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Phytohaemagglutinin (PHA) response and bill-hue wavelength increase with carotenoid supplementation in Diamond Firetails (*Stagonopleura guttata*)

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Abstract. Carotenoids cannot be synthesised by vertebrates and must be derived from the diet. A growing amount of research has focussed on the role of carotenoids in immune function, as well as the signalling function of carotenoids in the colour of the integument (skin, bill and legs) and feathers. We used an experimental approach to examine the effects of experimental reduction and supplementation of dietary carotenoids on phytohaemagglutinin (PHA) response (as an estimate of cell-mediated immune response) and colour of the bill in the Diamond Firetail (*Stagonopleura guttata*). To demonstrate the biological relevance of our study, we first show that carotenoid concentrations of our captive experimental birds did not differ significantly from those of wild birds. We found that carotenoids circulating in the plasma of captive Diamond Firetails dropped to 50% of the original concentration in less than 5 days when on a carotenoid-free diet. The mean level of total carotenoids in the plasma stabilised at 6% of the original concentration after 26 days. Finally, Diamond Firetails showed a significant increase in PHA response and increased bill-hue wavelength while on either a lutein or a zeaxanthin supplemented diet. Our results show rapid signalling of carotenoid concentration in the integument (as measured here in the bill) that we suggest complements the longer term signalling potential of feathers.

Introduction

Carotenoids are natural, fat-soluble products with antioxidant properties that modulate the immune response (Møller *et al*. 2000). In birds they are deposited in the integument (bill, skin and legs) and in feathers. The resulting colours often reflect the health of the individual, and may be used in visual displays because they are reliable indicators of quality (Lozano 1994; McGraw 2006). Vertebrates are unable to synthesise carotenoids and must obtain them from their diet (Goodwin 1984). Carotenoids obtained in the diet can be modified enzymatically to produce metabolically derived carotenoids (McGraw 2006). Each carotenoid has different biochemical properties and may be differentially involved in a variety of physiological processes (Goodwin 1984).

The literature on carotenoid-based colouration in birds has largely focussed on plumage (McGraw 2006), but this bias does not reflect the preponderance of carotenoid-based integumentary ornamentation. Forty percent of all birds have carotenoid-based plumage ornamentation, whereas 80% have carotenoid-based integumentary ornamentation (Bennett and Owens 2002). Carotenoids deposited in feathers are non-recoverable and their impact on the colour of a feather is fixed for the life of the individual feather (i.e. until the next moult; Norris *et al*. 2004; Serra *et al*. 2007), though it should be noted that feathers do degrade over time (McNett and Marchetti 2005). On the other hand, carotenoids deposited in the integument may be recovered for use elsewhere in the body (though this has yet to be shown). Thus, body parts with carotenoid-based colouration may provide a shorter term indication of the nutritive and health status of a bird than feather-colour. How quickly the carotenoid content, and thus signalling colours, of these integumentary parts change has rarely been measured.

Carotenoids are widely assumed to be in limited supply for maintenance of health, expression of sexual colouration, and reproduction (Grether *et al.* 1999; Hudon and Millie 1999; Hill *et al.* 2002). Carotenoids have many heath functions within the body, which is why carotenoid-based colouration is considered costly as colouration of body parts may result in a trade-off against health (reviewed in Olson and Owens 1998). Integumentary colours should reflect current health status, at least as it relates to carotenoid availability, and can be quickly compared with plumage colouration.

Animals may acquire several different xanthophylls and carotenes from their diet (Goodwin 1984). Among birds, lutein and zeaxanthin are the two most common carotenoids found in the diet and plasma of many birds (Goodwin 1984; McGraw and Schuetz 2004). Lutein and zeaxanthin are major precursors for the metabolic production of red ketocarotenoids that enrich the colour of the red beaks of many estrildid finches (McGraw *et al*. 2002; I. Stirnemann, unpubl. data). Zeaxanthin

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is expected to have greater antioxidant capacity than lutein owing to an extra conjugated double bond (Britton 1995). The complex interactions between dietary carotenoids, circulating carotenoids, antioxidant capacity, immune modulation and colouration (Møller *et al.* 2000; Chew 2004; Cohen and McGraw 2009) suggest that differences in the antioxidant potential of lutein and zeaxanthin may influence the trade-off between immune response and colouration (McGraw and Ardia 2004). Dietary supplementation is a widely used approach to examine physiological function rapidly (Casagrande *et al.* 2007). For example, many studies have used carotenoid manipulations in Zebra Finches (*Taeniopygia guttata*) to measure its impacts on signalling and physiology (e.g. Blount *et al.* 2003; Alonso-Alvarez *et al*. 2004; Bertrand and Faivre 2006), whereas only one study (McGraw and Schuetz 2004) has examined the effect of carotenoids in the Diamond Firetail (Estrildidae : *Stagonopleura guttata*), an Australian estrildid finch.

In this paper we examine the short-term signalling effects (bill-colour) and correlates of cell-mediated immune phytohaemagglutinin response (PHA response) of carotenoid supplementation in Diamond Firetails. We (1) compare the carotenoids in plasma from wild and captive birds feeding on seed containing carotenoids; (2) document the rate at which carotenoids are depleted in the plasma following removal of carotenoids from the diet; and (3) experimentally explore the separate influence of lutein and zeaxanthin on circulating carotenoids, PHA response, and colour of the bill. We used PHA as a measure of the degree to which carotenoids may modulate a cell-mediated immune response or an inflammatory response (Martin *et al*. 2006).

Methods

Study species

Estrildid finches have been widely used to study carotenoids in birds because: (1) many finches have carotenoid-based colours to feathers, legs and bills (e.g. McGraw and Schuetz 2004); (2) colouration of the bill is known to play a role in mate-attraction in finches (e.g. Zebra Finch; reviewed in Møller *et al*. 2000); and (3) estrildids exhibit some variation in their abilities to modify metabolically carotenoids derived from their diet, which may cause a difference in the level of carotenoids and therefore carotenoid colouration in various lineages in the group (McGraw *et al.* 2002; McGraw and Schuetz 2004).

The Diamond Firetail is a small (mean weight 17 g) estrildid finch that occurs in south-eastern Australia (Higgins *et al*. 2006). Both males and females have a crimson bill. Diamond Firetails breed from August to February, over the austral spring and summer (Higgins *et al*. 2006), though there is only scant information on its reproductive biology in the wild (see McGuire and Kleindorfer 2007). Diamond Firetails feed on seeds, which, over much of its current range, are predominately of introduced grasses that have replaced the original diet of seeds of native grasses (Read 1994). The species has declined in abundance and distribution in some areas of Australia since the 1980s (Ford *et al*. 2001).

Wild Diamond Firetails

We mist-netted wild unsexed Diamond Firetails ($n = 9$) from Monarto Zoo, Monarto (35°8′S, 139°8′E), South Australia, during May 2006 between 0600 and 1100 hours (Australian Central Standard Time). Between 80 and 150 µL of blood was drawn from the jugular vein of each bird using a 29-gauge needle (0.33 mm) attached to a 1-mL insulin syringe (BD Unltrafine, Becton, Dickinson and Co, Trenton, NJ, USA) previously flushed with heparin. Plasma carotenoid levels were measured from the blood samples (see below).

Captive housing and experimental design

Twenty three captive-born adult Diamond Firetails were purchased from private aviculturists in Adelaide, South Australia. The birds had been fed on a variety of commercial finch seed mixes before our study. Birds were housed in 550 mm wide × 320 mm deep × 370 mm high cages (3 birds per cage: 1 male and 2 females or 2 males and 1 female) in a room under a natural daily light-cycle at Adelaide Zoo. Two dowel perches (12- and 8-mm diameter) were placed in each cage. Throughout the study, all birds were fed an artificial base diet of Wombaroo Finch Diet (Passwell, Mt Barker, South Australia) (see Table 1 for composition of the diet). This artificial food was used because it allowed manipulation of the carotenoid composition of the diet. Water was available *ad libitum*.

Initially all birds were fed a commercial seed diet for 2 weeks. The birds were then transferred to the carotenoid-free diet on 18 May 2006 for 55 days to measure the rate at which carotenoids were flushed from circulation. Blood was sampled from all individuals at the onset of this period (Day 0, $n = 23$).

We subsequently collected blood from a randomly selected sub-sample ($n = 4$–7) of the group every 8–10 days as circulating carotenoids decreased. If a bird had been sampled on the immediately preceding occasion, it was not sampled again. This sampling continued until mean carotenoid levels showed no further significant reduction, as determined by the last three

Table 1. Composition of the artificial diet fed to Diamond Firetails
A moist crumble mix made up of dry food and 8% water was feed to the birds daily. Caroteniods were not present in the carotenoid-free base diet. In the carotenoid-supplemented diets, either the carotenoid lutein or zeaxanthin was added to the base diet depending on the experimental treatment. The metabolisable energy of the artificial base diet of Wombaroo Finch Diet was obtained using the equation of Lasiewski and Dawson (1967): for a body mass of 17 g we calculated that each bird would consume 3.9–5.8 g of prepared food per day

	Dry food	Prepared food (dry food + 8% water)
Protein	18.3%	16.9%
Fat	7.6%	7.0%
Carbohydrate	59.5%	55.1%
Fibre	6.4%	5.9%
Moisture	4.5%	11.7%
Ash	3.7%	3.4%
Metabolisable energy (kJ g^{-1})	14.3	13.2
Caroteniod (mg kg^{-1})	30	27.8

samples (range of 3.9–5.9 mg L^{-1} for the last three samples) (Fig. 1).

All birds were then randomly assigned to one of three experimental treatments on 12 July 2006. The control group (3 females, 4 males) continued on the carotenoid-free diet. The other treatments were fed a lutein supplemented diet (4 females, 4 males) or a zeaxanthin supplemented diet (4 females, 4 males). Both carotenoids were obtained from DSM Nutritional Products (Wagga Wagga, NSW, Australia). In the carotenoid supplemented diets, 30 mg kg^{-1} was added to the base diet (Table 1). We used the equation of Lasiewski and Dawson (1967); for a body mass of 17 g we calculated that each bird would consume 3.9–5.8 g of prepared food per day. All captive birds were sexed using molecular markers (DNA Solutions Laboratory, Melbourne). For all captive finches in each treatment, we measured circulating carotenoids and PHA response after 9 days on each treatment. Bill-reflectance was also measured for all captive finches after 26 days for each treatment. All measurements were done blind for the spectrometry and PHA study to eliminate the possibility of measurement bias between treatments. During the study, all experimental birds maintained good body condition and health.

Extraction and identification of carotenoids

Blood was drawn from the jugular vein of each bird, as described for wild birds. The blood was transferred to heparinised micro-haematocrit tubes and centrifuged at 13 000 rpm for 3 min in a Hettich Mikro 12–24 centrifuge (DJB Labcare Ltd., Newport Pagnell, UK). After centrifugation, the separated plasma was transferred to a 1.5 mL microfuge tube. If carotenoid extraction was not performed immediately, then the plasma was stored at −20°C.

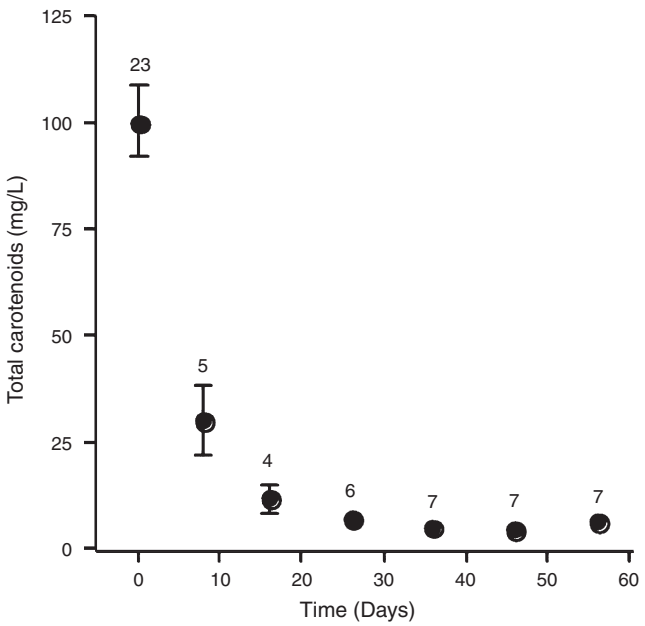

Fig. 1. Depletion of total circulating carotenoids in plasma (mg L^{-1}) of Diamond Firetails over time (days). Birds had been fed a commercial seed mix before being placed on an artificial diet containing no carotenoids on Day 0 of the depletion trial. Carotenoid concentration is shown as mean ± s.e. Sample sizes are shown above the data points.

A measured volume of plasma (up to 50 µL) was transferred to a clean Microfuge tube, extracted with 200 µL ethyl acetate and 200 µL absolute ethanol, vortexed and centrifuged at 1300 rpm for 1 min. The supernatant was removed to a clean Microfuge tube and the precipitated protein plug extracted with 500 µL hexane, vortexed and centrifuged at 1300 rpm for 1 min. The supernatant from the hexane extraction was added to that previously collected and the pooled extracts washed with 200 µL of demineralised water, vortexed and centrifuged at 13 000 rpm for 1 min. The organic layer was transferred to a clean Microfuge tube and the aqueous layer washed with 500 µL hexane, vortexed and centrifuged at 13 000 rpm for 1 min. The organic layer was added to that previously collected and the pooled organic extract dried over 100 mg of anhydrous sodium sulfate. The dried organic extract was vortexed, centrifuged at 13 000 rpm for 1 min and the supernatant removed to a 10 × 75 mm glass tube. The sodium sulfate residue was washed with 500 µL hexane, vortexed and centrifuged at 13 000 rpm for 1 min. This washing was added to the dried organic extract previously collected. The contents of the glass tube were evaporated to dryness at 65°C under vacuum. Reagents were Unilab (Auburn, Australia) analytical grade, and solvents Burdick and Jackson (Muskegon, MO, USA) HPLC grade.

The dried extract was dissolved in 55 µL of mobile phase and 50 µL was injected into a Varian Vista 5500 chromatograph (Varian, Mulgrave, Vic., Australia) fitted with an Alltech Apollo guard column (Alltech, Deerfield, IL, USA) (C18, 75 × 4.6 mm: particle size 5 µm) positioned ahead of an Apollo analytical column (C18, 250 × 4.6 mm: particle size 5 µm). The mobile phase consisted of 60% acetonitrile and 40% methanol and was applied at a flow rate of 1 mL min^{-1} for 8 min then graded to 24% acetonitrile, 16% methanol and 60% dichloromethane for a further 8.5 min. Carotenoids were detected at 450 nm. Chromatograph output was processed using Delta Chromatography Data Systems software (Delta Chromatography Data Systems, Fife, UK).

Carotenoids were identified and quantified from retention times and calibration curves for five reference carotenoids, which were were provided by DSM Nutritional Products: Astaxanthin, 5.5 min; Lutein, 7.2 min; Zeaxanthin, 7.6 min; Canthaxanthin, 10.6 min; β-carotene, 15.5 min. Calibration curves were constructed for each carotenoid by plotting the area under the curve (AUC) (uV s^{-1}) versus concentration (mg L^{-1}). Least-squares regression was performed to determine the slope, intercept and coefficient of determination for each carotenoid and mean values were taken as representative for all carotenoids quantified under the above chromatography conditions. Assay concentrations were calculated using the following formula (sample volume is in mL):

$$\text{Carotenoid concentration } (\mu\text{g mL}^{-1}) = (\text{AUC for assay} \times \text{standard concentration} \times \text{sample dilution}) / (\text{AUC for standard} \times \text{sample volume})$$

In addition to the amounts of the five referenced carotenoids we also measured total carotenoid concentration. These results are available upon request from the authors.

PHA response

PHA response is widely used as a measure of cell-mediated immuno-competence (Kennedy and Nager 2006; Martin *et al*. 2006). We measured PHA response using a standard protocol for birds (Smits *et al*. 1999). Feathers on the right wing were cleared with an alcohol swab to allow easy access to the wing web. Three measures of the patagium were taken before injection using a pressure sensitive spessimeter (accurate to nearest 0.01 mm; 573–191, Mitotoyo, Kawasaki, Kanagawa, Japan); we used the mean of these values for analysis. We injected 0.04 mL of a 5 mg mL^{-1} solution of PHA in phosphate buffered saline (PBS) subcutaneously using a 27-gauge needle. Each injection contained 0.2 mg PHA. Three measurements of patagium thickness were taken after 24 h to assess swelling, and the mean of these values was used for analysis. The reaction to PHA was assessed as the difference between the initial and final measurements of patagial thickness (Smits *et al*. 1999). PHA response was measured twice for each bird, on Day 0 of the onset of experiment (after birds had been fed on a carotenoid-free diet for 66 days), and on Day 9 after the experimental treatment began.

Bill-reflectance

The bill reflectance of Diamond Firetails was measured using a bifurcated fibre optical probe connected to an Ocean Optics USB4000 spectrometer and an Ocean Optics LS-1 tungsten halogen light source (Adelaide, SA, Australia), which provides reliable illumination in the human visible spectrum (400–750 nm). We recognise our measurement in the human visible range as a limitation of the current study. Given that aviculturists often use bill-colour during the breeding season as an indirect method of determination of sex, our findings are relevant to aviculture. Also, we are focusing specifically on carotenoid signalling. Very little work has been done on Diamond Firetails in general, and nothing is known about the possible role of UV reflectance for signalling in this species, which should be the focus of future study.

Reflectance measurements were expressed relative to a certified 99% diffuse white reference standard and the black current (electromagnetic disturbance) was subtracted from the spectrum. The fibre optical probe was mounted within an anodised aluminium probe holder which enabled repeatable measurements to be taken ~0.4 mm from the maxilla. Three consecutive scans (removing the bird from the probe between each scan) were taken and averaged; bill-reflectance was measured twice for each bird, at the beginning of the experiment (Day 0, after birds had been fed on a carotenoid-free diet for 66 Days) and on Day 26 after the experimental treatment had begun. All spectral data were averaged over each 5-nm wavelength-interval to facilitate manipulation of data. This also prevented noise from substantially affecting the colour calculations. All three replicates were averaged after calculating the colour indices.

From the spectral data we calculated three main indices of colour: hue, brightness and spectral purity. Hue (λR_{50}) was calculated as the wavelength halfway between the highest reflectance point and the lowest reflectance point ($\lambda R_{50} = \lambda R_{max} - \lambda R_{min})/2$). Brightness ($R_{avg}$) was calculated as the sum of all percentage reflectance values between 400 nm and 750 nm, divided by the number of values summed. Spectral purity (C_{max}) was measured as the maximum reflectance minus minimum reflectance divided by the average reflectance ($C_{max} = (R_{max} - R_{min})/R_{avg}$). Details on the methods used to analyse reflectance are provided in Montgomerie (2006). Measurements of the multiple scans for each of the three colour indices were highly repeatable ($r = 0.89$–0.93, $F_{50,60} = 47.2 - 75.8$, $P < 0.001$).

Statistical procedures

All data were normally distributed (normal Q–Q plots) and had equal variances (residual plots). The rate of carotenoid depletion was determined by fitting a power curve to a plot of circulating total carotenoids over time. A half-life of carotenoid depletion was calculated for these conditions. For the carotenoid experiment, we measured each bird's response as the difference between final and initial values. Differences in total circulating carotenoids, PHA response and bill-reflectance were analysed using univariate two-way analysis of variance (ANOVA) with experimental treatment and sex as the fixed factors. We used Tukey's *post hoc* test to examine pairwise comparisons. We acknowledge that the data are not independent because birds were housed together in cages, with up to three birds per cage. We therefore entered cage as a random effect in our mixed model. To assess repeatability of the PHA response at the individual level, we examined the maximum range for the difference in wing-web swelling values per bird, which was 0.15 mm with a range in standard deviation per bird of 0.026–0.072 mm across three samples, indicating high repeatability of the PHA response. We used two-tailed significance tests for all analyses in SPSS v14.0 (SPSS, Inc., Chicago, IL, USA). All descriptive statistics are reported as means ± standard error.

Results

Comparison of wild and captive Diamond Firetails

There was no significant difference in total circulating carotenoid concentration between wild Diamond Firetails sampled during May 2006 (78.61 ± 10.51 mg L^{-1}, $n = 9$) and captive birds fed on a commercial seed diet, at the same time, when we began a depletion trial (99.97 ± 8.22 mg L^{-1}, $n = 23$; ANOVA: $F_{1,31} = 2.00$, $P = 0.17$). There was also no significant difference between total carotenoid concentration in the wild birds and the captive birds on experimental carotenoid-enriched diets (both lutein and zeaxanthin) on Day 9 of the experiment (ANOVA: $F_{1,34} = 1.44$, $P = 0.24$; Table 1). Birds deprived of dietary carotenoids showed significantly lower circulating levels of carotenoids at the end of the depletion trial than wild and captive birds with access to carotenoids in their diet (Table 1).

Carotenoid depletion

At the beginning of the depletion trial, birds ($n = 23$) that had been fed on a commercial seed mix had 99.97 ± 8.22 mg L^{-1} of the total carotenoids circulating in the plasma (Fig. 1, Table 1). Most of this was lutein ($79\% \pm 6$) and zeaxanthin ($9\% \pm 1$), while the rest ($12\% \pm 1$) consisted of other,

metabolically derived carotenoids. Depletion of total circulating carotenoids showed a half-life of 4.75 Days. Total circulating carotenoids became constant after ~26 days at 6% of the original concentration (Fig. 1).

Effect of dietary carotenoids

Circulating carotenoids

Birds in the lutein and zeaxanthin treatments had significantly higher total carotenoid concentration than birds in the control group after 9 days (ANOVA: $F_{2,21} = 15.898$, $P < 0.001$; Table 1). Total carotenoid concentration did not differ between the lutein and zeaxanthin groups (Tukey's *post hoc* pairwise test, $P = 0.24$). There was no significant effect of sex or a significant sex × treatment interaction for total circulating carotenoids at the end of the experiment (all $P > 0.3$).

PHA response

A two-way ANOVA was used to examine the effects of sex and treatment group (control, lutein, zeaxanthin) on PHA response (the dependent variable) before treatment and during treatment. Before the treatment phase, all groups were on a non-carotenoid diet, with no significant effect of sex ($F_{1,21} = 0.73$, $P = 0.404$) or treatment group ($F_{2,21} = 0.16$, $P = 0.88$) on the PHA response. There was no significant difference between the pre-treatment PHA response and experimental control PHA response ($F_{1,29} = 1.31$, $P = 0.72$), which shows that the experimental control was valid. For the treatment phase we found no effect of sex on PHA response ($F_{1,21} = 0.89$, $P = 0.359$) but a significant effect of treatment group ($F_{2,21} = 14.99$, $P < 0.001$) (Fig. 2). The interaction term sex × treatment was not significant ($F_{2,21} = 1.23$, $P = 0.318$). The housing cage, entered as a random effect in the model, was not statistically significant ($F_{2,21} = 2.88$, $P = 0.109$).

Birds on a carotenoid-enriched diet (both lutein and zeaxanthin) had a significantly higher PHA response compared with birds in the control group (Fig. 2). Tukey's *post hoc* pairwise tests revealed a significant difference between the control and lutein ($P < 0.001$) and between the control and zeaxanthin ($P < 0.001$) groups. However, there was no significant difference between the PHA response in lutein and zeaxanthin treatment groups ($P = 0.87$). This was confirmed using a pairwise test for individuals within each treatment group: there was no significant difference between PHA response for individuals in the control group (comparing pre- *v.* post-treatment: paired *t* test: $t_6 = 0.15$, $P = 0.89$) but a significant difference for individuals in the lutein (paired *t* test: $t_7 = 8.53$, $P < 0.001$) and zeaxanthin (paired *t* test: $t_7 = -3.91$, $P = 0.006$) groups.

Bill colouration

At the onset of the experimental diet (Day 0; after birds had been fed on a carotenoid-free diet for 66 days), bill-brightness (R_{avg}) did not differ significantly between treatment groups before treatment (ANOVA: $F_{2,21} = 1.21$, $P = 0.29$) or during treatment (ANOVA: $F_{2,17} = 0.195$, $P = 0.824$). Bill-brightness was also not significantly different between the sexes before treatment (ANOVA: $F_{1,21} = 1.81$, $P = 0.0.21$) or during treatment (ANOVA: $F_{1,17} = 0.540$, $P = 0.473$).

The same pattern was found for bill-saturation (C_{max}), which did not differ significantly between treatment groups before treatment (Day 0) (ANOVA: $F_{2,21} = 0.70$, $P = 0.21$) or during treatment (ANOVA: $F_{2,17} = 0.17$, $P = 0.21$). There was also no significant effect of sex before treatment (ANOVA: $F_{1,21} = 0.1.2$, $P = 0.318$) or during treatment (ANOVA: $F_{1,17} = 0.540$, $P = 0.473$).

However, after 26 treatment days, bill-hue was significantly greater in groups fed lutein and zeaxanthin than in the control group (ANOVA: $F_{2,20} = 7.30$, $P = 0.007$; Fig. 3), and showed a non-significant trend for an effect of sex (ANOVA: $F_{1,20} = 3.6$, $P = 0.08$) (Fig. 3*a*, *b*). The interaction of sex × treatment was not significant (ANOVA: $F_{1,20} = 2.64$, $P > 0.1$). Bill-hue did not

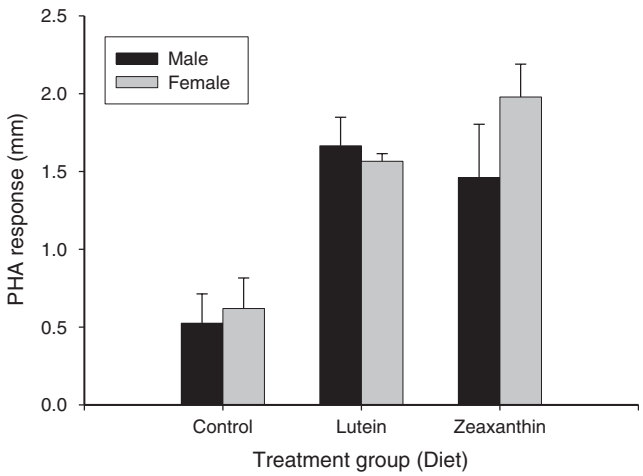

Fig. 2. Mean PHA response (±s.e.) of male and female Diamond Firetails after they had been on three artificial diets containing no carotenoids (control), 30 mg kg^{-1} lutein, or 30 mg kg^{-1} zeaxanthin for 9 days. Before the experiment all birds had been fed the control diet, which was carotenoid-free. The pre-treatment response was comparable to the response of the control group.

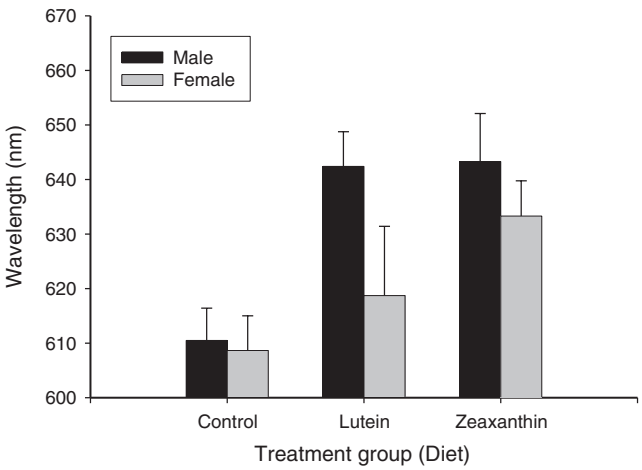

Fig. 3. Sexual differences in bill-hue (λR_{50}) for birds on different diets after they had been on three artificial diets containing no carotenoids (control), 30 mg kg^{-1} lutein, or 30 mg kg^{-1} zeaxanthin for 26 days. Before the experiment all birds had been fed the control diet, which was carotenoid-free. Bars show mean ± s.e. The pre-treatment response was comparable to the response of the control group.

differ between the lutein and zeaxanthin groups (Tukey's *post hoc* pairwise test; $P > 0.70$).

Discussion

Several elegant studies have confirmed Lozano's (1994) hypothesis that carotenoid-based colouration can provide a reliable indication of an individuals' quality to potential mates or competitors (e.g. Blount *et al.* 2003; Faivre *et al*. 2003; McGraw and Ardia 2003; Saks *et al*. 2003). However, most experimental studies have been done on captive birds with no clear reference to baseline data from the wild for comparison. In our study, the concentration of carotenoids in plasma was similar between wild and captive Diamond Firetails at the onset of the experiment, though we acknowledge the small sample size of wild birds.

The hypothesis that carotenoid-based colouration can provide a reliable indication of an individuals' quality (Lozano 1994; Møller *et al*. 2000; McGraw 2006) relies on the assumption that carotenoid-based colours provide a measure of the current quality of an individual. Plumage colouration at cessation of blood flow becomes fixed (though external stimuli can cause fading with time). Therefore, health and fitness can be signalled by plumage over the long term (months) while integumentary tissue may signal health and fitness over the short term (days or weeks).

Our experiment showed a clear effect of dietary carotenoids on circulating carotenoid concentration, PHA response, and the expression of bill-colour in Diamond Firetails. Increased availability of carotenoids resulted in larger amounts of carotenoids in blood plasma, higher PHA response, and increasing red pigmentation in the bill. These results are consistent with several other studies that have also found a positive relationship between carotenoid availability, various measures of immune response and colour in birds (Blount *et al.* 2003; Faivre *et al*. 2003; McGraw and Ardia 2003; Saks *et al*. 2003). These results suggest that bill-colour in this species can be an honest signal of quality. Using a focussed contrast, we tested the prediction that zeaxanthin should be a more potent immune enhancer than lutein owing to its higher antioxidant activity (McGraw and Ardia 2004), which was not supported. Our findings corroborate the findings of McGraw and Ardia (2005) who also found no effect of carotenoid type (lutein *v.* zeaxanthin) on immune response in the Zebra Finch.

Only one previous study has examined carotenoids in the Diamond Firetail. In contrast to our study, McGraw and Schuetz (2004) found ~80% of lutein and 20% of zeaxanthin in blood plasma and no other metabolic derivatives in Diamond Firetails on a seed diet. We found a similar percentage of lutein (79%), but slightly lower proportions of zeaxanthin (9%) for Diamond Firetails on dry seed. McGraw and Schuetz (2004) also found no metabolically derived carotenoids in blood plasma of Diamond Firetails, in strong contrast to our study (see Table 2), probably because McGraw and Schuetz (2004) focussed their study on yellow pigments. We found that ~12% of carotenoids in blood plasma of Diamond Firetails were made up of metabolic derivatives of dietary lutein and zeaxanthin (Table 2).

Table 2. Mean plasma concentration of carotenoids (mg L^{-1} ± s.e.) for Diamond Firetails after 9 days on commercial dry-seed mix, and three artificial diets containing no carotenoids (control), 30 mg kg^{-1} lutein, or 30 mg kg^{-1} zeaxanthin

n, sample size (number of birds)

Diet	*n*	Carotenoids			
		Lutein	Zeaxanthin	Other	Total
Seed	23	74.16 ± 5.77	8.39 ± 2.64	11.11 ± 2.26	99.97 ± 8.22
Control	7	3.13 ± 1.60	0.16 ± 0.46	0.09 ± 0.38	3.38 ± 1.60
Lutein	8	82.92 ± 6.48	1.21 ± 0.87	14.18 ± 1.87	98.31 ± 6.74
Zeaxanthin	8	0.16 ± 0.35	59.96 ± 4.23	12.05 ± 1.83	72.17 ± 4.47

Few studies have examined the rate of carotenoid depletion. In House Finches (*Carpodacus mexicanus*), carotenoids in the liver fell to near zero 7 days after the birds were deprived of dietary carotenoids (Inouye 1999). In this study, we found that carotenoids circulating in the plasma of Diamond Firetails dropped to 50% of the original concentration in under 5 days (Fig. 1). Carotenoids in the plasma stabilised at 6% of the original concentration after 26 days. In Diamond Firetails, this reduction in circulating carotenoids was accompanied by a reduction in bill-colour (compare control to carotenoid treatments in Fig. 3). Bill-colour recovered within 26 days of having access to carotenoids again.

During the depletion trial, low levels of circulating carotenoids persisted for at least 56 days after the birds were deprived of dietary carotenoids. These persistently low levels of circulating carotenoids may be the result of carotenoids being released from organs and fatty tissue after dietary input of carotenoids has ceased (Negro *et al*. 2001). This temporal buffering effect may allow functions for which carotenoids are important to continue during periods of carotenoid depletion. Previous studies on birds on a low carotenoid diet have shown variation in carotenoid storage abilities and resultant pigmentation (McGraw 2006). Future studies should be conducted to determine the level of variation in the rate at which circulating carotenoids become depleted in (1) different species, and (2) different tissues of the same species. Such studies would allow us to understand better the roles that carotenoids play in physiological homeostasis and social behaviour, and the trade-offs between these two functions.

Future research should address the effects of lutein and zeaxanthin on antioxidant capacity in birds, given recent findings that carotenoids may be less important as free-radical scavengers than previously thought (Hõrak *et al*. 2007; Costantini and Møller 2008). Furthermore, care is required in interpreting the PHA response as a measure of immuno-competence (Kennedy and Nager 2006). This is because the PHA response confounds the non-specific inflammatory response and the acquired immune response. Finally, we acknowledge that comparison of wild and captive birds in this study was limited by low statistical power. However, the data reveal an interesting finding: circulating carotenoids in our wild birds were slightly higher than those generally reported for other species. For example, we found 78 ± 11 mg L^{-1} circulating carotenoids, whereas the median concentration of total plasma carotenoids varied from 0.38 to 53.63 mg L^{-1} among 80 species of birds from Mexico and Spain (Tella *et al*. 2004). One might expect

granivorous estrildids to have low circulating levels of carotenoids because the carotenoid content of seeds is thought to be low (Olson 2006). However the carotenoid content of dry seed is much lower than that of green seed (G. Johnston and B. Rich, unpubl. data), and green seed is commonly eaten by wild Diamond Firetails (Read 1994). This may explain the higher amounts of circulating carotenoids we found, compared with other studies of granivores (Tella *et al*. 2004). The similar concentrations of carotenoids we found in plasma from wild and captive Diamond Firetails indicate that our carotenoid-supplementation protocols did not generate pharmacologically high levels of pigment for our study.

A substantial body of evidence suggests that carotenoid-based ornamentation evolved through the process of sexual selection (McGraw 2006). Sexual differences in circulating carotenoids, PHA response and integumentary colour have been reported for a variety of birds, including other estrildid finches (McGraw and Ardia 2005). These differences do not seem to be the result of differences in availability of dietary carotenoids to each sex, but rather relate to the physiological use by species and individuals (McGraw 2006).

We found a significant effect of carotenoid concentration for bill-hue, but no significant difference between the sexes (though this may be a result of small sample size). While males and females may sometimes be distinguished by bill-colour during the breeding season (O'Gorman 1980; Higgins *et al*. 2006; S. Kleindorfer pers. obs.), we are not aware of any scientific literature reporting sexual differences in bill-colour for Diamond Firetails.

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