MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 6177–6187 doi: 10.1111/mec.13460

Divergence in DNA photorepair efficiency among genotypes from contrasting UV radiation environments in nature

BROOKS E. MINER,*† PAIGE M. KULLING,†[1] KARLYN D. BEER‡§[2] and BENJAMIN KERR¶

*Department of Biology, Ithaca College, 953 Danby Rd., Ithaca, NY 14850, USA, †Department of Ecology and Evolutionary Biology, Cornell University, 215 Tower Road, E149 Corson Hall, Ithaca, NY 14853, USA, ‡Program in Molecular and Cellular Biology, University of Washington, Seattle, WA, USA, §Institute for Systems Biology, 401 Terry Ave N., Seattle, WA 98109, USA, ¶Department of Biology and BEACON Center for the Study of Evolution in Action, University of Washington, Box 351800, Seattle, WA 98195, USA

Abstract

Populations of organisms routinely face abiotic selection pressures, and a central goal of evolutionary biology is to understand the mechanistic underpinnings of adaptive phenotypes. Ultraviolet radiation (UVR) is one of earth's most pervasive environmental stressors, potentially damaging DNA in any organism exposed to solar radiation. We explored mechanisms underlying differential survival following UVR exposure in genotypes of the water flea *Daphnia melanica* derived from natural ponds of differing UVR intensity. The UVR tolerance of a *D. melanica* genotype from a high-UVR habitat depended on the presence of visible and UV-A light wavelengths necessary for photoenzymatic repair of DNA damage, a repair pathway widely shared across the tree of life. We then measured the acquisition and repair of cyclobutane pyrimidine dimers, the primary form of UVR-caused DNA damage, in *D. melanica* DNA following experimental UVR exposure. We demonstrate that genotypes from high-UVR habitats repair DNA damage faster than genotypes from low-UVR habitats in the presence of visible and UV-A radiation necessary for photoenzymatic repair, but not in dark treatments. Because differences in repair rate only occurred in the presence of visible and UV-A radiation, we conclude that differing rates of DNA repair, and therefore differential UVR tolerance, are a consequence of variation in photoenzymatic repair efficiency. We then rule out a simple gene expression hypothesis for the molecular basis of differing repair efficiency, as expression of the CPD photolyase gene *photorepair* did not differ among *D. melanica* lineages, in both the presence and absence of UVR.

Keywords: adaptation, *Daphnia*, photoenzymatic repair, photolyase, photoreactivation, ultraviolet radiation

Received 1 September 2014; revision received 1 November 2015; accepted 3 November 2015

Introduction

Understanding how extant populations of organisms have evolved to tolerate abiotic environmental stressors is important to understanding the scope and limits of evolutionary adaptation (MacColl 2011). Such knowledge is also essential for predicting future evolutionary responses to changes in environmental conditions due

Correspondence: Brooks E. Miner, Fax: 607-274-1131; E-mail: bminer@ithaca.edu

[1]Present address: Department of Pathology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

[2]Present address: Division of Foodborne, Waterborne and Environmental Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30329, USA

to global climate change (Bell & Collins 2008; Hoffmann & Sgrò 2011; Carlson *et al.* 2014). However, inferences of adaption to natural environmental stressors are often obscured by a lack of knowledge about the biological mechanisms that underlie observed differences in fitness among study populations. Conversely, evolution experiments in the laboratory (often with model organisms) can elucidate connections between phenotype, genotype and fitness under laboratory conditions, but the relevance of these connections to adaptation in nature is often unknown. An ideal combination is to explore the biological mechanism(s) responsible for clear fitness differences between closely related natural populations in response to a well-characterized environmental stressor that differs among populations.

Ultraviolet radiation (UVR) is an environmental stressor common to most aboveground terrestrial, freshwater and shallow marine habitats worldwide. The shortest wavelength radiation that reaches the surface of the earth, UV-B, is most harmful to life because it damages DNA by altering the structure of nucleotides in ways that interfere with transcription and replication (Cadet *et al.* 2005; Pfeifer *et al.* 2005). The harmful effects of UVR-caused DNA damage are both immediate (altering gene transcription and protein expression) and lasting (increasing the baseline mutation rate, which can raise mutational load). The mechanisms organisms employ to reduce the burden of natural UVR exposure include behavioural avoidance, photoprotective pigmentation and DNA repair pathways (Hansson & Hylander 2009).

Most species, including prokaryotes and eukaryotes, possess a repair mechanism specifically targeted at the two UVR-caused DNA damage structures, cyclobutane pyrimidine dimers and (6–4) photoproducts, both of which contain chemical bonds between adjacent pyrimidine bases in DNA. This widespread mechanism, known as photoreactivation, photoenzymatic repair or simply photorepair (Sutherland 1981; Heelis *et al.* 1993; Thoma 1999; Sinha & Häder 2002), employs a photolyase enzyme that binds to UVR-caused dimers and reverses the damage using energy derived from photons of visible or UV-A light. Photolyases are structurally related to cryptochromes, and contain a catalytic domain with a light-sensitive cofactor FAD and a DNA binding site. Repair of the DNA lesion occurs when the reduced form of the cofactor (FADH-) absorbs a light photon and subsequently injects an electron into the lesion, causing breakage of the double bond(s) between adjacent nucleotides (Heelis *et al.* 1993). In addition to the catalytic domain, photolyases also contain an *N*-terminal domain that in some, but not all, species contains an 'antenna' chromophore that broadens the action spectrum of the enzyme. However, the only characterized crystal structure of a eukaryotic photolyase lacked a bound antenna chromophore (Kiontke *et al.* 2011). Although the photorepair mechanism is dependent on the presence of light, it is much less energy-intensive for organisms than the more general excision repair pathways, which remove and replace damaged nucleotides entirely (Sinha & Häder 2002).

Zooplankton are among the most important primary consumers in both freshwater and marine habitats and are often exposed to levels of UVR that favour the evolution of defensive mechanisms. Cladocera and copepods have been intensively studied for their diverse adaptations to life in high-UVR environments such as shallow ponds and highly transparent lakes (Hansson & Hylander 2009). Several species of the freshwater microcrustacean *Daphnia* are among the most thoroughly studied, and differences exist among species and among populations in photoprotective melanin pigmentation (Hebert & Emery 1990; Hessen 1996; Scoville & Pfrender 2010) and the propensity to vertically migrate away from UVR (Leech *et al.* 2005; Williamson *et al.* 2011). In addition, DNA photorepair is a key UVR tolerance mechanism for zooplankton (Malloy *et al.* 1997; Zagarese *et al.* 1997; Grad *et al.* 2001, 2003; Williamson *et al.* 2002; Ramos-Jiliberto *et al.* 2004; Tartarotti *et al.* 2014), including *Daphnia* (Grad *et al.* 2001; Williamson *et al.* 2001; MacFadyen *et al.* 2004), where it differs in efficiency among species (Connelly *et al.* 2009). How existing interspecies differences in DNA photorepair efficiency arose is a question with considerable evolutionary significance.

The first step in the process of divergence in DNA photorepair efficiency among species was likely the formation of distinct populations of a single species that evolved in response to contrasting underwater UVR environments. We have previously identified a system of closely related populations of the water flea *Daphnia melanica* in shallow subalpine ponds of Olympic National Park, WA, USA that bears these features (Miner & Kerr 2011). Ponds inhabited by *D. melanica* differ dramatically in transparency to UVR as a result of dissolved organic matter concentrations, and *D. melanica* from these ponds differ in their ability to survive under laboratory UVR. *Daphnia* genotypes from ponds with higher UVR transparency exhibit greater survival following laboratory UVR exposure, presumably due to adaptation to the UVR threat in their native pond (Miner & Kerr 2011). Because the ponds are shallow (<1.5 m maximum depth), *Daphnia* in the most transparent ponds cannot escape UVR by migrating to deeper water, and our measured differences among populations in laboratory UVR tolerance also cannot result from behavioural avoidance. Therefore, behavioural mechanisms are not sufficient to explain divergent UVR-tolerance phenotypes in this system.

In most other clearwater UVR habitats worldwide, *Daphnia* individuals exhibit high concentrations of photoprotective melanin pigmentation (Hebert & Emery 1990; Hessen 1996; Hansson *et al.* 2007; Scoville & Pfrender 2010), yet our *D. melanica* have minimal melanin content that does not vary among populations (Miner & Kerr 2011; Miner *et al.* 2013). Another category of photoprotective compounds, mycosporine-like amino acids (MAAs), have more recently been discovered to be important for copepods, but not *Daphnia*, in high-UVR environments (Sommaruga & Garcia-Pichel 1999; Moeller *et al.* 2005; Hansson *et al.* 2007; Sommaruga 2010). Antioxidant enzymes such as catalase and glutathione transferase can also play a role in UVR defence for zooplankton, including *Daphnia* (Borgeraas & Hessen 2002; Hessen *et al.* 2002; Balseiro *et al.* 2008). Carotenoid accumulation is frequently found in copepods and has been shown to protect against UVR, but is not commonly found in *Daphnia* (Hansson *et al.* 2007; Hansson & Hylander 2009; Sommaruga 2010). However, carotenoids are present in *Daphnia* fat bodies and developing eggs (Green 1957), and carotenoid availability in the diet may influence antioxidant activity, providing an indirect link to UVR tolerance (Hessen 1993).

Here we describe investigations of the roles of pigmentation and DNA repair in determining differences in UVR tolerance among *Daphnia* genotypes derived from habitats differing in UVR exposure. We conducted a series of laboratory experiments to evaluate the importance of DNA photorepair, including measurements of UVR tolerance and the rate of removal of cyclobutane pyrimidine dimers (CPDs), the primary DNA damage structure resulting from UVR exposure (Sinha & Häder 2002; Cadet *et al.* 2005). We also measured expression of the gene *photorepair*, which encodes the CPD photolyase enzyme. Our findings demonstrate striking differences among genotypes from closely related populations in a basic biological mechanism necessary for coping with one of earth's most pervasive environmental stressors.

Materials and methods

Source of Daphnia genotypes and laboratory culture conditions

We collected live *Daphnia* from shallow ponds in the Seven Lakes Basin region of Olympic National Park, WA and propagated genotypes in laboratory culture as asexually reproducing clones. The five focal genotypes of the present study were collected in August 2009 from one pond with UVR transparency that was high relative to other ponds in the area (genotypes 2, 3 and 5); the other pond had relatively low UVR transparency (genotypes 1 and 4). For the former, which we here refer to as the 'High UVR Pond', the percentage of incident UV-B estimated at 10 cm depth averaged 85% across three years; the latter, identified as the 'Low UVR Pond', averaged 19% of incident UV-B at 10 cm depth (see Miner & Kerr 2011 for details; the ponds used here are labelled as 'C' and 'K' in Fig. 1 of that study). From our previous work we knew that these lineages have distinct multilocus genotypes at microsatellite loci and differ in their ability to survive UVR exposure in the laboratory, although they had minimal melanin content in the field and in laboratory culture (see Miner & Kerr 2011; Fig. 2B). We cultured *Daphnia* at 12 °C on a 16L:8D photoperiod in FLAMES zooplankton medium (Celis-Salgado *et al.* 2008) with regular feeding of vitamin-supplemented *Cryptomonas ozolinii*. The genotypes were cultured in the laboratory for over one year before the experiments detailed here were conducted.

Measurement of carotenoids and mycosporine-like amino acids (MAAs)

We measured carotenoid and MAA content of field-collected *Daphnia* from the two source ponds of the focal genotypes of this study, in addition to two other ponds in the region. We collected live *Daphnia* with plankton tow nets and isolated animals in 0.2 μm filtered water from the source pond for >1 h to clear the gut of potential carotenoid or MAA contributions from partially digested phytoplankton. We placed the animals in 1.7-mL centrifuge tubes and flash-froze immediately in a dry shipper containing liquid nitrogen, then stored in the laboratory at −80 °C. To measure carotenoid content, we combined 7–10 animals in each replicate extraction in 95% ethanol for 18 h in the dark after homogenizing the tissue with plastic pestles. We had 24 total replicate carotenoid extractions; six replicates from each of four source ponds. Source ponds included the two ponds from which our five focal genotypes were collected, plus a second high-UVR pond and a second low-UVR pond. We centrifuged samples at 14 000 *g* for 5 min and measured absorbance at 474 nm, the absorption peak for common carotenoids in zooplankton (Hairston 1979; Hansson *et al.* 2007), in a Beckman DU-50 spectrophotometer. Concentrations of pigments were normalized to dry mass, which we estimated from length measurements using an existing length–mass regression (Bottrell *et al.* 1976). For MAA measurement, we lyophilized frozen samples (~40 animals per replicate) and measured dry masses of each. We had 15 MAA replicates total: three from each of five ponds at our field site, including the two ponds from

which our five focal genotypes were collected. Extraction of MAAs and analysis via HPLC followed the methods of Sommaruga (2010) and took place in the laboratory of R. Sommaruga at the University of Innsbruck, Austria.

Experimental assessment of the role of photorepair in determining survival under UVR

To evaluate the importance of the photorepair mechanism for UVR tolerance, we took advantage of the fact that the mechanism can be disabled simply by depriving the animals of UV-A and visible wavelengths of radiation (together, 'photorepair radiation' or PRR). We conducted parallel UV-B-exposure trials in the presence and absence of PRR in a temperature-controlled incubator equipped with a UV-lamp phototron modified from the design of Williamson *et al.* (2001), Miner & Kerr (2011). One advantage (among many) of this design is that the lamps used to provide UV-A and UV-B radiation emit a broad range of wavelengths within the UV spectrum, rather than peak irradiance at a single wavelength (see Fig. 4 in Williamson *et al.* 2001). The apparatus consisted of a horizontal rotating wheel under a UV-B lamp (XX-15B; Spectronics Corporation) where beakers containing *Daphnia* were placed. Each experimental trial consisted of cohorts of egg-bearing adult *Daphnia* that were each 28–35 days old. A single experimental replicate consisted of an uncovered beaker containing 80 mL of FLAMES medium, *C. ozolinii* (~4.5–5.5 mg/L dry mass), and 10 *Daphnia* from either the high-UVR or low-UVR genotype. We placed these beakers on the outer rim of the rotating wheel in the phototron and exposed the animals to 12 h of UV-B radiation during a 16-h day at 12 °C, in either the presence or absence of PRR provided from the sides with cool white fluorescent and Q-Panel UVA-340 lamps. The total UV-B dosage in the treatments without PRR was ~27–33 kJ/m^2, quantified with a PMA2101 erythemally weighted UV-B detector attached to a PMA2100 radiometer (Solar Light, Glenside, PA, USA). We estimate this dose to be less than double the natural dose that our *Daphnia* genotypes would be exposed to near the surface of their native pond on an average summer day (using UV-B irradiance data for this region described by Palen *et al.* 2002). On the day following UV-B exposure, we moved each experimental unit of 10 animals into 500 mL of the same algae/medium mix and maintained these under continuing conditions of either +PRR or −PRR at 12 °C for an additional 7 days before counting survivors. We added fresh algae once, on the 3rd or 4th day following exposure, during the period between UV-B exposure and survival measurement. There were between 21 and 26 experimental replicates (beakers of 10 *Daphnia*) per genotype, per treatment, spread among four experimental blocks. All experiments included an overall control treatment in which animals were not exposed to UV-B; survival here was always 100%.

Quantification of the rate of repair of UVR-caused CPDs

To measure the rate at which UVR-caused CPDs are repaired via the photoenzymatic process, we used the same experimental apparatus described above, but with an acute dose of UV-B (without PRR) followed by 0, 3, 6 or 12 h for repair in either the presence or absence of PRR. There were 12 animals in each beaker, of which three individuals were collected at each time point (comprising a single experimental replicate). There were between 6 and 21 replicates per genotype, per time point in the +PRR treatment and 4–6 replicates per genotype, per time point in the –PRR (dark) treatment. The acute UV-B dose was ≈2 kJ/m^2 applied over 30 min (Experiment 1) or ≈4 kJ/m^2 applied over 60 min (Experiment 2) in the phototron using three XX-15B lamps (Spectronics Corporation), followed by exposure to PRR immediately afterwards (Experiments 1 and 2), or the absence of PRR (i.e. darkness; applied to a subset of replicates in Experiment 2, which we refer to as Experiment 3 for clarity). The instantaneous UV-B dose applied here was ≈25–50% greater than for the survival experiments described above, to generate detectable quantities of CPDs. We collected animals at 0, 3, 6 and 12 h after exposure, flash-froze them in liquid nitrogen and later extracted DNA using the CTAB method (Cristescu *et al.* 2006) and quantified DNA with the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Grand Island, NY, USA). We then measured the quantity of CPDs in 10 ng of extracted DNA using a monoclonal antibody for CPDs (clone TDM-2; Cosmo Bio Co., Tokyo, Japan; Mori *et al.* 1991) in the ELISA protocol described in the accompanying Supporting information.

Measurement of expression of the photorepair gene

We quantified expression of the *Daphnia* gene encoding the CPD photolyase, which we call *photorepair* to match its homolog in *Drosophila melanogaster* (Boyd & Harris 1987). We measured *photorepair* expression in four *D. melanica* genotypes (genotype labels correspond to those used above: genotypes 1 and 4 from the low-UVR pond and genotypes 2 and 5 from the high-UVR pond) under two radiation treatments: UV-B plus PRR, or visible light only (no UVR). Radiation treatments were applied in the phototron using the same experimental

setup as described in the survival experiments above, although the radiation treatments lasted only 6 h. Each experimental trial consisted of same-age cohorts born within a 7-day period, and all experimental organisms were egg-bearing adult females. Each experimental replicate consisted of five to eight animals in a 100-mL beaker with FLAMES medium and algal food as described above. There were 7–8 replicates per clone in the +PRR treatment and 5–6 replicates per clone in the visible light treatment. We exposed the beakers to the light treatments for 6 h, from 7:00 to 13:00, and then preserved all animals from each beaker into a single sample in RNA-Later (Qiagen), which was kept at 4 °C for two to three days and then stored at −20 °C until RNA extraction. RNA extraction and qPCR methods are described in detail in accompanying Supporting information, including our use of a geometric averaging method for normalizing expression of *photorepair* using three reference genes.

Statistical analyses

We tested the significance of experimental results using generalized linear models (GLMs), linear mixed-effects models (LMMs), and ANOVA models fit in R (R Core Team 2014). For the survival experiment, we fitted a GLM with a quasibinomial error distribution (due to overdispersed data) and a logit link function. We included PRR treatment (present or absent), genotype ID ('low UVR' and 'high UVR') and the interaction between the two as linear predictors of the survival response. The interaction explicitly addresses our hypothesis that the two *Daphnia* genotypes differ in the degree or direction in which they respond (in survival terms) to PRR; we tested its significance with an *F*-test.

For the DNA repair experiments, we used LMMs that included random effects of genotype ID within pond type (high-UVR or low-UVR), and experimental block. With absorbance at 492 nm of the ELISA product as our response variable, we modelled the decrease in DNA damage over time as an exponential decay process, in which the fraction of CPDs removed per unit time is constant. We used a two-parameter exponential decay model, for which the linear form is: $\ln(\text{absorbance}) = a - bt$, where t is time since DNA damage (in hours), a is the model intercept and b is the decay rate. Our null model included repair time and pond type as fixed effects. Our second model added a fixed effect for the interaction between repair time and pond type. We then evaluated the two models using a likelihood ratio test and by comparing values for Akaike's Information Criterion (AIC).

For our gene expression data, we performed a two-way analysis of variance on normalized *photorepair* expression values, with genotype ID (four genotypes), light treatment (UVR present or absent) and the genotype × light treatment interaction as fixed effects. We evaluated the significance of each fixed effect with *F*-tests.

Results

We did not detect even trace amounts of MAAs in any of the *Daphnia melanica* samples, and carotenoid concentration was below 0.015 μg/mg dry mass in all samples (Fig. S1, Supporting information). Together with our previous finding that melanin content is low and invariable among these *D. melanica* populations (Miner & Kerr 2011), very low carotenoid content and lack of MAAs suggest that differences in UVR tolerance (Miner & Kerr 2011) cannot be due to photoprotective pigmentation. We had also previously ruled out behavioural avoidance as a sufficient explanation (Miner & Kerr 2011), leaving only antioxidant enzyme activity and/or the capacity for repair of UVR-caused DNA damage as potential UVR tolerance mechanisms. We choose to focus solely on the latter in this study.

Because photoenzymatic repair is responsible for the majority of repair of UVR-caused DNA damage in multiple *Daphnia* species (Grad *et al.* 2001; Williamson *et al.* 2001; Connelly *et al.* 2009), we explored whether the light wavelengths necessary for photorepair (visible and UV-A, together known as 'photorepair radiation') influenced UVR tolerance in two *D. melanica* clonal genotypes ('Genotype 1', from the low-UVR pond in nature, and 'Genotype 2', from the high-UVR pond). We found that the survival advantage under UVR of Genotype 2, from the high-UVR pond, disappeared when we removed photorepair radiation (Fig. 1). The presence of a statistically significant interaction between light treatment and *Daphnia* genotype ($F_{1,62} = 16.76$, $P = 0.0001$) demonstrates that the two *D. melanica* clonal genotypes have dramatically different survival responses to light treatment. This result provides indirect evidence that the DNA photorepair mechanism likely differs among these two genotypes, providing a potential mechanistic explanation for among-population differences in UVR tolerance documented previously (Miner & Kerr 2011).

To further explore the potential for differences among *D. melanica* clonal genotypes in the efficiency of photorepair of UVR-caused DNA damage, we measured DNA damage and repair directly. Cyclobutane pyrimidine dimers (CPDs) are alterations to the structure of DNA that are generated by exposure to UV-B radiation, and they are an important reason that UVR is harmful to organisms (Kittler & Löber 1977; Ellison & Childs 1981). Using an ELISA method with an antibody that binds to CPDs, we measured the rate of repair of CPDs

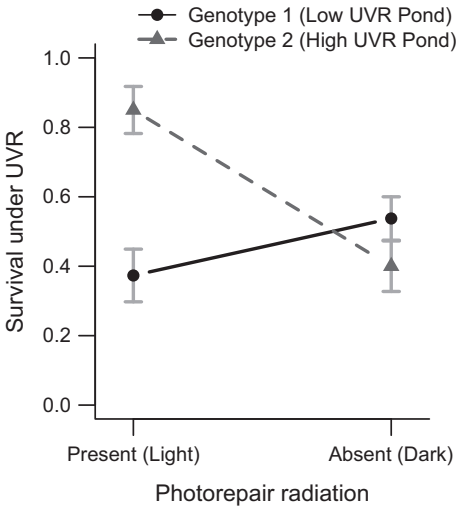

Fig. 1 Survival (mean ± SEM) under laboratory UV-B of two *Daphnia* clonal genotypes, one derived from a transparent pond with high UVR exposure and the other from a much less transparent pond with lower UVR exposure. The *x*-axis represents the presence or absence of photorepair radiation, the visible and UV-A light wavelengths necessary for photoenzymatic repair. The dramatic difference in survival in the presence of PRR disappears completely when PRR is removed, as demonstrated by a highly significant interaction term between PRR treatment and *Daphnia* source ($F_{1,62}$ = 16.76, P = 0.0001) in our GLM, with n = 66 observations.

in the DNA of animals following acute UV-B exposure. We measured repair of CPDs in two different experiments, using a total of five different clonal *D. melanica* genotypes (three from the high-UVR pond and two from the low-UVR pond).

Experiment 1 measured the rate of repair of CPDs in three genotypes (Genotype 1, from the low-UVR pond; and genotypes 2 and 3, both from the high-UVR pond) following an acute exposure to UV-B radiation. We found evidence for a faster rate of DNA repair in the genotypes from the high-UVR pond, although some samples from later in the repair trajectory approached the approximate detection limit of our ELISA (Fig. 2A). Therefore, we conducted a second experiment in which the initial damaging UV-B radiation dose was higher. In this second experiment, we used different *D. melanica* clonal genotypes than in the first experiment (Genotype 4, from the low-UVR pond, and Genotype 5, from the high-UVR pond). We used different genotypes in Experiment 2 for two reasons: (i) to increase our genotypic sampling, and (ii) because insufficient numbers of mature, same-age cohort females of the other genotypes were available at the time. The results of Experiment 2 again suggest a faster rate of DNA repair in the genotype derived from the high-UVR pond in nature (Fig. 2B).

To evaluate the statistical significance of the DNA repair results from the two experiments in a single analysis, we analysed the combined data set using linear mixed-effects models. We modelled the decrease in DNA damage over time as an exponential decay process. Our null model included repair time and pond type as fixed effects, with no interaction term. This allowed for genotypes from different pond types to have different starting points for the damage decay curves in Fig. 2A, B, but required that all genotypes have the same decay rate. Our second model allowed repair rate to depend on pond type, such that genotypes from high-UVR ponds could have a faster or slower decay rate than genotypes from low-UVR ponds (thus, the first model is strictly nested within the second). We compared the two models using a likelihood ratio test (χ^2 = 6.1096, P = 0.01345) and by comparing AIC values (Table 1; ΔAIC = 4.11). The significant difference between the two models indicates that *D. melanica* genotypes from the high-UVR pond repair CPDs faster than genotypes from the low-UVR pond in the presence of photorepair radiation.

We confirmed that the removal of CPDs following UV-B exposure was light-dependent by adding a treatment to Experiment 2 in which animals were kept in the dark following UV-B exposure, which we refer to as Experiment 3. For this 'dark repair' experiment, we used genotypes 1 and 2 (also used in Experiment 1 and the survival experiments described earlier). We chose these two genotypes to measure CPD removal in the absence of photorepair radiation in the same genotypes for which we had already measured survival under similar (dark) conditions (as shown in Fig. 1). The results of Experiment 3 indicate that very little repair of CPDs occurs in the dark, and the rate of dark repair does not differ between *D. melanica* genotypes (Fig. 2C).

As a first step towards uncovering the molecular mechanism responsible for the elevated rate of DNA photorepair in genotypes from high-UVR ponds, we measured expression of the *D. melanica* gene *photorepair*, which encodes the CPD photolyase enzyme responsible for the photoenzymatic repair of CPDs in DNA. We found similar *photorepair* expression among four *Daphnia* genotypes (two from the high-UVR pond and two from the low-UVR pond) in both the presence and absence of UVR (Fig. 3, $P > 0.68$ for all *F*-tests).

Discussion

We sought to identify the mechanistic underpinnings of previously documented differences in UVR tolerance among *D. melanica* populations from natural ponds that

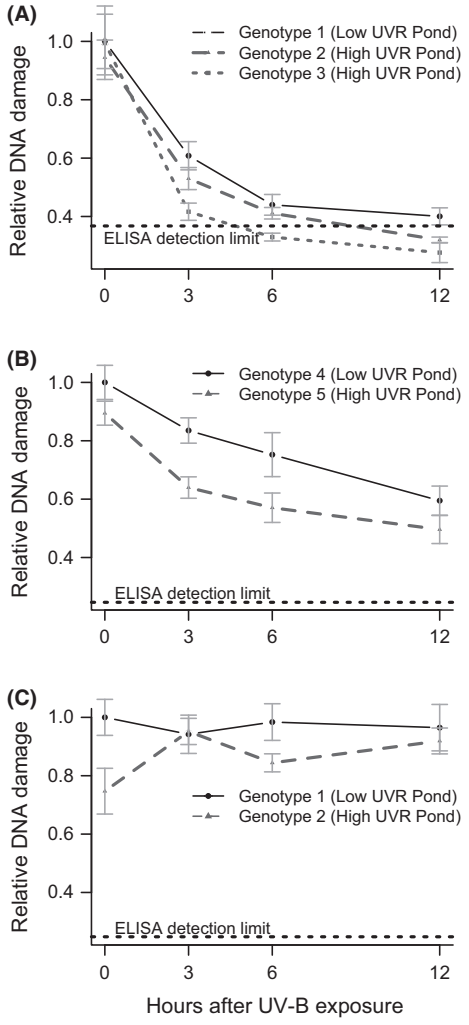

Fig. 2 The relative quantity (mean ± SEM) of UVR-caused CPDs in *Daphnia* DNA over 12 h following exposure to an acute dose of UV-B. Absorbance values from the ELISA for CPDs are normalized to the highest mean value in each panel for ease of presentation. (A) Experiment 1, organisms exposed to photorepair radiation (visible + UV-A); (B) Experiment 2, organisms exposed to photorepair radiation; (C) Experiment 3, organisms kept in the dark, preventing photorepair. Statistical analysis of these data is summarized in Table 1.

differ in UVR transparency (Miner & Kerr 2011). Three categories of zooplankton adaptations to reduce UVR-caused DNA damage exist: behavioural avoidance, photoprotective pigmentation and DNA repair (Hansson & Hylander 2009). Our previous work and our current findings allow us to rule out the first two of these strategies. Behavioural avoidance of UVR is insufficient to explain differences in UVR tolerance for animals from shallow ponds with high UVR transparency, especially because our tests of UVR tolerance did not provide a behavioural refuge. Pigmentation options for *Daphnia* include melanin, which is low in our study populations (Miner & Kerr 2011), carotenoids, which are also low in our study populations (Fig. S1, Supporting information), and perhaps MAAs, which in the present study we found to be entirely absent. Having ruled out behavioural and pigmentation strategies, we concentrated our study on the repair of UVR-caused DNA damage.

It is well known that the photoenzymatic repair pathway is important for the removal of UVR-caused DNA damage in many organisms, including multiple species of *Daphnia* (MacFadyen *et al*. 2004; Connelly *et al*. 2009). We took advantage of the light-dependence of this process by conducting UVR tolerance trials in the presence and absence of photorepair radiation. The survival advantage enjoyed by Genotype 2, from the high-UVR pond, disappeared when we removed photorepair radiation (Fig. 1), implying that a light-dependent mechanism such as DNA photorepair is involved. We therefore measured the rate of removal of UVR-caused CPD damage structures in DNA in five clonal *D. melanica* genotypes, and our results show that high-UVR pond genotypes repair CPDs significantly faster than low-UVR pond genotypes in the presence of photorepair radiation (Fig. 2A, B, Table 1), but not in the absence of photorepair radiation (Fig. 2C). We then asked whether expression of the *photorepair* gene that encodes the CPD photolyase responsible for CPD repair could explain differences in DNA repair rate among

Table 1 Linear mixed-effects models for DNA repair rate. The decrease in DNA damage over time is modelled as an exponential decay process, with ln(ELISA absorbance) as the response variable, and $n = 277$ observations. Both models contain random effects of genotype ID within pond type, and experimental block. A likelihood ratio test demonstrates that the models are significantly different ($\chi^2 = 6.1096$, $P = 0.01345$)

Model	Fixed effects	*k*	log-likelihood	AIC	ΔAIC
Repair rate depends on pond origin	Repair time, Pond origin, time × origin interaction	7	−101.93	217.87	0
Null	Repair time, Pond origin	6	−104.99	221.98	4.11

genotypes, but found gene expression to be similar in all genotypes, both in the presence and absence of UVR (Fig. 3).

Our primary finding here is that *D. melanica* genotypes differ in rates of light-dependent repair of UVR-caused DNA damage, highlighting a mechanism that may contribute to previously observed among-population differences in UVR tolerance. Our unbalanced design of measuring repair rates for different genotypes in different experiments (Fig. 2) is not ideal, and precludes any inference beyond the five genotypes studied here. Yet our in-depth assessment of DNA repair rates in a small number of clonal genotypes is not intended as a definitive demonstration that differences in DNA repair rates exist at the population level. A broader sampling of many more genotypes would be required to draw such a conclusion with certainty. Instead, we sought to evaluate the potential for significant variation in DNA repair rates among our five focal genotypes, whether any differences were light-dependent, and whether differences among genotypes correlated with the UVR threat in their native habitat. Our finding that genotypes from the high-UVR pond do indeed repair DNA faster than those from the nearby low-UVR pond, and via a photorepair mechanism, is a compelling demonstration of the evolutionary flexibility and adaptive importance of DNA repair. Although some studies have found variation in DNA repair efficiency among species (Karentz *et al.* 1991; Connelly *et al.* 2009), our findings demonstrate that variation in photorepair can exist within a single species. The match between DNA repair rate, UVR tolerance and the UVR threat in each genotype's native habitat draws an important connection between environment, fitness and molecular mechanism.

An intriguing unresolved issue in our study system is the identification of the specific molecular basis of elevated DNA photorepair. The CPD photorepair mechanism is quite simple at the molecular level, with only a single CPD photolyase gene and enzyme product rather than an elaborate protein pathway (Liu *et al.* 2015). This situation informs a small number of straightforward hypotheses for the molecular basis of elevated repair. In this study we were able to definitively reject the hypothesis that differences in CPD photorepair rate were due to differences in mRNA expression of the *photorepair* gene that encodes the CPD photolyase enzyme (Fig. 3). Although the *Daphnia pulex* genome (Colbourne *et al.* 2011) contains only one version of this gene, copy number variation could exist among genotypes (Eagle & Crease 2012) and may be worthy of investigation. However, our finding of similar expression of *photorepair* among genotypes does not suggest copy number variation as the most likely mechanism underlying differences in repair rate.

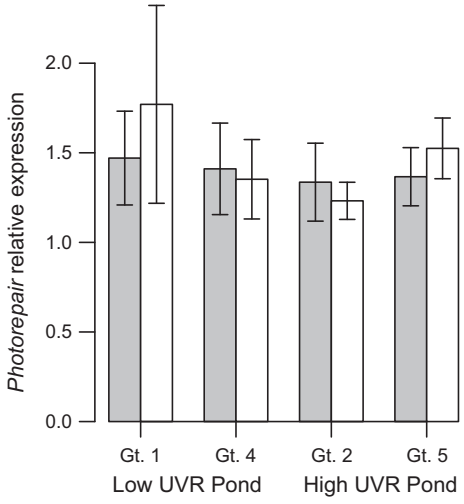

Fig. 3 Normalized *photorepair* expression (mean ± SEM) in the presence (shaded bars) and absence (open bars) of UVR, in four *D. melanica* clonal genotypes, two from the low-UVR pond and two from the high-UVR pond. 'Genotype' is abbreviated 'Gt'. and genotype identities correspond to those in earlier figures. *F*-tests for the effect of genotype, UVR treatment, and genotype × treatment interaction all resulted in *P* values >0.68, with $n = 54$ observations.

Several equally reasonable hypotheses remain to be investigated regarding the genetic basis of variation in CPD photorepair rate, which are not mutually exclusive. These include: (i) changes in the coding sequence of *photorepair* that alter the structure, function or longevity of the enzyme; (ii) availability or function of photolyase cofactors such as FAD; (iii) post-transcriptional processing of *photorepair* mRNA that affects the efficiency of the enzyme; (iv) *photorepair* expression differences that occur only at certain points within the circadian cycle, which we would have been unable to detect in the present study that measured expression at a single midday time point; and (v) differences in the expression of other genes, such as heat shock proteins, that may modulate efficiency of the photorepair process. A separate but related issue worthy of further investigation is the interaction between concentrations of dissolved organic matter (DOM) in the source ponds, UVR intensity, and reactive oxygen species (ROS) such as H_2O_2. High DOM concentrations reduce the amount of UVR to which zooplankton are exposed, but paradoxically may result in increased exposure to damaging ROS because of the photochemical reaction by which DOM is converted to H_2O_2 upon exposure to UV radiation (Richard *et al.* 2007). Any such investigation should prioritize measurement of *Daphnia* antioxidant enzyme

activity in addition to traits similar to those measured here.

Our *D. melanica* study populations from the Olympic mountains are closely related to populations in the Sierra Nevada (Miner *et al.* 2013) that have deep melanin pigmentation (Scoville & Pfrender 2010). This leads to the obvious question of why our Olympic *D. melanica* genotypes that experience higher levels of UVR in nature have adapted to this environment via a DNA repair pathway rather than the melanin pigmentation phenotype. At least two distinct possibilities exist: either the Olympic populations have always lacked the genetic variation necessary to induce concentrated melanin pigmentation in the carapace due to chance immigration and resulting founder effects, or the existing non-melanic genotypes with improved photoenzymatic repair have a selective advantage over melanic genotypes in even the clearest Olympic ponds. Both are reasonable hypotheses that warrant further investigation. The latter hypothesis is particularly appealing because melanin pigmentation carries a growth-rate cost in another *Daphnia* species (Hessen 1996). We do not know whether the elevated photorepair capacity of our high-UVR genotype carries an energetic cost relative to the low-UVR genotype, but the fact that high-UVR genotypes have not invaded nearby low-UVR ponds at our field site is consistent with a trade-off hypothesis.

Identifying the physiological and genetic mechanisms that underlie adaptive phenotypes in nature is fundamental to furthering understanding of how populations respond to natural selection. Identifying adaptive phenotypes is a necessary first step, but pushing down to the mechanistic details of such phenotypes can lead to novel insights about evolutionary process (e.g. Deng *et al.* 2010; Rosenblum *et al.* 2010). Adaptation in response to abiotic environmental stressors is particularly important given that the frequency and intensity of many environmental stressors will continue to change as a result of human activities. Studying natural populations that have adapted to existing gradients in abiotic stress, as we have done here, should inform expectations about future adaptive evolution in populations subject to changes in abiotic conditions due to global climate change. Exposure to UVR is a environmental stressor that must be included in this research agenda, given that summertime UVR levels in the mid-latitude regions may increase due to storm-related ozone depletion (Anderson *et al.* 2012), and recent observations of a dramatic and unprecedented increase in ozone depletion in the arctic (Manney *et al.* 2011) that may become frequent in coming decades (Sinnhuber *et al.* 2011). Our finding that differences can evolve among genotypes in the efficiency of an enzymatic pathway widely shared across the tree of life – photorepair of UVR-caused DNA damage – highlights the importance of understanding the organismal mechanisms that underlie adaptations to environmental challenges in nature.

Acknowledgements

We thank R. Sommaruga for assaying samples for the presence of MAAs, M. Badger and L. Schaffner for assistance with *Daphnia* care and experiments, F. Taub and R. Raguso for loans of UV radiometers, and H. Lindsey and L. Schaffner for invaluable laboratory support. We also thank five anonymous reviewers for valuable suggestions provided during the revision process, which improved the quality and presentation of this study. This work was supported by an NSF Doctoral Dissertation Improvement Grant (DEB-0808558), an NSF Graduate Research Fellowship, and a W. T. Edmondson award from the UW Biology Department (all to B.E.M.), a Cornell Biology Research Fellowship funded by the NSF (DBI-0933921) to P.M.K., and the BEACON Center for the Study of Evolution in Action under NSF Cooperative Agreement DBI-0939454.

References

Anderson JG, Wilmouth DM, Smith JB, Sayres DS (2012) UV dosage levels in summer: increased risk of ozone loss from convectively injected water vapor. *Science*, **337**, 835–839.

Balseiro E, Souza MS, Modenutti B, Reissig M (2008) Living in transparent lakes: low food P: C ratio decreases antioxidant response to ultraviolet radiation in daphnia. *Limnology and Oceanography*, **53**, 2383–2390.

Bell G, Collins S (2008) Adaptation, extinction and global change. *Evolutionary Applications*, **1**, 3–16.

Borgeraas J, Hessen DO (2002) Variations of antioxidant enzymes in Daphnia species and populations as related to ambient UV exposure. *Hydrobiologia*, **477**, 15–30.

Bottrell H, Duncan A, Gliwicz Z *et al.* (1976) A review of some problems in zooplankton production studies. *Norwegian Journal of Zoology*, **24**, 419–456.

Boyd JB, Harris PV (1987) Isolation and characterization of a photorepair-deficient mutant in Drosophila melanogaster. *Genetics*, **116**, 233–239.

Cadet J, Sage E, Douki T (2005) Ultraviolet radiation-mediated damage to cellular DNA. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **571**, 3–17.

Carlson SM, Cunningham CJ, Westley PAH (2014) Evolutionary rescue in a changing world. *Trends in Ecology & Evolution*, **29**, 521–530.

Celis-Salgado MP, Cairns A, Kim N, Yan ND (2008) The FLAMES medium: a new, soft-water culture and bioassay medium for Cladocera. *Vereinigung Internationale Verhandlungen Limnologie*, **30**, 265–271.

Colbourne JK, Pfrender ME, Gilbert D, *et al.* (2011) The Ecoresponsive Genome of Daphnia pulex. *Science*, **331**, 555–561.

Connelly SJ, Moeller RE, Sanchez G, Mitchell DL (2009) Temperature effects on survival and DNA repair in four freshwater cladoceran Daphnia species exposed to UV radiation. *Photochemistry And Photobiology*, **85**, 144–152.

Cristescu MEA, Colbourne JK, Radivojc J, Lynch M (2006) A microsatellite-based genetic linkage map of the waterflea,

Daphnia pulex: on the prospect of crustacean genomics. *Genomics*, **88**, 415–430.
Deng C, Cheng C-HC, Ye H, He X, Chen L (2010) Evolution of an antifreeze protein by neofunctionalization under escape from adaptive conflict. *Proceedings of the National Academy of Sciences*, **107**, 21593–21598.
Eagle SH, Crease TJ (2012) Copy number variation of ribosomal DNA and Pokey transposons in natural populations of Daphnia. *Mobile DNA*, **3**, 4.
Ellison MJ, Childs JD (1981) Pyrimidine dimers induced in *Escherichia coli* DNA by ultraviolet radiation present in sunlight. *Photochemistry and Photobiology*, **34**, 465–469.
Grad G, Williamson CE, Karapelou DM (2001) Zooplankton survival and reproduction responses to damaging UV radiation: a test of reciprocity and photoenzymatic repair. *Limnology And Oceanography*, **46**, 584–591.
Grad G, Burnett BJ, Williamson CE (2003) UV damage and photoreactivation: timing and age are everything. *Photochemistry And Photobiology*, **78**, 225–227.
Green J (1957) Carotenoids in Daphnia. *Proceedings of the Royal Society of London B: Biological Sciences*, **147**, 392–401.
Hairston N (1979) Relationship between pigmentation and reproduction in 2 species of Diaptomus (Copepoda). *Limnology And Oceanography*, **24**, 38–44.
Hansson LA, Hylander S (2009) Effects of ultraviolet radiation on pigmentation, photoenzymatic repair, behavior, and community ecology of zooplankton. *Photochemical & Photobiological Sciences*, **8**, 1266–1275.
Hansson LA, Hylander S, Sommaruga R (2007) Escape from UV threats in zooplankton: a cocktail of behavior and protective pigmentation. *Ecology*, **88**, 1932–1939.
Hebert PDN, Emery CJ (1990) The adaptive significance of cuticular pigmentation in Daphnia. *Functional Ecology*, **4**, 703–710.
Heelis PF, Kim S-T, Okamura T, Sancar A (1993) New trends in photobiology: the photo repair of pyrimidine dimers by DNA photolyase and model systems. *Journal of Photochemistry and Photobiology B: Biology*, **17**, 219–228.
Hessen DO (1993) DNA-damage and pigmentation in alpine and arctic zooplankton as bioindicators of UV-radiation. *Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie*, **25**, 482–486.
Hessen DO (1996) Competitive trade-off strategies in Arctic Daphnia linked to melanism and UV-B stress. *Polar Biology*, **16**, 573–579.
Hessen DO, Borgeraas J, Orbaek JB (2002) Responses in pigmentation and anti-oxidant expression in Arctic Daphnia along gradients of DOC and UV exposure. *Journal of Plankton Research*, **24**, 1009–1017.
Hoffmann AA, Sgrò CM (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–485.
Karentz D, Cleaver JE, Mitchell DL (1991) Cell survival characteristics and molecular responses of antarctic phytoplankton to ultraviolet-B radiation1. *Journal of Phycology*, **27**, 326–341.
Kiontke S, Geisselbrecht Y, Pokorny R *et al.* (2011) Crystal structures of an archaeal class II DNA photolyase and its complex with UV-damaged duplex DNA. *The EMBO Journal*, **30**, 4437–4449.
Kittler L, Löber G (1977) Photochemistry of the Nucleic Acids. In: *Photochemical and Photobiological Reviews* (ed. Smith KC), pp. 39–131. Plenum Press, New York, USA.
Leech DM, Padeletti A, Williamson CE (2005) Zooplankton behavioral responses to solar UV radiation vary within and among lakes. *Journal Of Plankton Research*, **27**, 461–471.
Liu Z, Wang L, Zhong D (2015) Dynamics and mechanisms of DNA repair by photolyase. *Physical Chemistry Chemical Physics*, **17**, 11933–11949.
MacColl ADC (2011) The ecological causes of evolution. *Trends in Ecology & Evolution*, **26**, 514–522.
MacFadyen EJ, Williamson CE, Grad G *et al.* (2004) Molecular response to climate change: temperature dependence of UV-induced DNA damage and repair in the freshwater crustacean Daphnia pulicaria. *Global Change Biology*, **10**, 408–416.
Malloy KD, Holman MA, Mitchell D, Detrich HW (1997) Solar UVB-induced DNA damage and photoenzymatic DNA repair in antarctic zooplankton. *Proceedings of the National Academy of Sciences*, **94**, 1258–1263.
Manney GL, Santee ML, Rex M *et al.* (2011) Unprecedented Arctic ozone loss in 2011. *Nature*, **478**, 469–475.
Miner BE, Kerr B (2011) Adaptation to local ultraviolet radiation conditions among neighbouring Daphnia populations. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 1306–1313.
Miner BE, Knapp RA, Colbourne JK, Pfrender ME (2013) Evolutionary history of alpine and subalpine Daphnia in western North America. *Freshwater Biology*, **58**, 1512–1522.
Moeller RE, Gilroy S, Williamson CE, Grad G, Sommaruga R (2005) Dietary acquisition of photoprotective compounds (mycosporine-like amino acids, carotenoids) and acclimation to ultraviolet radiation in a freshwater copepod. *Limnology And Oceanography*, **50**, 427–439.
Mori T, Nakane M, Hattori T *et al.* (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. *Photochemistry And Photobiology*, **54**, 225–232.
Palen WJ, Schindler DE, Adams MJ *et al.* (2002) Optical characteristics of natural waters protect amphibians from UV-B in the US Pacific Northwest. *Ecology*, **83**, 2951–2957.
Pfeifer GP, You Y-H, Besaratinia A (2005) Mutations induced by ultraviolet light. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **571**, 19–31.
R Core Team (2014) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
Ramos-Jiliberto R, Dauelsberg P, Zúñiga LR (2004) Differential tolerance to ultraviolet-B light and photoenzymatic repair in cladocerans from a Chilean lake. *Marine and Freshwater Research*, **55**, 193–200.
Richard LE, Peake BM, Rusak SA, Cooper WJ, Burritt DJ (2007) Production and decomposition dynamics of hydrogen peroxide in freshwater. *Environmental Chemistry*, **4**, 49–54.
Rosenblum EB, Römpler H, Schöneberg T, Hoekstra HE (2010) Molecular and functional basis of phenotypic convergence in white lizards at White Sands. *Proceedings of the National Academy of Sciences*, **107**, 2113–2117.
Scoville AG, Pfrender ME (2010) Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 4260–4263.

Sinha RP, Häder D-P (2002) UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences*, **1**, 225–236.

Sinnhuber B-M, Stiller G, Ruhnke R *et al.* (2011) Arctic winter 2010/2011 at the brink of an ozone hole. *Geophysical Research Letters*, **38**, L24814.

Sommaruga R (2010) Preferential accumulation of carotenoids rather than of mycosporine-like amino acids in copepods from high altitude Himalayan lakes. *Hydrobiologia*, **648**, 143–156.

Sommaruga R, Garcia-Pichel F (1999) UV-absorbing mycosporine-like compounds in planktonic and benthic organisms from a high-mountain lake. *Archiv für Hydrobiologie*, **144**, 255–269.

Sutherland BM (1981) Photoreactivation. *BioScience*, **31**, 439–444.

Tartarotti B, Saul N, Chakrabarti S *et al.* (2014) UV-induced DNA damage in Cyclops abyssorum tatricus populations from clear and turbid alpine lakes. *Journal of Plankton Research*, **36**, 557–566.

Thoma F (1999) Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *The EMBO Journal*, **18**, 6585–6598.

Williamson CE, Neale PJ, Grad G, De Lange HJ, Hargreaves BR (2001) Beneficial and detrimental effects of UV on aquatic organisms: implications of spectral variation. *Ecological Applications*, **11**, 1843–1857.

Williamson CE, Grad G, De Lange HJ, Gilroy S, Karapelou DM (2002) Temperature-dependent ultraviolet responses in zooplankton: implications of climate change. *Limnology And Oceanography*, **47**, 1844–1848.

Williamson CE, Fischer JM, Bollens SM, Overholt EP, Breckenridge JK (2011) Towards a more comprehensive theory of zooplankton diel vertical migration: integrating ultraviolet radiation and water transparency into the biotic paradigm. *Limnology and Oceanography*, **56**, 1603–1623.

Zagarese HE, Feldman M, Williamson CE (1997) UV-B-induced damage and photoreactivation in three species of Boeckella (Copepoda, Calanoida). *Journal of Plankton Research*, **19**, 357–367.

B.E.M. and B.K. designed the study. B.E.M. collected the genotypes, measured carotenoid concentration, conducted the survival and DNA damage experiments and performed the DNA damage assays. K.D.B. developed the DNA damage ELISA protocol. P.M.K. and B.E.M. conducted the gene expression experiments and qPCRs. B.E.M. analysed the data and wrote the manuscript, with contributions and revisions to the manuscript from all coauthors.

Data accessibility

The following data are available from Dryad Digital Repository (doi:10.5061/dryad.k78f6): (i) GPS coordinates of genotype collection locations, (ii) raw carotenoid content values used to generate Fig. S1 (Supporting information), (iii) survival under laboratory UVR data, (iv) raw ELISA absorbance values from DNA damage assays and (v) raw qPCR data (Ct values) for *photorepair* and all reference genes.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Carotenoid concentration in field-collected animals from four study ponds, including the two (C and K) from which the genotypes used in this study were collected.