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Author for correspondence:
Brooks E. Miner
e-mail: bminer@ithaca.edu

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Melanism protects alpine zooplankton from DNA damage caused by ultraviolet radiation

Cynthia K. S. Ulbing, Julia M. Muuse and Brooks E. Miner

Department of Biology, Ithaca College, 953 Danby Rd., Ithaca, NY 14850, USA

BEM, 0000-0002-6512-6582

Melanism is widely observed among animals, and is adaptive in various contexts for its thermoregulatory, camouflaging, mate-attraction or photoprotective properties. Many organisms exposed to ultraviolet radiation show increased fitness resulting from melanin pigmentation; this has been assumed to result in part from reduced UV-induced damage to DNA. However, to effectively test the hypothesis that melanin pigmentation reduces UV-induced DNA damage requires quantification of UV-specific DNA damage lesions following UV exposure under controlled conditions using individuals that vary in pigmentation intensity. We accomplished this using alpine genotypes of the freshwater microcrustacean *Daphnia melanica*, for which we quantified cyclobutane pyrimide dimers in DNA, a damage structure that can only be generated by UV exposure. For genotypes with carapace melanin pigmentation, we found that individuals with greater melanin content sustained lower levels of UV-induced DNA damage. Individuals with more melanin were also more likely to survive exposure to ecologically relevant levels of UV-B radiation. Parallel experiments with conspecific genotypes that lack carapace melanin pigmentation provide additional support for our conclusion that melanism protects individuals from UV-induced DNA damage. Finally, within-genotype comparisons with asexually produced clonal siblings demonstrate that melanin content influences DNA damage even among genetically identical individuals raised in the same environment.

1. Introduction

Pigmentation and melanism have long interested evolutionary biologists as potential adaptations in animals resulting from mechanisms as diverse as thermoregulation, crypsis/camouflage, mate attraction, and photoprotection [1]. The causes and consequences of melanism—the existence of visibly dark-coloured individuals within a species—have been investigated in a wide array of animals, including crustaceans [2–4], insects [5–7], amphibians [8], reptiles [9,10], terrestrial mammals [11–13] and whales [14]. In many cases, pigmentation levels in nature display positive correlations with environmental exposure to ultraviolet radiation (UVR) [7,8,10,14], suggesting that melanin protects organisms from damage that would result from UVR exposure. For example, populations of *Drosophila* in sub-Saharan Africa exhibit both latitudinal and elevational clines in pigmentation that correspond with ambient UVR levels [7], and blue whales increase melanin pigmentation on a seasonal basis that correlates with increasing UVR exposure [14]. These observations should inspire explorations of the underlying mechanisms, such as experimental tests of the hypothesis that melanin pigmentation reduces UVR-induced DNA damage.

To convincingly demonstrate that melanin pigmentation reduces DNA damage resulting from UVR exposure, a study must meet three minimal criteria: (1) variation among individuals in the quantity of melanin pigmentation; (2) exposure to UVR under controlled conditions to ensure that all individuals receive the same dose of UVR in the same environment; and (3) accurate quantification of a DNA damage structure that can only be generated by UVR exposure.

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For non-human animals, the mechanistic link between melanin and UVR-induced DNA damage has not previously been investigated in a manner that satisfies these three criteria. Evidence from humans suggests that exploration in other animals is warranted: a wide array of studies of human skin has generated conflicting results regarding the photoprotective properties of melanin pigmentation (reviewed in [15]). Recent examination of extreme phenotypes in humans (very dark versus very light skin types) exposed to UV radiation demonstrated an eightfold reduction in DNA damage resulting from melanin in dark skin [16]. These findings should inform hypotheses about non-human animals, but researchers should be wary of assuming a similar melanin–DNA damage relationship in other animals.

Two existing studies each meet two of the three criteria outlined above for testing the hypothesis that melanin reduces UVR-induced DNA damage. Hu *et al*. [17] showed that pigmented mutants of *Bombyx mori* larvae exposed to UV-A radiation had lower levels of a compound that is indicative of oxidative damage. This study satisfies the first two criteria (melanin variation and controlled UVR exposure), but the measured response related to oxidative damage, not DNA damage. In contrast, Martinez-Levasseur *et al*. [14] measured mitochondrial DNA damage in free-living blue whales and found that individuals with higher melanin had less damage. This study meets criterion 1 (melanin variation) and approaches criterion 3 (quantification of UVR-induced DNA damage), but could not ensure uniform UVR exposure under controlled conditions (criterion 2) for obvious reasons. In addition, quantification of DNA damage in this study was not specific to UVR-induced damage lesions [14], a requirement of criterion 3. To our knowledge, no existing study in non-human animals satisfies all three criteria, despite the widespread assumption that melanism is photoprotective against DNA damage for animals in high-UVR habitats (e.g. [1,18,19]).

For aquatic organisms such as zooplankton that live in pelagic (open water) habitats, spectral properties of the water they inhabit can dramatically affect the underwater UVR environment they experience. In alpine regions, underwater UVR levels can be high for two reasons: erythemal UV irradiance increases 18% with each 1000 m increase in elevation [20], and aquatic habitats in truly alpine regions have high UVR transparency due to low DOM concentrations [21] resulting from the absence of terrestrial vegetation. In high-UVR habitats such as alpine or arctic regions, species of the freshwater microcrustacean genus *Daphnia* often display intense melanin pigmentation [4,22–24]. Observations that melanic *Daphnia* are typically only found in high-UVR habitats and that melanin pigmentation is plastically upregulated in response to UVR exposure [3,25–27] suggest that melanin pigmentation is both costly to the organism and provides fitness benefits in the presence of UVR that are sufficient to outweigh these costs.

Despite numerous demonstrations that melanin pigmentation provides a survival benefit under UVR in multiple *Daphnia* species [2,4,23,25], two critical gaps in knowledge exist. First, tests of the benefit of melanism under UVR have previously treated pigmentation as a binary trait by comparing pigmented and transparent individuals [2,4,23,25], rather than as a quantitative trait, despite the fact that organisms exist in nature with a wide range of pigmentation intensities. Second, and more importantly, we lack information on the specific mechanism by which melanin pigmentation confers a fitness benefit, whether it be protection against UVR-induced DNA damage or something less direct. Shorter wavelengths within the ultraviolet spectrum (i.e. UV-B) are most harmful, as they induce lesions in DNA that interfere with replication and transcription [28,29]. UV-B and longer-wavelength UV-A also generate reactive oxygen species (ROS) within organismal tissues [29] and in the ambient aquatic environment [30]. Such ROS can then induce oxidative damage to proteins, lipids and nucleic acids [29–31]. Many authors have hypothesized that melanin pigmentation protects genomic DNA against direct damage caused by UV-B exposure and/or oxidative damage resulting from ROS formation, but such hypotheses have not yet been tested in any non-human animal.

Here we present the results of laboratory experiments designed to answer the following three questions. (1) Does melanin pigmentation protect against DNA damage lesions induced by exposure to UV-B radiation? (2) Does melanin pigmentation provide a survival benefit under UV-B exposure? (3) Do higher levels of pigmentation provide increased protection against DNA damage and/or increased survival benefits? Our study design meets all three of the criteria outlined earlier (melanin variation, UVR exposure under controlled conditions and quantification of UVR-induced DNA damage lesions). We hypothesize that melanin pigmentation reduces UVR-induced DNA damage and improves survival under UVR, and that the benefits conferred by pigmentation correlate directly with the melanin content of each individual. We quantify UVR-induced DNA damage by measuring cyclobutane pyrimidine dimers (CPDs) in *Daphnia* DNA. CPDs are the most prevalent form of DNA damage resulting from UVR exposure [28] and have previously been used to assess the repair of DNA damage in *Daphnia* [32–34]. In addition to comparisons among genotypes that differ in melanin content, we take advantage of the fact that carapace pigmentation is lower directly following moulting to conduct within-genotype comparisons among clonal siblings that vary in melanin content. Our finding that genetically identical individuals that differ in carapace melanin pigmentation (due to moult timing) differ in susceptibility to DNA damage provides a powerful demonstration of the mechanistic underpinnings of the fitness benefits of this important UVR-tolerance phenotype.

2. Material and methods

(a) *Daphnia* provenance and rearing conditions

We used laboratory-reared genotypes of *Daphnia melanica* descended from individuals collected from the Sierra Nevada of California (hereafter 'Sierra' genotypes) at elevations ranging from 3460 m to 3537 m, and from Olympic National Park in Washington state (hereafter 'Olympic' genotypes) at elevations ranging from 1276 m to 1463 m. Sierra genotypes were collected in August 2016 from Cony (37.251159° N, −118.690045° W) Grouse (37.24922° N, −118.68526° W), Pipit (37.24662° N, −118.68741° W) and Wahoo 3 (37.22693° N, −118.71400° W). Olympic genotypes were collected in September 2015 from four ponds (47.92192° N, −123.77994° W; 47.92213° N, −123.77790° W; 47.91184° N, −123.77223° W; 47.91004° N, −123.76803° W). Only Sierra genotypes possess the carapace pigmentation phenotype; all Olympic populations, which live at much lower elevations than Sierra populations, have non-melanized carapaces [35]. We have previously demonstrated that Olympic *D. melanica* do not possess detectable levels of carotenoids or MAAs [32], consistent with previous findings in other *Daphnia* species [36].

In our DNA damage experiment, we used eight genotypes in total: four Sierra and four Olympic. For our UVR tolerance experiment, we used only the four Sierra genotypes. Each genotype was maintained as an asexually reproducing population founded by a single female collected in nature. Each of the eight genotypes was collected from a different body of water, and all have unique multilocus genotypes assessed at four microsatellite loci (electronic supplementary material, table S1). We estimate that all genotypes had undergone at least 13 generations of asexual reproduction under controlled laboratory conditions before we conducted our experiment (preceding our first experimental trials, Sierra genotypes had been kept in the laboratory for roughly 6 months, or approx. 13–18 generations, whereas Olympic genotypes had been in the laboratory for around 18 months, or approx. 39–54 generations). While these *Daphnia* genotypes were kept in our laboratory, and during the experiments described below, we raised the animals in FLAMES medium [37] at 18–22°C under 16 : 8 Light : Dark photoperiod, and fed *Cryptomonas ozolini* (UTEX LB 2194; hereafter *Cryptomonas*) every 2–3 days. This alga is a highly nutritious food source rich in fatty acids, which in all of our experiments was provided in excess. As a result, animals in our experiments experienced optimal nutritional conditions and were not resource limited.

(b) Estimation of melanin concentration from photographs

Because melanin quantification requires digestion of the organism in high-molarity NaOH, we could not measure both melanin content and DNA damage in the same individuals. Instead, we estimated melanin concentration from photographs under standardized lighting and magnification. We photographed all individuals from a lateral perspective (animals laid on their side), including the reference cohorts used for standard curves, under dissecting microscopes at 20× magnification using identical lighting, exposure, aperture and white balance conditions using an S01-0801B camera (Science Supply, Schertz, TX). For the DNA damage experiment, we used an M5A stereomicroscope (Wild Heerbrugg AG, Switzerland) under brightfield transmitted illumination from an incandescent lamp, with the camera mounted in place of one of the ocular lenses. For the UVR tolerance experiment, we used a Nikon SMZ-745T stereomicroscope (Nikon Instruments, Melville, NY, USA) under brightfield transmitted illumination from an LED light source (Nikon C-LEDS Hybrid LED Stand), with the camera mounted to the phototube. We then used a python script to convert photographs to greyscale and calculated the mean pixel grey value (scale = 0–255) of all non-white pixels in the image (white and near-white pixels that comprised the background of the image were removed by excluding all pixels with grey values greater than 245). For ease of presentation, mean grey values were then inverted ($255 - x$) so that larger values represent darker individuals.

For both experiments, we created standard curves based upon sets of reference individuals ($n = 48$ or $n = 59$) for which we quantified melanin via extraction after photographing the individuals. We quantified melanin using established methods [2,27] by homogenizing individuals in 100 µl 5 M NaOH with a plastic pestle followed by incubation at 40°C for 72 h. We then measured absorbance at 350 nm of the supernatant using a plate reader (Infinite M200 PRO NanoQuant, Tecan, Männedorf, Switzerland), along with a dilution series of known concentrations of synthetic melanin (Sigma-Aldrich M8631) that we used to convert absorbance at 350 nm to µg melanin per individual. We then divided µg melanin by individual body length (measured from the base of the tailspine to the top of the carapace) to calculate individual melanin concentration (µg mm^{-1} body length). We chose these units to be consistent with the existing literature [27,32]. We then used assayed melanin concentrations and mean pixel grey values from these reference animals to establish standard curves to convert mean pixel grey values (from photographs) to inferred melanin concentration for animals used in experiments described below.

(c) Experimental UVR exposure

We conducted all exposures to UVR in a 'UV phototron' apparatus based upon the design of Williamson *et al*. [38] but with visible and UV-A illumination provided from the sides rather than from below (as in [32,35]). In this apparatus, we placed lidless glass containers containing *Daphnia* on the outer edge of a rotating wheel inside a growth chamber (I36-VL, Percival Scientific) at 15°C. The rotating wheel ensured uniform exposure to UV-B, UV-A and visible radiation over time, an essential feature of the UV phototron design. Lamp specifications were as follows: visible light, cool white fluorescent 4 ft lamps; UV-A radiation, Q-Panel UVA-340 lamps (4 ft) (Q-LAB, Westlake, OH); UV-B radiation, XX-15B lamp (Spectronics Corp.) suspended 24 cm above the rotating wheel. UV-B illumination was provided from above to ensure exposure, as the containers did not have lids. We measured total UV-B and UV-A exposure in all experimental trials with UV-3702-1 (UV-B; not erythema-weighted) and UV-3701-1 (UV-A) radiometric detectors connected to an X1-1 optometer (Gigahertz Optik, Türkenfeld, Germany). We verified that UV-A radiation provided from the sides transmitted through the glass containers that contained the *Daphnia*.

(d) DNA damage experiment

We raised individuals from birth to adulthood under standard laboratory conditions (three to five weeks) in cohorts born within a 7-day period. All individuals used in the DNA damage experiment were carrying subitaneous eggs of either the first or second clutch for that individual. We placed 100 ml Pyrex Vista tall-form beakers containing 10 individual *Daphnia* and 80 ml of FLAMES medium on the outer edge of the rotating wheel in the UV phototron apparatus. To induce DNA damage in the form of cyclobutane pyrimidine dimers (CPDs), we exposed individuals to an acute dose of UV-B radiation for one hour. Unlike the UV-B tolerance experiment described below, the acute UV-B exposure used here exceeded what organisms would experience in nature. This was necessary to generate sufficient quantities of CPDs to detect with our ELISA method. UV-B radiation was supplied by three XX-15B lamps; additional lighting conditions were as described above. Total UV-B dosage during the 1 h exposure was 23.6–27.4 kJ m^{-2} (dose rate: 6.56–7.61 W m^{-2}). We implemented this experimental design with four separate cohorts of organisms, representing four experimental blocks. Each block contained replicates of all eight genotypes, and we used multiple blocks to accommodate our target sample size. Immediately following the UVR exposure, we froze organisms in individual tubes at −20°C.

To quantify CPDs in *Daphnia* DNA, we first extracted genomic DNA using a 96-well plate extraction kit (Agencourt Genfind v. 2, Beckman Coulter Life Sciences). Individuals were crushed with plastic pestles in 100 µl of lysis buffer, then transferred into a round-bottom 96-well plate. Proteinase K (1.5 µl) was added to each well and samples incubated at room temperature for 30 min. Next, 50 µl of binding buffer was distributed to each well and the sample was incubated at room temperature for ten minutes. The 96-well plate was then placed onto a magnetic plate (Agencourt SPRIPlate 96R, Beckman Coulter Life Sciences), and the supernatant was removed while the binding buffer and DNA remained. A similar process was repeated for four wash steps before DNA was eluted in nanopure H_2O and frozen at −20°C.

Following genomic DNA extractions, we quantified CPDs ($n = 164$ individuals) via ELISA [32]. We coated 96-well flat-bottom polystyrene plates with protamine sulfate by adding 50 µl of 0.003% protamine sulfate solution per well and incubating

overnight at 37°C, followed by three rinses with 100 µl distilled water. We then thawed genomic DNA samples and quantified DNA concentrations using a Qubit 3.0 Fluorometer (ThermoFisher Scientific). From each sample's measured concentration, we calculated the volume to dilute each DNA sample to exactly 0.2 ng μl^{-1} in PBS. We added 50 µl of each sample (exactly 10 ng DNA) to wells of the coated plates, then denatured DNA by heating at 95°C for 10 min followed by chilling in an ice bath for 15 min, then incubated plates overnight at 37°C to bind DNA to the wells. Next, we performed a series of incubation steps for 30 min at 37°C, followed by five washes with 150 µl per well of PBS-T (0.05% Tween-20 in PBS). Incubations were performed as follows: (1) 150 µl per well 2% FBS in PBS, (2) 100 µl per well TDM-2 antibody (Cosmo Bio USA, [39]) diluted 1 : 2000 in PBS, (3) 100 µl per well Biotin-XX F(ab')2 fragment of anti-mouse IgG (H + L) (Life Technologies) diluted 1 : 35 000 in PBS, (4) 100 µl per well Pierce High Sensitivity Streptavidin-HRP (ThermoFisher Scientific) diluted 1 :25 000 in PBS. Following these steps, we added 150 µl per well citrate-phosphate buffer (24 mM citric acid, 41 mM sodium phosphate, pH 5.0), then discarded and immediately followed with 100 µl substrate solution (1-Step Turbo TMB-ELISA, Thermo Scientific). After 30 min incubation at room temperature (21° ± 1°C), we stopped the reaction by adding 50 µl per well 2 M H_2SO_4. We then measured absorbance at 450 nm using a plate reader (Infinite M200 PRO NanoQuant, Tecan, Männedorf, Switzerland). Absorbance values served as our measure of DNA damage for each individual *Daphnia*, as they correlates directly with the quantity of CPDs in the extracted DNA [32].

(e) UV-B tolerance experiment

We quantified survival following a 12 h UV-B exposure for the four Sierra genotypes, all of which have pigmented carapaces. While the earlier DNA damage experiment was designed to quickly generate substantial quantities of CPDs, the goal of the UV-B tolerance experiment was to evaluate survival following ecologically relevant UV-B exposure. Hence the former provided a high UV-B dose in a short time (1 h), while the latter provided a lower UV-B dose spread out over a full day (12 h). As with the earlier experiment, we raised animals from birth to maturity under the same standardized conditions. Shortly after individuals reached sexual maturity, they were placed in individual 60 ml glass jars and fed excess *Cryptomonas* algae for 3–5 days preceding UVR exposure. On the day before UVR exposure began, we photographed each individual to estimate melanin concentration. We are confident that for most animals, the carapace photographed was the same one the animals possessed during UV-B exposure on the following day, with the exception of the small number of animals that moulted during the intervening hours. UVR exposure occurred in the same UV phototron apparatus with UV-B radiation provided by a single XX-15B lamp illuminated over 12 h (within a 16 h day consisting of visible and UV-A radiation as described above). Across six experimental blocks (each of which contained replicates of all four genotypes), the mean UV-B dose was 42.0 kJ m^{-2} (range: 39.4–45.7 kJ m^{-2}) and mean UV-B dose-rate was 0.97 W m^{-2} (range: 0.91–1.10 W m^{-2}). Following UV-B exposure, we left individuals in the same jars on the rotating wheel of the phototron for six days in the continued presence of UV-A and visible radiation only (16 h day length). Feeding on the days following UV-B exposure matched our standard rearing conditions. On day 6 following UV-B exposure, we measured survival by recording each individual as alive or dead. We defined 'dead' as the complete lack of movement of swimming antennae in response to gentle disruption.

(f) Statistical analyses

We carried out statistical analyses using R v. 3.5.1 [40]. For all statistical tests that assume a normal error distribution, we tested response variables for normality. To evaluate the effectiveness of using photographs of individual *Daphnia* to quantify melanin concentration, we used least-squares linear regressions, with mean pixel grey value as the predictor variable and assayed melanin concentration (µg mm^{-1} body length) as the response.

For the DNA damage experiment, we tested for an overall difference in inferred melanin concentration between Sierra and Olympic animals using a nonparametric Mann–Whitney *U*-test because the melanin response variable was not normally distributed when data from both regions were analysed together. To test for within-region differences in melanin among genotypes, we used one-way ANOVAs with genotype as a fixed effect (the melanin response variable was normally distributed within regions, so nonparametric tests were not necessary here). For all analyses of DNA damage data, we analysed Sierra and Olympic genotypes separately because the populations differ in the presence/absence of carapace melanin pigmentation. We constructed linear models with DNA damage (ELISA absorbance) as the response variable and inferred melanin concentration, genotype and a melanin-by-genotype interaction as predictors; *F*-tests among nested models evaluated statistical significance. We also calculated coefficients of variation for DNA damage for the two regions.

For the UV-B tolerance experiment, we used one-way ANOVA to compare melanin concentration among genotypes, followed by a *post hoc* Tukey's HSD test. To analyse survival following UV-B exposure, we used logistic regression (generalized linear model with logit link); predictor variables included inferred melanin concentration and genotype. We tested the statistical significance of competing nested models with likelihood ratio (LR) tests. We followed this with genotype-specific survival models; this resulted in four separate logistic regressions, for which we tested the significance of melanin as a predictor variable using LR tests against null models. We controlled for false discovery rate among these tests following the procedure of [41].

3. Results

(a) Melanin estimation from photographs

We used different photography methods for organisms in the DNA damage and UVR tolerance experiments because we improved our methodology and throughput for the latter. With both methods, photographs allowed us to effectively infer melanin concentration for reference cohorts from which we extracted and assayed melanin. For photography methods used in the DNA damage experiment (figure 1*a*) and in the UVR tolerance experiment (figure 1*b*), mean pixel grey values calculated from photographs were significant predictors of melanin concentration assayed via extraction (figure 1*a*, $p < 0.0001$; figure 1*b*, $p < 0.0001$). For animals used in the experiments described below, we used the equations from these linear regressions to convert photograph mean pixel grey values to inferred melanin concentrations.

(b) DNA damage experiment

Genotypes from the Sierra Nevada region in California (hereafter 'Sierra') have visible carapace melanin pigmentation, whereas genotypes from the Olympic Mountains of Washington (hereafter 'Olympic') do not. Sierra animals had higher melanin than Olympic animals (Mann–Whitney $U = 372$, $p \ll 0.0001$). Within each region, inferred melanin concentration differed significantly among the four genotypes used from each region (Sierra: ANOVA $F_{3,83} = 4.382$, $p = 0.0065$; Olympic: ANOVA $F_{3,74} = 2.985$, $p = 0.037$).

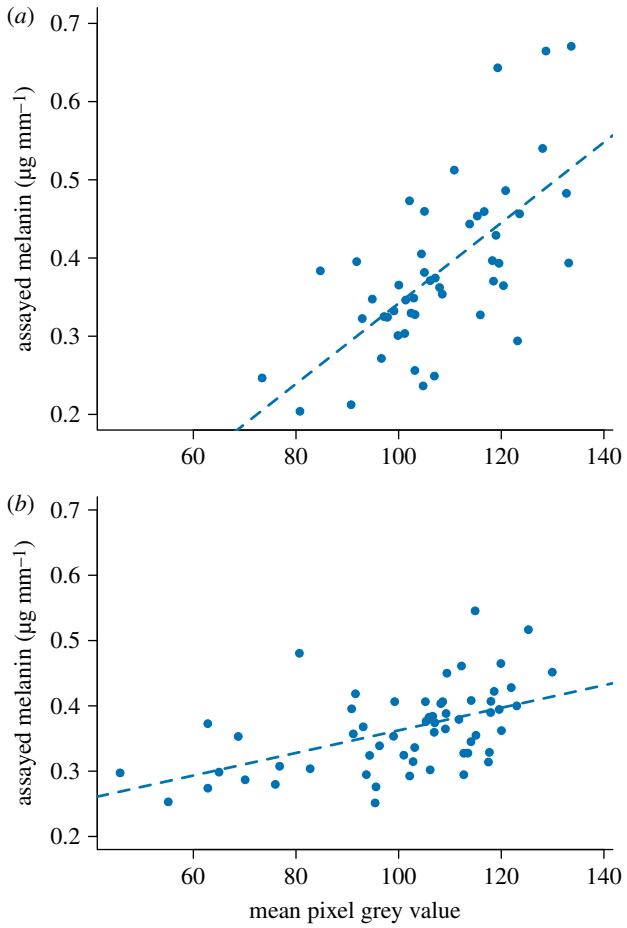

Figure 1. Estimation of melanin concentration from photographs. Assayed melanin concentration (microgram per millimetre body length) as a function of mean pixel grey value (averaged across all *Daphnia* pixels in each image). Grey value ranges from 0 (absolute white) to 255 (absolute black). Photography methods differed between the two experiments: (*a*) reference animals used for DNA damage experiment ($n = 48$). Linear regression (dashed line): $F_{1,46} = 35.4$, $p < 0.0001$, $R^2 = 0.44$. (*b*) Reference animals used for UV-B tolerance experiment ($n = 59$). Linear regression (dashed line): $F_{1,57} = 20.4$, $p < 0.0001$, $R^2 = 0.25$. (Online version in colour.)

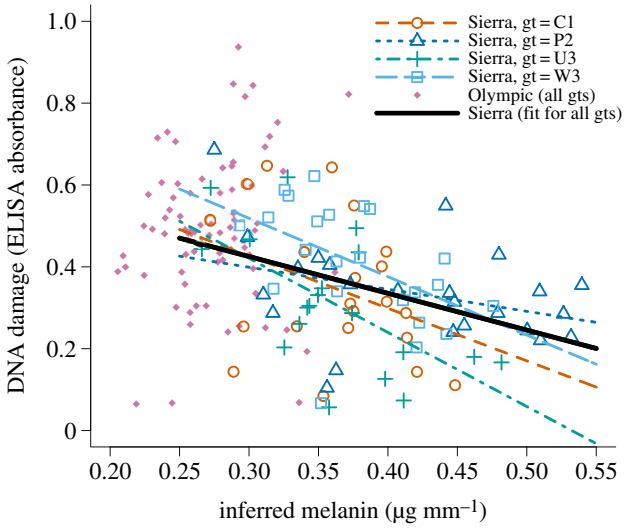

Figure 2. DNA damage as a function of melanin concentration for individual animals. Lines represent least-squares linear regressions. The thick black line represents a significant relationship for all four Sierra genotypes combined ($F_{1,85} = 14.57$, $p = 0.00026$, $R^2 = 0.15$). Genotype-specific regressions for Sierra genotypes appear as dashed lines (slope values: −0.54, −1.28, −1.43, −1.81). The regression for Olympic individuals is not significant ($F_{1,76} = 1.11$, $p = 0.29$, $R^2 = 0.014$). DNA damage is plotted as raw absorbance from the ELISA for CPDs; inferred melanin (units: microgram melanin per millimetre body length) was calculated from photograph mean pixel grey values using the fitted line shown in figure 1*a*. Sierra sample sizes: $n_{C1} = 21$, $n_{P2} = 24$, $n_{U3} = 19$, $n_{W3} = 23$; overall $n_{Sierra} = 87$. Olympic data include four genotypes with overall $N_{Olympic} = 78$. (Online version in colour.)

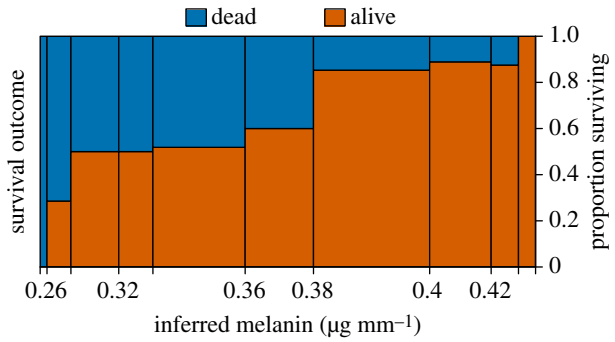

Figure 3. Survival proportions following UV-B exposure as a function of melanin concentration ($n = 145$). The *x*-axis has individuals binned by melanin concentration (bin sizes = 0.02); the width of each bar reflects the number of animals with melanin values within the bin boundaries. Inferred melanin concentrations (units: microgram melanin per millimetre body length) were calculated from photograph mean pixel grey values using the fitted line shown in figure 1*b*. See main text and figure 4 for statistical analyses. (Online version in colour.)

Our measurement of DNA damage quantifies CPDs that result from UV-B exposure via an ELISA method with a primary antibody to CPDs [32,39]. We quantified DNA damage as raw absorbance values (which were normally distributed; Shapiro–Wilk $W = 0.99$, $p = 0.34$) from the ELISA to quantify CPDs (as in [32]). For Sierra animals, inferred melanin was a significant predictor of DNA damage sustained from UV-B exposure (figure 2, solid black line; $p = 0.00026$), and melanin remained significant when we included genotype ID as a covariate ($F_{1,82} = 17.6$, $p = 0.000069$). Although melanin was a significant predictor of DNA damage, and melanin concentration differed significantly among Sierra genotypes, the predictive relationship between melanin concentration and DNA damage did not differ among these four genotypes, as demonstrated by a non-significant melanin-by-genotype interaction ($F_{3,79} = 1.5$, $p = 0.22$).

For Olympic animals (which lack visible carapace pigmentation), the outcome was quite different: inferred melanin was not a significant predictor of DNA damage (figure 2, filled pink diamonds; $p = 0.29$). This lack of a significant relationship also held when we included genotype ID as a covariate ($F_{1,73} = 0.11$, $p = 0.74$). Although the values for DNA damage may appear to be more variable among Olympic individuals than among Sierra individuals, we compared coefficients of variation and found the opposite to be the case (Sierra $\hat{c_v} = 0.43$, Olympic $\hat{c_v} = 0.36$).

(c) UV-B tolerance experiment

We measured survival following UV-B exposure for Sierra genotypes and found that animals with higher melanin concentration were more likely to survive (figure 3). Logistic regression demonstrated that inferred melanin concentration was a significant predictor of post-UV-B survival (figure 4;

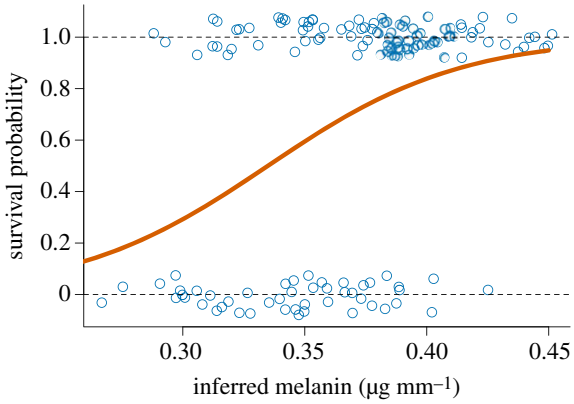

Figure 4. Survival following UV-B exposure as a function of inferred melanin concentration. Individual survival outcomes (open circles; $n = 145$) are either 1.0 (alive) or 0.0 (dead); random deviates along the *y*-axis aid visualization of points that would otherwise be overlaid. Melanin is a significant predictor of survival outcome (LR test against null model: deviance = 26.8, d.f. = 1, $p \ll 0.0001$). The curve represents predicted survival probability under the logistic regression fit to the data. (Online version in colour.)

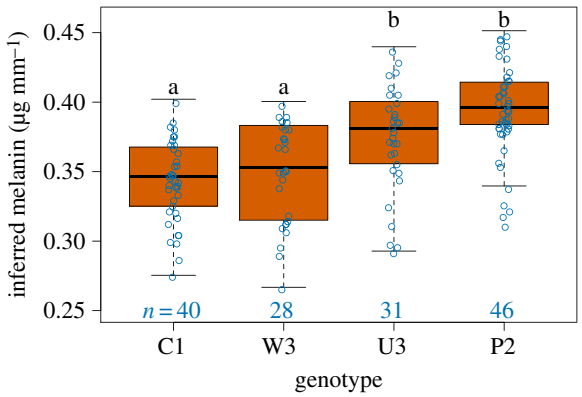

Figure 5. For animals used in the UVR tolerance experiment, inferred melanin concentration differed significantly among genotypes (ANOVA: $F_{3,141} = 18.47$, $p \ll 0.0001$). Genotypes with contrasting letters above boxes differ significantly (Tukey's HSD adjusted *p*-values: C1 versus U3: $p = 0.0016$, C1 versus P2: $p \ll 0.0001$, W3 versus U3: $p = 0.032$, W3 versus P2: $p \ll 0.0001$). Sample sizes appear below boxes. (Online version in colour.)

$p \ll 0.0001$). Although genotypes differed in average melanin concentration (detailed below), including genotype as a covariate did not significantly improve the logistic model (LR test: deviance = 1.8, d.f. = 3, $p = 0.62$), nor did including both genotype and a genotype-melanin interaction (LR test: deviance = 4.3, d.f. = 6, $p = 0.64$). In other words, the best-fit logistic model for post-UV-B survival included inferred melanin concentration as the only predictor variable.

Inferred melanin concentration differed significantly among the four Sierra genotypes for which we measured UVR tolerance (figure 5; $p \ll 0.0001$). In pairwise *post hoc* tests, genotypes C1 and W3 ($p = 0.90$) and genotypes U3 and P2 ($p = 0.07$) did not differ, but these two pairs of genotypes (C1 and W3; U3 and P2) differed significantly from each other (figure 5; *p*-values appear in figure legend).

Given the significant variation in melanin content among genotypes, we explored genotypic variation in the melanin-survival relationship. We constructed separate logistic regression models for each genotype and evaluated effect size by comparing the magnitude of β, the melanin slope coefficients (figure 6). We found that Genotype C1, which had the lowest average melanin concentration (figure 5), had the smallest coefficient and was the only genotype for which melanin was not a significant predictor of survival (LR test against null: $p = 0.32$). For the other three genotypes (W3, U3 and P2) melanin was a significant predictor of survival after controlling for false discovery rate (figure 6; *p*-values appear in the figure legend). Genotype P2, which had the highest melanin concentration (figure 5), also had the largest regression coefficient, indicating the strongest relationship between melanin and survival (figure 6).

4. Discussion

Observed fitness benefits of melanism in zooplankton exposed to UVR [2,4,23,25] and, in other animals, correlations between melanism and UVR exposure in nature [7,8,10,14] have led many researchers to hypothesize that melanism protects organisms from DNA damage caused by UVR. In the present study, we tested this hypothesis and demonstrated that melanin pigmentation reduced the quantity of DNA damage lesions (figure 2) and provided a fitness (survival) advantage under UVR (figures 3 and 4). Furthermore, we assessed melanism as a quantitative trait and found that greater levels of pigmentation conferred increased protection from DNA damage (figure 2) and improved survival (figures 3 and 4). Our findings provide support for a previously missing mechanistic connection between melanism and DNA damage protection that has long been assumed, but has not previously been demonstrated in a non-human organism.

An important element of our study design derives from the fact that *Daphnia* are cyclic parthenogens, providing the opportunity to raise cohorts of each genotype as asexually produced clones [42,43]. Although individual *Daphnia* from a clonal population are genetically identical (except for spontaneous mutations), they vary in melanin concentration as a result of the recurring moulting of their carapace, where the melanin is deposited [2]. Moulting occurs between juvenile instars and with the release of each clutch of offspring. Immediately following a carapace moult, the new carapace is visibly lighter in pigmentation and will gradually darken over a period of hours. Although all of the *Daphnia* in our experiment were egg-bearing adults, they differed from each other in the timing of their moult cycles, resulting in variation in carapace melanin content even within clonal populations. For our Sierra (i.e. melanic) genotypes, we found negative relationships between melanin concentration and DNA damage sustained (figure 2, dashed lines) within all four of these genetically identical populations.

In assessing the survival advantage of melanism, we used genotypes of *D. melanica* that differed in average pigmentation (figure 5), allowing us to investigate genotype-specific relationships between pigmentation intensity and survival under UVR. We cannot draw general conclusions from only four genotypes, yet it is noteworthy that we observed the strongest effect of melanin on survival in the genotype with the highest pigmentation, and the weakest effect in the genotype with lowest pigmentation (figure 6). This result raises the possibility that the relationship between melanin and survival may be stronger in genotypes with greater pigmentation. It also suggests that additional, non-pigmentation mechanisms of UVR tolerance probably influence survival, particularly for genotypes with lower melanin levels. The genotype with lowest pigmentation displayed ample variation in survival

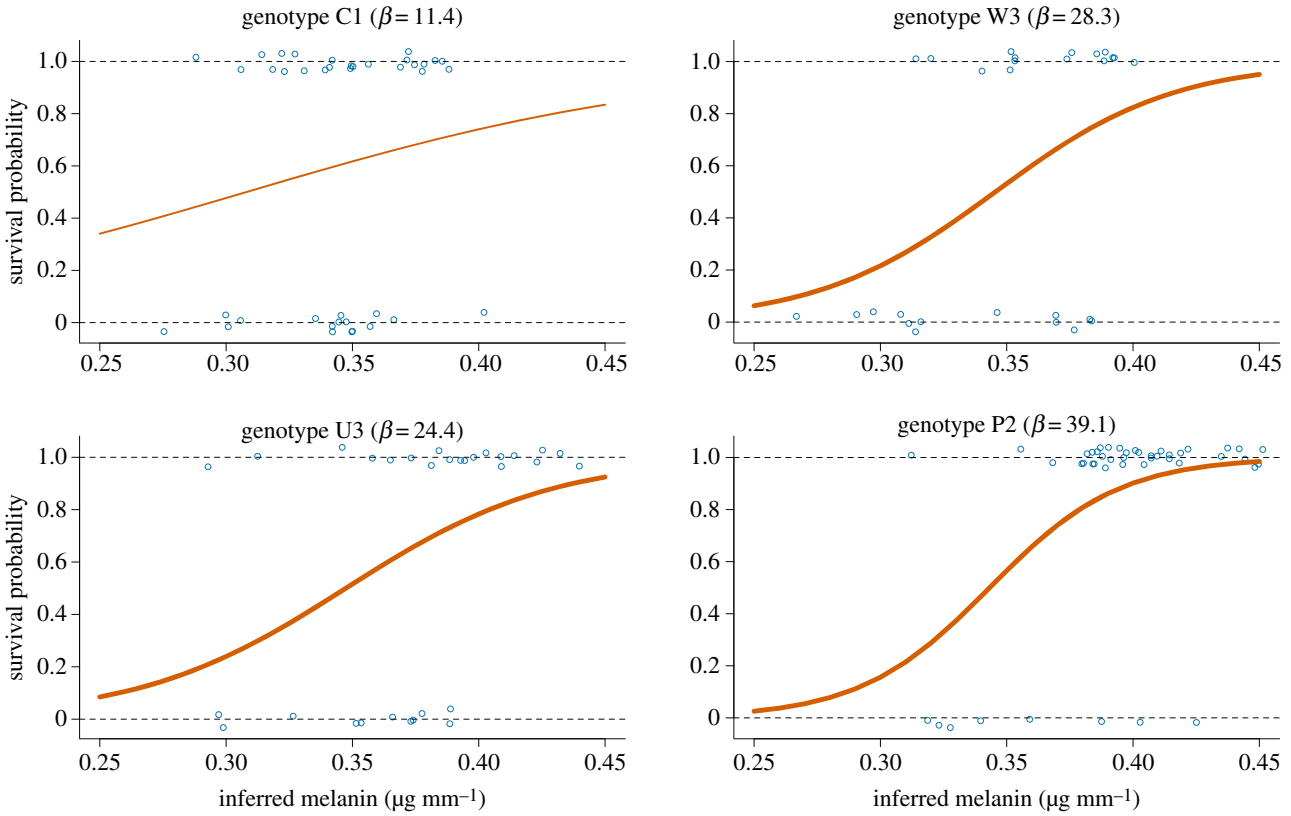

Figure 6. Melanin–survival relationships plotted separately for each genotype. Graphical details are as in figure 4. Effect sizes are evaluated by β, the regression coefficient for melanin, which determines the steepness of the logistic regression curve (β values appear above each graph). Genotype C1 lacks statistical significance (LR test: d.f. = 1, deviance = 1.0, $p = 0.31$). Melanin is a significant predictor of survival outcome (after controlling for false discovery rate following [41]) for genotypes W3, U3 and P2 (LR tests: d.f. = 1; deviance = 5.4, 5.9, 10.05; and $p = 0.02$, 0.015, 0.0015, respectively). (Online version in colour.)

outcomes following UV-B exposure (60% of individuals survived, while 40% died), yet pigmentation intensity was not a significant predictor of these outcomes (figure 6). One likely explanation for this variation is other mechanisms of UVR tolerance, such as the repair of UVR-induced DNA damage, which occurs via both light-dependent (i.e. photoenzymatic repair) and light-independent (i.e. nucleotide excision repair or 'dark repair') processes [32,36]. In addition, the production of antioxidant enzymes to reduce oxidative damage caused by UVR-generated ROS may vary among individuals, as modulation of antioxidant enzyme expression in response to UVR has been shown in numerous studies [25,31,44,45].

Melanic *Daphnia* are widely known to modulate carapace melanin pigmentation in response to UVR exposure [3,25,26] (including *D. melanica* collected from the same region as the Sierra genotypes studied here [27]), yet our present findings derive from individuals that were not exposed to UVR during juvenile development or in advance of our experimental tests. This was advantageous for two reasons. First, 'UVR-naive' individuals are less likely to differ from each other in additional, non-pigmentation phenotypes involved in UVR tolerance, allowing us to isolate melanin pigmentation as the explanatory variable in our experiment. Second, the fact that 'UVR-naive' Sierra animals displayed significant differences among genotypes in pigmentation (figure 5) indicates that the intensity of carapace melanin pigmentation must have a genetic basis, hinting at the possibility that this trait may be locally adapted among Sierra populations. This would provide an intriguing parallel to Olympic populations, which are locally adapted to the UVR level of their habitat [35], but via different trait(s) because they lack carapace pigmentation. We can speculate that if melanic individuals in the present study had instead been exposed to UVR in advance of the DNA-damage or UVR-tolerance experiments, their melanin levels would probably have been higher. That said, our fundamental finding that melanism protects against UVR-induced DNA damage would remain unchanged.

Hebert & Emery [2] speculated that melanism is energetically costly for *Daphnia*, as it must be synthesized *de novo* rather than sequestered from the diet, and any cost would recur with each successive carapace moult. Hessen [4] later demonstrated for arctic (Svalbard) *Daphnia pulex* that melanism carried a growth-rate cost, with non-melanic individuals outcompeting melanics in the absence of UVR. However, in both studies, the authors note the frequent (but not absolute) co-occurrence of polyploidy and melanism for arctic *Daphnia* [46], with polyploidy itself carrying a growth-rate cost [46]. Non-melanic *D. pulex* have been shown to have decreased UV-B tolerance under food restriction [47]; therefore, an investigation into the interactive effects of resource limitation and melanin content on UV-B tolerance would surely further our understanding of the mechanistic details of melanism's fitness costs as well as its benefits.

Our finding that melanism is an adaptive phenotype that protects individuals from DNA damage resulting from UVR exposure highlights the likelihood that further advances in our understanding of evolutionary mechanisms will be found in studies of photoprotective melanism in a wide range of animal species. Beyond *Daphnia* and other zooplankton, *Drosophila melanogaster* is an obvious candidate: pigmentation clines in nature correlate with variation in UV radiation resulting from latitudinal and elevational variation [7], and UV-B sensitivity of early embryos is lower in low-latitude populations (where incident UVR is greater), perhaps as a result of increased

expression of DNA repair genes [48]. Evidence of UVR–pigmentation correlations for vertebrates such as amphibians and lizards [8,10] raises the possibility that the evolutionary underpinnings of photoprotective melanism may be similar across divergent animal lineages. Investigations of such questions will surely increase our understanding of the evolutionary significance of both a widespread phenotype—melanism—and one of earth's most ubiquitous abiotic stressors, UVR radiation.

Data accessibility. Data described in this manuscript and R scripts used to conduct statistical analyses and generate figures are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.jwstqjq4n [49].

Authors' contributions. All authors contributed to the design of experiments. B.E.M. conceived of the study, assisted with data collection, analysed the data and drafted the manuscript with substantial contributions from C.K.S.U. and J.M.M. C.K.S.U. conducted the DNA damage experiments and collected all of the resulting data. J.M.M. conducted the UVR tolerance experiments and collected all of the resulting data. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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