DNA REPAIR AND RESISTANCE TO UV-B RADIATION IN WESTERN SPOTTED FROGS

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Abstract. We assessed DNA repair and resistance to solar radiation in eggs of members of the western spotted frog complex (*Rana pretiosa* and *R. luteiventris*), species whose populations are suffering severe range reductions and declines. Specifically, we measured the activity of photoreactivating enzyme (photolyase) in oocytes of spotted frogs. In some species, photoreactivation is the most important mechanism for repair of UV-damaged DNA. Using field experiments, we also compared the hatching success of spotted frog embryos at natural oviposition sites at three elevations, where some embryos were subjected to ambient levels of UV-B radiation and others were shielded from UV-B radiation. Compared with other amphibians, photolyase activities in spotted frogs were relatively high. At all sites, hatching success was unaffected by UV-B. Our data support the interpretation that amphibian embryos with relatively high levels of photolyase are more resistant to UV-B radiation than those with lower levels of photolyase. At the embryonic stage, UV-B radiation does not presently seem to be contributing to the population declines of spotted frogs.

Key words: amphibians; DNA repair; frogs and ultraviolet radiation; oocytes; photolyase; photoreactivation; Rana luteiventris; Rana pretiosa; *spotted frogs; ultraviolet radiation; UV-B resistance.*

Introduction

Atmospheric and associated global climate changes may have significant negative impacts on plants and animals. For example, ultraviolet (UV) radiation has been linked to decreased productivity in phytoplankton and terrestrial plants, coral bleaching, diminished populations of freshwater insect larvae, and egg mortality in amphibians (e.g., Bidigare 1989, Gleason and Wellington 1993, Blaustein et al. 1994*a*, 1997*a*, 1998, Bothwell et al. 1994, Caldwell et al. 1995, Häder 1997, Häder and Worrest 1997). In freshwater systems, increased exposure to UV radiation may occur because of stratospheric ozone depletion or as a result of climate warming and lake acidification that can lead to decreases in dissolved organic carbon concentrations (Schindler et al. 1996, Yan et al. 1996, Hader 1997).

Recent reports suggest that many amphibian species are exhibiting population declines and range reductions (e.g., Semb-Johansson 1989, Wake 1991, Crump et al. 1992, Richards et al. 1993, Pounds and Crump 1994,

Blaustein and Wake 1995, Stebbins and Cohen 1995, Fisher and Shaffer 1996, Laurance et al. 1996, Reaser 1996, Pounds et al. 1997). Mortality at the egg and embryo stage may be contributing to the population declines of certain amphibian species (Blaustein et al. 1994*a,b*, 1998). Indeed, accumulating data suggest that UV-B (280–315 nm) radiation is one factor that is contributing to such mortality in certain species, across a wide array of locations including North America (Blaustein et al. 1994*a*, 1995*a*, Anzalone et al. 1998), Europe (Lizana and Pedraza 1998), and Australia (Broomhall et al. *in press*). However, the eggs and embryos of some species are relatively resistant to damage by ambient levels of UV-B radiation (Blaustein et al. 1994*a*, 1995*a*, 1996, 1998, Blaustein and Kiesecker 1997, Ovaska et al. 1997, Anzalone et al. 1998, Corn 1998, Lizana and Pedraza 1998, Broomhall et al., *in press*).

For some species, hatching success in the field is greater for eggs that are shielded from UV-B radiation than for eggs in unshielded control conditions (Blaustein et al., 1998). Furthermore, there is a correlation between resistance to UV-B and the activity of the photoreactivating enzyme, photolyase. In some organisms,

photoreactivation is the most important mechanism for repair of cyclobutane pyrimidine dimers (CPDs), which are major cytotoxic and mutagenic photoproducts in DNA (Pang and Hays 1991). Species with the highest photolyase activities seem to be more resistant to UV-B radiation (Blaustein et al. 1994a, 1995a, 1996, 1997a).

Red-legged (*Rana aurora*) and Cascades (*R. cascadae*) frogs and members of the western spotted frog complex (*R. pretiosa* and *R. luteiventris*) have experienced range reductions in the western United States (McAllister et al. 1993, Blaustein et al. 1994a, 1996, Drost and Fellers 1996, Reaser 1997). UV-B radiation may be a contributing factor in the range reduction of *R. cascadae* because it lowers the hatching success of its eggs in nature (Blaustein et al. 1994a). However, the hatching success of *R. aurora* is not affected by ambient levels of UV-B radiation (Blaustein et al. 1996), and photolyase levels in *R. aurora* are higher than those in *R. cascadae* (Blaustein et al. 1994a, 1996). Furthermore, unusual egg mortality in nature has been reported in *R. cascadae* but not in *R. aurora* (Kiesecker and Blaustein 1997). There are no reports of unusual egg mortality for *R. pretiosa* and *R. luteiventris*, but these species have not been studied extensively. Egg mortality could have contributed to range reductions, but recent, detailed studies of reproduction in the western spotted frog complex are lacking. Thus, it is especially important to know if egg mortality is occurring in spotted frogs and if UV-B is a contributing factor.

To further extend our knowledge of the interrelationships among UV resistance, photolyase activities, and the potential impact of UV on amphibian populations, we examined resistance of eggs to ambient levels of UV-B radiation and photolyase activity in the Oregon spotted frog (*R. pretiosa*) and the newly recognized Columbia spotted frog (*R. luteiventris*) (Green et al. 1996, 1997). Members of this western spotted frog complex had an historical range from extreme southwestern Alaska south to central Nevada and central Utah, east to western Montana and northwestern Wyoming. However, *R. pretiosa* and *R. luteiventris* have experienced a dramatic range reduction in western Washington and Oregon (Leonard et al. 1993, McAllister et al. 1993, Blaustein et al. 1995b, McAllister and Leonard 1997). Populations of spotted frogs are patchy and rare west of the Cascade Range in Washington and have not been found west of the Cascade Range in Oregon for ~25 years (Leonard et al. 1993, McAllister et al. 1993). The status of spotted frogs in eastern Oregon and Washington is unknown. Causes for the severe range reduction in this complex of frogs are unknown and *R. pretiosa* is a candidate for rare and endangered status on the U.S. Fish and Wildlife Service Notice of Review (Marshall et al. 1996).

MATERIALS AND METHODS
Oocyte extract for photolyase activity assay

We collected oocytes from two female *Rana luteiventris* from Blue Lake, Washington, United States (Chelan County; elevation 1679 m), from two female *R. pretiosa* from Sand Creek, Nevada (Elko County; 2017 m), and from one female *R. pretiosa* from Trout Lake, Washington (Klickitat County; 596 m). To assess photolyase activities in oocytes of *R. luteiventris* and *R. pretiosa,* we used methods similar to those described in Blaustein et al. (1994a). Ovaries were dissected out and their lobes were sectioned and pulverized with three volumes of Modified Transcription Buffer (Glikin et al. 1984, Blaustein et al. 1994a). The resulting slurry was centrifuged in a Beckman TLA I 10. 2 rotor (Backman, Palo Alto, California, USA) at 4°C for 60 min at 60 000 × g. The exudate layer between the debris pellet and upper yolk layer was recovered and used for photolyase and protein assays. Protein concentrations were determined by the Bradford technique (Bradford 1976). Yields from ~400 oocytes per individual were ~2 mL of extract at a protein concentration of 12.0 mg/mL.

Aliquots of extract were diluted to 0.3 mg/mL in Modified Transcription Buffer and were assayed for blue-light-dependent removal of cyclobutane pyrimidine dimers (CPDs) from exogenous UV-irradiated DNA, using acid hydrolysis and thin-layer chromatography, as described by Blaustein et al. (1994a). Assays employing 1.5, 3.0, 4.5, and 6.0 μg total extract protein, respectively, were used and defined the linear range of response for the assay. Results from duplicate assays at each protein concentration in the linear range were used to determine the specific activities of the respective oocyte extracts (CPDs removed per hour per microgram of extract protein; Blaustein et al. 1994a).

Field experiments

Field experiments were conducted at natural oviposition sites in Washington State, USA, at three different locations and elevations. *Rana pretiosa* experiments were conducted at Dempsey Creek (Thurston County; elevation 37 m) from 4 to 16 March 1996 and Trout Lake Marsh (Klickitat County; 596 m) from 28 March to 4 April 1996. *Rana luteiventris* experiments were conducted at Blue Lake Trailhead (Chelan County; 1679 m) from 10 to 21 June 1996.

At Dempsey Creek and Trout Lake, we placed 150 newly deposited eggs (25 eggs from each of six clutches, <24 h old), with their jelly matrix intact, in each of 12 enclosures (38 × 38 × 7 cm) immersed in 5–10 cm of water, a depth at which eggs are often laid (Stebbins 1954, McAllister and Leonard 1997). The 12 enclosures were assigned to three sunlight treatments: unfiltered sunlight, sunlight filtered to remove UV-B and shorter wavelengths, and sunlight filtered to remove wavelengths shorter than ~290 nm (a control for placing filters over eggs). There were four replicates per treatment. Enclosures were placed in a linear array in a randomized block design at an oviposition site where numerous egg masses were laid. Enclosures had clear UV-B transmitting Plexiglas and floors of 1 mm² fiberglass mesh screen. These methods were repeated
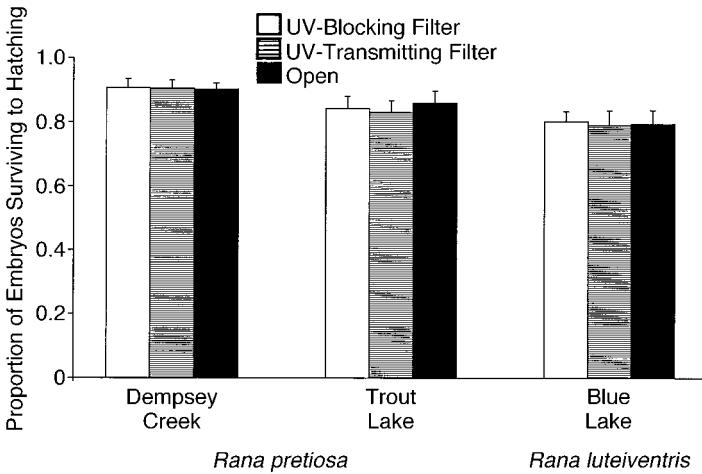
FIG. 1. Effects of UV-B radiation on hatching success ($\bar{X}$ + 1 SE) in the Oregon spotted frog (*Rana pretiosa*) and Columbia spotted frog (*R. luteiventris*).

at Blue Lake Trailhead, except that, because fewer eggs were laid at this site, we used 25 eggs from four clutches for a total of 100 eggs per enclosure.

A UV-B blocking filter (50 × 50 cm) made of Mylar-D was placed over one-third of the enclosures. A cellulose acetate filter (50 × 50 cm) that transmitted UV-B but not UV-C (wavelengths less than ~290 nm) was placed over another one-third of the enclosures. The remaining enclosures had no filters. The Mylar blocked 100% of UV-B (280–315 nm). The acetate allowed ~80% transmission of UV-B (Blaustein 1994*a*). The transmitting properties of Mylar and acetate were assessed by scanning a UV-B 313 (peak UV-B) lamp directly with an Optronics 752 spectroradiometer (Optronics, Chelmsford, Massachusetts, USA) and comparing the transmission with the same lamp covered with Mylar and acetate.

Experiments were terminated when all embryos had either hatched or died. All eggs were counted each day during the experiment. Survival was measured as the proportion of hatchlings produced per enclosure. Daily temperatures were taken within enclosures in each treatment. ANOVA was used to test for treatment effects on embryo survivorship. Post hoc comparisons (Tukey Test; Zar 1984) were performed to test for differences between means among the three regimes. We

used a power test (Cohen 1988) to assess our statistical ability to detect differences between treatments. Power values below 0.8 are considered inadequate for confidence in concluding that there were no differences between treatments.

RESULTS

Photolyase activity

Both *Rana pretiosa* and *R. luteiventris* showed similar, relatively high levels of photolyase activity. Photolyase activities (mean ± 1 SE) for *R. luteiventris* were 6.84 ± 0.3 and 6.31 ± 0.03 × 10^{11} CPDs·h^{-1}·µg^{-1} from Blue Lake, Washington and Sand Creek, Nevada, respectively. The average photolyase level for *R. pretiosa* from Trout Lake, Washington was 6.62 ± 0.01 × 10^{11} CPDs·h^{-1}·µg^{-1}.

Field experiments

Before survivorship was examined in field experiments, we first performed a preliminary analysis that indicated no significant block effects. Therefore, the block and error (mean squares and degrees of freedom) were pooled for all subsequent tests (Zar 1984). The survivorship data met all parametric assumptions (normality and homogeneity of variance) and transformations were not necessary.

Eggs and developing embryos were not affected by UV-B radiation in any of the field experiments. There were no significant differences in hatching success among the three regimes (Fig 1, Table 1). Power at an expected difference of 0.1 was 0.956 and 0.873 for *R. pretiosa* at Dempsey Creek and Trout Lake, respectively, and 0.814 for *R. luteiventris* at Blue Lake.

Temperatures did not differ significantly among treatments (ANOVA). Mean temperatures for the UV-B blocking, transmitting, and open regimes, respectively, were 11.6°, 11.4°, and 11.8°C ($F_{2,9} = 0.071$, $P = 0.917$) for *R. pretiosa* at Dempsey Creek; 13.8°, 13.9°, and 13.6 °C ($F_{2,9} = 0.107$, $P = 0.903$) for *R. pretiosa* at

TABLE 1. Univariate ANOVA of hatching success in western spotted frogs (*Rana pretiosa* and *R. luteiventris*).

Source of variation	MS	df	F	P
R. pretiosa at Dempsey Creek				
Treatment	0.00001	2	0.037	0.964
Error	0.00027	9		
R. pretiosa at Trout Lake				
Treatment	0.00080	2	0.937	0.427
Error	0.00086	9		
R. luteiventris at Blue Lake				
Treatment	0.00015	2	0.210	0.814
Error	0.00075	9		

Trout Lake; and 12.9, 12.4, and 12.7 °C ($F_{2,9} = 0.055$; $P = 0.947$) *R. luteiventris* at Blue Lake. All eggs were accounted for during the experiments. Predation of eggs did not occur.

DISCUSSION

Compared with other amphibians, *Rana pretiosa* and *R. luteiventris* have relatively high levels of photolyase activity. These species have more than four times the activity found in the western toad (*Bufo boreas*) and more than twice the activity found in a closely related congener, *R. cascadae* (Blaustein et al. 1994*a*). Both species of spotted frogs have somewhat higher levels of photolyase activity than another closely related congener, *R. aurora,* a species also with relatively high levels of photolyase activity (6.09×10^{11} CPDs·h^{-1}·µg^{-1}; Blaustein et al. 1996). Of the North American anurans that we have examined, only the Pacific treefrog (*Hyla regilla*) has higher levels of photolyase activity than do spotted frogs (Blaustein et al. 1994*a*).

The photolyase assays and results of our field experiments lend further support to the ''UV-Sensitivity Hypothesis'' (Blaustein et al. 1994*a*), which proposes that species with relatively high levels of photolyase activity are more resistant to ambient levels of UV-B radiation than are those species with lower levels. In our present and previous studies, embryos of four anuran species with the highest levels of photolyase activity in eggs and oocytes were resistant to ambient levels of UV-B radiation in field experiments (Blaustein et al. 1994*a*, 1996). Two anuran species and two salamander species (*Ambystoma gracile* and *A. macrodactylum*) with relatively low levels of photolyase activity had significantly reduced hatching success under UV-B transmitting and open (no filter) regimes in field experiments (Blaustein et al. 1994*a*, 1995*a*, 1997*b*). Furthermore, embryos of the California treefrog (*Hyla cadaverina*) are damaged by ambient UV-B radiation, and this species, too, has eggs with relatively low levels of photolyase activity (Anzalone et al. 1998).

It is generally assumed that UV levels are greater at higher altitudes (Blumthaler and Ambach 1990). In field experiments, however, spotted frog embryos were resistant to UV-B near sea level and at higher elevations. Nevertheless, there is great potential for eggs and embryos of spotted frogs to be exposed to UV-B radiation throughout development, because their eggs are usually laid in extremely shallow water and may be only partially submerged (Green and Campbell 1984, Leonard et al. 1993; A. R. Blaustein, *personal observations*).

Our study was designed to investigate whether *ambient* levels of UV-B radiation influenced hatching success in spotted frogs. Because ambient, rather than artificially enhanced, UV-B was the variable of concern, we believe that our study was ecologically relevant. Of course, it is possible that in other regions, or at our study sites at different times, with changing atmo-spheric conditions and fluctuating UV-B levels, western spotted frogs may experience a decrease in hatching success when exposed to ambient levels of UV-B radiation. Although measurements of UV-B would not influence the conclusions of this study or similar studies, such measurements may reveal the minimum doses that will cause egg mortality (discussed in Blaustein et al., 1998), another relevant question. However, this information would be more interesting with regard to species that are sensitive to UV-B radiation. The high photolyase levels in *R. pretiosa* and *R. luteiventris* suggest that, over evolutionary time, there seems to have been strong selection pressure for resistance in embryos to ambient UV-B radiation.

Numerous reports suggest that populations of spotted frogs are less numerous than they once were (e.g., Nussbaum et al. 1983, McAllister et al. 1993, McAllister and Leonard 1997, Reaser 1997). In fact, McAllister and Leonard (1997) suggest that the Oregon spotted frog (*R. pretiosa*) may have been lost from ~90% of its historic range and recommend that it be listed as a state endangered species in Washington. Reaser (1997) found that *R. luteiventris* has disappeared from >50% of historically occupied sites in Nevada. For several reasons, we suggest that populations of spotted frogs are not being significantly impacted by continued mortality at the embryo stage due to UV-B radiation. Spotted frogs (1) have relatively high levels of photolyase activity in eggs and oocytes; (2) their embryos are resistant to UV-B radiation in field experiments; and (3) no massive egg mortality in the field (other than that caused by freezing) has been reported. Of course, it is possible that UV-B radiation could be detrimental to spotted frogs at other stages of development. Moreover, the effects of UV-B radiation may only become manifest if there is an interaction with other agents (Kiesecker and Blaustein 1995, Long et al. 1995). It is also possible that populations of spotted frogs that were less resistant to UV-B have already disappeared, and the more resistant individuals have persisted. Nevertheless, based on the available data, we suggest that factors other than UV-B radiation are presently contributing to the decline of spotted frog populations.

Habitat destruction, introduced exotic species, and chemical pollution, including pesticides and fertilizers, are more likely than UV-B radiation to have contributed to the decline in spotted frog populations. For example, the shallow floodplain pools that are inhabited by spotted frogs in Washington have been drained, diked, and filled to accommodate human needs (McAllister and Leonard 1997). In the lowlands of Puget Sound (Washington state), wetlands have been lost in great number and exotic plants have changed the character of many existing wetlands, perhaps decreasing their suitability for spotted frogs (McAllister and Leonard 1997). Similar habitat alteration has occurred in Oregon (e.g., Olson et al. 1997).

Introduced predators such as bullfrogs (*Rana catesbeiana*) and fishes may have been responsible for the extirpation of several populations of spotted frogs in Washington (McAllister et al. 1993), as has been suggested for red-legged (*Rana aurora*) frogs in Oregon and foothill yellow-legged (*R. boylii*) frogs in California (Blaustein et al. 1996, Fisher and Shaffer 1996, Kiesecker and Blaustein 1997, 1998, Kupferberg 1997). Pesticides such as DDT may have contributed to mortality of *R. pretiosa* in Oregon in the mid 1970s (Kirk 1988) and may be a contributing factor to declining populations of this species. Other chemicals, such as nitrites and nitrates in fertilizers, can be toxic to amphibians (Oldham et al. 1997, Marco and Blaustein *in press*) and may contribute to losses in spotted frog populations.

Assessing the causes for declining amphibian populations is complex because numerous factors may contribute to population declines. Our field experiments provided a rigorous assessment of one factor, ambient UV-B radiation, on the embryonic stage. Additional experimental tests at various life stages are warranted.

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