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RESEARCH ARTICLE

Molecular and sensory mechanisms to mitigate sunlight-induced DNA damage in treefrog tadpoles

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ABSTRACT

The increased incidence of solar ultraviolet B (UVB) radiation has been proposed as an environmental stressor, which may help to explain the enigmatic decline of amphibian populations worldwide. Despite growing knowledge regarding the UV-induced biological effects in several amphibian models, little is known about the efficacy of DNA repair pathways. In addition, little attention has been given to the interplay between these molecular mechanisms with other physiological strategies that avoid the damage induced by sunlight. Here, DNA lesions induced by environmental doses of solar UVB and UVA radiation were detected in genomic DNA samples of treefrog tadpoles (*Hypsiboas pulchellus*) and their DNA repair activity was evaluated. These data were complemented by monitoring the induction of apoptosis in blood cells and tadpole survival. Furthermore, the tadpoles' ability to perceive and escape from UV wavelengths was evaluated as an additional strategy of photoprotection. The results show that tadpoles are very sensitive to UVB light, which could be explained by the slow DNA repair rates for both cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidone photoproducts (6,4PPs). However, they were resistant to UVA, probably as a result of the activation of photolyases during UVA irradiation. Surprisingly, a sensory mechanism that triggers their escape from UVB and UVA light avoids the generation of DNA damage and helps to maintain the genomic integrity. This work demonstrates the genotoxic impact of both UVB and UVA radiation on tadpoles and emphasizes the importance of the interplay between molecular and sensory mechanisms to minimize the damage caused by sunlight.

KEY WORDS: Amphibian decline, DNA photoproducts, Nucleotide excision repair, Photolyases, UV radiation

INTRODUCTION

The global increases in ultraviolet B (UVB) radiation incidence associated with stratospheric ozone depletion has been considered a leading hypothesis for amphibian decline, especially given its genotoxicity on the embryonic and larval life stages (Alton et al., 2012, 2011; Blaustein and Belden, 2003; Blaustein et al., 1994; Croteau et al., 2008a; Kerr and McElroy, 1993). Additionally, exposure to UVB radiation can cause spinal curvature in tadpoles, reduced growth and development rates, delayed metamorphosis, reduced locomotive ability, behavioral changes, and susceptibility to disease and predation (Alton et al., 2012, 2010, 2011; Ankley et al., 2000; Belden and Blaustein, 2002; Croteau et al., 2008a,b; Fite et al., 1998; Flamarique et al., 2000; Hays et al., 1996; Kiesecker et al., 2004; Kiesecker and Blaustein, 1995; Mitchell et al., 2012; van Uitregt et al., 2007).

In fact, cell death and mutagenesis are the most important effects elicited by the UV component of sunlight, which corresponds to UVB (280–315 nm) and ultraviolet A (UVA, 315–400 nm) wavelengths. These cellular effects are directly related to a chain of events that mainly involve the induction of DNA lesions by UV radiation. Therefore, sunlight is one of the main environmental genotoxic agents to which terrestrial and aquatic ecosystems are exposed (Häder et al., 2011; Schuch et al., 2013).

The chemical nature of the DNA lesions, as well as the efficiency of their formation, greatly depends on the wavelength of the incidental UV photons (Schuch et al., 2012). The direct absorption of UVB photons, mainly by the pyrimidine bases cytosine (C) and thymine (T), leads to the formation of distortive DNA lesions known as pyrimidine dimers. The main DNA photoproducts are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidone photoproducts (6,4PPs) (Rastogi et al., 2010; Schuch et al., 2009). However, sunlight can also indirectly damage DNA after photon absorption by other chromophores, thereby generating reactive oxygen species. Oxidatively generated DNA damage, such as the formation of 7,8-dihydro-8-oxoguanine (8oxodG), is more effectively induced by UVA than by UVB (Schuch and Menck, 2010; Schuch et al., 2012). However, studies indicate that UVA radiation induces CPDs directly on DNA more readily than it induces oxidized bases, suggesting that UVA may be more mutagenic than previously assumed (Jiang et al., 2009; Schuch et al., 2009). Additionally, although UVA absorption by DNA is poor, the presence of 6,4PPs in purified DNA samples and in human cell cultures after irradiation with UVA light has been demonstrated (Cortat et al., 2013; Schuch et al., 2009).

Despite growing knowledge in the field regarding the impact of UVB radiation on different amphibian species, there are only a few published works concerning the detection of UVB-induced DNA lesions and the efficacy of their removal by amphibian DNA repair mechanisms (Blaustein et al., 1999, 1994; Smith et al., 2000; Thurman et al., 2014). It is important to mention that photolyases play the main role in the repair of DNA photoproducts in many aquatic organisms. For instance, the photorepair in coral planulae is very efficient, as it repairs 50% of UVB-induced DNA damage in 23 min (Reef et al., 2009). Additionally, the photorepair of some species of echinoid embryos removes 50% of CPDs in 0.6 h (Lamare et al., 2006). In fishes, this DNA repair pathway also operates more quickly than nucleotide excision repair (NER), removing damage on a scale of minutes compared with hours or days for NER (Kienzler et al., 2013a). Furthermore, an *in vitro* study with fish cell lines showed that the half-time for the repair of CPDs is about 45 min, and within 3 h there is no significant difference

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List of abbreviations

6,4PPs	pyrimidine (6,4) pyrimidone photoproducts
8oxodG	7,8-dihydro-8-oxoguanine
CPDs	cyclobutane pyrimidine dimmers
NER	nucleotide excision repair
UV	ultraviolet

between UV-exposed cells and non-irradiated control cells. In contrast, NER activity is slow, showing a significant reduction in the amount of UV-induced lesions after only 24 or 48 h of irradiation (Kienzler et al., 2013b). In amphibian models, existing studies have also focused on the action of photolyases, but have disregarded the important role of the NER pathway in the removal of both CPDs and 6,4PPs. Consequently, although the UV-sensitivity hypothesis postulates that the differential sensitivity among amphibian species to UV radiation is linked to their DNA repair capacity (Blaustein et al., 1999, 1994), the role of photolyase activity in declining amphibian species is still a matter for discussion (Smith et al., 2000; Thurman et al., 2014). Furthermore, it is important to emphasize that there is an absence of studies showing the genotoxicity of UVA radiation in amphibian models, as well as their efficacy to repair the UVA-induced DNA damage.

Besides DNA repair pathways, there are other physiological mechanisms that help to minimize the hazard caused by solar UV radiation. At the cellular level, the formation of UV-induced DNA photoproducts drastically impairs DNA metabolism, culminating in the induction of cell death by apoptosis. This programmed cell death is triggered by the blockage of DNA replication and transcription processes due to the presence of unrepaired DNA damage (Batista et al., 2009). In addition, both UVB and UVA radiation can also induce apoptosis by increasing the expression of the cell surface receptor Fas, which leads to the clustering of Fas and the activation of the Fas/caspase 8 pathway (Bang et al., 2003, 2002). Therefore, apoptosis works to secure genomic integrity, avoiding the segregation of the UV-affected chromosome by the elimination of damaged cells (Rastogi et al., 2010).

Furthermore, choosing of oviposition sites protected from sunlight helps to reduce the exposure of embryos to UV levels below the lethal dose, thus reducing considerably the environmental risk imposed by solar UVB radiation (Palen and Schindler, 2010). This fact is reinforced by work showing that species that lay their eggs in places protected from sunlight are naturally more susceptible to UVB radiation than species that deposit the embryos on the water surface (Blaustein and Belden, 2003; Häder et al., 2007). In addition, the UVB-avoidance behavior of adult anurans was observed in two species of diurnal poison-dart frogs in Costa Rica (*Dendrobates pumilio* and *Dendrobates auratus*) that consistently preferred areas in the field and within experimental testing chambers that offered low levels of UVB radiation (Han et al., 2007). Another study has suggested that some frogs can use UVA light as a visual cue to avoid UVB damage. Artificially increasing the UVA levels in the environment induced males to move off their perches faster than when they were exposed to a control visible light source (Kats et al., 2012). These results suggest that the solar UV radiation-avoidance behavior may be very important for tropical frogs, even in rainforests where much of the solar radiation is shielded by the forest canopy. The same UVB-avoidance behavior has been observed in the larval stage of *Litoria aurea* and *Litoria peronni*. Controversially, tadpoles of *Litoria dentate* showed no significant preference between environments with or without UVB light (Van De Mortel and Buttemer, 1998). Additionally, no evidence for active UVB avoidance or changes in tadpoles' activity in the presence of UVB radiation were found for *Rana arvalis*, *Rana temporaria*, *Rana cascadae* and *Bufo bufo* (Belden et al., 2003; Pahkala et al., 2003), suggesting that these tadpoles do not perceive UVB radiation. Nonetheless, it is also important to emphasize that the spectral characteristic of natural waters may help to protect amphibian embryos by avoiding sunlight exposure (Palen et al., 2002).

To increase our understanding of the role of increased UVB radiation in the global decline of amphibian species, the ability of amphibians to behaviorally avoid UV-induced genotoxicity and the efficacy of their physiological defense mechanisms against it requires further investigation. In the present work, both molecular and biological experimental procedures were carried out to investigate how tadpoles of an endemic treefrog species from southern Brazil [*Hypsiboas pulchellus* (Duméril and Bibron 1841), Anura: Hylidae] deal with the deleterious effects induced by solar UVB and UVA radiation. First, we measured the incidence of daily UVB and UVA doses at the collection sites. Subsequently, we evaluated the induction of DNA lesions, DNA repair activity, apoptosis in blood cells and tadpole survival after irradiation with UVB and UVA doses at intensities intended to simulate environmental exposure, followed by a photoreactivation treatment to activate photolyases. Finally, the ability of tadpoles to sense and escape from UVB, UVA and natural sunlight was assessed.

By comparing the induction of DNA lesions by both UVB and UVA wavelengths, the efficacy of their removal by DNA repair pathways, and the observation of a behavioral avoidance of UV, we demonstrate new insights regarding the importance of different physiological mechanisms for the maintenance of genomic integrity of tadpoles during and after exposure to sunlight.

RESULTS

UVB- and UVA-induced DNA photoproducts and DNA repair rates

The daily solar UVB and UVA doses at the tadpole collection site (06:00 h to 19:00 h) corresponded to 53.25 and 2056.2 kJ m^{-2}, respectively. The overall incidence of UV light corresponded to 2.5% UVB and 97.5% UVA wavelengths. The induction of CPDs and 6,4PPs by the UVB and UVA doses applied in this study are presented in Figs 1 and 2, respectively.

Tadpoles were either exposed to photoreactivation treatment after UV irradiation (group L, light) or were kept in dark containers (group D, dark) and sampled at 3, 6, 24 or 48 h, or 7 days. The signal of the anti-CPD and anti-6,4PP antibodies generated by UVB light was statistically significant compared with the unexposed control samples up to 24 h after irradiation [anti-CPD signal ($F_{12,26}$=68.62) for control (D) versus: 5.0 kJ m^{-2} at 0 h, P<0.0001; 5.0 kJ m^{-2} at 3 h (D), P<0.0001; 5.0 kJ m^{-2} at 6 h (D), P<0.0001; 5.0 kJ m^{-2} at 24 h (D), P<0.0001; and for control (L) versus: 5.0 kJ m^{-2} at 0 h, P<0.0001; 5.0 kJ m^{-2} at 3 h (L), P=0.0003; 5.0 kJ m^{-2} at 6 h (L), P=0.001; 5.0 kJ m^{-2} at 24 h (L), P=0.0187); and anti-6,4PP signal ($F_{12,26}$=68.64) for control (D) versus: 5.0 kJ m^{-2} at 0 h, P<0.0001; 5.0 kJ m^{-2} at 3 h (D), P<0.0001; 5.0 kJ m^{-2} at 6 h (D), P<0.0001; 5.0 kJ m^{-2} at 24 h (D), P=0.0025; and for control (L) versus: 5.0 kJ m^{-2} at 0 h, P<0.0001; 5.0 kJ m^{-2} at 3 h (L), P=0.002; 5.0 kJ m^{-2} at 6 h (L), P=0.0006; 5.0 kJ m^{-2} at 24 h (L), P=0.0174]. There was a statistically significant difference in the repair of CPDs between the UV-exposed samples subjected to 3 h of photoreactivation treatment (group L) and the UV-exposed

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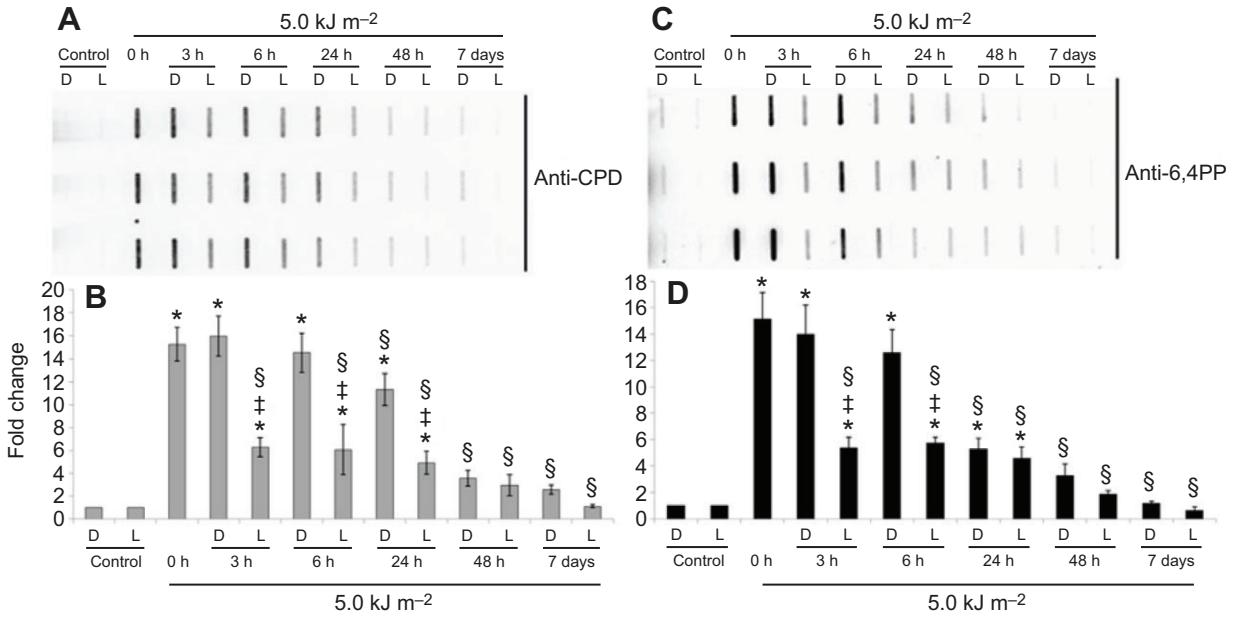

Fig. 1. UVB-induced DNA photoproducts and repair rates. (A) Immunological detection of cyclobutane pyrimidine dimers (CPDs), and (B) DNA repair rate for CPDs expressed as a fold change in relation to unexposed control samples. (C) Immunological detection of pyrimidine (6,4) pyrimidone photoproducts (6,4PPs), and (D) DNA repair rate for 6,4PPs expressed as a fold change in relation to unexposed control samples. D, tadpoles kept in the dark until the end of the experiment; L, tadpoles exposed to 3 h of photoreactivation treatment. *Statistically significant difference from the respective control; ‡statistically significant difference from the respective treatment without photoreactivation (D); §statistically significant difference from 5.0 kJ m^{-2} at 0 h (P<0.05).

samples kept in the dark (group D) at 3, 6 and 24 h after UVB irradiation ($F_{12,26}$=68.62; P<0.0001), and only at 3 and 6 h for 6,4PPs ($F_{12,26}$=68.64; P<0.0001). Immediately after the photoreactivation treatment, CPD-photolyase removed approximately 60% of UVB-induced CPDs, but 40% of this DNA lesion remained in the genome to be repaired by NER. Similarly, 6,4PP-photolyase removed approximately 66% of 6,4PPs. The NER pathway alone (group D) contributed to a slow

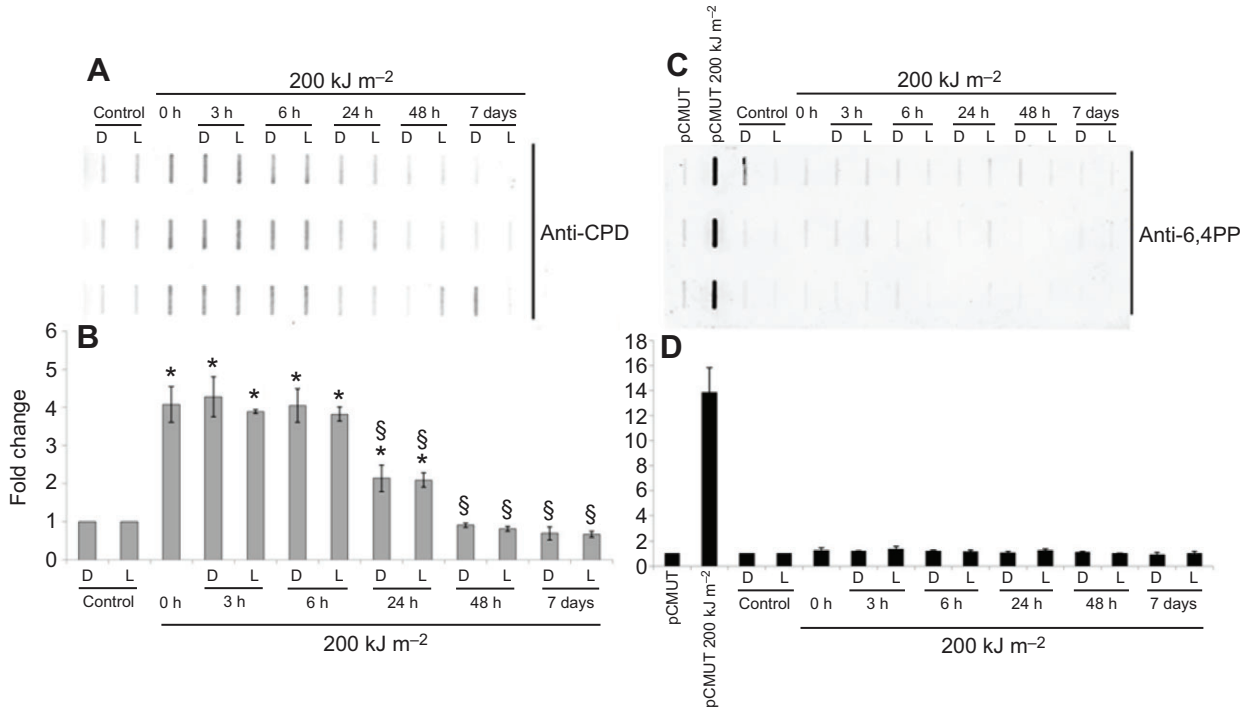

Fig. 2. UVA-induced DNA photoproducts and repair rates. (A) Immunological detection of CPDs, and (B) DNA repair rate for CPDs expressed as a fold change in relation to unexposed control samples. (C) Immunological detection of 6,4PPs, and (D) DNA repair rate for 6,4PPs expressed as a fold change in relation to unexposed control samples. D, tadpoles kept in the dark until the end of the experiment; L, tadpoles exposed to 3 h of photoreactivation treatment. pCMUT, purified plasmid DNA sample used as a positive control for the presence of UVA-induced 6,4PPs. *Statistically significant difference from the respective control; §statistically significant difference from 200 kJ m^{-2} at 0 h (P<0.05).

repair of UVB-induced CPDs and 6,4PPs, as demonstrated by a statistically significant decrease in the signal of anti-CPD and anti-6,4PP antibodies (in relation to 5.0 kJ m^{-2} at 0 h) only after 24 h of irradiation [anti-CPD signal ($F_{12,26}$=68.62) for 5 kJ m^{-2} at 0 h versus: 5.0 kJ m^{-2} at 24 h (D), P=0.0177; 5.0 kJ m^{-2} at 48 h (D), P<0.0001; 5.0 kJ m^{-2} at 7 days (D), P<0.0001); and anti-6,4PP signal ($F_{12,26}$=68.64) for 5 kJ m^{-2} at 0 h versus: 5.0 kJ m^{-2} at 24 h (D), P<0.0001; 5.0 kJ m^{-2} at 48 h (D), P<0.0001; 5.0 kJ m^{-2} at 7 days (D), P<0.0001].

The results also show that, despite the much smaller amount of UVA-induced CPDs in comparison to UVB treatment, there was also a statistically significant difference compared with the unexposed control samples up to 24 h after UVA irradiation [anti-CPD signal ($F_{12,26}$=98.34) for control (D) versus: 200 kJ m^{-2} at 0 h, P<0.0001; 200 kJ m^{-2} at 3 h (D), P<0.0001; 200 kJ m^{-2} at 6 h (D), P<0.0001; 200 kJ m^{-2} at 24 h (D), P=0.001; and for control (L) versus: 200 kJ m^{-2} at 0 h, P<0.0001; 200 kJ m^{-2} at 3 h (L), P<0.0001; 200 kJ m^{-2} at 6 h (L), P<0.0001; 200 kJ m^{-2} at 24 h (L), P=0.0018]. Removal of CPDs via NER activity after UVA irradiation was slow, as observed post-UVB irradiation, because the signal of anti-CPD antibody (in relation to 200 kJ m^{-2} at 0 h) significantly decreased only after 24 h of irradiation ($F_{12,26}$=98.34; P<0.0001). Interestingly, in contrast to observations made on a purified plasmid DNA sample (the positive control for the presence of UVA-induced 6,4PPs), the anti-6,4PP antibody failed to detect the presence of this DNA lesion in the genome of *H. pulchellus* after UVA irradiation.

Quantification of apoptotic cells and tadpole survival

The percentage of normal and apoptotic cells was calculated at different times after irradiation with UVB and UVA, and the results are shown in Fig. 3.

Both UVB and UVA treatment induced cell death via apoptosis, which was more pronounced at 24 and 48 h after irradiation. The percentage of apoptotic cells observed after UVB treatment was much higher than that observed after UVA irradiation. Additionally, photoreactivation proved to be effective in protecting against apoptosis at 24 h ($F_{12,23}$=272.1; P=0.0076), 48 h ($F_{12,23}$=272.1; P<0.0001) and 7 days ($F_{12,23}$=272.1; P<0.0001) after UVB irradiation (Fig. 3B), reducing the percentage of apoptotic cells significantly. Fig. 3C shows that there was a statistically significant difference between groups L and D, 7 days after UVA irradiation ($F_{12,26}$=59.54; P=0.001).

In parallel with these experiments on apoptosis induction, tadpole survival was checked daily until the 14th day after UVB and UVA irradiation. The results are presented in Fig. 4. The mortality rate over these 14 days is presented in supplementary material Table S1.

The treefrog tadpoles presented a high sensitivity to the UVB dose employed. In contrast, the same sensitivity was not observed for the UVA dose. Furthermore, a similar lack of sensitivity was observed even after exposure to a UVA dose of 400 kJ m^{-2}. The results clearly show that the photoreactivation treatment was important for a significant recovery of survival levels after exposure to UVB light, raising the average value from 13% in group D to 53% in group L ($F_{7,16}$=50.02; P=0.0013). In contrast, there was no difference between survival in groups D and L after UVA irradiation.

Ability of tadpoles to sense and escape from UVB and UVA light

The residence time under the visible and UV light sources was recorded and expressed in terms of the percentage of light exposure. The results are shown in Fig. 5.

Surprisingly, our findings clearly indicate the existence of a sensory mechanism that recognizes both UVB and UVA wavelengths independently and triggers the escape of tadpoles to the shaded environment ($F_{3,20}$=34.67; visible light versus: UVB, P<0.0001; UVA, P<0.0001; sunlight, P<0.0001). The same behavior was observed when tadpoles were exposed to natural sunlight. However, they did not react when exposed to a visible light

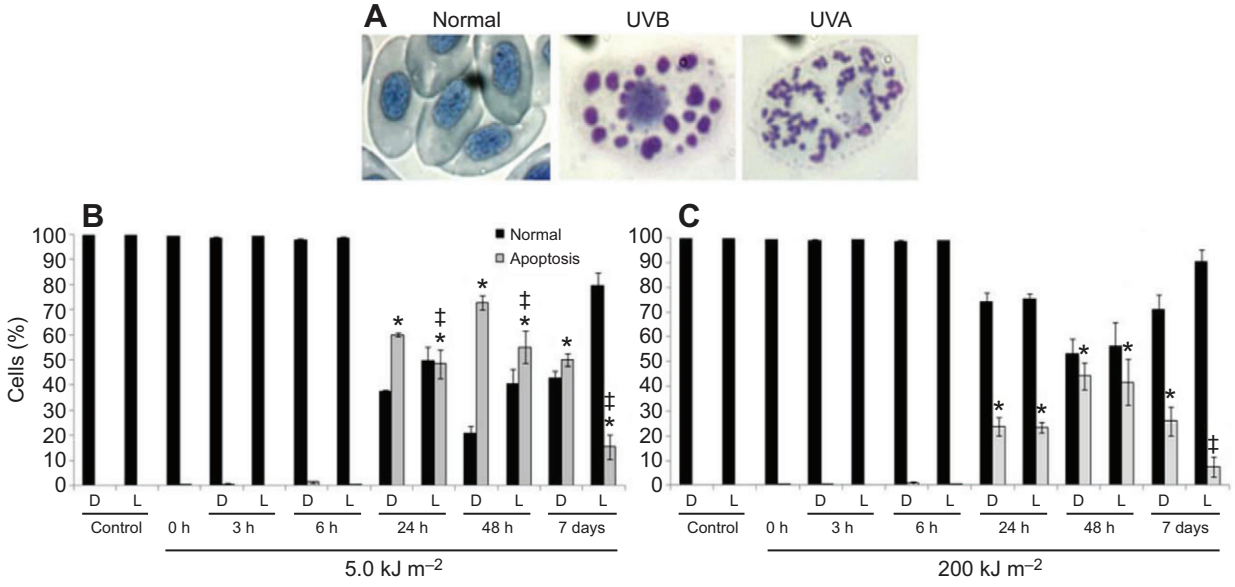

Fig. 3. Induction of apoptosis after UVB and UVA treatment. (A) Representative examples of normal blood cells and UVB- and UVA-induced apoptotic cells. (B) Percentage of normal and apoptotic cells 48 h after UVB irradiation. (C) Percentage of normal and apoptotic cells 48 h after UVA irradiation. D, tadpoles kept in the dark until the end of the experiment; L, tadpoles exposed to 3 h of photoreactivation treatment. Data are means±s.d. of three tadpoles analyzed at each point of the experiment (1000 cells counted per slide). *Statistically significant difference from the respective control; ‡statistically significant difference from the respective treatment without photoreactivation (D) (P<0.05).

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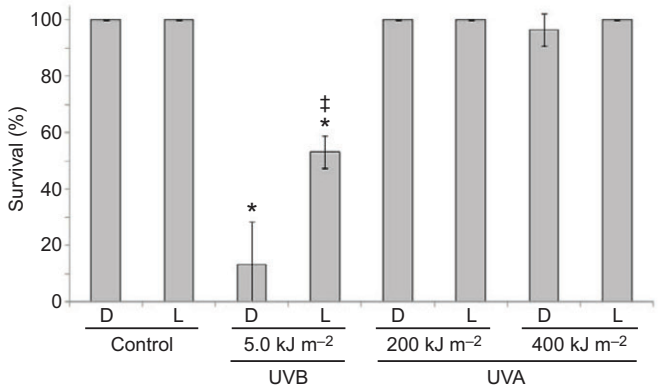

Fig. 4. Survival of tadpoles 14 days after UVB and UVA treatment. D, tadpoles kept in the dark until the end of the experiment; L, tadpoles exposed to 3 h of photoreactivation treatment. *Statistically significant difference from the respective control; ‡statistically significant difference from the respective treatment without photoreactivation (P<0.05).

lamp, remaining quiet and not seeking the shaded refuge, demonstrating a specific response to UV light. Representative examples of the tadpoles' UV radiation-avoidance behavior are presented in supplementary material Movie 1.

DISCUSSION

In recent years there has been an active debate concerning the importance of the increased incidence of solar UV radiation in the widespread decline of amphibians. Consequently, the detrimental effects of UVB radiation in several amphibian models have already been described. However, very little focus has been given to the effects of UVA light, which is also able to induce different types of DNA lesions, as well as deleterious mutations in many organisms (Häder et al., 2011). In addition, very little information has been published regarding the role of the NER pathway in the repair of UV-induced DNA lesions, although the activity of photolyases has been shown in some amphibian species (Blaustein et al., 1999, 1994; Smith et al., 2000; Thurman et al., 2014). Furthermore, there is an absence of research that correlates the DNA repair activity with other defense mechanisms that help to reduce the exposure of amphibians to UV light. Thus, this is the first study that shows the importance of the interplay between molecular and sensory mechanisms of tadpoles for the maintenance of genomic integrity against the DNA damage induced by UVB and UVA radiation.

Our results clearly indicate that high UVB and UVA doses reach the surface of the water at the tadpole collection site (see supplementary material Fig. S1). Although photolyases contribute to a rapid removal of UVB-induced DNA lesions, a considerable amount of both CPDs and 6,4PPs remained in the genome even after 3 h of photoreactivation (Fig. 1). These data emphasize the need for further studies to better investigate the efficiency of these photolyases, as well as those from other anuran species, in completely removing the DNA damage induced by environmental doses of UVB radiation. Furthermore, analyzing NER also revealed slow DNA repair rates for UVB-induced CPDs and 6,4PPs. In comparison to human cells, approximately 50% of CPDs are repaired in 4 h in the genome overall (although repair of the remaining lesions is not completed before 48 h after the treatment), in contrast with the removal of almost 100% of 6,4PPs in the same time period (Costa et al., 2003). In contrast, our results clearly indicate that, even though the repair of 6,4PPs was faster than for CPDs, the relative proportions of UVB-induced CPDs and 6,4PPs detected 24 h after irradiation (compared with 5.0 kJ m^{-2} at 0 h) were 74% and 35%, respectively.

As observed for UVB irradiation, the removal rate for UVA-induced CPDs was slow because the antibody signal began to decrease only after 24 h of irradiation. Nonetheless, the photorepair mechanism proved to be effective in addressing the low amount of UVA-induced DNA lesions (Fig. 2). However, there was no statistically significant difference between groups L and D in terms of CPD levels, thereby indicating that CPD-photolyase must have been activated during UVA irradiation, promoting an equal removal of CPDs in groups L and D. This photolyase activation was more evident with regard to 6,4PP levels because it was not possible to confirm the presence of this lesion in the genomic DNA after UVA irradiation, as we did in the purified plasmid DNA sample used as a positive control for the presence of UVA-induced 6,4PPs.

Quantification of apoptotic cells is very informative for this type of study because CPDs are one of the most important DNA lesions that induce apoptosis, although 6,4PPs also induce this cell death pathway. The cytotoxic effects of both lesions are commonly associated with the blockage of DNA transcription and replication,

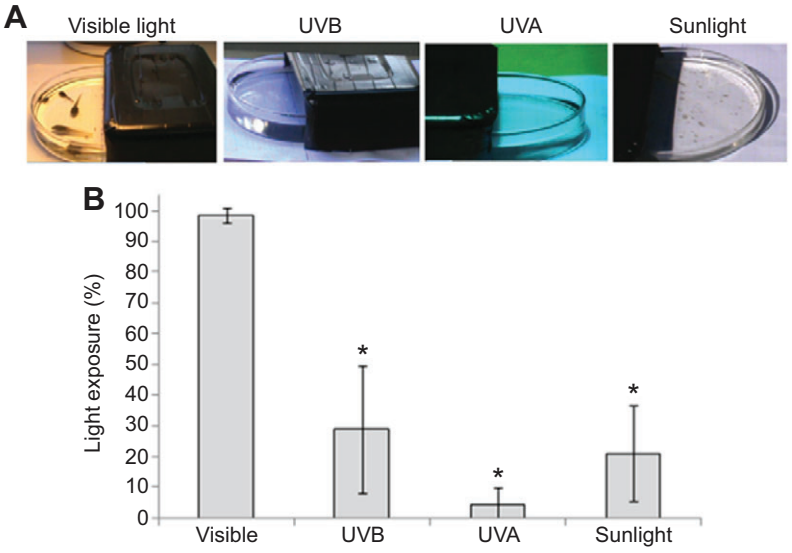

Fig. 5. Ability of tadpoles to sense and escape from UV wavelengths. (A) Representative example of tadpoles exposed to different light sources (visible light lamp, UVB lamp, UVA lamp and natural sunlight). (B) Relative percentage of the light exposure time tolerated by tadpoles under each light source. Data are means±s.d. of three independent experiments. *Statistically significant difference from the control samples (visible light source) (P<0.05).

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which results in a signal for the induction of programmed cell death (Batista et al., 2009). Both UVB and UVA generate CPDs, and the results show very clearly that both treatments induced cell death via apoptosis (Fig. 3). The amount of apoptotic cells peaked at 48 h after UV treatment and sharply declined thereafter to the values observed at 7 days after irradiation. Prevention of apoptosis by photoreactivation was observed 24 h, 48 h and 7 days after UVB irradiation (Fig. 3B), and only 7 days after UVA irradiation (Fig. 3C).

Several published studies demonstrate that amphibian embryos and larvae (tadpoles) suffer reduced survival when exposed to environmental doses of UVB radiation (Ankley et al., 2004, 2002, 2000; Anzalone et al., 1998; Belden and Blaustein, 2002; Belden et al., 2003; Blaustein et al., 1994, 1997; Diamond et al., 2002; Flamarique et al., 2000; Häkkinen et al., 2001; Hays et al., 1996). Our results agree with the previously published studies and demonstrate the deleterious impact of UVB light on the survival of tadpoles (Fig. 4; supplementary material Table S1). The fast removal of UV photoproducts by the photorepair system was essential to increase survival approximately 5-fold compared with samples treated with the same UVB dose but kept in the dark. In contrast, exposure to UVA light did not result in the reduced survival of tadpoles, which is probably directly associated with the activation of photolyases during UVA irradiation and the removal of UVA-generated DNA lesions immediately after their formation. Interestingly, notwithstanding that UVA treatment induced significant levels of apoptotic cells in comparison to untreated samples, the amount of apoptotic cells generated was apparently not enough to induce the mortality of tadpoles as observed for UVB irradiation.

Finally, the ability of tadpoles to sense and escape from UVB and UVA rays was evaluated. The results clearly indicate that treefrog tadpoles have a sensory photoprotection mechanism that triggers an avoidance behavior towards UVB and UVA wavelengths (Fig. 5; see supplementary material Movie 1). Interestingly, the average residence time of tadpoles under the UVA lamp and natural sunlight (consisting of 97.5% UVA wavelengths at the latitude of 29°43′S) was lower than that observed for the UVB lamp, although this did not reach statistical significance. Therefore, these results indicate that UV wavelengths specifically trigger this escape behavior from sunlight. Additionally, taking into account the relatively low efficacy of DNA repair pathways studied here, this sensory mechanism would be of paramount importance for avoiding a population decline in an environment with increased incidence of solar UVB due to ozone depletion or deforestation.

Conclusions

Although the impact of UVB radiation on amphibian models has been the subject of much research during decades of scientific work, our understanding of how the UV wavelengths of sunlight (especially UVA) act in the amphibian decline is still not clear. One reason for that is the complexity in evaluating the interplay between different types of photoprotection mechanisms that avoid DNA damage induced by this environmental genotoxic agent. In this work, we describe new (molecular and sensory) mechanistic insight regarding how treefrog tadpoles cope with the detrimental effects of solar UVB and UVA radiation. The results demonstrate that the relative inefficiency of DNA repair pathways in removing UV-induced DNA lesions is directly associated with the induction of apoptosis and reduction of tadpole survival. Surprisingly, a sensory mechanism that triggers the escape of tadpoles from solar UVB and UVA wavelengths complements this low DNA repair efficacy, avoiding the generation of DNA damage and maintaining the genomic integrity. Therefore, the application of both molecular and biological approaches for the evaluation of these physiological photoprotection mechanisms will aid in obtaining important information for future works focused on the investigation of the role of increased solar UVB radiation incidence in the decline of amphibian species.

MATERIALS AND METHODS

Animal collection and maintenance

Approximately 1000 *H. pulchellus* tadpoles were collected independently at different sites of a permanent pond at the Federal University of Santa Maria (29°43′16.84″S, 53°43′35.89″W) across three different days in January 2013 (summer in the Southern Hemisphere), with approximately 350 tadpoles collected on each day. Immediately following collection, tadpoles were transported to the laboratory where the experiments were performed. Prior to each experiment, tadpoles were selected as described below and kept in containers (10 tadpoles per container of 40×20×10 cm) filled with dechlorinated water (19±2°C) to a minimum depth of 40 mm (between 50 and 70 mm) and fed with boiled spinach *ad libitum*.

Incidence of solar UVB and UVA radiation at the collection site

Environmental measurements of solar UVB and UVA incidence were made on a clear-sky summer day (11 January 2013) at the tadpole collection site with a portable radiometer (UV Monitor MS-211-1, EKO Instruments, Japan) in order to define the UVB and UVA doses applied in this work. These measures of daily solar UVB and UVA doses covered the period from 06:00 h to 19:00 h. The daily incidence of solar UVB and UVA radiation is presented in supplementary material Fig. S1.

UVB and UVA irradiation, photoreactivation treatment and tadpole survival

Prior to exposure to UVB and UVA inside the laboratory, groups of tadpoles were selected based on their similarity in total body length (cm), mass (g) and stage (Gosner stage 29–31). These analyses were made with a stereomicroscope with 40× magnification (Nova Optical Systems, Brazil), an analytical balance (WTB 2000, Radwag, Poland) and a digital 0.01 mm precision caliper rule (Serie 500, Mitutoyo, Brazil). Firstly, the selected tadpoles were divided into three groups for the survival experiment (non-irradiated control, UVB and UVA), each containing 20 individuals. Then, each group of 20 tadpoles was again divided into two groups of 10 individuals each to test the effect of photoreactivation (see below). This tadpole selection procedure was performed three times, independently (on different days before each UV irradiation experiment).

The choice of the UVB and UVA doses of 5.0 and 200 kJ m^{-2}, respectively, was based on the environmental data of solar UV radiation measurements. This is equivalent to 10% of the daily solar UVB and UVA dose on a clear-sky summer day, or to the period of sun exposure from 12:30 h to 13:05 h (35 min) at the *H. pulchellus* collection site. For UVA treatment, an additional dose of 400 kJ m^{-2} was applied only for the survival analyses in order to investigate whether tadpoles would be resistant to a higher UVA dose. All UVB irradiation was performed with the same 15 W lamp (T15M, Vilber Lourmat, France) filtered with a polycarbonate sheet to block UVC wavelengths. For all UVA irradiation, tadpoles were exposed using the same 1000 W lamp (Osram Ultramed FDA KY10s) filtered with 3 mm-thick glass filter (BG39, Schott Glass, Germany). The dose rates of the UVB and UVA lamps were 3.2 and 72.3 J m^{-2} s^{-1}, respectively, and the exposure times to achieve the desired UV doses were 26 and 46 min, respectively. The amount of UVC contamination for the UVB lamp, and of UVC and UVB contamination for the UVA lamp, was below the detection limit (see supplementary material Fig. S2).

Immediately after UV exposure, one of the two groups of 10 tadpoles was subjected to 3 h of photoreactivation treatment to activate the photolyase enzymes (group L, light). This involved a second period of light exposure to two 40 W fluorescent lamps at a distance of 40 cm (General Electric, Brazil; see supplementary material Fig. S2). Tadpoles from group L were then

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maintained in transparent containers and exposed to a constant photoperiod (12 h light:12 h dark) until the end of the experiment. The other UV-irradiated group of 10 individuals was kept in the dark (containers were wrapped with aluminium foil) until the end of the experimental period (group D, dark). The same photoreactivation treatment was performed with tadpoles of the non-irradiated control group (control L), while the remaining non-irradiated control group was kept in the dark (control D). The UV irradiation and photoreactivation procedures were repeated three times independently (on different days). Then, tadpoles were monitored daily for survival until the 14th day, and the mean±s.d. were calculated based on the results of these three experiments performed independently.

Detection of UV-induced DNA damage, quantification of DNA repair rates and analysis of apoptosis induction

In parallel to the survival experiment, another set of tadpoles was selected and divided into three groups (non-irradiated control, UVB and UVA). The non-irradiated control group (10 tadpoles) was divided into two groups of five individuals, with one group exposed to the photoreactivation treatment (group L) and the other kept in a dark container (group D). The UVB and UVA groups were composed of 55 tadpoles each. These individuals were divided into groups of five tadpoles each, based on the time of genomic DNA extraction and collection of blood samples after UV irradiation, as well as on the exposure (or not) to the photoreactivation treatment, as follows: 0 h – 5 UVB-exposed tadpoles and 5 UVA-exposed tadpoles; 3, 6, 24 and 48 h, and 7 days – 5 UVB-exposed tadpoles (group L)+5 UVB-exposed tadpoles (group D) and 5 UVA-exposed tadpoles (group L)+5 UVA-exposed tadpoles (group D).

After treatment, tadpoles were anesthetized with Xylocaine® (lidocaine; 10%) and sectioned transversely at the height of the pelvis, so that the tail could be taken for genomic DNA extraction and blood samples could be collected for the cytogenetic analyses free of dirt originating from internal organs. The genomic DNA and blood samples were independently extracted from three tadpoles prior to UV irradiation (non-irradiated controls), as well as at the different periods of time after UVB, UVA and photoreactivation treatments (0, 3, 6, 24 and 48 h, and 7 days).

The formation of CPDs and 6,4PPs was confirmed immunologically: 200 ng of genomic DNA was mixed with 800 ng of salmon sperm DNA (Sigma-Aldrich, USA), boiled for 10 min at 100°C, immediately transferred to ice, and then spotted onto nitrocellulose membranes (Bio-Rad, USA) using a slot-blot apparatus (Omniphor, USA). The membranes were subsequently incubated with 5× SSC (750 mmol l^{-1} NaCl and 75 mmol l^{-1} sodium citrate) for 15 min at room temperature, dried (at room temperature) and baked for 2 h at 80°C. The membranes were blocked in 5% milk diluted in a PBS buffer (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 8 mmol l^{-1} Na_2HPO_4, 1.5 mmol l^{-1} KH_2PO_4, pH 7.6) for 18 h at 4°C and probed with anti-CPD and anti-6,4PP primary antibodies (Cosmo Bio Co., Ltd, Japan; diluted 1:2000 in 5% milk diluted in a PBS buffer) with constant shaking for 3 h at room temperature. All primary antibodies were removed, and the membranes were washed six times (5 min per wash) with PBST (0.1% Tween-20 in PBS). The secondary antibody, anti-mouse IgG HRP conjugate (R&D Systems, USA), was diluted 1:2000 in 5% milk-PBS, and the membranes were incubated with constant shaking for 2 h at room temperature. The secondary antibody was removed by six washes with PBST. DNA lesions were detected using a specific chemiluminescent reagent (Amersham ECL Western blotting detection reagents and analysis system, GE Healthcare, UK), followed by chemiluminescence detection (ImageQuant 300, GE Healthcare, UK). The DNA repair rates were calculated via the detection of antibody signals at different periods of time after UV treatment (0, 3, 6, 24 and 48 h, and 7 days), and expressed as fold change compared with the antibody signal in the unexposed control samples.

In order to confirm the cytotoxic potential of both UVB- and UVA-generated DNA lesions, blood samples were distributed over the entire area of clean and labeled glass slides, fixed in absolute methanol for 5 min and stained with Giemsa solution (15%) for 5 min. Finally, the slides were gently washed in running water and dried at room temperature. The analyses were performed with an Olympus BX41 optical microscope with a magnification of 1000×, and at least 1000 cells per slide were counted. The percentage of apoptotic and normal cells was determined in relation to the total number of cells counted per slide.

Determination of the ability of tadpoles to sense and escape from UV light

Sixty tadpoles were selected based on their morphological features (see above) and divided into four groups (visible light, UVB, UVA and sunlight) containing 15 individuals each. Prior to exposure to these different light sources, each group of 15 individuals was divided into three groups of five tadpoles each to be irradiated independently in each condition.

In order to investigate the existence of a sensory mechanism of photoprotection that triggers the behavior of UV-avoidance, half of a Petri dish (20 cm in diameter) was covered with a black plastic structure providing a shaded environment protected from light, while the other half was fully exposed to light. Three groups containing five tadpoles each (total of 15 tadpoles exposed in each light source) were exposed individually (independently) for 4 min to a specific light source. After 2 min of exposure, the position of the black structure was changed with the aim of exposing the two halves of the Petri dish equally during the irradiation, to avoid a possible preference of tadpoles for one of the Petri dish halves. The different light sources used in this work included a 15 W visible light (Taschibra, Brazil), the UVB and UVA lamps described above (see 'UVB and UVA irradiation, photoreactivation treatment and tadpole survival'), and also direct environmental sunlight. The UV dose rates for each light source were: 0 J m^{-2} s^{-1} for the visible light lamp (free of emission of UVB and UVA photons); 3.2 J m^{-2} s^{-1} of UVB; 72.3 J m^{-2} s^{-1} of UVA; and 1.0 J m^{-2} s^{-1} of UVB and 50 J m^{-2} s^{-1} of UVA for natural sunlight (April 2013, autumn in the Southern Hemisphere; irradiation began at 13:00 h on a clear-sky day and finished at 13:12 h; the environmental temperature during irradiation was 23°C). The light-avoidance behavior was recorded with a Sony DCR-SR21 video camera (Sony, Japan). The average residence time under each light source was assessed from all the recordings considering only the period when at least three tadpoles (50%+1) were exposed to light at the same time in order to eliminate the fast individual foraging movement between both UV-exposed and UV-protected environments. The average and standard deviation of the percentage of exposure time to each light source was then calculated after three independent experiments.

Statistical analyses

For the DNA damage and repair experiment, we used the antibody signal (expressed as fold change compared with the unexposed control samples) as the response variable, and exposure to the UVB or UVA doses, photoreactivation treatment (L or D), and time of DNA extraction (0, 3, 6, 24 and 48 h, and 7 days) as predictors.

For the apoptosis induction experiment, we used the number of apoptotic cells (expressed as a percentage) as the response variable, and the exposure to the UVB or UVA doses, photoreactivation treatment (L or D), and time of collection of blood cells (0, 3, 6, 24 and 48 h, and 7 days) as predictors.

For the tadpole survival experiment, we used the number of live tadpoles (expressed as a percentage) observed until the 14th day after irradiation as the response variable, and the exposure to the UVB or UVA doses, and photoreactivation treatment (L or D) as predictors.

For the determination of the tadpoles' UV-avoidance behavior, we used the residence time of tadpoles under each light source (expressed as percentage of time under the light) as the response variable, and the exposure to the visible, UVB and UVA lamps, and to the natural sunlight as predictors.

All the results produced in the experiments described above were analyzed and mutually compared by MANOVA (multivariate analysis of variance) because of the existence of multiple predictors, followed by Tukey's tests ($P<0.05$) using the software GraphPad Prism 6.0 (GraphPad Software, USA).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.P.S. is the main executor of this work. He participated directly in the design of the study and wrote the first version of this manuscript. He also participated in the development and implementation of all experiments performed in this work. V.M.L., M.B.S. and C.P.S. helped in conducting field work by collecting the animals and field data, as well as in the design of the study and initial draft of the manuscript. S.S.J. participated in the extraction of genomic DNA and in the DNA damage detection and DNA repair experiment. S.Z.C. participated in the design of the study and helped draft the manuscript. E.L.S.L. conceived and coordinated the study, helped design it and helped to write the manuscript. All authors gave final approval for publication.

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Supplementary material
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