Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1791–1795, March 1994
Ecology

UV repair and resistance to solar UV-B in amphibian eggs: A link to population declines?

(UV radiation/DNA repair)

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Communicated by George N. Somero, November 12, 1993 (received for review October 27, 1993)

ABSTRACT **The populations of many amphibian species, in widely scattered habitats, appear to be in severe decline; other amphibians show no such declines. There is no known single cause for the declines, but their widespread distribution suggests involvement of global agents—increased UV-B radiation, for example. We addressed the hypothesis that differential sensitivity among species to UV radiation contributes to these population declines. We focused on species-specific differences in the abilities of eggs to repair UV radiation damage to DNA and differential hatching success of embryos exposed to solar radiation at natural oviposition sites. Quantitative comparisons of activities of a key UV-damage-specific repair enzyme, photolyase, among oocytes and eggs from 10 amphibian species were reproducibly characteristic for a given species but varied >80-fold among the species. Levels of photolyase generally correlated with expected exposure of eggs to sunlight. Among the frog and toad species studied, the highest activity was shown by the Pacific treefrog (*Hyla regilla*), whose populations are not known to be in decline. The Western toad (*Bufo boreas*) and the Cascades frog (*Rana cascadae*), whose populations have declined markedly, showed significantly lower photolyase levels. In field experiments, the hatching success of embryos exposed to UV radiation was significantly greater in *H. regilla* than in *R. cascadae* and *B. boreas*. Moreover, in *R. cascadae* and *B. boreas*, hatching success was greater in regimes shielded from UV radiation compared with regimes that allowed UV radiation. These observations are thus consistent with the UV-sensitivity hypothesis.**

Populations of many amphibian species in widely distributed locations appear to have undergone declines and range reductions in recent times (1–4, ‡), with some apparently becoming extinct (4, 5). However, not all species within the same regions are affected (1, 2, 4, 6). No single cause for the amphibian population declines has been identified (1, 4). Although environmental degradation has been implicated, populations have declined in relatively undisturbed regions (1–4, 7, ‡). The diversity of the locations where amphibian populations have declined prompts consideration of atmospheric factors—e.g., increased terrestrial UV-B irradiance associated with depletion of stratospheric ozone. To elucidate possible relationships between UV-B stress and amphibian declines, we measured two critical factors that could ultimately play a role in amphibian population losses. First, we measured levels of enzymatic photoreactivation, a key activity for repair of UV-damaged DNA, in eggs and oocytes from nine species of amphibians from the Oregon Cascade and Coast Mountains (USA) and from laboratory-reared *Xenopus laevis*. These species differ considerably in exposure of their eggs to sunlight. Photoreactivating enzyme (photolyase) occurs in many organisms (8, 9); in some, photoreactivation is the most important mechanism for repair of cyclobutane pyrimidine dimers (CBPDs) (10), which are major cytogenic and mutagenic photoproducts in DNA. UV photoproducts impede gene expression by blocking transcription.

Second, we performed field experiments to measure the effects of solar UV-B on hatching success in three anuran species—*Hyla regilla, Rana cascadae*, and *Bufo boreas*. The eggs of these species are laid in open water, highly exposed to sunlight (11, 12). The populations of *R. cascadae* and *B. boreas* have undergone drastic declines (13, 14) and the eggs of *B. boreas* have experienced unusually high mortality at certain sites (15). Moreover, laboratory experiments have shown that *B. boreas* embryos from the Cascade Mountains of Oregon suffer increased mortality from prolonged exposure to UV (16). Populations of *H. regilla*, however, are not known to be in decline.

Amphibians are sensitive indicators of environmental change (17, 18, ‡). Their skin is not protected by hair or feathers; their eggs lack hard outer shells, thus exposing them to the environment (17). These characteristics may make amphibians especially sensitive to changes in atmospheric conditions, including changes in levels of UV radiation. At the terrestrial surface, most UV radiation of biological concern is in the 290 to 320-nm (UV-B) band (19–21); critical biomolecules absorb light of higher wavelength less efficiently and stratospheric ozone absorbs most light of lower wavelength (19–21).

Seasonal increases in UV-B irradiance linked to stratospheric ozone depletion (22) appear well documented at polar regions (23); there is some evidence for recent UV-B increase in relatively undisturbed temperate latitudes (24). Progressive expansion of impacted areas to lower latitudes is anticipated (25, 26). There are no data available for UV-B irradiances at specific locations where populations are declining. However, sustained small increases or temporary fluctuations in UV-B may affect especially sensitive species.

Elucidation of relationships between amphibian declines and UV-B exposure requires measurement of UV-exposure and UV-resistance parameters and comparisons of species whose populations appear to be in decline with species whose populations are persistent. The UV-sensitivity hypothesis predicts (*i*) significant differences among amphibian species with respect to UV-repair activities in eggs and differential hatching success of embryos exposed to solar radiation, (*ii*) correlation of these differences with expected exposure of eggs to sunlight, and (*iii*) higher repair activities for nondeclining species compared with those showing a decline, where exposures to sunlight are roughly similar.

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Abbreviation: CBPD, cyclobutane pyrimidine dimer.

‡Wake, D. B. & Morowitz, H. J., Report to National Research Council on Workshop on Declining Amphibian Populations, Feb. 19–20, 1990, Irvine, CA, p. 1–11.

MATERIALS AND METHODS

Collection of Amphibians and Eggs and Preparation of Egg and Oocyte Extracts. We collected whole clutches of eggs, within 12 hr after being laid, from field sites in the central Cascade and Coast Mountain Ranges of Oregon and/or removed oocytes from animals captured from these sites.

To stimulate egg laying by (laboratory-reared) *X. laevis* females, we injected them successively with 500 units of pregnant mare serum and, 24 hr later, with 1000 units of human chorionic gonadotrophin. We anesthetized female specimens by immersion in 1% aqueous tricaine (3-aminobenzoic acid ethyl ester) and then sacrificed them by subcutaneous injection with 1 ml of saturated tricaine solution. We removed oocytes or pro-eggs [arbitrarily defined as (originally internal) oocyte-like objects encapsulated in transparent jelly-like material] by dissection. To prepare extracts, we covered tissues with modified oocyte transcription buffer (7.5 mM potassium acetate/10 mM $MgSO_4$/4 mM ATP/2 mM EGTA/0.2 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/20 mM creatine phosphate/80 μg of creatine kinase per ml/10% glycerol/40 mM Hepes, pH 8.0) (27), crushed tissues by sedimentation in a Beckman TLA 100.2 rotor at 4°C for 60 min at 60,000 × *g*, and recovered the exudate, essentially as described by Glikin *et al.* (28). Pro-eggs and eggs were first dejellied by treatment for 30–120 min with 2% cysteine and then sedimented as above. We determined protein concentrations by the Bradford technique (29).

Chromatographic Assay for Photolyase Activity. We measured light-dependent removal of CBPDs from exogenous UV-irradiated DNA in egg/oocyte protein extracts. To minimize effects of differential DNase levels in extracts, we assayed bases hydrolyzed from DNA after photoreactivation. To ensure quantitative comparisons, we averaged values for several assays in the range where activities varied linearly with protein concentration (Fig. 1). For some species, we measured activities in eggs and pro-eggs. In four species, activities were measured in eggs sampled from two different years. Because oocytes store factors needed for rapid growth following fertilization (30), we expected early cell proliferation to have little effect on photolyase-specific activities in eggs.

Substrates were *Escherichia coli* DNAs, radiolabeled to 2.5–10 × 10^5 cpm/μg by overnight growth in glucose-minimal medium containing [^{3}H]thymidine (20 μCi/ml; 1 Ci = 37 GBq), extracted from cells and irradiated at 254 nm to 400 J/m^2. Typically 3–4% of thymine residues were converted to CBPDs. We incubated 200,000 cpm of irradiated [^{3}H]DNA (0.2–0.8 μg) with 1–10 μg of extract protein, in photoreactivation buffer (50 mM Tris·HCl, pH 7.4/1 mM EDTA/10 mM NaCl/10 mM dithiothreitol) in 30-μl volumes, for 0.5–6 hr at 22°C, with continuous blue-light irradiation (two 20-W Philips F20T 12/B lamps at 25 cm, filtered by two 3-mm glass plates).

To inhibit DNase activity, we added EDTA to 5 mM and EGTA to 2.5 mM. We purified DNA from reaction mixtures by phenol extraction and acid precipitation and then measured CBPDs by formic acid hydrolysis and one-dimensional silica-gel thin-layer chromatography, essentially as described by Reynolds *et al.* (31). When we treated the UV-irradiated DNA substrate with purified *E. coli* photolyase (gift of A. Sancar), or an excess of high-activity egg extract, the amount of radiolabel that chromatographed at the CBPD position was reduced to <0.1% of total thymine. Incubation times were chosen to make the numbers of CBPDs removed proportional to amounts of extract for at least three of four extract concentrations used.

Where sets of assays were performed on two different days, ranges were typically 10–15% of averages. Eggs (50–

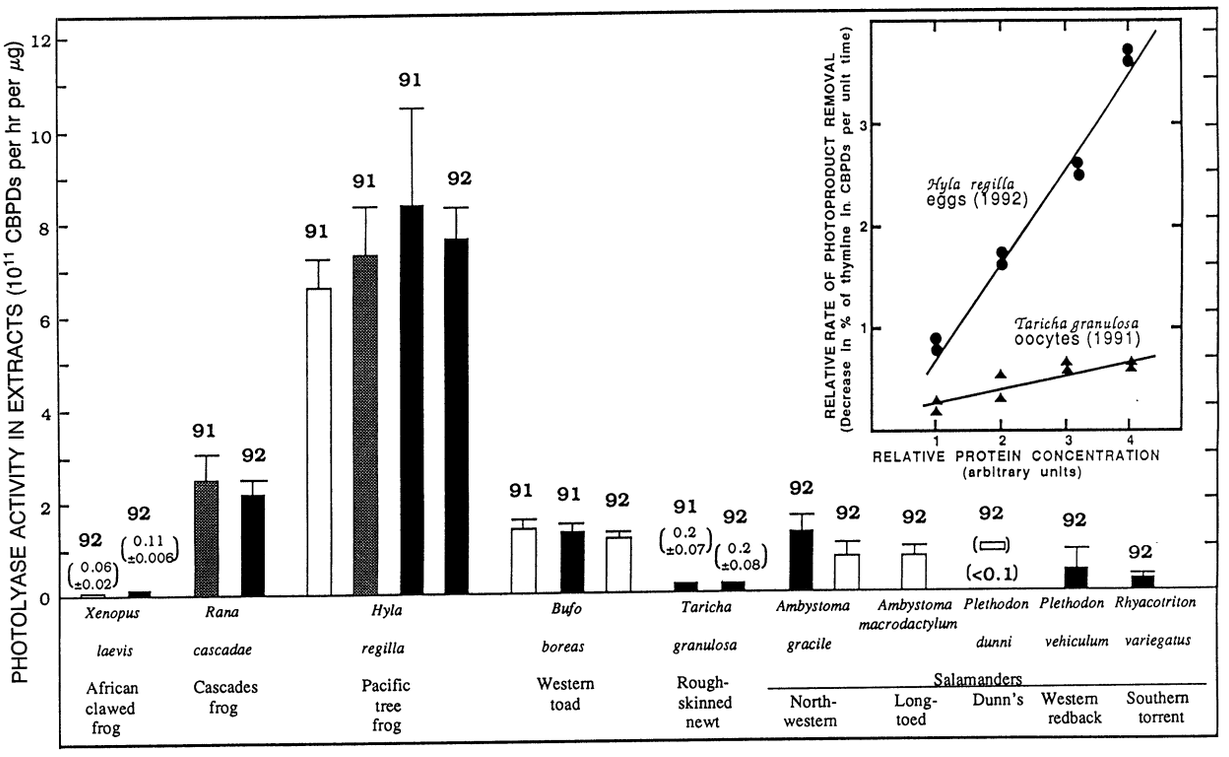

FIG. 1. Photolyase activities. Specific activities (rates of CBPD removal per μg of protein) are averages for six or eight simultaneous assays, at extract concentrations in the linear range (see *Inset*), for oocytes (open bars), pro-eggs (stippled bars; internal but encapsulated in jelly), and eggs (filled bars) (standard deviations indicated). Eggs and oocytes harvested in 1991 and 1992 are indicated by 91 and 92. Specific activities did not vary systematically with substrate concentration in the range of 200–800 ng, for five species tested, including *Rana*, *Bufo*, and *Hyla*. Differences between the values for the two concentrations were 4–25%. (*Inset*) Activity–concentration profiles for 800 μg of irradiated DNA substrate incubated 60 min with 2.2–9.0 μg of *H. regilla* extract and for 200 μg of substrate incubated 90 min with 4.0–16.2 μg of *T. granulosa* extract.

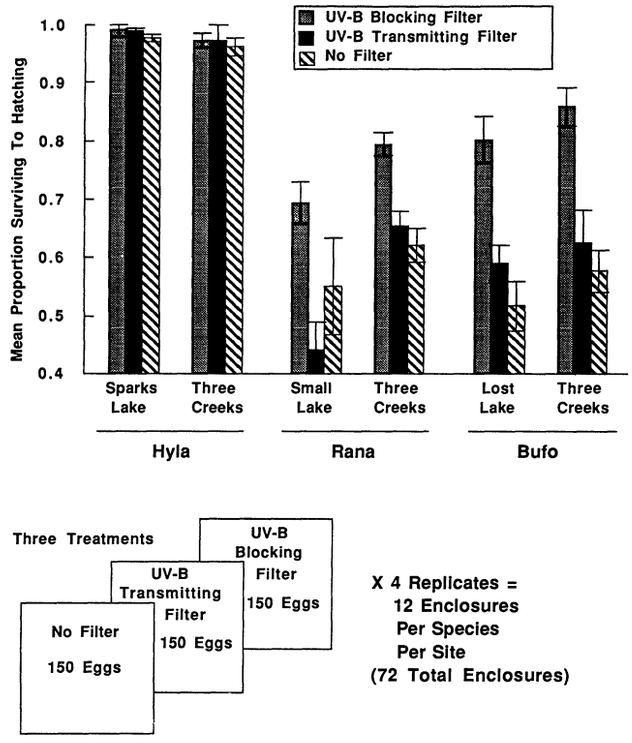

FIG. 2. Effects of UV-B on hatching success ($\bar{x} \pm$ SE) in three anuran amphibians and schematic representation of the experimental design.

200 per clutch) from three to five clutches were pooled. Oocytes/pro-eggs (12–200 per animal) from two to four animals (except one for *Plethodon dunni, Rhyacotriton variegatus, Ambystoma gracile*) were pooled.

Field Experiments. Field experiments were conducted in the Oregon Cascade Mountains. All species were tested at Three Creeks Lake (43 km west of Bend, Deschutes Co., Oregon, elevation 2000 m). Additionally, *H. regilla* was tested at Sparks Lake (Deschutes Co., Oregon, 42 km west of Bend, Oregon, elevation 1655 m), *R. cascadae* at Small Lake (Linn Co., Oregon, 92 km east of Albany, Oregon, elevation 1190 m), and *B. boreas* at Lost Lake (Linn Co., Oregon, 97 km east of Albany, elevation 1220 m).

We placed 150 newly deposited eggs (<24 hr old) in each of 12 enclosures at natural oviposition sites of each species (two sites per species). The 12 enclosures at each site were randomly assigned to three sunlight treatments: unfiltered sunlight, sunlight filtered to remove UV-B and shorter wavelengths, and sunlight filtered to remove wavelengths shorter than UV-B (a control for placing filters over eggs) (Fig. 2). Enclosures (38 × 38 × 7 cm) were placed in a linear array, parallel to the water's edge (depth of 5–10 cm) in a randomized block design (32). Enclosures had clear Plexiglas frames with floors of 1-mm^2 fiberglass mesh screen. For *R. cascadae* and *B. boreas*, 25 eggs from each of six different clutches (total = 150 eggs per enclosure) were placed in each enclosure. Because of their small clutch size, for *H. regilla* we used eggs from more than six clutches and randomly assigned 25 eggs from at least six clutches to each enclosure (total = 150 eggs per enclosure).

A UV-B blocking filter (50 × 50 cm) made of Mylar was placed over one-third of the enclosures. An acetate filter (50 × 50 cm) that transmitted UV-B was placed over another third of the enclosures. The remaining enclosures had no filters. There were four replicates per treatment for each species at each site (Fig. 2). The experiment was terminated when all of the original embryos either hatched or died. Survival was measured as the proportion of hatchlings produced per enclosure.

The transmitting properties of the Mylar and acetate used on enclosures were assessed before and after experiments by scanning a UV-B 313 lamp directly with an Optronics 752 spectroradiometer and comparing the transmission with the same lamp covered with acetate and Mylar. The Mylar blocked 100% of UV-B (290–320 nm). The acetate allowed about 80% transmission of UV-B.

RESULTS

Where photolyase was measured in both internal (oocytes, pro-eggs) and external (eggs) tissues (five species) or in two

Table 1. Univariate analysis (ANOVA) of hatching success in three amphibian species

Proportion hatched	Effect	MS	df	*F*	*P*
H. regilla					
Sparks Lake	Treatment	<0.001	2	0.732	0.507
	Error	<0.001	9		
Three Creeks	Treatment	<0.001	2	0.185	0.834
	Error	0.001	8		
R. cascadae					
Small Lake	Treatment	0.065	2	4.724	0.040
	Error	0.014	9		
Three Creeks	Treatment	0.034	2	13.808	0.002
	Error	0.002	9		
B. boreas					
Lost Lake	Treatment	0.089	2	15.393	0.001
	Error	0.006	9		
Three Creeks	Treatment	0.091	2	12.362	0.003
	Error	0.007	9		

A preliminary analysis indicated no significant block effects. Therefore, the block and error terms [mean squares (MS) and degrees of freedom (df)] were pooled for remaining tests (32). Post hoc comparisons (Tukey Test) (32) were performed to test for differences between means among the three regimes. Temperatures were taken within enclosures for each species in each treatment. Mean temperatures are given for each species at each site for the unfiltered, UV-B transmitting, and UV-B blocking regimes, respectively: *Hyla* at Three Creeks = 15.6°C, 15.8°C, and 15.8°C; *Hyla* at Sparks Lake = 21.4°C, 21.7°C, and 21.5°C; *Rana* at Three Creeks = 15.4°C, 15.2°C, and 15.3°C; *Rana* at Small Lake = 9.0°C, 9.3°C, and 9.2°C; *Bufo* at Three Creeks = 19.4°C, 19.2°C, and 19.6°C; *Bufo* at Lost Lake = 14.7°C, 14.5°C, and 14.6°C. Field experiments were conducted from 18 April–20 June (1993) corresponding to the anuran breeding cycles. *F* = F statistic; *P* = probability.

Table 2. Specific activity of photolyase, mode of egg laying, and relative exposure of eggs to sunlight in 10 amphibian species

Species	Specific activity of photolyase,* 10^{11} CBPDs per hr per μg	Egg-laying mode/exposure to sunlight	Ref(s).
P. dunni	<0.1	Eggs hidden/not exposed	11, 12
X. laevis	0.1	Eggs laid in laboratory; in nature, eggs laid under vegetation/limited exposure	35
T. granulosa	0.2	Eggs hidden/limited exposure	36
R. variegatus	0.3	Eggs hidden/not exposed	11, 37
P. vehiculum	0.5	Eggs hidden/not exposed	11, 37
A. macrodactylum	0.8	Eggs often laid in open water/some exposure	11, 37
A. gracile	1.0	Eggs often laid in open water/some exposure	11, 37
B. boreas	1.3	Eggs laid in open, often in shallow water/high exposure	12
R. cascadae	2.4	Eggs laid in open shallow water/high exposure	11, 12
H. regilla	7.5	Eggs laid in open shallow water/high exposure	12, 37

*Averages of values for tissues and years shown in Fig. 1 for each species.

different years (four species, including those used in field experiments), the levels were highly reproducible (Fig. 1). However, they varied by two orders of magnitude among species.

H. regilla eggs, which showed the highest photolyase activities, also appeared in field experiments to be highly resistant to UV-B-containing sunlight (Fig. 2; Table 1). The hatching success of *B. boreas* and *R. cascadae* was greater under sunlight lacking UV-B than under unfiltered sunlight or sunlight filtered to remove shorter wavelengths but not UV-B. At one *R. cascadae* site, the hatching success under UV-B blocking filters was significantly greater than success under UV-B transmitting filters but not statistically different from success under unfiltered sunlight (Fig. 2; Table 1).

DISCUSSION

We have found striking differences among amphibian eggs with respect to characteristic levels of photolyase, a key activity for repair of the major UV photoproduct in DNA, CBPDs. [We did not measure abilities to repair other important cytotoxic and mutagenic photoproducts—e.g., pyrimidine-(6-4')-pyrimidinone adducts (33).] The egg-laying behaviors and photolyase activities suggest that certain amphibian species have adapted so as to reduce exposure of their eggs to UV-B radiation.

The tendency of the salamanders to hide their eggs or lay them in relatively deep water affords them some shielding from UV radiation. However, other selective pressures, such as predation and thermal requirements for development, may have contributed to the evolution of this behavior, and protection from UV-B may be a secondary benefit. Nevertheless, increases in levels of UV-B could override these biochemical adaptations or, over evolutionary time, could select for increased photolyase activity.

The two anuran species with the lowest levels of photolyase activity, *R. cascadae* and *B. boreas*, have undergone such drastic population declines that they are candidates for designation as threatened species (14). *R. cascadae* populations have virtually disappeared from the southern portion of their range in California and have shown significant declines in Oregon (1, 13). *B. boreas*, a previously ubiquitous species in western North America, has undergone drastic declines in numbers throughout its range (14). Moreover, it has exhibited unusually high egg mortality at several locations (15). In laboratory experiments, *B. boreas* embryos taken from the Cascade Mountains of Oregon suffered developmental abnormalities and increased mortality from prolonged exposure to UV-B (16, 34). Since photolyase levels for *B. boreas* and *R. cascadae* were one-sixth and one-third of the *Hyla* activities (Fig. 1), respectively, this repair activity appears to be a significant determinant of survival under natural sunlight.

Photolyase levels were relatively high for the three anurans used in field experiments; they usually lay their eggs in shallow water, where subsequent evaporation exposes them to direct sunlight (Table 2). Levels were lower for most salamanders, which generally hide their eggs (Table 2). *Ambystoma*, which generally lay eggs in the open, but in deeper water, showed intermediate photolyase levels. Eggs from laboratory-reared *X. laevis* contained little photolyase (Fig. 1).

There was thus a general high-exposure, high-photolyase correlation among the amphibians (Table 2). Furthermore, eggs of the two anuran species exhibiting relatively low photolyase levels showed greater mortality under UV-B-containing sunlight. The population status of the three anuran species correlates strikingly with their repair proficiency and the corresponding resistance of their eggs to solar UV-B. These initial results support the UV-sensitivity hypothesis and suggest that stress can contribute to population declines in certain species. UV-repair and survival parameters should be measured for additional sets of amphibian populations.

Note Added in Proof. While this paper was in review, Kerr and McElroy (38) published a report that lends further credence to our results. Measurements they took at Toronto, Canada, which is at the same latitude (44°N) as our field sites, showed a 35% increase in UV-B per year in winter and a 7% increase per year in summer since 1989. These increases were caused by a downward trend in total ozone that was measured at Toronto during the same period. Moreover, Kerr and McElroy stated that increases in UV-B in the late spring may have a disproportionately larger effect on some species if they occur at critical phases of their development. The adverse effects of UV-B were apparent in our spring-conducted field experiments on developing anuran embryos.

We thank W. Ream and G. Somero for critically reviewing earlier drafts of the manuscript and B. Menge and R. O'Hara for discussions about experimental design. J. Beatty helped collect animals. We are grateful to J. Chapman and H. Lee II of the U.S. Environmental Protection Agency for measurements of the acetate and mylar shields. This research was supported by grants from the National Science Foundation, the U.S. Forest Service, and the National Geographic Society.

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