worms ad libitum on day 3 of the experiment.

To quantify darkening, we staged the degree of melanosome dispersion using the five stage index created by Hogben and Slome (1931). Stage one is total aggregation of melanosomes (light skin), and stage five is total dispersion (dark skin). This index is commonly used to quantify darkening responses in amphibians (e.g., Wilson and Morgan, 1979; Van Zoest et al., 1989; Rollag, 1996). Melanophores were staged five times during the five-day exposure (at 0, 12, 24, 36, and 120 h). For the first two days, readings were done within the half hour after the lights came on in the morning (0 and 24 h) and in the half hour before lights went off at night (12 and 36 h). The final reading was done half an hour before the lights went off at the end of day 5 (120 h). We used this schedule to examine initial diel changes as well as the longer term response to UV-B exposure, which was our main interest. Melanophore readings were done by the same person at each time and were done blind with regard to treatment.

Experiments for each species were completed at different times; thus, separate analyses were done for each species. Because we were mainly interested in the longer term responses to UV-B exposure, we analyzed the difference in the melanophore index between the three treatment groups only at the 120 h time point using an analysis of variance (ANOVA).

Experiment 2: Growth and survival.—To address whether skin darkening offers a survival advantage, we exposed *A. gracile* and *A. macrodactylum* larvae to UV-B while on either a dark or light background and recorded growth and survival after three weeks. For these experiments, five *A. gracile* egg masses were collected 25 km west of Corvallis, Benton County, OR, USA, returned to the laboratory and reared until large enough to test. After hatching, larvae were randomly assigned to 38-liter aquaria (5/aquaria) filled with dechlorinated tapwater. They were maintained in the laboratory at 18 C on a 12:12 h light:dark cycle, with complete water changes done once a week. Larvae were fed brine shrimp (*Artemia franciscana*) every other day until large enough to consume *Tubifex* worms, at which time they were switched to that diet. Mean length (± SD) of *A. gracile* at the time of testing was 26.4 (± 2.3) mm. *Ambystoma macrodactylum* larvae were collected from an ephemeral pond in the Cascade Mountains, Oregon, approximately 25 km south of Sisters, Oregon. These were maintained in the laboratory under the same conditions as the *A. gracile* larvae for two weeks prior to testing. For *A. macrodactylum*, the mean length (± SD) at the time of testing was 32.5 (± 1.6) mm.

One week prior to UV-B exposure, 28 larvae were placed in 15 cm diameter petri dishes filled with 1 cm depth of dechlorinated tapwater. These were then randomly assigned to either a black or white background. Background color was used to manipulate the skin color of the larvae, as many amphibians change color in response to background coloration (Bagnara and Hadley, 1973). Backgrounds were made of squares (20 cm^2) of corrugated, plasticized black or white cardboard that were placed beneath the dishes. Larvae remained in the laboratory on these backgrounds for one week to allow them to acclimate to the appropriate background color prior to UV-B exposure. Preliminary trials were completed to ensure that one week was a sufficient amount of time for both species to match the appropriate backgrounds. We used the same UV-B lighting regime and temperature as in experiment 1. For background coloration in the UV-B room, we used white or black plastic sheeting draped on all four sides and on the bottom of the test area for each background. The table was divided in half such that the same light tubes were used to illuminate both the black and white sides. The night before UV-B exposure began, seven larvae from each background group were randomly assigned to either UV-B exposed (acetate filter) or UV-B shielded (mylar filter) groups. This resulted in seven larvae in each of the 4 treatments: (1) black background with UV-B; (2) black background without UV-B; (3) white background with UV-B; and (4) white background without UV-B. Larvae were fed *Tubifex* worms every other day and complete water changes were done once a week. At the end of three weeks, the melanophore index and length was recorded for each individual. Growth was calculated for each individual as final length minus initial length.

We completed separate two-way ANOVAs on each response variable (growth and melanophore index) for each species, as they were tested at different times. The two explanatory factors used in the ANOVAs were UV-B exposure (yes/no) and background (black/white).

Results

Experiment 1: Skin darkening in response to UV-B exposure.—After five days, larval roughskin newts, *T. granulosa*, and Northwestern salamanders, *A. gracile*, that were exposed to UV-B were significantly darker than larvae exposed to full spectrum lighting without the UV-B component

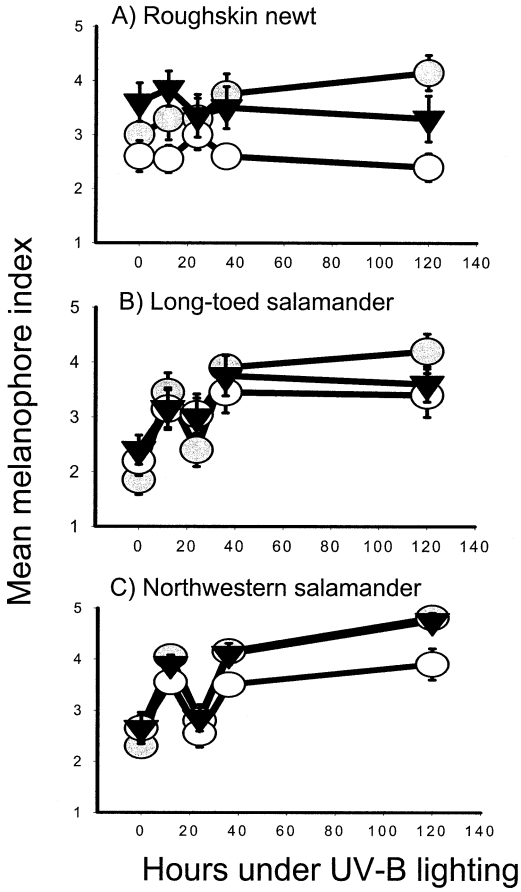

Fig. 1. Mean (±SE) melanophore index for (A) *Taricha granulosa*, (B) *Ambystoma macrodactylum*, and (C) *Ambystoma gracile* at 0, 12, 24, 36, and 120 h of UV-B exposure. Shaded circles represent larvae exposed to UV-B. Open circles represent larvae exposed to light without the UV-B component. Triangles represent larvae in dim light (black cover). Melanophore index from Hogben and Slome (1931) with 1 = lighter and 5 = darker. Statistics were performed only on larvae at the 120-h time point.

(overall ANOVA for *T. granulosa*, $P = 0.005$, Tukey posthoc between acetate and mylar groups, $P = 0.003$; overall ANOVA for *A. gracile*, $P = 0.015$, Tukey posthoc between acetate and mylar groups, $P = 0.03$; Fig. 1). In addition, long-toed salamanders, *A. macrodactylum*, showed the same trend for darkening with UV-B exposure, although it was not statistically significant (Overall ANOVA, $P = 0.259$; Fig. 1). In all three species, larvae in the low light treatment (black cover) were intermediate in coloration between the UV-B exposed and nonexposed individuals (Fig. 1). In addition, there were strong patterns of diel change during the first two days for both Ambystomatids (Fig. 1), with melanosome dispersion (darkening) occurring over the course of each day and melanosome aggregation occurring during the night. This pattern was not as strong for *T. granulosa*.

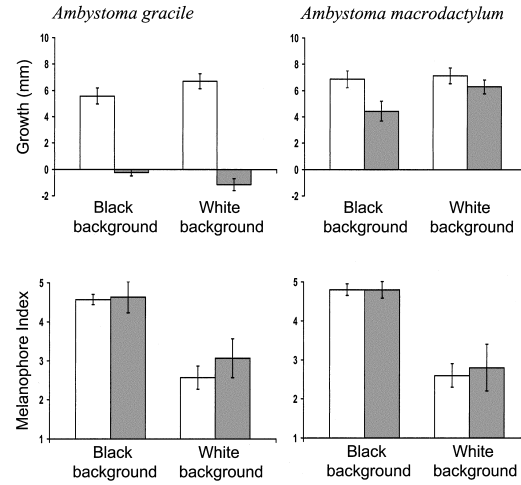

Fig. 2. Mean (± SE) growth (millimeters) and mean (± SE) melanophore index of *Ambystoma gracile* and *Ambystoma macrodactylum* on white/black backgrounds with/without UV-B after three weeks. Melanophore index from Hogben and Slome (1931) with 1 = lighter and 5 = darker. White bars represent larvae exposed to light without the UV-B component. Gray bars represent larvae exposed to UV-B.

Experiment 2: Growth and survival.—In experiment 2, for both species, variation in skin color was explained best by background coloration (*A. gracile*, $P < 0.005$ for background; *A. macrodactylum*, $P < 0.005$ for background; Fig. 2). Larvae in the black environment were significantly darker than larvae in the white environment. The interaction between background coloration and UV-B was nonsignificant for all tests, although there was a trend ($P = 0.083$) for an interaction for growth of *A. gracile*. Differences in growth after three weeks were best explained by UV-B exposure for both species (*A. gracile*, $P < 0.005$ for UV-B; *A. macrodactylum*, $P = 0.016$ for UV-B; Fig. 2). For both species, larvae that were exposed to UV-B were grew less after three weeks; in fact, many exposed *A. gracile* larvae actually shrank with exposure. Only three larvae (of 56) died during the three-week exposure and these were all *A. gracile* larvae exposed to UV-B on a black background.

Discussion

We did not find support for our hypothesis that dark larvae would be less impacted than

light larvae in terms of growth and survival when exposed to UV-B radiation. In fact, the only larvae in our second experiment that died were exposed to UV-B on dark backgrounds, and all larvae exposed to UV-B suffered from reduced growth, regardless of background color. We focused on the role of dermal melanophores, however, and epidermal melanophores may have a role in UV protection as well. But the results of our study do suggest that the protective role of skin darkening for larval amphibians exposed to UV-B requires further investigation. Indeed, another recent study has found that in spotted salamander, *A. maculatum*, embryos, DNA damage occurs in response to UV irradiation despite increased melanin production in the presence of UV-B (Lesser et al., 2001).

We did demonstrate in our first experiment that UV-B exposure can induce skin darkening in larval salamanders on a neutral background. However, the results from our second experiment indicate that background color can have a much stronger impact than UV-B exposure on skin color. If UV-B was critical in determining skin color, we would have expected individuals on white backgrounds exposed to UV-B to darken in experiment 2. Although individuals exposed to UV-B on white backgrounds were slightly darker than nonexposed individuals on white backgrounds, this result was not statistically significant. There might be strong factors present in the field that encourage selection for background matching in salamanders. For instance, work by Storfer et al. (1999) provides evidence that predation pressure can influence larval salamander coloration.

Background coloration is well known as a factor controlling the lightening and darkening of amphibians (e.g., Bagnara and Hadley, 1973). A few amphibian studies have also correlated skin darkening in response to dark backgrounds with increased circulating levels of α-melanocyte stimulating hormone (α-MSH), a peptide hormone that is synthesized and released by the *pars intermedia* of the pituitary gland (Wilson and Morgan, 1979; Fernandez and Bagnara, 1991). The darkening we saw in experiment 2 in response to background coloration is likely controlled by this mechanism, although we have been unable to measure this response because of the small size of our larvae. Daily oscillations in color change, similar to those we observed for the two Ambystomatids in experiment 1, are thought to be controlled in large part by melatonin, which is released at night and causes nocturnal blanching of the skin (Bagnara, 1965; Camargo et al., 1999).

We have previously observed reduced growth in *A. macrodactylum* exposed to UV-B as compared with unexposed controls (Belden et al., 2000), and we now report a similar response in *A. gracile* larvae. Growth reduction in *A. gracile* was more severe than in *A. macrodactylum*, with many individuals actually shrinking in body length. A similar reduction in vertebrate length has been documented previously in marine iguanas and has been associated with energetic stress (Wikelski and Thom, 2000). The energetic stress of repairing UV damage may be the cause of growth reductions observed in our study. Reduced growth as a result of UV-B exposure is unlikely to be a consequence of the energetic cost of skin darkening or pigment production, because larvae on both backgrounds incurred damage, regardless of skin color. Sublethal effects of UV-B exposure on growth can be important because larval amphibians must reach a minimum size threshold for metamorphosis (Wilbur and Collins, 1973). This is particularly critical for larval amphibians developing in temporary ponds, where they must metamorphose prior to pond drying. Larger amphibian larvae also are generally larger when they metamorphose, which can have positive consequences for adult fitness (e.g., Smith, 1987; Semlitsch et al., 1988).

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

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