The Journal of Experimental Biology 211, 1257-1261
Published by The Company of Biologists 2008
doi:10.1242/jeb.015065

Carotenoid intake does not mediate a relationship between reactive oxygen species and bright colouration: experimental test in a lizard

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Accepted 27 January 2008

SUMMARY

We performed experiments on male Australian painted dragon lizards (*Ctenophorus pictus*) to test the hypothesis that carotenoids can scavenge reactive oxygen species (ROS), protecting the organism from oxidative stress, and that this capacity is reflected in skin colours involved in signalling. Subsequent to 4 weeks of carotenoid treatment we used flow cytometry to analyse unspecified ROS (H_2O_2, singlet oxygen, superoxide and peroxynitrite level), hereafter termed ROS, and baseline superoxide specifically (bSO in peripheral blood cells). Mean background levels of ROS and bSO did not differ between carotenoid-treated and control males. bSO, which represents the superoxide level in un-manipulated blood, was negatively correlated with colour development in all males, regardless of carotenoid treatment. Thus, carotenoid intake does not reduce circulating levels of ROS or bSO, suggesting that carotenoids are inefficient antioxidants *in vivo* and, therefore, are unlikely to provide a direct link between oxidative stress and colouration.

Key words: reactive oxygen species (ROS), carotenoids, colouration, lizards.

INTRODUCTION

In a paper well ahead of its time, von Schantz et al. (von Schantz et al., 1999) introduced the idea that the expression of male secondary sexual traits may be constrained by oxidative stress of toxic metabolic by-products, e.g. reactive oxygen species (ROS), and that tolerance towards these compounds initiated by the adaptive immune and detoxication systems may be a criterion for female mate choice. This idea is logically appealing, since male colouration may function as an indicator to females of male 'quality' on these vital aspects of organismic function (von Schantz et al., 1999).

More than ten major ROS are potential health hazards to vertebrates (Peto et al., 1981; Mayne, 1996; Whalley, 2001; Matsuo and Kakato, 2000) and are countered by two principally different systems: antioxidation and oxidation damage repair (Matsuo and Kakato, 2000). The antioxidant defence, which we are primarily concerned with in the current paper, is made up of three different components: (i) preventive antioxidant enzymes (e.g. superoxide dismutase), (ii) metal sequestration (e.g. transferrin), and (iii) dietary antioxidants [e.g. α-tocopherol (vitamin E), carotenoids and ascorbic acid (vitamin C)] (Matsuo and Kakato, 2000). The latter category, the most relevant for this study, can be further categorised with respect to where they primarily occur. Carotenoids and vitamin E (lipid soluble) are mostly active in biological membranes and lipoproteins, whereas ascorbic acid (water soluble) is mostly active extracellularly and in the cytosol (Matsuo and Kakato, 2000). The relative capacity of these systems for antioxidation of ROS is unstudied in reptiles, but in humans, for example, ascorbic acid is recognised as the most effective plasma antioxidant (Alexandrova and Bochev, 2005).

The function of carotenoids as an indicator of male quality in sexual displays and evolution of mate preferences relies on relatively straightforward, positive cytological carotenoid effects. The published literature, however, seems to reveal a more complex picture. Although plasma and tissue levels of vitamin C and E can be reduced by 35–75% following immune responses to infectious disease (Omenn et al., 1996), these effects may arise in more than one way, of which some but not all may link antioxidation and secondary sexual trait expression. For example, carotenoids may have strongly detrimental effects, e.g. significantly increasing the risk of lung cancer and cardiovascular disease, and the concomitant risk of mortality in humans (Omenn et al., 1996). Thus, if carotenoids even have negative health and viability effects in some circumstances, their link between health and colouration may be a 'red herring' to researchers in sexual selection signalling [i.e. in this scenario, not accurately reflecting underlying health status (Hartley and Kennedy, 2004; Isaksson et al., 2007)].

Previous work has successfully demonstrated positive carotenoid effects on immune function (Matsuo and Kakato, 2000), reproductive output (Blount, 2004) and behaviour, for example flight capacity (Blount and Matheson, 2006). Few studies have, however, focused on assessing circulating ROS levels and their effects on colouration [but see McGraw et al. (McGraw et al., 2005b) for an example of TBARS test demonstration of lipid peroxidation effects on colouration, and Isaksson et al. (Isaksson et al., 2007) for links between colouration and total antioxidant activity]. In the current paper, we tested the prediction, using flow cytometry, that ingestion of carotenoids at biologically realistic levels and time frames may reduce the level of ROS in the blood, and have concomitant effects on male colouration in the polychromatic (red *versus* yellow) painted dragon lizard (*Ctenophorus pictus*) from Australia. This species was chosen because it is one of the most brilliantly coloured lizards on the Australian continent (Cogger, 2000), and therefore a suitable

model system for assessing carotenoid effects on ROS, and for illustrating ROS level effects on colouration.

To analyse ROS, we used flow cytometry in combination with two probes that freely diffuse into cells, accumulate within mitochondria, and become fluorescent when oxidised by different ROS: dihydrorhodamine 123 (DHR) is oxidised by singlet oxygen, superoxide, H_2O_2 or peroxynitrite [hereafter unspecified ROS (Spence, 2005; Vowells et al., 1995)], while MitoSOX Red is sensitive to superoxide specifically, and was used to measure basal superoxide level (bSO).

MATERIALS AND METHODS

Study species and husbandry

We studied ROS removal by carotenoids in whole blood in the Australian painted dragon lizard (*Ctenophorus pictus* Peters 1866), a polymorphic species in which males occur with red and yellow heads (Cogger, 2000). Painted dragons are sexually dimorphic (females are cryptically greyish-brown), and the dazzling colours of the adult males are used for signalling to deter approaching rivals (Healey et al., 2007; Olsson et al., 2007). Polymorphism is correlated with differences in behavioural traits, such as dominance (Healey et al., 2007). The lizards in our experiments (N=43) were all caught a week before the onset of the experiments (October, 2005) by noose or by hand in homogeneous habitat with respect to colour morph spatial distribution (desert *Spinifex* and mallee) at Yathong Nature Reserve, New South Wales (145°35′E; 32°35′S) and were brought back to holding facilities at the University of Wollongong. Specific sample sizes are given for each statistical test (see Results), since two samples were accidentally lost during flow cytometry preparations. The lizards were kept in cages (330 mm×520 mm×360 mm), on a 12 h:12 h light regime (light:dark), with a spotlight at one end of the cage to allow thermoregulation to the preferred body temperature, and fed crickets and meal worms to satiation at 09:00–10:00 h every second day. Males were weighed to the nearest 0.01 g and measured snout to vent to the nearest 1.0 mm, and body condition was calculated as residuals from a mass–snout–vent length regression. There were no differences between colour morphs or treatment groups in male mass, snout–vent length or body condition (P>0.19 for all analyses), nor was there an effect of their interaction on any of these variables (P>0.11 for all three analyses). Blood was sampled at 09:00–10:00 h on the day of flow cytometry in random order of male colour and treatment category (see below for description). A blood vessel in the corner of the mouth, *vena angularis*, was punctured with a syringe (1.20 mm×50 mm BC/SB) and the blood collected in a capillary tube (for details, see Olsson, 1994), then transferred to PBS buffer and put on ice until flow cytometry analysis. Apart from seven animals that were killed for biochemical analysis, all lizards were returned to the wild subsequent to the laboratory trials and released unharmed at the place of capture.

Carotenoid treatment

Our choice of carotenoid supplement was limited by current market availability and we therefore looked for support of its appropriateness in two ways. Firstly, we examined what carotenoids were deposited into the skin of male painted dragon lizards using HPLC (see below). This analysis verified that two of the main carotenoids in our supplement (lutein and zeaxanthin) were indeed deposited into the skin (see Results). Secondly, we calculated the concentration of the carotenoid supplementation (0.1 mg ml^{-1}) based on liver concentrations in another insectivorous lizard of the same approximate body size (Czeczuga, 1980), following methodology developed for supplementary studies in birds (Latscha, 1990). The carotenoid supplement contained trans-lutein (yellow), trans-capsanthin (red), beta-carotene and zeaxanthin (8 g kg^{-1}, OroGlo 8 Liquid, Kemin Industries, Inc., USA), diluted in sunflower oil (with no or only small traces of carotenoids). Using a pipette, males were fed 100 μl of the solution or sunflower oil (controls). Each supplementation took less than 10 s. No lizards were confirmed to regurgitate the carotenoid solution. Over 4 weeks, eleven supplementations were administered (Monday–Wednesday–Friday). This resulted in a total daily intake of 20 μg day^{-1} or 220 μg in total. All vitamin mixtures, which can potentially contain carotenoids, were excluded during the experiment.

Measurements of plasma and skin colour

Plasma colour

Blood samples (100 μl) were taken once, after the experimental treatment; 30 μl of this sample was used for assessing plasma colour, whilst 70 μl was used for flow cytometry analysis (see below). Plasma colour was qualitatively measured blindly by matching the colour of plasma from whole blood centrifuged at 5000 r.p.m. for 6 min to Munsell colour charts (Olsson, 1994). Seven randomly selected samples were analysed for spectrophotometric (Smartspec Plus, BioRad) congruence with ocular inspection at wavelengths where peak absorption is expected for lutein (λ=448 nm) and zeaxanthin (λ=552 nm), and correlated with their corresponding Munsell scores under the directional prediction of a positive relationship. This was verified in both cases (r=0.75, P=0.026, and r=0.74, P=0.030, respectively, one-tailed test). This analysis was admittedly less sensitive than for example HPLC but was only performed to qualitatively confirm the well-established fact that carotenoids are taken up from the diet and circulated in the bloodstream (e.g. Mayne, 1996). This was also clear from our data (see Results). No samples contained lysed erythrocytes (which could influence colouration).

Head colour

Skin colour measurements were made twice (at the beginning and end of the experiment) at the preferred lizard body temperature (~37°C) with a USB2000 spectrometer system (Ocean Optics Inc., Dunedin, FL, USA), using a PX-2 pulsed xenon lamp as a light source together with an R200 fibre optic reflectance probe fitted with a cylindrical plastic sheath to block out external light. A dark and a reference scan from a WS-2 white standard (>98% reflectance within wavelengths of 300–800 nm) were obtained before each individual was measured. The fibre optic probe was held at a 90° angle against the skin, using a fixed stand as a landmark to increase sampling repeatability. Using OOIBase32 spec software (Ocean Optics Inc.), we sampled three times in the same location on the head (removing the probe between each measurement) and used the average measurement for our analyses.

Colorimetric calculations

From the raw spectral reflectance data, we computed spectral purity ('chroma'; $(R_{max}–R_{min})/R_{average}$) in the area from 420 nm to 700 nm for head colour, which is an indication of carotenoid pigments incorporated into the integument (Johnsen et al., 2003; Andersson and Prager, 2006).

Carotenoid extraction and HPLC analysis of painted dragon skins

Seven lizards were killed by an overdose of Brietal and the skin was immediately excised from the body, weighed and then stored

overnight in 0.5 ml acetone. All skin carotenoids dissolved in the acetone phase. The following day the acetone was filtered (0.2 μm syringe filter, 13 mm GHP Acrodisc) into a new tube. For saponification, 100 μl ascorbic acid (10%) and 200 μl KOH were added and kept at 70°C for 30 min. The yellow upper phase was evaporated to dryness under nitrogen gas. The carotenoid residue was finally dissolved in 20 μl tetrahydrofuran (THF) and 80 μl of the mobile phase (70:30 acetonitrile:methanol), and immediately analysed by high performance liquid chromatography (HPLC, see below).

Part of the sample (60 μl) was injected with the isocratic mobile phase into an RP-18 column (ODS-AL, 150 mm×4.0 mm i.d., YMC Europe GmbH, Schermbeck, Germany), fitted on a ThermoFinnigan (San Jose, CA, USA) HPLC system with PS4000 ternary pump, AS3000 autosampler and UV6000 diode-array UV/VIS detector. Column temperature was maintained at 30°C and the flow-rate at 0.6 ml min^{-1}. Two- (at 450 nm) and three-dimensional (300–700 nm) chromatograms were obtained and analysed with ChromQuest 4.0 software (ThermoFinnigan). The major pigment fractions were identified and quantified by comparison to internal standards and calibration curves of lutein (β,ε-carotene-3,3′-diol) and zeaxanthin (β,β-carotene-3,3′-diol), kindly provided by Roche Vitamims Inc. (Basel, Switzerland). All concentrations were calculated as μg g^{-1} dry skin.

After the carotenoid extraction, the red-headed morph still had visible red pigments, whereas yellow-headed skin patches were colourless. To investigate whether or not the pigments were of carotenoid origin we continued by using a separation method described by McGraw and Ardia (McGraw and Ardia, 2003) and McGraw et al. (McGraw et al., 2005a). Small pieces of skin from the pigmented areas of the head were digested by adding acidified pyridine, into which the pigments were released. Adding an organic solvent to the solution makes it possible to separate the lipid-soluble carotenoids from other pigments. If carotenoids were present, the upper phase should be coloured, which we verified for yellow pigments. Red pigments, however, became deposited in the lower (water) phase, suggesting that they were not carotenoids [possibly pteridines (Steffen and McGraw, 2007)], and, in hindsight, that our treatment possibly did not provide a component for directly boosting red colour production [since pteridines are synthesised from basic purine, e.g. guanine (Steffen and McGraw, 2007)].

Measuring ROS of blood cells by flow cytometry

After administering the final diet treatment, the single sample of peripheral blood (70 μl) was diluted immediately with 9 volumes of phosphate buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 8 mmol l^{-1} Na_2HPO_4, pH 7.4) and stored on ice prior to analyses, which were completed within 4 h of sampling. Prior to staining, diluted blood was diluted a further 50-fold with PBS and then centrifuged (300 ***g*** for 5 min) to pellet cells; each cell pellet corresponded to 10 μl of whole blood. Cells were resuspended in 100 μl of PBS containing one of the following: no additions (unstained control), 0.1 mmol l^{-1} dihydrorhodamine 123 (DHR; Molecular Probes, Invitrogen, USA) or 5 μmol l^{-1} MitoSOX Red (MR; Molecular Probes). DHR and MR were added from stock solutions in dimethylsulphoxide (DMSO); the final concentration of DMSO was 0.2% (v/v) or less. Cells were subsequently incubated at 37°C for 30 min, then washed with PBS by centrifugation as described above and held on ice until analysed by flow cytometry; 50 000 events were acquired for all samples. Flow cytometry was performed using a Becton Dickinson LSR II (Becton Dickinson, Sydney, Australia), with excitation at 488 nm and emitted fluorescence collected using bandpass filters of 515±10 nm (DHR) and 575±13 nm (MR). Data were acquired and analysed using FACSDiva v4.0.1 and CellQuest Pro v5.1.1 software (Becton Dickinson), respectively. On the basis of forward angle laser scatter and side angle laser scatter, a number of blood cell populations were discerned; the results obtained were similar for all these populations. For each sample, the arithmetic mean fluorescence for all 50 000 cells acquired was determined using CellQuest software and used to compare between samples and treatments. Repeatability of the flow cytometry result for samples from the same individuals was measured in a separate experiment.

RESULTS

Plasma colouration

Plasma chroma was significantly higher in carotenoid-treated males compared with controls (mean ± s.e.m. score 5.8±0.34, N=19, *vs* 3.5±0.32, N=17, respectively), while there was no significant effect of male morph (two-factor ANOVA, R^2=0.40, model $F_{3,31}$=6.8, P=0.0012; type III statistics, treatment (carotenoid *vs* control) F=20.4, P=0.0001, d.f.=1; colouration (red *vs* yellow) F=0.01, P>0.9, d.f.=1; treatment × colour morph $F\approx0.0$, P>0.99, d.f.=1). Thus, carotenoid levels were successfully elevated in carotenoid-treated males.

Test of flow cytometry accuracy

In a separate experiment involving 14 males, we took two blood samples (A and B) independent of each other and looked for a correlation between samples A and B. For bSO, the between-sample correlation was r=0.97 (P<0.0001), and for ROS it was r=0.80 (P=0.0006). Thus, our flow cytometry technique was highly repeatable.

Effects of carotenoids and male colouration on ROS

bSO and ROS, body mass (g) and body condition (residuals from a mass–snout–vent length regression) were all normally distributed (Wilks lambda, W, normal: 0.97, P=0.67, ROS, N=34; 0.96, P=0.35, bSO, N=35; 0.94, P=0.06, mass, N=34; and 0.97, P=0.58, condition, N=34). Body mass and body condition were not significantly correlated with ROS or bSO (Pearson's product moment correlations, $-0.33<r<0.15$, $0.22<P<0.94$).

A two-factor ANOVA (carotenoid treatment, colour morph) with interaction term showed no effect on bSO of treatment, colour morph or their interaction (Table 1; mean ± s.e.m. bSO counts, 6.2±0.29, N=18, and 6.0±0.18, N=17, for carotenoid-treated and control males, respectively). For ROS, a similar result was obtained with no effect of treatment, colour morph, or treatment × colour morph (Table 1; mean ± s.e.m. ROS counts, 396±16, N=19, and 405±19, N=15, carotenoid-treated and control males, respectively).

For the above analyses, Tukey's studentised range tests were performed as *post hoc* tests but revealed no difference in means between the two treatments or the two colour morphs for bSO or ROS (P>0.05 for all comparisons; the following values are the minimum significant differences, MSD, at α=0.05 followed by the real differences between trait categories: bSO, treatment MSD=0.73 *vs* 0.26, colour MSD=0.74 *vs* 0.12; ROS, treatment MSD=52.1 *vs* 10.3, colour MSD=52.1 *vs* 9.0).

Skin carotenoids and colouration

Seven males were analysed using HPLC, resulting in identification of a mean content of lutein of 3.06 μg g^{-1} dry skin (±1.66 s.d.; λ_{max}=448, retention time=4.3 min) and 1.06 μg g^{-1} zeaxanthin (±0.94 s.d.; λ_{max}=452, retention time=4.6 min).

Table 1. Effects of carotenoids and male colouration on ROS

	d.f.	SS	MS	*F*	*P*
Response variable: bSO; model $F_{3,30}$=0.76, *P*=0.52					
Treatment	1	0.32	0.32	0.29	0.59
Colour	1	0.11	0.11	0.10	0.75
Treatment × colour	1	1.84	1.84	1.68	0.20
Response variable: ROS; model $F_{3,29}$=0.39, *P*=0.76					
Treatment	1	524.3	524.3	0.10	0.76
Colour	1	360.3	360.26	0.07	0.80
Treatment × colour	1	4738.3	4738.3	0.89	0.35

The table shows the results of two-factorial ANOVAs with interaction terms testing the effects of two fixed factors, male natural head colour (yellow or red) and treatment (carotenoid supplement or control), on the levels of reactive oxygen species (ROS) in red blood cells of male painted dragons after 4 weeks of treatment (ROS: H_2O_2, singlet oxygen, superoxide and peroxynitrite; bSO, basal superoxide level). Type III test statistics are given for independent tests of factors and interaction terms. SS, sum of squares; MS, mean squares.

The effects of carotenoid treatment, natural colouration and their interaction on the change in head chroma through the experiment were analysed in a two-factor ANCOVA with the estimate of chroma at the onset of the experiment as a covariate. None of the factors, or their interaction, significantly influenced head chroma at the end of the treatment, whereas the covariate (colour at onset) did have a significant effect (two-factor ANCOVA, model $F_{4,28}$=7.86, *P*=0.0002, R^2=0.53; type III statistics, treatment *F*=0.73, *P*=0.40, d.f.=1; colour morph *F*=0.78, *P*=0.38, d.f.=1; treatment × colour morph *F*=1.8, *P*=0.19, d.f.=1; chroma at onset of experiment, *F*=25.6, *P*<0.0001, d.f.=1).

We then analysed the change in chroma in response to bSO levels and colour morph with the covariates chroma at onset of experiment and body condition (two-factor ANCOVA, model $F_{5,24}$=8.85, *P*<0.0001, R^2=0.65; type III statistics, colour morph *F*=1.43, *P*=0.40, d.f.=1; bSO *F*=8.22, *P*=0.008, d.f.=1; colour morph × bSO *F*=2.05, *P*=0.16, d.f.=1; chroma at onset of experiment, *F*=40.2, *P*<0.0001, d.f.=1; body condition *F*=0.22, *P*=0.64, d.f.=1). Thus, this analysis demonstrated a significant effect of bSO on colouration, as also reflected in a negative correlation coefficient between bSO and head chroma development (*r*=–0.45, *P*=0.012, *N*=31, controlling for body condition in a Pearson's partial correlation analysis, since condition may reflect resource availability necessary for the expression of colouration; Fig. 1).

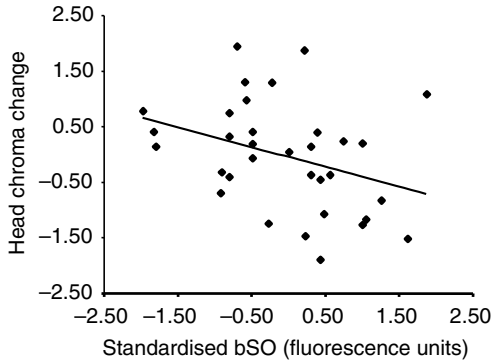

Fig. 1. Male baseline levels of superoxide (bSO) at the end of the carotenoid experiment and its relationship with head chroma change during the corresponding time (mean after – before treatment reflectance measurements; see Materials and methods). Since male natural colour morphs (red, yellow) differ in these trait values, we used measurements standardised for natural colour. The relationship was also statistically significant when we controlled for body condition (*r*=–0.45, *P*=0.012, *N*=31).

The corresponding analysis for a change in chroma in response to ROS, however, showed no effect of ROS or other predictors except chroma at the onset of the experiment (two-factor ANOVA, model $F_{5,23}$=6.48, *P*=0.0007, R^2=0.58; type III statistics, colour morph *F*=0.07, *P*=0.79, d.f.=1; ROS *F*=2.01, *P*=0.17, d.f.=1; colour morph × ROS *F*=0.00, *P*=0.95, d.f.=1; chroma at onset of experiment, *F*=28.2, *P*<0.0001, d.f.=1; body condition *F*=0.20, *P*=0.66, d.f.=1). The corresponding series of analysis on change in hue from before to after the experiment showed no significant effects of any other variables (*P*>0.20) except for hue at the onset of the experiment, which was significant in both analyses (*P*<0.0003 in all cases).

DISCUSSION

Previous work has shown that carotenoid supplementation may lead to sexually selected fitness benefits, manifested *via* increased immunocompetence (Alonso-Alvarez et al., 2004; McGraw, 2005). For example, Blount et al. (Blount et al., 2003) demonstrated that carotenoid-treated male zebra finches had better cell-mediated immune function and were more sexually attractive than controls; this was later replicated by others (McGraw and Arida, 2003). However, in humans, large prospective studies have failed to show an effect of β-carotene on the health of well-nourished people with a balanced diet (Hughes, 2001) and subsequent analysis of carotenoid-induced protection against oxidative stress in birds have provided similar results (Hõrak et al., 2006; Constantinti et al., 2007). Other studies with a very similar treatment duration to ours (3–4 weeks) suggest that carotenoids may even have directly harmful effects under some conditions [e.g. in vitamin C-deprived patients (Omenn et al., 1996)]. This has contributed to the unclear role of ROS as moderators of sexually selected traits, and of carotenoids in countering their systemic effects on health in general and colour in particular (Hartley and Kennedy, 2004; Isaksson et al., 2007). The summed negative effects of ROS on aspects of immunocompetence have previously been measured (Kurtz et al., 2006), and one study has assessed the effects of lipid peroxidation in the context of sexual signalling (McGraw et al., 2005b). However, not a single study has to the best of our knowledge measured the circulating levels of ROS subsequent to antioxidant treatment and concomitant effects on colouration. Thus, a crucial assumption of ROS-induced ill-health and colouration, namely that ROS levels decline following carotenoid administration, remains untested.

Our study demonstrates no overall effect of carotenoid treatment on circulating ROS levels or bSO. Thus, there is no evidence that dietary carotenoids reduce circulating levels of free radicals, thereby questioning the efficiency of carotenoids as antioxidants *in vivo*. Nevertheless, since there was still a negative correlation between the change in skin chroma across the experimental period and bSO across all males, which could indicate that males already had sufficient carotenoids for colour development, this suggests that some additional factor(s) to carotenoids links colouration and superoxide exposure. We do not know the underlying causative mechanism, but it appears less likely to be a direct effect of superoxide on immune function, with concomitant pathogenicity,

since these males were all in good health and showed no signs of disease or parasite infestation (whether they would react differently if unhealthy or not in physiological homeostasis we do not know). However, one potential explanation is that males varied in superoxide levels due to events prior to our experiments (and that our carotenoid treatment did not counter this effect). Such variation could come about through the production of ROS during innate immune responses, with potential tissue damage effects and reduced colouration as a result (Bertrand et al., 2006). However, for this to be important, it seems likely that superoxide effects would have to remain high for longer periods of time than is likely in this study (and in the wild). Alternatively, variation in superoxide among males could be due to variation in the production of superoxide dismutase (SOD), i.e. the endogenously produced antioxidant controlling superoxide levels. If SOD production is costly, and/or traded off against active deposition of carotenoids in the integument, then this may also contribute to reduced colouration in males with higher superoxide levels.

In conclusion, consumption of carotenoids has no or limited effect on circulating baseline levels of ROS or superoxide in physiologically unchallenged lizards. Thus, although basal superoxide indeed influences the maintenance of colouration, it remains to be demonstrated that animals in natural populations can access and utilise natural carotenoids to the extent that this depresses circulating levels of ROS, in particular bSO, and that it has concomitant effects on sexually selected traits.

We thank E. and G. Snaith for logistic support, G. Swan for field assistance, and the Australian Research Council (M.O.) and Wenner-Gren Foundations (T.U.) for funding support. All experimental work complies with the current laws of Australia (under permit from the Animal Ethics Committee, University of Wollongong, AE04/03-05). We thank two anonymous reviewers for comments that improved the quality of the manuscript.

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