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Effect of Exposure of Ultraviolet B Radiation on Two Anuran Species, *Polypedates cruciger* and *Duttaphrynus melanostictus* under Empirical Conditions

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ABSTRACT

Increase in ultraviolet (UV)-B radiation has been identified as a suspect in amphibian deformities and decline. Eggs of two amphibian species, *Polypedates cruciger* and *Duttaphrynus melanostictus* were exposed to UV-B radiation (312 nm) to receive erythemal dose (0.08 relative response) of the action spectra under laboratory conditions. Biologically estimated doses were calculated to match 50% (high), 25% (medium) and 10% (low) of the average daily irradiance given for the Asian region (17.92×10^{-3} Wm^{-2}). Hatching success of the eggs and survival, growth of tadpoles and development of malformations were recorded until metamorphosis. Although the exposure had no effect on the hatching success of the eggs of the two species, the survival of tadpoles of both species was significantly reduced compared to the tadpoles coming from unexposed eggs in the control. Tadpoles of both species experienced growth retardations due to UV-B exposure. Exposed individuals were smaller at metamorphosis and they took longer to metamorphose. In addition, malformations such as ulcers, edemas, abnormally pigmented skin were common among the exposed tadpoles. Species-specific differences in the growth were observed between *D. melanostictus* and *P. cruciger* but not in the other measured parameters. This study shows that the two anuran species are highly sensitive to levels of UV-B radiation that is present in their natural environment and suffered both lethal and sub-lethal effects.

Keywords: survival, growth, malformations, hatching success

INTRODUCTION

Depletion of the stratospheric ozone due to anthropogenic activities affects the natural shield of the earth against harmful ultraviolet (UV) radiation, especially UV-B radiation (Kerr and McElroy, 1993). This is considered as a matter of importance and it was recommended that urgent research be conducted to examine the effects of increased UV-B radiation on organisms. In this context, amphibians are likely to be more susceptible than most other taxa due to their life-cycle strategies. Amphibians lay unshelled eggs which are often exposed to direct sunlight and the larvae develop in the same environment. Many amphibian species prefer clear water with intense solar irradiance and low riparian cover making them even more vulnerable to radiation (Langhelle *et al*., 1999). The potential impacts of UV-B radiation on animals act mainly on their physiology by means of DNA damage, direct protein damage and by producing oxygen free radicals (Formicki *et al*., 2003). The ultimate effect of this damage is cell death (Jurkiewicz and Buettner, 1994). Apart from these direct effects, amphibians particularly suffer the consequences of low oxygen consumption which might affect their survival by restricting behavioral responses such as anti-predator behavior, thermoregulation and foraging (Formicki *et al*., 2003). Furthermore, the synergistic effect of UV-B and other natural factors such as pathogenic fungi and pesticides too, act as additional stress factors (Hatch and Blaustein, 2000).

Ultraviolet -B radiation present in natural sunlight can increase the mortality and malformation rates in embryos of amphibians (Blaustein *et al*., 1999). In the early stages UV-B has been reported to cause higher mortality in *Bufo boreas* and *Rana*

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cascadae in North America (Blaustein *et al*., 1994). Tadpoles of two other North American species, *Hyla regilla* and *R. auroa* exposed to UV-B radiation developed lens opacities (cataracts) and skin burns under laboratory settings (Flamarique *et al*., 2000). A laboratory simulation of UV-B in *B. bufo* tadpoles was reported to decrease oxygen consumption, which in turn may affect development and defense against stochastic stress factors (Formicki *et al.,* 2003). Furthermore, these authors reported that eggs under UV-B shields had a higher hatching rate (85%,vs. 65% in embryos exposed to UV-B), fewer deformities (19%, vs.21%) and show more rapid development than those exposed to UV-B. Many such studies have focused on early life stages of amphibians, during which the most critical developmental processes take place. Upon the exposure to UV radiation *R. pipiens* showed bilateral ectromelia and ectrodactyly in the hind limbs of developing tadpoles (Ankley *et al.*, 1998). The same authors later revealed that direct sunlight caused approximately 50% mortality during early larval development of *R. pipiens* (Ankley *et al*., 2002). These studies suggest that damage caused by UV radiation in amphibians may differ among species. Amphibians live in habitats which are constantly exposed to UV radiation are more tolerant compared to those exposed to UV intermittently (Blaustein *et al*., 1999).

Trematode infections, ultraviolet radiation and biocides have all been identified as major causes of amphibian malformations (Ankley *et al*., 2002; Blaustein and Johnson, 2003; Johnson *et al*., 1999; Kiesecker, 2002). Recent reports of malformed frogs from a nature reserve in Sri Lanka (Rajakaruna *et al*., 2007) led to laboratory studies exploring the effect of pesticides (Jayawardena *et al*., 2010; 2011) and parasites (Rajakaruna *et al*., 2008; Jayawardena *et al*., 2010; 2013). Tadpoles of *Polypedates cruciger* (common hour glass tree frog) and *Duttaphrynus melanostictus* (Asian common toad) exposed to these stress factors developed severe malformations, leading to high mortality. More recently, Wijesinghe *et al.* (2011) investigated the potential of natural solar radiation to alter the toxicity of carbofuran, a commonly used carbamate pesticide, on tadpoles of *D. melanostictus*, showing that photo-irradiation markedly reduced the toxicity of carbofuran as evident by its effects on mortality, growth and swimming activity.

Polypedates cruciger and *D. melanostictus* are widely distributed amphibians in Sri Lanka, predominantly in human altered habitats in the island's south-western wet zone. The tadpoles of *P. cruciger* and *D. melanostictus* develop in clear ponds and pools that are directly exposed to the sun. Here we investigate the effect of biologically estimated doses (BED) of UV-B radiation on the hatching success, survival, growth and malformations of the tadpoles of *P. cruciger* and *D. melanostictus* under laboratory conditions.

MATERIALS AND METHODS

Eggs of *P. cruciger* and *D. melanostictus* were collected from ponds and pools in the Peradeniya University Park (Sri Lanka). Each egg clutch (Gosner 11-12) was separated into four masses having a similar number of eggs in each mass (approximately 50 eggs). Egg masses of *P. cruciger* were attached to the wall of a glass trough containing 500 mL of dechlorinated tap water while egg strands of *D. melanostictus* were submerged in similar troughs containing the same amount of water.

Biologically estimated doses (BED) of radiation for the exposure were calculated (Table 1).The doses were estimated such that the UV lamp mimics 50% (high), 25% (medium) and 10% (low) of the total irradiance (≈ 258 kJm-2), expected in Sri Lanka, daily for a period of four hours (10.00-14.00 hr). Eggs of both species were exposed to a 312 nm UV lamp (VL-115 M, Vilber Lourmat, spectral region 280-320 nm, spectral peak 312 nm, 30 W) inside a UV chamber. The UV source was placed at 30 cm above the water level to give a surface irradiance of 2.5 Wm-2 (Table 1). The exposure was done in 12:12 hr light: dark cycles. Hence, after the UV exposure tadpoles were exposed to full spectrum florescence light to complete the 12 hr light period. This exposure was continued daily until metamorphosis. Throughout the experiment the pH of the medium was kept constant by renewing the water on a daily basis. Soon after hatching, the number of tadpoles in each setup was recorded and hatching success calculated by dividing the number of hatched tadpoles by the initial number of eggs in each (20-30) exposure. Mortality was recorded daily and the snout vent length (SVL) and the body weight of the metamorphs were measured. Metamorphs were observed under binocular microscope and malformations were recorded. The malformed individuals were first euthanized in MS222 and then preserved in 70% alcohol for further investigations. Survival of the tadpoles was calculated as a percentage of successfully hatched tadpoles that survived to metamorphosis. The time required for forelimb emergence of half the number of tadpoles (TE_{50}) in a particular regime was calculated and used as a measure for assessing the length of the development period. The experiment was repeated four times per species using separate egg clutches (n=487 for *D. melanostictus* and n=342 for *P. cruciger*).

Data were analyzed using MINITAB 14.0 for Windows. The existence of a linear relationship between different doses of UV-B radiation and hatching success, survival, growth parameters and development of malformations was tested using a Pearson correlation. The effect of different doses of UV-B radiation on hatching success, survival, growth parameters and development of malformations was analyzed using one-way ANOVA (analysis of variance) and individual comparisons were made using Tukey post hoc test (Sokal and Rohlf 1999). The differences in hatching success, survival, growth parameters and malformations between the two anuran species were compared using an analysis of co-variance (ANCOVA).

RESULTS

Hatching success

The eggs of both species began hatching within 3−5 days after exposure (Figure 1a). Although the hatching success decreased with increasing radiation dose, there was no significant correlation in both species (Pearson correlation; *P. cruciger,* r=-0.278, p= 0.722; *D. melanostictus,* r=-0.378, p=0.622). The decrease in hatching success was not consistent in *P. cruciger* (Fig. 1a). Exposure to UV-B had no effect on the hatching success of in the exposed eggs of both species (One way ANOVA, df= 3, p> 0.05). Moreover, there was no significant difference in the overall hatching success between the two species (ANCOVA; $F_{1,3}$= 2.66, p= 0.119).

Table 1. Different exposure regimes and dose estimation at each regime of the study.

Exposure Regime	Percentage of total daily dose*(%)	Total dose (kJm^{-2})	Time of irradiance (sec)	Biologically estimated dose (BED, kJm^{-2})	Treatment no. given in Figures 1 & 2
Control	0	--	--	0	1
Low	10	25.8	10	1.5×10^{-2}	2
Medium	25	64.5	26	3.8×10^{-2}	3
High	50	129.0	52	7.6×10^{-2}	4

* Total daily dose (258. 048 kJm^{-2}) was estimated by multiplying mean intensity of UV (17.92×10^{-3} Wm^{-2}) by 14, 400 sec. (for a period of 4 hr during day time (10 a.m.-2 p.m.). A 60 W white light fluorescent bulb was used for the control group.

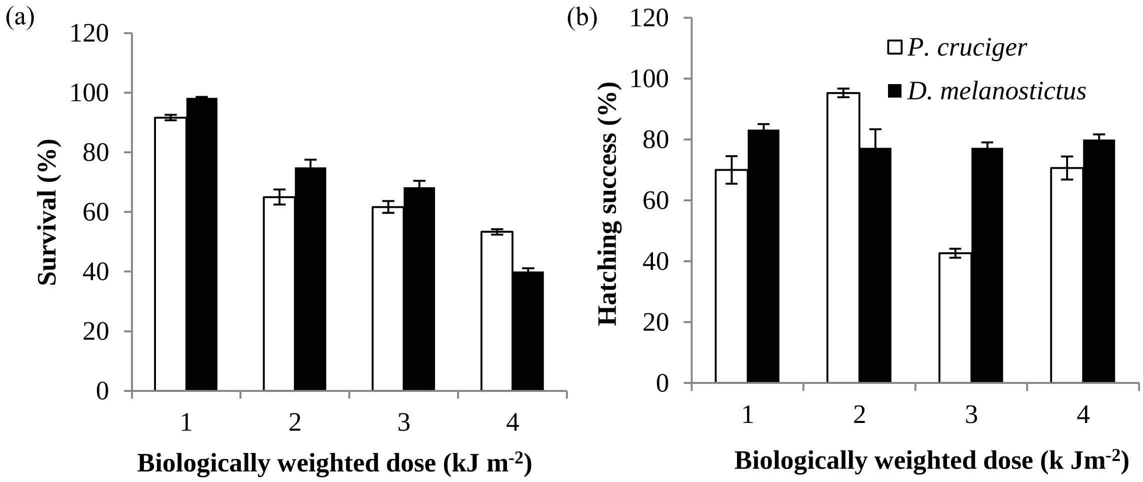

Figure 1. (a) Hatching success of eggs and (b) survival of tadpoles of *P. cruciger* and *D. melanostictus*, exposed to UV-B radiation (312 nm) at different exposure regimes with the standard error of the mean.

Survival

The percentage survival of the metamorphs decreased with increasing radiation dose (Fig. 1b) showing a statistically significant linear relationship in *D. melanostictus*, (Pearson correlation, r =-0.960, p = 0.040) but not in *P. cruciger* (Pearson correlation, r = - 825, p=0.175). However, exposure to UV had a significant effect on the survival of tadpoles of both species (One way ANOVA, df=3, $p< 0.05$). Individual comparisons between different doses and control showed significantly lower survival in all the exposed tadpoles of both species (Tukey's post hoc test, df=1, $P<0.05$). There was no significant difference in the survival of tadpoles between the two species (ANCOVA; $F_{1,3}= 0.17$, p= 0.681).

Growth

All three parameters of growth (SVL, weight and TE_{50}) were affected in the two species exposed to radiation. The overall growth was retarded and the metamorphs that emerged were smaller than those of the control (Fig. 2). Although there was a decrease in growth parameters with increase in the dose, the relationship was not linear (Pearson correlation; SVL of *P. cruciger,* r= - 0. 782, p= 0.218, SVL of *D. melanostictus,*r = -0.745, p = 0.255; weight of *P. cruciger,* r = -0.866, p= 0.134, weight of *D. melanostictus,* r = -0.826, p= 0.174). Radiation exposure had a significant effect on the size of both species compared to that of the non-exposed animals in the control groups (One way ANOVA; *P. cruciger*- SVL $F_{3,164}= 175.86$, $p<0.001$, body weight $F_{3,164}= 71.3$, $p<0.001$, and *D. melanostictus*- SVL $F_{1,164}= 97.87$, $p<0.001$, body weight, $F_{1,164}= 27.00$, $p<0.001$). Individual comparisons between different doses showed that exposed tadpoles of both species were significantly smaller than those in the control (Tukey's post hoc test, df=1, $P<0.05$). Between the two species, *P. cruciger* tadpoles experienced a significantly higher reduction in size compared to *D. melanostictus* tadpoles (ANCOVA; $p< 0.001$).The lengthening of the development period,$TE_{50}$was also higher in UV-B treated animals with average of 67 days for *P. cruciger* (56 days in the control) and 54 days for *D. melanostictus* (32 days in the control).

Malformations

The malformations were easily detectable to the naked eye. The most common type was edema in the abdominal region. It was more prominent in *P. cruciger* (Fig. 3a) than in *D. melanostictus* (Fig. 3d). Tadpoles with edema swam upside down at the water surface. A swollen abdominal region was also observed to be a common malformation in both species (Fig. 3b, e). These specimens possessed distended, prominent and transparent abdomens compared to the normal individuals. Lighter skin color with abnormal pigmentation was observed in exposed *P. cruciger* (Fig. 4).

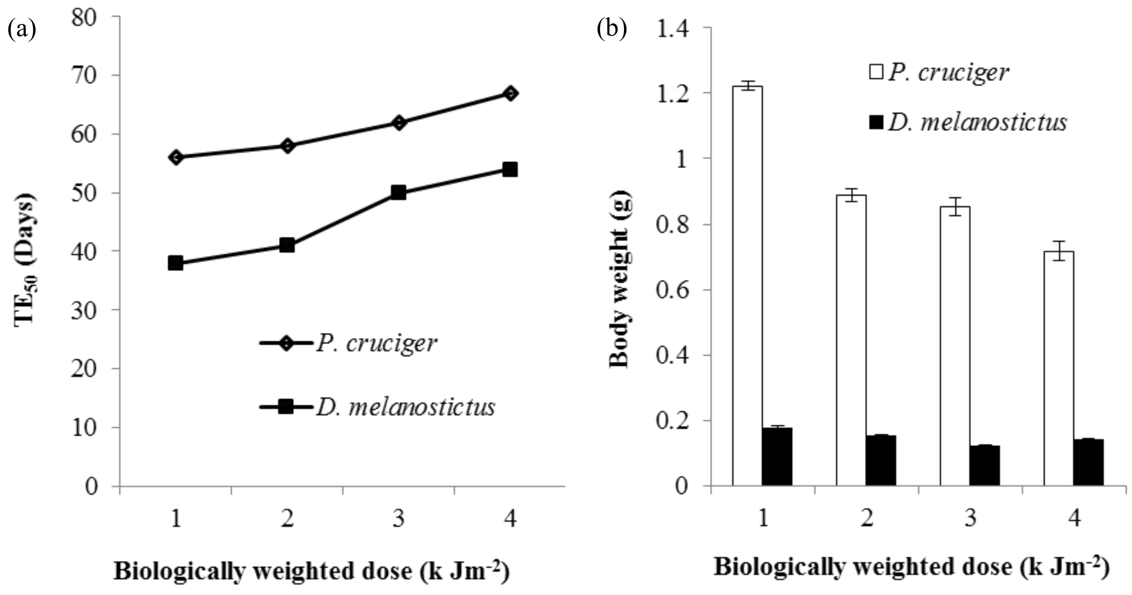

Figure 2. (a) Time required for the metamorphosis of half the number of tadpoles in a treatment (TE50) in days and (b) the body weight of the metamorphs in grams of *P. cruciger* and *D. melanostictus*, exposed to UV-B radiation (312 nm) at different exposure regimes with the standard error of the mean. (1 = the control experiment, 2= $1.5x10^{-2}$, 3 = $3.8x10^{-2}$, 4 = $7.6x10^{-2}$ k Jm^{-2})

Curvature of the vertebral column was also common to both species (Figure 3 c and f). Exposure to UV-B radiation had a significant effect on inducing malformations in both *P. cruciger* (One-way ANOVA; dose, $F_{2,12}$= 6.04, p= 0.037)and *D. melanostictus* (ANOVA; dose, $F_{2,12}$= 7.09, p= 0.026).There was no significant difference in the percentage malformations between the two species (ANCOVA; $F_{1,14}$= 1.78, p= 0.203).There were no malformations in any of the control animals.

DISCUSSION

Exposure to UV B radiation has a significant effect on the survival, growth and development of malformations in both *P. cruciger* and *D. melanostictus*. UV-B irradiance used in the laboratory corresponds to the average daily dose received in a tropical country on a sunny day (WOUDC, 2007). At the time of the study UV-B intensities of the test area (Peradeniya, Sri Lanka) has not been estimated, hence, measurements for the region (17.92×10^{-3} Wm^{-2}, WOUDC, 2007) was taken as the basis for calculation of the exposure time to UV lamps. The biologically estimated doses (BED) were also calculated under the exposure system according to erythema action spectrum. The BED under the UV lamp ranged from $1.5\text{-}7.6 \times 10^{-2}$ $kJm^{-2}$and this value was substantially low in comparison to several other studies conducted in Europe and America. In most of the studies UV-B intensities were taken as high as 1.5-2.5 kJm^{-2}.

Figure 3. Malformations found in tadpoles of *P. cruciger* and *D. melanostictus*, exposed to UV-B radiation (312 nm) at different exposure regimes. (a) Edema at the left side of the body (b) swollen abdomen and (c) curvature at the tail region of *P. cruciger* tadpoles; (d) edema at the right side of the body (e) swollen abdomen and (f) curvature at the tail region of *D. melanostictus* (G) tadpoles.

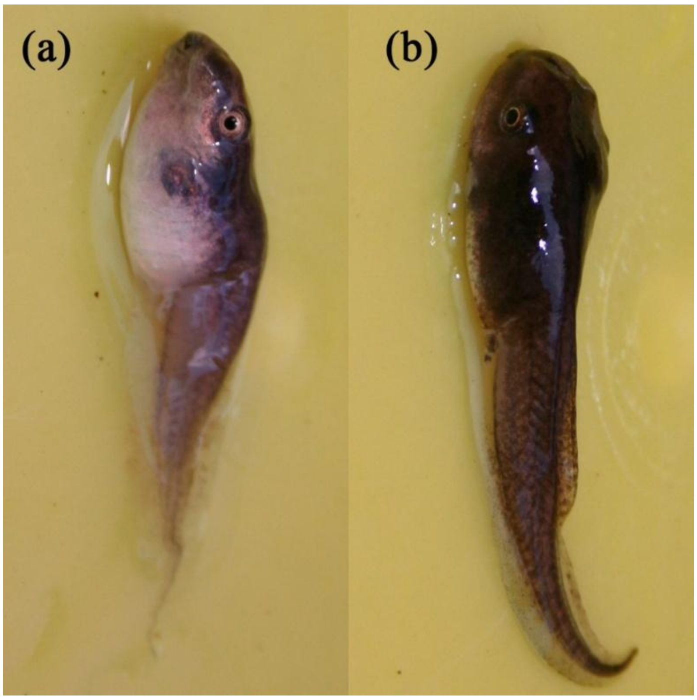

Figure 4. (a) Abnormal pigmentation in *P. cruciger* compared to (b) a normal tadpole of the same species.

Studies have shown that different amphibian species respond differently to exposure to UV radiation. Hatching success of eggs of both *P. cruciger* and *D. melanostictus* were not affected by exposure to UV-B radiation. Lower hatching success upon exposure to UV radiation has been observed in many amphibian species including *Bufo boreas* and *Rana cascadae* (Blaustein *et al.,* 1994), two salamander species *Ambystoma gracile* (Blaustein *et al*., 1996) and *Ambystoma macrodactylum* (Blaustein *et al.,* 1997). The long-toed salamander, *A. macrodactylum* experienced a 14.5 % decline in hatching rate when exposed to UV compared to UV-shielded counterparts (Blaustein *et al.,*1997). Although the hatching success was unaffected in both *P. cruciger* and *D. melanostictus,* survival of the tadpoles showed a significant reduction upon exposure in both species. Field experiments conducted in Oregon, USA showed *B. boreas* and *R. cascadae* exposed to UV had low hatching rates as well as higher mortality (Blaustein *et al.,* 1994). Similarly, embryos of the salamander *Ambystoma gracile* exposed to UV-B had a higher mortality in addition to lower hatching rates (Blaustein *et al*., 1996).

However, several studies have shown that exposure of eggs or tadpoles to UV have no effect on hatching success or survival. For example, Langhelle *et al*. (1999) reported no negative effect on survival or hatching success of *Rana temporaria*, *Bufo bufo*, *Bufo calamita* and *Hyla arborea* exposed to UV-B radiation. Moreover, mortality among *R. arvalis* eggs and larvae even after the exposure to 1.254−1.584 kJm^{-2} levels was not affected (Pahkala *et al*., 2001). Similarly, embryos of *R. temporaria* showed neither mortality nor developmental abnormalities upon exposure to weighted doses of 1.19-1.29 kJm^{-2} for 3-15 days (Cummins *et al*., 1999). Field experiments conducted in Oregon reported that the hatching success of *Hyla regilla* was not affected upon exposure to UV-B (Blaustein *et al.,* 1994).

Elongation of the growth period was common to the exposed tadpoles of both species. Their metamorphs were significantly smaller in size compared to those in the controls. The longer time taken for metamorphosis and smaller size of adults can have many adverse impacts on their biology and ecology. In the natural context, smaller size at metamorphosis makes anurans less competitive in foraging and other resource utilization, leading to reduced fitness in comparison with the larger individuals (Carey and Bryant, 1995). Nonetheless, delaying metamorphosis may have a crucial impact on amphibian survival, as they often breed in temporary water bodies, particularly in rice fields and human altered habitats (Manamendra-

Arachchi and Pethiyagoda, 2006), which may dry up before completion of metamorphosis. Rapid colonization, reproduction and growth are important for the survival of organisms living in rapidly changing ecosystems, such as rice fields (Fernando, 1995; Wells, 2007).

A high frequency of malformations was observed in the exposed individuals of both species, edemas in the abdominal region being the most common type. Exposure to UV-B radiation has been reported inducing different types of malformations such as eye-cataracts (Flamarique *et al*., 2000), edema (Jagger, 1985), tail curvature and abnormal pigmentation (Licht and Grant,1997) in different amphibian species. Similarly, *Bufo americanus*, *Rana clamitans* and *Rana sylvatica* tadpoles have suffered UV-B induced pigmentation followed by direct mortality (Grant and Licht, 1995). *Rana pipiens* showed induction of bilateral ectromelia and ectrodactyly in the hind limbs under UV exposure (Ankley *et al.,*1998). Ankley and colleagues (2002) further reported a significant elevation (21-53%) of hind limb and digit malformations of *R. pipiens* upon exposure to laboratory UV-B (290-320 nm) radiation.

Most of the malformations consisted of deletions and truncations where missing and shortened digits and digit segments were observed. There are a few studies reporting the occurrence of eye abnormalities such as varying degrees of micro/anophilmia (80%) reported by Ankley *et al*. 2002. Jablonski (1998) reported UV induced neural tube defects due to folate deficiency resulted by UV light induced photolysis of folate. Moreover, Worrest and Kimelderf (1976) reported malformations such as epidermal hyperphasia, lordosis (curvature of the vertebral column), abnormal development of presumptive cornea together with high mortality levels in *B. boreas* upon laboratory exposure to UV radiation (290-315 nm with 7.5 kJm^{-2}- 41 kJm^{-2}).

Even though, some species such as Oregon cascade amphibians are able to develop normally under intense solar radiation (Kats *et al*., 2000) but some other species such as *R. casecadae* and *B. boreas* suffer high mortality due to radiation (Blaustein *et al*., 1999). Damage caused by UV radiation is dependent on intrinsic factors such as photolyase levels, egg laying behaviors and pigmentation (Licht and Grant, 1997) and abiotic factors such as water depth, turbidity or vegetation cover, which alter the amount of radiation received by the animals in their habitat. Hence, some species are more vulnerable to radiation even though they share the same habitat with other low-sensitivity species. The physical nature of the amphibian egg is an important factor in the context of radiation damage. The jelly capsule around the egg is an effective covering from UV-B radiation (Grant and Licht, 1995). Eggs with 5−6 mm^2 jelly cover of 3 mm thickness absorb 6−14 % of UV B radiation in *B. americanus*, *R. aurora* and *R. sylvatica* eggs (Grant and Licht, 1995). In addition to the above, life history characteristics such as length of the development period is an important factor. Tadpoles of *D. melanostictus* were smaller than the tadpoles of *P. cruciger* at the time of hatching. Fast -living species (Jonhson *et al*., 2012) which attain smaller body sizes at metamorphosis appear to suffer more compared to those that develop slowly and attain larger body sizes at metamorphosis. Between the two species tested, *D. melanostictus* is a fast living species (45±5 days for the completion of metamorphosis) compared to the slow-developing *P. cruciger* (60±7.5 days for the completion of metamorphosis). Hence, *P. cruciger* seems to have more time to repair UV B radiation related damage, and hence suffer less mortality, compared to the common toad.

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