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Original Article

Dietary carotenoid availability affects avian color discrimination

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Carotenoid pigments are found in the retinas of many vertebrate species, where they serve a range of functions. In birds, carotenoid-containing retinal oil droplets act as optical filters, modifying the light reaching the underlying visual pigment and thereby enhancing color vision. Dietary carotenoid manipulation is known to affect the allocation of carotenoids to the retina, although the effects this has on vision are less well understood. Using dietary manipulations, in which juvenile Japanese quail (*Coturnix japonica*) received either a high- or a low-carotenoid diet, we tested the effects of carotenoid availability on the ability to perform a color discrimination task. Birds on both diet treatments were able to make a relatively coarse discrimination between colors that appeared to humans as yellow-orange and orange; however, only high-carotenoid diet birds were able to make a finer-scale discrimination involving intermediate colors, showing that dietary carotenoid availability can directly affect the ability of birds to make chromatic discriminations. This finding has implications for our understanding of trade-offs in carotenoid allocation between vision and other key functions such as sexual ornamentation and health maintenance, and suggests that variation in dietary carotenoid availability may affect the ability of animals to make ecologically pertinent color discriminations, such as between sexual signals or cryptic food items.

Key words: **carotenoids, color vision, resource allocation, retinal oil droplets, visual ecology.**

INTRODUCTION

The biological function of carotenoids, and in particular their role as immunostimulants, antioxidants, and the constituents of some color signals, has attracted considerable attention (Lozano 1994; Olson and Owens 1998; von Schantz et al. 1999; Moller et al. 2000; Blount 2004). However, carotenoids are also found in the eyes of many, if not all vertebrate species, where they provide photoprotection and facilitate visual function (Kirschfeld 1982; Douglas and Marshall 1999). For example, in Japanese quail (*Coturnix japonica*), dietary carotenoid supplementation has been shown to increase retinal carotenoid levels and protect against light-induced photoreceptor death (Thomson et al. 2002). In many species, including various turtles, birds, and primates, carotenoids are also used as pigments in retinal oil droplets where they are thought to enhance color discrimination and color constancy in variable lighting environments (Goldsmith and Butler 2003; Toomey et al. 2015). They achieve this by selectively filtering light reaching the photoreceptor's visual pigment-containing outer segment, thereby reducing the spectral overlap between spectrally adjacent photoreceptors and so improving the range of colors seen and color discrimination (Vorobyev et al. 1998; Goldsmith and Butler 2003; Vorobyev 2003; Hart and Hunt 2007), and by removing photoreceptors' short-wavelength beta peaks (Govardovskii et al. 2000).

In birds, the carotenoid concentration of oil droplets has been shown to be labile in response to a number of ecological and physiological factors (Hart et al. 2006). For example, microspectrophotometry of individual oil droplets from chickens (*Gallus gallus*) revealed that birds raised under filtered sunlight showed a reduction in the concentration of carotenoids in their oil droplets (Hart et al. 2006). There is also growing evidence that the dietary availability of carotenoids, which cannot be synthesized de novo by vertebrates (Goodwin 1984), can directly affect retinal allocation (Partridge 1989). For example, recent studies have found that retinal carotenoid concentrations in house finches (*Carpodacus mexicanus*) were variable between seasons and positively correlated with plasma carotenoid concentration (Toomey and McGraw 2009); that experimental manipulation of dietary carotenoids affected allocation to the retina (Toomey and McGraw 2010); and that experimental activation of their immune system caused a reduction in retinal carotenoid levels (Toomey et al. 2010). Knott et al. (2010) have shown, through direct measurement of oil droplet absorption spectra, that carotenoid allocation can be affected by dietary availability in zebra finches (*Taeniopygia guttata*) and crimson rosella parrots (*Platycercus elegans*), and dietary effects of carotenoids on oil droplets have also been found in Japanese quail (Wallman 1979; Bowmaker et al. 1993)—although it should be noted that both these latter studies involved rearing the offspring of carotenoid-deprived mothers on a carotenoid-free diet and so represents

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a condition unlikely to be found in nature. Nonetheless, collectively these studies demonstrate that carotenoid allocation to retinal oil droplets is variable, although whether this affects vision has received little experimental attention. In the only empirical study to address this question, Toomey and McGraw (2011) demonstrated, using an elegant foraging experiment on house finches, that diet-induced changes in retinal carotenoid accumulation were related to the birds' ability to use color to locate seeds under various different lighting conditions. In this study, we experimentally tested whether dietary access to carotenoids could affect color discrimination ability in Japanese quail, using a psychophysical experiment in which birds were trained to discriminate between colored targets that differed in their predicted discriminability under standardized lighting conditions. Based on the previous work (Knott et al. 2010; Toomey and McGraw 2011), we hypothesized that increased access to carotenoids would lead to improved color discrimination and thus better performance in the visual task.

MATERIALS AND METHODS

Subjects and experimental design

The Japanese quail used in this experiment were derived from breeding stock maintained at the University of Exeter. Eggs were incubated in-house, and on hatching chicks were brooded communally for 1 day. They were then randomly allocated to groups of 5–7 birds, each of which was assigned to either a high-carotenoid diet or a low-carotenoid diet, and transferred to the rearing cage. Because we wanted all birds to be approximately the same age at testing, practical constraints meant that birds were bred and tested in 5 consecutive cohorts. The number of groups per cohort varied, but we always ensured there was at least 1 group on each diet treatment. In total 71 chicks were used, $n = 37$ on high carotenoids and $n = 34$ on low carotenoids. Although the sex of the birds was not known, hatching sex ratios in this population were typically equal (Pike TW, personal observation), and so there is no reason to assume that the relative proportion of male and female chicks differed between groups. The rearing cage (160 × 65 cm and 25 cm high) was divided width-wise into 4 sections of equal size, each of which housed 1 group of birds, and was illuminated by fluorescent ceiling lights (see Supplementary Figure S1 for their spectral output) with a flicker rate of ~100 Hz. Each group had a 25-W "electric-hen"-style brooder (that emitted no light) and received custom-made chick crumbs (Target Feeds, Whitechurch, UK), which contained only trace quantities of carotenoids (Orledge et al. 2012), and water ad libitum throughout the experiment. In order to provide carotenoids to each group in differing concentrations, the drinking water was supplemented with water-soluble FloraGLO Lutein (Kemin Health, Des Moines, IA), which contains 20% lutein and 0.86% zeaxanthin (Biard et al. 2005), at either 100-µL carotenoids/L water (high-carotenoid diet) or 10 µL/L (low-carotenoid diet). Dietary lutein and zeaxanthin are known to act as metabolic precursors to carotenoids present in quail's retinal oil droplets (Bhosale et al. 2007; Toomey and McGraw 2007), and the supplemented levels are well within the range used previously to manipulate carotenoids in quail (McGraw 2006). Drink bottles were covered with aluminum foil to avoid degradation and photo-oxidation of carotenoids, and supplemented water was changed daily. Birds were maintained on a 16L:8D photoperiod at 19 ± 1 °C.

During the first week, food crumbs were scattered on the floor. One day prior to the beginning of training (day 7 posthatch), food was provided in open-topped semicircular feeders (62 mm width × 35 mm radius) in order to allow the birds to become used to using them. For training and testing, these feeders were covered around the outside and inside with paper containing a printed stimulus pattern (described below), and chicks had to learn to use the chromatic components of the pattern for the discrimination task. The opening to the feeder was also covered with the stimulus paper, and had 6 parallel slits 25-mm-long cut in it in order to allow the chicks to peck inside the container and learn about its contents.

Stimuli construction

Stimuli were designed following Osorio et al. (1999). A chequered pattern consisting of 2 × 2 mm squares were printed onto white paper using an RICOH Aficio MP C2800 color printer at 600 dpi. 30% of the squares were colored and the remainder gray (see Supplementary Figure S2 for an example of a representative stimulus pattern). Because chromatic cues vary in their perceived luminance (for instance, yellow typically appears brighter than orange to humans), the use of luminance as a cue was excluded by varying the perceived luminance (L) of the colored and gray elements with a uniform random distribution of contrast range 0.3 (i.e., $[L_{max} - L_{min}]/[L_{max} + L_{min}] = 0.3$, where L_{max} and L_{min} are maximum and minimum recorded luminance values, respectively; see below for their calculation), while maintaining a constant mean luminance across all stimulus types. It is assumed that this ruled out the possibility that stimuli were distinguishable using achromatic cues alone (as is the case for the closely-related chicken, *Gallus domesticus*; Osorio et al. 1999; Olsson et al. 2015). Stimuli patterns were randomly generated in Matlab (MathWorks; Natick, MA) using RGB values that, when printed, were known to result in colors that elicited particular patterns of cone excitation when viewed by quail, determined from spectrophotometric measurements combined with psychophysical models of the quail's visual system, as follows.

The reflectance spectra of colored and achromatic color patches were measured with an Ocean Optics USB2000 UV-visible spectrophotometer, coupled with a pulsed xenon light source (PS-2) and a bifurcated 400-µm fiber optic probe, at 1-nm intervals, relative to a spectrally flat 99% reflecting Spectralon standard (Labsphere, North Sutton, NH). The tip of the fiber optic probe was housed in a hollow, black plastic sheath with a 45° angled tip to reduce specular reflection (Endler 1990).

Japanese quail have 4 single-cone types with peak sensitivities (λ_{max}) at 418 nm (V cone), 450 nm (S cone), 505 nm (M cone), and 567 nm (L cone), and a double (D) cone with a peak at 567 nm (Bowmaker et al. 1993). The D, L, M, and S cones are associated with carotenoid-pigmented oil droplets (Bowmaker et al. 1993). Effective cone sensitivity functions were modeled using the rhodopsin visual pigment template of Govardovskii et al. (2000) and incorporated the transmittance spectra of the combined ocular media from peafowl (Hart 2002) and estimated quail oil droplet transmission spectra calculated following Hart and Vorobyev (2005) and data from Bowmaker et al. (1993) (Supplementary Figure S3). Quantum catches for each of the different cones types viewing a given color patch were then estimated as the summed product of the color patch's spectral reflectance (Supplementary Figure S4), the spectral output of the fluorescent ceiling light, and the effective spectral sensitivity of each cone class, summed across all quail-visible wavelengths (300–700 nm) (Supplementary Equation S1). We assumed that cones adapt to the nominally achromatic gray of the stimulus pattern (Supplementary Figure S4) following a von

Kries coefficient law (Vorobyev and Osorio 1998). Perceived luminance was assumed to be equal to the response of the double cones (Osorio et al. 1999).

In total, 5 stimulus types were generated differing in how orange or yellow the chromatic elements appeared (at least to human observers) (see Figure 1 for the location of these colors in a quail chromaticity space constructed from cone quantum catch data following Osorio et al. 1999). We also used the log form of the tetrachromatic version of Vorobyev and Osorio's (1998) model (Supplementary Equation S2) to estimate how discriminable pairs of stimuli would be to quail. This model assumes that receptor noise limits visual discrimination, and used a Weber fraction value of 0.06 for the most abundant cone type (Olsson et al. 2015), and relative proportions of cone types for the peafowl of 0.45:0.86:1:0.95 (V:S:M:L) (Hart 2002). The model calculates just noticeable differences (jnds) between the color stimuli; we assumed that jnds < 1 meant that 2 stimuli were indistinguishable, with higher values indicating that 2 stimuli were increasingly distinguishable (Vorobyev and Osorio 1998). The 2 most perceptually different colors (labeled A and E in Figure 1) appeared to human observers as relatively yellow (A) and relatively orange (E) and were very likely to be discriminable by quail (being separated by ~3 jnds). The other 3 colors fell along a straight line in chromaticity space, linking colors A and E, such that C was approximately equally discriminable from A and E, B fell between A and C, and D between C and E (Figure 1). Colors A and B, and E and D were unlikely to be discriminable (both pairs being separated by <1 jnd).

The above discriminability estimates assume that the experimental quail have carotenoid-rich oil droplets filtering light before it reaches the visual pigment (Bowmaker et al. 1993). Less carotenoid-rich oil droplets would filter less light, therefore increasing the spectral overlap between spectrally adjacent cone classes and reducing the ability to discriminate certain colors. If we assume that, hypothetically, the experimental quail have carotenoid-deplete (i.e., fully transparent) oil droplets, then we would predict that stimuli A and E are just distinguishable (being separated by ~1.3 jnds), but all other pairs are indistinguishable (being separated by <1 jnd).

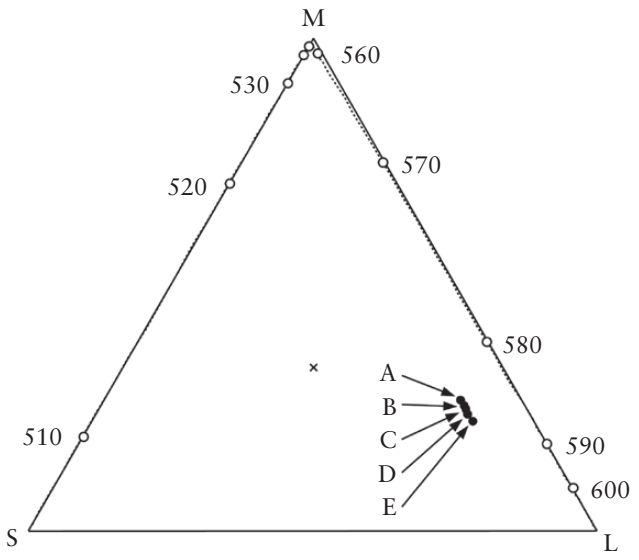

Figure 1
Chromaticity diagram representing Japanese quail chromaticity space, showing the location of the 5 stimulus colors, A–E, after adaptation to the background. The achromatic point is denoted by "x," and the apices of the triangle represent stimulation of the L, M, and S cones (the V cone showed negligible stimulation and so has been omitted for clarity). The monochromatic locus (dotted line) is also plotted, with symbols placed at 10-nm intervals between 510 and 600 nm; numbers give the wavelength (nm) of some of these points.

Training procedure

Chicks were trained to discriminate between the 2 most different stimulus colors (A and E) for 3 days, from days 8 to 10 post-hatch. One of these stimuli was rewarded with food, and the other contained no food reward. Which stimulus color was rewarded was initially randomly selected for each group and this designation remained constant throughout the experiment. Each group of chicks had access to 4 feeders, 2 of which were rewarded. The positions of the feeders were changed randomly 6 times during the day (at approximately hourly intervals between 0900 and 1700), to prevent the chicks from learning to associate a reward with the position of a feeder, at which time rewarded feeders were refilled. The stimuli paper covering the feeders was also changed daily. At night, the birds were provided with an additional 2 rewarded feeders and 1 unrewarded feeder to ensure a sufficient food supply. The length of the training procedure (i.e., 3 days) was determined by pilot trials as being effective in allowing chicks to learn to discriminate between feeders on the basis of the associated stimulus color under test conditions.

Testing procedure

Following training, the color discrimination ability of individual subjects was tested from day 11 in a separate experimental cage (Supplementary Figure S5). Each cage (160 × 65 cm) was divided into 4 equal-sized areas by partitions. The central partition was opaque and allowed 2 test procedures to be carried out simultaneously, one on each side. The remaining 2 partitions were made of wire mesh and allowed the focal chick in the center-most compartment to be separated from, but in constant contact with, the 4–6 chicks from its rearing group in the outer-most compartment. This was necessary as chicks were distressed by social isolation or when held with unfamiliar conspecifics.

Each chick was required to complete 3 consecutive discrimination trials, each involving a single forced choice between 2 feeders. Both feeders contained food but a thin layer of black nylon netting was placed underneath the lid of the unrewarded stimulus to inhibit access, but control for the presence of olfactory cues. Feeders were placed 12 cm apart on the same wall of the arena and their relative positions (left or right) were randomized for each trial by the flip of a coin. At the start of each trial, birds were placed centrally against the opposite wall to the feeders, equidistant from each one. Subjects were not food deprived prior to the testing trial, but the only food available to them during the test was in the rewarded feeder. The first test trial involved a coarse discrimination comparing stimulus A against stimulus E. They were then given 2 further discriminations consisting of the trained rewarded stimulus (A or E) against colors predicted to be more perceptually similar: that is, stimulus A versus C or E versus C (medium discrimination) and A versus B or E versus D (fine discrimination), depending on their rewarded training color. A bird's feeder preference was scored in a binary manner by recording their first feeder choice as "correct" (a peck at the rewarded feeder) or "incorrect" (a peck at the unrewarded feeder). We also recorded the latency between release and contact with their first choice of feeder (s) as a measure of motivation to

complete the task. As soon as the birds had pecked once at a feeder, it was removed and the next trial commenced.

Statistical analysis

Color discrimination ability was tested using a generalized linear mixed-effects model, using the glmer function in the lme4 package for R (Bates et al. 2013), with a binary response variable (correct or incorrect choice). Differences in the latency to approach the first choice of feeder was tested using a linear mixed-effects model (implemented using the lmer function; Bates et al. 2013), with square root-transformed latency as the response variable. Both models included diet (high or low carotenoids), discrimination level (coarse, medium, or fine), and their interaction as fixed factors, and random effects terms of rewarded stimulus (A or E), cohort, and chick identity. This allowed changes in an individual chick's performance to be assessed over successive discriminations. *P* values were calculated by comparing the full model to a reduced model lacking the term of interest using likelihood ratio tests (Crawley 2002). Pairwise comparisons between discrimination levels were assessed using orthogonal repeated contrasts (coarse vs. medium, medium vs. fine), and whether group performance differed significantly from chance (i.e., a success rate of 0.5) was tested using binomial tests. Where multiple tests were performed, presented *P* values have been Bonferroni corrected (Rice 1989). All analyses were conducted in R version 2.15.2.

Ethical statement

The experiment presented here met the ABS/ASAB ethical guidelines for the use of animals in research and strictly adhered to the legal requirements of the UK. The study was approved, as part of HL's Masters project, by an internal ethics committee at the University of Exeter. All chicks successfully completed their test trials, after which they were incorporated back into the stock population.

RESULTS

There were no effects of diet ($\chi^2_1 = 1.91$, $P = 0.167$), discrimination level ($\chi^2_2 = 2.46$, $P = 0.292$), or their interaction ($\chi^2_2 = 2.206$, $P = 0.332$) on the latency to approach the feeder. However, color discrimination success was significantly predicted by both diet ($\chi^2_1 = 7.08$, $P = 0.008$) and discrimination level ($\chi^2_2 = 19.81$, $P < 0.001$), but not their interaction ($\chi^2_2 = 1.12$, $P = 0.572$), with chicks on the high-carotenoid diet performing best overall (Figure 2). Low-carotenoid diet chicks showed a significant decline in performance between successive discrimination levels as the discrimination task became harder (coarse vs. medium: $z = 2.39$, $P = 0.017$; medium vs. fine: $z = 2.75$, $P = 0.006$), whereas chicks on the high-carotenoid diet showed consistent performance between the coarse and medium discrimination ($z = 1.45$, $P = 0.148$) and a significant decline in performance between the medium and fine discrimination ($z = 3.47$, $P = 0.001$). Both groups performed significantly better than chance for the coarse discrimination and no better than chance for the fine discrimination, but only the high-carotenoid diet chicks performed better than chance for the medium discrimination (Figure 2). Consistent with this, there were no significant differences in color discrimination success between diet treatments on either the coarse ($\chi^2_1 = 1.21$, $P = 0.271$) or the fine discrimination ($\chi^2_1 = 1.83$, $P = 0.176$); there was, however, a significant difference on the medium discrimination ($\chi^2_1 = 5.93$, $P = 0.015$).

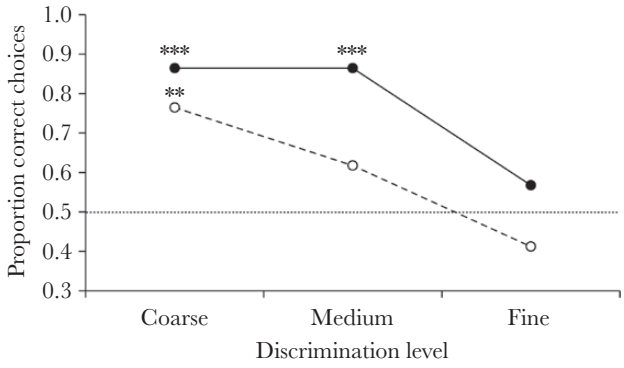

Figure 2
Proportion of correct choices made by chicks in the high-carotenoid (solid line, black points) and low-carotenoid (dashed line, white points) diet groups on the coarse, medium, and fine discrimination tasks (see text for full details). The horizontal dotted line indicates random choice. Asterisks above data points indicate a Bonferroni-corrected significant difference from chance: ***$P < 0.001$, **$P < 0.01$.

DISCUSSION

The results of this study show that access to dietary carotenoids can affect the ability of Japanese quail chicks to discriminate between stimuli on the basis of color. Birds in both the high- and low-carotenoid diet groups performed well on the coarse discrimination between orange- and yellow-colored stimuli (A and E in Figure 1), probably in part because these 2 stimulus colors were fairly easy for the birds to discriminate (Osorio et al. 1999), and partly because these were the 2 colors they had been trained on. The high level of success demonstrates that training was effective and that birds in both groups could successfully manage the task. In contrast, neither group performed well on the finest discrimination, with chicks in both groups performing no better than chance. The most interesting result was found on the medium-level discrimination, in which only birds in the high-carotenoid group performed significantly better than chance.

These results could have arisen through any of at least 3 non-mutually exclusive mechanisms. First, variation in the composition or concentration of retinal carotenoids may have directly affected visual discrimination. In species for which suitable data exist, it is clear that the composition of carotenoids in oil droplets is complex (Toomey et al. 2015); for example, at least 8 types of carotenoid have been identified in the retina of Japanese quail (Toomey and McGraw 2007), many of them (notably galloxanthin and astaxanthin, the 2 most abundant carotenoids) derived directly from the metabolic transformation of ingested lutein and zeaxanthin (Bhosale et al. 2007). Variation in dietary availability will necessarily affect both the absolute quantity of carotenoids available for allocation to the retina and, though trade-offs between specific bioconversion pathways, the relative abundance of each class of carotenoid. This may have resulted in biased allocation of carotenoids between oil droplets associated with particular classes of photoreceptor (Knott et al. 2010) or a bias in which carotenoids were allocated to particular types of oil droplet (Toomey and McGraw 2009). Toomey and McGraw (2010), for example, found that following immune system activation in house finches, some (but not all) types of retinal carotenoids appeared depleted. A shift in the composition of carotenoids allocated to an oil droplet may have a pronounced effect on its transmission spectrum, and therefore on the composition of light reaching the underlying visual pigment.

Both less efficient filtering of incoming light (resulting in broader photoreceptor sensitivity spectra) and changes to the transmission spectra of oil droplets (resulting in shifts to the photoreceptors' peak sensitivity) have the potential to influence color discrimination (Vorobyev 2003).

Knott et al. (2010) found that only the transmission spectra of oil droplets associated with double cones were affected by dietary carotenoid manipulation in zebra finches and crimson rosella which, if oil droplet density contributes to the luminance detection function of double cones (which has not yet been demonstrated), suggests that their luminance vision could be primarily affected (Osorio and Vorobyev 2005). The design of our stimuli would suggest that quail could not have discriminated between them on the basis of luminance cues, although we cannot be certain that double cones were not involved in the color discrimination (Osorio et al. 1999; Goldsmith and Butler 2005). It should be noted that, for ethical reasons, we did not directly measure carotenoid allocation to retinal oil droplets, and so we cannot be certain that our dietary manipulations directly affected retinal allocation, although previous studies would suggest that this assumption is plausible (Toomey and McGraw 2009; Knott et al. 2010; Toomey et al. 2010; Toomey and McGraw 2010).

Second, lower levels of photoprotective carotenoids in the retina could have allowed increased light-induced damage to the photoreceptors, perhaps affecting the overall abundance of photoreceptors and thereby negatively affecting their color discrimination ability. For example, Thomson et al. (2002) have shown that the number of apoptotic rods and cones in light-damaged eyes correlated significantly and inversely with the concentrations of both lutein and zeaxanthin in the retina, suggesting a key role for these carotenoids in protecting the underlying photoreceptors from photodamage. However, the conditions needed to elicit this phenomenon were fairly extreme (high levels of light and long periods of carotenoid deprivation), and so it is unclear how relevant this mechanism would be in our study, or to wild bird populations. We consider it unlikely, though, given the relatively low light levels emitted from the ceiling lights. Given carotenoids' antioxidant properties (El-Agamey et al. 2004), a related mechanism could involve photoreceptor (or other retinal) damage as a result of reduced antioxidant activity in low-carotenoid birds; however, without further study we have no evidence to support this conjecture.

Finally, it is possible that variation in behavior between groups (e.g., carotenoid-mediated variation in locomotory behavior; Blount and Matheson 2006), and not dietary-induced sensory constraints, was responsible for the results. However, we consider this unlikely as there was no evidence for differences in physical ability or motivation between groups—for example, there was no difference in the latency birds took to make their choice during the test phase of the experiment. Moreover, there was no significant difference in success rate between groups on the coarse discrimination task, suggesting that they were equally able/motivated to make the discrimination. This would suggest that it is unlikely that a difference in ability and/or motivation was responsible for the difference seen in the medium discrimination. Further work, including a direct assessment of the carotenoids present in the retina, is needed to identify the underlying mechanisms.

In conclusion, our results show that the dietary availability of carotenoids can directly affect the ability of a bird to perform chromatic discriminations. Although the precise mechanism underlying this finding is unclear, we consider it very plausible that it arose from variation in the quantity or composition of carotenoids allocated to retinal oil droplets. Regardless of the mechanistic basis, if such variation in color discrimination ability is present in wild birds, then it may have implications for the successful discrimination of colorful sexual signals Toomey and McGraw (2012) and prey items (e.g., against a heterogeneously colored background) (Toomey and McGraw 2011), and suggests that foraging (and more specifically carotenoid-acquisition) ability may affect visual performance such that only high-quality individuals can make accurate visual discriminations.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Lim and Pike (2016).

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