

Avian Retinal Carotenoid Accumulation:
Ecophysiological Constraints and Behavioral Consequences

by

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ABSTRACT

The elaborate signals of animals are often costly to produce and maintain, thus communicating reliable information about the quality of an individual to potential mates or competitors. The properties of the sensory systems that receive signals can drive the evolution of these signals and shape their form and function. However, relatively little is known about the ecological and physiological constraints that may influence the development and maintenance of sensory systems. In the house finch (*Carpodacus mexicanus*) and many other bird species, carotenoid pigments are used to create colorful sexually selected displays, and their expression is limited by health and dietary access to carotenoids. Carotenoids also accumulate in the avian retina, protecting it from photodamage and tuning color vision. Analogous to plumage carotenoid accumulation, I hypothesized that avian vision is subject to environmental and physiological constraints imposed by the acquisition and allocation of carotenoids. To test this hypothesis, I carried out a series of field and captive studies of the house finch to assess natural variation in and correlates of retinal carotenoid accumulation and to experimentally investigate the effects of dietary carotenoid availability, immune activation, and light exposure on retinal carotenoid accumulation. Moreover, through dietary manipulations of retinal carotenoid accumulation, I tested the impacts of carotenoid accumulation on visually mediated foraging and mate choice behaviors. My results indicate that avian retinal carotenoid accumulation is variable and significantly influenced by dietary carotenoid availability and immune system activity. Behavioral studies suggest that retinal carotenoid

accumulation influences visual foraging performance and mediates a trade-off between color discrimination and photoreceptor sensitivity under dim-light conditions. Retinal accumulation did not influence female choice for male carotenoid-based coloration, indicating that a direct link between retinal accumulation and sexual selection for coloration is unlikely. However, retinal carotenoid accumulation in males was positively correlated with their plumage coloration. Thus, carotenoid-mediated visual health and performance may be part of the information encoded in sexually selected coloration.

DEDICATION

I dedicate this work to my parents Eileen and Brian Toomey who have inspired and supported my curiosity no matter where it has taken me. I submit this dissertation with a deep and abiding respect for the birds that made this work possible.

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PREFACE

The brilliant coloration of animals and complexity of the eye present enduring and inspiring challenges to the theory of evolution that have led to profound progress in our understanding of the natural world (Darwin 1859; Fox & Vevers 1960; Land & Nilsson 2002; Hill & McGraw 2006). As components of a communication system that mediates essential activities in the lives of animals, including feeding, mating, aggression, and predator avoidance, coloration and vision are inexorably entwined. The conventional view of the coevolution of these traits is a step-wise process where natural selection outside of the context of signaling shapes the sensitivities of the visual system that subsequently selects for specific signal characteristics (Ryan 1990; Endler 1992; Endler & Basolo 1998). However, colorful signals and the visual system are both costly to develop and maintain (Maynard-Smith and Harper 2003; Wangsa-Wirawan and Linsenmeier 2003). Therefore, coloration and vision may be constrained by common environmental and physiological factors that could significantly influence the pace and direction of their coevolution.

Costly signal production

Signals are the behavioral or morphological traits that convey information from one organism to another (Searcy & Nowicki 2005). A particularly important piece of information is the quality of an individual as a mate or rival. The direct

costs of producing a signal may limit its expression and are proposed as a mechanism of honestly encoding information about individual quality (Zahavi 1975; Grafen 1990). These signals take a variety of forms, with different physiological mechanisms of expression controlling different aspects of quality that signals can reveal (e.g. Moller & Pomiankowski 1993; McGraw & Hill 2000; Hebets 2004). For example, bird song complexity is linked to diet quality during key periods of ontogeny (Nowicki et al. 1998; Buchanan et al. 2003), while song production depends upon current nutritional status (Searcy 1979). Yet, determining the precise information content of a signal like song is often difficult to determine because the complex neurophysiological mechanisms involved in its production. Carotenoid-based colors offer a unique opportunity to trace the mechanisms and costs of signal production through a single currency that is directly linked to diet and influences specific physiological processes (Blount and McGraw 2008).

Carotenoids are a class of tetraterpenoid organic molecules consisting of a series of conjugated double bonds that allow these molecules to absorb light and quench free radicals (Goodwin 1984). Carotenoids initially evolved in archeobacteria as structural components of the cell wall and subsequently became important accessory pigments in the photosystems of photosynthetic organisms (Vershinin 1999). Animals have evolved to utilize carotenoids, but cannot produce them *de novo* and depend on the direct or indirect consumption of plants,

fungi and bacteria to acquire carotenoids (Goodwin 1984, but see Moran et al. 2010). Thus, the expression of carotenoid-based traits may be limited by the environmental availability of carotenoids (Grether et al. 1999, Hill et al. 2002). In addition to generating external coloration, carotenoids also offer several physiological benefits, including serving as vitamin precursors (Bauernfeind 1981), promoting immune function (Chew & Park 2004), providing photoprotection (Krinsky et al. 2003), and alleviating oxidative stress (Alonso-Alvarez et al. 2004; but see Costantini & Møller 2008). The potentially limited pool of carotenoids in the diet is likely to be traded-off among these various functions (Lozano 1994, von Schantz et al. 1999). Therefore, carotenoid-based colors can communicate specific information about an individual's diet and health, and sexual selection favoring intense carotenoid-based coloration has been demonstrated in a number of taxa and birds in particular (reviewed in McGraw & Blount 2009).

Carotenoid-based signaling in the house finch

My study species, the house finch (*Carpodacus mexicanus*), is a model organism for the study of the ecology, physiology, and evolution of carotenoid-based coloration (Hill 2002). Male finches display carotenoid-based plumage color that varies from drab yellow to deep red. Redder males have superior dietary access to carotenoid pigments, deposit more of these pigments into ornamental

feathers, and are in better nutritional and health condition (Hill 2002). The reddest males also tend to provide higher levels of parental care and have greater reproductive success than drab males (Hill 2002). Dietary supplementation with carotenoids enhances plumage color (Hill 1992), while experimental infection with parasites can limit color expression (Brawner et al. 2000). Females from populations throughout North America show strong and consistent mating preferences for the reddest males (Hill 2002, Oh & Badyaev 2006). Visual discrimination of carotenoid-based coloration is an obvious and essential part of this mate choice, but if females vary in their ability to visually discriminate male coloration, it has the potential to alter their choice of mates and ultimately the pace and direction of sexual selection for color plumage.

Costly signal reception

Like elaborate signals, sensory systems can be costly to develop and maintain, and their function may depend upon an individual's current or developmental condition. One of the best-understood neural/sensory systems is the song system of passerine birds, where the sensory and motor neurons responsible for song learning and production have been identified. Nowicki et al. (1998) proposed that the development of these regions may be shaped by nutritional stress, making song an indicator of condition during development. Food restriction during key developmental periods limits the growth of the song

control brain region and impairs song learning in some species (Nowicki et al. 2002; MacDonald et al. 2006) and may also affect song reception. Leitner and Catchpole (2002) observed that the size of a song control brain region in female canaries (*Serinus canaria*) is positively correlated with their ability to discriminate male song quality. A similar phenomenon may also occur among anurans. The fundamental frequency of the sexually selected male advertisement calls of many frog species are dependent upon body size, and females prefer most often lower frequencies indicative of larger male body size (Ryan 1980, Keddy-Hector et al. 1992, Castellano et al. 1999). Within populations of cricket frogs (*Acris crepitans*), male call frequencies, as well as the tuning of the female auditory system, are correlated with body size (Keddy-Hector et al. 1992). In painted reed frogs (*Hyperolius marmoratus*), female body size is correlated with their selection of preferred lower frequency calls (Jennions et al. 1995). Therefore, determinants of growth in anurans (i.e. foraging success) may shape both sexual signal production *and* reception.

Physical and chemical properties of the eye determine the upper limits of visual discrimination (Vorobyev & Osorio 1998) and shifts in photoreceptor sensitivities and neural noise can significantly change the discriminability of signals (Lind & Kelber 2009). The eye is one of the most metabolically active organs in the body, requiring large amounts of energy and nutrients while generating oxidative by-products that must be eliminated (Wangsa-Wirawan &

Linsenmeier 2003; Nolan et al. 2006). Vision also requires diet-derived vitamin A, which is an essential component of the visual pigments of all photoreceptor cells (Shichida & Matsuyama 2009). Therefore the maintenance and function of the eye may be constrained by diet and the allocation of energy and nutrients in the body. Diet-derived carotenoids are abundant in the eyes of many animals and play a key role in visual health and function (Douglas & Marshall 1999).

Carotenoids are a precursor for vitamin A, absorb light, protect against photodamage, and can alleviate oxidative stress within the eye by quenching free radicals (Krinsky et al. 2003). Carotenoids are particularly important in the avian visual system because they directly shape visual sensitivity (Vorobyev 2003).

Carotenoids in the avian retina

In the avian retina, there are four types of single-cone photoreceptor cells and a double-cone photoreceptor that each contains a carotenoid-pigmented oil droplet of a specific color (Fig. 1; Goldsmith et al 1984; Bowmaker et al. 1997). Color vision is achieved through the opponent processing of the relative stimulation of the four single cones, while the double cone is thought to mediate achromatic motion detection (Osorio et al. 1999). The carotenoid-pigmented cone oil droplets filter light that reaches the visual pigment, thereby narrowing the spectral sensitivity, reducing overlap between spectrally adjacent photoreceptors, and ultimately enhancing color discrimination and color constancy in variable lighting

environments (Fig 2.; Vorobyev et al. 1998, Vorobyev 2003). Changes in the concentrations of carotenoids within the oil droplets are predicted to alter spectral sensitivities and color discrimination (Lind & Kelber 2009). Therefore, avian color vision could be constrained by availability of carotenoids in the environment and their allocation within the body in much the same way as carotenoid-based plumage coloration.

This hypothesis is supported in part by evidence from domesticated species indicating that carotenoid accumulation in the avian retina can be affected by dietary carotenoid intake (Duecker & Schulze 1977, Wallman 1979, Bowmaker et al. 1993, Thompson et al. 2002ab; Knott et al. 2010). Additionally, pharmacological manipulations of domestic quail (*Coturnix japonica*) producing birds with carotenoid-free clear oil droplets result in altered innate color preferences (Duecker & Schulze 1977, Bowmaker et al. 1993) and optomotor responses (Wallman 1979). However, it is not clear how retinal carotenoid accumulation varies among birds in the natural environment and what impact this variation would have on visually mediated behaviors.

Indirectly, carotenoids may also influence vision by protecting cells in the eye from photodamage and oxidative stress. In domestic quail, dietary elevation of retina carotenoid levels reduced light-induced photoreceptor death and the formation of *N*-retinyl-*N*-retetinylidene ethanolamin (A2E), which is a marker of light-induced oxidative damage (Thomson et al. 2002ab; Bhosale et al. 2009).

Birds may even be able to facultatively increase carotenoid accumulation in the retina to protect against light-induced damage. For example, chickens reared in bright environments developed more intensely pigmented oil droplets than birds raised in a dim environment (Hart et al. 2006). However, the disparity between bright and dim conditions was much greater than wild birds would typically experience, and it remains to be determined if and how retinal carotenoid accumulation responds to natural variation in light exposure.

Hypothesis

Given the importance of carotenoid pigments in avian vision and their links to the ecology and physiology of the individual, I hypothesize that *avian vision is subject to environmental and physiological constraints imposed by the acquisition and allocation of carotenoids*. These constraints are known to limit the expression of carotenoid-based colors in the integument, and, if present for avian vision, would provide a unique biochemical linkage between the signal and sensory system. Such a linkage could have significant implications for the pace and direction of the evolution of carotenoid-based color signals.

Dissertation outline

To test my hypothesis, I have coupled biochemical analyses, physiological and nutritional manipulations, and behavioral tests to investigate the variation in,

constraints on, and functions of retinal carotenoid accumulation in the house finch. My dissertation studies are organized around three main questions:

1) Is there significant variation in retinal carotenoid accumulation among free-living wild house finches?

In Toomey & McGraw (2009; Appendix A), I sought to answer this question and generate observations to guide subsequent studies. In a year-long correlational study of wild house finches, I compared retinal carotenoid accumulation among seasons as well as between the sexes, and examined correlations with body condition, circulating plasma carotenoid levels, and male plumage coloration. If retinal accumulation was constrained by environmental and physiological factors similar to carotenoid-based plumage coloration, I predicted that retinal accumulation would be positively correlated with body condition and circulating carotenoid levels. Because carotenoid accumulation in the retina may be subject trade-offs with other functions I predicted that retinal levels would vary seasonally between the sexes as demands of egg production (spring) and plumage pigmentation (fall) could shift carotenoid allocation away from the eye.

2) What are the proximate environmental and physiological constraints on retinal carotenoid accumulation?

In Toomey & McGraw (2009), I observed significant environmentally and physiologically relevant variation in retinal carotenoid accumulation among wild house finches. To determine the specific factors driving this variation, I carried out a series of manipulative experiments with wild-caught captive house finches.

In Toomey & McGraw (2010; Appendix B), I detailed a series of experiments testing the influence of different types and concentrations of dietary carotenoids on retinal accumulation. Based on my hypothesis and previous studies of domesticated species (e.g. Knott et al. 2010), I predicted that retinal carotenoid accumulation would be positively related to dietary carotenoid levels.

In Toomey et al. (2010; Appendix C), I investigated the effect of long-term experimentally induced immune system activity on retinal carotenoid accumulation. Because carotenoids play an important role in the avian immune system (e.g. McGraw et al. 2011), immune system activation may place additional demands on carotenoid resource and limit allocation to the eye. Therefore, I predicted that immune-challenged birds would have reduced retinal carotenoid accumulation when compared to unchallenged birds.

In Chapter 1, I designed two experiments to test the influence of light exposure on retinal carotenoid accumulation. Hart et al. (2006) demonstrated that light exposure significantly increased absorbance (and presumably carotenoid content) of the cone oil droplets that may be a facultative response to increase photoprotection in the retina. However, their manipulation of light was extreme

and well-beyond what my study species would experience in the natural environment. Therefore, I used a more biologically relevant light exposure treatment by manipulating the duration of direct sun exposure and predicted that birds exposed to longer durations of direct sun would accumulate higher levels of retinal carotenoids.

3) What are the behavioral consequences of variable retinal carotenoid accumulation?

The goal of chapters 2 and 3 was to determine if and how variations in retinal carotenoid accumulation that I described in the previous four studies influenced color vision in a way that alters behaviors essential for survival and reproduction. In chapter 2, I investigated the influence of diet-manipulated retinal carotenoid levels on visual foraging behavior under varying lighting conditions. Because carotenoid-pigmented cone oil droplets are predicted to enhance color discrimination (Vorobyev 2003; Lind & Kelber 2009), I predicted that birds with diet-enhanced retinal carotenoid levels would have increased foraging success under challenging light-contrast conditions.

In chapter 3, I directly examined the relationship between retinal carotenoid accumulation and sexual selection for carotenoid-based coloration. I manipulated the retinal carotenoid levels of female birds through the diet and examined their mate selection behaviors (e.g. preference, discrimination, interest)

toward male finches of varying color. Because increased retinal carotenoid accumulation may enhance color discrimination, I predicted that females with increased levels of retinal carotenoids would be more efficient, discriminating, and repeatable in their choices of colorful males.

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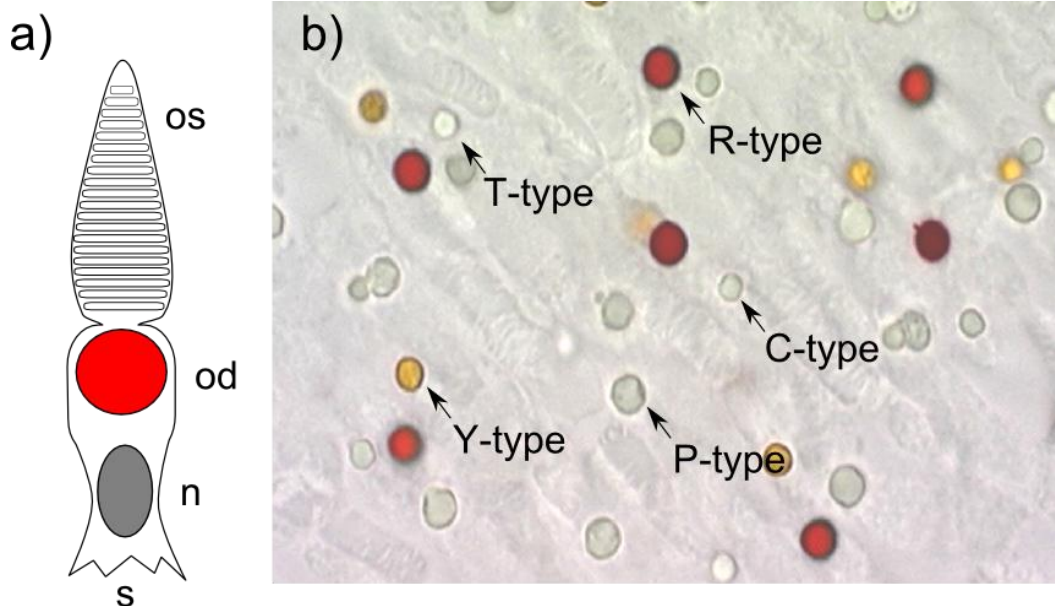


Figure 1. a) A schematic drawing of a typical long-wavelength sensitive single cone in the avian retina. The red carotenoid-pigmented oil droplet (od) is located within the inner segment and filters light before it reaches the visual pigment in the outer segment (os). For orientation the synapse (s) is located at the bottom of the image and the nucleus (n) is located within the inner segment. b) House finch cone oil droplets imaged at 1000x magnification. Each color of oil droplet is coupled to a different cone receptor type and pigmented with specific carotenoid types. Red (R-type) oil droplets are part of the long wavelength sensitive cones (LWS) and pigmented with astaxanthin, Yellow (Y-type) are part of the medium wavelength sensitive (MWS) cone and pigmented with xanthophyll carotenoids, C-type oil droplets are pigmented with galloxanthin and part of the short wavelength sensitive (SWS) cone; similar colored and pigmented P-type oil droplet is part of the double cone, and the transparent (T-type) oil droplet does not contain carotenoids and is part of the UV sensitive cone.

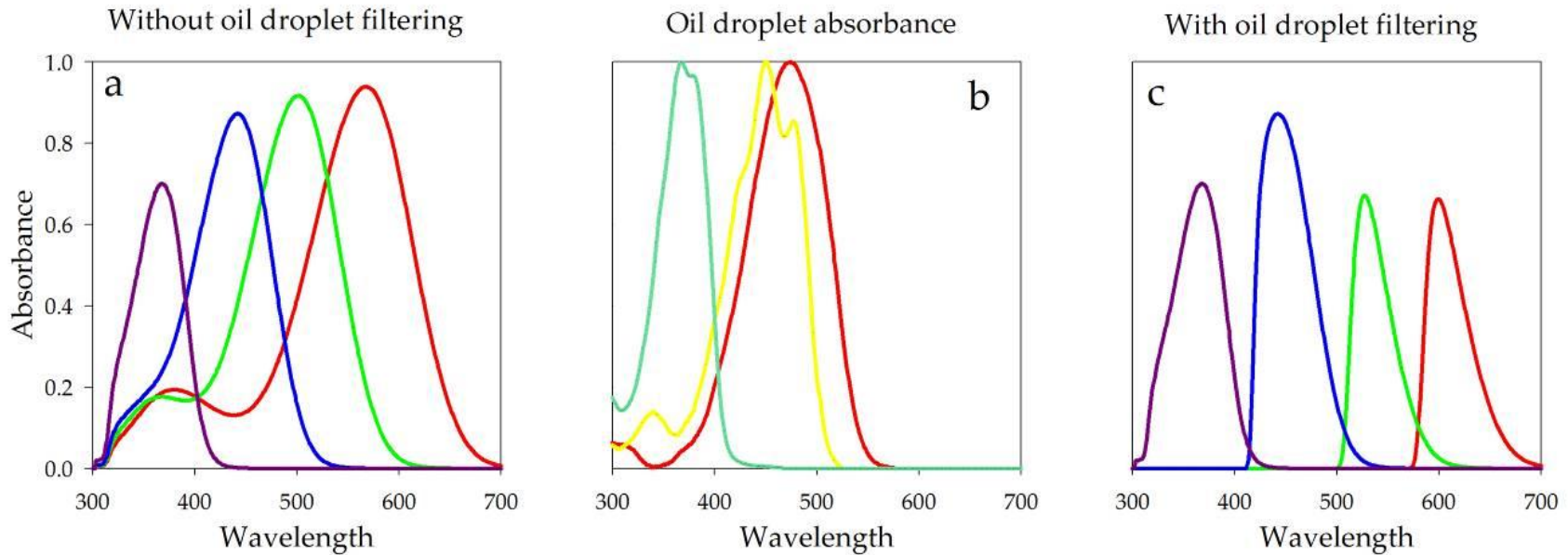


Figure 2. **a)** The absorbance spectra of the LWS (red), MWS (green), SWS (blue), and UVS (violet) photoreceptors modeled without the filtering effects of carotenoid-pigmented oil droplets, **b)** the cone oil droplet carotenoid pigment absorbance spectra (red – R-type, yellow – Y-type, and green – C-type), and **c)** the absorbance spectra of the photoreceptors with the filtering effects of carotenoid-pigmented oil droplets included (Das et al. 1998, Govardovskii et al. 2000, Hart & Vorobyev 2005). Physiological measures of house finch spectral sensitivities are not available, so these figures are based upon data from the canary (*Serinus canaria*) - a species closely related to the house finch.

Chapter 1

EFFECTS OF LIGHT EXPOSURE ON CAROTENOID ACCUMULATION AND OXIDATIVE STRESS IN THE RETINA OF A WILD BIRD

Diet-derived carotenoid pigments accumulate in the retinas of a diversity of animals, where they can provide photoprotection and antioxidant defense as well as tune color vision. Carotenoids are highly concentrated in the retinas of birds, and have been shown to provide photoprotection in the retinas of domesticated quail (*Coturnix coturnix japonica*). However, these observations were gathered using relatively extreme manipulations of retinal carotenoid accumulation and light exposure. The purpose of my study was to examine the influence of an ecologically relevant manipulation of light on carotenoid accumulation and oxidative stress in the retina of a wild bird species, the house finch (*Carpodacus mexicanus*). In a series of two experiments, I manipulated the duration that adult house finches were exposed to direct sunlight (8 v. 3 hrs./day) for two months during the winter (i.e. a low-light time of year), and measured the resulting carotenoid accumulation and lipid peroxidation levels in the retina. In the second experiment conducted during summer, when days are longer and solar irradiance is more intense, I examined if and how dietary carotenoid levels and light exposure interact to affect retinal carotenoids and oxidative damage, by coupling the aforementioned light manipulation with a carotenoid supplementation treatment. In both experiments, I found no significant effects of

light exposure on carotenoid or lipid peroxidation levels in retina. Dietary carotenoid supplementation in experiment two led to significantly higher retinal carotenoid accumulation, but did not significantly affect lipid peroxidation levels. Carotenoid accumulation differed significantly between the winter and summer experiments, even among birds with identical diets and similar housing condition. These results suggest that light exposure at my experimental levels does not affect retinal carotenoid accumulation, but that retinal carotenoid accumulation may track other seasonal changes in physiology (e.g. hormones) and the environment (e.g. photoperiod).

Introduction

Diet-derived carotenoid pigments accumulate in the retinas of a wide diversity of animals, from lamprey to turtles to humans, and play an essential role in the health and function of the visual system (Douglas & Marshall 1999, Krinsky et al. 2003). Carotenoids protect the retina directly by absorbing short-wavelength, high-energy light and indirectly as antioxidants that counter oxidative stress (Krinsky et al. 2003). The effectiveness of these protective mechanisms depends upon the types and concentration of carotenoids that are ultimately depleted by these processes. Therefore, efficient accumulation and maintenance of carotenoid levels may be essential for long-term retina health and function.

The importance of carotenoids in the human eye is supported by a number of epidemiological studies that demonstrate significant negative correlations of dietary carotenoid intake and retinal accumulation with the incidence of age-related macular degeneration (Seddon et al. 1994, Bernstein et al. 2006). However, the specific mechanism of this protection remains unresolved. In primate models, dietary supplementation with carotenoids increases retinal accumulation and directly protects the retina from photodamage (Barker et al. *in press*). Studies of cultured photoreceptor cells also demonstrate that carotenoids can provide dose-dependent antioxidant protection (Chucair et al. 2006, Nakajim et al. 2009). However, when generalizing these results to other species, and to birds in particular, it is important to note that carotenoids in the primate retina accumulate throughout the photoreceptor axons and are only highly concentrated in the fovea (Snodderly 1984). In contrast, carotenoids in the avian retina are highly localized within specialized structures – oil droplets – that are widely distributed throughout the retina (Goldsmith et al. 1984).

Oil droplets are located between the cone inner and outer segments of the avian retina, with a distinct carotenoid composition and concentration (and human-perceivable color) in each photoreceptor type (Goldsmith et al. 1984). In this position the carotenoid pigments alter the composition and intensity of light reaching the visual pigment and provide both spectral tuning and photoprotection benefits (Hart 2001; Vorobyev 2003). Similar to primates, dietary enhancement of

carotenoid accumulation in the avian retina reduces light induced photoreceptor death and the formation of *N*-retinyl-*N*-retetinylidene ethanolamin (A2E), which is a marker of light-induced oxidative damage (Thomson et al. 2002ab; Bhosale et al. 2009). However, photoprotection through high levels of carotenoid accumulation may come at a cost to visual function, and birds may modulate accumulation to meet these competing demands.

In the avian retina, oil droplet filtering can be quite extensive, absorbing more than 50% of the light reaching the photoreceptor and potentially limiting color vision under low light conditions (Bowmaker 1977; Vorobyev 2003; Hart and Vorobyev 2005, Chapter 2). Thus, there may be a trade-off between photoprotection from bright light and color vision under dim conditions. Consistent with this hypothesis, many nocturnal bird species tend to have pale oil droplets with presumably improved transmittance and low-light sensitivity (Bowmaker & Martin 1979; Hart et al. 2006). Recently, Hart et al. (2006) observed that domestic chickens (*Gallus gallus*) reared under bright conditions developed more intensely pigmented oil droplets than birds raised in a dim environment. This result suggests that birds can up- or down-regulate retinal carotenoid accumulation to match their light environment and visual demands.

In contrast, long-term light exposure may limit carotenoid accumulation in the retina through photodegradation. Exposure to ultraviolet light (UV) has been shown to deplete carotenoids from plasma (Roe 1986; White et al. 1988; Biesalski

et al. 1996) and, without compensatory mechanisms of accumulation, this systemic depletion of carotenoids along with the direct degradation in the retina could cause significant declines in retinal carotenoid levels. Consistent with this prediction, I have observed that the retinal carotenoid levels of a wild species of bird – house finches (*Carpodacus mexicanus*) – are lowest in the late spring and summer, when the animals are exposed to the longest days with the most intense light levels (Toomey & McGraw 2009; Appendix A). However, I cannot rule out a number of other factors (e.g. diet, egg production) that could be responsible for this seasonal decline. Thus, the evidence reviewed here offers contrasting predictions about the influence of light on retinal carotenoid accumulation in the avian retina. If retinal carotenoid accumulation is tuned to environmental light levels, I would predict a positive relationship between light and accumulation, but if the photodegradation of carotenoids is an important determinant of accumulation I would expect the opposite pattern. However, these predictions are largely based on studies of domesticated species that involve manipulations of light intensity well outside the natural range of variation for a wild desert bird.

The goal of my studies presented here were to determine if and how ecologically relevant variations in light exposure influence carotenoid accumulation and oxidative damage in the retina of an adult wild bird and test the contrasting predictions of accumulation and degradation. I carried out two separate experiments where I exposed wild-caught captive house finches to short

or long daily bouts of direct sunlight exposure for two months and measured their resulting plasma and retinal carotenoid levels with high-performance liquid chromatography (HPLC) and retinal lipid peroxidation levels with a thiobarbituric acid reactive substances assay (TBARS). My manipulation resulted in a two to four fold difference in light exposure between the treatment groups, which is much smaller than previous studies (e.g. Hart et al. 2006), but was intended to approximate a range of light exposure experienced by house finches in the natural environment. My first experiment was conducted over the winter months (Jan.-Mar.) and included both male and female birds. The second experiment was done in the late spring - summer (May-July a period characterized by long, cloud-free days, with intense irradiance; AZMet 2011), used only male birds, and included a manipulation of dietary carotenoid levels to test for a possible interaction between light exposure and dietary carotenoid availability.

Methods

Experiment 1

Capture and housing of study animals

In October 2007, I captured 16 male and 13 female house finches on the campus of Arizona State University (ASU) in baited basket traps following the methods described in Toomey & McGraw (2009). These birds were housed as male/female pairs or singly (n = 3 males) in small cages (0.6 × 0.4 × 0.3 m) on top

shelves of movable racks. These racks were kept in an outdoor enclosure within an animal run designed for large mammals. This space included areas of direct sun exposure and shaded areas under a metal roof. The birds were provided with *ad libitum* access to tap water for drinking and a maintenance diet (ZuPreem small bird maintenance diet, Premium Nutritional Products Inc. Mission, KS, USA) that contained two predominant carotenoid types – lutein ($1.15 \pm 0.12 \mu\text{g g}^{-1}$) and zeaxanthin ($0.52 \pm 0.06 \mu\text{g g}^{-1}$).

Light exposure manipulation

To manipulate light exposure among the birds, I controlled the amount of time during each week day that birds were exposed to direct sunlight vs. shade. I randomly assigned the caged birds to one of two racks and placed one rack in the direct sun for a period of three hours per day (low-light exposure, n = 8 M, 7 F), while the other rack was kept in direct sunlight for eight hours per day (high-light exposure, n = 8 M, 6 F). The 8 hr. sun exposure period was from 0830 to 1630 hrs, while the 3 hr. period was randomized among days to occur sometime within that same 8 hr. time span. On weekends, both treatment groups were kept in the shade for the entire day; all birds were moved to the shaded side of the cage each night as well. To track the levels of light exposure that each treatment group received, I attached data loggers (HOBO UA-002-64, Onset Computer Co. Bourne, MA) to each rack and recorded light intensity and temperature at four

min intervals throughout the study. A sample of the daily light intensity and temperature profile is given in Fig. 3ab. The light exposure manipulation continued for eight weeks, at which point I euthanized the birds and collected tissue for carotenoid and lipid peroxide analyses.

Body mass, food consumption, and carotenoid measurements

To examine the possible influence of the light exposure manipulation on the body mass and food intake of the birds, which might affect carotenoid intake/use in ways independent from direct light exposure, I weighed the birds prior to beginning the manipulation (week 0), in the middle of the study (week 4), and at the conclusion of the study (week 8). In week three of the manipulation, I measured the mass of food consumed by each pair of birds in a 24 hr period. On weeks 0, 4, and 8, I collected plasma samples (~40 μ l) from each bird and determined circulating carotenoid levels with high-performance liquid chromatography (HPLC) following Toomey & McGraw (2009). At the conclusion of the study, I euthanized all birds, dissected out the retina of the left eye, and measured retinal carotenoid concentrations with HPLC (*sensu* Toomey & McGraw 2009). As in previous studies, I observed six major types of carotenoids in the house finch retina and I report concentration per whole retina (Toomey & McGraw 2009; 2010).

2.1.4 *Oxidative stress measurement*

Oxidative stress in the retina was measured using a miniaturized thiobarbituric acid reactive substances (TBARS) assay modified from a commercially available kit (Oxi-Tek TBARS assay kit, ZeptoMetrix Corp., Buffalo, NY). The TBARS provides a measure of oxidative stress by quantifying levels of lipid peroxidation products, specifically malondialdehyde (MDA), a major marker of oxidative stress (Janero 1990). Briefly, whole retinas were dissected out of the right eye, weighed to the nearest 0.0001 g with a digital balance, and then homogenized in 500 μL of phosphate buffered saline. A 30 μL of aliquot of this homogenate was mixed with 30 μL of 8.1% sodium dodecyl sulfate (SDS) and 750 μL of thiobarbituric acid (TBA) buffer reagent. Samples were then incubated at 95°C for 60 min. The samples were then placed on ice for 10 min and centrifuged at 3000 rpm for 15 min. I measured absorbance of the supernatant at 540 nm and calculated concentration by comparison to a standard curve of known concentrations of MDA and are expressed in $\text{nmol}\cdot\text{mg}^{-1}$ of MDA equivalents.

Statistical analyses

I compared the mean daily light intensities and temperatures and the food consumption of the high- and low-light exposure treatments using a Student's *t*-test. I compared changes in body mass and plasma carotenoid levels over time

between the sexes and treatment groups in repeated-measures analyses of variance (rmANOVA). I compared retinal carotenoid levels between the sexes and treatment groups with a multivariate analysis of variance (MANOVA), with the six major retinal carotenoid types as the dependent variable. I compared lipid peroxidation levels between the sexes and treatment groups using analysis of covariance (ANCOVA), with total retinal carotenoid concentration as the covariate. Lipid peroxidation values were natural log transformed to meet the assumptions of normality. All statistical analyses were carried out in R 2.12 (R Development Core Team 2010), values are reported as mean \pm S.E., and the alpha level was set at 0.05.

Experiment 2

Capture and housing of study animals

In April 2010, I captured 32 male house finches at a private residence ~0.75 mi from the ASU campus as described above. I limited the sample to male birds in this study because I found no significant difference between the sexes in experiment 1 (see more below) and because I wanted to avoid taking females that were actively laying eggs at this time of year (Hill 1993). The birds were housed as pairs in the same cage types and on the same base diets as described above. However, for this experiment, the cage racks were kept in a large outdoor aviary at the same facility as experiment 1 that offered similar areas of sun and shade.

Light exposure manipulation

The light exposure manipulation mimicked experiment 1, with 16 males receiving three hours per day of direct sun (low-light exposure) and 16 males receiving eight hours per day (high-light exposure). However, this experiment was conducted in the summer when outdoor temperatures in the direct sun can rise well above 45 °C. To counter these extreme temperatures, I used a combination of fans and a misting system to cool the birds in the direct sunlight. Unfortunately, on one day during week 6 of the study, this cooling system failed and resulted in the death of five birds in the high-light treatment. After this incident, the birds were monitored continuously for signs of heat stress (e.g. gaping, lethargy) and removed from the direct sun for 30 min intervals if necessary. These cooling bouts were infrequent, occurring at a maximum of three per day, and did not compromise treatment differences in light exposure; data from these bouts are included in the mean light and temperature calculations presented in Table 1.

Carotenoid supplementation

To examine if and how dietary carotenoid levels might interact with light exposure to influence retinal carotenoid accumulation; I supplemented eight of the birds in each light treatment with zeaxanthin. I chose zeaxanthin because it is the

putative dietary precursor for many of the carotenoid types in the avian retina (Schiedt 1991; Bhosale et al. 2007). Zeaxanthin ($17.5 \mu\text{g ml}^{-1}$, OptiSharp™, DSM Inc. Heerlen, Netherlands) was given in the drinking water along with a vitamin supplement (Vita-Sol, United Pet Group EIO, Tampa, FL) for carotenoid-treated birds; control animals received only the vitamin supplement in their water. These drinking-water treatments were administered each weekday evening, after the light exposure manipulation was finished and when birds from both light treatments were in the shade. I replaced the supplemented water with plain tap water each morning, prior to the light exposure manipulation, to ensure that differences in carotenoid accumulation were not driven by water consumption while the birds were differentially exposed to direct sun.

Body mass, food consumption, carotenoid, and oxidative stress measurements

Body mass, plasma, and retinal carotenoid levels, and retinal oxidative stress were measured as described for experiment 1. Food consumption was measured as in experiment 1, but measurements were made in week two and week six of the light manipulation.

Statistical analyses

Statistical analyses were carried out as described for experiment 1 (§2.1.5), with the inclusion of carotenoid supplementation level as an independent

variable. Also, as all of the birds in experiment 2 were males, sex was not included as a factor in ANOVA models.

Comparisons between experiments 1 and 2

Among wild house finches, retinal carotenoid levels vary among the seasons, with a minimum in the early spring (March) and a peak in the late fall (November; Toomey & McGraw 2009). However, it is not clear from such correlational data what factors (e.g. diet, health, reproductive status) drive these seasonal differences. A comparison of individuals from experiments one and two offers me the opportunity to examine the influence of season, while controlling for the influence of dietary carotenoid availability. For this comparison, I limited my analyses to male finches that were not carotenoid-supplemented, leaving us with 16 males from experiment one and 15 from experiment two. The only differences between the experimental groups were the year, date, time in captivity prior to the light manipulation (66 and 35 days respectively), and the sex of their cage mate. In experiment one the birds were housed as mixed-sex pairs and in experiment two only male finches were studied. I compared retinal carotenoid accumulation between the experiments and light-exposure treatments in a MANOVA and used univariate ANOVA to compare total plasma carotenoid levels and retinal lipid peroxidation levels.

Results

Experiment 1

Light intensity and temperature

Mean intensity of light to which birds from the high-light treatment were exposed was significantly greater than for the low-light group (Table 1). Similarly, birds from the high-light treatment were exposed to 3.4 °C higher temperatures, on average, than low-light birds (Table 1).

Body mass and food consumption

There was no significant effect of light treatment on body mass of the finches (rmANOVA: treatment \times date – $F_{2,51} = 0.34$, $p = 0.71$) or food consumption in a 24 hr period ($t = -0.99$, $p = 0.34$). Body mass did not differ significantly between the sexes ($F_{1,26} = 0.49$, $p = 0.49$); however, it did change significantly over the course of the study for birds of both sexes in both treatments (rmANOVA: date – $F_{2,51} = 16.78$, $p < 0.0001$, Fig. 4a). The loss and recovery of body mass commonly occurs when wild house finches are brought into captivity (e.g. Toomey et al. 2010) and is unlikely to be related to the specific conditions in this study.

Retinal and plasma carotenoid accumulation

Retinal carotenoid concentration did not differ significantly between the sexes (Table 2) or between the high- and low-light exposed birds (Table 2, Fig.

5a). Total plasma carotenoid levels did not differ significantly between treatment groups (rmANOVA: treatment \times date – $F_{2,51} = 0.23$, $p = 0.80$) or sexes ($F_{1,26} = 2.03$, $p = 0.17$). However, plasma carotenoid levels did change significantly over the course of the study for birds of both sexes in both treatments (rmANOVA: date – $F_{2,52} = 4.23$, $p = 0.020$, Fig. 6a), with a significant increase from week four to week eight (Tukey's post hoc, $p = 0.010$).

Retinal oxidative stress

Retinal lipid peroxidation levels did not differ significantly between light exposure treatments or the sexes and was not significantly correlated with total retinal carotenoid concentrations (Table 3; Fig. 7a).

Experiment 2

Light intensity and temperature

Birds in the high-light treatment experienced significantly greater mean light intensities and temperatures than did those in the low-light group (Table 1). However, the difference in mean temperatures among treatments was < 1 °C (Table 1).

Body mass and food consumption

Body mass again declined over the course of the experiment, and there was a significant three-way interaction between date, carotenoid supplementation, and light treatment (diet \times treatment \times date: $F_{2,50} = 5.07$, $p < 0.0099$; Fig 4b). However, within sampling periods, there were no significant differences in body mass among dietary and light treatment groups (Tukey post-hoc, $p > 0.98$, Fig. 4b). Consistent with the decline in mass, food consumption declined significantly between the May and June sampling periods from 9.74 ± 0.40 to 6.91 ± 0.18 g day⁻¹ cage⁻¹ ($F_{1,15} = 57.80$, $p < 0.0001$), but did not differ significantly between diet treatments ($F_{1,13} = 0.045$, $p = 0.83$) or light treatments ($F_{1,13} = 5.29$, $p = 0.55$).

Retinal and plasma carotenoid accumulation

Retinal carotenoid accumulation was significantly higher in zeaxanthin-supplemented birds than in control, unsupplemented birds (Table 2, Fig. 5b). Zeaxanthin supplementation also resulted in significantly higher levels of galloxanthin ($F_{1,29} = 4.23$, $p = 0.049$) and zeaxanthin ($F_{1,29} = 14.68$, $p < 0.001$) in the retina. There was no significant effect of light exposure on retinal carotenoid accumulation, or a significant interaction of light exposure and carotenoid supplementation (Table 2). Zeaxanthin supplementation significantly increased circulating plasma carotenoid levels (rmANOVA: diet \times date – $F_{2,52} = 7.09$, $p = 0.0019$, Fig. 6b), but there was no significant effect of light exposure ($F_{1,28} = 0.78$,

$p = 0.38$) or interaction between light exposure and diet on total plasma carotenoid levels ($F_{1,28} = 1.56, p = 0.22$).

Retinal oxidative stress

Retinal lipid peroxidation levels did not differ significantly between light exposure or diet treatments (Fig. 7bc), and there was no significant interaction between light exposure and zeaxanthin supplementation (Table 3).

Comparisons between experiments 1 and 2

Retinal carotenoid accumulation differed significantly between males in experiment one and two (Wilks' $\lambda = 0.15, df = 6,22, p < 0.0001$) and males in experiment two had significantly higher levels of astaxanthin, an unknown carotenoid, and ϵ -carotene (Table 4, Fig. 8a). In contrast, birds in experiment one circulated significantly higher levels of carotenoids in their plasma than birds in experiment two ($F_{1,25} = 25.26, p < 0.0001, \text{Fig. 8b}$). There was no significant difference in the levels of retina lipid peroxidation ($F_{1,25} = 0.023, p = 0.88$) or body mass at week eight ($F_{1,25} = 2.99, p = 0.096$) between experiments one and two.

Discussion

In a previous study (Toomey & McGraw 2009; Appendix A), I observed significant variation in the accumulation of carotenoids in the retinas of wild

house finches, both among individuals and across seasons. My goal in this series of experiments was to examine if and how light exposure and dietary carotenoid availability influence retinal carotenoid accumulation. My experiments generated three main results: 1) manipulating direct sunlight exposure did not significantly affect retinal carotenoid accumulation or oxidative stress, 2) dietary carotenoid supplementation increased retinal carotenoid accumulation but did not influence retinal oxidative stress, and 3) retinal carotenoid accumulation differed significantly between experiments one and two, even for individuals with the same diet and similar housing conditions.

Direct sunlight exposure did not influence retinal carotenoid accumulation or oxidative stress

Contrary to my predictions and other previous studies (Hart et al. 2006), there were no significant differences in retinal carotenoid accumulation or retinal oxidative stress between birds exposed to long vs. short daily bouts of direct sunlight exposure in either of my experiments. The lack of an effect of light exposure on carotenoid accumulation and oxidative stress suggests that this may not be an important factor determining carotenoid accumulation in the retina and that the house finch is well-adapted to the stresses of intense light in its typical open, desert habitat. However, interpreting these negative results requires careful consideration of the experimental design and biology of the house finch.

Although there was a more than two-fold difference in light exposure among treatment groups, this manipulation was quite conservative compared to previous studies demonstrating physiological changes in the avian eye. For example, Hart et al. (2006) observed significant changes in the carotenoid pigmentation (i.e. light-absorbance properties) of the cone oil droplets of chickens reared in bright and dim environments. However, in their study, the birds were maintained under relatively constant exposure levels and the bright treatment averaged 70,250 lux, while the birds in dim light received only 14 lux, a >5000 fold difference in light intensity (Hart et al. 2006). Even more dramatic changes in the gross morphology of the eye have been observed among domesticated chickens reared under dim artificial lighting conditions. Significant increases in eye mass (Blatchford et al. 2009) and size (Deep et al. 2010), as well as declines in corneal thickness (Harrison et al. 1968), have been observed among chickens reared under very dim (< 5 lux) compared to normal indoor lighting conditions (> 50 lux), representing a > 10 fold intensity difference between treatments. Therefore, the lack of a significant effect of light exposure on retinal carotenoid accumulation in my study may have resulted from the relatively bright (even if natural) conditions in the experiments and the limited differences (~2-4×) between treatments. Additionally, I manipulated the duration of light exposure, rather than the maximum intensity; thus all birds experienced intense sunlight at least a portion of the time. Despite these limitations, my study is informative

because my treatments are more consistent with the conditions experienced by birds as they move through their natural environment. With the exception of the densest forests (Endler 1993), day-time conditions in terrestrial habitats are much brighter than those used in previous studies, especially for desert-dwelling house finches.

Although the consistency of my findings across repeated experiments suggests the results are reliable, there are aspects of the study design that may have limited my ability to detect the effects of light on the retina. I cannot rule out the possibility that the birds behaviorally compensated for the light exposure by seeking out the very small and few shaded microenvironments within the cage (e.g. shadows cast by thin cage bars, perches, food/water dishes, or the body of their cage mates). It is possible that the effects of light exposure were localized to specific regions of the retina and that I was unable to detect these with a whole retina measurement. Dietary carotenoid supplementation tends to enhance accumulation specifically in the dorsal retina (Knott et al. 2010), and the effects of light exposure on retinal oil-droplet absorbance are most pronounced in the ventral retina (Hart et al. 2006). Therefore the localized effects of light exposure on retina warrant further investigation. The time course of the study may also have limited my ability to detect light-induced changes. I manipulated light exposure over the course of eight weeks in adult birds, which is a significantly shorter period than the 30-week manipulation of young chickens employed by

Hart et al. (2006). Carotenoids in the avian retina are quite stable (Toomey & McGraw 2020; Appendix B), and it is possible that much longer-term changes in light exposure are required to alter accumulation. A gradual response to long-term changes is consistent with the relatively gradual onset of many of the diseases associated with light exposure. For example, age-related macular degeneration, a disease against which carotenoid accumulation may provide protection, develops over the course of decades in humans (Young 1987, Seddon 1994). Finally, studies demonstrating light-mediated effects on the avian retina (e.g. Harrison 1968; Hart et al. 2006; Blatchford et al. 2009) have all used young domesticated chickens. It is possible that the influence of light is limited to the developmental period and would explain why I did not detect changes among adult house finches. Consistent with this hypothesis, there is growing evidence linking developmental conditions (i.e. dietary carotenoid levels) to adult carotenoid assimilation and accumulation efficiency in birds (Blount et al. 2003, Butler and McGraw 2010).

Also contrary to my predictions, duration of direct sunlight exposure did not significantly affect oxidative stress levels in the retina. Although my light exposure manipulation may not have lasted long enough to influence retinal carotenoid accumulation, it may have been too long to detect an effect on oxidative stress. Shibuki et al. (2000) observed that lipid peroxide levels in the rat retina peak three hours after the application of a stressor (ischemia-reperfusion)

and return to normal 48 hours after the stressor has been removed. Thus, the extended duration of my study may have allowed for adaptation to and recovery from photostress (i.e. each evening following the light treatments). It is also important to consider that the house finch is native to the Sonoran desert (Hill 1993) and is likely to have evolved physiological mechanisms to protect against the intense sunlight that is characteristic of this open habitat.

Dietary supplementation enhanced retinal carotenoid accumulation

Consistent with my previous study of house finch retinas (Toomey & McGraw 2010; Appendix B), dietary carotenoid supplementation led to significantly higher retinal carotenoid levels. Specifically, dietary zeaxanthin supplementation increased galloxanthin and zeaxanthin levels in the retina. Previously I had speculated that the specificity of these diet-driven increases may be attributable to differing rates of carotenoid turn-over in the retina (Toomey & McGraw 2010; Appendix B). For example, quail fed labeled zeaxanthin precursors tend to accumulate higher levels of labeled galloxanthin and zeaxanthin in retina than other carotenoids (Bhosale et al. 2007). This leads to a prediction that light exposure should hasten the degradation of galloxanthin and zeaxanthin and an interaction between light exposure and dietary carotenoid levels. However, this was not the case; thus the photodegradation of specific

carotenoids in the retina is unlikely to be driving this pattern of specific diet-enhanced carotenoid accumulation.

I found no significant relationship between retinal carotenoid accumulation and lipid peroxidation, and this may reflect the nature of carotenoid accumulation in the avian retina. Because many carotenoids in the avian retina are esterified and bound up in lipid rich oil droplets (Goldsmith et al. 1984, Bhosale et al. 2007), they may not be available to function as rapidly mobilized antioxidants. It would now be interesting to track the relationship between oxidative stress levels and concentrations of free and esterified carotenoids in the avian retina. My results contrast with studies of domesticated quail showing that retinal carotenoid accumulation promotes photoprotection and eye health (Thomson et al. 2002ab; Bhosale et al. 2009). However, these studies quantified photoreceptor death (Thomson et al. 2002ab) and A2E accumulation (Bhosale et al. 2009) as measures of health that are not necessarily associated with the production of lipid peroxides.

Carotenoid accumulation differs among seasons despite identical dietary conditions

Under identical dietary and similar housing conditions, male finches in experiment two (May-Jul) had significantly higher retinal carotenoid levels, but lower plasma carotenoid concentrations, than the males in experiment one (Jan-

Mar). This pattern of seasonal retinal accumulation in captive birds is consistent with observations of wild birds (Toomey & McGraw 2009; Appendix A). However, in this comparison, I have controlled for the effects of diet and reproductive effort, suggesting that abiotic environmental factors such as daylength or temperature influence the accumulation of carotenoids in the retina.

The timing of two experiments corresponds to distinct phases in the reproductive cycle of the house finch, which suggests that a proximate hormonal mechanism may mediate these seasonal differences in carotenoid accumulation. Experiment one occurred at the beginning of the breeding season, when birds are pairing and testosterone levels are highest in males (Hammer 1966, Duckworth et al. 2004), whereas experiment two took place during the nestling and post-nesting phase, when house finches become photorefractory and testosterone levels drop (Hammer 1968, Duckworth et al. 2004). Additionally, the males in experiment one were housed with females while the males in experiment two were housed only with other males, which may have led to differences in reproductive state between the experiments. Recently, Gautier et al. (2008) have shown that housing male zebra finches (*Taeniopygia guttata*) in mixed versus single sex conditions significantly shifts the allocation of carotenoid, with males in mixed groups showing increased carotenoid-based bill coloration (Gautier et al. 2008). This leads to the hypothesis that reproductive state and specifically, testosterone, may influence the accumulation of carotenoids in the retina.

A role for testosterone in carotenoid accumulation is supported by a growing body of evidence linking this sex steroid with carotenoid bioavailability. In both zebra finches (*Taeniopygia guttata*) and red-legged partridges (*Alectoris rufa*), experimentally increased testosterone levels result in the increased production of plasma lipoprotein carotenoid carriers and a concomitant rise in circulating carotenoid levels (McGraw et al. 2005; Blas et al. 2006). Because carotenoids can promote immune function, this enhancement of carotenoid bioavailability has been hypothesized as a means to counter the immunosuppressive effects of testosterone (McGraw & Ardia 2007, Peters 2007). However, increases in plasma carotenoid levels do not necessarily translate to other tissues. For example, testosterone has been shown to inhibit the accumulation of carotenoids in house finch plumage (Stoehr & Hill 2001). Thus, testosterone may not increase the overall levels of carotenoids but rather shift allocation among tissues and systems in the body. Consistent with a shift in carotenoid allocation, I found that the birds in experiment one circulated relatively high levels of carotenoids in their plasma, but had relatively low levels in their retinas, while the pattern was reversed for the birds in experiment two.

The males in experiments one and two differed primarily in their accumulation of astaxanthin in the retina, which is a red ketocarotenoid that is metabolized from dietary carotenoid precursors (i.e. zeaxanthin; Scheidt 1991; Bhoslae et al. 2007). This contrasts with previous studies (Toomey & McGraw

2010; Toomey et al. 2010; Appendix B & C) where I have found astaxanthin accumulation to be much more stable to dietary changes and immune system perturbations than other retinal carotenoid types (i.e. galloxanthin and zeaxanthin). This result suggests that these different components of the avian retinal carotenoid profile are subject to different environmental and physiological constraints. It is interesting to note that astaxanthin and other ketocarotenoids are a major component of sexually attractive and metabolically derived red male house finch plumage. Because red pigmentation in house finches is inhibited by testosterone (Steohr & Hill 2001), the metabolic production of ketocarotenoids may be particularly sensitive to sex steroids.

Although the comparison of experiments one and two suggests an intriguing relationship between daylength, gonadal hormones, and retinal carotenoid accumulation, it is important to acknowledge the limitations of this comparison. Experiments one and two were carried out two years apart and the birds were collected from different locations (although < 1 mi apart). Therefore, the differences could reflect population-level differences rather than a response to season. I also lack direct measures of hormone titer or testicular development; therefore I can only infer a link between hormone status and retinal carotenoid accumulation from the seasonal profiles available in the literature (e.g. Hamner 1966). To address these limitations, additional studies, specifically testosterone

manipulations, are needed to tease apart the influences of day length, temperature, and testosterone on retinal carotenoid accumulation.

Conclusion

Taken together, these studies indicate that the duration of intense light exposure does not influence the accumulation of carotenoids in retinas of adult house finches. The house finch retina also appears to be buffered against the oxidative stresses of intense light exposure, which may reflect an adaptation to its bright desert environment. However, the comparison of experiments one and two suggests a role for seasonal cues in shaping retinal carotenoid accumulation.

There are several potential mechanisms that could drive these seasonal differences, including the direct influence of day length on the retina and/or hormone-mediated shifts in carotenoid allocation. Given the importance of carotenoids in visual health and performance of both birds and humans, unraveling these mechanisms may provide important insight into the evolution of the eye and the maintenance of visual health.

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Table 1. Light and temperature conditions among treatment groups in the two captive experiments in which I manipulated duration of light exposure for wild-caught house finches.

Experiment	Dates	Mean day length (mins)	Treatment	Hours of direct light exposure	Mean light intensity (Lux)*	Mean Temperature (°C)*
1	7Jan – 3Mar 2008	642.88 ± 3.69	High	8	12746.93 ± 197.76	15.69 ± 0.059
			Low	3	5714.55 ± 176.73	14.37 ± 0.047
2	10May – 5Jul 2010	852.10 ± 1.44	High	8	21561.29 ± 369.69	31.42 ± 0.066
			Low	3	5584.77 ± 183.33	31.07 ± 0.061

* differed significantly between treatment groups and experiments ($t > 4.02$, $p < 0.0001$)

Table 2. Results of MANOVA analyses testing the effect of light exposure, sex, and their interaction on retinal carotenoid accumulation in experiments 1 and 2. Significant terms are in bold.

Factor	Wilks' λ	df	<i>p</i>
Experiment 1			
light treatment	0.89	6, 18	0.90
sex	0.78	6, 18	0.58
light treatment \times sex	0.82	6, 18	0.69
Experiment 2			
light treatment	0.71	6, 23	0.21
diet	0.57	6, 23	0.033
light treatment \times diet	0.81	6, 23	0.50

Table 3. Results of ANOVA analyses testing the effects of light exposure, sex, and their interaction on retinal lipid peroxidation in experiments 1 and 2.

Factor	<i>F</i>	df	<i>p</i>
Experiment 1			
light treatment	0.0098	1,23	0.92
sex	0.55	1,23	0.46
total retinal carotenoids	0.060	1,23	0.81
Experiment 2			
light treatment	0.027	1,21	0.55
diet	0.0093	1,21	0.72
light treatment × diet	0.0058	1,21	0.94

Table 4. . Results of ANOVA analyses testing the effects of light exposure, experiment, and their interaction on the accumulation of specific types of retinal carotenoids. Significant terms are in bold.

Factor	<i>F</i>	df	<i>p</i>
Astaxanthin			
light treatment	1.68	1,27	0.21
experiment	64.76	1,27	< 0.0001
light treatment × experiment	0.084	1,27	0.77
Galloxanthin			
light treatment	0.44	1,27	0.51
experiment	1.49	1,27	0.23
light treatment × experiment	0.55	1,27	0.46
Lutein			
light treatment	1.10	1,27	0.30
experiment	0.064	1,27	0.80
light treatment × experiment	1.28	1,27	0.27
Zeaxanthin			
light treatment	0.0045	1,27	0.95
experiment	1.38	1,27	0.25
light treatment × experiment	1.21	1,27	0.28
Unknown			
light treatment	1.03	1,27	0.32
experiment	8.37	1,27	0.0074
light treatment × experiment	0.12	1,27	0.73
ε-carotene			
light treatment	1.69	1,27	0.21
experiment	31.22	1,27	< 0.0001
light treatment × experiment	1.23	1,27	0.28

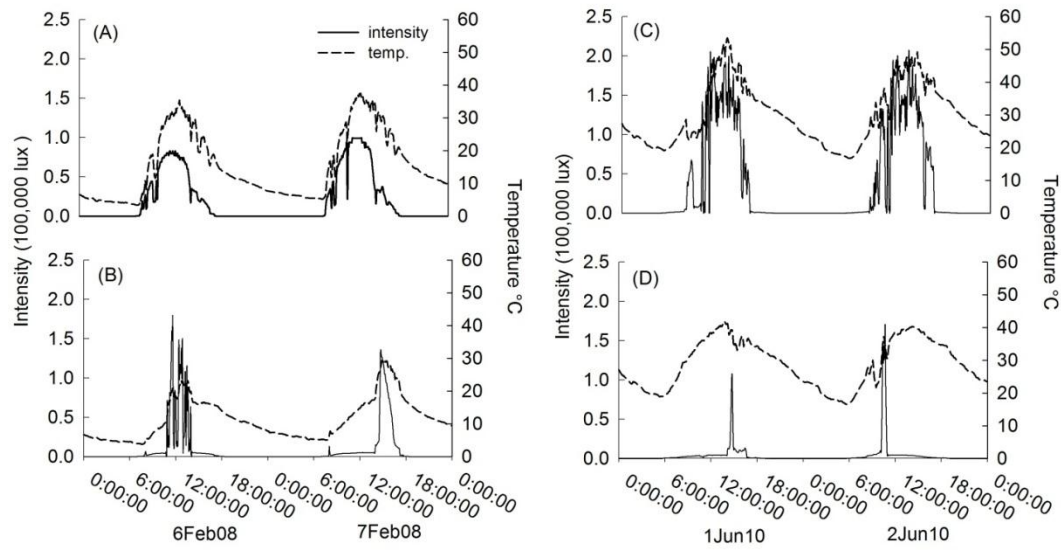


Figure 3. Examples of the temporal light intensity and temperature profiles for (A) high-light treatment group from experiment 1, (B) low-light treatment from experiment 1, (C) high-light treatment from experiment 2, and (D) low-light treatment from experiment 2.

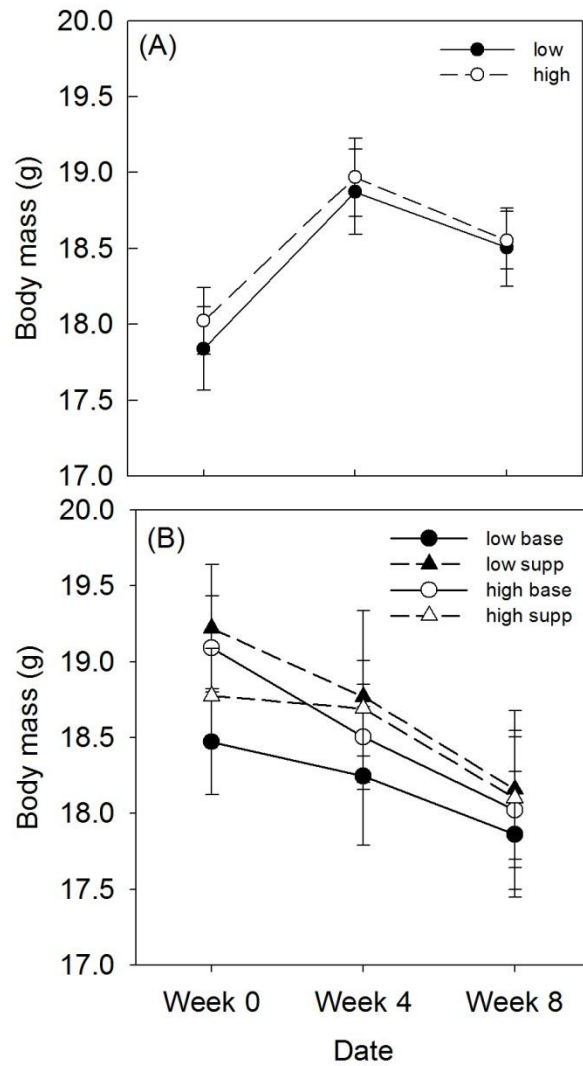


Figure 4. Mean \pm S.E. body mass of house finches over the course of experiments 1 (A) and 2 (B). Week 0 measurements were performed just prior to the beginning of light exposure and dietary (experiment 2 only) manipulations. In experiment 2 (B) “supp.” birds received a zeaxanthin supplement in their drinking water.

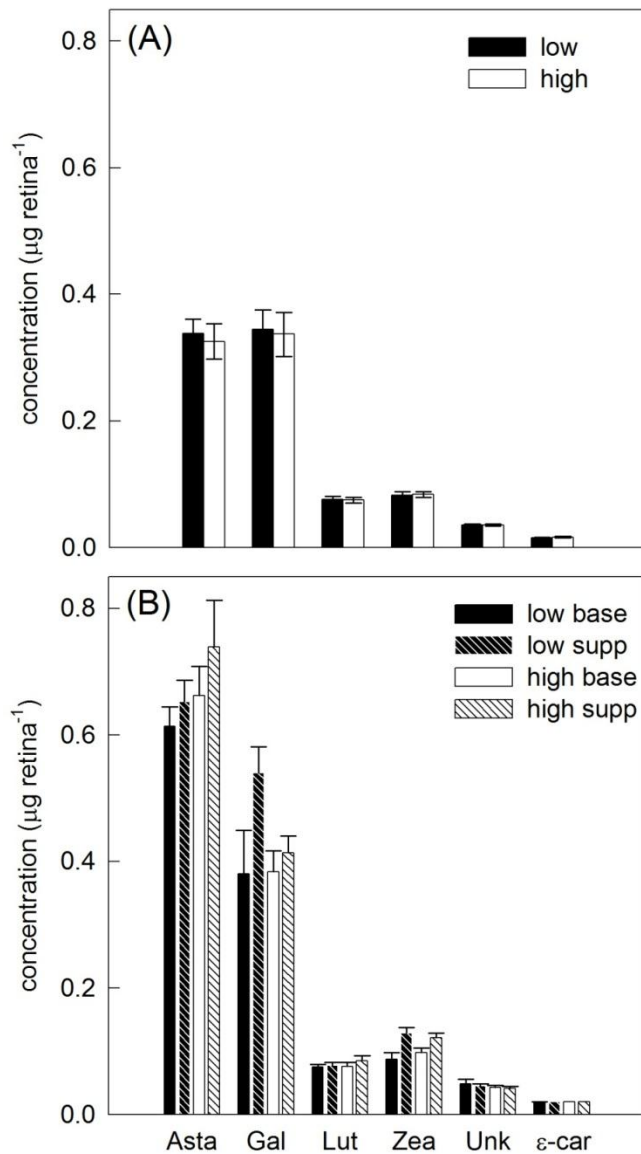


Figure 5. Mean \pm S.E. retinal carotenoid concentration of house finches in experiments 1 (A) and 2 (B). In experiment 2 (B) “supp.” birds received a zeaxanthin supplement in their drinking water. As I have previously shown (Toomey & McGraw 2009, 2010), the major retinal carotenoid types measured in house finches were astaxanthin (Asta), galloxanthins (Gal), lutein (Lut), zeaxanthin (Zea), an unidentified carotenoid (Unk), and ϵ -carotene (ϵ -car).

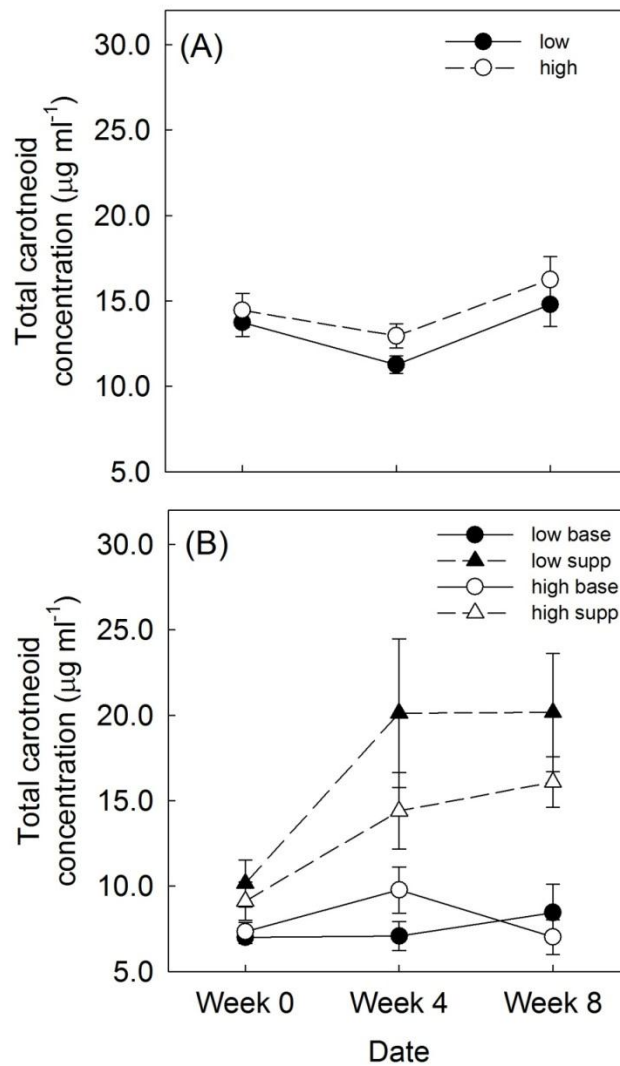


Figure 6. Mean \pm S.E. total plasma carotenoid concentrations of house finches over the course of experiments 1 (A) and 2 (B). Week 0 measurements were performed just prior to the beginning of light exposure and dietary (experiment 2 only) manipulations. In experiment 2 (B) “supp.” birds received a zeaxanthin supplement in their drinking water.

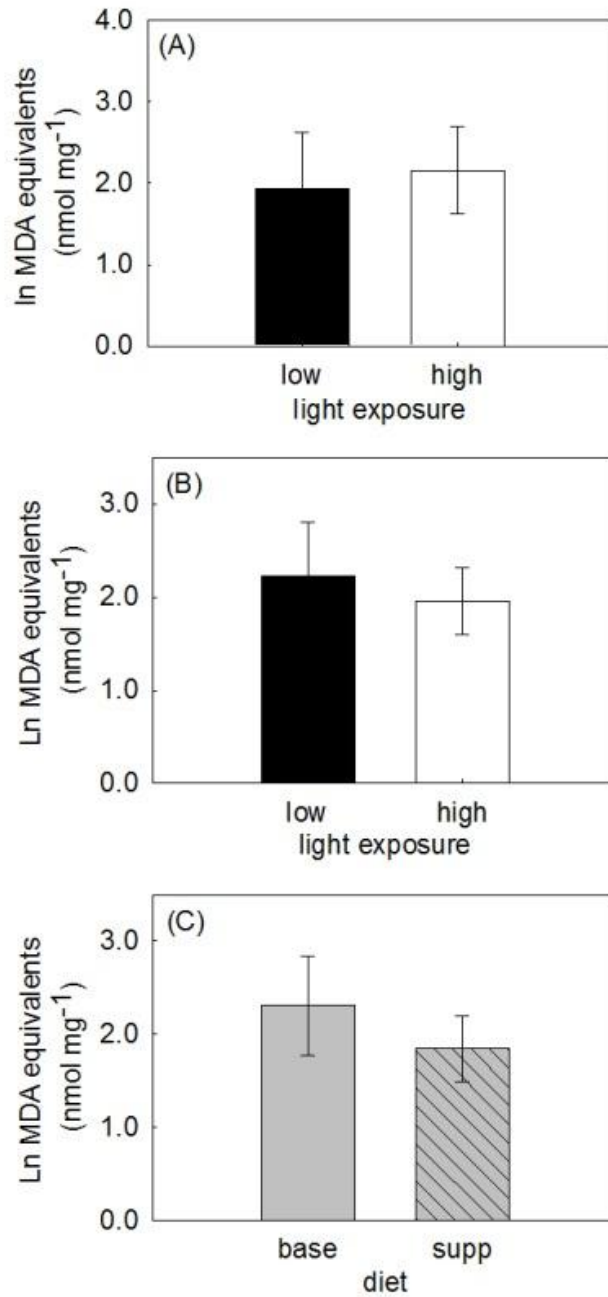


Figure 7. Mean \pm S.E. natural log transformed retina lipid peroxidation (MDA equivalents) levels in retinas of low- and high-light exposed house finches in (A) experiment 1 and (B) experiment 2. (C) Lipid peroxidation levels of house finches receiving the low carotenoid base diet (base) or a zeaxanthin supplement (supp).

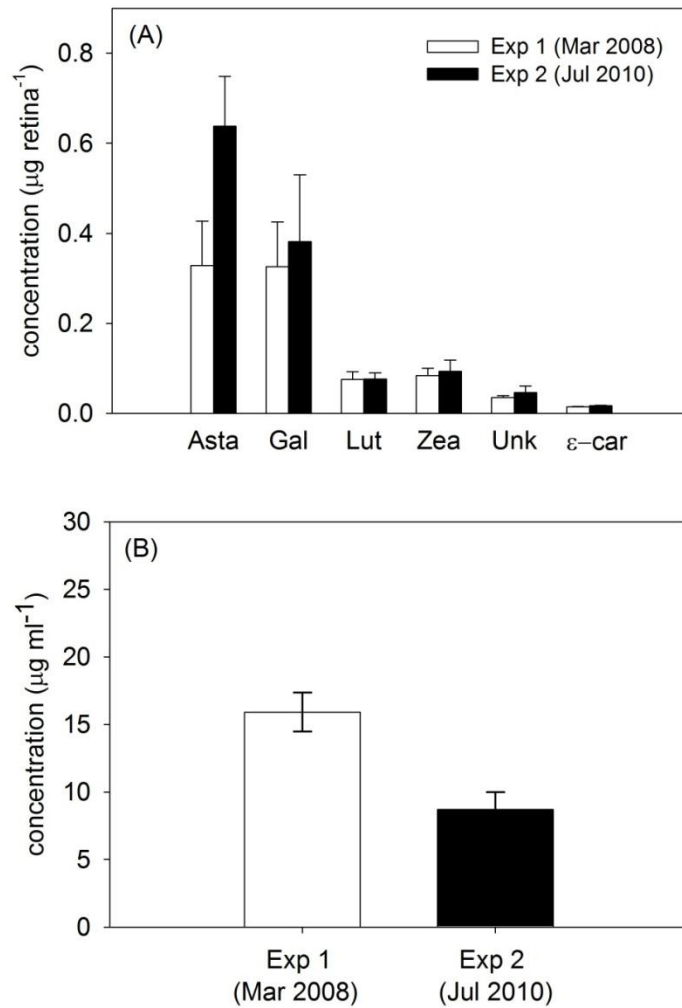


Figure 8. (A) Mean \pm S.E. retinal carotenoid concentration of unsupplemented male house finches in experiments one and two. Carotenoid types abbreviated as in figure 3. (B) Mean \pm S.E. plasma carotenoid concentrations of unsupplemented male house finches in experiments one and two at the conclusion (week 8) of each study.

Chapter 2

THE EFFECTS OF DIETARY CAROTENOID SUPPLEMENTATION AND RETINAL CAROTENOID ACCUMULATION ON VISION-MEDIATED FORAGING IN THE HOUSE FINCH

For many bird species, vision is the primary sensory modality used to locate and assess food items. The health and spectral sensitivities of the avian visual system are influenced by diet-derived carotenoid pigments that accumulate in the retina. Among wild House Finches (*Carpodacus mexicanus*), I have found that retinal carotenoid accumulation varies significantly among individuals and is related to dietary carotenoid intake. If diet-induced changes in retinal carotenoid accumulation alter spectral sensitivity, then they have the potential to affect visually mediated foraging performance. In two experiments, I measured foraging performance of house finches with diet manipulated retinal carotenoid levels. I tested each bird's ability to extract visually contrasting food items from a matrix of inedible distracters under high-contrast (full) and dimmer low-contrast (red-filtered) lighting conditions. In experiment one, zeaxanthin-supplemented birds had significantly increased retinal carotenoid levels, but declined in foraging performance in the high-contrast condition relative to astaxanthin-supplemented birds that showed no change in retinal accumulation. In experiments one and two combined, I found that retinal carotenoid concentrations were positively correlated with relative foraging performance in the low- vs. high-contrast light

conditions for birds with low to medium levels of carotenoid accumulation. However, in experiment two, foraging performance was negatively related to retinal carotenoid accumulation in the zeaxanthin supplemented birds with very high retinal carotenoid levels. This result suggests that carotenoid-mediated spectral filtering enhances color discrimination, but that this improvement is traded off against a reduction in sensitivity that can compromise discrimination. Thus, retinal carotenoid levels may be optimized to meet the visual demands of specific behavioral tasks and light environments.

Introduction

Food detection is a major selective pressure shaping the visual systems of animals, and a primary goal of visual ecologists is to understand the links between the environment, foraging behavior, and the physiology and function of the visual system (Lythgoe 1979). For example, the evolution of trichromatic color vision in primates is thought to be driven by selection for the detection of red fruits against green foliage (Osorio & Vorobyev 1996), and the spectral sensitivities of numerous aquatic species are precisely matched to the light spectra available in their habitats (Partridge & Cummings 1999). Natural selection on the visual system, in the foraging context, can subsequently shape sexually selected signals in animals through the process of sensory drive (Endler 1992). By favoring signals matched to the adaptation of the visual system, sensory drive can lead to

the evolution of elaborate coloration and the emergence of new species (e.g. Seehausen et al. 2008).

Foraging may also have a much more direct influence on the performance of the visual system because it determines the availability of nutrients necessary for the development, maintenance, and function of the eye. For example, retinal (or vitamin A aldehyde) is an essential component of the photopigments of all animals and must be acquired from food, and diet-derived carotenoid pigments act as intraocular filters to protect the eye and tune spectral sensitivities of photoreceptors in many species (Douglas & Marshall 1999). Therefore, the visual capabilities of an individual may not only be shaped by natural selection for the ability to find food on an evolutionary time scale, but also the quality and quantity of that food consumed within the individual's lifetime.

Among vertebrates, birds have some of the most complex and capable visual systems and are a model for the study of visual ecology (Bennett & Thery. 2007). Avian color vision is based upon the response of four types of single-cone photoreceptors that range in sensitivity from the ultraviolet through the entire human-visible spectrum (Fig. 1b, Hart 2001). A separate class of long-wavelength-sensitive double cones is thought to be responsible for achromatic (luminance) discrimination (Osorio & Vorobyev 2005), and scotopic (i.e. low-light) vision depends upon rod photoreceptors. Carotenoids accumulate within the cone photoreceptors in oil droplets located between the inner and outer segments

(Goldsmith et al. 1984) and filter the light reaching the visual pigment. The types and concentrations of carotenoids in the oil droplets are specific to the cone types (Fig. 9a, Goldsmith et al. 1984)) and thus act as matched filters that enhance color discrimination, improve color constancy, provide photoprotection, but also reduce the quantum catch of the photoreceptor (Fig. 9b, Vorobyev 2003).

Carotenoids are particularly interesting because their accumulation 1) is dependent upon environmental availability and acquisition, and 2) may be traded off among multiple functions in the body, including antioxidant protection, immune system performance, and body coloration (Blount 2004). Birds cannot produce carotenoid pigments *de novo*, but must acquire them through their diet, and carotenoid accumulation in the retina is sensitive to recent dietary pigment intake (Toomey & McGraw 2010), as well as, immune system activation (Toomey et al. 2010). These shifts in retinal carotenoid accumulation have the potential to shift cone oil-droplet filtering and alter visual performance (Lind & Kelber 2009). Recently, Knott et al. (2010) examined the influence of dietary carotenoid supplementation on cone oil droplet filtering of zebra finches (*Taeniopygia guttata*) and crimson rosellas (*Platycercus elegans*) and observed subtle shifts in the absorbance of specific types of oil droplets in specific regions of the retina. They concluded that these small changes were unlikely to affect spectral sensitivity; however this was not tested directly.

In this study, I examined the influence of dietary carotenoid supplementation and retinal carotenoid accumulation on the visually mediated foraging behavior of the house finch (*Carpodacus mexicanus*). The house finch is a common North American passerine and a model species for the study of sexual selection and the evolution of elaborate ornaments (Hill 2002). Male finches display sexually selected carotenoid-based plumage coloration that varies from drab yellow to deep red, depending upon dietary carotenoid access and health (Hill 2002) and I have found that retinal carotenoid accumulation follows much the same pattern as plumage carotenoids. For example, retinal carotenoid levels are positively correlated with body condition and plumage coloration (Toomey & McGraw 2009; Appendix A), immune challenges deplete carotenoids from the retina (Toomey et al. 2010; Appendix C), and levels of some carotenoid types (e.g. galloxanthin) are dependent upon dietary carotenoid intake (Toomey & McGraw 2010; Appendix B). Color vision plays an important role in foraging in this species, as house finches actively discriminate among food items based upon color (Stockton-Shields 1997; Bascuñán et al. 2009). Therefore, if changes in retinal carotenoid accumulation alter color vision, they may also impact visual foraging behavior.

In my first experiment, I tested this prediction by measuring the foraging performance of captive finches before and after supplementing them with dietary carotenoids. I tested foraging by presenting birds with red-dyed food items in a

matrix of achromatically variable inedible distracters under two lighting conditions that produced high or low chromatic contrast conditions with similar levels of achromatic contrast. I predicted that dietary carotenoid supplementation would enhance carotenoid-mediated spectral tuning in retina, thereby improving food detection and foraging. Specifically, I predicted that carotenoid-supplemented birds would find more food items in both lighting conditions and that the difference in foraging performance between the high- and low-contrast lighting conditions would diminish following supplementation as compared to the low-carotenoid birds. I also examined the influence of carotenoid supplementation on food color preferences by measuring the consumption of sunflower seeds dyed various colors (Bascuñán et al. 2009), with the prediction that carotenoid supplementation would improve discrimination and strengthen existing color preferences.

Because dietary supplementation has a relatively limited effect on the accumulation of retinal carotenoids in previous studies, and levels may vary in response to a number of other factors (Toomey & McGraw 2010), I included data from a second experiment and took a correlational approach to investigate the relationship between direct measures of retinal carotenoid accumulation and visual foraging performance. I predicted that the relative number of food items eaten in the low- vs. high-contrast condition would be positively correlated with direct measures of retinal carotenoid accumulation.

Methods

Study animals and carotenoid supplementation

Experiment 1

In June 2009, I captured 14 adult male and 14 adult female house finches on the campus of Arizona State University in Tempe, Arizona, USA in baited basket traps (for details see Toomey & McGraw 2009). I housed the birds individually in small wire cages (0.6 m x 0.4 m x 0.3 m) in two greenhouse rooms with *ad libitum* access to tap water and a very low carotenoid (0.078 ± 0.031 $\mu\text{g/g}$) base diet of sunflower seeds. The greenhouse was illuminated with sunlight, and throughout the study the birds were maintained on a natural photoperiod. The birds were fed the base diet for eight weeks to minimize retinal carotenoid variation stemming from dietary differences in the wild. In weeks seven and eight of the initial depletion period, I tested foraging performance (see below) and in week nine I randomly assigned birds to one of three diet treatments: 1) control – four males and four females received the base diet and tap water with a non-carotenoid vitamin supplement (Vita-Sol®, United Pet Group EIO, Tampa, FL); 2) zeaxanthin – five males and five females received a supplement of zeaxanthin beadlets (35 $\mu\text{g/ml}$ of OptiSharp® DSM, Heerlen, Netherlands) suspended in their drinking water and the vitamin supplement; and 3) astaxanthin - five males and five females received a supplement of astaxanthin beadlets (35 $\mu\text{g/ml}$ of

Carophyll Pink® DSM, Heerlen, Netherlands) suspended in their drinking water and the vitamin supplement. The birds were given the supplements *ad libitum* each weekday for eight weeks (weeks 9-16), with plain tap water provided on weekends. At the start of week 17 and continuing through week 18, all birds were returned to the base seed and tap-water diet and I again tested foraging performance (see below). Carotenoids deplete from the retina relatively slowly compared to other tissues, requiring ≥ 4 weeks of deprivation to cause significant declines (Toomey & McGraw 2010); thus this final depletion period was an effort to decouple any immediate effects that carotenoid supplementation might have on health state (and perhaps foraging motivation) from the effects of carotenoid accumulation in the retina. At end of 18 weeks, I euthanized all birds and collected retinas to directly measure carotenoid accumulation (see below).

Experiment 2

In November 2009, I captured and housed 27 female house finches to study the influence of dietary carotenoid supplementation on female mate choice behavior (Chapter 3). I trapped these finches as described in experiment one and maintained them on a sunflower seed diet. In January 2010, I randomly selected 13 females and supplemented their drinking water with carotenoids (zeaxanthin: $17.5 \mu\text{g ml}^{-1}$ OptiSharp® DSM, Heerlen, Netherlands), while the remaining 14 birds continued on the unsupplemented sunflower seed diet. Supplementation

continued for eight to ten weeks and, following a depletion period as described in experiment one, I tested the foraging performance of all birds (see below) and conducted a series of mate choice trials for male plumage coloration (Chapter 3). At the conclusion of the behavioral tests, I euthanized all birds and collected retinas to directly measure carotenoid accumulation (see below).

Foraging performance test

I developed a foraging task based upon the methods of Caine and Mundy (2000) and Maddocks et al. (2001), in which birds were challenged to pick out food pellets from a contrasting matrix. Although more precise behavioral tests of color vision are available (e.g. Goldsmith & Butler, 2005), I chose this method because it offers three advantages: 1) it does not require extensive training and can be rapidly learned by wild birds, 2) it is easily scaled to test a relatively large number of individuals and, 3) this task is analogous to ground foraging for seeds, the primary mode of foraging in the house finch (Salt, 1952).

I presented each bird with 30 rice pellets (3.5 mm diam., Careline rice diet, Roudybush, Woodland, CA) dyed with red food coloring (McCormick & Company Inc., Sparks, MD; Figs. 9 & 10) in a matrix of inedible distracters varying from tan to black of similar shape and size as the food pellets (Kaytee Soft-Sorbent, Kaytee Products Inc. Chilton, WI). The food pellets and distracters were presented on white paper plates (15.3 cm diam.) in the housing cage of each

bird, with water, but not food, available throughout each trial. Birds were tested three times under two lighting conditions before (weeks 9-10) and after (weeks 17-18) carotenoid supplementation. Trials lasted 20 min. and were carried out only once per day and began at 0800 hrs following overnight food deprivation, to ensure that birds were motivated to forage. After each trial, I collected plates, recovered any spilled pellets and distracters, and counted the number of food pellets remaining as a measure of foraging performance. The number of pellets eaten in each of the three trials was moderately repeatable ($R = 0.578$; Lessells & Boag, 1987) and for subsequent analyses, I calculated mean number of the three repeated trials in each lighting condition at each time point. To investigate possible treatment-group and lighting-condition differences in activity levels of the birds, I video recorded the foraging behavior of a subset of birds (4 per treatment group) in both lighting conditions during the post-supplementation period and measured the amount of time they spent actively foraging.

Foraging tests were carried out in a windowless indoor room under two lighting conditions: (1) full, unfiltered fluorescent light (Sylvania, 34W, T12 rapid start Super Saver, Osram-Sylvania, Danvers, MA, USA), or (2) red-filtered-light created by placing filters (Roscolux Fire #19, Rosco Laboratories Inc., Stamford, CT, USA) over the fluorescent lights (Fig. 9 & 10). The filters were set up the night before the trials at ~1800 hrs to allow the birds time to acclimate to the new conditions. To assess how lighting conditions affected food-pellet

conspicuousness, I measured 15 reflectance spectra from the food pellets and distracters, as well as three irradiance spectra of the filtered and unfiltered-light using an Ocean Optics USB2000 spectrophotometer (Ocean Optics Incorporated, Dunedin, FL, USA; for methods see electronic supplementary material). I then used the noise-limited receptor model (Goldsmith & Butler. 2005; Osorio et al. 2004; Siddiqi et al. 2004), with the spectral sensitivities of the Canary (*Serinus canaria*, a cardueline-finch relative of house finches, Das et al. 1999), to calculate the chromatic and achromatic contrasts between the food pellets and distracters and among the distracters under both lighting conditions (supplementary methods). These measures confirmed that the food items contrasted significantly with the background distracters and that this contrast differed between the lighting conditions (Table 5). Specifically the chromatic contrast of the food items against the background distracters was significantly greater than the contrast among the distracters, while the achromatic contrast was not significantly different between food and background distracters compared to the contrast among the distracters (Table 1). To estimate the effects of the relatively dim light conditions in the experiment, I also calculated the visual contrasts with an estimate of photon noise for dim environments (Vorobyev et al. 1998). The inclusion of photon noise in the model reduced the magnitude of the contrasts but did not alter the pattern of contrast between food and distracters relative to the contrast among the distracters (Table 5).

Food color preference test

In experiment one, prior to the second foraging performance test (week 16), I measured the food color preferences of all birds following the methods of Bascuñán et al. (2009), with the following modifications to match the timing and duration of the foraging performance tests. The test began at 0800 hrs, lasted 20 mins, and 20 of each red, green, yellow and orange dyed sunflower seeds were presented on the same paper plates used in the foraging performance tests. However, no distracters were present during the food color preferences tests, and the tests were carried out under the semi-natural lighting conditions of the greenhouse housing room. I measured the number of seeds of each color eaten by counting the seeds remaining at the end of the trial.

Carotenoid analyses

I quantified amounts of specific carotenoid types in the left retina of each bird using high performance liquid chromatography (HPLC). Extraction procedures, analytical methods, and the results of experiment 1 are reported in Toomey & McGraw (2010; Appendix B).

Statistical analyses

Analyses were carried out in SPSS13 (SPSS inc., Chicago, IL), and values are reported as mean \pm SE throughout. To examine the influence of lighting conditions on the number of food pellets eaten, I used repeated-measures analyses of variance (rmANOVA), with the number of food pellets eaten in each lighting condition as the within-subjects factor and sex as a between-subjects factor. Because the number of pellets eaten differed significantly between lighting conditions (§3b), I tested the effects of dietary carotenoid supplementation on foraging performance in separate rmANOVAs for full and red-light, with the number of pellets eaten before and after supplementation as within-subjects factors and sex and supplementation treatment as between-subjects factors. Food color preferences were tested using rmANOVA, with seed color as the within-subjects factor and sex and supplementation treatment as the between-subjects factors. Non-significant interaction terms were removed from the models, Greenhouse-Geisser corrections were used when the models deviated from the assumptions of sphericity, and the significance level was set to $\alpha < 0.05$.

To test the relationship between direct measures of retinal carotenoid accumulation and changes in foraging performance, I carried out separate repeated-measures analysis of covariance (rmANCOVA), with the number of food pellets eaten before and after supplementation as the repeated measure, sex as a between-subjects factor, and total retina carotenoid concentration as a covariate, under each lighting condition. Concentrations of all six retinal

carotenoid types were significantly intercorrelated (Toomey & McGraw 2010), but because they are associated with different photoreceptors (Goldsmith et al. 1984) they may influence visual function in different ways. To explore the individual association between each of the six different retinal carotenoid types and the change in foraging performance, I calculated separate Pearson's correlations.

Because dietary supplementation had a relatively limited effect on the accumulation of retinal carotenoids (Toomey & McGraw 2010), I took a correlational approach to further investigate the relationship between retinal carotenoid accumulation and visual foraging performance. I fitted linear models of total retinal carotenoid concentration against the number of pellets eaten in the low-contrast relative to the high-contrast condition. I limited these analyses to the foraging tests in the post-diet-manipulation period of experiments one and two, just prior to taking the direct measures of retinal carotenoids.

Results

Dietary supplementation and retinal carotenoid accumulation

Experiment 1

The effects of dietary supplementation on retinal carotenoid accumulation are reported elsewhere (Experiment 3 in Toomey & McGraw 2010). To summarize, birds supplemented with zeaxanthin had significantly higher levels of retinal galloxanthin and ϵ -carotene than birds receiving the astaxanthin and

control diets. There were no significant differences in the accumulation of any retinal carotenoids between the astaxanthin-supplemented or control birds. Carotenoid supplementation did not significantly affect accumulation of astaxanthin, zeaxanthin, or lutein in the retina, and there were no significant sex differences in retinal carotenoid accumulation.

Experiment 2

Female finches receiving the zeaxanthin-supplemented diet had significantly higher retinal carotenoid levels than birds maintained on the low-carotenoid diet (Wilk's $\lambda = 0.29$, $F_{6,20} = 7.89$, $p = 0.00018$, Fig. 11). Specifically, retinal astaxanthin, galloxanthin, zeaxanthin and ϵ -carotene levels were significantly higher in the high-carotenoid treatment ($F_{1,25} = 6.90$, $p = 0.014$, $F_{1,25} = 43.40$, $p < 0.0001$, $F_{1,25} = 9.71$, $p = 0.0046$, $F_{1,25} = 10.51$, $p = 0.0033$ respectively). All retinal carotenoid types were significantly positively intercorrelated ($r > 0.40$, $p < 0.037$), with the exception of galloxanthin and an unidentified carotenoid ($r = 0.30$, $p = 0.13$)

Effects of lighting condition on foraging performance

Experiment 1

Prior to carotenoid supplementation, birds ate significantly fewer food pellets in the low-contrast, red-filtered-lighting condition than in unfiltered full-

light (rmANOVA lighting: $F_{1,24} = 49.24$, $p < 0.0001$, Fig. 12). This effect was stronger for females than males (rmANOVA lighting \times sex: $F_{1,24} = 4.95$, $p = 0.036$, Fig. 12). Prior to supplementation, treatment groups did not differ significantly in foraging performance in either lighting condition (rmANOVA lighting \times treatment: $F_{1,24} = 0.39$, $p = 0.676$). The number of food pellets eaten in individual trials ranged from 0-24 under red light, and 3-27 under full light and all individuals consumed pellets under each lighting condition in at least one of the three trials.

Experiment 2

Female finches ate significantly fewer food pellets in the low-contrast, red-filtered-lighting condition than in unfiltered full-light (rmANOVA lighting: $F_{1,25} = 5.72$, $p = 0.025$, Fig. 12).

Effect of dietary carotenoid supplementation on foraging performance

There was a significant effect of dietary carotenoid supplementation on number of food pellets eaten in the full-light condition (rmANOVA time \times treatment: $F_{2,24} = 5.25$, $p = 0.013$, Fig. 13). The number of food pellets eaten by zeaxanthin-supplemented birds in full-light declined significantly following supplementation ($t = 2.49$, $p = 0.034$) and differed significantly from the astaxanthin-supplemented group (Tukey's post-hoc $p = 0.014$), but not control

birds (Tukey's post-hoc $p = 0.708$). Supplementation had no significant effect on foraging in the red-light condition (rmANOVA time \times treatment: $F_{2,24} = 1.84$, $p = 0.620$, Fig. 13). The change in the number of food pellets eaten in full-light differed significantly between the sexes (rmANOVA time \times sex: $F_{1,24} = 8.50$, $p = 0.008$); females declined over time (pre: 15.00 ± 0.93 vs. post: 12.4 ± 0.95), while males remained relatively constant (pre: 11.3 ± 0.93 vs. post: 12.0 ± 0.95). There was a significant increase in the number of food items eaten in the red-filtered-light condition over time across all diet treatments (rmANOVA time: $F_{1,24} = 18.92$, $p < 0.0001$, Fig. 13); this increase did not differ between the sexes (rmANOVA time \times sex: $F_{1,24} = 1.59$, $p = 0.219$). In the subset of birds for which I observed behavior during the trials, the mean amount of time spent actively foraging did not differ significantly between lighting conditions ($F_{1,8} = 0.590$, $p = 0.465$), the sexes ($F_{1,8} = 0.027$, $p = 0.873$), or among treatment groups ($F_{2,8} = 2.88$, $p = 0.114$). Over the course of these trials, I occasionally observed the birds making errors, picking up the distracters, manipulating them in their bills, and subsequently rejecting them.

Consistent with the treatment effects described above, retinal carotenoid levels, measured at the conclusion of experiment one, significantly predicted the change in the number of food pellets eaten in full-light before and after supplementation (total carotenoids: $F_{1,25} = 5.19$, $p = 0.032$). In separate analyses of the different retinal carotenoid types, concentrations of retinal galloxanthin and

ϵ -carotene were significantly negatively correlated with the change in the number of food pellets eaten in full-light ($r = -0.480, p = 0.014$ and $r = -0.435, p = 0.021$ respectively). Concentrations of other retinal carotenoid types were not significantly correlated with the decline in foraging performance (asta: $r = -0.377$, lut: $r = -0.138$, zea: $r = -0.329$, unk: $r = -0.163$). The temporal improvement in foraging performance in red-filtered-light was not significantly related to retinal carotenoid accumulation ($F_{1,25} = 0.78, p = 0.387$).

Dietary supplementation and food color preference

Seed consumption differed significantly by color type ($F_{1,19,26.14} = 56.17, p < 0.0001$), with finches eating significantly more red dyed seeds than all other colors (Tukey post-hoc test, $p < 0.0001$; Fig. 14). Food color preferences did not differ between the sexes ($F_{1,18,26.14} = 0.21, p = 0.694$). There was no significant effect of dietary carotenoid supplementation on seed color preference ($F_{2,37,26.14} = 0.25, p = 0.813$) or on the total amount of food eaten ($F_{2,22} = 0.71, p = 0.502$).

Retinal carotenoid accumulation and foraging performance in high vs. low contrast conditions

Experiment 1

Foraging performance, measured as the relative number of pellets eaten in the low- vs. high- contrast condition in the post-supplementation period, did not

differ significantly among diet treatments or between the sexes ($F_{2,24} = 1.93$, $p = 0.17$ and $F_{1,24} = 2.83$, $p = 0.11$ respectively). However, across sexes and treatment groups, total retinal carotenoid concentration was a significant positive predictor of relative foraging performance in the low contrast condition ($r^2 = 0.185$, $F_{1,26} = 5.92$, $p = 0.022$, Fig. 15a). The correlation between retinal carotenoid accumulation and foraging performance was not specifically driven by the experiment-induced decline in foraging performance in the high-contrast condition. When I removed zeaxanthin-supplemented birds from the analysis, total retinal carotenoid concentration remained significantly positively correlated with foraging performance ($r^2 = 0.335$, $F_{1,26} = 8.06$, $p = 0.012$).

Experiment 2

In the second experiment, foraging performance did not differ significantly between diet treatments ($F_{1,25} = 0.97$, $p = 0.33$). There was no significant linear relationship between retinal carotenoid accumulation and foraging performance ($r^2 = 0.017$, $F_{1,26} = 0.45$, $p = 0.51$). However, there was a significant interaction between diet treatment and retinal carotenoid accumulation ($F_{1,23} = 12.58$, $p = 0.0017$, Fig. 15b). Consistent with experiment 1, there was a positive correlation ($r = 0.80$) between retinal carotenoid levels and foraging performance for the control diet birds (Fig. 15b). In contrast, retinal carotenoid levels and foraging performance were negatively correlated ($r = -0.39$) among the zeaxanthin

supplemented birds (Fig. 15b). It is important to note that the zeaxanthin supplemented birds in this experiment had significantly higher retinal carotenoid levels that are commonly observed among wild birds (Fig 15b).

Discussion

This study provides the first evidence linking retinal carotenoid accumulation to visually mediated foraging behavior. Contrary to my predictions, dietary carotenoid supplementation and the subsequent increase in retinal carotenoid accumulation did not improve the foraging performance of house finches. Rather, birds with experimentally elevated retinal carotenoid levels showed a significant decline in foraging in the high-contrast condition, while all birds, regardless of diet treatment, improved in the low-contrast condition. Surprisingly, I found that retinal carotenoid accumulation positively correlated with foraging performance at low and intermediate levels of accumulation, but was negatively correlated with performance at very high levels of accumulation. Although unexpected, these results are consistent with a carotenoid-mediated trade-off between color discrimination and photon noise.

The diet-driven decline in foraging performance is consistent with putative effects of retinal accumulation on visual function. Carotenoid-pigmented cone oil droplets are predicted to enhance color discrimination (Vorobyev 2003; Vorobyev et al. 1998), but this enhancement comes at the cost of reduced quantum catch and

the potential for increased photon noise (Vorobyev et al. 1998). In dim conditions, contrast sensitivity declines with the square root of light intensity (Rovamo et al. 2001), and increased carotenoid filtering essentially reduces the intensity of light reaching the photoreceptors. Increased receptor noise levels can significantly reduce chromatic discriminability (Lind & Kelber 2009) and thus could limit the detectability of food items. Direct measures of oil droplet absorbance, coupled with behavioral tests at varying light intensities, are now needed to clarify mechanisms underlying these changes in visual foraging performance.

Although the diet-related changes in foraging are consistent with a visual mechanism, I cannot rule out more general influences of diet and learning. Regardless of dietary treatment, all birds improved their foraging efficiency in the low-contrast red light condition, suggesting that the birds learned to discriminate food more effectively and/or use different cues. The significant difference in full-light foraging performance that arose between zeaxanthin- and astaxanthin-supplemented birds may be attributable to changes in foraging motivation. For example, dietary carotenoid availability has been shown to influence color-based foraging preferences of guppies (*Poecilia reticulata*, Grether et al. 2005) and may have altered the motivation of the birds in the study to feed on red food items. Additionally, astaxanthin-supplemented birds received this red-colored carotenoid in their drinking water and may have become accustomed to consuming red material, which may have increased motivation to feed on the red food items in

the experimental context. However, I found no difference in food color preferences or foraging effort between the diet treatments. I also observed significant differences in foraging behavior between the sexes over time, suggesting that foraging behavior is influenced by sex-specific physiological changes (the experimental period included a transition from breeding to molt). Thus, I am left with an intriguing pattern, but further studies are needed to address these confounding factors and clarify the links between dietary carotenoids, retinal carotenoids, and visual foraging behavior.

Despite the unresolved relationship between dietary carotenoid supplementation and visual foraging performance, I found a significant relationship between direct measures of retinal carotenoid accumulation and visual foraging performance. As predicted, performance was positively correlated with retinal carotenoid accumulation at low and medium levels of accumulation. However, this correlation did not hold and was actually negative for individuals with very high concentrations of retinal carotenoids. These very high levels fall outside of the 90th percentile of accumulation in wild birds examined in an earlier study (Toomey & McGraw 2009; Appendix A), and suggests that performance may be optimized at a specific retinal carotenoid level. Optimization is consistent with a trade-off between chromatic discrimination and sensitivity that has been hypothesized for cone oil droplet filtering (Vorobyev et al. 1998). Under the relatively dim conditions of the low-contrast treatment,

carotenoid accumulation may promote discrimination through the narrowing of spectral sensitivity, but at high levels may compromise discrimination by reducing photon catch and increasing photon noise (see above). Because photon noise levels depend upon the intensity of light (Rovamo et al. 2001), the carotenoid level, at which the costs and benefits of accumulation are balanced, should increase with increasing light intensities and this trade-off may disappear at high intensities. Although the light intensities used in this study are low compared to the natural, desert habitats of the house finch, they are comparable to conditions found under dense forest canopies (Endler 1993). An important next step will be to explore this trade-off in visual performance across the broad range of natural light intensities and among species that inhabit diverse light environments.

A carotenoid-mediated trade-off in avian visual function is supported by patterns of retinal carotenoid accumulation observed among species and individuals reared under varying light intensity. The retinas of nocturnal species (e.g. owls) have relatively pale oil droplets that presumably contain lower concentrations of carotenoids, which is hypothesized to improve their visual sensitivity under low light conditions (Hart 2001). In chickens (*Gallus gallus*), retinal carotenoid accumulation is developmentally plastic in response to light environment, such that chicks reared in dim environments develop less absorbent oil droplets with presumably lower carotenoid levels (Hart et al. 2006). Thus, the

demands of dim light vision may set a functional upper limit on the accumulation of carotenoids in the avian retina. Interestingly, very few (<10%) wild house finches exceed the “optimal” retinal carotenoid level identified in this study (Toomey & McGraw 2009), yet I was able to push captive birds beyond this point with dietary supplementation. This suggests that the mechanisms of retinal accumulation are tuned to natural dietary carotenoid availability and/or birds use cues not available in captivity to regulate accumulation.

Linking visual foraging performance to retinal carotenoid accumulation is particularly intriguing because carotenoid-based male plumage coloration plays an important role in house finch mate choice (Hill 2002). Among wild house finches, I have found that retinal carotenoid levels are significantly positively correlated with male plumage redness (Toomey & McGraw 2009; Appendix A), suggesting a potentially unique link between a sexually selected signal and the sensory system. Although dietary carotenoid supplementation (Toomey & McGraw 2010; Appendix B) and immune system challenges (Toomey et al. 2010; Appendix C) can cause small changes in retinal accumulation, much of the variation I have observed among wild birds remains unexplained. If retinal carotenoid accumulation is developmentally or genetically determined, then it could be linked with plumage color through common heritable variation in the mechanisms of carotenoid uptake and metabolism (e.g. lipoprotein production (McGraw & Parker 2006)). Alternatively, foraging could environmentally link

vision and color signal expression, if vision-mediated food choice affects development of ornamental color. House finches have distinct food color preferences (Stockton-Shields 1997; Bascuñán et al. 2009) and may use color to select carotenoid- and/or antioxidant-rich foods (e.g. desert cactus fruits). Fruit color, for example, is a reliable indicator of antioxidant content (but not necessarily carotenoid levels (Schaefer et al. 2008), and the increased consumption of antioxidants can enhance the expression of carotenoid-based colors (Pike et al. 2007; Perez et al. 2008). However, my results indicate that benefits of retinal carotenoid accumulation are not monotonic, and understanding their adaptive value will require a better understanding of the light environments in which foraging and mate choice occur.

The visual pigment sensitivities of birds are considered to be highly conserved among species (Osorio & Vorobyev 2008), which has led to the widespread application of avian visual models based upon a relatively limited set of physiological parameters (e.g. Eaton 2005). My results indicate that, within a species, visual discrimination can vary considerably in response to the physiological state of the eye. This complicates the interpretation of visual modeling results because discrimination may be influenced by the interacting effects of individual- and species-specific differences in retinal carotenoid accumulation with light intensity. This could be a particularly important

consideration when assessing signaling and crypsis in dim environments, such as with colorful eggs and nestling mouths in cavity nests (e.g. Aviles et al. 2008).

The trade-off between chromatic and luminance detection is an important force shaping the evolution of the visual system (Lythgoe & Partridge 1991; Cummings 2004; Osorio & Vorobyev 2005). To date, visual ecologists have focused on how the genetically determined photoreceptor diversity and opsin-based spectral tuning mediate this trade-off (Lythgoe & Partridge 1991; Cummings 2004; Osorio & Vorobyev 2005). However, my results suggest that inter-ocular filters (retinal carotenoids) mediate a similar trade-off in avian vision, opening up a range of new questions. Because retinal carotenoid accumulation is sensitive to alterations in diet, health, and developmental light environment (Hart et al. 2006; Toomey & McGraw 2010; Toomey et al. 2010), visual performance may also be shaped by the environment, not just over the course of generations, but throughout an individual's lifetime.

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Table 5. Total irradiance and predicted visual contrasts between food pellets and background distracters under the experimental lighting conditions modeled assuming either bright or dim (photon-noise limited) conditions.

Lighting	Total irradiance ($\mu\text{mol s}^{-1} \text{m}^{-2}$)	Vision model	Contrast between food and distracters (jnds) \pm st. dev.		Contrast within distracters (jnds) \pm st. dev.	
			chromatic ^{a,c}	achromatic ^b	chromatic ^c	achromatic
Full	12.92 \pm 6.47	bright	21.41 \pm 6.22	9.66 \pm 6.48	4.39 \pm 3.26	8.93 \pm 6.86
		dim	5.32 \pm 1.52	2.44 \pm 1.67	1.03 \pm 0.75	2.46 \pm 1.76
Red	5.10 \pm 1.94	bright	19.86 \pm 5.58	7.56 \pm 5.63	5.40 \pm 4.08	9.16 \pm 7.01
		dim	2.94 \pm 0.83	1.45 \pm 1.07	0.99 \pm 0.76	1.89 \pm 1.35

^a > 1 jnd difference between lighting conditions ($p < 0.001$) for both vision models

^b > 1 jnd difference between lighting conditions ($p = 0.007$) for bright vision model only

^c > 1 jnd difference between food/distracter contrast and distracter/distracter contrast ($p < 0.001$) in both vision models

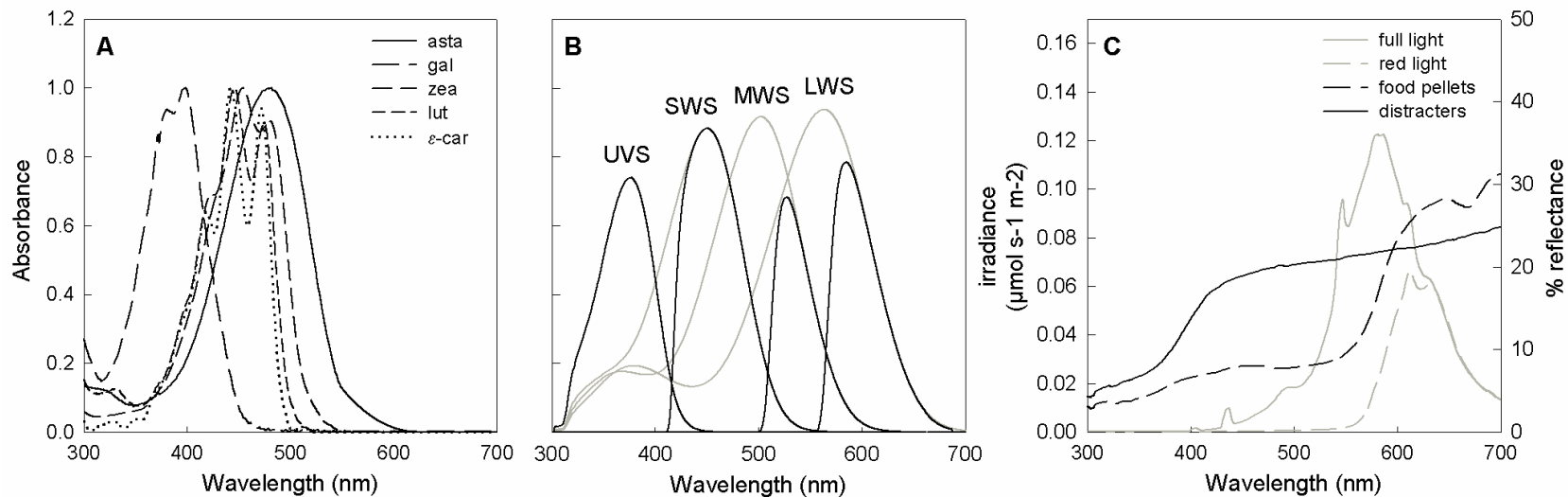


Figure 9. (A) Normalized absorbance spectra of carotenoids found in the house finch retina: astaxanthin (asta), galloxanthin (gal), zeaxanthin (zea), lutein (lut), and ϵ -carotene (ϵ -car). (B) Absorbance spectra of single-cone photoreceptors before (gray lines) and after (black lines) carotenoid-pigmented cone oil-droplet filtering. Spectral sensitivities are based upon measures from the canary (*Serinus canaria*; (Das et al. 1999)), the house finch's closest relative for which these values are known. Microspectrophotometric studies (Goldsmith et al. 1984) suggest that the long-wavelength-sensitive cone (LWS) is filtered by an oil droplet pigmented with astaxanthin, the medium-wavelength-sensitive cone (MWS) is filtered by a zeaxanthin-pigmented oil droplet, the short-wavelength-sensitive cone (SWS) is filtered by a galloxanthin-containing oil droplet, and the ultraviolet-sensitive cone (UVS) has a transparent oil droplet. (C) Sample irradiance spectra from the full and red-filtered room lights and reflectance spectra of the food pellets and distracters. Irradiance spectra are presented in gray and are associated with the y-axis on the left. Reflectance spectra are presented in black and associated with the y-axis on the right.

A



B



Figure 10. (a) A sample image of the red food pellets and inedible gray paper distracters presented to the birds; (b) Unfiltered full-lighting conditions in the study room (left panel) compared to the red-filtered-lighting conditions (right panel).

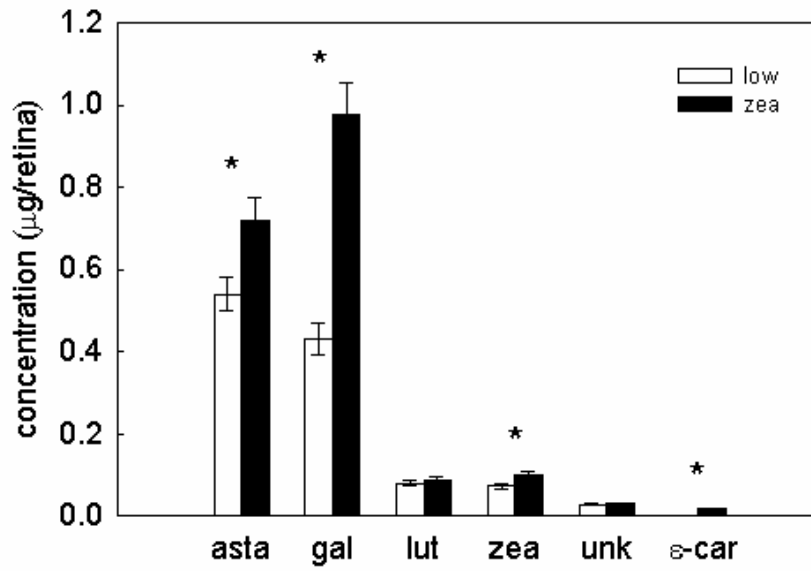


Figure 11. Mean \pm S.E. concentration of the six carotenoid types in retinas of female finches receiving a low-carotenoid (white bars) or zeaxanthin-supplemented (black bars) diet in experiment two. *indicate significant treatment differences.

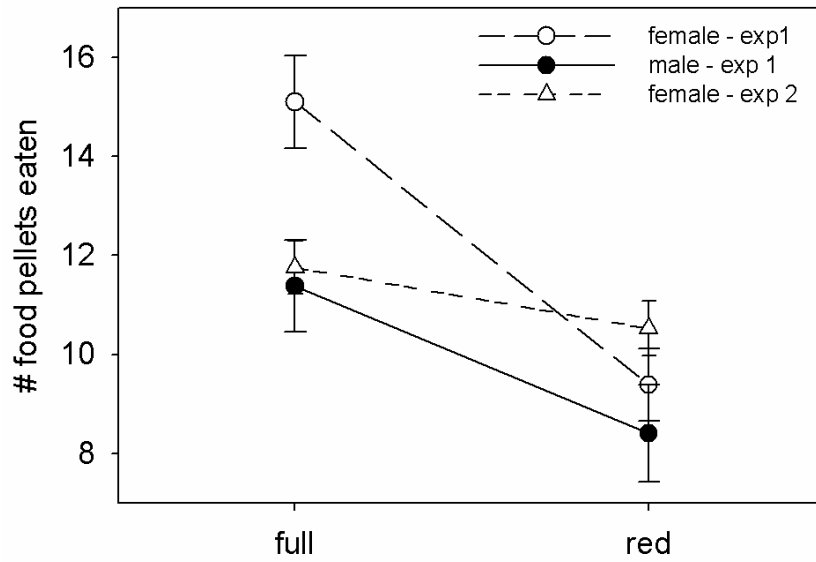


Figure 12. Mean \pm S.E. number of food pellets eaten by male and female house finches in experiment one, and by female house finches in experiment two under high-contrast full-light vs. low-contrast red-light conditions.

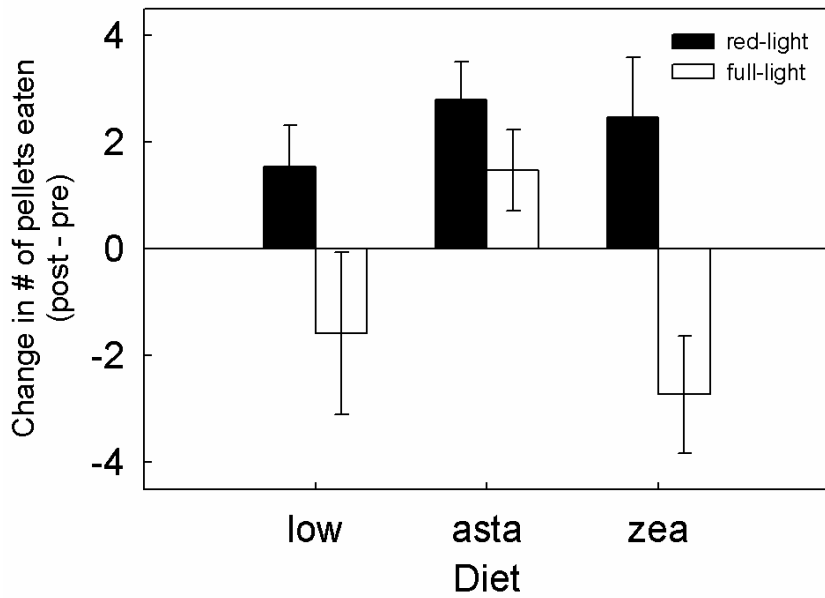


Figure 13. Mean \pm S.E. change in the number of food pellets eaten by finches in the red-filtered light (solid bars) and the full light (open bars) following eight weeks on a low carotenoid, astaxanthin- (asta) supplemented, or zeaxanthin- (zea) supplemented diet.

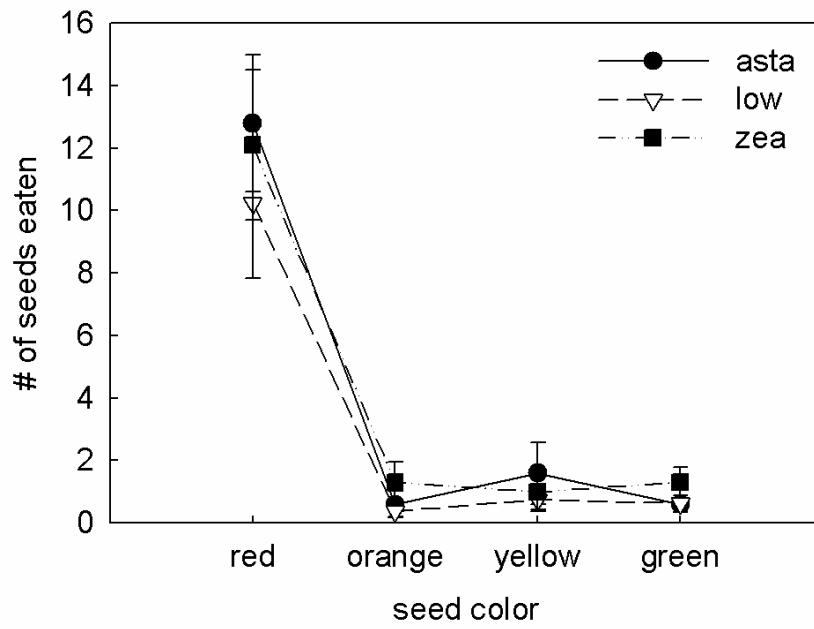


Figure 14. Mean \pm S.E. number of seeds dyed each of four colors eaten during the 20 min food preference trial. Diet treatments are denoted with different symbols.

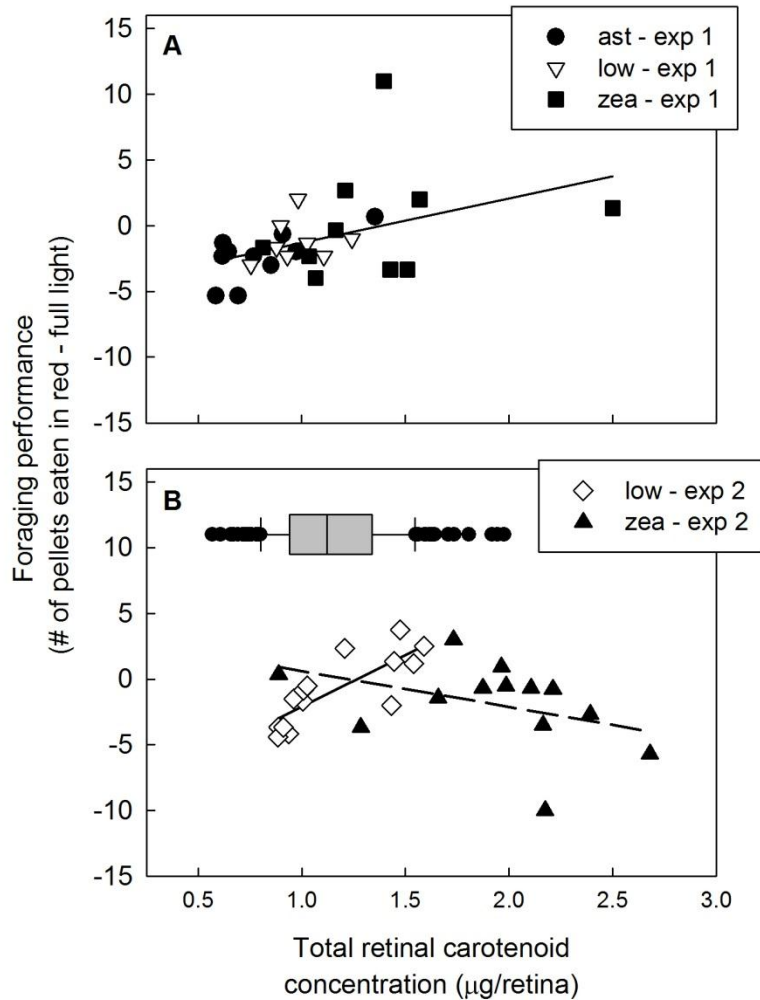


Figure 15. Relative number of food pellets eaten in the low-contrast red-light, as compared to high-contrast full-light, in the post-supplementation period for (A) experiment one, (B) experiment two, and (C) experiments one and two combined. The diet treatments within experiments one and two are denoted with different symbols. The box plot in at the top of figure C represents the natural range of variation in house finch retinal carotenoid levels reported by Toomey & McGraw (Toomey & McGraw. 2009).

Supplementary methods

Reflectance spectra measurement:

I measured reflectance spectra of food pellets and background distracter matrix, relative to a white standard (Spectralon; Labsphere Inc., North Sutton, NH, USA), with an Ocean Optics USB2000 spectrophotometer with a PX-2 pulsed xenon light source (Ocean Optics Incorporated, Dunedin, Florida, USA). Fifteen spectra each were collected from the food pellets and background distracters at coincident-normal geometry at a distance of 1 cm from the surface. The reflectance spectra were then binned to 1 nm intervals for subsequent analyses.

Irradiance spectra measurement:

I measured irradiance spectra under the two lighting conditions (full and red-filtered) using an Ocean Optics USB2000 spectrophotometer with a single fiber-optic probe (P400-1-UV-VIS; Ocean Optics) and a cosine-correcter with a 180° acceptance angle and a measurement surface of 6 mm in diameter (CC-3-UV; Ocean Optics). I calibrated the spectrophotometer with a standard light source (LS-1-CAL; Ocean Optics) and measured the downwelling irradiance spectra three times under each lighting condition within the housing cages of the birds.

Chromatic contrast calculation:

To calculate the avian visual system chromatic contrast between the food pellets and distracters, I followed the models proposed by Vorobyev et al. (1998) as modified by Aviles et al. (2008). I calculated the photoreceptor quantum catch for each cone class with the following equation:

$$Q_i = \int_{\lambda} R_i(\lambda)S(\lambda)I(\lambda)d\lambda$$

where λ indicates wavelength, Q_i is the quantum catch for the *ith* photoreceptor, $R_i(\lambda)$ is the spectral sensitivity of the *ith* photoreceptor, $S(\lambda)$ is the reflectance spectrum of the color patch, and $I(\lambda)$ is the irradiance spectrum. I calculated photoreceptor sensitivity based on physiological data from the canary is detailed in the table below. To assess the influence of lighting conditions on chromatic contrast, I repeated these calculations using the irradiance spectra from either full or filtered red-light.

I then calculated the log ratio of the quantum catches for the food pellets against the background distracters:

$$\Delta f_i = \log \frac{Q_i}{Q_b}$$

where Q_b is calculated with the reflectance spectrum of the background distracters.

I calculated the chromatic contrast in just-noticeable differences as follows:

$$\Delta S = \sqrt{\frac{((e_1 e_2)^2 (\Delta f_4 - \Delta f_3)^2 + (e_1 e_3)^2 (\Delta f_4 - \Delta f_2)^2 + (e_1 e_4)^2 (\Delta f_3 - \Delta f_2)^2 + (e_2 e_3)^2 (\Delta f_4 - \Delta f_1)^2 + (e_2 e_4)^2 (\Delta f_3 - \Delta f_1)^2 + (e_3 e_4)^2 (\Delta f_2 - \Delta f_1)^2)}{((e_1 e_2 e_3)^2 + (e_1 e_2 e_4)^2 + (e_1 e_3 e_4)^2 + (e_2 e_3 e_4)^2)}}$$

To account for the potential effects of the relatively dim conditions in the experiment, I calculated the light intensity independent noise (e_i) for a given photoreceptor following Vorobyev et al. (1998)

$$e_i = \frac{v_i}{\sqrt{n_i}}$$

and light intensity dependent noise (e_i) following Osorio et al. (2004)

$$e_i = \sqrt{\frac{(1/(\log(1000*(Q_{iA}+Q_{iB})/2)))^2 + v_i^2}{n_i}}$$

Q_{iA} and Q_{iB} are the quantum catches of the i th photoreceptor for the food items and background relative to a 100% reflecting surface. v_i is the noise in a single photoreceptor that I set to 0.05 in my model and n_i is the number of receptors of type i , which are given in table S2.

Achromatic contrast calculation:

I modeled avian perception of the achromatic contrast between food pellets and distracters following Siddiqi et al. (2004) as presented by Loyau et al. (2007). I calculated the quantum catch of the double-cone photoreceptors as follows:

$$Q_{dc} = \int_{\lambda} R(\lambda)S(\lambda)I(\lambda)d\lambda$$

I then calculated the log ratio of the quantum catches for the food pellets and background distracters:

$$\Delta f_{dc} = \log \frac{Q_{dc}}{Q_{dcb}}$$

where Q_{dcb} is calculated with the reflectance spectrum of the background distracters.

I calculated the achromatic contrast as follows:

$$\Delta S = \frac{\Delta f_{dc}}{e}$$

where the receptor noise e is calculated as follows:

$$e_i = \sqrt{\frac{1}{(\log(1000 * (Q_{iA} + Q_{iB}) / 2))^2 + v_i^2}}$$

Modeling spectral sensitivity:

I modeled the absorbance spectra of the photoreceptors based upon the λ_{\max} values reported by Hart and Vorobyev (2005) given in table S2 below and the visual pigment template of Govardovskii et al. (2000) as follows:

$$S_{vp}(\lambda) = \frac{1}{\exp[69.7(a - \lambda) + \exp[28(0.922 - \lambda)]] + \exp[-14.9(1.104 - \lambda) + 0.674]} + S_{\beta}(\lambda)$$

where:

$$a = 0.8795 + 0.0459 \cdot \exp\left[-\frac{(\lambda_{max} - 300)^2}{11940}\right]$$

and $S_{\beta}(\lambda)$ equals the absorbance of the β -band of the opsin absorbance spectrum:

$$S_{\beta}(\lambda) = 0.26 \cdot \exp\left\{-\left[\frac{\lambda - (189 + 0.315 \cdot \lambda_{max})}{-40.5 + 0.195 \cdot \lambda_{max}}\right]^2\right\}$$

To account for the spectral tuning of the cone oil droplets, I used the cut-off values given in table S2 and model template proposed by Hart and Vorobyev (2005) as follows:

$$T(\lambda) = \exp[-2.93 \cdot \exp[-2.89 \cdot B_{mid}(\lambda - \lambda_{cut})]]$$

For all models I used the ocular media transmission ($T_{ocular}(\lambda)$) of the starling (Hart et al. 1998). Therefore, spectral sensitivity for a given photoreceptor was defined as:

$$S(\lambda) = S_{vp}(\lambda)T(\lambda)T_{ocular}(\lambda)$$

Visual system parameters based upon the canary (*Serinus canaria*; Das et al. 1999)

parameter	value
Cone oil droplet -	
λ_{cut} and (B_{mid})	
P-type*	413 (0.095)
C-type	414 (0.095)
Y-type	506 (0.054)
R-type	578 (0.054)
Visual pigment	
(λ_{max})	
UVS/VS	363
SWS	440
MWS	501
LWS	567
Cone ratios	1,1,2,2
(u,s,m,l)	

*P-type oil droplet parameters were not available for the canary; therefore I used values from the blue tit (Hart et al. 2000). Published b_{mid} values are not available for the P-type oil droplet; therefore I set this to the same value as the C-type oil droplet.

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Chapter 3

FEMALE CHOICE FOR A CAROTENOID-BASED ORNAMENT IS LINKED TO CAROTENOID AVAILABILITY AND ACCUMULATION IN THE HOUSE FINCH (*CARPODACUS MEXICANUS*)

The coevolution of male traits and female preferences has led to the elaboration and diversification of sexually selected traits; however the mechanisms that mediate trait-preference coevolution are largely unknown. Carotenoid acquisition and accumulation are key determinants of the expression of male sexually selected carotenoid-based coloration and a primary mechanism maintaining the honest information content of these signals. Carotenoids also influence female health and reproduction in ways that may alter the costs and adaptive value of mate choice behaviors and thus provide a potential biochemical link between the expression of male traits and female preferences. To test this hypothesis, I manipulated the dietary carotenoid levels of captive female house finches (*Carpodacus mexicanus*) and assessed their mate choice behavior in response to color-manipulated male finches. Carotenoids did not influence the directional preference for red males; however, females receiving a high-carotenoid diet were more responsive to males, and discrimination among colorful males was linked to a female's ability to accumulate carotenoids. This carotenoid-related variation in mating responsiveness and discrimination may significantly alter how preferences

are translated into choice and promote the coevolution of female choice and carotenoid-based male plumage coloration.

Introduction

Directional female mating preferences in many animals have led to the emergence of extremely elaborate and diverse male ornamentation (reviewed in Andersson 1994). A robust framework exists for understanding how traits and preferences coevolve at the population genetic level (Fisher 1930; Lande 1981; Grafen 1990; Kokko et al. 2003). These models predict that sexual selection and the assortative mating of attractive males and choosy females inevitably leads to a positive genetic covariance between male trait and female preference. Yet, the physiological pathways that translate genetic information to behavioral decisions are largely unknown. These physiological mechanisms have the potential to profoundly shape the rate and direction of coevolution if they have mutually pleiotropic effects on the expression of trait and preference.

Sexually selected carotenoid-based male coloration appears in a diversity of taxa, from crabs (*Callinectes sapidus*; Baldwin and Johnson 2009) and fish (*Poecilia reticulata*; Kodric-Brown 1989) to birds (*Carpodacus mexicanus*; Hill 1990), and has become a model system for understanding the costs, benefits, and evolution of male sexual trait expression (Blount & McGraw 2008). Carotenoids are a class of pigment molecules that vertebrates are unable to produce endogenously and must acquire directly or indirectly from plants, bacteria, or

fungi (Goodwin 1984). Carotenoids can promote immune function (Chew & Park 2004) and alleviate oxidative stress (Alonso-Alvarez et al. 2004; but see Costantini & Møller 2008). Thus, carotenoid-based coloration reveals information about male quality (i.e. diet, health) and female preferences for intense carotenoid-based coloration have been demonstrated in a number of taxa (especially in birds; reviewed in McGraw & Blount 2009).

Although the costs and benefits of male coloration have dominated the attention of behavioral ecology researchers, carotenoids can also influence female condition and fitness in ways that complement their role in male reproduction. Similar to males, carotenoid supplementation of female birds has been shown to enhance immune system responsiveness and antioxidant protection (e.g. McGraw et al. 2011; Blount et al. 2002). Carotenoids are particularly important to breeding female birds because they deposit large amounts in the yolks of their eggs, and yolk allocation of carotenoids is linked to embryo development and offspring quality (Blount et al. 2003; Biard et al. 2005; McGraw et al. 2005; Berthouly et al. 2008). Thus, carotenoids, essential for the expression of male coloration, have complementary benefits for female birds, and I hypothesize that the traits that underlie carotenoid acquisition and accumulation provide a mechanistic link between female choice and male coloration.

At a proximate level, carotenoids can shape female choice behavior through their direct influence on female physiology. Locating, assessing, and

discriminating among potential mates can be a physiologically demanding process (Byers et al. 2005; Vitousek et al. 2007), and in many species female choice is a condition-dependent trait (reviewed in Cotton et al. 2006). The condition-dependence has been demonstrated through general manipulation of nutrition or rearing environment (e.g. Hebets et al. 2008; Riebel et al. 2009), making it difficult to pinpoint the specific mechanisms linking condition and behavior. Recently, integrative biologists have begun to realize that specific nutrients may constrain life-history and sexually selected traits (Morehouse et al. 2010, Isaksson et al. 2011) and I suggest that carotenoids may facilitate costly mate choice behaviors. Through their immunomodulatory and antioxidant actions, carotenoids may reduce the costs of the physical activity associated with mate choice. For example, carotenoid supplementation improves the escape flight performance of zebra finches (*Taeniopygia guttata*, Blount & Matheson 2006), and antioxidant supplementation, including carotenoids, alleviates flight-induced lipid peroxidation and DNA damage in budgerigars (*Melopsittacus undulates*; Larcombe et al. 2008). Indirectly, carotenoids may also facilitate female choice behavior through their positive effects on health (reviewed in McGraw 2006), by reducing the intensity and duration of sickness and freeing up resources that might otherwise be devoted to immune activity. Thus, females that efficiently acquire and accumulate carotenoids should be able to devote more effort to finding, assessing, and discriminating among mates.

Carotenoids also have the potential to shape mate choice for colorful traits through their direct influence on the visual systems of birds. In the avian retina, carotenoid pigments accumulate within oil droplets located between the inner and outer segments of the single cone photoreceptors (Goldsmith et al. 1984; Hart 2001). In this position, carotenoids modify the spectral sensitivity of the cone in a way that is predicted to enhance color discrimination and color constancy (Vorobyev 2003). Similar to plumage coloration, carotenoid accumulation in the avian retina is constrained by diet and health (Schiedt et al. 1991; Thompson et al. 2002; Bhosale et al 2009; Knott et al. 2010; Toomey & McGraw 2010; Toomey et al. 2010), and variation in retinal accumulation is linked to some aspects of visual discrimination (e.g. foraging; Chapter 2). Therefore, carotenoid accumulation in the retina may directly influence a female's ability to discriminate the coloration of potential mates.

At an ecological level, changes in the availability of dietary carotenoids can change the value of carotenoid-based colors as indicators of male quality and the benefits to females for choosing those traits (Grether 2000; Grether et al. 2005). Specifically, carotenoid-based male signals in a carotenoid-rich environment may not be useful indicators of quality because it may be relatively easy for males to acquire all of the carotenoids they need to become colorful. Consistent with this hypothesis, Grether et al. (2005) found that female guppies reared on a carotenoid-limited diet had significantly stronger preferences for male

carotenoid-based coloration then females reared on a carotenoid-rich diet. This result suggests that dietary carotenoid levels provide females with information about environmental carotenoid availability that they somehow use to weigh the value of carotenoid-based male signals. However, physiological mechanisms behind this environmentally tuned response are unknown.

To investigate effects of carotenoids on female mate choice, I manipulated dietary carotenoid intake, quantified physiological accumulation of carotenoids, and examined mate selection behaviors of female house finches (*Carpodacus mexicanus*) for male plumage coloration. The house finch is a model species for the study of sexually selected carotenoid-based coloration; males have plumage that varies from drab yellow to brilliant red depending upon dietary carotenoid access, health, and genetic quality (Hill 2002). Male coloration is a positive predictor of both paternal investment and incubation feeding of females (Hill 1991; but see Badyeav & Hill 2002). Thus, male coloration is considered an honest indicator of both direct and indirect benefits to mates and, in nearly all populations, females prefer brilliant red males (Hill 1994; Oh & Badyeav 2006; but see Badyeav & Hill 2002). Although these population-level preferences for male coloration are clear, individuals within a population may vary in their responsiveness, discrimination, and strength of their preferences, in ways that can alter the intensity and direction of sexual selection (Jennions & Petrie 1997; Widemo & Sæther 1999; Cotton et al. 2006). My goal was to examine how

individual variation in dietary carotenoid level and the accumulation of carotenoids in the plasma and retina relate to females choice behaviors. Because mate choice is a complex behavior with components that may be differentially influenced by physiological or environmental conditions, I examined three specific components.

Mate choice behavior can be initially divided into two general parts: 1) the preference function and 2) choosiness (Jennions & Petrie 1997; Widemo & Sæther 1999; Brooks & Endler 2001). The preference function is the slope of the relationship between a female's response and the level of expression of the male ornament (Jennions & Petrie 1997; Widemo & Sæther 1999; Brooks & Endler 2001); steeper slopes indicate stronger preferences. There is evidence in some taxa that the slope of the preference function may be influenced by female condition (Bakker et al. 1999; Hunt et al. 2005; Burley and Foster 2006), but this is by no means a universal pattern (Syriatowicz & Brooks 2004; Woodgate et al. 2010). Choosiness reflects the effort invested into mate choice by the female and can be further divided into two components: 1) responsiveness and 2) discrimination (Brooks & Endler 2001). Responsiveness is the mean level of response by a female to all males (i.e. general mating interest), and discrimination is the variance in the female's response among the males she has sampled (Brooks and Endler 2001). A high level of discrimination indicates that the female is biasing her response toward a specific male, while a low level of discrimination

indicates a similar response to all males. Because choosiness captures the energetically demanding process of searching for and visiting potential mates, it is not surprising that choosiness exhibits both heritable and condition-dependent variation (Brooks & Endler 2001; Syriatowicz & Brooks 2004; Hebets et al. 2008).

To examine the influence of carotenoids on these components of mate choice, I captured female finches prior to the breeding season, maintained them in captivity, and fed them high- or low-carotenoid diets. I then presented these females with males that were manipulated to vary from drab yellow to brilliant red and measured the association time of the females with the males, which has been shown to be a reliable indicator of female choice in this species (Hill 1990). From these observations, I calculated the preference function, responsiveness, and discrimination for each female. I measured plasma carotenoid levels before and after carotenoid supplementation and retinal carotenoid levels at the conclusion of the study. Because all females were maintained on a very low carotenoid diet for two months before carotenoid supplementation, I considered the initial measure of plasma carotenoid levels to be an indicator of a female's ability to accumulate carotenoids from a limited diet (*sensu* McGraw 2005). Therefore, if female preference for carotenoid-based coloration is linked to carotenoid accumulation and availability, I predicted that both the preference function and choosiness would be positively correlated with pre-supplementation plasma carotenoid levels

and significantly increased among carotenoid-supplemented females. If retinal carotenoids affect a female's ability to discriminate among potential mates, I predicted that retinal carotenoid levels would be positively correlated with the level of discrimination and with the repeatability of the preference function among repeated choice trials. Alternatively, the environmentally contingent carotenoid indicator model of Grether et al. (2005) predicts that female preferences should be negatively related to carotenoid availability and accumulation.

Methods

Experimental Animals

At the beginning of their first molt into nuptial plumage (July 2009), I captured 13 hatch-year male house finches to serve as stimulus birds for the mate-choice experiment, which is a number that is consistent with previous studies of mate choice in house finches ($n = 12$; Hill 1994). I trapped the males on the campus of Arizona State University in Tempe, Arizona, USA (details available in Toomey & McGraw; Appendix A) and housed them in groups of 2 in wire cages ($0.6 \times 0.4 \times 0.3$ m); in a greenhouse room that provided a natural photoperiod and semi-natural spectrum of light (i.e. the greenhouse glass blocked ultraviolet light). I fed the birds a standard maintenance diet (ZuPreem small bird maintenance diet, Premium Nutritional Products Inc. Mission, KS, USA) and tap water with a

vitamin supplement (Vita-Sol, United Pet Group EIO, Tampa, FL) *ad libitum*. Because this diet contained low levels of a limited diversity of carotenoids (lutein: $1.15 \pm 0.12 \mu\text{g g}^{-1}$ and zeaxanthin: $0.52 \pm 0.06 \mu\text{g g}^{-1}$), all of the males molted into uniformly drab yellow plumage that I subsequently manipulated for the mate choice trials (see below).

In November 2009, I captured 27 female house finches and housed them singly in the same greenhouse as the males, in a separate room where they were visually and acoustically isolated from the males. The females were initially maintained on a low-carotenoid sunflower seed diet for two months and then supplemented with carotenoids for the experimental treatments (see below).

Female dietary carotenoid manipulation and carotenoid measurement

To limit the influence of previous dietary history and storage on carotenoid availability, I maintained all of the female finches on a very low carotenoid diet of sunflower seeds ($0.078 \pm 0.031 \mu\text{g g}^{-1}$, lutein:zeaxanthin, 3.2:1) for the first two months after capture. This diet results in the >95% depletion of both circulating plasma carotenoids and liver carotenoid stores (Toomey & McGraw 2010). In January 2010, I randomly selected 13 females and began supplementing their drinking water with carotenoids (zeaxanthin: $17.5 \mu\text{g ml}^{-1}$, OptiSharpTM, DSM Inc. Heerlen, Netherlands), while the remaining 14 birds continued on the low-carotenoid diet. The supplement was given on five days per

week (Monday – Friday) up until two weeks prior to the beginning of mate choice trials, at which point all birds were returned to the low-carotenoid diet. This depletion period was included in an effort to isolate the effects of increased retinal carotenoid accumulation that persists through short-term depletion from the influence of circulating carotenoids that decline rapidly (Toomey & McGraw 2010). However, contrary to my previous studies (Toomey & McGraw 2010), the effects of the supplementation on circulating carotenoid levels persisted through the depletion period (see results).

To determine the effect of the diet manipulation on circulating carotenoid levels, I collected plasma samples at three time-points: 1) in January, after birds spent eight weeks on the low carotenoid diet and prior to carotenoid supplementation, 2) in March, after eight weeks of carotenoid supplementation, and 3) at the conclusion of the mate choice trials in April 2010. To measure carotenoid levels in the retina, I euthanized all females at the conclusion of the study and collected the left retina from each bird. I measured carotenoid levels in plasma and retinal tissue using high performance liquid chromatography (HPLC) following previously established protocols (Toomey & McGraw 2009; Appendix A). Plasma carotenoid levels are reported as $\mu\text{g ml}^{-1}$, and retinal carotenoid levels are reported per whole retina ($\mu\text{g retina}^{-1}$).

Stimulus male color manipulation and measurement

To assess the influences of carotenoid accumulation and supplementation on female choice for male plumage coloration, I presented females with sets of four stimulus male finches that had their plumage coloration experimentally manipulated to vary from drab yellow to brilliant red. Following McGraw and Hill (2000), I used Prismacolor® art markers (Newell Rubbermaid Office Products, Oak Brook, IL, USA) to color the plumage of each male using one of four colors: red (carmine red PM-4), orange/red (yellowed orange PM-15 with carmine red PM-4), orange/yellow (yellowed orange PM-15 with canary yellow PM-19), and yellow (canary yellow PM-19). Because this coloration faded over the course of the mate choice trials, I reapplied the color treatments every two weeks.

Although the coloration of the stimulus males can easily be categorized with the human visual system, these categories are unlikely to reflect how female house finches perceive male coloration (Bennett et al. 1994, Cuthill 2006). To capture a more relevant measure of coloration and determine the best predictor of female choice, I used noise-limited receptor and cone-capture models of the avian visual system to calculate the contrast values and tetrahedral color-space location of the color of each male, then examined how well these measures predicted female choice. First, I measured the spectral reflectance of the feathers from 300 to 700 nm with an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer and a PX-2 pulsed xenon light source. I collected a total of

nine spectra, three each from the crown, breast, and rump, and then calculated average spectra for each bird. Because the birds faded and were recolored biweekly, I measured the fresh and faded coloration, and calculated average spectra for the two-week period. I then used the noise-limited receptor model (Vorobyev et al, 1998; Siddique et al. 2004) and the visual system parameters from the canary (*Serinus canaria*; Das et al. 1999) to calculate the chromatic and achromatic contrast of the ornamental coloration of each male in a given mate choice trial relative to the reddest male in that trial, who was given a contrast of zero (supplementary methods). I calculated the tetrahedral color space location of the manipulated plumage color of each male following the methods of Stoddard et al. (2008; supplementary methods). This method defines a color as a vector in spherical coordinates, where the radius corresponds to saturation of the color, ϕ indicates the relative stimulation of the ultraviolet sensitive cone, and the relative stimulation of the long- and medium-wavelength sensitive cones is indicated by the θ value. For comparison, I also calculated a traditional tristimulus hue value for each male following Andersson et al. (2002). To examine how well each color metric predicted female choice, I used a linear-mixed model analysis, with the natural log of female association time as the dependent variable; trial nested within female id as a random effect, and compared the R^2 values of separate models with each of the color-metrics as covariates. I found that θ was the best

avian visual system predictor of female preference (see results) and used this measure of coloration in all subsequent analyses.

Choice trials and measures of choice

I quantified each female's response to the color-manipulated males in repeated (3×) mate choice trials with different sets of males but the same combination of plumage colors. The trials were conducted in a custom-built aviary (see Tobias and Hill 1998) that is partitioned into four visually separated flight cages that housed the stimulus males, while the female moved freely in a larger adjoining cage that gave her free visual and auditory access to the males. The female cage also contained a partition that created a “no choice” zone, where the female was out of visual contact with the males. Food (sunflower seeds) and water was available *ad libitum* at the back of the male cages and in the female “no choice” area. Each mate-choice trial lasted one hour and all trials were carried out between 0800 and 1300 hrs, from 15 March - 16 April 2010. Each female was tested only once each day, and all three trials were completed within an average of five days and a maximum of eight. Approximately 10 minutes prior to the beginning of each trial, one male of each color (red, orange/red, orange/yellow and yellow) was placed within the separate partitions of the aviary. The identity and location of the males within the aviary was randomized, such that females viewed unfamiliar males in each trial and the combinations of stimulus males differed between each female. Females were also taken from their housing cages

10 min before the trials in which they participated, and at the start of the trials were immediately released into the mate choice aviary and video recorded for one hour via four cameras, each focused on one male cage. After one hour, the female and male finches were returned to their housing cages and the next trial with different males and females was setup.

I reviewed the video recordings of each trial using the program Cowlog 1.0 (Hänninen & Pastell 2009) and quantified the amount of time, to the nearest second, that the female associated with each of the four stimulus males. I considered the female to be associating with a male when she was < 0.5 m away from him, a distance consistent with previous studies in this and other finch species (Burley et al. 1982, Hill 1990). When an association ended, I recorded whether the male or female moved away. I also recorded the amount of time that females engaged in flying, sitting, preening, or were out of view in the “no choice” area. From these observations, I calculated three components of mate choice behavior: 1) responsiveness, 2) preference function, and 3) discrimination. I calculated responsiveness as the mean association time of each female across all males and trials. I calculated the preference function as the t -value of the regression of the natural log of association time against male coloration (θ value) following Brooks & Endler (2001) and Forstmeier & Birkhead (2004). To calculate the t -values, I used R 2.10 (R Development Core Team 2010) and the nlme package (Pinheiro et al. 2010) to calculate the linear model lme (association

time $\sim\theta$ *female ID, random = ~ 1 |trial). The random factor of trial number is included to account for the non-independence of female association times within the repeated trials. I measured discrimination as the standard deviation of the mean association time for each female across all males and trials (Brooks & Endler 2001).

Statistical analyses

All statistical analyses were carried out with R 2.10 (R Development Core Team 2010) using the nlme package (Pinheiro et al. 2010). To examine the effect of dietary carotenoid supplementation on plasma carotenoid levels and body mass, I used separate repeated-measures analyses of variance (rmANOVA) with time as the within-subjects factor. To examine the effect of dietary carotenoid supplementation on retinal carotenoid concentrations, I compared levels of all six retinal carotenoid types between the dietary treatments in a multivariate analysis of variance (MANOVA). I compared average female activity in three mate choice trials (flying, sitting, and preening), per total time in view, between the diet groups in a MANOVA. Because male responsiveness to a female could significantly bias my measures of female choice, I compared the frequency (mean of the three repeated trials) that males terminated associations with high and low-carotenoid diet females with a Student's *t*-test.

I natural-log-transformed association time to meet the assumptions of parametric statistics and examined the effect of dietary carotenoid supplementation on female association time in a linear, mixed-effects model, with log association time as the dependent variable, diet treatment as an independent variable, male coloration (θ) and trial start time as covariates, and trial number nested within female identity as a random effect. With the exception of the diet \times θ interaction, I removed all non-significant interaction terms from the final model. I then examined the relationship between the specific components of mate choice (responsiveness, preference function, and discrimination, averaged over the three mate choice trials for each female) and plasma and retinal carotenoid levels in a multivariate analysis of covariance (MANCOVA). I considered plasma carotenoid levels just prior to the diet manipulation, when all birds had been maintained on a uniform low-carotenoid diet for 2 months, to be representative of carotenoid accumulation efficiency (*sensu* McGraw 2005) and compared these levels to the components of mate choice in a MANCOVA, with diet treatment as an independent variable and pre-supplementation plasma carotenoid levels as a covariate. Because retinal carotenoid concentrations differed significantly among the diet treatments (see results), I compared total retinal carotenoid accumulation to the components of mate choice in separate MANCOVAs for each diet treatment.

I calculated the repeatability of each female's choice for male coloration among the three trials following Lessells and Boag (1987), by calculating separate analyses of variance for each female with association time as the dependent variable, trial number as an independent variable, and male coloration (θ) as the covariate. From these ANOVAs, I took the mean square (MS) value of θ as the within-measure error (MS_w) and the MS of the trial term as the among-measure (MS_A) error to calculate the repeatability. I compared repeatability between the diet treatment groups in an ANOVA and examined the Spearman rank correlations with total retinal carotenoid level within each treatment group.

Results

Female carotenoid accumulation, body condition, and activity rate during mate choice

In January, prior to carotenoid supplementation, plasma carotenoid levels did not differ significantly between the treatment groups (Tukey's post hoc $p = 1.00$, Fig 16a). The high-carotenoid diet significantly increased plasma carotenoid levels in females compared to initial levels and those fed a low-carotenoid diet (time \times diet: $F_{2,50} = 108.12$, $p < 0.0001$, Fig. 16a). Following the eight-week carotenoid supplementation, I returned all birds to a low-carotenoid diet in an effort to minimize differences in circulating carotenoid levels during the mate choice trials, but the supplementation differences persisted and high-carotenoid

birds retained significantly higher plasma carotenoid levels during the mate choice test (April, Tukey's post hoc $p < 0.001$, Fig 16a). The effects of diet supplementation on the retinal carotenoid levels of these birds are presented as part of a separate study (Chapter 2), but, to summarize these results, birds receiving the high-carotenoid diet had significantly higher retinal carotenoid levels (specifically of astaxanthin, galloxanthin, zeaxanthin and ϵ -carotene) than those fed the low-carotenoid diet.

There was no significant effect of carotenoid supplementation on body mass (time \times diet: $F_{1,25} = 0.14$, $p = 0.71$, Fig. 16b); however body mass changed significantly over time ($F_{3,75} = 14.92$, $p < 0.0001$). Body mass declined following capture (November vs January, Tukey's post hoc $p < 0.001$) for both groups, then remained stable for the rest of the study (January – April, Tukey's post hoc $p > 0.845$).

Female activity levels differed significantly between the diet treatments (Wilk's $\lambda = 0.29$, $F_{1,24} = 5.72$, $p = 0.0042$), and this difference was driven primarily by a significant increase in movement of the high-carotenoid females ($F_{1,24} = 14.92$, $p = 0.034$). High-carotenoid females spent a mean \pm S.E. 19.43 ± 1.82 min. flying and or climbing during the trials, compared to the low-carotenoid females that spent 14.11 ± 2.09 min. moving. The treatment groups did not differ significantly in the amount of time preening ($F_{1,24} = 0.76$, $p = 0.39$) or sitting ($F_{1,24} = 1.024$, $p = 0.32$).

Male coloration measures as a predictor of female choice

Females spent significantly more time in association with the reddest males ($F_{1,241} = 15.71, p = 0.0001$, Fig. 17), and all of the color measures I tested, with the exception of r , were significant predictors of female association time (Table 6). Because of its particular link to visual perception, I chose to use the color-space vector component θ for subsequent analyses.

Carotenoid status and female choice behavior

Female preference for red males did not differ significantly between the diet treatments ($\theta \times \text{diet}: F_{1,241} = 0.95, p = 0.33$). However, females fed the high-carotenoid diet were significantly more responsive than were the low-carotenoid-diet females (Table 7, Fig 18a), spending significantly more time associating with males generally. Mate preference functions and discrimination did not differ between the diet treatments (Table 7). However, pre-supplementation plasma carotenoid levels were a significant predictor of female mate discrimination (Table 7), such that females who circulated relatively higher levels of carotenoids discriminated more among males (Fig 18b). Female responsiveness and the mate preference function were not significantly associated with pre-supplementation plasma carotenoid levels (Table 7, Fig. 18c). Time of day was a significant predictor of female association time, with females spending more time with males

earlier in the day ($F_{1,241} = 6.02, p = 0.018$). However, there was no significant effect of time of day on color preference ($\theta \times \text{Time}: F_{1,241} = 0.57, p = 0.45$).

Within each treatment group, total retinal carotenoid accumulation was not a significant predictor of mate choice behavior (High: Wilk's $\lambda = 0.79, F_{3,9} = 0.78, p = 0.53$, Low: Wilk's $\lambda = 0.52, F_{3,10} = 3.02, p = 0.080$). The repeatability of the preference for male plumage coloration did not differ significantly between diet treatments ($F_{1,25} = 1.52, p = 0.23$) or with retinal carotenoid accumulation within each treatment (High: $F_{1,11} = 0.025, p = 0.88$, Low: $F_{1,12} = 0.60, p = 0.45$).

I found no evidence that males responded differently to females from different diet treatment groups. Males were much less likely than females to terminate associations ($t = 7.093, df = 27.94, p < 0.0001$), and the frequency of male terminations did not differ significantly between the female diet treatments ($t = -1.15, df = 22.44, p = 0.26$). Males terminated association a mean of 5.19 ± 0.72 times per trial, while females ended associations 32.15 ± 3.73 times/trial.

Discussion

Here I provide evidence that carotenoids, a dietary component essential for the expression of sexually selected male coloration, also significantly influence female mate choice behavior. Specifically, female house finches supplemented with dietary carotenoids significantly increased their mating responsiveness to male finches, and the degree of discrimination among colorful

males was positively correlated with a female's ability to accumulate carotenoids from a limited diet. In contrast, the directional preference for red male plumage coloration was unaffected by supplementation and was not significantly linked to carotenoid accumulation ability. I propose that the observed carotenoid-dependent changes in specific components of mate choice behavior should be viewed in light of: 1) physiological costs and constraints of mate choice behavior, and/or 2) the context-dependent adaptive value of specific choice behaviors (Cotton et al. 2006).

Carotenoids and mate choice constraints

In many species, mate choice is a complex and costly process requiring the location, assessment, and comparison of potential mates (Pomiankowski 1987; Reynolds and Gross 1990; Cotton et al. 2006). Moving among and interacting with potential mates depletes energetic resources (Byers et al. 2005; Vitousek et al. 2007), and this activity is likely to generate oxidative stress (Powers et al. 2004). Carotenoids may facilitate active choice by countering the oxidative stress resulting from physical activity (i.e. flight; Blount & Matheson 2006). The behavioral effects of carotenoids extend beyond locomotion, and recently van Hout et al. (2011) found that carotenoid supplementation of male starlings (*Sturnus vulgaris*) enhanced song production. Specifically, nest-oriented song production was increased, suggesting a link between carotenoids and reproductive

behaviors in particular (van Hout et al. 2011). Consistent with this direct physiological role of carotenoids, I observed that carotenoid supplementation increased female responsiveness to males and general movement in the mate choice context, suggesting that supplemented females may have been better able to meet the costs of active mate choice.

I observed that a female house finch's level of mate discrimination correlated positively with her ability to efficiently accumulate high levels of carotenoids from a carotenoid-limited diet. Carotenoid accumulation efficiency may be associated with mate discrimination because it is related to a female's ability to meet the cognitive demands of mate choice that allow for the rapid discrimination of mates. Assessing, comparing, and recalling a pool of potential mates is a cognitively demanding task, and a female's ability to efficiently discriminate among potential mates may be limited by her cognitive capacity. In birds, cognitive traits have been shown to depend upon both developmental (Nowicki et al. 1998; Nowicki et al. 2002; Fisher et al. 2005) and current conditions (Pravosudov et al. 2002), and in humans some studies suggest a positive link between carotenoid levels and cognitive health (e.g. Jama et al. 1996; Akbaraly et al. 2007). The initial plasma carotenoid measure represents a rather general integration of female condition including, food consumption, assimilation efficiency, and the production of lipoprotein carriers in the plasma (McGraw and Parker 2006), in balance with the allocation of carotenoids to

physiological demands (e.g. immune function, antioxidant protection). Thus, poor carotenoid accumulators may be cognitively limited and require longer repeated assessments to discriminate among males. However, given the complex processes that determine carotenoid accumulation and cognitive ability, the mechanism that underlies this correlation remains to be determined.

The discrimination of mates is also potentially constrained by the performance of the sensory system (Endler and Basolo 1998). I have previously found that retinal carotenoid accumulation is a significant predictor of visual discrimination in a foraging context (Chapter 2) and hypothesized that carotenoid availability may influence female choice for colorful males through retinal-carotenoid-mediated changes in color discrimination. However, I found no support for this idea in the current study. Although carotenoid supplementation increased retinal accumulation as well as significantly influenced mate choice behavior, when I looked within diet treatment groups I found no relationship between mate choice behavior and retinal carotenoid accumulation. Although a direct experimental manipulation of retinal carotenoids would be a stronger test of this, my data suggest that it is unlikely that a retinal-carotenoid-specific mechanism is driving the changes in mate choice behavior between treatment groups. This is not altogether surprising, because the color differences between stimulus males were relatively large, easily discriminated by human observers, and likely outstripped any of the carotenoid-related shifts in discriminability. A

much finer-scale manipulation of male coloration is now needed to test for more subtle effects of retinal carotenoids on visually mediated mate selection based on coloration.

Carotenoid status and the adaptive value of choice

The cost and benefits of mate choice are dependent upon the context in which reproduction occurs, and females may adaptively shift choices to balance these costs and benefits (e.g. Qvarström et al. 2000; Oh and Badyeav 2006). Carotenoid availability and accumulation can change the context of mate choice because they can significantly influence a female's reproductive potential. Through their antioxidant and immunomodulatory effects (reviewed McGraw 2006), carotenoids can enhance female health and condition and can directly promote fecundity and offspring quality (Blount et al. 2000; Saino et al. 2002; Blount et al. 2003; Biard et al. 2005; McGraw et al. 2005). Therefore, the increased responsiveness of carotenoid-supplemented females that I observed may be an adaptive response to a relatively enhanced potential of supplemented females to produce many high-quality offspring. Such changes in receptivity are likely to be mediated through reproductive hormones, and there is evidence, in male birds, that dietary carotenoids facilitate the elevation of circulating testosterone by countering its immunosuppressive effects (Blas et al. 2006; McGraw and Ardia 2007). It would be particularly interesting to examine if and

how female reproductive hormones, such as estradiol, respond to carotenoid supplementation.

In contrast to responsiveness, female mate discrimination was not affected by carotenoid supplementation but was positively related to the female's ability to accumulate carotenoids from a limited diet. This correlation may represent an adaptive balance between the costs and consequences of mate choice. As mentioned above, plasma carotenoid levels are a broad integrator of a number of aspects of female condition, and these factors are likely to play an important role in determining a female's ability to allocate maternal resources to eggs (Saino et al. 2002; Hargatai et al. 2009) and provide care for young (Pike et al. 2007). In fact, a positive carotenoid balance over the course of a breeding season is a significant predictor of reproductive success in barn swallows (*Hirundo rustica*; Safran et al. 2010). Female house finches have been shown to increase the allocation of carotenoids to eggs when mated to unattractive drab males, in what is considered a compensatory strategy (Navara et al. 2006). Therefore, if females that efficiently accumulate carotenoids are better able to compensate for mate quality through maternal egg allocation and parental care, then the need to assess and carefully discriminate among a large pool of males is reduced and these females can make quick and decisive mate choices that minimize energetic and opportunity costs. In contrast, females with limited carotenoid accumulation ability may need to devote more effort to finding a compatible mate to ensure

reproductive success. For example, there is evidence that zebra finches pair assortatively on the basis of current condition (Burley and Foster 2006) and developmental history (Riebel et al. 2009).

Grether (2000; Grether et al. 2005) proposed that the value of male carotenoid-based colors as quality indicators is negatively related to the availability of carotenoids in the environment. Thus, carotenoid-limited females should show stronger preferences for male carotenoid coloration than carotenoid replete females (Grether et al. 2005). However, I found that color preferences of female house finches were not affected by dietary carotenoid supplementation or correlated with the carotenoid accumulation ability of females. This result contradicts the predictions of Grether et al. (2000; 2005); however my carotenoid manipulation occurred only during the adult stage and lacked the concomitant limitation of food availability during development that revealed these patterns in guppies (Grether et al. 2005). The stability of the mate preference function is not surprising because these are generally considered innate and/or developmentally determined components of mate choice (Widemo & Sæther 1999), which tend to vary less among individuals (Brooks & Endler 2001), and therefore may be less sensitive to female condition (Syriatowicz & Brooks 2004). In previous work, Hill (1994) found that female house finches from distinct populations, with very different patterns of male coloration, all show a common preference for red males,

suggesting that this preference is maintained despite very different social and environmental conditions and is an ancestral trait in the house finch.

Implications for trait preference coevolution

I hypothesized that traits mediating carotenoid acquisition and accumulation could promote the coevolution of male ornaments and female preferences by facilitating both the production of sexually selected male coloration and female choice for those ornaments. This coevolutionary process is typically envisioned as a linkage between male ornament expression and the female directional preference for that ornament (Fisher 1930; Lande 1981; Grafen 1990; Kokko et al. 2003). However, I found no significant relationship between carotenoid acquisition and accumulation and the directional preferences of female house finches for male plumage coloration. Yet, the carotenoid-dependence of female responsiveness and discrimination has the potential to influence the intensity and direction of sexual selection on male carotenoid-based coloration.

Dietary carotenoid supplementation enhanced female responsiveness and efficient carotenoid accumulators were more discriminating amongst potential mates. If we extend this observation to the natural context, I would expect carotenoid-replete females to pair earlier in season, secure access to the highest-quality (and reddest) mates in the population (Hill et al. 1998; McGraw et al. 2003), and strongly express their inherent preferences for red males. Because

early-breeding house finches also enjoy higher reproductive success (McGraw et al. 2003), such pairings are likely to result in the production of relatively more offspring. Following this scenario, there should be significant selection on the traits that mediate carotenoid acquisition and accumulation in both males and females promoting the evolution of elaborate carotenoid-based coloration.

Conclusion

I found that the acquisition and accumulation of carotenoids, which are nutrients important for health, female reproduction, and the production of sexually selected male coloration, are positively related to female mate responsiveness and discrimination. I suggest that the behavioral and physiological traits mediating carotenoid acquisition and accumulation provide a mechanism that may promote the coevolution of carotenoid-based sexual ornaments and female choice for those traits. The common and complementary benefits of carotenoid accumulation in males and in females (Biard et al. 2009) may underlie the ubiquity and elaboration of carotenoid-based sexual signals.

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Table 6. Mean \pm SE values for color measures acquired from stimulus males, as well as results from linear regressions of female association time with each measure of male plumage color.

Measure	Male color				β	t	p	R^2
	red	red / orange	orange / yellow	yellow				
r	0.12 \pm 0.01	0.12 \pm 0.007	0.10 \pm 0.006	0.11 \pm 0.006	3.81	1.54	0.12	0.191
ϕ	1.28 \pm 0.08	-1.39 \pm 0.06	-0.616 \pm 0.73	-1.01 \pm 0.15	0.099	2.60	0.0098	0.216
θ	-0.48 \pm 0.02	0.02 \pm 0.06	0.15 \pm 0.04	0.49 \pm 0.10	-0.59	-4.60	<0.0001	0.236
chromatic contrast (jnds)	-	11.0 \pm 1.5	11.49 \pm 0.9	15.6 \pm 1.4	-0.032	-4.20	<0.0001	0.233
achromatic contrast (jnds)	-	3.1 \pm 0.7	3.6 \pm 0.73	6.5 \pm 1.1	-0.055	-3.21	0.0015	0.233
hue (nm)	575.9 \pm 6.7	553.2 \pm 1.8	548.8 \pm 1.0	524.3 \pm 6.4	0.011	4.50	<0.0001	0.244

Table 7. Results of univariate ANCOVAs testing the effects of dietary carotenoid supplementation and pre-supplementation plasma carotenoid levels on the three components of female choice for male plumage coloration.

Mate choice component	Diet			Pre-supplementation plasma carotenoid levels		
	<i>F</i>	df	<i>p</i>	<i>F</i>	df	<i>p</i>
Responsiveness	9.37	1,24	0.0054	0.081	1,24	0.78
Discrimination	0.24	1,24	0.62	15.71	1,24	0.0058
Preference function	1.44	1,24	0.24	3.44	1,24	0.076

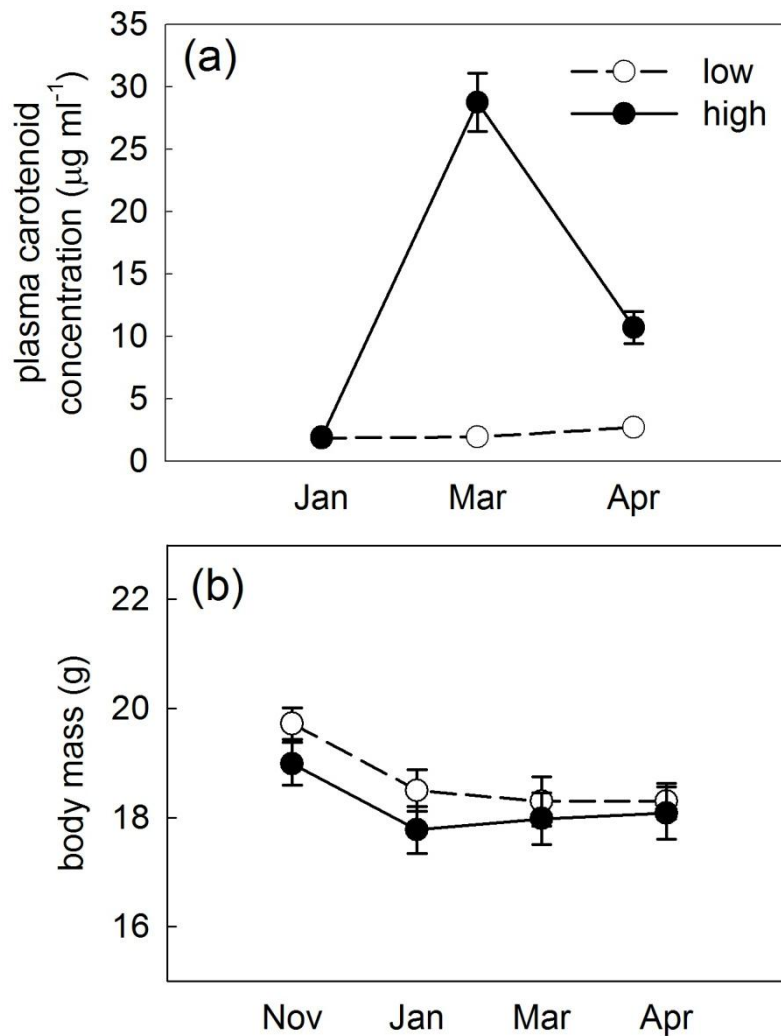


Figure 16. (a) Mean \pm S.E. plasma carotenoid concentrations of the high- and low-carotenoid diet female house finches throughout the study. Carotenoid supplementation began after the first sample was obtained (Jan.), and the mate choice tests were carried out in the period between the Mar. and Apr. samples. (b) Body mass of the high- and low-carotenoid finches at capture (Nov.) and throughout the study. Open symbols represent the low-carotenoid females and closed symbols represent the high-carotenoid females.

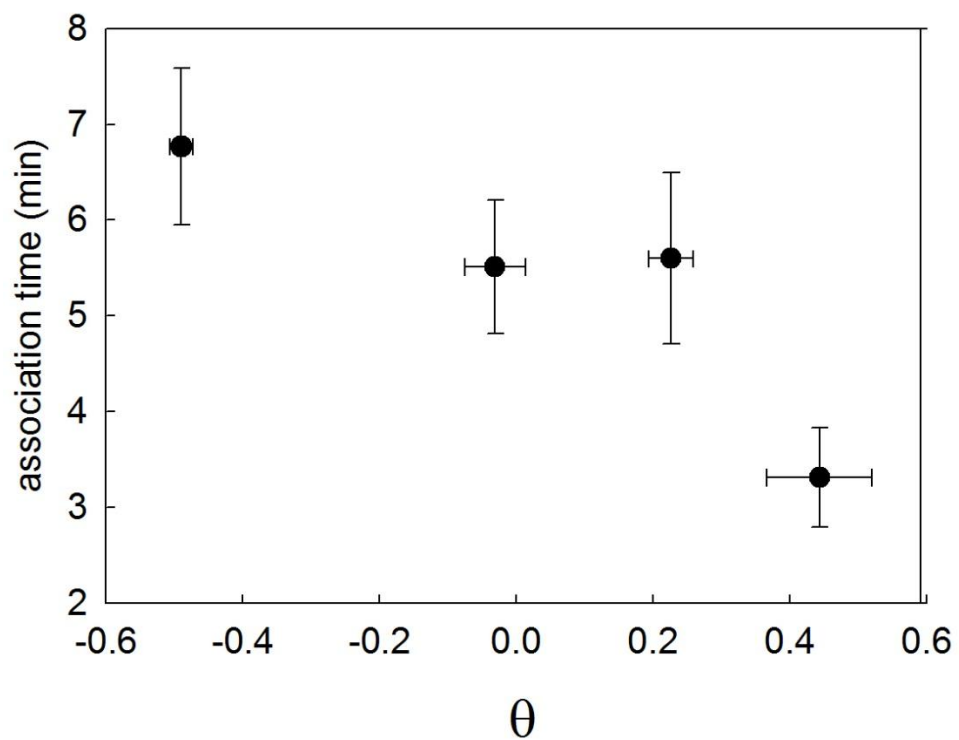


Figure 17. Mean \pm S.E. association time of females with stimulus males of varying color. For presentation the males were grouped into four color categories, with the mean θ value of each category presented on the x-axis. Lower θ values indicate redder males.

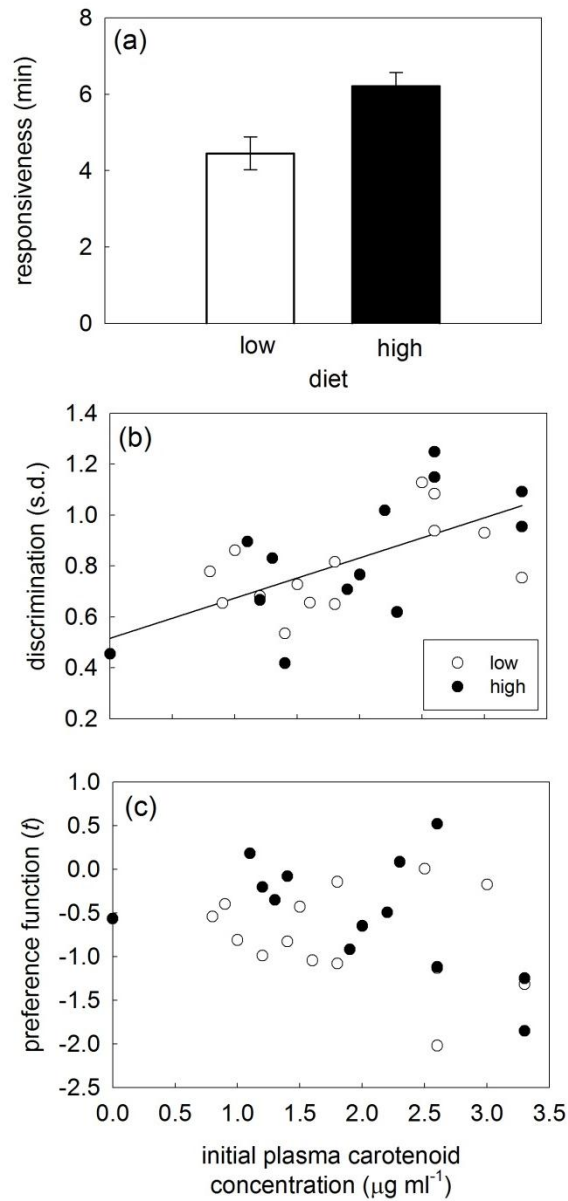


Figure 18. (a) Mean \pm S.E. mate responsiveness of high- and low-carotenoid females. (b) Scatterplot illustrating the relationship between female mate discrimination and plasma carotenoid levels prior to carotenoid supplementation. (c) Scatterplot showing the female preference functions and plasma carotenoid levels prior to carotenoid supplementation. Open symbols represent the low-carotenoid females and closed symbols represent the high-carotenoid females.

Supplementary methods

Reflectance spectra measurement:

I measured reflectance spectra of food pellets and background distracter matrix, relative to a white standard (Spectralon; Labsphere Inc., North Sutton, NH, USA), with an Ocean Optics USB2000 spectrophotometer with a PX-2 pulsed xenon light source (Ocean Optics Incorporated, Dunedin, Florida, USA). Fifteen spectra each were collected from the food pellets and background distracters at coincident-normal geometry at a distance of 1 cm from the surface. The reflectance spectra were then binned to 1 nm intervals for subsequent analyses.

Chromatic contrast calculations:

To model avian visual perception carotenoid pigmented tissues I followed the models proposed by Vorobyev et al. 1998 as modified by Aviles et al. 2008. I calculated the photoreceptor quantum catch for each cone class with the following equation:

$$Q_i = \int_{\lambda} R_i(\lambda)S(\lambda)I(\lambda)d\lambda$$

where λ indicates wavelength, Q_i is the quantum catch for the i th photoreceptor, $R_i(\lambda)$ is the spectral sensitivity of the i th photoreceptor, $S(\lambda)$ is the reflectance spectrum of the color patch, and $I(\lambda)$ is the irradiance spectrum. I calculated photoreceptor sensitivity based on physiological data from the canary as detailed below.

I then calculated the log ratio of the quantum catches for the color patches against a background of adjacent melanin pigmented plumage:

$$\Delta f_i = \log \frac{Q_i}{Q_b}$$

where Q_b is calculated with the reflectance spectrum of the background patches.

I calculated the chromatic contrast in just-noticeable differences as follows:

$$\Delta S = \sqrt{\frac{((e_1 e_2)^2 (\Delta f_4 - \Delta f_3)^2 + (e_1 e_3)^2 (\Delta f_4 - \Delta f_2)^2 + (e_1 e_4)^2 (\Delta f_3 - \Delta f_2)^2 + (e_2 e_3)^2 (\Delta f_4 - \Delta f_1)^2 + (e_2 e_4)^2 (\Delta f_3 - \Delta f_1)^2 + (e_3 e_4)^2 (\Delta f_2 - \Delta f_1)^2)}{((e_1 e_2 e_3)^2 + (e_1 e_2 e_4)^2 + (e_1 e_3 e_4)^2 + (e_2 e_3 e_4)^2)}}$$

Where e_i is the Weber fraction for the given photoreceptor calculated as follows;

$$e_i = \frac{v_i}{\sqrt{n_i}}$$

v_i is the noise in a single photoreceptor that I set to 0.05 in the model and n_i is the number of receptors of type i which are given in table S2.

Achromatic contrast calculations:

I model avian perception of the achromatic contrast of the carotenoid pigmented patches following Siddiqi et al. 2004 as presented by Loyau et al. 2007. I

calculated the quantum catch of the double cone photoreceptors as follows:

$$Q_{dc} = \int_{\lambda} R(\lambda) S(\lambda) I(\lambda) d\lambda$$

I then calculated the log ratio of the quantum catches for the color patches against a background of adjacent melanin pigmented plumage:

$$\Delta f_{dc} = \log \frac{Q_{dc}}{Q_{dcb}}$$

where Q_{dcb} is calculated with the reflectance spectrum of the background patches.

I calculated the achromatic contrast as follows:

$$\Delta S = \frac{|\Delta f_{dc}|}{e}$$

where the Weber fraction e is set to 0.05.

Color space parameter calculations:

I calculated the location of the carotenoid pigmented patches in avian perceptual color space following the model proposed by Stoddard and Prum (2008). I calculated the quantum catch for each of the photoreceptor types Q_i the relative stimulation against the background Δf_i as above. I then calculated the relative stimulation of each photoreceptor:

$$p_i = \frac{\Delta f_i}{\sum_i \Delta f_i}$$

I then plotted these relative stimulations in Cartesian space:

$$X = \frac{1 - 2s - m - u}{2} \sqrt{\frac{2}{3}}$$

$$Y = \frac{-1 + 3m + u}{2\sqrt{2}}$$

$$Z = u - \frac{1}{4}$$

where u is the relative stimulation of the UVS/VS cone, s – SWS cone, m – MWS cone, l – LWS cones. I then projected these points into a spherical coordinate system as follows:

$$r = \sqrt{X^2 + Y^2 + Z^2}$$

$$\varphi = \arctan \frac{\sqrt{X^2 + Y^2}}{Z}$$

$$\theta = \arctan \frac{Y}{X}$$

Modeling spectral sensitivity:

I modeled the absorbance spectra of the photoreceptors based upon the λ_{\max} values reported by Hart and Vorobyev (2005) given in the table below and the visual pigment template of Govardovskii et al. (2000) as follows:

$$S_{vp}(\lambda) = \frac{1}{\exp[69.7(a - \lambda) + \exp[28(0.922 - \lambda)]] + \exp[-14.9(1.104 - \lambda) + 0.674]} + S_{\beta}(\lambda)$$

where:

$$a = 0.8795 + 0.0459 \cdot \exp\left[-\frac{(\lambda_{\max} - 300)^2}{11940}\right]$$

and $S_{\beta}(\lambda)$ equals the absorbance of the β -band of the opsin absorbance spectrum:

$$S_{\beta}(\lambda) = 0.26 \cdot \exp\left\{-\left[\frac{\lambda - (189 + 0.315 \cdot \lambda_{\max})}{-40.5 + 0.195 \cdot \lambda_{\max}}\right]^2\right\}$$

To account for the spectral tuning of the cone oil droplets, I used the cut-off values given in table S2 and model template proposed by Hart and Vorobyev (2005) as follows:

$$T(\lambda) = \exp[-2.93 \cdot \exp[-2.89 \cdot B_{mid}(\lambda - \lambda_{cut})]]$$

For all models I used the ocular media transmission ($T_{ocular}(\lambda)$) of the starling (Hart et al. 1998). Therefore, spectral sensitivity for a given photoreceptor was defined as:

$$S(\lambda) = S_{vp}(\lambda)T(\lambda)T_{ocular}(\lambda)$$

Visual system parameters based upon the canary (*Serinus canaria*; Das et al. 1999)

parameter	value
Cone oil droplet - λ_{cut} and (B_{mid})	
P-type*	413 (0.095)
C-type	414 (0.095)
Y-type	506 (0.054)
R-type	578 (0.054)
Visual pigment (λ_{max})	
UVS/VS	363
SWS	440
MWS	501
LWS	567
Cone ratios (u,s,m,l)	1,1,2,2

*P-type oil droplet parameters were not available for the canary; therefore I used values from the blue tit (Hart et al. 2000). Published b_{mid} values are not available for the P-type oil droplet; therefore I set this to the same value as the C-type oil droplet.

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Chapter 4

CONCLUDING REMARKS

The goal of my dissertation research has been to test the hypothesis that *avian vision is subject to environmental and physiological constraints imposed by the acquisition and allocation of carotenoids*. The role of carotenoids in avian vision is particularly intriguing, because many bird species have carotenoid-based coloration that is similarly constrained and functions as an honest signal of quality (McGraw & Blount 2009). Thus, carotenoid-mediated vision could have far-reaching implications for the evolution of visual systems and these colorful signals. To test this hypothesis, I studied the house finch, which is a common passerine bird with sexually selected carotenoid-based coloration, and focused my studies on three questions:

- 1) Is there significant, biologically relevant variation in retinal carotenoid accumulation among free-living wild house finches?
- 2) What are the proximate environmental and physiological constraints on retinal carotenoid accumulation?
- 3) How does retinal carotenoid accumulation influence visually mediated behaviors, such as foraging and mate choice, of house finches?

Consistent with the predictions of my hypothesis, I found that carotenoid accumulation in the house finch retina is a variable trait that correlates with body condition and male plumage coloration and is causally influenced by an

individual's recent diet and health state (Appendices A-C). Moreover, retinal carotenoid accumulation was positively correlated with visual foraging performance under specific lighting conditions (Chapter 2) and may also indirectly influence vision by promoting the health of the eye through photoprotection (Thomson et al. 2002ab). However, I did not find support for a direct role of retinal carotenoids in shaping female preferences for carotenoid-based male coloration (Chapter 3). Therefore, it is unlikely that the carotenoid-mediated constraints on vision provide a direct linkage between signal production (carotenoid-based coloration) and mate discrimination, as I had initially speculated. Yet, it is possible that there is an indirect connection between coloration and vision because male coloration is positively correlated with retinal carotenoid accumulation (Toomey & McGraw 2009; Appendix A). Male carotenoid-based coloration therefore has the potential to communicate information about a male's visual health and performance to a potential mate.

The unique nature of carotenoid accumulation in the avian retina

Although the patterns of carotenoid accumulation in retina share some similarities with other carotenoid-based traits, my research also has revealed characteristics that are unique to this tissue. In contrast to other tissues (e.g. liver, plasma), carotenoid levels in the avian retina are relatively stable in the face of dietary depletion, and extended deprivation is required to cause significant

declines (Toomey & McGraw 2010; Appendix B). The relatively muted effects of diet on retinal carotenoid accumulation are not necessarily a surprise, given the nature of accumulation in the retina and functional importance in vision.

Carotenoids in the avian retina are highly esterified and largely localized within cone oil droplets that are composed of neutral lipids (Johnston & Hudson, 1976; Goldsmith et al. 1984; Bhosale et al. 2007), which may afford them enhanced stability and make them inaccessible for mobilization to other body tissues or functions. Although highly concentrated, the total amount of carotenoid in the retina is relatively small ($\sim 1 \mu\text{g}$ in a house finch retina) compared to other tissues like feathers, which may contain an order of magnitude, more carotenoid (McGraw et al. 2006). Therefore, it may be relatively easy to quantitatively maintain retinal carotenoid levels even when availability is severely limited, and the functional benefits are likely to out-weight the costs of allocating those carotenoid resources away from other functions.

Given the comparatively long-term stability of carotenoids in the avian retina, the availability of carotenoids during development (including embryonic allocation from mother, in the form of egg yolk) may be a particularly important determinant of retinal carotenoid accumulation throughout an individual's life. Recently, Biard et al. (2010) suggested that the maternal transfer of carotenoids is an important mechanism in the evolution of carotenoid-based colors because early-life availability can influence adult carotenoid assimilation and coloration.

Hammond (2008) hypothesized that carotenoid availability during development could shape the development, function, and visual health of humans. In the avian eye, the carotenoid pigmentation of cone oil droplets initially develops *in ovo* (Wai and Yew 2002), and these maternally derived carotenoids are maintained in the retina through extended periods of dietary carotenoid deprivation (Meyer 1971). Therefore, the maternal allocation of carotenoids to eggs and availability may influence accumulation throughout life and warrants further investigation.

In contrast to the results of my diet studies, I found that carotenoid accumulation in retina was more sensitive to immune system activity than were other body tissues. I observed that a long-term immune system challenge led to significantly lower levels of carotenoids in the retina, but that plasma, liver, and plumage carotenoid accumulation were unaffected (Toomey et al. 2010; Appendix C). Because other tissues were unaffected, it is unlikely that the retinal decline was driven by global depletion or systemic shifts in carotenoid allocation. Thus, immune system activity may have specific effects on carotenoid physiology of the avian retina. For example, carotenoids may be depleted or fail to accumulate because they are utilized to reduce inflammation within the eye (e.g. Jin et al. 2006). Carotenoid consumption and accumulation are associated with reduced risks of age related macular degeneration (AMD) and other eye diseases in humans (Seddon et al. 1994). Recently, a significant association between inflammatory processes and AMD has been discovered (Bok 2005). Therefore,

determining the mechanisms of these immune-induced changes in avian retinal carotenoid accumulation may provide us with insights into the complex interaction of carotenoids, inflammation, and eye disease in humans.

The influences of the environmental and physiological constraints on accumulation differed among the types of carotenoids in the avian retina. Retinal levels of galloxanthin and zeaxanthin responded rapidly to dietary changes, were depleted by immune challenges, and positively correlated with plasma carotenoid levels. One possible explanation for the heightened sensitivity of galloxanthin zeaxanthin is that these carotenoids degrade more rapidly and with a higher rate of turnover than other types. Consistent with a high rate of turnover, quail fed labeled zeaxanthin precursors; tend to accumulate higher levels of labeled galloxanthin and zeaxanthin than other retinal carotenoids (Bhosale et al. 2007). In contrast, astaxanthin levels in the retina rarely responded to dietary supplementation (but see Chapter 2), were not influenced by immune challenge, and were not correlated with plasma carotenoid levels. However, astaxanthin accumulation did differ among seasons (Toomey & McGraw 2009; Chapter 1) suggesting the possibility of gonadal hormones influencing accumulation of this carotenoid. These differing sensitivities may reflect differences in the mechanisms of accumulation and/or functional role of these carotenoid types. Both galloxanthin and astaxanthin are metabolically derived from a dietary zeaxanthin precursor; however this metabolism likely involves different enzymes (Schiedt

1991) whose expression and activity could be differentially regulated. At an ultimate level, the different functional roles of galloxanthin and astaxanthin may explain their differing sensitivities. Galloxanthin predominantly pigments the pale oil droplet of the principle member of the double cone, a photoreceptor that is thought to be primarily responsible for luminance detection (Jones and Osorio 2004). Because the opsin of the double cone is tuned to absorb long wavelength ($\lambda_{\max} \sim 560$ nm), the absorbance of galloxanthin ($\lambda_{\max} = 402$ nm) has little impact on the spectral tuning of this photoreceptor. However, galloxanthin may be important for photoprotection in these cells. In contrast, astaxanthin pigments the red oil droplet of the long wavelength sensitive (LWS) cone which is involved in the opponent processes of color vision (Goldsmith et al. 1984). The absorbance of astaxanthin ($\lambda_{\max} = 480$ nm) overlaps considerably with the opsin of the LWS cone ($\lambda_{\max} \sim 560$ nm), especially at the very high concentrations astaxanthin is found in the red oil droplet and significantly alters the sensitivity of the LWS cone. Therefore, changes in the concentrations of astaxanthin may have greater functional consequences than the same level of variation in the accumulation of galloxanthin. Knott et al. (2010) have hypothesized that excess carotenoids in the retina are metabolized to galloxanthin and shunted to the pale oil droplets to maintain precise levels of other carotenoids in the retina. However, to determine the adaptive significance of these differential patterns of accumulation, a better

understanding of the specific role of each retinal carotenoid type in visual function is needed.

Retinal carotenoid accumulation as a determinant of visual performance

The visible world is extremely complex and light intensities can vary over more than seven log units making it impossible for any single eye design to be optimized for all conditions (Lythgoe 1979). Therefore, trade-offs must be made between aspects of visual function. For example, color discrimination is optimized with many spectrally distinct and narrowly tuned photoreceptor types, but dim light vision is best with achieved with a single class of broadly tuned photoreceptors (Osorio & Vorobyev 2005). This trade-off has generally been examined in a comparative context, however my results suggest that carotenoid accumulation may mediate a similar trade-off among individuals within a population. In Chapter 2, I found that foraging performance, which relied on color discrimination, was positively correlated retinal carotenoid accumulation for birds with low to average levels of carotenoids but tended to decline in finches that had very high levels. This pattern is consistent with a carotenoid-mediated trade-off between spectral tuning and low-light sensitivity that has been predicted by model calculations of carotenoid-pigmented cone oil droplet filtering (Vorobyev 2003). Therefore, species adapted for low-light sensitivity may be limited in the color discrimination and vice versa. A key next step will be to take the refined

behavioral methods discussed above and apply them to conditions of varying light intensity to rigorously test this hypothesized trade-off.

A full understanding of the direct impacts of retinal carotenoid accumulation on vision will require the refinement of biochemical manipulations and behavioral tests. A limitation of my approach has been that I used diet to manipulate retinal carotenoid accumulation, which resulted in only limited changes in the retina and generated potential knock-on effects on health and behavior. A definitive test of the role of carotenoids in avian vision will require the development of tools to directly manipulate carotenoids in cone oil droplets, in isolation from other aspects of physiology and in concert with operant conditioning paradigms that allow for the precise testing of visual discrimination thresholds (e.g. Goldsmith & Butler 2005).

Mathematical models of visual discrimination have become a common feature of studies of avian color signaling (e.g. Delhey et al. 2010). These models, based upon physiological measurements that include the carotenoid-pigmented cone oil droplet tuning of spectral sensitivity, are used to predict the discriminability and conspicuousness of color signals in birds. However, the physiological parameters used in these models are derived from a limited number of species and individuals and carry the assumption that the avian visual system varies little among species and individuals. My dissertation research suggests that this assumption is unlikely to hold because carotenoid levels in the retina vary

considerably among individuals and even within an individual's lifetime.

Therefore, the wide application of these models to evolutionary questions of sexual signaling, predator avoidance, and nest parasitism should be reconsidered and validated to account for the potential impacts of individual variation on the predictions of these models.

Conclusion

The study of sensory systems has long focused on elucidating the mechanisms of action and probing the limits of performance for a species (Dusenbery 1992). In so doing, the variation among individuals within a species has largely been overlooked, and thus opportunities to understand how selection has shaped sensory systems have necessarily been limited. My dissertation research has revealed significant individual variation in a single component of the avian visual system (carotenoid accumulation); however the potential exists for similar individual variation in sensory system components across broad diversity of taxa and sensory modalities. For example, the spectral sensitivities of the mantis shrimp (*Haptosquilla trispinosa*) are tuned to the local light environment through developmental changes to filtering structures analogous to cone oil droplets (Cronin & Marshall 2001). Among adult animals, the visual sensitivities of female túngara frogs (*Physalaemus pustulosus*) change with reproductive state in a way that makes male signals more conspicuous (Cummings et al. 2008).

Therefore, understanding the causes and consequences of variation in animal sensory systems has the potential yield profound insights into the evolution of signaling traits and adaptive radiation of species (Dangles et al. 2009). The challenge now for sensory ecologists is to develop the tools to quantify the relevant axes of variation within each sensory system and track their adaptive consequences.

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APPENDIX A
SEASONAL, SEXUAL, AND QUALITY RELATED VARIATION IN
RETINAL CAROTENOID ACCUMULATION IN THE HOUSE FINCH
(*CARPODACUS MEXICANUS*)

Seasonal, sexual, and quality related variation in retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus*)

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Summary

1. Studies of visual ecology generally focus on the tuning of the eye to the spectral environment. However, the environment may also shape vision if the availability of nutrients or other extrinsic stressors impact eye structure or function.

2. Carotenoids are diet-derived pigments that accumulate in the retinas of birds, where they provide photoprotection and tune colour vision. In domesticated species, carotenoid accumulation in retina is dependent on dietary intake, but little is known about the variability in or control of these pigments in the eyes of wild animals.

3. Carotenoids are also deposited in the integument of many animals, where they generate colourful sexually selected displays that communicate information about individual health and nutrition. We hypothesize that retinal carotenoid accumulation is subject to the same health and nutritional constraints as the use of carotenoids in colour signals.

4. As a first test of this hypothesis, we examined retinal carotenoid accumulation in relation to season, sex, body condition, circulating plasma carotenoid concentrations, and plumage colouration in a free ranging population of house finches (*Carpodacus mexicanus*) – a model species for studies of carotenoid ecology.

5. Retinal carotenoid accumulation varied considerably among individuals and differed significantly among seasons, with the highest levels observed in late fall and winter. Body condition and plasma zeaxanthin levels were significantly positively correlated with retinal carotenoid accumulation, but retinal carotenoid concentrations did not differ between the sexes. Plumage redness covaried positively with retinal carotenoid concentration as well, though this relationship was no longer significant when accounting for seasonal variation.

6. Our results, although correlational, do suggest that retinal carotenoid accumulation is a variable trait that may be influenced by environmental and physiological conditions, raising the intriguing possibility that plumage colouration and colour vision could be linked through a common biochemical mechanism.

Key-words: colour vision, HPLC, visual ecology

Introduction

Studies of the ecology and evolution of animal signals have surged in recent decades, and we now have detailed understandings of the function, efficacy, and information content for many forms of visual, acoustic, chemical, and electric communication (Bradbury & Vehrencamp 1998; Espmark *et al.* 2000; Maynard *et al.* 2003). Comparatively less attention has been paid to the evolutionary ecology of signal reception.

Sensory systems are traditionally considered a property of the species that is optimized to detect specific stimuli (e.g. prey, predators, mates) under particular environmental conditions (Ryan 1990; Endler 1992; Endler & Basolo 1998) and are often modelled as a constant component of the signalling interaction (e.g. Endler & Mielke 2005; Delhey & Peters 2008). Because sensory systems are complex and finely tuned, there is great potential for individual variation arising from the costs of developing and maintaining sensory organs and neural networks. Growing evidence indicates that the developmental environment can influence the structure and function of sensory systems (Cronin *et al.* 2001; Kröger *et al.* 2003;

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Fuller *et al.* 2005; Shand *et al.* 2008). However, much less is known about how environmental conditions, including resource availability and other environmental stressors, affect mature sensory systems.

Vision is the dominant sensory system for many bird species and is a model for the study of sensory ecology and evolution (Bennett & Thery 2007). Birds have six classes of retinal photoreceptors: four classes of cone photoreceptors producing tetrachromatic colour vision, as well as achromatic sensitive double cones, and rods (Hart 2001). Within the cone photoreceptors and the principle member of the double cones, there are carotenoid-pigmented oil droplets that absorb incoming light before it reaches the visual pigment (Goldsmith *et al.* 1984; Bowmaker *et al.* 1997). Here, carotenoids protect the photoreceptor from harmful ultraviolet (UV) radiation and narrow the spectral sensitivity of the photoreceptor, enhancing colour discrimination and colour constancy in variable lighting environments (Vorobyev *et al.* 1998; Vorobyev 2003). Birds cannot synthesize carotenoids but must acquire them from their diet (Goodwin 1984), and complete exclusion of carotenoids from the diet of domesticated quail (*Coturnix japonica*) produces individuals with colourless oil droplets (Meyer 1971; Duecker & Schulze 1977; Wallman 1979; Bowmaker *et al.* 1993). These carotenoid-free individuals, although still capable of colour vision, have altered colour preferences (Duecker & Schulze 1977; Bowmaker *et al.* 1993) and spectral sensitivities (Wallman 1979). Based on these observations from domesticated species, it is possible that environmental availability of carotenoids may affect retinal accumulation and colour vision in wild birds. However, nothing is known about how retinal carotenoid accumulation varies in any wild animal species.

In contrast, a considerable amount is known about the accumulation of carotenoids in the sexually selected ornaments of wild animals like birds. Carotenoid-based avian colours have emerged as popular subjects for investigating the costs and content of animal signals (Blount & McGraw 2008). Elaborate colouration is linked to dietary carotenoid intake in wild birds and fishes (Grether *et al.* 1999; Hill *et al.* 2002). Carotenoid pigments also serve other roles, including antioxidant defence and immune-system enhancement (Vershinin 1999; McGraw 2006), and in many species carotenoid colouration is an honest indicator of individual health and quality (reviewed in Hill 2006). Because plumage and retinal carotenoids share a common dietary source, their accumulation may be subject to the same environmental and physiological constraints. In effect, carotenoid accumulation in the eye may be condition-dependent allowing only the best individuals to obtain key visual benefits of retinal carotenoid deposition.

In our first study on this topic, we sought to correlate the accumulation of retinal carotenoids in individuals of a free-ranging animal species with several traditional, environmentally variable metrics, such as season, body condition, and carotenoid-based plumage colouration. We did this in house finches (*Carpodacus mexicanus*)—a model avian species for studying the costs and benefits of carotenoids and colouration in animals (Hill 2002). The red, orange, and yellow

carotenoid-based plumage colour of male house finches is a classic example of sexually selected carotenoid-based colouration, and much is known about carotenoid physiology and signalling behaviour of this species (reviewed in Hill 2002). Males with redder (more carotenoid enriched) plumage have fewer parasites (Thompson *et al.* 1997), grow their feathers faster (Hill & Montgomerie 1994), and have higher concentrations of carotenoids in their diet (Hill *et al.* 2002), plasma, and liver (McGraw *et al.* 2006) during feather growth. The condition dependence of plumage colouration has been confirmed with experimental manipulations of dietary carotenoids (Hill 1992), caloric intake (Hill 2000), and parasite load (Hill & Brawnner 1998; Brawnner *et al.* 2000; Hill *et al.* 2004). Females prefer to mate with males with the reddest plumage (Hill 1990; Hill 1991; Hill *et al.* 1999; Oh & Badyaev 2006), such that they acquire mates that are in the best nutritional and health condition.

We measured retinal and plasma carotenoid levels and body condition of male and female adult house finches, as well as male plumage colouration, throughout the year. We predicted that retinal carotenoids levels would correlate positively with body condition and male plumage colouration. We also hypothesized that retinal carotenoid accumulation would be a function of plasma carotenoids being delivered to the eye, as is the case for other tissues (e.g. liver) in house finches (McGraw *et al.* 2006). Therefore, we predicted that accumulation of carotenoids in house finch retinas would vary seasonally, like plasma carotenoid levels do in house finches (K.J. McGraw & L.L. Washington unpublished data) and other songbirds (Isaksson *et al.* 2007; Deviche *et al.* 2008), and peak when plasma carotenoids are at their annual high. Sex differences in plasma carotenoids are minor in this species, occurring only for a few carotenoids during the molt period (McGraw *et al.* 2006); based on this limited information, we did not anticipate dramatic sex differences in retinal carotenoid accumulation.

Methods

SAMPLE COLLECTION

We captured wild house finches on the Arizona State University campus in Tempe, Arizona, USA in basket traps at baited feeding stations between the hours of 08.00 and 13.00 h (*sensu* McGraw *et al.* 2006). We captured male (M) and female (F) adult finches during five different time periods in 2006–2007: 2–10 March 2006 ($n = 15M, 15F$), 2–10 June 2006 (19M, 19F), 17 July–14 August 2006 (18M, 15F), 24–28 November 2006 (15M, 15F), and 3–4 February 2007 (15M, 15F). In the March, June, and July–August sampling periods, adult birds were readily identified by plumage characteristics (Hill 1993) and hatch year birds were excluded from the sample. A subset of the adult birds were banded in previous years with United States Fish and Wildlife Service bands and could be assigned to one of two age classes: 1 year old (19M, 11F), or 2 or more years old (4M, 4F). In the July–August period, we also sampled six hatch year (HY) birds to assess age related variation in retinal accumulation; however, these individuals were excluded from all other analyses. From all individuals, we collected a plasma sample (~80 μ L) from the wing

Table 1. The retention times (R_t), absorbance maxima (λ_{max}), putative identity, and mean \pm S.E. concentration of carotenoids in the house finch retina ($n = 161$)

Peak No.	R_t (min)	λ_{max} (nm)			Carotenoid	Concentration ($\mu\text{g}/\text{retina}$)
1	3:12	(356:4)	373:0	393:5	galloxanthin (A) ¹	0.042 \pm 0.0007
2	3:32		380:2	394:0	galloxanthin (B) ¹	0.267 \pm 0.007
3	3:55		381:3	397:1	galloxanthin (C) ¹	0.146 \pm 0.004
4	6:13		336:1	457:6	astaxanthin (A) ²	0.014 \pm 0.002
5	6:68		375:4	468:5	astaxanthin (B) ²	0.019 \pm 0.0004
6	7:10		368:2	469:7	astaxanthin (C) ²	0.042 \pm 0.001
7	7:94			478:2	astaxanthin (D) ²	0.309 \pm 0.008
8	8:24			477:0	astaxanthin (E) ²	0.031 \pm 0.002
9	10:0			474:6	astaxanthin (F) ²	0.062 \pm 0.002
10	8:17	(423:0)	446:7	475:8	3'-epilutein ³	0.086 \pm 0.002
11	8:87	(421:3)	446:7	474:6	lutein ²	0.047 \pm 0.001
12	11:05		453:9	479:4	zeaxanthin ²	0.107 \pm 0.002
13	14:40	(422:5)	447:9	477:0	unknown	0.041 \pm 0.0006
14	18:21	(420:0)	443:0	472:1	ϵ -carotene ⁴	0.017 \pm 0.0001

The λ_{max} values in parentheses denote the location of shoulders in the absorbance spectra and letters following the carotenoid name denote different isomers.

¹Identified based on the descriptions of Goldsmith *et al.* (1984) and Toyoda *et al.* (2002) and quantified based on the calibration curve for lutein.

²Identified and quantified by comparison to purified standards. ³Identified based on the descriptions of Khachik *et al.* (2002). ⁴Identified based on the descriptions of Goldsmith *et al.* (1984) and quantified based on calibration curve for β -carotene.

vein, morphological measurements (body mass and tarsus length), and scored the hue of the crown, breast, and rump plumage of males with a visible-light Colortron™ reflectance spectrophotometer (Light Source Inc. San Rafael, California; see McGraw & Hill 2000 for description and justification). In our analyses, we used the mean hue value from the three colourful plumage patches, and calculated body condition as the residual of the linear regression of body mass on tarsus length (Duckworth *et al.* 2001). We euthanized the birds within 1 h of capture and collected the retinas from both eyes. Retinas and plasma samples were stored at -80°C for up to 6 months prior to carotenoid analysis. We collected birds under United States Fish and Wildlife Service permit #MB088806-0, and Arizona State Game and Fish scientific collecting permit SP797514. All procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University (protocol #06-874R).

CAROTENOID ANALYSIS

We measured carotenoid accumulation in the left retina of each individual with high performance liquid chromatography following previously described methods (Toomey & McGraw 2007). We chose to analyze a single retina because preliminary analyses indicated that accumulation in the left and right retina was significantly positively correlated ($r^2 = 0.55$, $F_{1,160} = 34.68$, $P < 0.0001$, unpublished data). Briefly, we ground each retina for 3 min at 30 Hz in a ball mill (MM200, Retsch GmbH & Co. KG, Haan, Germany) in 2 mL of 1 : 1 hexane: *tert*-butyl methyl ether (MTBE). The ground retinas and solvent were centrifuged for 5 min at 3000 r.p.m., at which point the solvent fraction was transferred to a fresh tube, evaporated to dryness under a stream of nitrogen, resuspended in 1 : 1 (vol/vol) MTBE, split into two equal samples, and dried again. We split the samples in half to maximize our recovery of retinal carotenoids by performing two different saponification procedures: one half of the sample was resuspended in 1 mL of a 0.02 M solution of NaOH in methanol (a weak base treatment for analysis of ketocarotenoids) and the other was resuspended in 1 mL of a 0.2 M solution of NaOH in methanol (a strong base treatment for analysis of xanthophylls;

Toomey & McGraw 2007). The samples were then capped under nitrogen gas and incubated in the dark at room temperature for 4 h. After this time, we extracted the carotenoids from the saponification solution with 3 mL hexane : MTBE, dried the samples under nitrogen, and resuspended them in 200 μL of HPLC mobile phase consisting of 44 : 44 : 12 (vol/vol/vol) methanol : acetonitrile : dichloromethane. We injected 50 μL of each sample into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, Massachusetts) fitted with a Waters YMC Carotenoid 5.0- μm column (4.6 mm \times 250 mm). Retinal carotenoids were identified by comparing their retention times and absorbance spectra to those of external standards of purified zeaxanthin (DSM Inc., Heerlen, Netherlands), astaxanthin (Sigma, St. Louis, MO), lutein, and β -carotene (CaroteNature, Lupsingen, Switzerland). When external standards were not available, we identified carotenoids based upon published absorbance values and quantified them with external standard curves of available reference carotenoids (see Table 1 for details). We measured plasma carotenoid levels following the methods of McGraw *et al.* (2006).

STATISTICAL ANALYSES

Consistent with previous studies (Khachik *et al.* 2002; Bhosale *et al.* 2007), we report retinal carotenoid concentrations as μg of carotenoid per whole retina. For our analyses, we summed the concentrations of all isomers of a given carotenoid type (Table 1) and assessed the intercorrelations among retinal carotenoid types with a series of Pearson's product-moment correlations. The concentrations of all retinal carotenoid types were significantly intercorrelated (Table 2), so we summed them and used total retinal carotenoid concentration for subsequent analyses.

We used analysis of covariance (ANCOVA) to explore variation in total retinal carotenoid accumulation in relation to sampling period, sex, body condition, and the plasma concentrations of lutein and zeaxanthin. In our study, lutein and zeaxanthin made up > 91% of total circulating carotenoids and were the only plasma carotenoid types consistently observed in every individual (β -cryptoxanthin

Table 2. Pearson product-moment correlation coefficients for the relationships among carotenoid concentrations in the retinas of wild house finches ($n = 161$)

	Astaxanthin	Galloxanthin	Lutein	Zeaxanthin	Unknown	ϵ -carotene
Astaxanthin	–	0.27	0.39	0.41	0.37	0.19
Galloxanthin		–	0.68	0.70	0.49	0.40
Lutein			–	0.77	0.51	0.30
Zeaxanthin				–	0.86	0.39
Unknown					–	0.40
ϵ -carotene						–

Isomers of each carotenoid type were pooled for analysis. The concentrations of all carotenoid types were significantly intercorrelated ($P \leq 0.016$).

and β -carotene were also present in the plasma of 66 and 117 of 161 individuals, respectively, on average making up < 5% of total carotenoid concentration). There were no significant interaction terms in any of our models ($P > 0.05$), so we retained only the main effects in final models. We used Tukey–Kramer *post hoc* comparisons to compare retinal carotenoid levels among the five sampling periods. We also repeated this analysis using a multivariate approach (MANCOVA) with the six retinal carotenoid types as dependent variables (see Appendix S1 in Supporting Information); however these results were similar to our analyses with total retinal carotenoid concentration and we chose to present only the univariate analyses.

To explore the influence of age on retinal carotenoid accumulation, we used a one-way ANOVA to compare levels among age classes of the subsample of known age birds and repeated the above ANCOVA analysis within the largest known age-class in our sample (1 year). Because plumage colouration was only measured for male finches, we used a separate ANCOVA model to analyze the relationship between mean plumage hue, sampling period, plasma concentrations of lutein and zeaxanthin, and retinal carotenoid accumulation. Because sampling period explained much of the variation in retinal carotenoid accumulation (see results), we also investigated seasonal variation in body condition, plumage hue, and plasma zeaxanthin with ANOVA. Our data met the assumptions of parametric statistics (normality and equal variance), an alpha level of $P = 0.05$ was used throughout, and statistical analyses were carried out with SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

RETINAL CAROTENOID TYPES

We detected six major carotenoid types in the house finch retina, with a varying number of isomers (Fig. 1, Table 1). The house finch retina was dominated by astaxanthin (five isomers, 39% of total) and galloxanthin (three isomers, 37%), with lesser amounts of lutein (two isomers), zeaxanthin, ϵ -carotene, and one unidentified carotenoid (Table 1). Galloxanthin and ϵ -carotene are apparently unique to the retina of birds and have not been observed in the diet or other tissues of house finches (McGraw *et al.* 2006).

INTERINDIVIDUAL VARIABILITY IN RETINAL CAROTENOID CONCENTRATION

There was considerable variation in total retinal carotenoid accumulation among individuals in our sample, ranging from

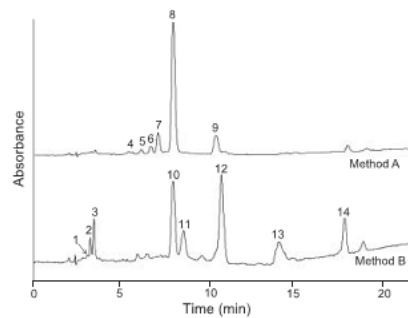


Fig. 1. Representative HPLC chromatograms (at 448 nm) of major carotenoids present in the retinas of house finches, recovered using method A (weak base) and method B (strong base) saponification. Numbers above peaks correspond to those listed in Table 1. Absorbance units are not relevant for this qualitative depiction and are omitted.

0.569 to 1.975 $\mu\text{g}/\text{retina}$, with a mean of 1.165 $\mu\text{g}/\text{retina}$ and coefficient of variation (CV) of 24.5. This level of variation is consistent with sexually selected traits (Cuervo & Møller 1999) and much greater than presumably naturally selected traits measured in this study, such as body mass (CV = 7.4) and tarsus length (CV = 2.9), but less than the variation in total plasma carotenoid concentration (CV = 73.9) or total plumage carotenoid concentration (CV = 65.1; data used from McGraw *et al.* 2006).

PREDICTORS OF RETINAL CAROTENOID ACCUMULATION

Retinal carotenoid accumulation differed significantly among sampling periods, with the highest levels in November 2006 and February 2007 (Table 3, Fig. 2a) – a period that includes prebreeding mate choice (Hill 1993). Although our study was limited to 1 year, we collected seven additional birds (5M, 2F) in May 2007 for another study, and these birds had relatively low retinal carotenoid levels (mean $0.936 \pm 0.072 \mu\text{g}/\text{retina}$), which is consistent with a cyclic seasonal pattern of retinal carotenoid accumulation (Supplementary Fig. S2a). Seasonal

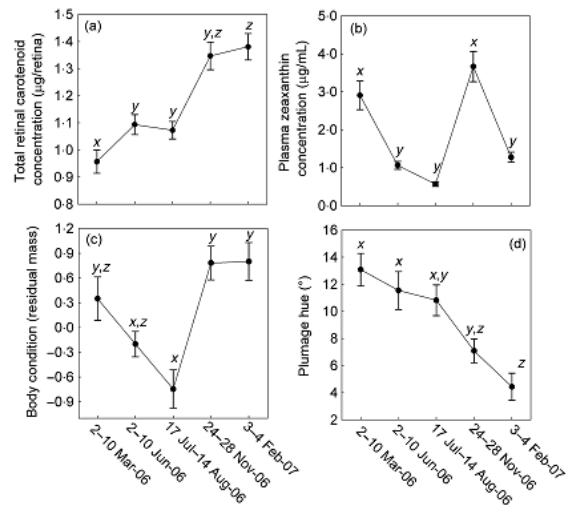


Fig. 2. Comparisons among sampling periods for: (a) mean \pm SE retinal carotenoid concentrations, (b) mean \pm SE plasma carotenoid concentrations, (c) mean \pm SE mass/tarsus length residuals (a measure of body condition), and (d) mean \pm SE male plumage hue (lower values indicate redder plumage). Within each panel, points that do not share a letter in common are significantly different (Tukey–Kramer *post hoc* tests, $P < 0.05$).

Table 3. ANCOVA table depicting the effects of body condition, sampling period, sex, and circulating plasma carotenoid concentrations on retinal carotenoid accumulation

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1,159	4.07	0.045
Sampling period	4,159	16.96	<0.001
Sex	1,159	0.48	0.48
Plasma lutein	1,159	0.01	0.91
Plasma zeaxanthin	1,159	10.29	0.0016

variation could have resulted from differences in the ages of birds sampled in each period. However this is unlikely because there was no significant difference in retinal accumulation among known age birds (HY, 1 year, & ≥ 2 years, $F_{2,42} = 1.87$,

$P = 0.17$) and if we limit our analysis to a single age-class (1 year olds), season remains a significant predictor of retinal accumulation ($F_{4,22} = 9.66$, $P = 0.0006$) and follows a qualitatively similar pattern to the whole sample (Supplementary Fig. S2b).

Body condition was a significant predictor of retinal carotenoid accumulation (Table 3), and body condition varied significantly among sampling periods with the highest levels in November 2006 and February 2007, when retinal carotenoid accumulation was also highest ($F_{4,160} = 9.66$, $P < 0.0001$, Fig. 2a,c). Across sampling periods, individuals in better condition had higher concentrations of retinal carotenoids (Fig. 3a). Retinal carotenoid accumulation also was significantly positively related to plasma zeaxanthin concentrations (Table 3, Fig. 3b). Plasma zeaxanthin concentrations differed

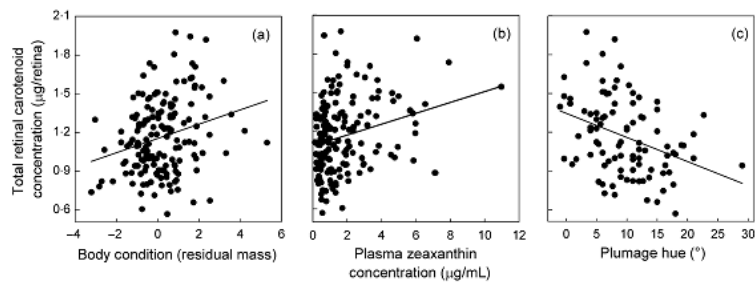


Fig. 3. Predictors of retinal carotenoid accumulation: (a) body condition, (b) circulating plasma zeaxanthin levels, (c) male plumage hue (lower hue values indicate redder plumage).

significantly across sampling periods ($F_{4,160} = 28.61, P < 0.0001$), but this pattern was not consistent with temporal differences in retinal accumulation (Fig. 2a,b).

Retinal carotenoid accumulation did not differ significantly between the sexes (Table 3), and male plumage hue was not a significant predictor when sampling period was taken into consideration ($F_{1,79} = 0.19, P = 0.67$). However, plumage hue differed significantly among sampling periods ($F_{4,79} = 9.69, P < 0.0001$) in a pattern that was consistent with retinal accumulation. Across sampling periods, there was a significant correlation between retinal carotenoid accumulation and male plumage hue, with redder males having higher levels of retinal carotenoids ($r_s = -0.378, P = 0.0005$, Fig. 3c). However, within any given season, mean plumage hue was not a significant predictor of retinal carotenoid accumulation ($P > 0.19$).

Discussion

Here we present the first ecological study of retinal carotenoid variation and correlates in a population of wild animals. Prior work on these natural pigments in animal eyes has been restricted to domesticated species (Khachik *et al.* 2002; Toyoda *et al.* 2002; Bhosale *et al.* 2007) or has been qualitative and inferential, by deducing light absorption properties of retinal oil droplets (which contain carotenoids) using microspectrophotometry (e.g. Goldsmith *et al.* 1984; Hart *et al.* 2006). Recent visual ecological studies have shown environmental sensitivity of eye morphology in vertebrates; for example, developmental lighting conditions affect the frequency of retinal cone types in several fish species (Kroger *et al.* 2003; Fuller *et al.* 2005; Shand *et al.* 2008) as well as the absorbance properties of cone oil droplets in chickens (*Gallus gallus domesticus*; Hart *et al.* 2006) and of visual filtering pigments in mantis shrimp (Cronin *et al.* 2001). However, an ecological approach has yet to be applied to carotenoid nutrients in the eye or to the plasticity of the mature visual system. Here, we found that retinal carotenoid accumulation varied among adult house finches from the same population, differed significantly among seasons, and correlated with body condition and colouration.

Qualitatively, the carotenoids we detected in house finch retinas were similar to those reported from HPLC studies of Japanese quail (*Coturnix coturnix japonica*; Bhosale *et al.* 2007; Toomey & McGraw 2007) and match the absorbance spectra of cone oil droplets reported in microspectrophotometry studies of a number of bird species (Goldsmith *et al.* 1984; Bowmaker *et al.* 1997). Astaxanthin and galloxanthin were dominant carotenoids in the house finch retina but not present in the diet (unpublished data) or circulation. These retinal carotenoids are likely produced through the metabolic conversion of specific dietary precursors (Davies 1985). Because of the conserved nature of absorbance profiles in retinal oil droplets interspecifically, and now similar carotenoid profiles in two unrelated species – one domesticated and one wild – it may be that the types of carotenoids in the retina are highly conserved and genetically controlled among birds (Bowmaker

et al. 1997). In fact, the presence of carotenoid pigmented oil droplets appears to be phylogenetically conserved even beyond birds. Animals ranging from very primitive lungfish (Bailes *et al.* 2006) to fish, turtles, and lizards possess colourful cone oil droplets (Douglas & Marshall 1999) and it will now be exciting to determine retinal carotenoid types in these groups for comparison to birds.

Quantitatively, however, we discovered considerable inter-individual variation in retinal carotenoid concentration in wild house finches, along with several ecological and physiological predictors of this variation. The strongest predictor of retinal carotenoid levels was season, with highest levels seen in late fall (November) and winter (February). As several extrinsic and intrinsic factors change with season, it is difficult to assess the precise source of this variation, especially in a correlational study, but diet has traditionally been targeted as a key modulator of carotenoid accumulation in animals. Dietary carotenoid intake predicts plumage colouration in molting wild house finches (Hill *et al.* 2002), and based on diet and plasma analyses (Beal 1907; Hill 1995) carotenoid accumulation appears to peak during molt (September–October) in this species, a few months before retinal carotenoid accumulation reached an annual high in our study. In fact, in a pilot experiment with captive house finches, we found that it took two months of a dietary carotenoid manipulation to appreciably affect carotenoid levels in retina (K.J. McGraw, E.A. Tourville & M.B. Toomey, unpublished data), which suggests together with data from wild animals that high carotenoid supplies in the molting diet could influence retinal carotenoid levels a few months later. This long-term dietary mechanism would be in line with evidence from domesticated birds that multi-generational carotenoid deprivation produces individuals with carotenoid-free colourless oil droplets (Meyer 1971; Bowmaker *et al.* 1993) and that long-term dietary carotenoid supplementation increases retinal carotenoid levels (Toyoda *et al.* 2002; Thomson *et al.* 2002a; Thomson *et al.* 2002b).

Often correlated with dietary carotenoid intake is plasma carotenoid concentration (e.g. Negro *et al.* 2000), and we found that levels of one carotenoid type in plasma – zeaxanthin – significantly and positively predicted retinal carotenoid concentration in wild house finches. This was true at the individual level, controlling for season and other factors. Based on radio-labelling and diet experiments, zeaxanthin also has been touted as particularly important, compared with other carotenoids, for retinal carotenoid accumulation and maintenance in domesticated species like chickens and quail (Davies 1985; Schiedt *et al.* 1991; Toyoda *et al.* 2002; Bhosale *et al.* 2007). Zeaxanthin is deposited directly into cone oil droplets but is also the putative precursor for the formation of other retinal carotenoids, including astaxanthin and galloxanthin (Davies 1985; Schiedt *et al.* 1991; Bhosale *et al.* 2007). Thus, it is possible that both dietary and physiological/metabolic factors (Brush 1990) underlie retinal carotenoid accumulation. This could be analogous to the control of plumage colouration in molting house finches, which has a dietary component (Hill *et al.* 2002) but also involves

conversion of yellow dietary pigments into red forms (Inouye *et al.* 2001) and is affected by carotenoid-independent nutritional state (i.e. has an 'energetic cost'; Hill 2000). The fact that male plumage redness was positively correlated with retinal carotenoid accumulation in our study is consistent with the notion that they have similar control mechanisms.

Consistent with our hypothesis, retinal carotenoid accumulation was positively correlated with body condition and male plumage colouration. However, to establish if retinal carotenoid accumulation is 'condition-dependent' *per se*, the particular physiological processes associated with carotenoid uptake, transport, conversion, and use should now be examined in relation to the eye, and in the context of the many other allocations that have been previously studied (e.g. to colouration, egg yolk, antioxidant defence, immunomodulation, and internal tissue storage; McGraw 2006). The idea of carotenoid trade-offs has received increased attention recently in the literature on life-history evolution (Blount 2004; Clotfelter *et al.* 2007; Fitz *et al.* 2007; Peters 2007), but this work has yet to include the retina as an important allocation site for carotenoids. Health roles have stood at the centre of these studies (Lozano 1994), and we are now anxious to analyze how carotenoids may be diverted between health and retinal functions. However we do not suspect rapid health/retina trade-offs, if they exist, because avian retinal carotenoids appear to be stable to short-term changes in dietary carotenoid levels (Wang *et al.* 2007; authors' unpublished data).

Amidst all we attempted to account for in this correlational and ecological study of retinal carotenoids, it is also plausible that retinal carotenoids track more than internal supplies and needs, but also external demands like environmental light exposure. Carotenoids absorb harmful ultraviolet radiation and protect the retina from light-induced photodamage in quail (Thomson *et al.* 2002a; Thomson *et al.* 2002b). Hart *et al.* (2006) found that absorbance spectra of the retinal oil droplets of chickens reared in bright environments were shifted toward longer wavelengths compared with individuals from a dim environment. This spectral shift is likely the result of increase carotenoid accumulation; therefore, individuals may up-regulate retinal carotenoid accumulation to enhance photoprotection. In our study, however, we found that retinal carotenoid levels were highest in November and February, a period when solar radiation is relatively low (~400 mJ m⁻²) compared to May and June (> 800 mJ m⁻², AZMET 2008, Phoenix Greenway station), when retinal carotenoid levels were low. The low levels of retinal carotenoid levels we see during the spring and summer months could instead be linked to (at least in part) photodegradation of retinal carotenoids (Mortensen & Skibsted 1999); however, the photostability of retinal carotenoids is not known. The variety of factors potentially affecting retinal accumulation provide ample opportunity for experimental manipulation and study, but what makes this variation in retinal carotenoid physiology particularly intriguing is its potential to affect colour vision and behaviour.

Functionally, retinal carotenoids accumulate in cone oil droplets and enhance colour vision by absorbing short-

wavelength light and reducing the overlap of spectrally adjacent single cone photoreceptors (Vorobyev *et al.* 1998; Vorobyev 2003). Variation in carotenoid concentration should shift the cut-off wavelengths of the oil droplets, change the spectral overlap among photoreceptors, and alter an individual's colour vision. The absence of retinal carotenoids is known to alter the spectral sensitivity (Wallman 1979) and innate colour preferences of quail (Duecker & Schulze 1977; Bowmaker *et al.* 1993), though no empirical studies have been conducted within the natural range of variation in retinal carotenoid accumulation. We envision two key effects that retinal carotenoids could have on colour vision and sexual selection in house finches: (i) enhancing a male's ability to acquire colourful carotenoid-rich foods and thus develop colourful plumage, and (ii) facilitating female mate choice for colourful plumage in males. In our study, we uncovered two results consistent with these predictions. First, there was an overall correlation between retinal carotenoid levels and male plumage colouration, suggesting that better colour vision in a male may be associated with superior carotenoid intake and colouration. Second, retinal carotenoid accumulation was highest in the late fall and winter, coinciding with the pre-breeding period when house finches choose mates (Hill 1993). On this basis, one might also expect the sexes to accumulate different levels of carotenoids in retinas to facilitate these season-specific foraging or mating activities, but we found no evidence of sex differences in our study. Surely, to truly understand the functional importance of retinal carotenoid accumulation to avian colour vision and sexual selection, we need to merge microspectrophotometric assessments and visual models with the critical behavioural experiments and evidence. However, our initial correlational results do suggest that retinal carotenoid accumulation may be limited by environmental and physiological conditions, raising the intriguing possibility that plumage colouration and colour perception may be linked through a common biochemical mechanism.

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Supporting Information

The following Supporting Information is available for this article:

Appendix S1. MANOVA analyses of accumulation of the six retinal carotenoid types in relation to body condition, sampling period, sex, and circulating lutein and zeaxanthin levels.

Fig. S2. Mean \pm SE retinal carotenoid accumulation across sampling periods (a) for a sample including May 2007 that shows low levels consistent with data from 2006 and (b) a sample limited to 1-year-old birds.

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Appendix S1. Results of the MANCOVA with separate values of astaxanthin, galloxanthin, lutein, zeaxanthin, unknown carotenoid, and ϵ -carotene as dependent variables and body condition, sampling period, sex, and circulating lutein and zeaxanthin concentrations as independent variables. Similar to the analysis presented in the text, sampling period was a significant predictor of retinal carotenoid accumulation in the overall MANCOVA and ANCOVA analyses for each type of retinal carotenoid. Body condition was a marginal predictor in the overall model and a significant predictor of retinal lutein, zeaxanthin, and unknown carotenoid accumulation. The concentrations of both plasma lutein and zeaxanthin were significant predictors of retinal accumulation in the overall model, but this pattern differed among separate retinal carotenoid types,

Factor	Wilks' λ	d.f.	<i>F</i>	<i>P</i>
Body condition	0.92	6, 146	2.10	0.057
Sampling period	0.17	24,510	14.20	< 0.0001
Sex	0.96	6, 146	0.97	0.45
Plasma lutein	0.83	6, 146	4.87	0.0001
Plasma zeaxanthin	0.76	6, 146	7.90	< 0.0001

ANCOVA results for each retinal carotenoid type:

Astaxanthin

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	2.73	0.10
Sampling period	4, 151	12.54	< 0.001
Sex	1, 151	0.27	0.60
Plasma lutein	1, 151	0.08	0.78
Plasma zeaxanthin	1, 151	1.87	0.17

Galloxanthin

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	0.66	0.42
Sampling period	4, 151	14.59	< 0.001
Sex	1, 151	2.73	0.10
Plasma lutein	1, 151	0.41	0.52
Plasma zeaxanthin	1, 151	12.69	0.0005

Lutein

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	5.73	0.018
Sampling period	4, 151	23.09	< 0.0001
Sex	1, 151	2.06	0.15
Plasma lutein	1, 151	0.02	0.89
Plasma zeaxanthin	1, 151	5.40	0.021

Zeaxanthin

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	9.48	0.0025
Sampling period	4, 151	6.51	< 0.0001
Sex	1, 151	0.28	0.60
Plasma lutein	1, 151	4.46	0.036
Plasma zeaxanthin	1, 151	18.38	< 0.0001

Unknown

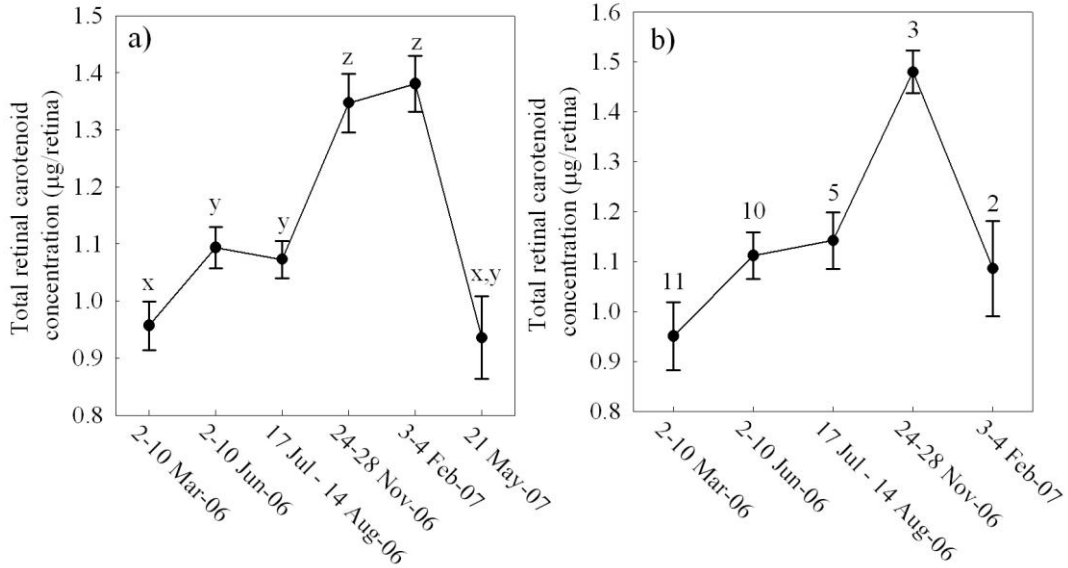
Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	9.64	0.0023
Sampling period	4, 151	5.10	0.0007
Sex	1, 151	0.20	0.66

Plasma lutein	1, 151	1.94	0.17
Plasma zeaxanthin	1, 151	6.71	0.011

β -carotene

Factor	d.f.	<i>F</i>	<i>P</i>
Body condition	1, 151	1.60	0.21
Sampling period	4, 151	3.48	0.0095
Sex	1, 151	0.53	0.47
Plasma lutein	1, 151	8.46	0.0042
Plasma zeaxanthin	1, 151	0.87	0.35

Figure S2. a) Mean \pm S.E. retinal carotenoid concentration by sampling period with the addition of seven individuals collected on 21May2008 as part of a separate study. Retinal carotenoid concentrations differed significantly with sampling period ($F_{4,22} = 7.49$, $P = 0.0006$) and points that do not share a letter in common are significantly different (Tukey-Kramer post-hoc test $P < 0.05$). b) Mean \pm S.E. retinal carotenoid concentration by sampling period for 1 yr old birds only. Sample size for each period is given above each point.



Toomey & McGraw. Seasonal, sexual, and quality related variation in retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus*)

APPENDIX B
THE EFFECTS OF DIETARY CAROTENOID INTAKE ON CAROTENOID
ACCUMULATION IN THE RETINA OF A WILD BIRD, THE HOUSE FINCH
(*CARPODACUS MEXICANUS*)



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The effects of dietary carotenoid intake on carotenoid accumulation in the retina of a wild bird, the house finch (*Carpodacus mexicanus*)

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ABSTRACT

Carotenoid pigments accumulate in the retinas of many animals, including humans, where they play an important role in visual health and performance. Recently, birds have emerged as a model system for studying the mechanisms and functions of carotenoid accumulation in the retina. However, these studies have been limited to a small number of domesticated species, and the effects of dietary carotenoid access on retinal carotenoid accumulation have not been investigated in any wild animal species. The purpose of our studies was to examine how variation in dietary carotenoid types and levels affect retinal accumulation in house finches (*Carpodacus mexicanus*), a common and colorful North American songbird. We carried out three 8-week studies with wild-caught captive birds: (1) we tracked the rate of retinal carotenoid depletion, compared to other body tissues, on a very low-carotenoid diet, (2) we supplemented birds with two common dietary carotenoids (lutein + zeaxanthin) and measured the effect on retinal accumulation, and (3) we separately supplemented birds with high levels of zeaxanthin – an important dietary precursor for retinal carotenoids – or astaxanthin – a dominant retinal carotenoid not commonly found in the diet (i.e. a metabolic derivative). We found that carotenoids depleted slowly from the retina compared to other tissues, with a significant (~50%) decline observed only after 8 weeks on a very low-carotenoid diet. Supplementation with lutein + zeaxanthin or zeaxanthin alone significantly increased only retinal galloxanthin and *ε*-carotene levels, while other carotenoid types in the retina remained unaffected. Concentrations of retinal astaxanthin were unaffected by direct dietary supplementation with astaxanthin. These results suggest highly specific mechanisms of retinal carotenoid metabolism and accumulation, as well as differential rates of turnover among retinal carotenoid types, all of which have important implications for visual health maintenance and interventions.

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Introduction

Diet-derived carotenoids acting as provitamins, immunomodulators, antioxidants, and/or photoprotectants play an important role in the health and normal physiological function of humans and a diversity of animals [1,2]. Carotenoids are particularly important for visual health, and increased retinal carotenoid accumulation is associated with a reduced risk of age-related macular degeneration (AMD)¹ and cataract in humans [3,4]. Carotenoid accumulation in the retina is influenced by carotenoid intake [5,6], raising the possibility of dietary interventions to promote visual health. However, testing such interventions in humans is difficult, and thus birds have been proposed as a model system for the study of the controls and functions of retinal carotenoids because they

accumulate levels of retinal carotenoids comparable to humans [7–9]. In young quail (*Coturnix japonica*), for example, dietary carotenoid supplementation elevates retinal carotenoid concentrations, enhances photoprotection, and reduces the accumulation of A2E – a marker of eye disease [8–10]. However, the mechanisms of accumulation and functions of carotenoids in the avian retina differ from humans in several important aspects. The avian retina contains metabolically derived apo- and keto-carotenoids not found in the human retina, and the majority of carotenoids in the avian retina accumulate in an esterified form [11]. Therefore carotenoid accumulation in the avian retina may be subject to constraints that are not shared by humans.

In the avian retina, carotenoids accumulate within cone oil droplets that are located between the inner and outer segments of the cone photoreceptors. In this position the light absorbance of carotenoids modifies the spectrum and intensity of light reaching the visual pigment. The types and concentrations of carotenoids in the oil droplets are specific to the type of photoreceptor. For example, the long-wavelength-sensitive cone contains a red oil droplet pigmented with astaxanthin, while the short-wavelength sensitive cone oil droplets contain galloxanthin that

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¹ Abbreviations used: AMD, age-related macular degeneration; HPLC, high-performance liquid chromatography; MANOVA, multiple analyses of variance; rmANOVA, repeated-measures analyses of covariance; zea, zeaxanthin; asta, astaxanthin; gal, galloxanthin; lut, lutein; *ε*-car, *ε*-carotene.

absorbs light at shorter wavelengths [12]. Thus carotenoid-pigmented oil droplets act as matched filters, enhancing color discrimination and improving color constancy [13]. This specific and matched carotenoid accumulation is achieved through the metabolism and selective accumulation of dietary carotenoids. Avian retinal carotenoids, like galloxanthin, are found only in the eye, suggesting highly localized carotenoid metabolism [11,14,15].

Because of their essential role in color vision and photoprotection in the avian eye, variation in retinal carotenoid accumulation could impact color vision, retinal health, and have consequences for an individual's survival and fitness. However, the influence of diet on retinal carotenoid accumulation is not known for any wild bird or other wild animal species. There have been a number of comparative microspectrophotometric studies of the carotenoid-pigmented cone oil droplets of wild birds [16], and the majority of experimental studies of retinal carotenoid dynamics have been limited to young birds of domesticated species like the chicken (*Gallus domesticus*) and quail. Studying these selectively bred domesticated species makes it difficult to place these results in a natural context and limits our ability to examine the evolutionary forces shaping retinal carotenoid accumulation.

To expand these investigations to wild birds, we have initiated a series of studies on retinal carotenoids in the house finch (*Carpodacus mexicanus*), a common North American passerine bird. This species is sexually dimorphic, with sexually selected carotenoid-based male plumage coloration, and has become a model species for the study of carotenoid physiology in wild birds [17]. We have found that carotenoid levels in the retinas of wild house finches vary among seasons, and positively correlate with body condition and male coloration [18]. The purpose of the current study is to examine how dietary carotenoid intake affects accumulation of specific retinal carotenoids and may shape the variation we have seen in the wild population. We carried out three separate experiments with captive wild-caught house finches to determine: (1) the time course of retinal carotenoid depletion, as animals are being fed a very low-carotenoid diet, (2) how supplemental levels of two common dietary carotenoids (lutein and zeaxanthin) affect retinal carotenoid accumulation, and (3) the effect of separate high doses of two abundant carotenoids in the avian retina – zeaxanthin (the main carotenoid in medium-wavelength-sensitive cones and precursor of other metabolically derived carotenoids in the quail retina [11]) or astaxanthin (the metabolic derivative that pigments the oil droplets associated with the long-wave-sensitive cones) – on retinal carotenoid accumulation.

Methods

Chemicals

Solvents for carotenoid extraction and high-performance liquid chromatography (HPLC) analyses were purchased from Fisher Scientific (Waltham, MA). Purified standards of lutein and β -carotene were acquired from CaroteNature (Lupsingen, Switzerland), astaxanthin from Sigma–Aldrich (St. Louis, MO), and zeaxanthin from DSM (Heerlen, Netherlands). A diet supplement of combined lutein and zeaxanthin (OroGlo-15[®] containing 15 g/kg of 8.2:1, lutein:zeaxanthin) was donated by Kemin Inc. (Des Moines, IA). Zeaxanthin (OptiSharp[®] 5% by weight) and astaxanthin (Carophyll[®] Pink 10% by weight) dietary supplements were donated by DSM Inc. (Heerlen, Netherlands).

Experimental animals

Wild house finches were captured on the campus of Arizona State University in Tempe, Arizona, USA in basket traps at baited feeding stations following the methods of Toomey and McGraw [18]. The

birds were housed in small wire cages (0.6 × 0.4 × 0.3 m) in two greenhouse rooms with *ad libitum* access to tap water and food (diets detailed below).

Experiment 1 – Depletion

To examine the response of retinal carotenoid accumulation to dietary carotenoid deprivation, we maintained 25 males, 10 females, and 15 juveniles (of unknown sex) in captivity on a very low-carotenoid diet of black oil sunflower seeds (0.078 ± 0.031 μg/g, lutein:zeaxanthin, 3.2:1, methods see Carotenoid extraction) for up to eight weeks. On weeks 0, 2, 4, 6, and 8, we randomly selected and euthanized five males, two females, and three juveniles to collect the left retina and liver tissue for carotenoid analysis. To track circulating carotenoid levels, we collected a blood sample (~100 μl) from the ulnar vein of all remaining birds every 2 weeks throughout the study and measured plasma carotenoid levels with HPLC (see Methods: Carotenoid extraction and HPLC analyses).

Experiment 2 – Lutein and zeaxanthin supplementation

Lutein and zeaxanthin are the most common carotenoids in the plasma of house finches [19], and the purpose of this experiment was to examine how varying levels of these two carotenoids in the diet influenced retinal carotenoid accumulation. In September 2008, we brought 28 adult male and 26 adult female house finches into captivity and maintained them on a low-carotenoid diet (Caroline rice diet, Roudybush Inc. Woodland, CA; 0.31 ± 0.03 μg/g, lutein:zeaxanthin, 4:1, methods see Carotenoid extraction) for five weeks to wash out any individual differences that may have stemmed from variation in their diet in the wild. We then randomly assigned the birds to one of three diet treatments: (1) low – consisting of the low-carotenoid base diet (n = 9 males and 8 females), (2) medium – consisting of the base diet supplemented with OroGlo[®] to a level of 10 μg/g lutein:zeaxanthin (8.2:1) (n = 10 males and 9 females), and (3) high – consisting of the base diet supplemented with OroGlo[®] to a level of 30 μg/g lutein:zeaxanthin (8.2:1) (n = 9 males and 9 females). This carotenoid manipulation falls within the range of variation in carotenoid concentrations of the gut contents from wild house finches reported by Hill et al. [20] (mean ca. = 8 μg/g, range = <1–>70 μg/g). The birds were given *ad libitum* access to these diets for eight weeks, then all birds were returned to the base diet for a period of 2 weeks for behavioral experiments (not presented here), at which point they were euthanized so that the left retina of each bird could be collected for carotenoid analyses. Results of Experiment 1 indicated that this two-week return to the base diet was unlikely to affect retinal carotenoid levels. Blood samples were collected from all birds in weeks 0, 4, and 8 of the diet manipulation to track changes in plasma carotenoid levels (see below).

Experiment 3 – Zeaxanthin or astaxanthin supplementation

Because the effects of the dietary treatments in Experiments 1 and 2 were relatively limited (see Results), we designed the third experiment to maximize our likelihood of detecting a dietary effect by supplementing the birds with high levels of zeaxanthin, the putative dietary precursor for many of the carotenoids in the avian retina [11,14,15], and astaxanthin, which is not found in circulation, but is metabolically derived and the dominant carotenoid in the house finch retina [18]. In June 2009, we brought 14 adult male and 14 adult female house finches into captivity and fed them a low-carotenoid sunflower seed diet for 8 weeks. We then randomly assigned birds to one of three carotenoid treatments: (1) control – four males and four females continued to receive the

sunflower seed diet and tap water with vitamin supplement (Vita-Sol[®], United Pet Group EIO, Tampa, FL), (2) zeaxanthin – five males and five females received a supplement of zeaxanthin beadlets (35 µg/ml of OptiSharp[®]) suspended in their drinking water along with the vitamin supplement, and (3) astaxanthin – five males and five females received a supplement of astaxanthin beadlets (35 µg/ml of Carophyll Pink[®]) suspended in their drinking water along with the vitamin supplement. These supplements were given for 8 weeks *ad libitum* each weekday and plain tap water was given on weekends. Then all birds were fed the control diet for two weeks during behavioral experiments (not presented here), at which point they were euthanized so that the left retina of each bird could be collected for carotenoid analyses. Blood samples were collected on weeks 0, 4, and 8 of the diet manipulation to track changes in plasma carotenoid levels.

Carotenoid extraction

One whole retina from each individual was extracted three times in hexane:methyl tert-butyl ether (MTBE) 1:1 (v:v) by homogenization in a ball mill (MM200, Retsch GmbH & Co. KG, Haan, Germany) for 3 min at 30 Hz and then evaporated to dryness under a stream of nitrogen. Because avian retinal carotenoids are esterified, we saponified the extracts following methods we previously developed to maximize the recovery of avian retinal carotenoids [21]. We split each sample from an individual into two separate tubes and then resuspended the extract in 0.2 or 0.02 M KOH in methanol, capped them under nitrogen, and incubated for 6 h at room temperature in the dark. To complete the saponification, we added 1 ml of a saturated solution of NaCl in water, 2 ml of distilled water, then extracted the free carotenoids with 3 ml of hexane:MTBE and dried the split extracts separately under a stream of nitrogen and prepared them for HPLC analyses by resuspending them in 200 µl mobile phase (methanol:acetonitrile:dichloromethane, 44:44:12, v:v:v).

Plasma and liver carotenoids were extracted following McGraw et al. [19]. Briefly, 10 µl plasma were combined with 100 µl ethanol and 100 µl hexane:MTBE, vortexed, then centrifuged for 3 min at 9000g. The supernatant was collected, evaporated to dryness under a stream of nitrogen, and then resuspended in mobile phase for HPLC analysis. The right lobe of the liver was excised (0.11–

0.51 g) and ground three times using a mortar and pestle in the presence of 3 mL hexane:MTBE. The extract was saponified with 0.2 M KOH in methanol for 6 h at room temperature in the dark. We then extracted the free carotenoids and prepared them for HPLC as described for the retina.

Carotenoids were extracted from the sunflower seed and Carline rice diet by separately grinding three samples of each (approximately 0.5 g) in a mortar and pestle in the presence of 2 ml tetrahydrofuran. This procedure was repeated three times, at which point we collected the solvent, transferred it to a fresh tube, and centrifuged for 5 min at 3000 rpm. The supernatant was then collected, evaporated to dryness under a stream of nitrogen, and resuspended in mobile phase for HPLC analyses.

HPLC analyses

Carotenoid analyses were carried out on a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) with a Waters YMC Carotenoid 5 µm column (4.6 × 250 mm) heated to 30 °C, and a Waters 2996 photodiode array detector. Retinal carotenoids were separated using a flow rate of 1.2 ml/min and a mobile phase of 44:44:12 (v:v:v) methanol:acetonitrile:dichloromethane run isocratically for the first 11 min, then a linear gradient up to 42:23:35 (v:v:v) through 21 min, holding at this condition until 25 min, followed by a return to the initial isocratic conditions from 25 to 30 min. HPLC conditions for plasma, liver, and diet carotenoid analyses were similar to those for the retina, but the initial mobile phase was 42:42:16 (v:v:v). External standards of lutein, zeaxanthin, astaxanthin, and β-carotene were also run on the HPLC for comparison to our samples; for other carotenoids where standards were not available, we used published absorbance and relative retention time values for identification (Table 1).

Statistical analyses

Reported values are mean ± standard error (SE), and statistical analyses were carried out in SPSS13 (Chicago, IL). We compared concentrations of different retinal carotenoids between the sexes and among treatments or times with separate multiple analyses of variance (MANOVA) for each experiment and Tukey post hoc tests for between-treatment comparisons. Non-significant interaction

Table 1
Putative identity, retention times (R_t), absorbance maxima (λ_{max}), and percentage of total carotenoids in house finch retina, plasma, and liver measured in week zero of Experiment 1 ($n = 10$). The λ_{max} values in parentheses denote the location of shoulders in the absorbance spectra. Retention times differ between tissue types because of differences in chromatographic conditions (see Methods).

Tissue	Carotenoid	R_t (min)	λ_{max} (nm)	% of total
Retina	Galloxanthin ^{ab}	3.55	381.3	41.9 ± 0.3
	Astaxanthin ^c	7.94	478.2	35.3 ± 0.3
	Lutein ^c	8.87 (421.3)	446.7	10.0 ± 0.4
	Zeaxanthin ^c	11.05	453.9	7.8 ± 0.4
	Unknown ^b	14.40 (422.5)	447.9	3.4 ± 0.3
	ε-Carotene ^{d,e}	18.21 (420.0)	443.0	1.6 ± 0.1
Plasma	Lutein ^c	6.61 (421.3)	446.7	82.0 ± 0.5
	Zeaxanthin ^c	7.94	453.9	12.4 ± 0.2
	β-Cryptoxanthin ^b	14.05 (424.0)	453.9	2.7 ± 0.3
	β-Carotene ^c	21.04 (426.5)	456.4	2.8 ± 0.2
Liver	Lutein ^c	6.61 (421.3)	446.7	55.8 ± 3.5
	Zeaxanthin ^c	7.94	453.9	9.6 ± 0.5
	β-Cryptoxanthin ^b	14.05 (424.0)	453.9	4.6 ± 0.6
	β-Carotene ^c	21.04 (426.5)	456.4	23.6 ± 0.7
	α-Carotene ^d	21.80 (427.8)	450.9	4.5 ± 0.3

^a Identified based on the descriptions of Goldsmith et al. [11] and Toyoda et al. [32].

^b Quantified based on the calibration curve for lutein.

^c Identified and quantified by comparison to purified standards.

^d Identified based on the descriptions of Goldsmith et al. [11] and Goldsmith and Butler [39].

^e Quantified based on calibration curve for β-carotene.

terms were removed from the models. Concentrations of many of the carotenoids in plasma and liver of house finches are significantly intercorrelated [19]; therefore we analyzed total concentrations in Experiments 1 and 2. Because we had multiple plasma samples over time from each individual, we used repeated-measures analyses of covariance (rmANOVA) to examine the effects of sex, treatment, and time on the plasma concentrations of specific carotenoids. Two-way interactions were included in all of the models but were removed when non-significant. Significance level for all tests was set at $\alpha = 0.05$.

Results

Retina and plasma carotenoid types

Consistent with our previous studies [18,22], we observed six major carotenoid types in house finch retinas from all three experiments (Table 1). The retinal carotenoid profile was dominated by astaxanthin and galloxanthin, together making up on average >75% of total retinal carotenoids. The plasma carotenoid profile of wild house finches was dominated by lutein and zeaxanthin, with small amounts of β -cryptoxanthin and β -carotene making up <5% of total at the time of capture (Table 1). On the captive diets, the birds circulated only lutein and zeaxanthin; however, when we supplemented the birds with astaxanthin in Experiment 3, we found significant amounts of astaxanthin in circulation (see Experiment 2 – lutein and zeaxanthin supplementation). The liver predominantly contained lutein, β -carotene, zeaxanthin and small amounts of β -cryptoxanthin and α -carotene (Table 1).

Experiment 1 – Depletion

Retinal carotenoid levels declined significantly over the course of the 8 weeks that birds were fed a very low-carotenoid diet (MANOVA week: Wilks' $\lambda = 0.188$, $F_{24,133.8} = 3.43$, $p < 0.0001$, Fig. 1A). Retinal astaxanthin concentrations were significantly lower in birds sampled at weeks 4 and 8 compared to those at week 0 (Tukey's post hoc, $p \leq 0.025$). Retinal galloxanthin and ϵ -carotene levels were significantly lower in birds collected at week 8 than birds collected at week 0 (Tukey's post hoc, $p \leq 0.012$). However, there was not a consistent temporal decline in retinal carotenoid levels, and birds collected on week 6 did not differ significantly from those in week 0 (Tukey's post hoc, $p \geq 0.574$, Fig. 1A). There was a significant difference in overall retinal carotenoid accumulation among juvenile, adult male, and adult female house finches (Wilks' $\lambda = 0.217$, $F_{12,76} = 7.27$, $p < 0.0001$). Regardless of time-point of sampled, juvenile finches had significantly higher levels of galloxanthin and ϵ -carotene than adult males (Juvenile: Gal – $0.393 \pm 0.031 \mu\text{g}/\text{retina}$, ϵ -car – $0.0154 \pm 0.0001 \mu\text{g}/\text{retina}$, Adult Male: Gal – $0.190 \pm 0.024 \mu\text{g}/\text{retina}$, ϵ -car – $0.0147 \pm 0.0002 \mu\text{g}/\text{retina}$; Tukey's post hoc, $p \leq 0.032$). Retinal carotenoid levels, of any type, did not differ significantly between juveniles and adult females or between adult females and adult males (Tukey's post hoc, $p \geq 0.140$).

Plasma carotenoid levels declined rapidly and significantly in captive birds fed a very low-carotenoid diet (ANOVA week: $F_{4,43} = 34.04$, $p < 0.0001$, Fig. 1B). Birds collected in weeks 2–8 had significantly lower total plasma carotenoid levels than birds collected at week 0 (Tukey's post hoc, $p < 0.0001$). There were no significant differences in total plasma carotenoid concentrations among birds collected in weeks 2–8 (Tukey's post hoc, $p > 0.812$). Liver carotenoid concentrations followed a pattern similar to plasma and declined significantly in response to dietary carotenoid depletion (ANOVA week: $F_{4,43} = 13.00$, $p < 0.0001$, Fig. 1C). Finches collected in weeks 2–8 had significantly lower total liver carotenoid concentrations than birds collected at week 0 (Tukey's post hoc, $p < 0.0001$). There were no significant differences in total liver carotenoid concentrations among birds collected in weeks 2–8 (Tukey's post hoc, $p > 0.99$).

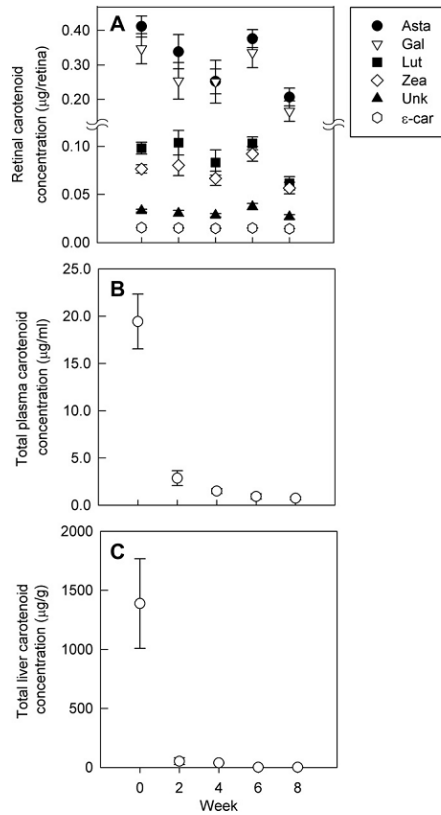


Fig. 1. Mean \pm S.E. (A) retinal, (B) total plasma, and (C) total liver carotenoid concentrations of house finches maintained in captivity on a very low-carotenoid sunflower seed diet for 0, 2, 4, 6, or 8 weeks. At each time-point, 10 different individuals were collected and measured.

oid concentrations than birds collected at week 0 (Tukey's post hoc, $p < 0.0001$). There were no significant differences in total liver carotenoid concentrations among birds collected in weeks 2–8 (Tukey's post hoc, $p > 0.99$).

Experiment 2 – Lutein and zeaxanthin supplementation

Dietary supplementation with lutein + zeaxanthin for eight weeks significantly affected retinal carotenoid levels of captive house finches (MANOVA treatment: Wilks' $\lambda = 0.290$, $F_{12,90} = 6.44$, $p < 0.0001$, Fig. 2A). Birds receiving the highest dietary carotenoid level had significantly higher retinal galloxanthin concentrations than birds on the medium diet, and both high- and medium-diet birds had significantly higher levels than birds receiving the low diet (Tukey's post hoc, $p < 0.001$, Fig. 2A). Birds on the high-carotenoid diet had significantly higher levels of ϵ -carotene than

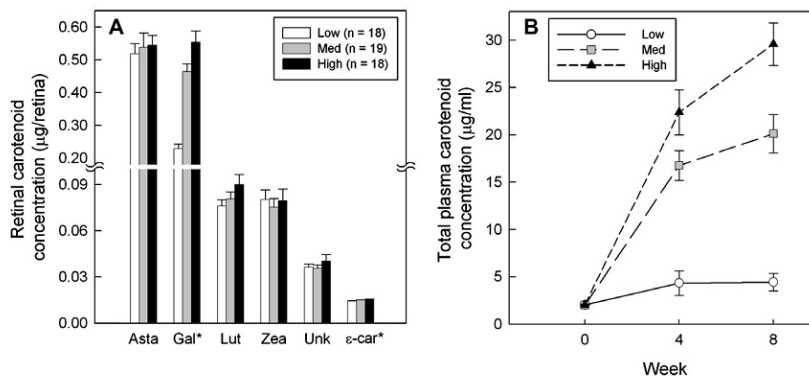


Fig. 2. (A) Mean \pm S.E. retinal carotenoid concentrations of adult house finches supplemented with low (0.31 μ g/g), medium (10 μ g/g), or high (30 μ g/g) levels of lutein:zeaxanthin for eight weeks. Asterisks indicate significant pairwise differences between the low-carotenoid diet and other diet treatments for that particular retinal carotenoid type (see text). (B) Total plasma carotenoid concentrations of house finches across the 8 weeks of supplementation.

birds on the low-carotenoid diet (Tukey's post hoc, $p < 0.001$, Fig. 2A). There were no significant differences in retinal carotenoid accumulation between male and female finches (Wilks' $\lambda = 0.933$, $F_{6,45} = 0.537$, $p = 0.777$).

Plasma carotenoid levels changed over the course of the experiment and as a function of dietary carotenoid treatment (rmANOVA time \times treatment: $F_{2,56,76.86} = 37.75$, $p < 0.0001$, Fig. 2B). Treatment groups did not differ significantly at the beginning of the experiment (week 0 – prior to diet manipulation; Tukey's post hoc, $p \leq 0.782$, Fig. 2A). In week 4, high- and medium-diet birds had significantly higher plasma carotenoid levels than low-diet birds (Tukey's post hoc, $p < 0.001$, Fig. 2B). In week 8, plasma carotenoid levels of high-, medium-, and low-diet groups all differed significantly, with the highest levels in the high-diet group (Tukey's post hoc, $p \leq 0.004$, Fig. 2B).

Experiment 3 – Zeaxanthin or astaxanthin supplementation

Dietary supplementation with zeaxanthin significantly affected retinal carotenoid levels of captive house finches (MANOVA treatment: Wilks' $\lambda = 0.290$, $F_{12,90} = 6.44$, $p < 0.0001$, Fig. 3). Zeaxanthin-

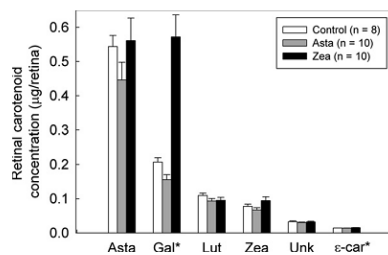


Fig. 3. Mean \pm S.E. retinal carotenoid concentrations of adult house finches supplemented with 35 μ g/ml of zeaxanthin (Zea) or astaxanthin (Asta) for eight weeks and an unsupplemented (Control) group. Asterisks indicate significant pairwise differences between the control and other treatment groups for that particular retinal carotenoid type (see text).

supplemented birds had significantly higher levels of retinal galloxanthin and ϵ -carotene than unsupplemented birds (Tukey's post hoc, $p \leq 0.028$, Fig. 3). Supplementation with dietary astaxanthin had no significant effect on the accumulation of any retinal carotenoid types, and retinal levels of astaxanthin-supplemented birds did not differ significantly from those of the low-carotenoid (control) group (Tukey's post hoc, $p > 0.332$, Fig. 3). There were no significant differences in retinal carotenoid accumulation between male and female finches (Wilks' $\lambda = 0.795$, $F_{6,19} = 0.815$, $p = 0.572$).

Because we administered specific purified carotenoid supplements in Experiment 3, we used separate statistical tests to analyze the three plasma carotenoids (lutein, zeaxanthin, and astaxanthin) and determine whether and how these purified supplements were taken up into circulation. Plasma levels of all three carotenoids were significantly affected by carotenoid supplementation (rmANOVA time \times treatment: $F_{3,23,37.18} \geq 17.69$, $p < 0.0001$, Fig. 4). In week 0, prior to supplementation, plasma lutein levels were low (mean 0.26 ± 0.044 μ g/ml) for all treatment groups, but levels increased significantly in zeaxanthin-supplemented birds compared to controls in weeks 4 and 8 (Tukey's post hoc, $p < 0.0001$, Fig. 4A). Plasma lutein levels of astaxanthin-supplemented birds did not differ from controls (Tukey's post hoc, $p > 0.9$). Prior to supplementation, all birds had low levels of zeaxanthin in circulation (mean 0.29 ± 0.056 μ g/ml). As expected, supplementation with zeaxanthin significantly increased circulating zeaxanthin levels in weeks 4 and 8 of the study (Tukey's post hoc, $p < 0.0001$, Fig. 4B). Plasma zeaxanthin levels of astaxanthin-supplemented birds did not differ from those in controls (Tukey's post hoc, $p > 0.9$). Consistent with observations from wild house finches [18,19], none of the birds were circulating astaxanthin in their plasma at the beginning of the study. However, birds supplemented with astaxanthin did circulate significant astaxanthin levels through plasma in weeks 4 and 8 of the study (Tukey's post hoc, $p < 0.0001$, Fig. 4C).

Discussion

Carotenoid types and concentration

The carotenoid profile of the wild house finch retina was dominated by astaxanthin and galloxanthin, making up 42% and 35% of

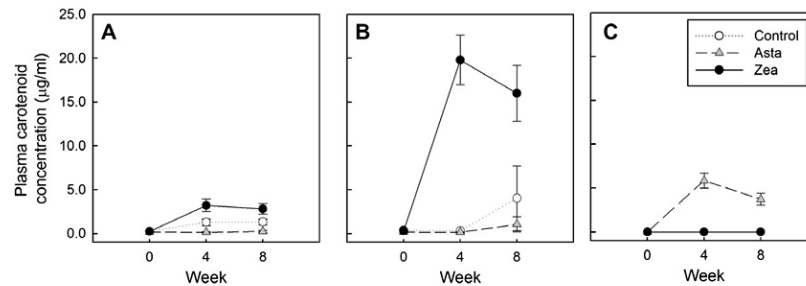


Fig. 4. Mean \pm S.E. plasma concentrations of (A) lutein, (B) zeaxanthin, and (C) astaxanthin in adult house finches supplemented with 35 μ g/ml of zeaxanthin (Zea) or astaxanthin (Asta) for eight weeks and an unsupplemented (Control) group.

total, respectively. This is roughly similar to the turkey (*Meleagris gallopavo*), consisting of 23% astaxanthin and 28% galloxanthin [14], but contrasts with results from domesticated chickens (*Gallus domesticus*) with 71% and 7%, respectively and quail with 29% and 13%, respectively. These species-specific differences could stem from: (1) variation in the frequency of retinal cone types, (2) differences in rearing and dietary conditions among studies, and/or (3) species-specific patterns of retinal carotenoid accumulation within cone types. The frequency of astaxanthin pigmented R-type and galloxanthin pigmented P-type oil droplets are unlikely to explain differences among these species, as they are relatively similar: chickens have an R to P-type ratio of 1:2.5 [23], quail 1:3.1 [24], and house finches 1:3.0 [unpublished data]. Recent studies of chickens, zebra finches (*Taeniopygia guttata*), and crimson rosellas (*Platycercus elegans*) suggest that galloxanthin pigmentation of the P-type oil droplet is particularly sensitive to environmental light conditions and dietary carotenoid access [25,26]. The results of our three experiments and another recent study on house finches confirm that galloxanthin is particularly sensitive to dietary carotenoid input and influenced by immune system activity [22]. Therefore, the differences among studies and species in the relative accumulation of astaxanthin and galloxanthin may be influenced by lighting, diet, and the general health of the experimental animals. However, we cannot rule out genetically based species differences in the uptake, metabolism, and deposition of carotenoids into the retina. Qualitative comparisons indicate that interspecific variation in oil droplet pigmentation variation may be functionally relate to differences in spectral environment and foraging ecology [16]. For example, nocturnal species, like owls, tend to have relatively pale oil droplets, with presumably lower carotenoid concentrations, which should improve sensitivity in low light conditions [16].

Carotenoid depletion

Maintaining house finches on a very low-carotenoid diet depleted retinal levels of astaxanthin, galloxanthin, and ϵ -carotene. This contrasts with the reported stability of retinal carotenoids to dietary depletion, ranging from 4 weeks in young chickens to several months in adult quail [27,28]. However, Wang et al. [28] only measured retinal lutein and zeaxanthin in chickens, which we also found to be unaffected by diet depletion, and Meyer et al. [27] only qualitatively examined the coloration of cone oil droplets of quail and were unlikely to detect subtle variation in concentration. Despite depletion, retinal carotenoid levels in house finches were much more stable than plasma and liver carotenoid levels. Significant depletion of retinal carotenoids was detected only after 4 weeks for astaxanthin and 8 weeks for galloxanthin and ϵ -caro-

tene, while total plasma carotenoid levels dropped by >85% and liver carotenoid levels declined >95% in the first two weeks on the very low-carotenoid diet. Moreover, the final magnitude of the decline in retinal carotenoid concentration was much less than that of plasma and liver. By week 8, retinal levels had declined to ca. 50% of initial levels, while circulating and liver carotenoids had dropped to 4% and 2% of initial values, respectively. Although we observed a decline in retinal carotenoid levels over the course of the depletion study, there was considerable variation among weeks that was not attributable to dietary depletion. For example, the retinal carotenoid concentrations of birds collected in week six did not differ significantly from birds collected in week zero and deviates from the trend of decline seen in other weeks. This deviation is unlikely to have resulted from systematic error, because when we repeated our analyses on extracts from the other whole eye of each individual (data not reported) we found the same patterns as we present from the retina. Because the measurement of retinal carotenoids required destructive sampling of retinal tissue, we were limited to a cross-sectional analysis of different birds over time. We expect that this unexplained variation would diminish if we were able to measure retinal carotenoid accumulation longitudinal in the same individuals.

The relatively limited effects of dietary depletion suggest that inter-individual differences other than diet can influence carotenoid accumulation and retention in the avian retina. Retinal carotenoid accumulation may be influenced by an individual's health and condition. In wild house finches, we found retinal carotenoid accumulation to be positively correlated with body condition [18], and recently we showed that long-term immune system challenges deplete retinal carotenoids in finches [22]. Retinal carotenoid accumulation may also reflect an individual's genetic predisposition to accumulate retinal carotenoids. Among humans there is up to an 85% heritability of retinal carotenoid accumulation (macular pigment density) [29]. In birds, the heritability of retinal carotenoid accumulation has not been examined; however, the carotenoid-based plumage coloration of male house finches is positively correlated among fathers and sons [30], and the carotenoid-based bill color of male zebra finches is heritable ($h^2 = 0.33$) [31].

Effects of dietary carotenoid supplementation

Adult house finches supplemented with lutein + zeaxanthin in Experiment 2 or with zeaxanthin alone in Experiment 3 had significantly higher levels of retinal galloxanthin and ϵ -carotene than birds maintained on the low-carotenoid diet. The strong response of galloxanthin to supplementation is consistent with the results

of Knott et al. [26], who found that dietary supplementation of crimson rosella and zebra finches with lutein and zeaxanthin caused shifts in the absorbance properties of cone oil droplets, with the largest effects occurring in the galloxanthin-pigmented, P-type oil droplets. We found no significant effect of carotenoid supplementation on the accumulation of other retinal carotenoids, including lutein and zeaxanthin, which have been shown to respond positively to supplementation in quail and chickens [8,9,11,32]. However, these studies involved dietary manipulation of young growing birds, which, in some cases [8,9,32], began life entirely devoid of carotenoids through multi-generational carotenoid depletion. The maturing retina may be particularly sensitive to dietary manipulations, as the development and pigmentation of cone oil droplets, initiated *in ovo*, is completed [33]. The adult retinas we examined may be less sensitive to diet manipulation because they are fully developed and only requiring dietary inputs to replace degraded carotenoids.

Changes in retinal carotenoid accumulation were seen only in house finches supplemented with zeaxanthin, indicating that there is selective uptake into the retina and specific precursor-product relationships. Astaxanthin is the dominant carotenoid in the house finch retina; however, direct supplementation with dietary astaxanthin had no effect on retinal carotenoid accumulation in adult house finches, despite the fact that they circulated this pigment in plasma and accumulated it in liver and feathers [unpublished data]. Zeaxanthin has been suggested as the primary dietary precursor for many of the metabolically derived retinal carotenoids in birds, like astaxanthin and galloxanthin [14,15]. This product-precursor relationship was recently confirmed in a study of retinal carotenoid accumulation in quail utilizing labeled (deuterated) dietary carotenoids. Bhosale et al. [11] found that feeding young quail with labeled zeaxanthin for 16 weeks led to significant labeling of zeaxanthin, astaxanthin, galloxanthin, and ϵ -carotene in the retina.

Although zeaxanthin is the likely precursor for many of the carotenoids found in the retina, our supplementation experiments only influenced the accumulation of two retinal carotenoids – galloxanthin and ϵ -carotene. There are several possible explanations for this specificity: (1) Galloxanthin and ϵ -carotene may turn over more rapidly than other retinal carotenoid types. This could occur if these carotenoids were more prone to photodegradation than the other retinal types, but the relative stability of these carotenoids *in vivo* is not known. (2) The potential for increased accumulation may be limited if cone oil droplets are already saturated with carotenoids. Across a number of bird species, R-type oil droplets contain very high concentrations of astaxanthin and have absorbance values >20. Y-type oil droplets contain lutein and zeaxanthin and range up to 10 absorbance units, while P-type oil droplets containing galloxanthin and ϵ -carotene tend to have absorbance values less than two [12]. Therefore, P-type oil droplets, with their presumably lower concentration of carotenoids, have the greatest potential to accumulate carotenoids, while the R-type oil droplets may be saturated with astaxanthin, limiting the potential for further increase.

On a functional level, the carotenoid-specific responses to dietary change may be related to their role in color vision. Knott et al. [26] suggested that birds shunt excess carotenoids into P-type oil droplets to preserve spectral tuning and sensitivity of the single cone photoreceptors. The P-type oil droplet is part of the double-cone photoreceptor that affects motion and luminance detection, but does not play a role in color vision [34]. In the P-type oil droplet, there is relatively little overlap between the carotenoid absorbance and the visual pigment, whereas the absorbance spectra of the single cone visual pigments overlap considerably with the carotenoid absorbance of their respective oil droplets. Thus, increased carotenoid accumulation in the single cones would result

in reduced sensitivity, while the double-cone sensitivity may be less affected by the increased carotenoid accumulation.

Conclusion

Carotenoid accumulation in the retina varies significantly among adult wild house finches and correlates with condition and male carotenoid-based plumage coloration [18]. The studies presented here suggest that, although dietary carotenoid access does affect retinal accumulation, diet is not the primary source of variation observed among wild birds. Compared to other body tissues, retinal accumulation in adult house finches is relatively stable to dietary changes over time, and carotenoid supplementation only affects the accumulation of a small subset of retinal carotenoid types. In some sense, this stability is not surprising, because retinal carotenoids play an essential role in color vision [13] and photoprotection [8,9]. Rapid changes in retinal carotenoids could compromise visual health and function and thus drive selection for greater stability and a decoupling of retinal accumulation away from current dietary intake. However, we are still left to account for the variation we see amongst wild house finches. In contrast to our results, studies of young growing domesticated chickens and quail indicate that dietary access can have profound effects on retinal accumulation [8,9,11,32]. In zebra finches, neonatal diet quality has been shown to affect the ability of adult birds to assimilate carotenoids into circulation [35]. Therefore, adult retinal carotenoid accumulation could be shaped by conditions during early life and reflect carotenoid access during key developmental periods. The maternal transfer of carotenoids to eggs may be particularly important because oil-droplet development and pigmentation occurs *in ovo*. Similarly, in humans, early-life carotenoid access may also be important for visual health and function. Among human infants, retinal carotenoid levels are highly variable [36] and recently it has been suggested that dietary carotenoid intake during development may be important for retinal development and life-long visual health [37,38]. Although there are limitations of birds as model organisms [15,21], because of their high levels of retinal carotenoid accumulation and relatively short life-spans, birds still provide an excellent opportunity to examine the life-long effects of carotenoid availability during the developmental period. Dietary carotenoid manipulations at various stages of development in a rapidly maturing species, like quail, could provide clues to the organizational effects of dietary carotenoid intake on adult retinal accumulation and visual health.

Acknowledgments

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APPENDIX C
IMMUNE-SYSTEM ACTIVATION DEPLETES RETINAL CAROTENOIDS
IN HOUSE FINCHES (*CARPODACUS MEXICANUS*)

Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*)

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SUMMARY

The costs of developing, maintaining, and activating the immune system have been cited as an important force shaping life-history evolution in animals. Immunological defenses require energy, nutrients and time that might otherwise be devoted to other life-history traits like sexual displays or reproduction. Carotenoid pigments in animals provide a unique opportunity to track the costs of immune activation, because they are diet-derived, modulate the immune system, and are used to develop colorful signals of quality. Carotenoids also accumulate in the retinas of birds, where they tune spectral sensitivity and provide photoprotection. If carotenoid accumulation in the retina follows the patterns of other tissues, then immune activation may deplete retinal carotenoid levels and impact visual health and function. To test this hypothesis, we challenged molting wild-caught captive house finches (*Carpodacus mexicanus*) with weekly injections of lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) over the course of 8 weeks. Immunostimulated adult males and females produced significant antibody responses and molted more slowly than uninjected control birds. After 8 weeks, immune-challenged birds had significantly lower levels of specific retinal carotenoid types (galloxanthin and zeaxanthin), but there were no significant differences in the plasma, liver or feather carotenoid levels between the treatment groups. These results indicate that immune-system activation can specifically deplete retinal carotenoids, which may compromise visual health and performance and represent an additional somatic and behavioral cost of immunity.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/213/10/1709/DC1>

Key words: lipopolysaccharide, phytohaemagglutinin, trade-off, vision.

INTRODUCTION

The ability of an individual to resist parasites and disease is essential for survival, but this resistance comes at a cost. Fever, phagocytosis, antibody production, and the entire spectrum of immune-system responses requires energy, nutrients, and time that could otherwise be devoted to other traits such as growth or reproduction (Klasing, 2004; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). Because these resource allocation patterns can have profound effects on individual fitness, the physiological costs of immune system function have come to be viewed as a key force shaping the evolution of life histories (Lochmiller and Deerenberg, 2000; Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). Consistent with this hypothesis, recent empirical studies have demonstrated the predicted costs of immune activation on self-maintenance, sexual attractiveness, reproductive output, and parental care (e.g. Bonneaud et al., 2003; Martin, 2005; Peters et al., 2004; Sanz et al., 2004). However, identifying specific mechanisms and resource currencies that link the immune system to life history traits has remained a challenge.

Carotenoid pigments are nutrient resources that have diverse biological functions and may provide a direct window into life-history trade-offs (Blount, 2004) as well as the costs of immune activity (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Animals cannot synthesize carotenoids *de novo*, but must acquire them from their diet through the direct or indirect consumption of plant material (Goodwin, 1984). Therefore, the supply of carotenoids to an animal may be limited by the environment and/or physiological uptake (Olson and Owens, 1998). Carotenoids can modulate immune

function by stimulating T-cell and antibody production, facilitating gap-junction communication, influencing gene expression, and may also act as antioxidants, protecting vulnerable cells and tissues from reactive oxygen species generated during immune response (reviewed in Chew and Park, 2004). Carotenoids also generate sexually selected colors in many animals that can serve as reliable signals of individual health if carotenoids are traded off between coloration and immune function (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Consistent with this hypothesis, experimentally induced immune challenges have been shown to fade the carotenoid-based bare-part ornaments in a number of bird species [blackbirds, *Turdus merula* (Favre et al., 2003); zebra finches, *Taeniopygia guttata* (McGraw and Ardia, 2003); mallard ducks, *Anas platyrhynchos* (Peters et al., 2004); and blue-footed boobies, *Sula nebouxi* (Torres and Velando, 2007)]. In the realm of behavioral ecology and ecophysiology, the immune, antioxidant and color signaling functions of carotenoids have received almost exclusive attention, but carotenoids also accumulate in the eyes of many animals where they provide photoprotection and facilitate visual function (Douglas and Marshall, 1999).

Carotenoids are found in the retinas of a diversity of vertebrates, including turtles, birds and primates (Douglas and Marshall, 1999) and have been extensively studied in humans, where accumulation is dependent upon dietary carotenoid intake, and linked to reduced risk of age-related macular degeneration (Seddon et al., 1994; Whitehead et al., 2006). In birds, retinal carotenoids are thought to have similar protective properties. For example, Thomson et al. (Thomson et al., 2002a; Thomson et al., 2002b) found that dietary supplementation increased retinal carotenoid levels and protected

Japanese quail (*Coturnix japonica*) against light-induced photoreceptor death. Retinal carotenoids also influence avian color vision by selectively filtering light reaching the photoreceptors to reduce overlap in the absorbance spectra of spectrally adjacent types, resulting in enhanced color discrimination and color constancy in variable lighting environments (Goldsmith and Butler, 2003; Vorobyev et al., 1998; Vorobyev, 2003). If immune system activity affects retinal carotenoid accumulation, it could impact visual health and performance.

The eye is extremely sensitive to immune activity because inflammation of ocular tissues can alter the precise spatial arrangement of elements within the eye [e.g. lens and photoreceptor mosaics (Streilein, 1999)] and thus compromise visual acuity. To counter these detrimental effects, the eye employs a complex of protective immunosuppressive mechanisms and is considered an 'immune privileged' tissue (Streilein, 1999). Despite these mechanisms, systemic immune challenges can still induce ocular inflammation (Bhattacharjee et al., 1983; Rosenbaum et al., 1980) and recently carotenoids have been identified as significant anti-inflammatory agents in the eye (Jin et al., 2006; Ohgami et al., 2003; Shiratori et al., 2005). Therefore immune activation may directly influence the accumulation of carotenoids in the eye.

The goal of our study was to examine if immune-system activation affects retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus* Muller 1776), a passerine species whose carotenoid physiology and carotenoid-based color signaling have been investigated extensively (reviewed by Hill, 2002). Male house finches display carotenoid-based plumage coloration that varies from drab yellow to chromatic red, with females generally preferring the reddest males (Hill, 2002). Male coloration is dependent upon the metabolism and deposition of carotenoids into the feathers, and both dietary carotenoid intake and individual health influence coloration. Plumage redness is negatively correlated with the intensity of avian pox infection and feather mite parasitism (Thompson et al., 1997), and experimentally infecting molting birds with *Isospora* coccidians and *Mycoplasma gallisepticum* both result in reduced coloration of male plumage (Brawner et al., 2000; Hill et al., 2004). From these studies, we cannot separate the effect of immune-system activation from disease pathology, but they do suggest that immune challenges influence carotenoid allocation, metabolism, and/or deposition in feathers. It is not known in this or any other species how immune activity affects retinal carotenoid accumulation. In wild house finches, retinal carotenoid levels vary seasonally and are positively correlated with plumage coloration and body condition (Toomey and McGraw, 2009). Therefore, individual variation in immune activity and seasonal variation in disease prevalence (e.g. McClure, 1989) could be contributing to the observed variation in retinal carotenoid levels.

To examine the effects of immune-system activation on retinal carotenoid accumulation, we brought wild adult male and female house finches into captivity during the molt period (June–Sept), maintained them on a standardized diet, challenged a subset of these birds for 8 weeks with weekly injections of lipopolysaccharide and phytohaemagglutinin, then measured carotenoid levels in the plasma, liver, feathers and retina. We chose to use these two antigens over a long time course because the retina appears to be relatively stable to short-term perturbations. For example, we found that 8 weeks of dietary manipulation were required to cause a significant decline in retinal carotenoid levels in house finches, compared with the rapid depletion (within 2 weeks) from the plasma and liver (M.B.T. and K.J.McG., unpublished). The time course of the study is justifiable because long-term infections are common among wild house

finches. The symptoms of *Mycoplasma gallisepticum* (MG) infection, which include extreme inflammation of the conjunctiva of the eye, last on average 10 weeks and up to 24 weeks in captive experimentally infected house finches (Kollias et al., 2004; Sydenstricker et al., 2006). Active infections of avian pox can last as long as 3 months (McClure, 1989).

MATERIALS AND METHODS

Bird capture and captive housing conditions

In May 2008, we captured 17 male and 19 female adult house finches in baited basket traps on the Arizona State University (ASU) campus in Tempe, Arizona (*sensu* McGraw et al., 2006). Birds were housed individually in wire cages (0.6 m × 0.4 m × 0.3 m) in a greenhouse room on the ASU campus that allowed for natural light levels and day length. We fed finches a standard maintenance diet (ZuPreem small bird maintenance diet, Premium Nutritional Products Inc. Mission, KS, USA) and tap water *ad libitum* for the duration of the study. The maintenance diet contained two carotenoids: lutein (1.15 ± 0.12 μg g⁻¹) and zeaxanthin (0.52 ± 0.06 μg g⁻¹). These levels are relatively low compared with the gut contents of wild birds [mean ≈ 8 μg g⁻¹ total carotenoids (Hill et al., 2002)]; however, the circulating plasma carotenoid levels of our captive birds were equal to, or greater than, wild birds during the same period [mean ≈ 12 μg ml⁻¹ total carotenoids (McGraw et al., 2006)] indicating that our captive birds were not dietarily limited.

Antigens

To stimulate the immune system we used two antigens commonly used in eco-immunological studies with a broad range of effects on carotenoid physiology: lipopolysaccharide (LPS; from *S. typhimurium*, Sigma #L7261, St Louis, MO, USA) and phytohaemagglutinin (PHA; from *P. vulgaris*, Sigma #L8754). LPS is isolated from the cell wall of bacteria and inoculation simulates bacterial infection, stimulating both the innate and adaptive arms of the immune system (Klasing, 2004). Inoculation with LPS depletes circulating plasma carotenoid levels in chickens (*Gallus gallus*) (Koutsos et al., 2003), jungle fowl (McGraw and Klasing, 2006), and zebra finches (Alonso-Alvarez et al., 2004) and fades the carotenoid-based coloration of bare part ornaments in the blue-footed boobies (Torres and Velando, 2007) and zebra finches (Alonso-Alvarez et al., 2004). Systemic inoculation with LPS has also caused significant ocular inflammation in rats (Bhattacharjee et al., 1983; Rosenbaum et al., 1980). PHA is a mitogen that causes T cells to proliferate *in vitro* and results in the recruitment and activation of other leukocytes, resulting in edema (Martin et al., 2006). PHA-induced immune activity has been shown to reduce circulating carotenoid levels in blackbirds (*Turdus merula*) (Baird et al., 2009) and red-legged partridges (*Alectoris rufa*) (Perez-Rodriguez et al., 2008), increase circulating levels in Eurasian kestrels (*Falco tinnunculus*) (Costantini and Dell'omo, 2006), but to have no effect on plasma carotenoids in the greenfinches (*Carduelis chloris*) (Hörak et al., 2007).

Experimental treatment

Beginning on 7 July 2008, we injected 10 randomly selected male and 10 randomly selected female birds intramuscularly (pectoralis major) with 0.1 mg of LPS and 0.1 mg of PHA dissolved in 0.05 ml of phosphate-buffered saline (PBS). These injections were repeated each week for 8 weeks. Seven male and nine female control birds did not receive injections of any kind. As in other studies (Adler et al., 2001), we omitted a sham injection treatment because sham injections can elicit an immune response, confounding our ability

to detect the effects of immune activation. To minimize differences in the levels of stress between the treatment and controls, all birds were housed, handled, and sampled such that the only difference between groups was the presence or absence of the injection. To track body condition, we weighed the birds (to the nearest 0.001 g) each week and recorded which primary feather was actively growing (1–9) on weeks 7 and 8. To track antibody titer and circulating carotenoid levels throughout the study, we collected a blood sample (~100 µl) from the ulnar vein on weeks 0, 2, 4 and 8 prior to administering the weekly antigen injection. These samples were immediately centrifuged (3 min at 7550 g), and plasma was collected and stored at –80°C for up to 14 months prior to analysis. Unlike many previous studies utilizing PHA (e.g. Smits et al., 1999), we did not measure a swelling response because our injection site was not amenable to this assessment. One week after the final injection, all birds were killed and the retinas and liver tissue collected, using the method of Toomey and McGraw (Toomey and McGraw, 2009) and McGraw et al. (McGraw et al., 2006), respectively. By this time, all of the male birds had begun to molt into their ornamental plumage, but the rate and location of this molt varied among individuals; therefore we were not able to collect standardized reflectance measures for color analysis. We did, however, pluck 9–25 recently grown breast feathers from each male at the conclusion of the study to analyze feather carotenoid concentration (see below).

Carotenoid analyses

Carotenoid types and concentrations were determined using high-performance liquid chromatography (HPLC). Our HPLC system consists of a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) with a Waters YMC carotenoid 5 µm column (4.6 mm × 250 mm) heated to 30°C, and a Waters 2996 photodiode array detector. We used a constant solvent flow rate of 1.2 ml min⁻¹, but different three-step solvent gradients, to elute carotenoids particular to each tissue type (see below). We identified and quantified carotenoids by comparing their respective retention times and absorbance maxima (λ_{max}) to those of external standards [as in Toomey and McGraw (Toomey and McGraw, 2007; Toomey and McGraw, 2009)].

Plasma and liver carotenoids were extracted following McGraw et al. (McGraw et al., 2006) and eluted with a mobile phase solvent gradient of 42:42:16 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, then a linear gradient up to 42:23:35 (v/v/v) for 21 min, held isocratically at this condition until 25 min, and then returned to the initial condition from 25 to 30 min. Feather carotenoids were extracted by repeated (3 ×) grinding with a ball mill (MM200, Retsch GmbH and Co. KG, Haan, Germany) in 1 ml of methanol. These recently deposited feather carotenoids were highly esterified and required saponification for analysis in our HPLC system. To

do this, we dried the feather extract under nitrogen, resuspended the pigment in 1 ml of 0.2 mol l⁻¹ methanolic KOH, capped the solution under nitrogen, and held the samples in the dark at room temperature for 6 h. We then added 1 ml of saturated NaCl and 2 ml of distilled H₂O, and vigorously shook the samples with each addition. To re-extract carotenoids from this solution, we then added 3 ml of hexane:tert-butyl methyl ether (1:1) to each tube, shook them for 1 min to mix the layers thoroughly, centrifuged the samples for 5 min at 1500 g, and collected the supernatant. These saponified feather extracts (containing xanthophylls, based on the xanthophyll diet they were fed) (Inouye et al., 2001) were then analyzed under the same HPLC conditions as were the plasma and liver samples.

We extracted carotenoids from whole retinas following the method of Toomey and McGraw (Toomey and McGraw, 2009) and used a two-step saponification procedure to ensure reliable quantification of the entire suite of retinal carotenoids (for details, see Toomey and McGraw, 2007). Retinal carotenoids were eluted with a mobile phase solvent gradient of 44:44:12 (v/v/v) methanol:acetonitrile:dichloromethane for 11 min, then a linear gradient up to 42:23:35 (v/v/v) by 21 min, held isocratically at this condition until 25 min, and then returned to the initial condition from 25 to 30 min. Retinal carotenoid concentrations are presented as micrograms of carotenoid per whole retina [as in Toomey and McGraw (Toomey and McGraw, 2007; Toomey and McGraw, 2009)].

Carotenoid types and distribution

Our captive birds contained carotenoid types and concentrations in plasma and liver that were consistent with observations from wild house finches. Within each tissue, the concentrations of different carotenoid types were significantly positively intercorrelated; therefore, we used total plasma carotenoid concentration for all subsequent analyses (supplementary material Table S1) (McGraw et al., 2006; Toomey and McGraw, 2009). The recently grown feathers of our captive male house finches contained canary xanthophylls A and B, lutein, zeaxanthin, and β-cryptoxanthin (supplementary material Table S2). Owing to the diet they received, and as commonly occurs with captive molting house finches (Hill, 2002), these feathers were yellow and lacked any traces of carotenoids responsible for the production of red color (e.g. astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, adonirubin, and 4-oxo-rubixanthin) (Inouye et al., 2001). Concentrations of all feather carotenoids were significantly positively intercorrelated (supplementary material Table S2), and we used total feather carotenoid concentration for all subsequent analyses. Consistent with wild birds, the retinas of our captive birds contained six major types of carotenoids (Table 1) (Toomey and

Table 1. Concentration of and Pearson's correlations between retinal carotenoid types for treatment and control groups combined

	Concentration (µg retina ⁻¹)	Pearson's correlation (r_p)				
		Astaxanthin	Galloxanthin	Lutein	Zeaxanthin	Unknown
Astaxanthin	0.47±0.022	–				
Galloxanthin	0.42±0.017	0.27	–			
Lutein	0.065±0.0031	-0.033	0.37	–		
Zeaxanthin	0.093±0.0036	0.26	0.65	0.16	–	
Unknown	0.037±0.0010	0.17	0.61	0.22	0.93	–
ε-Carotene	0.015±0.000082	-0.08	0.45	0.079	0.44	0.48

Bold type indicates significant correlations ($P \leq 0.007$). For detailed descriptions of the spectral properties and HPLC retention times of these carotenoids see Toomey and McGraw (Toomey and McGraw, 2009).

McGraw, 2009). The concentrations of some, but not all, retinal carotenoid types were significantly positively intercorrelated (Table 1). However, because the accumulation of the different retinal carotenoid types are photoreceptor specific (Goldsmith and Butler, 2003; Goldsmith et al., 1984) and differentially affected by dietary carotenoid manipulations (Bhosale et al., 2007; Scheidt et al., 1991), we statistically analyzed each of the retinal carotenoid types separately.

Antibody titer measurement

We measured antibody response to LPS and PHA to quantify the immunostimulatory effects of each antigen. This method differs from many previous eco-immunology studies (e.g. Smits et al., 1999) that combine multiple measures of immunocompetence into a single metric (e.g. adaptive and innate immunity within a PHA-induced swelling response) (Martin et al., 2006), and provides the advantage of directly measuring the immunogenicity for both antigens in a single type of assay. To measure antibody response we used an enzyme-linked immunosorbent assay (ELISA) (*sensu* Butler and Duffy, 2007). First, we incubated 96-well plates (Fisher 468667, Hampton, NH, USA) at 4°C overnight with 0.5 mg ml⁻¹ LPS or 0.5 mg ml⁻¹ PHA suspended in sodium carbonate (pH 9.6) buffer. We then washed the wells with PBS and Tween 20 (0.05% by volume) and blocked the wells with blocking buffer (PBS with 1% bovine serum albumin and 0.1% sodium azide) at room temperature for 2 h. We washed the plates three times then added 100 µl of plasma diluted 1:12,800 in blocking buffer, and incubated overnight at 4°C. Plasma dilutions for both antibodies (LPS and PHA) were previously determined in a separate assay to ensure that concentrations of antibody fell within the linear range of the assay. We then washed the plates, added 80 µl of HRP-labeled secondary anti-avian IgG antibody (A140-110P; Bethyl Laboratories, Montgomery, TX, USA) diluted 1:10,000 in blocking buffer, and incubated at room temperature for 2 h. After washing the plates, we added 100 µl of tetramethylbenzidine (TMB). We stored the plates at room temperature, protected from light, for 20 min and then added 50 µl of H₂SO₄. Within 10 min, we measured the absorbance of the contents of each well at a wavelength of 450 nm using SOFTmax PRO (Molecular Devices, CA, USA). Absorbance values were averaged across triplicate samples (intra-assay variation ranged from 3.07 to 4.41) and used in all subsequent analyses.

Statistical analyses

We analyzed temporal changes in antibody titer, body mass, molt progress and plasma carotenoid levels between treatment groups with repeated-measures analyses of variance (RMANOVA), with

sex and treatment (control *versus* challenged) as the between-subjects factors. There were no significant interactions between the between-subjects factors, so we omitted these from final models. We used univariate analysis of variance (ANOVA) to examine the effects of experimental treatment on feather, liver and retinal carotenoid levels measured at the conclusion of the study. Sex and treatment were the independent factors in analyses, and there were no significant interactions between these terms so we omitted these from our final ANOVA models. Because retinal carotenoid levels differed significantly between treatments (see below), we examined how the extent of immune response among challenged birds related to retinal accumulation with an additional analysis of covariance (ANCOVA) for the immune-challenged birds only, with sex as the independent factor and the maximum LPS and PHA titer as covariates and retinal carotenoid levels as dependent variables. We examined the residuals of the ANOVA models and found no major deviations from normality or equal variance.

RESULTS

Response to immune challenge

As expected, immunization with LPS and PHA caused a significant increase in circulating antibodies to each of these antigens (Fig. 1; LPS-week: $F_{3,54}=27.66$, $P<0.001$; PHA-week: $F_{3,54}=12.31$, $P<0.0001$). However, the temporal and sex-specific effects of the immune-system treatment differed for these two antigens. LPS antibody titer increased significantly between weeks 0, 2 and 4 (*post-hoc* Bonferroni, $P\leq 0.03$), but did not differ significantly between weeks 4 and 8. Male finches had a significantly stronger antibody response to LPS than did female finches (Fig. 1A; ANOVA, sex: $F_{1,18}=6.61$; $P=0.018$). PHA antibody levels increased significantly from week 0 to 2 (*post-hoc* Bonferroni, $P=0.001$), with a non-significant decline throughout the remainder of the study (Fig. 1B). The maximum measured LPS and PHA antibody titers were marginally positively intercorrelated in immune-challenged male finches ($r_F=0.62$, $P=0.058$), but not females ($r_F=0.19$, $P=0.60$).

Effects of immune challenge on body mass and molt progress

There was no significant effect of immune challenge on body mass (RMANOVA – week×treatment: $F_{1,92,63,51}=1.46$, $P=0.24$). The mass of both challenged and control birds significantly increased between weeks 2, 4 and 8 (Fig. 2A; RMANOVA week×week: $F_{1,92,63,51}=13.02$, $P<0.0001$; *post-hoc* Bonferroni, $P<0.0001$). In week 7, immune-challenged birds had molted significantly fewer primary feathers than controls (Fig. 2B; ANOVA treatment: $F_{1,33}=4.71$; $P=0.037$); however, in week 8 the treatment groups converged in molt progress (Fig. 2B; ANOVA treatment: $F_{1,33}=0.40$; $P=0.53$).

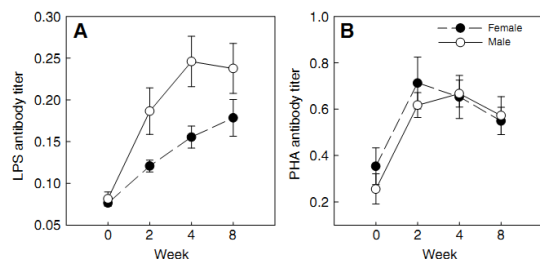


Fig. 1. Response to immune challenge. Antibody titer of (A) lipopolysaccharide (LPS) and (B) phytohaemagglutinin (PHA; measured as optical density at 450 nm) of immune-challenged house finches by sex before injection (week 0) and weeks 2, 4 and 8 of the study. Values are means \pm s.e.m.

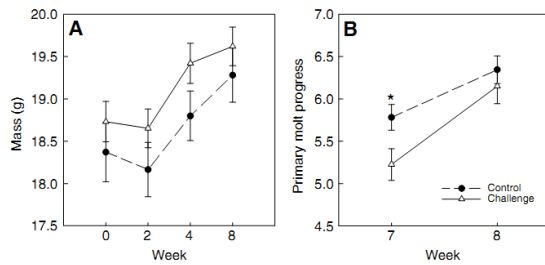


Fig. 2. Effects of immune challenge on body mass and molt progress. (A) Body mass of immune-challenged (weekly with LPS and PHA) and uninjected control house finches before injection (week 0) and at weeks 2, 4 and 8 of the study. (B) Number of molted primary feathers for immune-challenged control birds on weeks 7 and 8 of the study. *Significant difference between challenged and control groups (see text). Values are means \pm s.e.m.

Effects of immune challenge on carotenoid accumulation

Immune challenge significantly depleted retinal carotenoid concentrations, and these changes were specific to retinal carotenoid types. Injected birds had significantly lower levels of retinal galloxanthin, zeaxanthin and an unknown carotenoid than control birds (Fig. 3, Table 2), but there were no significant differences in astaxanthin, lutein or ϵ -carotene levels (Table 2). Among challenged birds, retinal carotenoid levels of any type were not significantly related to maximum LPS or PHA antibody titers ($P > 0.18$). Throughout the study, there were no significant differences in total plasma carotenoid levels between challenged and control birds (RMANOVA, week \times treatment: $F_{2,49,79,59} = 0.28$; $P = 0.80$); however, levels differed significantly among weeks, with the highest levels occurring in week 8 of the study (Fig. 4A; RMANOVA, week: $F_{2,49,79,59} = 10.68$; $P < 0.0001$). From liver samples collected at the conclusion of the experiment, we found no significant differences in total liver carotenoid concentrations between treatment groups (ANOVA treatment: $F_{1,33} = 0.09$; $P = 0.77$; Fig. 4B). The carotenoid concentration in growing ornamental breast feathers did not differ between challenged and control male house finches ($t = -1.013$; d.f. = 15; $P = 0.33$, Fig. 4B).

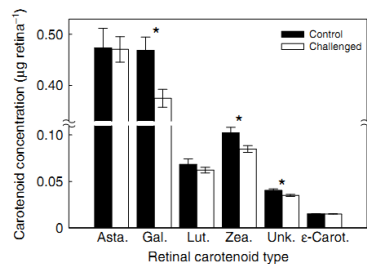


Fig. 3. Effects of immune challenge on carotenoid accumulation. Concentration of each retinal carotenoid type for immune challenged (weekly for 8 weeks with LPS and PHA) and uninjected control house finches measured at the conclusion of the study. *Significant difference between challenged and control birds (Table 2). Asta., astaxanthin; Gal., galloxanthin; Lut., lutein; Zea., zeaxanthin; Unk., unknown; ϵ -Carot., ϵ -carotene. Values are means \pm s.e.m.

DISCUSSION

We administered a sustained immune-system challenge to captive molting house finches to determine its effect on carotenoid allocation to retinal tissue. Our weekly LPS and PHA injections successfully elevated circulating levels of LPS- and PHA-specific antibodies. Prolonged immune-system activation also had other notable effects on finch physiology and morphology, namely by slowing molt progress and thus presumably limiting resources available for feather growth. This is consistent with trade-offs between immune response and feather growth observed in house sparrows (*Passer domesticus*) (Martin, 2005) and pied flycatchers (*Ficedula hypoleuca*) (Sanz et al., 2004). Although one might expect a costly immune response to cause a loss of body mass, we found no such effect. However, a number of other studies have reported significant changes in carotenoid physiology in response to experimentally induced immune activity with no significant changes in body mass (e.g. Alonso-Alvarez et al., 2004; Biard et al., 2009; Hörak et al., 2006; Perez-Rodriguez et al., 2008; Peters et al., 2004).

Our prolonged immune-system challenge significantly depleted retinal carotenoid levels of our captive house finches. This is the first study to show an association between an animal's immune-system activity and retinal carotenoid accumulation. One possible concern when interpreting our results is that the level and duration of immune stimulation in our experiment was much greater than that experienced by finches in nature. However, several lines of evidence suggest that our immune challenge was not an unnaturally stressful manipulation: (1) birds maintained and even increase in body mass over the course of the study; (2) although slowed in the challenged birds, molt progressed within the natural time frame for the house finch (Hill, 1993); (3) the carotenoid levels in tissues known to be sensitive to immune challenge (e.g. plasma) were not

Table 2. Results of ANOVAs for retinal carotenoid concentration in relation to immune challenge and sex

	Treatment		Sex	
	F	P	F	P
Astaxanthin	0.003	0.96	0.041	0.84
Galloxanthin	9.055	0.005	0.046	0.83
Lutein	1.29	0.26	3.15	0.085
Zeaxanthin	6.68	0.014	0.41	0.53
Unknown	7.74	0.009	0.002	0.96
ϵ -Carotene	0.63	0.43	2.39	0.13

The non-significant treatment \times sex interaction term was omitted from the model. d.f. = 1,33 for all comparisons.

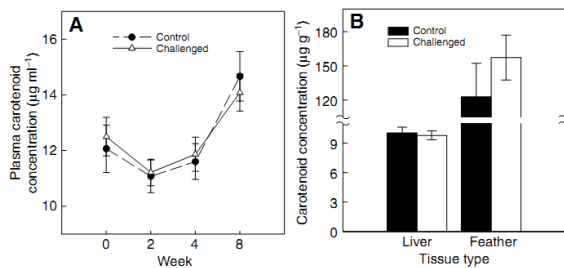


Fig. 4. Effects of immune challenge on carotenoid accumulation. (A) Plasma carotenoid concentrations of immune-challenged (weekly with LPS and PHA) and uninjected control house finches before injection (week 0) and at weeks 2, 4 and 8 of the study. (B) Liver tissue carotenoid concentration of control and immune-challenged birds of both sexes and the carotenoid concentrations of male ornamental breast feathers from control and challenged male birds at the conclusion of the study. Values are means \pm s.e.m.

affected by our manipulation, and (4) our 8-week manipulation falls well within the natural duration of common infections such as *Mycoplasma gallisepticum* and avian pox (see Introduction). Therefore, our manipulation is probably representative of challenges birds may face in the wild.

Carotenoid depletion was specific to the retina and we found no significant effect of immune system activation on plasma, liver and feather carotenoid levels. This result contrasts with a number of previous studies that have shown significant declines in carotenoid levels in the blood and colorful ornaments following an immune challenge (e.g. Biard et al., 2009; Faivre et al., 2003; Peters et al., 2004; Perez-Rodriguez et al., 2008; McGraw and Klasing, 2006). However, Biard et al. (Biard et al., 2009) recently determined that the depletion of circulating carotenoids during a PHA-induced immune response in blackbirds is relatively short-lived and the effects disappear after >2 days. Therefore, transient depletion of plasma carotenoids may have occurred in our immune-challenged birds, but we were not able to detect it because our sampling occurred 7 days after the injections. The lack of an effect of immune challenge on feather carotenoid accumulation is consistent with studies of goldfinches (*Carduelis tristis*) (Navara and Hill, 2003) and great tits (*Parus major*) (Fitze et al., 2007) and suggests that feather carotenoid deposition may be somehow buffered against the relatively rapid changes in carotenoid availability and allocation. However, because of the limited concentrations and types of dietary carotenoids available in our study, which induced all males to grow pale yellow feathers, we must cautiously interpret this result and perform follow-up studies using proper metabolic precursors (e.g. β -cryptoxanthin) (McGraw et al., 2006) that allow development of sexually preferred, elaborate red plumage coloration.

The fact that immunological effects on carotenoid accumulation were retina-specific suggests that we isolated a particularly strong stressor for the eye and its immunomodulatory components. Generally, retinal carotenoids are considered relatively stable when compared with other tissues (e.g. Wang et al., 2007), but may be particularly affected by immune challenges because the eye exhibits a high level of immunosuppression to counter the detrimental effects of inflammation (see Introduction). Systemic immune challenges with LPS are known to cause significant inflammation, and nitric oxide (a damaging pro-oxidant) production in the rat eye (Bhattacharjee et al., 1983; Goureau et al., 1995; Rosenbaum et al., 1980). Carotenoid supplementation has been shown to reduce inflammation and inhibit the production of nitric oxide in the rat eye following LPS challenge (Jin et al., 2006; Ohgami et al., 2003; Shiratori et al., 2005). Therefore, we may have seen a decline in retinal carotenoid levels because these pigments were being allocated

to and consumed by anti-inflammatory and anti-oxidant functions in the eye. However, in birds, the immunosuppressive properties of the eye and the fine-scale molecular relationships between immune activation and carotenoid physiology have yet to be explored. Regardless of the mechanism, our results do suggest that long-term health perturbations represent significant costs toward producing or maintaining carotenoid pigments in retina.

Not only were the effects of immune challenges retina specific, but within the retina only certain types of carotenoids were affected by our treatment. Retinal galloxanthin, zeaxanthin and an unknown carotenoid were significantly depleted in the immune-challenged birds, whereas the concentrations of other carotenoid types remained unchanged. These carotenoid-specific changes may be related to differences in accumulation mechanisms and turnover rates among retinal carotenoid types. In wild house finches, retinal levels of the carotenoids that were immune-treatment-sensitive in this study (e.g. galloxanthin and zeaxanthin) were significantly correlated with circulating plasma carotenoid levels, while retinal astaxanthin and *e*-carotene levels, which were unaffected by our immune challenge, did not significantly correlate with plasma carotenoids (Toomey and McGraw, 2009). This suggests that concentrations of some retinal carotenoid types may be maintained through replenishment from the circulating pool, while other types are buffered against short-term changes and turn over relatively slowly. Further support for this idea comes from Bhosale et al. (Bhosale et al., 2007), who tracked the accumulation of isotopically labeled dietary zeaxanthin in quail retina and found that the degree of labeling differed among retinal carotenoid types. After 16 weeks on the labeled diet, zeaxanthin and galloxanthin showed high levels of labeling (94% and 58%, respectively), whereas retinal astaxanthin was relatively low (28%) (Bhosale et al., 2007).

The specificity of these changes is particularly interesting because these retinal carotenoids differ not only in their patterns of accumulation, but have distinct functions in the avian eye. Retinal carotenoids in birds accumulate in colorful oil droplets located between the inner and outer segments of the cone photoreceptors. Each of the five cone photoreceptor types contain a different colored oil droplet containing specific types of carotenoids. For example, the long-wavelength-sensitive cone contains a red oil droplet pigmented with astaxanthin, whereas the medium-wavelength-sensitive cone has a yellow oil droplet pigmented with zeaxanthin (Goldsmith et al., 1984; Goldsmith and Butler, 2003). These oil droplets filter light that reaches the retina, not only providing photoprotection, but also enhancing color vision. Behavioral studies of domesticated birds suggest that the complete exclusion of carotenoids from the avian retina can shift color preferences and

spectral sensitivity (Bowmaker et al., 1993; Duecker and Schulze, 1977; Wallman, 1979). A recent modeling study by Lind and Kelber (Lind and Kelber, 2009) indicates that even subtle shifts (10 nm) in retinal oil droplet filtering can significantly change predicted color matches and chromatic contrast values within a given visual system. Carotenoids are also important photoprotectants and, in quail, increased retinal zeaxanthin accumulation prevents light-induced photoreceptor death (Thomson et al., 2002a; Thomson et al., 2002b). Therefore, immune system activity induced depletion of retinal carotenoids could have direct impacts on both visual health and function.

Studies of carotenoids and life-history evolution (Blount, 2004) have primarily traced pigment allocation between two competing functions – health and ornamentation – to identify the information content and honesty-maintaining mechanisms of sexual signals (Lozano, 1994; Møller et al., 2000; von Schantz et al., 1999). Our results indicate that immune-system activation may also divert carotenoid resources away from the visual systems that receive these colorful signals. If retinal carotenoid depletion brought about by immunological challenge compromises the health and performance of the eye, then this represents a previously unappreciated carotenoid-mediated cost of immune activity. Moreover, the reliability with which visual signals are received and perceived may differ with a receiver's health status. Sensory phenotype has been implicated as an important factor shaping individual preference functions in the context of mating (Widemo and Sæther, 1999), introducing the possibility of a common physiological mechanism (carotenoid allocation/accumulation) mediating both a sexual trait (colorful plumage) and preference.

ACKNOWLEDGEMENTS

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APPENDIX D

APPROVAL LETTER FROM INSTITUTE FOR ANIMAL CARE AND USE
COMMITTEE

*Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University*

Tempe, Arizona 85287-1103
(480) 965-2179 FAX: (480) 965-7772

Animal Protocol Review

ASU Protocol Number: 09-1054R
Protocol Title: Mechanisms and functions of carotenoid accumulation in the retina and testes of House finches
Principal Investigator: Kevin McGraw
Date of Action: March 30, 2009

The animal protocol review was considered by the Committee and the following decisions were made:

- The original protocol was APPROVED as presented.
- The revised protocol was APPROVED as presented.
- The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be reconsidered when these issues are clarified and the revised protocol is submitted.
- The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- The Committee requests you to contact _____ to discuss this proposal.
- A copy of this correspondence has been sent to the Vice President for Research.
- Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENT:

Approved # of Animals: 156 **Pain Level:** C
Species: Carpodacus mexicanus (house finch)
Approval Period: 3/30/2009 - 2/29/2012
Funded: Unfunded

Signature: 
IACUC Chair or Designee

Date: 3/30/09

Original: Principal Investigator
cc: IACUC Office
IACUC Chair
ORSPA/SPS

APPENDIX E

COAUTHOR PERMISSIONS FOR INCLUSION OF PUBLISHED WORKS

June 14, 2011

To whom it may concern,

I, Kevin J. McGraw, grant Matthew Toomey permission to include, as part of his dissertation, our manuscripts:

Toomey, M. B. & McGraw, K. J. 2010. The effects of dietary carotenoid intake on carotenoid accumulation in the retina of a wild bird, the house finch (*Carpodacus mexicanus*). Archives of Biochemistry and Biophysics, **504**, 161-168.

Toomey, M. B., Butler, M. W. & McGraw, K. J. 2010. Immune-system activation depletes retinal carotenoids in house finches. Journal of Experimental Biology, **213**, 1709-1716.

Toomey, M. B. & McGraw, K. J. 2009. Seasonal, sexual, and quality related variation in retinal carotenoid accumulation in the house finch (*Carpodacus mexicanus*). Functional Ecology, **23**, 321-329.

Sincerely,

A handwritten signature in black ink, appearing to read 'KJ McGraw', with a horizontal line extending to the right.

Kevin J. McGraw

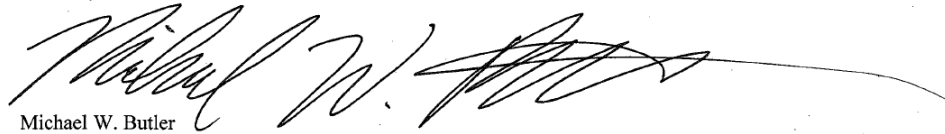
June 14, 2011

To whom in may concern,

I Michael W. Butler grant Matthew Toomey permission to include, as part of his dissertation, our manuscript:

Toomey, M. B., Butler, M. W. & McGraw, K. J. 2010. Immune-system activation depletes retinal carotenoids in house finches. *Journal of Experimental Biology*, **213**, 1709-1716.

Sincerely,

A handwritten signature in black ink, appearing to read "Michael W. Butler", with a long horizontal flourish extending to the right.

Michael W. Butler

