Do high temperatures enhance the negative effects of ultraviolet-B radiation in embryonic and larval amphibians?

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Summary

For the embryos and tadpoles of amphibian species, exposure to ultraviolet-B radiation (UVBR) can be lethal, or cause a variety of sublethal effects. Low temperatures enhance the detrimental effects of UVBR and this is most likely because the enzyme-mediated processes involved in the repair of UVBR-induced damage function less effectively at low temperatures. Whether these repair processes are also impaired, and thus the negative effects of UVBR similarly enhanced, at high temperatures is not known, but is an ecologically relevant question to ask given that organisms that inhabit environments where the temperature fluctuates widely on a daily timescale are likely to experience high doses of UVBR when temperatures are high. Here we examined the thermal-dependence of UVBR effects in the context of an ecologically-relevant fluctuating UVBR and temperature regime to test the hypothesis that exposure to peak UVBR levels while the temperature is high (35°C) is more detrimental to embryonic and larval *Limnodynastes peronii* than exposure to peak UVBR levels while the temperature is moderate (25°C). Embryos exposed to peak UVBR levels at 35°C hatched 10 h later than those exposed to peak UVBR levels at 25°C and, as tadpoles, were smaller and consequently swam more slowly but, in an environment with predators, exhibited no difference in survival time. There was also no effect of experimental treatment on the hatching success of embryos, nor on the post-hatch survival of tadpoles. These findings, therefore, are not sufficiently strong to support our hypothesis that high temperatures enhance the negative effects of UVBR in embryonic and larval amphibians.

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Key words: Amphibian declines, Ultraviolet radiation, Temperature, Locomotor performance, Predation

Introduction

Global increases in ultraviolet-B radiation (UVBR) associated with stratospheric ozone depletion are potentially contributing to the loss and decline of numerous amphibian species around the world (Bancroft et al., 2008; Blaustein et al., 1998; Blaustein et al., 2003; Croteau et al., 2008a; Crump, 2001; Stuart et al., 2004). UVBR is absorbed primarily by DNA, but also by proteins and lipids, causing damage that can lead to mutations and cell death (Tevini, 1993). Accumulation of this UVBR-induced damage at the cellular level is lethal to the embryos and tadpoles of several amphibian species, and also manifests as a range of sublethal effects including reduced growth, reduced rate of development, delayed metamorphosis, reduced locomotor performance, developmental abnormalities, behavioral changes, and increased susceptibility to disease (e.g. Alton et al., 2010; Ankley et al., 2000; Belden and Blaustein, 2002; Blaustein et al., 1994; Croteau et al., 2008b; Kats et al., 2000; Kiesecker and Blaustein, 1995; van Uitregt et al., 2007). The effects of UVBR have been shown to be temperature-dependent in amphibians with UVBR effects greater at lower temperatures (Broomhall et al., 2000; Grant and Licht, 1995; van Uitregt et al., 2007). It has been suggested that at lower temperatures the negative effects of UVBR are enhanced because lower temperatures slow the rate of growth of developing embryos and tadpoles thereby prolonging their exposure to harmful UVBR (Grant and Licht, 1995). A longer exposure period to UVBR, however, is not the sole reason for greater UVBR effects at lower temperatures. Using a controlled laboratory study, van Uitregt et al. found that the negative effects of UVBR on the survival, growth, development and locomotor performance of striped marsh frog *Limnodynastes peronii* embryos and tadpoles were greater at 20°C than at 30°C independent of exposure period (van Uitregt et al., 2007). van Uitregt et al. therefore proposed that the thermal-dependence of UVBR effects may also be due to the reduced activity of the enzymes responsible for the repair of UVBR-induced damage to DNA, proteins and lipids at lower temperatures (van Uitregt et al., 2007).

Absorption of UVBR by DNA causes adjacent pyrimidine nucleotide bases to join together to form a pyrimidine dimer (Friedberg et al., 2006). These dimers compromise DNA replication and transcription and thus pose a significant threat to the viability and functional integrity of the cell (Friedberg et al., 2006). In a number of taxa, including amphibians, the dimerisation of adjacent pyrimidine nucleotide bases can be

reversed in a single-step reaction known as enzymatic photoreactivation (EPR), which is catalysed by enzymes known as photolyases that utilise 350–450 nm light as an energy source (Blaustein et al., 1994; Friedberg et al., 2006). An alternative, but more complex, DNA repair mechanism found in all taxa, is nucleotide excision repair (NER) which, rather than reversing the damage, removes the section of DNA harbouring the pyrimidine dimer using an ATP-dependent multi-subunit enzyme system called excision nuclease; the gap in the DNA strand is subsequently filled using DNA polymerase and ligated (Friedberg et al., 2006; Sancar and Tang, 1993). EPR and NER, being catalysed by enzymes, have both been shown to be temperature-dependent in a range of organisms with rates of repair being slower at lower temperatures (Lamare et al., 2006; Li et al., 2002; MacFadyen et al., 2004; Pakker et al., 2000). This therefore suggests that the temperature dependence of UVBR effects in amphibians is, in part, mediated by the temperature dependence of the reaction rates of the enzymes involved in the repair of UVBR-induced DNA damage, and most likely also by those involved in the degradation and resynthesis of proteins and lipids.

In controlled laboratory studies, the thermal-dependence of UVBR effects in amphibians have mostly been examined using experimental treatments where the temperature remains either constantly low or constantly high (Grant and Licht, 1995; Searle et al., 2010; van Uitregt et al., 2007). In the natural environment, a number of amphibian species develop in shallow, ephemeral water bodies that experience wide fluctuations in temperature (e.g. Fig. 1) and UVBR irradiance on a daily time scale (Alford, 1999). Embryonic and larval amphibians in these environments are therefore more likely to experience maximal UVBR exposures when temperatures are high in the middle of the day. Given that enzymes typically become denatured at high temperatures, exposure to peak levels of UVBR at high temperatures potentially compromises the ability of early life stage amphibians to adequately cope with the detrimental effects of UVBR. In the present study, we examined the thermal-dependence of UVBR effects in the context of an ecologically-relevant fluctuating UVBR and temperature regime to test the hypothesis that exposure to a peak in UVBR levels while the temperature is high enhances the negative effects of UVBR in embryonic and larval amphibians. In a controlled laboratory experiment, we reared striped marsh frog *Limnodynastes peronii* embryos and tadpoles under a diurnally-fluctuating UVBR-irradiance regime that had a peak in UVBR levels at the photoperiod midpoint. The temperature in this experiment fluctuated between 25℃ and 35℃ on a daily timescale, and *L. peronii* individuals either experienced synchronous fluctuations in UVBR and temperature, such that they experienced peak UVBR levels at 35℃ (SYNC fluctuation regime), or asynchronous fluctuations in UVBR and temperature, such that they experienced peak UVBR levels at 25℃ (ASYNC fluctuation regime). To assess whether exposure to peak UVBR levels at 35℃ was more detrimental to embryos and tadpoles than exposure to peak UVBR levels at 25℃ we measured the time-to-hatch and hatching success of *L. peronii* embryos, and the post-hatch survival, locomotor performance, and morphology of *L. peronii* tadpoles. We then measured the survival time of tadpoles in an environment with predators, using freshwater shrimp *Macrobrachium australiense* as predators, to determine whether changes in locomotor performance and morphology were correlated with improved chances of survival in an environment with predators.

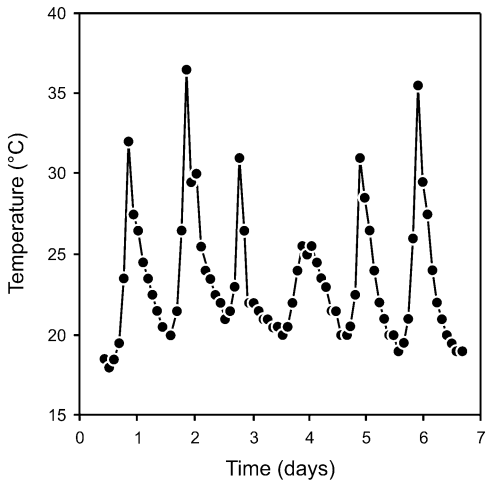

Fig. 1. Thermal variation in *Limnodynastes spp.* habitats. A typical daily pattern of temperature for an open pool where *Limnodynastes spp.* were observed (reproduced from Niehaus et al., 2012).

Results

SYNC embryos hatched significantly later than ASYNC embryos ($\chi^2=184.7$, d.f.=1, $P<0.001$), with the median hatch time of ASYNC and SYNC embryos being 1.7 and 2.1 days, respectively, a delay of 10 h (Fig. 2).

There was no significant effect of fluctuation regime on embryonic hatching success ($\chi^2=1.1$, d.f.=1, $P=0.30$), or post-hatch tadpole survival ($\chi^2=0.7$, d.f.=1, $P=0.42$).

SYNC tadpoles had a burst swimming speed of 158±4 mm s^{-1} (mean ± S.E.), which was significantly slower than the burst swimming speed of ASYNC tadpoles, which was 175±4 mm s^{-1} ($F_{1,81}=10$, $P=0.002$ in the analysis with tadpole total length not included as a covariate) (Fig. 3A). Burst swimming speed was positively associated with tadpole total length ($F_{1,80}=34.2$, $P<0.001$ in the analysis with tadpole total length as a covariate), however, and when the effect of tadpole total length was accounted for, there was no effect of fluctuation regime on tadpole burst swimming performance ($F_{1,80}=2.1$, $P=0.15$ in the analysis with tadpole total length included as a covariate) (Fig. 3A), indicating that the effect of fluctuation regime on burst swimming performance was attributable to differences in tadpole total length.

SYNC tadpoles were significantly smaller than ASYNC tadpoles (PC 1: $F_{1,99}=16.1$, $P<0.001$) (Fig. 3B) with the total length, body length, body width, and tail muscle width (Fig. 4) of SYNC tadpoles being 10.9±0.1 mm, 3.9±0.04 mm, 2.6±0.03 mm and 0.58±0.04 mm, respectively, and the total length, body length, body width, and tail muscle width of ASYNC tadpoles being 11.5±0.1 mm, 4.1±0.05 mm, 2.7±0.03 mm and 0.63±0.01 mm, respectively.

There was no significant effect of fluctuation regime on tadpole survival time in predation trials ($F_{1,87}=1.05$, $P=0.31$) with the SYNC and ASYNC tadpoles surviving for 19±4 min and 13±2 min, respectively (Fig. 3C).

Discussion

Low temperature enhances the negative effects of UVBR (Broomhall et al., 2000; Grant and Licht, 1995; van Uitregt et

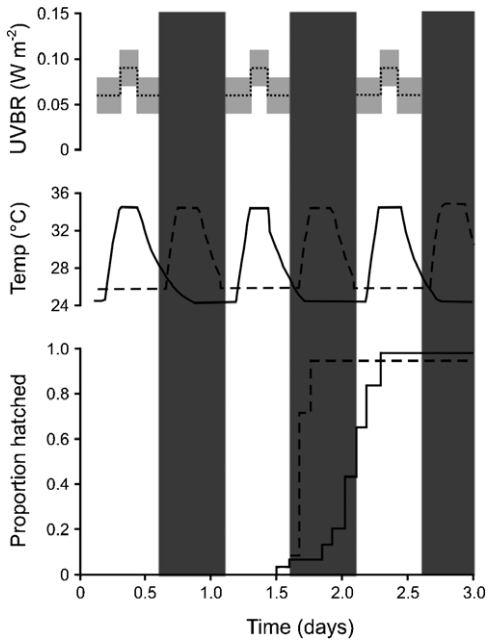

Fig. 2. Fluctuating ultraviolet-B radiation (UVBR) and temperature treatments and their effect on the hatching of *Limnodynastes peronii* embryos. Top panel: *L. peronii* embryos and tadpoles were exposed to a diurnally-fluctuating UVBR-irradiance regime (12 h per day, 0830 to 2030) that had a peak in UVBR levels at the photoperiod midpoint (4 h per day, 1230 to 1630). The dotted line is the mean absolute irradiance of UVBR (W m^{-2}) of 10 spectral irradiance measurements taken at the surface of the water and the light grey shading around this dotted line is the S.D. (Table 1). Middle panel: All *L. peronii* individuals were exposed to temperatures that fluctuated between 25±1℃ and 35±1℃ on a daily timescale, and *L. peronii* individuals either experienced synchronous fluctuations in UVBR and temperature, such that they experienced peak UVBR levels at 35℃ (SYNC fluctuation regime: solid line), or asynchronous fluctuations in UVBR and temperature, such that they experienced peak UVBR levels at 25℃ (ASYNC fluctuation regime: dashed line). Bottom panel: SYNC embryos (solid line) hatched later than ASYNC embryos (dashed line) ($P>0.001$) but there was no effect of fluctuation regime on the hatching success of embryos ($P=0.30$). Time 0 represents 0600 when egg masses were collected and the dark grey shading shows the time when the lights were off. Note that SYNC embryos experienced two full cycles of temperature fluctuations prior to hatching, whereas ASYNC embryos only experience one.

al., 2007). This relationship between UVBR and temperature, however, has mostly been examined under constant temperature regimes (Grant and Licht, 1995; Searle et al., 2010; van Uitregt et al., 2007). In the present study we used UVBR and temperature regimes that fluctuated on a daily time scale to assess whether exposure to peak UVBR levels while the temperature was high (35℃; SYNC fluctuation regime) was more detrimental to *L. peronii* embryos and tadpoles than exposure to peak UVBR levels while the temperature was moderate (25℃; ASYNC fluctuation regime). *L. peronii* embryos reared under the SYNC fluctuation regime hatched 10 h later than those reared under the ASYNC fluctuation regime (Fig. 2), and, as tadpoles, were smaller (Fig. 3B) and consequently had slower burst swim speeds (Fig. 3A). Although these effects suggest that simultaneous exposure to a high level of UVBR and a high temperature is detrimental to embryonic and larval amphibians, as we hypothesised, we must first consider two important points.

Firstly, the effect of fluctuation regime on hatching time is confounded by the timing of temperature fluctuations, i.e. SYNC

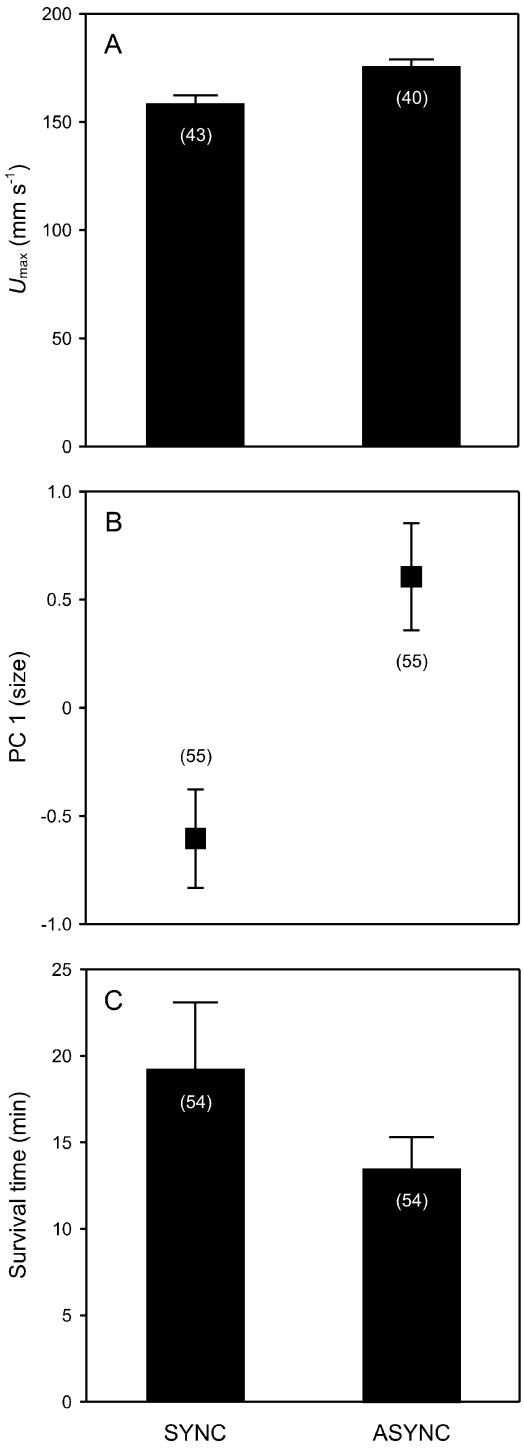

Fig. 3. The effect of fluctuation regime on *Limnodynastes peronii* tadpole burst swimming performance, size, and survival time in an environment with predators. SYNC and ASYNC denote the synchronous and asynchronous fluctuation regimes, respectively. (**A**) SYNC tadpoles had a burst swimming speed (U_{max}, mm s^{-1}) that was significantly slower than ASYNC tadpoles ($P=0.002$), but when the effect of tadpole total length was accounted for, there was no effect of fluctuation regime on tadpole burst swimming performance ($P=0.15$), indicating that the effect of fluctuation regime on burst swimming performance was attributable to differences in tadpole total length. (**B**) SYNC tadpoles were significantly smaller (PC 1) than ASYNC tadpoles ($P<0.001$), and (**C**) there was no significant effect of fluctuation regime on tadpole survival time (min) in predation trials ($P=0.31$). Data represent means ± SE and numbers in parentheses are the sample size.

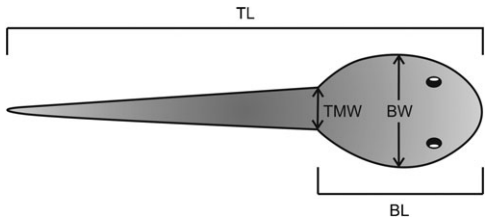

Fig. 4. Diagrammatic representation of the four morphological measurements taken from each tadpole. The four measurements taken from the dorsal view were total length (TL), body length (BL), body width (BW), and tail muscle width (TMW).

embryos may have hatched later simply because they experienced their first detrimental increase in temperature 12 h before ASYNC embryos, and also because they experienced two full cycles of temperature fluctuations prior to hatching whereas ASYNC embryos experienced only one (Fig. 2). Given that we are limited in our ability to attribute differences in hatching time to experimental treatments, we must therefore acknowledge that the effect of fluctuation regime found on tadpole size and locomotor performance may suffer the same confound as a consequence of knock-on effects associated with differences in hatching time. Following hatching, however, tadpoles experienced 13 days of exposure to experimental treatments before the response variables of locomotor performance, morphology, and survival time in an environment with predators were measured, and it is reasonable to propose that this exposure period following hatch is long enough to outweigh any effects that may have been caused by differences in hatching time associated with the timing of temperature fluctuations. Future studies, however, may avoid this confound by offsetting the timing of lighting fluctuations as opposed to temperature fluctuations as in the present study, but this then creates a new confound because one treatment group would suffer a change in circadian rhythm while the other would not, and this may have its own negative effects. An alternative to both these experimental designs would be to exclude examinations of treatment effects on the embryonic life stage all together and only expose individuals to experimental treatments once they have all hatched, or all reached Gosner stage 25 (Gosner, 1960).

The second point that needs to be considered before we draw definitive conclusions regarding the interactive effects of UVBR and temperature, is that there was no effect of fluctuation regime on tadpole survival time in an environment with predators (Fig. 3C), which has implications for the biological significance of the effect of fluctuation regime on tadpole size and consequently burst swimming speed. It is interesting that differences in size, and consequently burst swimming speed, appear not to be the mechanisms by which tadpoles gain a fitness advantage in the particular predator system used in the present study. This lack of correlation between burst swimming speed and survival time has been found previously for *L. peronii* (Alton et al., 2011). In our previous study on *L. peronii* that used the same protocol for predation trials as the present study, we found that exposure to increased UVBR had no effect on tadpole burst swimming performance, but caused tadpoles to have reduced survival times in predation trials (Alton et al., 2011). We did, however, also find that *L. peronii* tadpoles exposed to predatory chemical cues (cues that signal risk of predation) had faster burst swimming speeds and survived for longer in predation trials (Alton et al., 2011), which suggests that an increase in locomotor performance does confer some benefit to survival in an environment with predators, as has been shown in other predator-prey systems (Arendt, 2009; Hawlena et al., 2011; Langerhans, 2009; Walker et al., 2005; Watkins, 1996), but clearly other factors (e.g. tadpole behavior) (Warkentin, 1999) must contribute to tadpole survivorship in an environment with predatory shrimp. Also, given that in our previous study we were able to detect differences in tadpole survival time using the same protocol for predation trials as the present study, we think that the predation trials used in the present study were an adequate tool by which to evaluate the effect of fluctuation regime on tadpole survival time.

Why ASYNC tadpoles did not survive for longer in predation trials compared to SYNC tadpoles, we do not know, but regardless, we think that the results from the present study are not sufficiently strong to support our hypothesis that high temperatures enhance the negative effects of UVBR in embryonic and larval amphibians. We do, however, feel that the question of whether the detrimental effects of UVBR exposure are enhanced at extremely high temperatures warrants further investigation. It is a question that is ecologically relevant for organisms that inhabit environments where the temperature fluctuates widely on a daily timescale, because organisms in such environments are likely to experience high doses of UVBR when temperatures are extremely high. Designing an experiment to address this question, however, is difficult because exposing organisms to a constant temperature that is extremely high is likely to result in high levels of mortality, irrespective of the UVBR dose to which they are exposed. The ecological relevance of a high and stable temperature is also questionable. In order to maintain adequate levels of survival to examine the interaction between UVBR and temperature, a fluctuating temperature regime must be adopted, but designing an appropriate control for such an experimental treatment presents its own challenges. Using a stable temperature that is equivalent to the mean of the fluctuating temperature treatment is not an adequate control because physiological rates vary approximately exponentially with temperature (Gillooly et al., 2001; Gillooly et al., 2002; Krogh, 1914). An appropriate control could be the temperature at which physiological rates equal the mean physiological rate of the fluctuating temperature regime, but if the temperature dependence of physiological rates varies for different processes then the appropriate constant temperature is likely to be different for different processes.

In the present study we attempted to avoid these issues by having two experimental treatments that differed only in the temperature at which individuals experienced exposure to peak levels of UVBR while keeping the daily temperature fluctuations the same. In addition to the issue of embryonic hatching time being confounded with the timing of temperature fluctuations, there are other problems that mean that this design is also not an ideal way to address the question. For instance, if no significant effect is found between the two fluctuation treatments (i.e. SYNC and ASYNC) it may not necessarily mean that the repair mechanisms involved in the repair of UVBR-induced damage are not compromised at extremely high temperatures, but rather it could mean that the repair mechanisms that operate during the night (i.e. NER) are able to compensate for the reduced repair capabilities during the day. In our particular experiment, it could also mean that the reaction rates of repair enzymes are the same

at 25℃ and at 35℃, with the optimal temperature for repair rates being between 25℃ and 35℃. UVBR-induced DNA damage is also known to be temperature-dependent, with greater amounts of damage occurring at higher temperatures (Li et al., 2002; MacFadyen et al., 2004); therefore in a SYNC treatment a study organism may suffer more damage than in an ASYNC treatment, but if repair enzymes function better at higher temperatures this may compensate for the higher level of damage, which, again, may lead to a non-significant treatment effect.

Given the problems associated with designing an appropriate control for a fluctuating temperature treatment and the SYNC/ASYNC experimental design used in the present study, as an alternative, researchers might adopt an experimental design with two fluctuating temperature treatments that vary only in peak temperature and fluctuate synchronously with a fluctuating UVBR irradiance regime. While the two fluctuating temperature treatments would presumably lead to differences in growth rate and size, these effects could be accounted for using appropriate statistical analytical techniques (Freckleton, 2002; García-Berthou, 2001; Packard and Boardman, 1999) and thus the interaction between UVBR and temperature could still be considered. Otherwise, researchers may choose to only measure response variables that are not confounded with temperature-related differences in size (e.g. binominal data, such as survival). Such an experimental design might offer the best means to determining whether the detrimental effects of UVBR exposure are enhanced at extremely high temperatures.

Materials and Methods

Animal collection and maintenance

Ten freshly laid *Limnodynastes peronii* foam egg masses were collected at 0600 from an ephemeral creek near The University of Queensland, Brisbane, Australia (27°30′22.81″S, 152°59′22.99″E). Egg masses were transported to The University of Queensland where an equal number of eggs were randomly selected from each mass and divided amongst experimental treatments (n=60 per treatment). Immediately following collection, *L. peronii* individuals were exposed to experimental treatments (see later) for 15 days with all individuals reared separately in containers filled with 12 mL (water depth of 20 mm) of water that was purified by reverse osmosis (RO water) and supplemented with ocean salt (Aquasonic, Wauchope, Australia) to give a salinity of approximately 0.3 ppt. Upon hatching (2–4 days after collection), tadpoles were fed boiled spinach *ad libitum* and 100% water changes were performed daily to maintain water quality. During the 15-day exposure period the position of each tadpole within treatments was assigned randomly and changed daily. After being exposed to experimental treatments for 15 days, tadpoles were maintained at a constant temperature of 25±1℃ without exposure to UVBR for two more days during which tadpole locomotor performance and morphology were measured on the first day (day 16) and predation trials were performed on the second day (day 17).

UVBR treatment

All *L. peronii* individuals were exposed to UVBR, ultraviolet-A radiation (UVAR) and visible light emitted from two 40 W linear fluorescent light bulbs (Repti Glo 8.0, Exo Terra®, Montreal, Canada) that were on for 12 h each day (0830 to 2030). At the photoperiod midpoint, embryos and tadpoles were exposed to additional UVBR, UVAR, and visible light emitted from two 40 W linear fluorescent light bulbs (two Repti Glo 8.0, Exo Terra®, Montreal, Canada) that were on for 4 h each day (1230 to 1630) (Fig. 2).

The spectral irradiance (W m^{-2} nm^{-1}) produced by our peak (photoperiod midpoint) and non-peak lighting was measured using a cosine corrector (CC-3-UV-S, Ocean Optics, Dunedin, Florida, USA) and UV-VIS fibre optic cable (400 µm Premium Fiber, Ocean Optics, Dunedin, Florida, USA) attached to a spectrometer (USB2000+ Miniature Fiber Optic Spectrometer, Ocean Optics, Dunedin, Florida, USA) at 10 fixed positions at the level of the water surface. The spectrometer was calibrated using a NIST-traceable UV-NIR (220–1050 nm) calibration source (DH-2000-CAL Deuterium Tungsten Halogen Calibration Standard, Ocean Optics, Dunedin, Florida, USA). The absolute irradiance (W m^{-2}) of UVBR (Fig. 2) and UVAR at each of the 10 fixed positions was calculated by integrating the spectral irradiance data between 300 and 320 nm, and 320 and 400 nm, respectively. To account for the wavelength dependency of ultraviolet radiation (UVR) effects, spectral irradiance data were weighted according to the McKinlay-Diffey erythemal action spectrum (McKinlay and Diffey, 1987), and erythemal UVR (UVR_{ery}) was calculated by integrating the weighted spectral irradiance data between 300 and 400 nm (Table 1). Although the McKinlay-Diffey weighting function describes the wavelength dependency of damage to human skin, it may be considered representative of other biological UVBR effects (e.g. DNA damage) (Setlow, 1974) because the action spectra of these effects are similar to that of the erythemal action spectrum (McKenzie et al., 2003). To calculate the daily dose (J m^{-2}) of UVBR and UVAR at the 10 fixed positions, spectral irradiance data for peak and non-peak lighting were multiplied by exposure time (4 h for peak lighting and 8 h for non-peak lighting) and added together (Table 1). To calculate the daily dose (J m^{-2}) of UVR_{ery} at the 10 fixed positions, erythemally-weighted spectral irradiance data for peak and non-peak lighting were multiplied by exposure time (4 h for peak lighting and 8 h for non-peak lighting) and added together (Table 1). The UV Index (UVI) was calculated by multiplying UVR_{ery} by 40 m^2 W^{-1} (WHO, 2002) (Table 1).

The absolute irradiance of UVBR in ambient midday sunlight during the peak breeding season of *L. peronii* (i.e. summer) in Brisbane, Australia, has been measured previously as 5 W m^{-2} (van Uitregt et al., 2007) which corresponds to a UV index (UVI) of 11 (ARPANSA, UV-Index Summary for Brisbane, 10th December 2005, http://www.arpansa.gov.au/uvindex/historical/images/bri10122005.gif) (WHO, 2002). The peak absolute irradiance of UVBR generated by our UVBR lighting regime was 0.09±0.02 W m^{-2} (mean ± S.D.) and the UVI was less than 1 (Table 1). Over the course of a cloudless day in Brisbane during summer (December–February), the UVI is below 1 only in the early morning (before 0700) and in the late afternoon (after 1700), with the average summer (December–February) daily maximum UVI between 2004 and 2010 being 11 (ARPANSA, Monthly UV-Index Summaries for Brisbane, http://www.arpansa.gov.au/uvindex/monthly/brimonthlysumm.htm).

Temperature treatments

All *L. peronii* individuals were exposed to temperatures that fluctuated between 25±1℃ and 35±1℃ on a daily timescale (Fig. 2). *L. peronii* individuals either experienced synchronous fluctuations in UVBR and temperature (SYNC fluctuation regime), such that they experienced peak UVBR levels at 35℃, or asynchronous fluctuations in UVBR and temperature (ASYNC fluctuation regime), such that they experienced peak UVBR levels at 25℃ (Fig. 2). The peak temperature of 35℃ was chosen because it is likely to be a temperature that is highly stressful for *L. peronii* embryos and tadpoles since a constant stable temperature of 34℃ is known to be lethal to *L. peronii* embryos and tadpoles (Niehaus et al., 2012). The peak temperature of 35℃ was also chosen based on maximum daily temperatures that had been recorded previously in open habitats in southeastern Queensland during the summer when *L. peronii* breed (Niehaus et al., 2012) (Fig. 1). Although these same temperature recordings in *L. peronii* habitats indicated that the minimum temperatures experienced by *L. peronii* are between 18℃ and 21℃ (Niehaus et al., 2012) (Fig. 1), we chose a minimum temperature of 25℃. This is because exposure to UVBR at 20℃ is detrimental to *L. peronii* embryos and tadpoles (van Uitregt et al., 2007), and since the purpose of our study was to assess whether high temperatures exacerbate the negative effects of UVBR, we needed to make comparisons between a high temperature (35℃) and a more moderate temperature (25℃).

To generate our fluctuating temperature regimes, containers holding *L. peronii* individuals were placed in to one of two water baths that were maintained at 25±1℃ using a 300 W aquarium heater. The water bath containing *L. peronii* individuals that were assigned to the SYNC treatment had four 55 W aquarium heaters that turned on at 1000 and one 55 W aquarium heater that turned on 1200 using electronic timers. These aquarium heaters heated the water at a rate of approximately 4℃ h^{-1} until the water temperature reached 35±1℃ at 1230. The water temperature was then maintained at 35±1℃ for 4 h, during which time individuals were exposed to peak UVBR levels. At 1630 the aquarium heaters turned off and the water cooled at a rate of 2℃ h^{-1} by having the temperature of the room at 20±1℃. The water bath containing *L. peronii* individuals that were assigned to the ASYNC treatment heated and cooled in the same way as the SYNC treatment water bath only it was maintained at 25±1℃ during exposure to peak UVBR levels and heated to 35±1℃ between 2200 and 0030 and remained at 35±1℃ between 0030 and 0430.

Time to hatch, hatching success, and post-hatch survival

Embryos were checked daily to record time to hatch and hatching success. Hatching commenced on the second day of the experimental period. Once hatching commenced, embryos were checked every 2 h. Time to hatch was considered to be the midpoint between the time at which hatch was first recorded and the preceding check time. Following hatching, tadpole survival was checked daily until the termination of the experiment, which occurred on day 15 of the experimental period.

Table 1. Ultraviolet radiation levels used in experimental treatments. The mean (± S.D.) absolute irradiance (W m^{-2}) and daily dose (J m^{-2}) of ultraviolet-B radiation (UVBR: 300–320 nm), ultraviolet-A radiation (UVAR: 320–400 nm), erythemal ultraviolet radiation (UVR_{ery}: 300–400 nm), and mean (± SD) UV index (UVI) of 10 spectral irradiance measurements. Peak absolute irradiance and peak UVI is the measure of UVBR at the photoperiod midpoint, and non-peak absolute irradiance and non-peak UVI is the measure of UVBR outside this time period.

Measurement	Exposure time (h)	Mean ± S.D.
Peak absolute irradiance (W m^{-2})		
UVBR	4	0.09±0.02
UVAR	4	1.86±0.41
UVR_{ery}	4	0.009±0.003
Non-peak absolute irradiance (W m^{-2})		
UVBR	8	0.06±0.02
UVAR	8	0.36±0.08
UVR_{ery}	8	0.006±0.002
Daily dose (J m^{-2})		
UVBR	12	3064±674
UVAR	12	37229±7847
UVR_{ery}	12	300±80
UVI		
Peak	4	0.37±0.12
Non-peak	8	0.23±0.08

Locomotor performance and morphology

Following exposure to experimental treatments, the locomotor performance (i.e. burst swimming performance) and morphology of each tadpole was measured on day 16. Burst swimming performance was assessed by filming a minimum of three startle responses for each individual with a high-speed digital camera (TroubleShooter LE 500, Fastec Imaging, San Diego, CA, USA) recording at 250 Hz. Swims were performed in a temperature controlled room at 25±1℃ without exposure to UVBR and were conducted in a container filled to 10 mm deep with RO water supplemented with ocean salt (Aquasonic, Wauchope, Australia) to give a salinity of approximately 0.3 ppt. Startle responses were induced by a small electrical stimulus (S88 Grass Stimulator) and recordings were played back using Redlake software (Redlake Motionschope Media Player Version 2.21). Only burst swimming sequences that consisted of a C-start response in which the tadpole started from a stationary position and continued in a straight line parallel to the bottom were analysed. Briefly, a C-start response is a fast-escape response that has been characterised in fishes and amphibians: it initially involves a tight bend to one side, causing a C-shaped curve in the body, and then a propulsive movement out of the C-shape (Domenici and Blake, 1997; von Seckendorf Hoff and Wassersug, 2000). The average velocity over 100 ms was used as the measure for burst swimming performance (U_{max}) and was calculated by digitising the snout tip at the end of C-start manoeuvre and again 25 frames (100 ms) forward and measuring the total straight-line distance travelled. Of all the recordings taken for each individual, the fastest was taken as a measure of maximum performance and was used for statistical analysis.

Immediately after being filmed for burst swimming performance, the dorsal view of each tadpole was photographed using a digital camera mounted through a dissecting microscope. Four morphological measurements (total length, body length, body width, and tail muscle width) (Fig. 4) were taken using SigmaScan Pro 5.0 (Systat Software, California, USA).

Predation trials

Ten 5 L aquaria, each containing four starved shrimp (*M. australiense*), were used as predator tanks and were maintained at 25±1℃ in a temperature-controlled room without exposure to UVBR. Predation trials were performed on day 17 giving tadpoles a minimum of 12 h rest following burst swimming performance and morphology measurements. A single predation trial consisted of an individual tadpole, selected at random from the treatment groups, being introduced to one of the predator tanks by gently passing it through a pipette. Following entry, the tadpole was observed and once the tadpole had been successfully caught and had begun to be consumed by a shrimp, the trial was terminated and survival time was calculated from the time of entry. One SYNC tadpole was not successfully caught after 2.3 h and this trial was omitted from the dataset.

Table 2. Principal components analysis matrix showing the factor loadings of each measured morphological variable and in which direction they contribute towards principal component (PC) 1 (tadpole size) and PC 2 (tadpole shape).

Morphological variable	PC 1	PC 2
Total length	0.942	−0.066
Body width	0.937	−0.195
Tail muscle width	0.887	0.457
Body length	0.961	−0.167

Statistical analyses

Time to hatch data was analysed using a parametric survival analysis with a Weibull distribution and fluctuation regime (SYNC and ASYNC) as a fixed factor. Hatching success and tadpole post-hatch survival data were analysed using logistic regression with fluctuation regime (SYNC and ASYNC) as a fixed factor. Burst swimming performance data were analysed both with and without tadpole total length as a covariate using an ANCOVA and ANOVA, respectively, (to determine if the effect of fluctuation regime was driven solely by differences in tadpole length) with fluctuation regime (SYNC and ASYNC) as a fixed factor. Egg mass was not included in the statistical model as a random effect as it accounted for negative variance when it was included.

To assess changes in body size and shape associated with treatments, a principal components analysis (PCA) was performed on the correlation matrix of the four morphological measurements of all treatment groups combined. This generated a new set of standardised uncorrelated morphological variables with principal component (PC) 1 accounting for 87% of the variation (eigenvalue of 3.5) and PC 2 accounting for 7% of the variation (eigenvalue of 0.3). The PCA matrix of component loadings (Table 2) shows the correlation between the original morphological measurements and the two principal components. PC 1 represents a measure of tadpole size because all morphological dimensions load strongly and positively on this axis (Table 2), and PC 2 represents tadpole shape. The PC factor scores for PC 1 were analysed using a mixed-model ANOVA with fluctuation regime (SYNC and ASYNC) as a fixed factor and egg mass as a random effect to identify how each treatment affected PC 1 (size). Since PC 2 had an eigenvalue less than 1, the PC factor scores for PC 2 were not retained for further analysis (Quinn and Keough, 2002).

For predation trials, survival time data were cube root transformed to satisfy assumptions of normality and homogeneity of variance, and analysed using a mixed-model ANOVA with fluctuation regime (SYNC and ASYNC) as a fixed factor, and egg mass and predator tank as random effects.

All statistical analyses were performed using JMP 8.0.2 (SAS Institute Inc., Cary, NC, USA), except the PCA which was performed using StatistiXL 1.8 (www.statistixl.com).

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Competing Interests

The authors have no competing interests to declare.

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