

Deleterious effects of light exposure on immunity and sexual coloration in birds

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Summary

1. Sunlight ultraviolet (UV) exposure can be highly damaging to biological tissues, being known to cause cellular damage and immunosuppression in humans and rodents, and depletion of carotenoid-based sexual plumage coloration in birds (i.e. photobleaching). However, it remains unknown whether sunlight may cause photobleaching in living tissues that comprise sexual signals such as bare parts in birds. It seems possible that any carotenoids depleted from bare parts by sunlight could be replenished, but if so, this could impact the availability of carotenoids for other functions such as immunity and antioxidant defence. Such trade-offs seem particularly likely in individuals that have a low dietary intake of carotenoids, or small amounts of carotenoids in body storage.

2. We investigated the effects of exposure to simulated sunlight UV (UV+) on bill coloration, blood plasma and liver (i.e. body storage) carotenoids and pro-inflammatory immune responses in male zebra finches (*Taeniopygia guttata*) *in vivo*, compared with individuals maintained in an identical environment but with the UV wavelengths removed by a filter (UV-). We also investigated whether any deleterious effects of UV exposure were mitigated by dietary carotenoid supplementation (UV+ Car+).

3. Exposure to simulated sunlight UV was associated with low levels of carotenoids in liver, and elevated levels of carotenoids in blood plasma, suggesting the mobilisation of stored carotenoids. Simulated sunlight UV also caused impaired pro-inflammatory immune responses, whereas this was not seen in carotenoid-supplemented birds. We found no effects of simulated sunlight UV or carotenoid supplementation on oxidative damage in blood plasma or bill.

4. Bill 'carotenoid chroma' diminished, and bill 'UV chroma' correspondingly increased, in all groups during the experiment (i.e. even in UV+ Car+ birds, and in UV- birds). This likely arose because the illuminance in the experimental cages was far higher than in standard laboratory housing conditions and suggests that carotenoid pigmentation is highly susceptible to photobleaching even under exposure to human-visible wavelengths.

5. These results highlight the potential for sunlight exposure to invoke a range of deleterious consequences for birds, including photobleaching and depression of immunity via pathways mediated by carotenoid allocation trade-offs. The expression of carotenoid-based sexual signals may therefore reveal an individual's history of sunlight exposure and its deleterious effects on immunity.

Key-words: carotenoid coloration, inflammatory response, oxidative damage, photobleaching

Introduction

Sexual selection often promotes the evolution of elaborately coloured phenotypes. Such traits have traditionally been considered to be static, but it is becoming increasingly clear

that signal expression often changes over time. This has commonly been attributed to biotic processes, such as changes in aspects of physiological condition (Pérez-Rodríguez 2008), activation of the immune system (Faivre *et al.* 2003; Alonso-Alvarez *et al.* 2004), and bacterial degradation of feathers (Shawkey, Pillai & Hill 2009) and has been linked to ageing and competition for mates in fish (e.g.

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Lindström *et al.* 2009; Svensson *et al.* 2009). However, abiotic factors such as abrasion (e.g. Willoughby, Murphy & Gorton 2002), dust and dirt accumulation (e.g. Griggio, Serra & Pilastro 2011), and exposure to sunlight have also been hypothesised to cause colour changes (Burt 1986; McGraw & Hill 2004; Blanco *et al.* 2005; Figuerola & Senar 2005; McNett & Marchetti 2005). However, we are aware of only two studies that have experimentally demonstrated that pigment loss (i.e. photobleaching) in ornamental traits can be attributed to sunlight exposure (Test 1940; Surmacki 2008). Carotenoids have an extended system of conjugated double bonds, which enables them to absorb light and impart colour, but also renders them susceptible to light-induced oxidation (Mortensen & Skibsted 1999). Despite the fact that all diurnal species of wild animals are exposed to sunlight, little is known about the mechanisms that determine the susceptibility of individuals to light-induced colour change. Moreover, how sunlight exposure may in parallel influence physiological state to shape the information content of signals has not been studied previously.

Cuticle and feathers, which often form a platform for the presentation of sexual coloration, are metabolically inert after development, and therefore, photobleaching of pigments may be inevitable. However, whether exposure to sunlight may similarly affect sexual coloration in living tissues is not known. In many animal species, areas of skin or specific structures, such as bills in birds, are often pigmented with carotenoids that are diet-derived compounds with the potential to function as antioxidants and immunostimulants *in vivo* (recently reviewed by Catoni, Peters & Schaefer 2008). In fact, across bird species, carotenoids are more commonly responsible for the coloration of bare parts than feathers (Olson & Owens 2005). Such traits have the potential to provide relatively up-to-date information about individual quality. It seems possible that any carotenoids depleted by sunlight could be replenished, but if so, this could impact the availability of carotenoids for other functions *in vivo*. Such a trade-off seems particularly likely in individuals that have a low dietary intake of carotenoids, or small amounts of carotenoids in body storage. In addition, carotenoids might perform a local photoprotection role in living tissues, which are exposed to sunlight. For example, the epidermis of the bill comprises cornified cells that synthesise β -keratin and contain considerable amounts of lipids (Alibardi 2002) and may therefore be susceptible to UV-induced lipid peroxidation. Studies of humans and animal models have shown that sunlight ultraviolet radiation (UV) can induce inflammation, oxidative damage, gene mutation and photocarcinogenesis in skin and initiate systemic suppression of adaptive and innate immunity (e.g. Roberts & Beasley 1997; Yeun, Nearn & Halliday 2002; Dinkova-Kostova 2008; Halliday & Rana 2008; Murphy 2009; Schwartz 2010). Carotenoids in skin function as a form of 'sunscreen' by absorbing and scattering light and have also been hypothesised to function as photoprotectants via antioxidant activity, and by modulating gene expression and cellular signalling (e.g. Alaluf *et al.* 2002; Sies & Stahl 2007; Dinkova-Kostova 2008). It therefore seems possible

that carotenoid-based ornaments initially evolved utilising pigments that were already found in epidermis for the function of photoprotection. However, no previous study has considered whether exposure to sunlight may simultaneously affect carotenoid-based sexual coloration, oxidative damage and immunity in any species.

Here, we investigate the effects of exposure to simulated sunlight UV, coupled with dietary carotenoid supplementation, on carotenoid coloration, and blood and tissue levels of carotenoids, oxidative damage and immunity in zebra finches (*Taeniopygia guttata*) *in vivo*. Bill coloration is carotenoid based in this species (McGraw & Toomey 2010). Previous studies have shown that bill coloration is influenced by dietary carotenoid supply, and bill coloration and blood carotenoid levels predict immunity (e.g. McGraw & Ardia 2003; Alonso-Alvarez *et al.* 2004) and female mate choice (Blount *et al.* 2003). We hypothesised that carotenoid-based bill coloration would be susceptible to photobleaching, but such carotenoids may be replenished from circulation and other body tissues. This could impact carotenoid availability for other functions *in vivo*, particularly in carotenoid-limited individuals. We specifically predicted that exposure to simulated sunlight UV would (i) deplete bill coloration and/or (if individuals compensated for such depletion) would deplete blood and liver levels of carotenoids; (ii) impair immunity; and (iii) cause increased oxidative damage in bill tissue and blood plasma. We also predicted (iv) that dietary carotenoid supplementation would mitigate such deleterious effects of exposure to simulated sunlight UV.

Materials and methods

Birds used in the experiment were bred in-house under standardised conditions, as described in the Data S1, Supporting information. Changes in the social environment may result in changes in bill pigmentation in zebra finches (Gautier *et al.* 2008). However, males used in this study were housed in single-sex groups prior to and during the experiment. Zebra finches can breed from *c.* 80 days of age (Zann 1996). Sexually mature, young adult males were allocated to an experimental group for the next 16 weeks: birds not exposed to simulated sunlight UV (UV-; $n = 10$); birds exposed to simulated sunlight UV (UV+; $n = 10$); or birds exposed to simulated sunlight UV and on a carotenoid-supplemented diet (UV+ Car+; $n = 10$). The experiment was low throughput and was therefore run in two, consecutive cohorts with $n = 5$ birds per treatment in each cohort. At the start of the experiment, there were no significant differences amongst treatments in the ages of birds, body mass, tarsus length, blood plasma carotenoid concentration, bill carotenoid chroma or bill UV chroma as determined by spectrometry (Table S1, Supporting information). Measurements were made as described later. All birds received mixed seeds (J. E. Haith, Cleethorpes, UK), grit and cuttlefish, and ultrapure drinking water (Milli-Q Synthesis; Millipore UK Ltd, Watford, UK) *ad libitum*. Carotenoid-supplemented birds received the same water containing $10 \mu\text{g mL}^{-1}$ carotenoids [Oro Glo™ liquid, 11 mg mL^{-1} lutein and zeaxanthin (20 : 1, w/w); Kemin Europa N.V., Herentals, Belgium]. These are amongst the major plasma carotenoids found in captive zebra finches on a seed diet (McGraw, Adkins-Regan & Parker 2002). Drinkers were sheathed in aluminium foil to prevent the light-induced oxidation of

carotenoids (to standardise presentation, this was performed for all treatments).

Birds were housed indoors under ambient artificial light (Phillips TL-D lamps, 16L : 8D cycle; Phillips Lighting, Guildford, UK) in a laboratory maintained at 21 °C. Experimental groups were housed in cages (60 × 30 × 39 cm, L × W × H; with wire fronts) that were furnished identically, including one high perch positioned 26 cm above the cage floor, and one low perch 15.5 cm above the cage floor. Cage ceilings comprised welded mesh (2.5 × 1 cm, 19 gauge), above which was a polished aluminium hood housing four UVA-340 fluorescent lamps (Q-Lab Europe Ltd., Farnworth, UK), spanning the full length of the cage in parallel. Either a UV transmitting filter (UV+ and UV+ Car+: 130 Lee Clear), or a UV blocking filter (UV-: 226 Lee UV) was placed between the lamps and the mesh ceiling of cages (Lee Filters, Andover, UK). UVA-340 lamps provide an accurate simulation of sunlight in the short-wavelength region of 365 nm to the solar cut-off of 295 nm ($\lambda_{\text{max}} = 340$ nm) and produce only wavelengths found in sunlight (Brown *et al.* 2000; Fig. S1, Supporting information). Power to the lamps was provided by a QUV/se Accelerated Weathering Tester (Q-Lab Europe Ltd.), which is designed to test the colour stability of manufactured products (e.g. plastics, textiles) when exposed to simulated sunlight UV. The QUV/se was set to provide a constant irradiance at its 'Solar Eye Irradiance Controllers', which adjust the power to lamps to compensate for deterioration in lamp performance over time and therefore ensure constant irradiance; two such controllers were positioned 40 mm from the lamps. At weeks 0, 5, 10 and 15 of the experiment, temperature within cages was measured using a digital thermometer (± 0.1 °C), illuminance was measured using a HI97500 Luxmeter (Hanna Instruments Ltd., Leighton Buzzard, UK) and irradiance was measured using a CR10 radiometer (Q-Lab Europe Ltd.). At 5 cm above the upper perch, i.e. the approximate head-height of zebra finches, the temperature was 20.73 ± 0.025 °C (mean \pm SE), the illuminance was 0.68 ± 0.01 Klx (mean \pm SE) and the irradiance was 0.28 ± 0.006 W m⁻² (mean \pm SE). This temperature is almost identical to the ambient laboratory temperature, whilst this illuminance is considerably higher than in our standard laboratory cages for zebra finches, which only receive ambient light from overhead room lights (range, 0.05–0.35 Klx). The irradiance in UV+ and UV+ Car+ cages was within the range recorded at Alice Springs, Australia (monthly mean UV: 0.115–0.325 W m⁻²; Gies *et al.* 2004), which is within the geographic range of wild zebra finches (Zann 1996). For comparison, the summer sunlight maximum at terrestrial surfaces is 0.68 W m⁻² at 340 nm (Kock 1989). UVA-340 lamps were set to illuminate daily (8L : 16D cycle) during the second half of the room's normal 'daylight' phase, therefore providing a maximum cumulative irradiation dose of 9.032×10^5 J m⁻¹. The level of irradiance that we chose was therefore within the range that birds would likely experience in the wild but was relatively conservative because we were interested to study chronic rather than acute deleterious effects. All areas of the cage were directly exposed to UV, i.e. there were no refugia where birds could seek shade.

At the start of the experiment (week 0) and again at week 16, body mass was measured to the nearest 0.1 g using an electronic balance, tarsus length was recorded with a sliding calliper to the nearest 0.5 mm and bill reflectance was measured using a USB2000 spectroradiometer (Ocean Optics, Duiven, The Netherlands). Light from a halogen light source was transferred to the bill through a quartz fibre optic cable reaching the surface at 90°. The sampling optic was placed at 90° to the upper surface of the upper bill and connected to the spectrophotometer by a quartz fibre optic cable. Measurements were made three times from different locations at 1-nm intervals in the

range of 300–750 nm and referred to a standard white reference (WS-2) and to the dark. Spectral reflectance data were smoothed using a 10 point running average and then used to compute objective colorimetric measures. As the surface of the bill is not flat, small variation in the angle of the reflectance probe may cause variation in the proportion of light reflected at different wavelengths. Therefore, spectra were standardised by dividing reflectance at each wavelength by total brightness before calculating chroma and hue (Endler 1990; Pérez-Rodríguez 2008). We calculated 'carotenoid chroma' (C_{car}), $(R_{700} - R_{450})/R_{700}$, which is one of the best spectroscopic estimates of carotenoid pigmentation (Andersson & Prager 2006). We also calculated 'UV chroma' (C_{UV}), $(R_{400} - R_{300})/R_{400}$, because the bill of the zebra finch shows a relatively small peak of reflectance in the UV (e.g. Pérez-Rodríguez 2008). Finally, we calculated 'hue' (λ_{R50}), λ halfway between the wavelength of peak reflectance and the wavelength of minimal reflectance (Andersson & Prager 2006). Values for λ_{R50} and C_{car} were significantly correlated (Pearson's correlation: $r = 0.43$, $n = 60$, $P < 0.001$), and therefore analyses are presented for C_{car} only. The three measurements from each individual were averaged prior to analysis.

A small blood sample (c. 100 μ L) was collected in week 0 and week 16, respectively. Whole blood was collected from the brachial vein into heparinised capillary tubes, centrifuged for 5 min, and then plasma was transferred to a microtube and snap frozen in N₂. In week 16, immediately after blood sampling, we assessed the capacity of birds to mount an inflammatory immune response following intradermal injection with phytohaemagglutinin (PHA). PHA induces an inflammatory response leading to cellular infiltration, which can be measured as a swelling. PHA responses are reported as the post-minus pre-injection thickness of the PHA-injected wing-web, minus any change in thickness of the other (control) wing-web; the thickness of both wing-webs were measured before injection and 24 \pm 1 h later (Methods S1, Supporting information). The response provides a measure of inflammatory potential (Vinkler, Bainova & Albrecht 2010). Studies of humans and animal models have shown that UV exposure suppresses immunity, as measured for example by cutaneous hypersensitivity responses, i.e. inflammatory potential (reviewed by Schwartz 2010), whilst PHA responses in birds are known to be affected by carotenoid availability, likely through effects on signalling cascades (e.g. Blount *et al.* 2003; McGraw & Ardia 2003). In common with most other studies that have used the PHA test, we assayed responses on one occasion following the experimental manipulation. The reason for this is that the PHA test involves acquired as well as innate immune components (reviewed by Vinkler, Bainova & Albrecht 2010), and after repeated exposure acquired immunity can develop in birds (Tella *et al.* 2008). Therefore, repeated exposure to PHA would make it difficult to separate effects of differences in immunological memory and differences in responses to light exposure and dietary carotenoids. Birds were then killed by cervical dislocation and immediately dissected; the liver and upper bill were collected and snap frozen in N₂. All samples were stored in a -80 °C freezer within 4 h of collection until analyses of plasma and liver carotenoids, and plasma and bill concentrations of malondialdehyde (MDA) – a product of the breakdown of oxidised polyunsaturated lipids (e.g. Nussey *et al.* 2009), which were carried out using standard methods (see Methods S1).

DATA ANALYSES

Data were checked for normality, homoscedasticity, and, in the case of repeated measures, sphericity and compound symmetry.

Within-subject changes in body mass, bill coloration metrics and plasma carotenoid concentrations were analysed using general linear mixed models (GLMM), with treatment (UV⁻, UV⁺, UV⁺ Car⁺) and measurement (week 0, week 16) as fixed factors, and the treatment \times measurement interaction. Random factors were included to account for repeated measures (male identity) and experimental replicates (cohort). Our aims were to test whether simulated sunlight UV reduced carotenoid coloration and immunity, and increased oxidative damage (UV⁻ vs. UV⁺), and whether carotenoid supplementation mitigated any such negative effects (UV⁺ vs. UV⁺ Car⁺). Therefore, following any significant treatment \times measurement interaction, ANOVA contrasts were carried out comparing UV⁻ vs. UV⁺, and UV⁺ vs. UV⁺ Car⁺, respectively. Similarly, variation in PHA responses, plasma and bill concentrations of MDA, and liver concentrations of carotenoids were analysed using GLMMs, with treatment as a fixed factor and cohort as a random factor. Significant overall tests were followed by contrasts between specific groups, as mentioned earlier. Data for plasma MDA and liver carotenoids were positively skewed and heteroscedastic, which was resolved by log₁₀-transformation. Data for bill UV chroma (C_{UV}) were negatively skewed, which was resolved by square root transformation. All explanatory terms were entered into initial models. Models were developed by the backward elimination of the fixed factors, starting with the interaction, removing terms where $P > 0.05$ until only significant terms remained. Significance was calculated using the Wald statistic, which approximates the χ^2 distribution. We report any non-significant P -values associated with the treatment \times measurement interaction, for clarity, because this test is central to our hypotheses about expected effects of light exposure and carotenoid supply. Birds gained body mass slightly (*c.* 5%) but significantly over the course of the experiment, to a similar extent in all treatments [GLMM: treatment, $\chi^2 = 0.75$, d.f. = 2, $P = 0.69$; measurement, $\chi^2 = 9.94$, d.f. = 1, $P = 0.004$; treatment \times measurement, $\chi^2 = 0.73$, d.f. = 2, $P = 0.70$; cohort: 5.71 ± 8.27 (estimate \pm SE); male identity, 1.15 ± 0.62 (estimate \pm SE)], presumably because there was enhanced foraging efficiency in the relatively brightly lit experimental cages compared with the standard stock cages where birds were housed previously. We checked whether any such changes in body mass were related to changes in bill coloration metrics, plasma carotenoids, liver carotenoids, PHA responses, plasma MDA or bill MDA, respectively, in GLMMs with treatment and body mass change as fixed factors, and their interaction, and with cohort as a random factor; these analyses showed no significant effects of changes in body mass (body mass change, χ^2 range, 0.01–1.94, d.f. = 2, P range, 0.94–0.16; body mass change \times treatment interaction, χ^2 range, 0.11–4.20, d.f. = 2, P range, 0.95–0.14). Therefore, changes in body mass are not included in the main Results presented below. Data are presented as means \pm 1 SE. All analyses were performed in GENSTAT v. 12.

Results

Bill carotenoid chroma declined over the course of the experiment, and bill UV chroma correspondingly increased, but the slope of these changes did not differ significantly amongst treatments (Fig. 1 and Table 1). Thus, depletion of carotenoid pigmentation was observed even where birds were exposed only to human-visible light, and even where supplemental dietary carotenoids were provided. Changes in plasma carotenoid concentrations during the experiment differed significantly amongst treatments (Fig. 2a and Table 1).

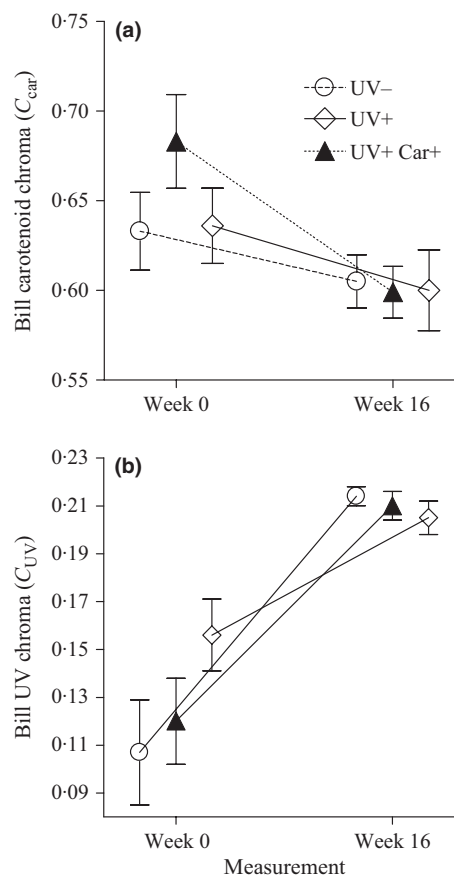


Fig. 1. Effects of simulated sunlight UV and dietary carotenoid supplementation on carotenoid-based bill coloration in male zebra finches. (a) Changes in bill carotenoid chroma; (b) changes in bill UV chroma. Values are means \pm SE. See Table 1 for statistical analyses.

Plasma carotenoids increased in birds exposed to simulated sunlight UV (UV⁺) compared with controls (UV⁻). A similar increase in plasma carotenoids was found in birds exposed to simulated sunlight UV that received supplemental carotenoids (UV⁺ Car⁺), the slope of this change not differing significantly compared with UV⁺ birds. Therefore, both simulated sunlight UV exposure and carotenoid supplementation were independently associated with an increase in circulating concentrations of carotenoids.

Liver carotenoid levels at the end of the experiment differed significantly amongst treatments (GLMM on log₁₀-transformed values: $\chi^2 = 12.76$, d.f. = 2, $P = 0.006$; cohort: -0.00118 ± 0.00033 (estimate \pm SE)). Liver carotenoid concentrations were lower in UV⁺ compared with UV⁻ birds and were higher in UV⁺ Car⁺ birds compared with UV⁺ birds (Fig. 2b). Thus, exposure to simulated sunlight UV resulted in depleted liver stores of carotenoids, but this depletion was not observed in birds exposed to simulated sunlight UV that received supplemental carotenoids. In all experimental groups, individuals that had low liver carotenoid concentrations had low plasma carotenoid concentrations at the end of the experiment (GLMM with plasma carotenoids as the dependent variable: treatment, $\chi^2 = 4.74$, d.f. = 2, $P =$

Table 1. Variation in bill coloration and plasma carotenoid concentrations arising from GLMMs, with treatment (UV−; UV+; UV+ Car+) and measurement (weeks 0 and 16) as fixed factors and male identity and cohort as random factors (see Materials and methods for details)

Dependent	Treatment			Measurement			Treatment × measurement			Male identity	Cohort
	χ^2	d.f.	<i>P</i>	χ^2	d.f.	<i>P</i>	χ^2	d.f.	<i>P</i>	Estimate ± SE	Estimate ± SE
Bill C_{car}	1.36	2	0.52	10.00	1	0.004	2.54	2	0.30	0.00054 ± 0.00080	0.00003 ± 0.00018
Bill C_{UV}	2.94	2	0.25	51.42	1	<0.001	4.95	2	0.10	0.00008 ± 0.00013	0.00006 ± 0.00007
Plasma carotenoids	7.96	2	0.031	9.43	1	0.005	7.30	2	0.04*	27.94 ± 11.96	35.01 ± 53.39

*ANOVA contrasts for changes in plasma carotenoid concentrations: UV− vs. UV+, *P* = 0.033; UV+ vs. UV+ Car+, *P* = 0.13.

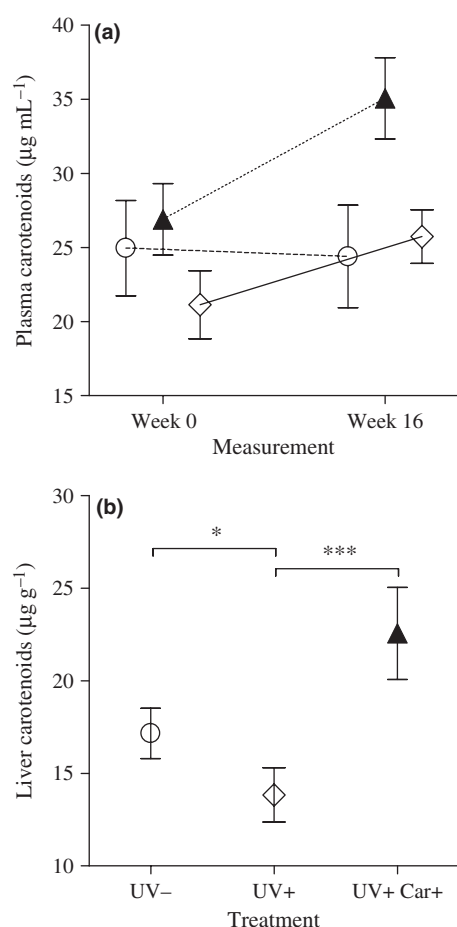


Fig. 2. Effects of simulated sunlight UV and dietary carotenoid supplementation on blood plasma and liver carotenoid concentrations in male zebra finches. (a) Changes in plasma carotenoid concentrations; (b) liver concentrations of carotenoids at the end of the experiment. Values are means ± SE. See Table 1 and text of Results for statistical analyses. In figure (b), asterisks indicate significant differences between groups as determined by ANOVA contrasts (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

0.11; liver carotenoids, $\chi^2 = 12.05$, d.f. = 1, *P* = 0.002; treatment × liver carotenoids, $\chi^2 = 2.43$, d.f. = 2, *P* = 0.32; cohort: 26.32 ± 42.83 (estimate ± SE); liver carotenoids, 0.74 ± 0.21 (estimate ± SE).

PHA responses at the end of the experiment differed amongst treatments (GLMM: $\chi^2 = 10.79$, d.f. = 2, *P* = 0.011; cohort: 0.05363 ± 0.08162 (estimate ± SE); Fig. 3a).

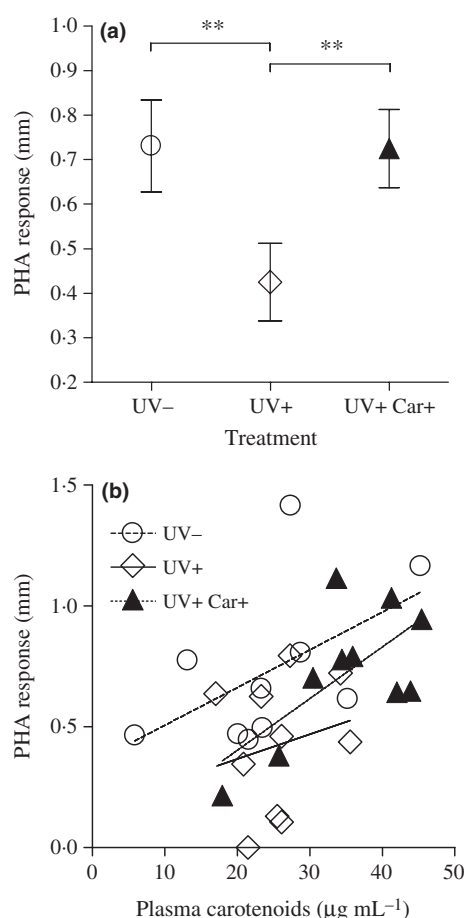


Fig. 3. Effects of simulated sunlight UV and dietary carotenoid supplementation on cell-mediated immunity in male zebra finches. (a) PHA responses (means ± SE); asterisks indicate significant differences between groups as determined by ANOVA contrasts (***P* < 0.01); (b) relationship between plasma carotenoid concentrations and PHA responses; each data point is an individual bird. See text of Results for statistical analyses.

PHA responses were lower in UV+ compared with UV− birds and were higher in UV+ Car+ compared with UV+ birds (Fig. 3a). Thus, exposure to simulated sunlight UV was associated with impaired PHA responses, but this diminishment was not observed in simulated sunlight UV-exposed individuals that received supplemental dietary carotenoids. PHA responses were positively correlated with plasma

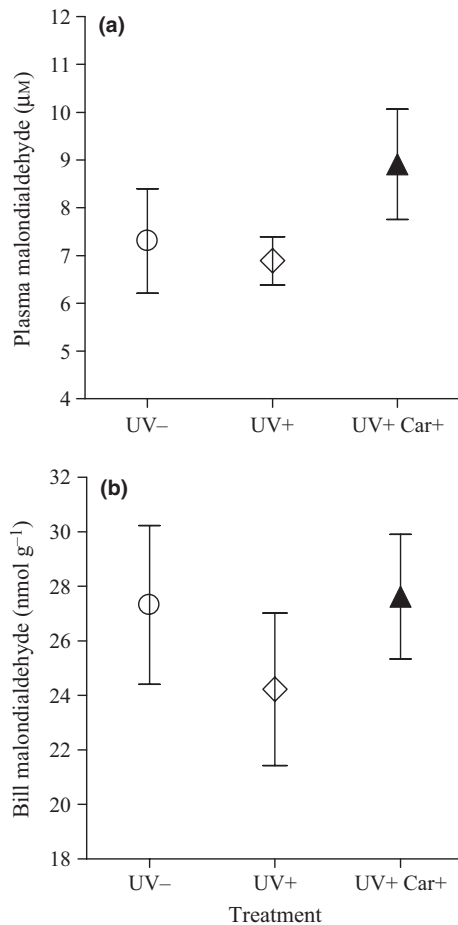


Fig. 4. Effects of simulated sunlight UV and dietary carotenoid supplementation on blood plasma and bill concentrations of malondialdehyde (MDA) in male zebra finches. (a) Plasma MDA; (b) bill MDA. Values are means \pm SE. See text of Results for statistical analyses.

carotenoid concentrations at the end of the experiment in all groups [treatment, $\chi^2 = 7.89$, d.f. = 2, $P = 0.032$; plasma carotenoids, $\chi^2 = 8.45$, d.f. = 1, $P = 0.007$; treatment \times plasma carotenoids, $\chi^2 = 0.34$, d.f. = 2, $P = 0.84$; cohort: 0.02908 ± 0.04764 (estimate \pm SE); Fig. 3b].

Plasma malondialdehyde (MDA) levels at the end of the experiment did not differ significantly amongst treatments (GLMM on \log_{10} -transformed values: $\chi^2 = 2.15$, d.f. = 2, $P = 0.34$; cohort: 0.00231 ± 0.00074 ; Fig. 4a) and were not significantly related to current plasma carotenoid concentrations [treatment, $\chi^2 = 2.37$, d.f. = 2, $P = 0.32$; plasma carotenoids, $\chi^2 = 0.00$, d.f. = 1, $P = 0.99$; treatment \times plasma carotenoids, $\chi^2 = 3.45$, d.f. = 2, $P = 0.20$; cohort: 0.600 ± 0.186 (estimate \pm SE)]. Similarly, bill MDA levels did not differ significantly amongst treatments (GLMM: $\chi^2 = 0.96$, d.f. = 2, $P = 0.62$; cohort: 3.92 ± 1.97 , Fig. 4b) and were not significantly related to current bill carotenoid chroma [treatment, $\chi^2 = 0.96$, d.f. = 2, $P = 0.62$; bill C_{car} , $\chi^2 = 0.11$, d.f. = 1, $P = 0.75$; treatment \times bill C_{car} , $\chi^2 = 2.22$, d.f. = 2, $P = 0.35$; cohort: 3.92 ± 1.97 (estimate \pm SE)] or bill UV chroma [treatment, $\chi^2 = 0.726$, d.f. = 2, $P = 0.70$; bill C_{UV} , $\chi^2 = 0.58$, d.f. = 1, $P = 0.45$;

treatment \times bill C_{UV} , $\chi^2 = 1.24$, d.f. = 2, $P = 0.55$; cohort: 3.74 ± 1.90 (estimate \pm SE)].

Discussion

Sunlight exposure must to some extent be unavoidable for diurnal animal species, and our results suggest that this could be damaging for both carotenoid-based coloration and immunity. Exposure to simulated sunlight UV, but also human-visible wavelengths, was associated with reductions in carotenoid coloration, and this was apparent *in vivo* even where birds simultaneously received supplemental dietary carotenoids. Exposure to simulated sunlight UV also resulted in reduced immunity, which was mitigated by dietary carotenoid supplementation.

Sunlight exposure has been hypothesised to cause fading of carotenoid-based feather coloration (Burt 1986; McGraw & Hill 2004; Figuerola & Senar 2005; McNett & Marchetti 2005), and this has been confirmed by two experimental studies (Test 1940; Surmacki 2008). Indeed, Surmacki (2008) showed that, whilst sunlight UV radiation was responsible for most deterioration in pigmentation in carotenoid-based great tit (*Parus major*) feathers, a decline in carotenoids was also observed when feathers were exposed only to human-visible wavelengths found in sunlight. In our experiment, zebra finch bill carotenoid chroma diminished, and UV chroma correspondingly increased, in all birds – even those that were exposed only to human-visible light (UV–), and those that were exposed to UV whilst at the same time receiving supplemental carotenoids (UV+ Car+). We think the most likely explanation for these findings is that carotenoid pigmentation in bill tissue, like feathers (Surmacki 2008), is susceptible to photobleaching caused by both UV and human-visible wavelengths. The source of simulated sunlight used in our experiments provides an accurate match for UV wavelengths in the solar spectrum but does not correspond well in the human-visible wavelength range (Brown *et al.* 2000), providing a restricted range of wavelengths and a lower relative irradiance than that found in natural sunlight (Fig. S1, Supporting information). Therefore, it is important to note that our UV– condition was not analogous to natural sunlight minus the UV component. In fact, in natural conditions, birds are exposed to relatively high levels of human-visible light, and therefore, the carotenoid pigmentation of bills should be expected to be even more susceptible to photobleaching than we observed in the laboratory. That said, it seems possible that wild zebra finches avoid prolonged direct exposure to sunlight. Ultimately, field studies will be required to ascertain the cumulative irradiation dose experienced by wild birds and the effect this has on bill pigmentation.

Zebra finches housed in conventional laboratory cages and fed a standard diet do not typically exhibit diminutions of bill pigmentation over time, whilst carotenoid supplementation studies have invariably reported increased bill carotenoid pigmentation in this species (e.g. Blount *et al.* 2003; McGraw & Ardia 2003; Alonso-Alvarez *et al.* 2004). Why then did UV– birds, and UV+ Car+ birds, apparently exhibit

photobleaching? A likely explanation is that the illuminance in our experimental cages was far higher than that in standard laboratory cages, which receive only ambient light from overhead room lights (see Materials and methods). Unlike feathers, which are metabolically inert after moult, bill tissue grows continuously and therefore individuals may replenish any carotenoids destroyed by sunlight. However, the process of carotenoid allocation to bills takes place over several weeks in zebra finches (e.g. Blount *et al.* 2003; McGraw & Ardia 2003; Alonso-Alvarez *et al.* 2004), as keto-carotenoids must first be metabolised from dietary precursors (McGraw & Toomey 2010). Our results indicate that exposure to light prevented birds from increasing, or even maintaining, levels of carotenoids in bill tissue despite unlimited dietary access to carotenoids.

Given our findings in relation to changes in bill coloration, it is difficult to conceive of a suitable negative control for exposure of live birds to light; clearly, birds cannot be maintained in darkness over days or weeks. For this reason, we cannot altogether exclude the possibility that the changes in bill coloration arose because of some other time-related factor, such as stress caused by the social environment (Gautier *et al.* 2008). However, we think this is very unlikely to explain our results, because (i) males were housed in single-sex groups prior and during the experiment, i.e. the social environment was stable; and (ii) there was no loss of body mass during the experiment, as might be expected under conditions of a general stress response (see Materials and methods).

Exposure to simulated sunlight UV (UV+) was associated with decreased PHA responses compared with birds exposed only to human-visible wavelengths (UV-). It is well established that exposure of even small areas of skin to sunlight UV can initiate systemic immunosuppression in humans and animal models, affecting both innate and acquired immunity. At the molecular level, UV radiation can cause the formation of genetic lesions, such as cyclobutane pyrimidine dimers (CPDs) and 8-hydroxy-2'-deoxyguanine, which in turn can impair signalling pathways and cell functions (e.g. Roberts & Beasley 1997; Yeun, Nearn & Halliday 2002; Dinkova-Kostova 2008; Halliday & Rana 2008; Murphy 2009; Schwartz 2010). CPDs have been linked to the suppression of T memory cells and thus impaired immune surveillance (Kuchel, Barnetson & Halliday 2005). In addition, sunlight UV can induce T cells with suppressor activity, trigger keratinocytes and mast cells to release immunosuppressive cytokines such as interleukin 10 (IL-10), cause the isomerisation of *trans*-urocanic acid to its *cis*-isomer – a systemic immunosuppressant which impairs contact hypersensitivity, and inhibit the function of antigen-presenting cells (Dinkova-Kostova 2008; Halliday & Rana 2008; Murphy 2009; Schwartz 2010). We did not, however, observe UV-induced immunosuppression in birds exposed to simulated sunlight UV whilst receiving supplemental carotenoids (UV+ Car+). Similarly, in humans, UV-induced immunosuppression can be prevented by dietary carotenoid supplementation (e.g. Alaluf *et al.* 2002; Sies & Stahl 2007). This latter effect is believed to arise because carotenoids are allocated to integument where they

directly protect against UV. In contrast, in our study, carotenoid supplementation failed to maintain or increase bill carotenoids in UV-exposed zebra finches. It therefore seems likely that the observed mitigatory effect of carotenoids on UV-induced immunosuppression arose through direct interactions between carotenoids and immune cells in circulation. Positive effects of carotenoids on inflammatory immune responses have been reported previously in birds, including zebra finches (e.g. Blount *et al.* 2003; McGraw & Ardia 2003; McGraw, Nolan & Crino 2011). Indeed, we found that plasma carotenoid concentrations predicted the size of PHA responses in all treatment groups. This could explain why exposure to simulated sunlight UV appeared to trigger the mobilisation of carotenoids from liver storage in UV+ birds (see Fig. 1b,c), but if this was the purpose of such mobilisation, it was insufficient to maintain immunity to the same level seen in birds exposed only to human-visible wavelengths.

Carotenoids have been hypothesised to confer photoprotection to tissues through localised antioxidant activity *in vivo*, in addition to absorbing and scattering light (Alaluf *et al.* 2002; Sies & Stahl 2007; Dinkova-Kostova 2008). Both processes can result in destruction of carotenoids, and experimental evidence for a specific antioxidant protection role is lacking. The prevailing view is that carotenoids are in general only minor antioxidants in birds *in vivo* (e.g. Isaksson *et al.* 2007; Costantini & Møller 2008). Consistent with this, we found no relationships between bill coloration metrics and concentrations of MDA, or between plasma concentrations of carotenoids and MDA. We cannot exclude the possibility that other biomolecules (e.g. proteins) in bill tissue could be susceptible to oxidative damage caused by sunlight. However, as carotenoids are lipophilic molecules, their antioxidant activity (if any) in tissue seems most likely to be revealed by assaying lipid peroxidation than any alternative marker of oxidative damage.

In conclusion, the results of this study suggest that sunlight exposure is likely to be an important cause of depletion of immunity and carotenoid coloration in birds. Both human-visible and UV wavelengths apparently caused reductions in carotenoid coloration, and UV specifically depressed immunity. Carotenoid-based sexual signals in bare parts could therefore be indicators of sunlight exposure and its effects on immunity. Diurnal species of birds depend on sunlight for vision and to perform essential tasks such as foraging and mate choice (Cuthill *et al.* 2000). The hypothesis that animals may face physiological trade-offs in the allocation of carotenoids to sexual signals vs. immune defence (Lozano 1994) may explain, at least in part, the evolution and information content of carotenoid-based sexual signals. However, the capacity for light exposure to influence the physiological availability and utilisation of carotenoids raises some interesting new questions. For example, do birds behaviourally regulate their exposure to sunlight to avoid excessive damage to signals and immunity, seeking greater access to shade where carotenoid supply is limiting, or where the risk of disease is higher? If so, might this trade-off against important activities such as the time spent signalling? It would be interesting to

investigate whether photobleaching of carotenoids in metabolically inert tissue such as grown feathers, as affected by sunlight exposure, may correlate with immunity. More generally, explanations for interspecific patterns of carotenoid coloration have been elusive (Olson & Owens 2005). Given that sunlight exposure is ubiquitous, yet may vary markedly according to life history and ecology (e.g. latitude, altitude, habitat openness), it would be interesting to assess the evolutionary relationships between sunlight exposure, carotenoid coloration and immunity across species.

Acknowledgements

We thank S. Pickett, W. Schuett, L. Shaw and N. Westbury-Harris for help with bird husbandry, N.J. Royle for supplying parental stock, and C. Mitchell for assistance with laboratory analyses. JDB was supported by a Royal Society Research Fellowship, and TWP by a Natural Environment Research Council Fellowship. Experimental procedures were carried out under license from the UK Home Office. We are grateful to John A. Endler and James Allen for stimulating discussions, and the anonymous referees for helpful comments on an earlier draft.

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Received 12 April 2011; accepted 16 September 2011

Handling Editor: Kevin McGraw

Supporting Information

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

Methods S1. Full methodological details.

Table S1. Age, bill colouration, body mass, tarsus length, and plasma carotenoid concentrations at the start of the experiment.

Fig. S1. Normalised irradiance spectrum of the UVA-340 lamp (faint solid line), and the standard D65 illuminant defined by the International Commission on Illumination (CIE) which corresponds approximately to midday sunlight in Western Europe (thick solid line).

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