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Thea F. van de Mortel
Southern Cross University

William A. Buttemer

Peter Hoffman

John Hays

Andrew Blaustein

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A Comparison of Photolyase Activity in Three Australian Tree Frogs

Thea van de Mortel*, William Buttemer•, Peter Hoffman[Δ], John Hays[Δ], and Andrew Blaustein[Δ].

Address for correspondence:* Southern Cross University, P.O. Box 157, Lismore, 2480, NSW, Australia. email: tvandemo@scu.edu.au Fax: (61-2) 66203022

• University of Wollongong, Wollongong, Australia.

[Δ] Oregon State University, Oregon, USA.

Abstract Ultraviolet-B (UV-B) irradiation of DNA generates mutagenic photoproducts such as cyclobutane pyrimidine dimers (CPDs) which can affect the growth and development of amphibian embryos. Differential ability to repair UV-B-induced DNA damage may be responsible for differences in population stability between some amphibian species. Photoreactivation via the enzyme photolyase is a major mechanism used to remove CPDs from DNA. The aim of this study was to determine if photolyase activity differed in three sympatric Australian amphibian species; one of which has suffered marked population declines (*Litoria aurea*) and two whose populations do not appear to be in decline (*L. dentata* and *L. peronii*). The specific activity of photolyase was measured in each species, and compared to the hatching success of eggs of these species under unfiltered summer sunlight. The mean specific activities of photolyase were $1.10 \pm 0.18 \times 10^{11}$, $5.76 \pm 1.01 \times 10^{11}$, and $2.66 \pm 0.15 \times 10^{11}$ CPDs repaired per hour per μg of egg protein extract, for *L. aurea*, *L. dentata* and *L. peronii,* respectively. When intrinsic differences in hatching success between species were controlled for, the relative percentage hatching success under unfiltered sunlight of *L. aurea* (77%) was lower than that of *L. peronii* (91%) and *L. dentata* (98%), however, these values did not differ significantly. *Litoria aurea* had the lowest photolyase activity of the three species and showed a non-significant trend of reduced hatching success under UV-B exposure.

Key words amphibians, ultraviolet-B radiation, photolyase, photoreactivation

Introduction

Damage to DNA is a normal consequence of exposure to UV-B radiation (UV-BR) in all living organisms (Heelis et al. 1993). Ultraviolet-B irradiation of DNA generates mutagenic photoproducts such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone dimers (Kim et al. 1993; Thomas and Kunkel, 1993; Yasui et al. 1994). Dimers cause mutations because they disrupt replication of the DNA (Thomas and Kunkel, 1993; Lindenauer and Darby, 1994). These mutations may be lethal if they affect the normal growth and development of an organism (Heelis et al. 1993).

Major intracellular mechanisms for repairing UV-B-induced DNA damage include excision repair via exinucleases, and photoreactivation via enzymes called photolyases which split the pyrimidine dimers using photoinduced electron transfer (Heelis et al. 1993; Kim et al. 1993; Li et al. 1993). Photoreactivation of CPDs takes place most effectively at wavelengths between 360-460 nm (Heelis et al. 1993). Photolyase contains two associated chromophores or light-absorbing groups (Sancar and Sancar, 1988). One chromophore donates an electron to the dimer following absorption of a photon, causing formation of an unstable dimer anion. This dimer then collapses into a pyrimidine monomer thereby directly reversing the previous chemical change (Sancar and Sancar, 1988).

Photolyase enzymes are found in many organisms (Pang and Hays, 1991; Yasui et al. 1994), and levels of photolyase activity differ substantially between amphibian species (Blaustein et al. 1994). Such interspecific differences in capacity to repair DNA damaged by UV-BR may contribute to the differences in embryo mortality and ultimately to population stability in various amphibian species (Blaustein et al. 1994). Species with

lower levels of photolyase activity may be particularly sensitive to increases in UV-BR. In three North American amphibian species, Blaustein et al. (1994) demonstrated an inverse relationship between photolyase activity and amphibian embryo mortality when embryos were exposed to unfiltered sunlight. Two species that had comparatively low levels of photolyase activity and high embryo mortality under unfiltered sunlight were also those which were suffering population declines.

The Green and Golden Bell Frog (*Litoria aurea*) lays its spawn in shallow water exposed to sunlight (pers. obs.), and is suffering population declines in New South Wales (White, 1995). By contrast, sympatric congeners, *L. dentata* and *L. peronii*, with similar spawning behaviour appear to have stable populations (Tyler, 1991). Previous field experiments (van de Mortel, 1996; van de Mortel and Buttemer, 1996) showed a trend that hatching success of *L. aurea* eggs under unfiltered sunlight was lower than under sunlight filtered to prevent UV-B penetration although the difference between treatments was not statistically significant ($p=0.06$). The aim of this study was to measure photolyase activity in three species: *L. aurea*, *L. dentata* and *L. peronii*. If UV-BR was having a negative impact on the hatching success of *L. aurea* but not of the latter two species, one would expect that levels of photolyase activity would be lower in the eggs of *L. aurea* than in those of *L. dentata* and *L. peronii*. The specific activity of photolyase was defined as the number of CPDs converted to monomers per hour per μg of egg protein extract.

Materials and Methods

The study was carried out on three aliquots of 50 eggs of *L. aurea*, and two aliquots of 50 eggs each of *L. dentata* and *L. peronii*. The methods used to conduct the photolyase assay were described in detail by Blaustein et al. (1994); the major steps are summarized below. Eggs in each sample were dejellied prior to being crushed by sedimentation in a TLA 100.2 rotor at 65,000G. The exudate was recovered and the protein concentration was determined. The assay measured light-dependent removal of CPDs from UV-irradiated *Escherichia coli* DNA in the presence of egg protein extracts. The substrate DNA was radiolabeled by overnight growth of bacteria in a medium containing tritiated thymidine, then extracted from the bacteria and irradiated with 254 nm light to 400 Joules/m^2. The irradiated DNA was incubated with egg extract under continuous blue-light irradiation for 10 minutes. TCA precipitation was used to purify DNA from the reaction mixtures. Formic acid hydrolysis and silica gel thin layer chromatography were used to measure CPDs. Four to six assays were carried out per sample and the data presented are the means ± a standard error (Table 1). Six determinations represent three extract concentrations assayed in duplicate, while four[1] represent two concentrations assayed in duplicate. Specific activities were measured at various concentrations in order to ensure that activity was directly proportional to the amount of extract.

Ideally, the aliquots of eggs should have been collected within a short time of one another in case enzyme activity decreased over time. However, the difference in time of collection was probably unimportant as Blaustein et al. (1994) conducted photolyase

[1]Four determinations were carried out per *L. aurea* sample as the low level of activity required the use of large quantities of extract in order to obtain a level of activity high enough to score. The limited number of

assays on amphibian egg samples collected in different years and found no trend towards a decline in enzyme activity over time. The assay conducted on samples from our study also showed no trend related to time of sample collection.

Results

The mean specific activity of photolyase of *L. aurea* egg extract was $1.10 \pm 0.18 \times 10^{11}$ while those of *L. dentata* and *L. peronii* were $5.76 \pm 1.01 \times 10^{11}$, and $2.66 \pm 0.15 \times 10^{11}$ CPDs repaired per hour per μg of protein (Table 1).

eggs limited the number of assays. Only four data points are recorded for the first *L. dentata* sample because two which were outside the linear range of the assay had to be discarded.

Table 1. Specific activity of photolyase (no. of cyclobutane pyrimidine dimers removed x 10^{11} per hour per µg of egg protein extract) in eggs of *L. aurea*, *L. dentata* and *L. peronii*. Six determinations represent three extract concentrations done in duplicate, while four represent two concentrations done in duplicate. Assay data are rounded to one decimal place.

Species	**Date sample collected**	**Assay No. 1**	**2**	**3**	**4**	**5**	**6**	**Mean ± s.e.m.**
L. aurea	26/12/94	0.9	1.0	0.9	1.0			0.95 ± 0.05
	06/01/95	1.7	1.4	1.4	1.4			1.46 ± 0.06
	06/01/95	0.9	1.0	0.9	0.8			0.88 ± 0.03
L. dentata	21/01/95	6.7	6.8	6.9	6.7			6.77 ± 0.13
	25/10/96	4.5	5.0	4.8	5.2	4.7	4.5	4.75 ± 0.11
L. peronii	21/12/94	3.2	2.0	2.6	2.8	2.2	2.3	2.51 ± 0.18
	06/01/96	3.3	2.6	2.9	1.9	1.8	4.4	2.81 ± 0.34

Discussion

There may be a physiological basis for differential hatching success between *L. aurea*, *L. dentata* and *L. peronii*, as assays of egg protein extracts showed that photolyase activities were 2.4 and 5.3 times lower in *L. aurea* extracts than in those of *L. peronii* and *L. dentata,* respectively. In addition, the mean photolyase activity seen in *L. aurea* samples was similar to that of the North American species *Ambystoma gracile* (Table 2) which showed reduced hatching success under unfiltered sunlight (Blaustein et al., 1995). *L. aurea* showed a non-significant trend towards reduced hatching success under UV-B radiation, whereas the hatching success of *A. gracile* was significantly reduced under UV-B. Therefore, although the hatching success under different UV-B regimes was not statistically different in *L. aurea*, it may be biologically significant.

Table 2. The specific activity of photolyase (no. of dimers removed x 10^{11} per hour per μg of protein) and population status for amphibian species tested by Blaustein et al. (1994, 1995 and 1996).

Species	**Specific Activity**	**Reduced hatching success under UV-B radiation**	**Population status**
Bufo boreas	1.3	yes	Declining
Rana cascadae	2.4	yes	Declining
Hyla regilla	7.5	no	Apparently Stable
Rana aurora	6.1	no	Declining
Ambystoma gracile	1.0	yes	Unknown

However, the relationship between photolyase specific activity and hatching success may not be a simple one. When one controls for intrinsic differences in hatching success between species by expressing hatching success under unfiltered sunlight as a proportion of hatching success under the UV-B-blocking filter, the relationship between specific activity and relative hatching success is not linear (Fig 1).

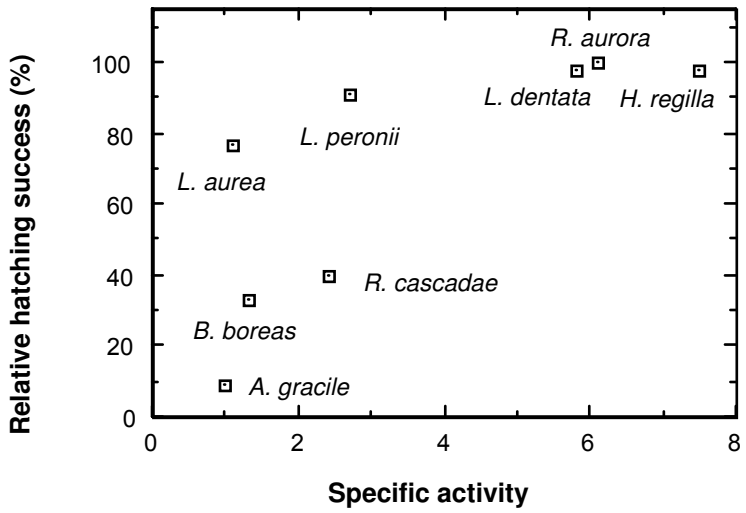

Fig 1. The relationship between the specific activity of photolyase (number of dimers removed x 10^{11} per hour per µg of protein), and relative hatching success (no. of hatchlings under unfiltered sunlight/ no. hatched under the UV-B-blocking filter x 100). Data on hatching success for *A. gracile*, *B. boreas*, *H. regilla*, *R. cascadae* and *R. aurora* were obtained from Blaustein et al. (1994, 1995 and 1996).

To illustrate this, *L. aurea* had a dimer repair rate similar to *A. gracile*, but relative hatching success was markedly higher amongst *L. aurea* eggs (Fig 1). In addition, *L. peronii* eggs had a dimer repair rate similar to that of *Rana cascadae*, a declining species which showed a significant improvement in hatching success when UV-B wavelengths were blocked (Blaustein et al. 1994). However, *L. peronii* does not appear to be declining, and did not show an improvement in hatching success in the field when exposure of eggs to UV-BR was reduced (van de Mortel, 1996; van de Mortel and Buttemer, 1996).

There may be doubts about these comparisons as the species were tested on different continents, under different abiotic and biotic conditions. However, comparisons between

species whose hatching success was tested at the same sites also showed that differences in photolyase activity do not necessarily manifest themselves as proportional differences in hatching success. To illustrate this, the hatching success of *L. peronii* was similar to that of *L. dentata,* although the latter had a level of photolyase activity double that of *L. peronii*. Data collected by Blaustein et al. (1994), also showed a two-fold difference in photolyase activity between *Bufo boreas* and *R. cascadae* that did not manifest itself in a similar difference in hatching success under unfiltered sunlight.

There are several possible explanations for these disparate results. Firstly, specific activity represents the rate of CPD repair per μg of egg protein in extract, but in practice total protein per egg may vary between species, which may also affect the *in vivo* rate of DNA repair. Total protein could not be compared among the three study species due to technical difficulties. Secondly, photolyase activity may also not be limiting until it is below some threshold value, i.e. even relatively low photolyase levels may suffice for repair of DNA damage incurred by amphibian embryos in the field.

Thirdly, the assay of photolyase activity was carried out *in vitro* under controlled conditions of temperature and pH which may differ substantially from those present in field experiments. Water temperature at the time of the field experiments may have affected the *in vivo* activity of photolyase (Pang and Hays, 1991). Physiological pH may also differ between species either intrinsically or as a result of the environmental pH. The activity of photolyase is affected by changes in pH as the enzyme-substrate complex forms most efficiently at a pH of 7.5 (Sancar and Sancar, 1988). Long et al. (1995) demonstrated a pH/UV-B synergism in *R. pipiens*. Hatching success decreased by 24 - 46% when embryos were exposed to current and elevated doses of UV-B in concert with

a pH of 4.5. At a higher pH, there were no significant effects of UV-BR on hatching success.

Kiesecker and Blaustein (1995) found an interaction between UV-B and the fungus *Saprolegnia*. When the hatching success of *R. cascadae*, *B. boreas* and *Hyla regilla* was compared under unfiltered sunlight and under a UV-B-blocking filter, in an environment free of *Saprolegnia*, there was no appreciable increase in hatching success in the treatments which excluded UV-B. However, the two declining species showed a significant increase in mortality under unfiltered sunlight when *Saprolegnia* was not excluded from the environment. The response of a given species to UV-BR may differ depending on levels of pathogens present at the time of field experiments.

Time taken for the hatching of eggs between species also differs vastly. Eggs of *L. aurea* generally hatch within 3 days whilst eggs of *A. gracile* can take up to 60 days to hatch (Blaustein et al. 1995; van de Mortel and Buttemer, 1996). Thus, eggs of the latter species may experience a much greater cumulative exposure to UV-B radiation.

Lastly, the ability to utilize other repair mechanisms such as excision repair (Sancar and Sancar, 1988) may differ between species, compensating for species differences in photoreactivation rate. It is also possible that some completely different mechanism is at work. Differences in UV-B-absorbing pigments may affect UV-B penetration to the tissues so that the rate of dimer production differs between species. Interestingly, the percentage transmittance of UV-BR between 280 and 300 nm is lower through *L. aurea* skin than in *L. peronii* (0% vs. 2%; W. Buttemer, unpublished data).

Despite the differences in the *in vitro* specific activity of photolyase between species, these differences did not result in a significant improvement in the hatching success of *L. aurea* eggs when UV-BR levels were reduced. These results, however, do not fully

exclude UV-BR as a cause of *L. aurea* population declines, as synergistic effects between UV-BR and other environmental factors may influence survivorship. For example, the activity of photolyase in *L. aurea* may have been further reduced due to pH and/or thermal conditions associated with periods of decline which were not present during the field experiment. There may also be synergistic effects between UV-B and aquatic pathogens which were not present at the time of the field studies. Evaluation of pH and temperature effects on photolyase activity, and interaction between UV-B and pathogens in various species may shed light on these uncertainties.

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