Avian retinal oil droplets: dietary manipulation of colour vision?

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Avian vision is highly developed, with bird retinas containing rod and double-cone photoreceptors, plus four classes of single cones subserving tetrachromatic colour vision. Cones contain an oil droplet, rich in carotenoid pigments (except VS/ultraviolet-sensitive cones), that acts as a filter, substantially modifying light detected by the photoreceptor. Using dietary manipulations, we tested the effects of carotenoid availability on oil droplet absorbance properties in two species: *Platyercus elegans* and *Taeniopygia guttata*. Using microspectrophotometry, we determined whether manipulations affected oil droplet carotenoid concentration and whether changes would alter colour discrimination ability. In both species, increases in carotenoid concentration were found in carotenoid-supplemented birds, but only in the double cones. Magnitudes of effects of manipulations were often dependent on retinal location. The study provides, to our knowledge, the first experimental evidence of dietary intake over a short time period affecting carotenoid concentration of retinal oil droplets. Moreover, the allocation of carotenoids to the retina by both species is such that the change potentially preserves the spectral tuning of colour vision. Our study generates new insights into retinal regulation of carotenoid concentration of oil droplets, an area about which very little is known, with implications for our understanding of trade-offs in carotenoid allocation in birds.

Keywords: avian vision; retinal oil droplets; carotenoids; colour vision; visual ecology; resource allocation

1. INTRODUCTION

The effects of diet on sexually selected colour signals have been studied in great detail in many taxa (Hill 1991; Andersson 1994; Grether *et al.* 2005; Pike *et al.* 2007), but virtually no studies have tested whether diet can affect visual discrimination of these signals. This is puzzling because animals such as birds possess brightly coloured ‘carotenoid’-rich retinal oil droplets that play a significant role in their colour vision (Hart 2001; Hart & Hunt 2007). The functional effects of the oil droplets are well understood: by absorbing short wavelengths, they reduce overlap between spectrally adjacent photoreceptors, thereby improving the range of colours seen, and colour discrimination (Bowmaker 1977; Vorobyev *et al.* 1998; Vorobyev 2003; Hart & Hunt 2007). Additionally, the droplets eliminate short-wave sensitivity by removing the visual pigment beta peak (Govardovskii *et al.* 2000). However, whether ecological factors, such as dietary availability of carotenoids, affect the concentration of carotenoids within these droplets, and thus whether diet can directly impact on colour vision, has been overlooked. Variation in the dietary carotenoid intake of individuals would be predicted to affect the carotenoid content of these oil droplets, thus altering an individual’s colour vision and, subsequently, the

perception of colour signals and performance of colour-based behaviours in comparison to other individuals.

Birds have developed one of the most elaborate visual systems among vertebrates, with most avian species thought to possess six different types of photoreceptors within their retinas (Bennett & Théry 2007; Hart & Hunt 2007): low-light sensitive rods; four spectral classes of single cone thought to produce tetrachromatic colour vision (Maier & Bowmaker 1993; Osorio *et al.* 1999; Goldsmith & Butler 2005); and long-wave sensitive (LWS) double cones that appear to be involved in luminance and motion detection (Campenhausen & Kirschfeld 1998; Vorobyev *et al.* 1998). The oil droplets occupy almost the entire diameter of the distal end of the inner segment of all cone types and are classified into five types: R-type, Y-type, C-type and T-type droplets in LWS, middle-wave-sensitive, short-wave-sensitive and ultraviolet-sensitive single cones, respectively, and a P-type droplet in the principal member of the double cones (Bowmaker *et al.* 1997; Hart 2001).

Carotenoids are biologically important pigments that animals can only obtain through the diet (Goodwin 1986). Thus, carotenoid availability can be manipulated by controlling dietary intake, and many studies have observed the effects this manipulation has on carotenoid-dependent systems in different animals. Much work has been undertaken showing avian coloration responding to natural and experimental changes in dietary intake of

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carotenoids (e.g. Hill *et al.* 2002; McGraw *et al.* 2003b; Shawkey *et al.* 2006). However, data linking dietary carotenoids to vision and the discrimination of colour signals are much scarcer. Outside of birds, in Diptera, the connection between carotenoid deprivation and vision has been tested: Zimmerman & Goldsmith (1971) raised *Drosophila* on a carotenoid-free diet and supplemented some individuals with the carotenoid β -carotene, and observed a significant reduction in the sensitivity of the visual pigments in deprived individuals. In birds, oil droplet carotenoid concentration has been shown to be labile in response to ecological factors such as ambient light. Hart *et al.* (2006) raised chickens under natural and filtered sunlight. Microspectrophotometry (MSP) of individual oil droplets revealed that the birds raised under filtered sunlight showed a reduction in the concentration of carotenoids in their oil droplets, thought to be caused by the trade-off between maintaining colour discrimination and maintaining absolute photoreceptor sensitivity in the reduced light conditions. Hart *et al.* (2006) demonstrated that intraspecific variation in the oil droplets can occur in response to environmental factors, and so it is reasonable to expect dietary carotenoid availability to lead to a similar outcome. Toomey & McGraw (2009) found that retinal carotenoid concentration was variable between seasons and was positively correlated with plasma carotenoid concentration. While Toomey & McGraw (2009) did not test for a correlation between plasma and oil droplets carotenoid concentrations, their study offers further support for the hypothesis that oil droplets will be labile in response to dietary carotenoids. Furthermore, in a MSP study of 15 species from five orders, Partridge (1989) suggested that interspecific variation in oil droplets was better explained by ecology, specifically in relation to diet, than by phylogeny although, to date, there has been no experimental test of this hypothesis.

Two papers have focused on the relationship between diet and oil droplets in birds: both Wallman (1979) and Bowmaker *et al.* (1993) used carotenoid-free diets to obtain clear oil droplets in quail. However, this required a long-term treatment raising chicks on a carotenoid-free diet, and, moreover, unpigmented oil droplets were only achieved in the F₁ generation following breeding from the carotenoid-deprived birds (Wallman 1979). This would neither represent a condition likely to be found in nature nor do these two studies shed light on whether dietary variation over several weeks (a time scale known to affect plumage) can affect the oil droplets. To date, no work has been undertaken to experimentally test the short-term effect of dietary carotenoid manipulation on the perception of visual signals through changes in the carotenoid concentrations present in retinal oil droplets. The research reported here aims to determine whether changes in oil droplet carotenoid concentration occur over a short time period, in response to dietary carotenoid availability, in two species of bird. If variation in carotenoid concentration, and subsequent changes in the absorption properties of the oil droplets, can be linked to the carotenoids available through the diet, this would suggest that dietary quality could cause changes in colour vision and, subsequently, differences in behaviours using colour information such as mate choice, identity and dominance signalling, detection

of nest parasitism, begging, foraging and predation (Andersson 1994; Bennett *et al.* 1994; Jourdie *et al.* 2004; Cherry *et al.* 2007).

To test the effect of dietary quality on carotenoid concentration of the retinal oil droplets, we used two different avian species: a parrot, the crimson rosella *Platycercus elegans* (Joseph *et al.* 2008; Ribot *et al.* 2009), and a passerine, the zebra finch *Taeniopygia guttata*. These species have notably different carotenoid usage within their bodies: *T. guttata* uses carotenoid pigments in display signals in the beak, a trait shown to be sexually selected (Burley & Coopersmith 1987; Collins & ten Cate 1996) and a trait that varies through dietary carotenoid manipulation (Blount *et al.* 2003; McGraw *et al.* 2003b). *Platycercus elegans* is not thought to use carotenoid pigments in its plumage as, being a parrot, it instead has a unique pigmentary coloration system consisting of psittacofulvin pigments (Stradi *et al.* 2001; McGraw & Nogare 2005). However, while carotenoids are not thought to be present in parrot plumage, they are present in parrot retinas (Bowmaker *et al.* 1997), and so may therefore affect colour perception. Daily carotenoid intake was manipulated for *P. elegans* and *T. guttata* using a carotenoid supplement, containing xanthophylls lutein and zeaxanthin. These carotenoids are two of the three main circulating carotenoids in bird plasma (McGraw *et al.* 2002), and also appear to be present as, or act as metabolic precursors to, the carotenoids in retinal oil droplets (Goldsmith *et al.* 1984; Schiedt *et al.* 1991). This allowed us to test the effect of increased dietary carotenoid intake on the concentration of carotenoids in the oil droplets. If oil droplet concentration is dependent on dietary intake, we predicted that the differences in dietary carotenoid intake would be reflected in changes in circulating carotenoids of experimental birds and, subsequently, an increased absorption in oil droplets in the retinas of 'carotenoid-supplemented' birds and a decreased absorption in birds on dietary carotenoid restriction when compared with 'control' groups.

2. MATERIAL AND METHODS

(a) *Experimental design*

(i) *Platycercus elegans*

Eighteen outdoor aviaries (ca 1.5 × 1 × 2 m high, part-shaded by garden netting, and with access to natural light) were separated into six blocks of three adjoining aviaries, with one aviary per block randomly assigned to each of the three diet treatments. Each block contained a single sex (three male blocks and four female blocks), and birds were housed singly. Birds were sexed from DNA samples, using the method of Griffiths *et al.* (1998) and randomly assigned to aviaries. There was an initial 'wash-in' period of four weeks, during which all birds were fed an ad libitum seed mix comprising one part Barrow Mill 'Parrot' mix (with peppers and dried fruit removed) and two parts Barrow Mill 'Parakeet/cockatiel' mix (Barrow Mill, Bristol), plus two unshelled peanuts and 10 g Granny Smith apple per day. Birds were then started and maintained on the assigned experimental diet described in §2b until sacrifice at 5–6 months. Birds were weighed twice a week.

(ii) *Taeniopygia guttata*

Twenty-four male zebra finches were obtained from captive bred UK sources and randomly assigned to indoor individual cages ($0.5 \times 0.5 \times 0.5$ m, room lighting: Osram 6 ft 58 W fluorescent tubes on 12 h light/dark cycle). Birds were maintained on an ad libitum seed diet (Countrywide foreign finch seed, Barrow Gurney, UK) before a wash-in period in which the birds were introduced to (over 14 days), and maintained on (mean 46 days; range 30–60 days) a carotenoid-restricted diet comprising 3.5 g seed, plus 6.5 g carotenoid-free food customized for this experiment (TestDiet: diet no. 5C7V) until the start of the diet manipulation detailed in §2b. This seed content was below the mean daily intake of the birds on an ad libitum diet and reduced the bird's daily seed (and therefore carotenoid) intake, but did not cause weight loss (birds weighed every 2 days, loss defined as 10% reduction from weight while on ad libitum seed diet).

(b) *Diet manipulation*(i) *Platycercus elegans*

The experiment comprised three treatments: (i) 'control' was the maintenance diet described in §2a with ad libitum unmanipulated water; (ii) 'carotenoid' was the described maintenance diet plus an ad libitum lutein/zeaxanthin carotenoid supplementation added to tap water at $100 \mu\text{g ml}^{-1}$ (Oro Glo-8, Kemin Agrifoods Europe, Herentals, Belgium); and (iii) 'energy-restricted' was 38 g of a mix comprising one part maintenance diet plus four parts oat husk (J. E. Haith Ltd, Grimsby, UK) by volume (4:3 seed:husk by weight) with unmanipulated water. The energy-restricted treatment was used as part of a larger experiment and represents a carotenoid-restricted treatment owing to the reduced food available to the subjects. Food and water were replaced daily until sacrifice.

(ii) *Taeniopygia guttata*

Cages were paired, and one subject in each was randomly assigned to one of the two treatments: (i) 'carotenoid-supplemented' or (ii) 'control'. At experiment week 0, subjects assigned to the carotenoid treatment had a lutein/zeaxanthin carotenoid supplementation (Oro Glo-11, Kemin Agrifoods Europe, Herentals, Belgium) added to drinking water at $50 \mu\text{g ml}^{-1}$ following the protocol of Blount *et al.* (2003). Subjects in the control treatment were given unmanipulated drinking water. Subjects were maintained on the treatments for six weeks, and regularly weighed to ensure welfare. Three birds were removed from the experiment owing to 10 per cent weight loss, prior to commencing manipulation.

(c) *Plasma carotenoid quantification*

Blood samples were taken from all subjects for plasma carotenoid analysis; 300–400 μl was taken from *P. elegans* at the start of the experiment (week 0) and during weeks 4 and 12; 70–100 μl was taken from *T. guttata* at the beginning and end of the experimental treatment (weeks 0 and 6). Immediately after collection, blood samples were centrifuged for 15 min at 12 000g, and the plasma removed and stored at -20°C until analysis. Carotenoid concentration was quantified by high-performance liquid chromatography (HPLC), based on the ethanol–hexane method used by Blount *et al.* (2003). Carotenoids were identified as a single peak at 445 nm and quantified using lutein (Sigma–Aldrich, UK) as a standard.

(d) *Microspectrophotometry*

The retinal oil droplets of birds were assessed using MSP and, in both experiments, the MSP operator (B.K.) was blind to the experimental treatment of the subject being studied. For *P. elegans*, each block of three subjects was assessed at the end of the experimental treatment, in the order in which the experimental blocks were started. Subjects from the appropriate block were randomly selected by animal technicians, and sacrificed using approved humane methods. For *T. guttata*, pairs of subjects were sacrificed after exactly six weeks of experimental treatment, and the first of each pair assessed by MSP randomly selected by J.K.B. All oil droplet measurements were completed within 36 h of sacrifice.

Eyes were enucleated, and the lens and cornea removed. The eyecup was oriented using the pecten. *Platycercus elegans* eyecups were then cut into anterior, posterior, dorsal and ventral sectors. Owing to their smaller size, *T. guttata* eyecups were only cut into dorsal and ventral sectors. For each retinal segment, an approximate 2 mm^2 sector of retina was separated from the pigment epithelium, teased apart on a coverslip using razorblades and mounted in saline containing 10 per cent dextran. The sample was squashed with a second coverslip, which was sealed with wax. The absorbance spectra of oil droplets were measured using a computer-controlled Liebman dual-beam microspectrophotometer as described by Bowmaker *et al.* (1997). The measuring beam, with a diameter smaller than the oil droplet, was lined up to pass through the oil droplet, with the reference beam passing through tissue free of droplets. Spectra were measured from 750 to 350 nm, then back from 351 to 749 nm in 2 nm intervals. The order in which retinal segments were analysed was randomized. For each sector of *P. elegans* retina, spectra from eight individual droplets were measured for each droplet type. For each sector of *T. guttata* retina, spectra from 15 droplets were measured for each droplet type, with all measurements in both experiments made within 36 h of death. The unpigmented T-type droplets were not measured in either species.

(e) *Calculation of λ_{cut}*

The λ_{cut} , a theoretical wavelength at which no light of shorter wavelengths is transmitted, was calculated using a standard method suggested by Lipetz (1984), which calculates the wavelength value of the intercept at maximum measured absorbance of a tangent fitted to the absorbance curve at 50 per cent of maximum measured absorbance. P-type droplets often show a shoulder on the long-wave arm of the absorbance spectra in addition to the main absorbance peak (figure 2b). If this shoulder was greater than or equal to 50 per cent of the maximum measured absorbance, the programme gave a λ_{cut} value calculated from the second peak. For shoulders less than 50 per cent, the value was calculated from the main peak. Based on the shape of the absorbance curve, P-type droplets were therefore analysed as two separate groups defined by the presence ('double peak') or absence ('single peak') of a shoulder greater than or equal to 50 per cent of the maximum measured absorbance. The effects of λ_{cut} on visual pigment sensitivity spectra were modelled by subtraction of normalized droplet spectra from a Govardovskii template curve (Govardovskii *et al.* 2000) for *P. elegans* LWS visual pigment.

(f) Statistical analyses

Data were analysed using SPSS 15 (SPSS Inc.). We analysed MSP data for each droplet type using linear mixed models (LMMs), with restricted maximum likelihood estimation. These models included fixed effects for treatment and retina sector, and a random intercept comprising subject. The random effect was included to account for non-independence of oil droplets measured from each subject. We report estimates of fixed effects and pairwise comparisons based on estimated marginal means resulting from these models. Significance of fixed effects was assessed using type-III sums of squares. Changes in Akaike's information criteria (AIC) are presented to express the relative contributions of each predictor to the final model. Degrees of freedom from LMM were rounded to whole numbers where applicable.

3. RESULTS**(a) *Platycercus elegans* weight**

At week 0, there was no significant weight difference between any of the treatment groups (ANOVA $F_{2,17} = 0.14$, $p = 0.86$, figure 1a). Subjects in the energy-restricted group and control groups showed a significantly higher weight change between weeks 0 and 4 of the treatment period (ANOVA $F_{2,17} = 5.7$, $p = 0.013$), showing a loss, compared with a gain in the carotenoid-supplemented group. The weight of subjects in the energy-restricted groups did not significantly change later in the experiment. The initial weight decrease observed in the control group in weight between weeks 0 and 4 was recovered by week 8. Between weeks 8 and 12, no significant weight changes occurred in any treatment group (ANOVA $F_{2,17} = 1.9$, $p = 0.18$).

(b) Plasma carotenoid concentration**(i) *Platycercus elegans***

At week 0, there were no significant differences in plasma carotenoid concentration between groups (ANOVA $F_{2,15} = 1.3$, $p = 0.3$, figure 1b). The change in plasma carotenoid concentration did not differ significantly between any treatment groups between weeks 0 and 4 (ANOVA $F_{2,15} = 0.847$, $p = 0.446$). However, between weeks 0 and 12, the carotenoid-supplemented group showed a significant increase in plasma carotenoid concentration compared with the energy-restricted and control groups (ANOVA $F_{2,17} = 3.7$, $p = 0.047$, figure 1b).

(ii) *Taeniopygia guttata*

Carotenoid-supplemented birds showed a significant increase in plasma carotenoid concentration between week 0 and the end of the experiment at week 6 (paired t -test: $t_9 = -3.67$, $p = 0.003$, figure 1c). Control birds showed no significant change in plasma carotenoid concentration (paired t -test: $t_{10} = -0.06$, $p = 0.48$) throughout the treatment.

(c) Oil droplet microspectrophotometry**(i) *Platycercus elegans***

Spectra for five types of oil droplet were found in *P. elegans*: all measured single-cone droplets (R, Y and C); plus two varieties of P-type droplet that either demonstrated or lacked a long-wave shoulder (figure 2). The λ_{cut} of all droplet types showed significant main effects

Figure 1. Effect in *P. elegans* subjects of carotenoid-supplemented (solid black line), energy-restricted (light grey line) and control (light grey dashed line) treatments on (a) weight and (b) plasma carotenoid concentration. (c) *Taeniopygia guttata* plasma carotenoid concentration for each treatment at the beginning of the experimental treatments and sacrifice at six weeks: carotenoid group (solid black line) and control group (light grey line). Error bars in both panels represent ± 1 s.e.

of retinal sector, and significant interactions between sector and treatment (table 1).

In R-, Y-, C- and single-peak P-type droplets, carotenoid-supplemented birds consistently had a higher, though non-significant, main effect of treatment on λ_{cut} than the energy-restricted and control groups, when taking all λ_{cut} values for all four sectors for each individual (table 1 and figure 3). The magnitudes of the differences in the λ_{cut} of R-, Y- and C-type droplets were small (approx. 2 nm). In single-peak P-type droplets, the λ_{cut} differences between

Figure 2. Normalized absorbance spectra of *P. elegans* oil droplets: (a) single cones and (b) double cones. In (a), C-type (light grey line); Y-type (dark grey line); R-type (solid black line). In (b), single peak (light grey dashed line); double peak (dark grey dashed line); 100% absorbance double peak (solid black line). Single-cone spectra from (a) are also representative of those measured in *T. guttata*. In (b), only single-peak spectra (light grey dashed line) were measured in double cones of *T. guttata*.

Table 1. Mean λ_{cut} values for all individuals, and summary of statistical analyses from linear mixed model (LMM) on oil droplet λ_{cut} values in both experimental species. (ΔAIC : change in AIC, determined by subtraction of the predictor from the full model.)

species	droplet type	predictor	ΔAIC	F	d.f.	p
<i>Platycercus elegans</i>	R-type	treatment	43.18	1	2, 15	0.38
		sector	72.65	13.8	3, 546	<0.001
		treatment $\times$ sector	39.42	6.7	6, 546	<0.001
	Y-type	treatment	54.98	0.8	2, 17	0.48
		sector	101.18	20.1	3, 281	<0.001
		treatment $\times$ sector	50.03	8.2	6, 281	<0.001
	C-type	treatment	29.74	0.5	2, 15	0.62
		sector	33.05	2.7	3, 538	0.044
		treatment $\times$ sector	26.67	3.4	6, 538	0.003
	P-type (single peak)	treatment	64.08	2.8	2, 17	0.086
		sector	80.06	8	3, 281	<0.001
		treatment $\times$ sector	51.74	5.3	6, 281	<0.001
	P-type (double peak)	treatment	74.07	0.3	2, 15	0.76
		sector	79.60	3.6	3, 246	0.014
		treatment $\times$ sector	63.59	6.3	6, 246	<0.001
<i>Taeniopygia guttata</i>	R-type	treatment	22.51	0.05	1, 19	0.82
		sector	5.01	20.8	1, 607	<0.001
		treatment $\times$ sector	24.94	2.4	1, 607	0.13
	Y-type	treatment	18.19	0	1, 19	0.99
		sector	8.91	11.4	1, 606	<0.001
		treatment $\times$ sector	21.30	4	1, 606	0.046
	C-type	treatment	58.26	0.1	1, 19	0.58
		sector	22.70	197.1	1, 539	<0.001
		treatment $\times$ sector	65.18	29.5	1, 539	<0.001
	P-type	treatment	204.80	3.6	1, 19	0.074
		sector	32.72	41.1	1, 603	<0.001
		treatment $\times$ sector	207.64	13.4	1, 603	<0.001

treatment groups were of larger magnitudes (approx. 7 nm, figure 3). Spatial variation in the droplet λ_{cut} was determined by comparing values between retinal sectors, independent of treatment effects. R- and Y-type droplets showed similar patterns of spatial variation in λ_{cut} , with higher λ_{cut} values in the posterior and ventral sectors (table 2).

The differences in λ_{cut} of carotenoid- and energy-restricted treatments from the control treatment varied between retinal sectors (figure 3). When considering λ_{cut} in relation to sector, R- and Y-type droplets showed a consistent pattern of variation, controlling for treatment, with droplets in the ventral sector having the highest λ_{cut} values. C-type and single-peak P-type

droplets did not show a consistent pattern of variation within treatments, but the data suggest that λ_{cut} in the ventral is above the mean in C-type and single-peak P-type droplets, as well as in R- and Y-type droplets.

Carotenoid supplementation produced higher λ_{cut} in R- and Y-type droplets compared with restricted and control groups, with the effect being largest (2–4 nm) in the posterior and ventral sectors. λ_{cut} was closer between all treatment groups in anterior and dorsal sectors. C-type droplets showed large effects of carotenoid supplementation in the posterior and anterior sectors. In single-peak P-type droplets, carotenoid supplementation produced higher λ_{cut} values in all sectors compared with

Figure 3. Mean difference in λ_{cut} ($\Delta\lambda_{\text{cut}}$) of carotenoid (black shaded boxes) and energy-restricted (grey shaded boxes) treatments from control (treatment minus control) shown separately for each droplet type in *P. elegans*. Single cones: (a) R-type, (b) Y-type and (c) C-type; double cones: (d) single-peak P-type and (e) double-peak P-type. Data are grouped by retinal sector (anterior, dorsal, posterior and ventral). Error bars represent ± 1 s.e.

Table 2. Mean λ_{cut} for each droplet type that showed significant spatial variation independent of treatment in table 1, shown for all droplets from all subjects (full retina) and for droplets from each retinal sector (anterior, dorsal, posterior and ventral) across all subjects, showing significant spatial variation independent of treatment (table 1). (*Taeniopygia guttata* retina was only cut into dorsal and ventral sectors.)

species	droplet type	mean λ_{cut} (nm) by section				
		full retina	anterior	dorsal	posterior	ventral
<i>Platycercus elegans</i>	R	573.8	573.8	573.2	573.7	574.5
	Y	512.9	512.8	512.2	512.6	514.1
	C	420.8	421.3	420.4	420.6	421
	P (single peak)	428.3	430.7	426.3	426.8	429.5
	P (double peak)	485.6	482.3	487.9	486.2	486.2
<i>Taeniopygia guttata</i>	R	574.9	—	574.4	—	575.4
	Y	512.5	—	511.6	—	513.3
	C	415.1	—	413	—	417.3
	P	433	—	430.8	—	435.2

other treatment groups. The largest effect size was observed in these droplets, ranging from 2 to 8 nm, with the largest differences occurring between carotenoid and restricted treatments in the anterior (approx. 8 nm) and carotenoid and control treatments in the ventral sector (approx. 9 nm).

In double-peak P-type droplets, the additional long-wave peak was highly variable in both absorbance (as a proportion of the main peak) and presence or absence in individual sectors. All individuals produced droplets with spectra showing second peaks, which accounted for 47 per cent of all spectra measured. Most sectors measured showed only one variety of P-type droplet, with mixed droplet types occurring in 10 of the 72 analysed sectors. Statistical analyses showed highly

significant effects on λ_{cut} values of both carotenoid and restricted treatments with large effect sizes (table 1), but the treatment group showing the highest concentration in each sector was not consistent (figure 3).

(ii) *Taeniopygia guttata*

Spectra for four types of oil droplet were found in *T. guttata*: all measured single-cone droplets (R, Y and C); plus a single variety of P-type droplet (figure 2). No P-type droplets with a long-wave peak were measured. The λ_{cut} of all droplet types showed significant main effects of sector, and significant interactions between sector and treatment (table 1). The differences in λ_{cut} between carotenoid-supplemented and control treatments for each droplet type are shown in figure 4.

Figure 4. Difference in mean λ_{cut} ($\Delta\lambda_{\text{cut}}$) between carotenoid and control treatments (carotenoid minus control) shown separately for each droplet type in *T. guttata*: (a) R-type, (b) Y-type, (c) C-type and (d) P-type. Data are grouped by retinal sector (dorsal and ventral). Error bars represent ± 1 s.e.

All droplet types showed significant differences in λ_{cut} between the dorsal and ventral sectors, independent of treatment, with ventral sectors showing consistently higher λ_{cut} than dorsal sectors (table 2). Y- and C-type droplets showed significant interactions between sector and treatment, with carotenoid-supplemented birds showing higher λ_{cut} in dorsal sectors, but control birds showing higher λ_{cut} in ventral sectors though the magnitude of the differences was small (approx. 2 nm). R-type droplets showed no significant main effects of treatment and no significant interaction between sector and treatment.

P-type droplets showed no significant main effects of treatment, but significant effects of eye sector, and significant effects of the interaction between sector and treatment. The magnitude of the difference was much larger than for single-cone droplets, with carotenoid-supplemented birds showing, approximately, a λ_{cut} value of 2 nm higher in the ventral sector and 7 nm higher in the dorsal sector.

(iii) Effect on photoreceptor absorbance spectra

The effect of the post-treatment oil droplet absorption on the overall photoreceptor sensitivity spectra is negligible (figure 5). The curves represent the spectral sensitivity of the LWS cone cell after factoring in the filtering effects of R-type oil droplets (from each experimental group) on the LWS visual pigment template. The curves show very small differences in the peak sensitivity of the absorbance curve (approx. 1 nm). The carotenoid treatment also shows a negligible reduction in absolute sensitivity when compared with the restricted treatment. The *P. elegans* R-type curves shown are representative of the effects on spectral sensitivity curves in both study species, and across all cone types for which spectral sensitivity data are available.

Figure 5. Effect of oil droplet filtering on visual pigment spectra for R-type droplets for each treatment: Govardovskii template curve for *P. elegans* LWS visual pigment (black dashed line); cone sensitivity spectrum after filtering by mean λ_{cut} from carotenoid-supplemented treatment (dark grey line); cone sensitivity spectrum after filtering by mean λ_{cut} from energy-restricted treatment (light grey line). The curves shown are for mean λ_{cut} values from *P. elegans* posterior sector, which showed the largest difference in λ_{cut} after the experimental treatments (figure 3).

P-type oil droplet absorbance spectra with associated LWS visual pigment template are shown in figure 6. All P-type droplets have λ_{cut} below the maximum sensitivity for the LWS pigment, but greater than the additional beta peak present at short wavelengths, thus rendering the cell insensitive to these short wavelengths. The droplets with spectra in which the additional long-wave peak is the dominant λ_{cut} (greater than 50% maximum measured absorbance) also cut off a section of the left-hand limb of the pigment absorbance curve.

Figure 6. P-type oil droplet spectra from *P. elegans*: single peak (light grey line); double peak (dark grey line); 100% absorbance double peak (solid black line), with associated Govardovskii template curve for *P. elegans* LWS visual pigment spectrum (black dashed line).

4. DISCUSSION

These experiments have demonstrated, in two avian lineages, hitherto unreported patterns of variation in the absorbance of retinal oil droplets, related to dietary intake, droplet type and retinal location. Given that carotenoids are only available from the diet, it is not surprising that droplet carotenoid density should vary with diet. However, in several cases, the patterns of variation were not uniform across droplet types or across the retina, but these patterns did show a remarkable consistency between the two species. Specifically, the experiments have produced two new findings consistent in both species studied: first, carotenoid supplementation had increased oil droplet λ_{cut} ; the magnitude and direction of the change in λ_{cut} was dependent on the location of the oil droplet within the retina and, in Y- and C-type droplets, the spatial pattern of treatment effects differed between the two species. Second, in both species, the effects of dietary carotenoid supplementation are most pronounced in the P-type droplets found in the double cones. Our results demonstrate, to our knowledge, for the first time, a direct and rapid link between dietary carotenoid intake of birds and their visual physiology. These results could have broad implications for visual function in birds as similar outcomes were observed in the two species studied, despite their major differences in carotenoid usage in display coloration, and that they are not closely related avian taxa.

(a) Effect of eye sector

Oil droplet concentration is known to show natural spatial variation depending on retinal location. For example, droplets in the red and yellow fields of the pigeon *Columba livia* (Bowmaker 1977) have higher λ_{cut} than other retinal regions, and dorsoventral variation in λ_{cut} in the European starling (Hart *et al.* 1998). Hart *et al.* (2006) also found that droplets in the dorsal and ventral retina of the chicken differed in λ_{cut} depending on location, although which sectors had the droplets containing higher concentration differed between droplet types. Our experiments using *P. elegans* and *T. guttata* have also demonstrated that variation based on carotenoid

availability is likely to be focused in specific regions of the retina. All R- and Y-type droplets showed notably similar patterns of variation in the experiment using *P. elegans*, where the largest variation in λ_{cut} occurred in the posterior and ventral sectors. These areas will be viewing, respectively, the areas in front of and above the bird. Knowing the orientation of the eye *in situ*, it is clear that if the head is tipped forward slightly, these regions will view the area directly ahead, suggesting that they could therefore represent the most important visual fields, and be used for stereoscopic vision. Therefore, changes in vision owing to alteration of oil droplet concentration could be mediated by receiving visual information from both eyes, suggesting that the posterior and ventral regions are absorbing variation to preserve more important regions, such as the centre of the retina, that act independently of the other eye. Whether this detail should be relevant to carotenoid concentration in the oil droplets, and the response to dietary intake, remains open, as the reasons why a particular area of the retina/visual field should be more susceptible to carotenoid availability than other regions remain to be resolved. The variation could simply be a consequence of the physiological pathway used to incorporate carotenoids into the retina, or a more complex adaptive strategy suited to the visual ecology of a particular group of birds. The shape of the spectral sensitivity curve of a given photoreceptor has been shown to have important consequences on colour discrimination (e.g. He & Shevell 1995; Vorobyev 2003). Examination of the effects of the treatments on photoreceptor spectral sensitivity suggests that any change is unlikely to be an adaptive one for visual ecology, as the shape of the filtered sensitivity curves resulting from the two most extreme treatment effects between groups appears nearly identical (figure 6); this suggests that there has been little diet-dependent variation in overall spectral sensitivity on which selection could act to favour birds that maintain an optimum oil droplet carotenoid concentration. The similarity of the curves produced by these experiments also suggests that a dietary carotenoid deprivation sufficient to alter colour vision between individuals would probably lead to other more severe welfare consequences for wild birds. A corollary is that, as the shape does not seem to be altered by the treatments, there seems to be selection for stability in colour vision, despite changed levels of circulating carotenoid.

(b) Variation in P-type droplets

We have demonstrated that larger changes occur in the λ_{cut} of P-type droplets in both species. The P-type droplets are located in the double cones that contain the same LWS pigment as the red-sensitive single cone, with a peak sensitivity of around 570 nm. Therefore, the observed experimental variation in the λ_{cut} of these droplets does not affect the peak sensitivity of the pigment (figure 6), although these droplets do render the cells insensitive to short wavelengths, and the further reduction of short-wave light is likely to have some effect on overall spectral sensitivity. Double cones are thought to be involved in the detection of luminance and motion detection (Campenhausen & Kirschfeld 1998; Vorobyev *et al.* 1998; Bennett & Théry 2007). Therefore, the smaller changes in the ventral retina

compared with the dorsal could represent a strategy to maintain optimum luminance and motion detection when looking up to detect aerial predators. However, the role of the P-type oil droplets in double-cone function is not currently understood. The large effect sizes seen in the P-type droplets could also represent a strategy to preserve colour vision, by attempting to absorb the majority of variation in carotenoid intake into the droplet types that do not appear to cause significant variation in visual sensitivity. This strategy could potentially work against both an increase and a decrease in retinal carotenoid levels: if carotenoid intake is limited, the variation in P-types may prevent a reduction in concentration in the single-cone droplets, thus maintaining the advantages produced by the spectral filtering; if carotenoid intake is in excess, absorbing additional carotenoid into the P-type droplets may prevent an excess concentration in the single-cone oil droplets, which could impair photoreceptor sensitivity.

Alternatively, if carotenoid resources are prioritized towards the colour vision system, the smaller changes observed in the R-, Y- and C-type droplets could be owing to all treatment groups receiving enough carotenoids to reach a maximum concentration in these droplets, whereas the P-type droplets have yet to receive adequate carotenoids, such that any differences are only observed in these droplets. Much is known about the physiological processes producing carotenoid-based display colours (McGraw *et al.* 2003a; McGraw 2004). However, while some of the carotenoids present within different droplet types are known (Goldsmith *et al.* 1984), and dietary increases in specific carotenoids will alter ratios of the carotenoids present in the chicken retina (Schiedt *et al.* 1991), the mechanisms transferring carotenoids from the plasma into the oil droplets are poorly understood. Nevertheless, the research reported here using two different species has shown that short-term changes in dietary availability of carotenoids produced complex effects on carotenoid content of oil droplets, dependent on both the spatial location of a droplet and the droplet type.

The presence of the additional long-wave peak found in many P-type droplets of *P. elegans* did not appear to be correlated with either treatment or retinal location. Hart *et al.* (2006) found that raising chickens under bright light significantly increased the contribution of the secondary peak to the λ_{cut} of the droplets. The ambient light results combined with the results of our study reported here suggest the possibility that the secondary peak is an active response to the brightness of light to which the birds are exposed. The chickens generally had higher carotenoid concentrations in the ventral retina, thought to be owing to this sector of retina receiving brighter light from above (Hart *et al.* 2006). This effect was also observed in this experiment, with generally higher concentrations in the ventral retinal sectors.

Our study provides, to our knowledge, the first experimental confirmation of dietary intake affecting short-term carotenoid concentration of retinal oil droplets. Oil droplets are known to enhance avian colour discrimination, and the apparent lack of change in the single-cone droplets, despite reduced carotenoid availability, suggests that these droplets are highly prioritized to maintain this important sensory system.

All experimental work was performed under licence from the UK Home Office (Project licence PPL 30/2340).

The authors wish to thank Ian D. Bull and James M. Williams for assistance with HPLC, and NERC for funding the mass spectrometry facilities at Bristol (contract no. 821 R8/H12/15; <http://www.lsmsf.co.uk>). The authors also thank Ian Stewart and the staff of the NERC-funded Sheffield Molecular Genetics facility for assistance in *P. elegans* sexing. We also thank Ellis Loew and John A. Endler for useful comments on the manuscript, and Di Flower, Rob Massie and Sadie Iles-Ryan of Bristol University for care of the birds. B.K. was funded by a BBSRC strategic studentship no. BBS/S/H/2005/11891. M.L.B., K.L.B. and A.T.D.B. were funded by BBSRC grant B01568, and A.T.D.B. by the Leverhulme Trust and NERC.

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