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Ketocarotenoid circulation, but not retinal carotenoid accumulation, is linked to eye disease status in a wild songbird

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ABSTRACT

Pathogenic or parasitic infections pose numerous physiological challenges to organisms. Carotenoid pigments have often been used as biomarkers of disease state and impact because they integrate multiple aspects of an individual's condition and nutritional and health state. Some diseases are known to influence carotenoid uptake from food (e.g. coccidiosis) and carotenoid use (e.g. as antioxidants/immunostimulants in the body, or for sexually attractive coloration), but there is relatively little information in animals about how different types of carotenoids from different tissue sources may be affected by disease. Here we tracked carotenoid accumulation in two body pools (retina and plasma) as a function of disease state in free-ranging house finches (*Haemorhous mexicanus*). House finches in eastern North America can contract mycoplasmal conjunctivitis (*Mycoplasma gallisepticum*, or MG), which can progress from eye swelling to eye closure and death. Previous work showed that systemic immune challenges in house finches lower carotenoid levels in retina, where they act as photoprotectors and visual filters. We assessed carotenoid levels during the molt period, a time of year when finches uniquely metabolize ketocarotenoids (e.g. 3-hydroxy-echinenone) for acquisition of sexually selected red plumage coloration, and found that males infected with MG circulated significantly lower levels of 3-hydroxy-echinenone, but no other plasma carotenoid types, than birds exhibiting no MG symptoms. This result uncovers a key biochemical mechanism for the documented detrimental effect of MG on plumage redness in *H. mexicanus*. In contrast, we failed to find a relationship between MG infection status and retinal carotenoid concentrations. Thus, we reveal differential effects of an infectious eye disease on carotenoid types and tissue pools in a wild songbird. At least compared to retinal sources (which appear somewhat more temporally stable than other body carotenoid pools, even to diseases of the eye evidently), our results point to either a high physiological cost of ketocarotenoid synthesis (as is argued in models of sexually selected carotenoid coloration) or high benefit of using this ketocarotenoid to combat infection.

Introduction

Immunologists and epidemiologists have long been interested in identifying biomarkers for diseases of wildlife and commercial animals, which may permit rapid clinical intervention and slowing or prevention of the spread of devastating infectious diseases [1–3]. Many physiological, morphological, and behavioral parameters are now known to honestly reveal infection state of organisms, and carotenoid pigments are among the animal nutrients with biomarker potential that are of interest to both basic and applied disease biologists [4–8].

Carotenoids are unique chemicals in this sense, as they can both endogenously and exogenously reveal an animal's disease status [9]. These compounds are acquired exclusively from food, circulated through the body, and can act as antioxidants to combat oxidative stress as well as enhance immune-system responsiveness in the face of pathogenic challenges [10]. Tracking carotenoid levels in internal (e.g. human macula, immune tissues) and external tissues (e.g. integumentary coloration) often can indicate antioxidant mobilization/depletion during infection [11], severity/clearance of infection [12], and even survival of individuals [13,14]. Immune challenges, disease administrations, and carotenoid supplementations have been powerful experimental tools in examining carotenoid/health interactions [15–18], but assessment of carotenoids and disease in free-ranging animals also has provided key insights into naturally and sexually selected priorities for carotenoids in the face of pathogenic challenges [19–21].

Different tissue types in the body can vary in their carotenoid content as well as their immunological role. For example, in

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chickens (*Gallus gallus domesticus*), some tissue pools of carotenoids (e.g. plasma, liver, thymus) are altered more rapidly or intensely during immune responses than others (e.g. bursa, shank) [11]. Certain tissues like retina are thought to possess very stable pools of carotenoids, which remain unaffected for weeks by physiological perturbations [22,23]. Moreover, many animals acquire and harbor several different carotenoid types, with major classes including hydroxycarotenoids (e.g. lutein, zeaxanthin), oxocarotenoids (e.g. canthaxanthin, astaxanthin), and carotenes (e.g. β-carotene). As it relates to combatting disease, relatively little is known about how varying carotenoid types play different roles in modulating health status [24]. Degree of bond conjugation has been argued to affect antioxidant potential for some carotenoids [25]. Moreover, in a few animal systems, infectious/immunological challenges affect the absorption or depletion of specific carotenoids (e.g. xanthophylls and human macular degeneration: [26]; thymic zeaxanthin during the acute phase response in chickens: [11]).

To improve our understanding of possible differential links between infectious challenges and carotenoid types/tissue-sources, we investigated carotenoid accumulation as a function of disease status in a free-ranging avian model for the study of carotenoids and disease. The carotenoid-based plumage coloration of house finches (*Haemorhous mexicanus*) serves as a classic example of a sexually selected signal of diet and health in animals [27]. Moreover, since the mid-1990's, there has been considerable epidemiological, immunological, and genetic research on the mycoplasmal conjunctivitis outbreak that has swept through the house finch population of eastern North America [28–31]. This disease (*Mycoplasma gallisepticum*, or MG)[1] causes infectious sinusitis and manifests in swollen eyes, often leading to blindness and death [32]. Mycoplasmosis impairs development of sexually attractive red plumage [33], and plumage redness positively predicts resistance to [12] and survival through an infection [28]. To date, however, types and amounts of carotenoids have not been measured per se as a function of MG infection in this species.

We studied the relationship between eye disease status and carotenoids in the plasma and retina of molting male and female house finches. We focused on these two tissue types for the following reasons: (1) molting males uniquely circulate ketocarotenoid pigments (e.g. 3-hydroxy-echinenone) through blood at this time of year, which they metabolize from dietary sources for developing red plumage coloration [34,35]; hence at this time of year we can investigate the widest pool of available carotenoids, as they relate to the key process of male pigmentation, when MG incidence is at its annual peak [36]; and (2) retinal carotenoids are understudied compared to other tissue pools of carotenoids [37], and disease-related shifts in retinal carotenoid allocation would have consequences not just for health state or color signaling, but also color vision, given the role of oil-droplet carotenoids in tuning avian spectral sensitivity [23]. Few links have been made previously in the literature between pathogenic infections and vision in non-human animals [38] and, despite evidence for relatively stable pools of carotenoids in retina (as above), an eye-specific disease might be among the most likely modulators of eye carotenoid status. Finally, because carotenoids can be linked to immunity and disease through oxidative stress (e.g. need to quench free radicals produced during infection; [39,40]), we also measured oxidative stress levels in the plasma samples of our birds. We predicted that carotenoid levels would be higher, and oxidative stress levels lower, in birds showing no (or less severe) MG symptoms. We also predicted to find a negative relationship between plasma oxidative stress and carotenoid levels, perhaps only (or stronger) in plasma compared to retina.

Methods

Bird capture, measurement, and sample collection

From 3 August to 2 September 2011, 43 female and 26 male house finches were captured on the campus of Auburn University, Alabama, USA using basket traps hung around seed feeders [35]. At capture, we determined sex (based on presence of newly molted patches of carotenoid plumage in males), estimated degree of MG infection for each eye using a 5-point integer scale (0 = no eye swelling; 4 = complete eye closure; *sensu* [41]), measured body mass to the nearest 0.01 g with a digital scale, and drew a small (<100 μl) blood sample from the wing vein for later determination of levels of plasma carotenoids and oxidative stress (see below). We then euthanized the birds following approved IACUC procedures and dissected out the eyes. The left eye of each bird was placed in RNAlater for a different study and the right eye was placed in a screw-cap Eppendorf tube with no preservative and immediately frozen at −80 °C. We used the frozen right eyes for later separation of the retina and analysis of retinal carotenoids. Plasma and eyes were stored at −80 °C for 4–6 months prior to analysis.

Carotenoid analyses

Plasma and retinal carotenoids were extracted and quantified using our previously published reverse-phase high performance liquid chromatographic (HPLC) techniques [22,35]. Briefly, we added 100 μl ethanol to 20 μl thawed plasma in a fresh Eppendorf tube and vortexed for 5 s. Then we added 100 μl of a 1:1 hexane:methyl *tert* butyl ether (MTBE) solution to the mix and vortexed again for 5 s. For retinas, we weighed dissected tissue to the nearest 0.01 mg with an electronic balance and ground it in a screw-cap Eppendorf tube in the presence of 1 ml of 1:1 hexane:MTBE and 6 stainless steel grinding balls for 15 min. at 30 Hz. At this point, for both types of extracts, we centrifuged the tubes for 3 min at 10,000 rpm, transferred the supernatant to a fresh tube, and evaporated the solution to dryness under a stream of nitrogen.

For HPLC analysis, we resuspended the extract in 200 μl mobile phase (42:42:16 methanol:acetonitrile:dichloromethane, v/v/v for plasma samples; 46:46:8 mix of the same respective solvents for retinas) and injected 50 μl into a Waters Alliance autosampler HPLC equipped with a C-30 YMC™ 5 μm Carotenoid column (4.6 × 250 mm ID) and a column heater set to 30 °C. In previous work, we discovered that pre-treating the column with a weak acid (1% *ortho*-phosphoric acid) in methanol permits successful recovery of highly polar ketocarotenoids (e.g. astaxanthin in bird retina; [42]). We did this for plasma and retinal samples here, and in fact for the first time identified red ketocarotenoids (e.g. 3-hydroxy-echinenone) in the plasma of molting house finches. We used a 29.5-min. gradient run to elute plasma carotenoids [43] and a 26.5-min. gradient run to elute retinal carotenoids [42]. Carotenoids were identified by comparison to authentic reference carotenoids or to published HPLC values for retention time and absorbance maxima [37]; we quantified pigments based on external standard curves.

Oxidative stress analyses

Similar to previous studies of songbirds [17,43,44] and other animals [45–49], we measured thiobarbituric acid-reactive

[1] *Abbreviations used:* MG, *Mycoplasma gallisepticum*; HPLC, high performance liquid chromatographic; MTBE, methyl *tert* butyl ether; TBARS, thiobarbituric acid-reactive substances; TBA, thiobarbituric acid; 3HE, 3-hydroxy-echinenone; OS, oxidative stress; MDA, malondialdehyde; SDS, sodium dodecyl sulfate, HSD, honestly significant difference.

substances (TBARS) in plasma as an index of oxidative stress. We used a miniaturized TBARS assay modified from a commercially available kit (Oxi-Tek TBARS assay kit, ZeptoMetrix Corp., Buffalo, NY). The TBARS assay quantifies oxidative stress by measuring levels of lipid peroxidation, a major biomarker of oxidative stress in animal tissues. Specifically, this assay involves the reaction of malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, with thiobarbituric acid (TBA) under conditions of high temperature and acidity to generate an adduct that can be measured by spectrophotometry. Briefly, 20 μL thawed plasma was mixed with 20 μL 8.1% sodium dodecyl sulfate (SDS) and 500 μL TBA buffer reagent. The TBA buffer reagent was prepared by mixing 50 mg thiobarbituric acid with 10 ml acetic acid and 10 ml NaOH. Samples were then vortexed and incubated at 95 °C in capped tubes for 60 min. Thereafter, the sample was placed on ice for 10 min and centrifuged at 3000 rpm for 15 min. After centrifugation, the supernatant was removed and absorbance of the sample was measured in duplicates at 532 nm (Bio-Tek μQuant microplate spectrophotometer). Sample concentrations from the absorbance averages were calculated by interpolation from a standard curve of MDA in concentration from 0 to 100 nmol/ml and are expressed in nmol/ml of MDA equivalents.

Statistics

We used two metrics of MG infection state in our statistical analyses: (1) presence v. absence (hereafter referred to as MG presence), and (2) mean infection score of both eyes, for those birds with at least one non-zero eye score. We used separate analyses of variance (ANOVA) to examine the effect of MG presence/absence, sex, and their interaction on individual plasma carotenoid concentrations, plasma oxidative stress levels, retinal carotenoid concentrations, and body mass. These response variables all met the assumptions of parametric statistics, and we used Tukey–Kramer honestly significant difference (HSD) post hoc tests to evaluate pairwise differences when terms were significant. Neither capture date ($F_{1,65} = 0.47$, $p = 0.50$) nor body mass ($F_{1,41} = 0.06$, $p = 0.81$) were significant predictors of MG presence/absence (sex effects also insignificant in these models, both $p > 0.40$), so we did not further consider these variables in analyses. For the 32 birds that had some degree of MG infection, we ran correlational analyses (Fisher's *r*-to-*z* tests) to compare mean MG infection score with each of the aforementioned response variables. Sample sizes vary by analysis due to the fact that plasma and eye samples were not available (or were not in sufficient amounts for multiple analyses) from every individual; we had plasma carotenoid data for 59 birds, plasma oxidative stress data from 42 birds, and retinal carotenoid data for 31 birds.

Results

Effect of MG presence on plasma carotenoids, retinal carotenoids, and oxidative stress

Twenty-two of the 43 females and 10 of the 26 males in our study were infected with MG. We found no effects of MG presence (both $p > 0.67$) or sex (both $p > 0.08$) on circulating levels of lutein and zeaxanthin, but in both cases the MG presence ∗ sex interaction was significant ($p < 0.035$; Fig. 1a and b). Males circulated significantly less lutein than did females, but only among uninfected birds (Fig. 1a). Uninfected males also circulated the lowest levels of zeaxanthin among the groups (Fig. 1b). In contrast to these patterns for hydroxycarotenoids, we found strong effects of MG presence on red ketocarotenoid circulation ($F_{1,55} = 13.19$, $p = 0.0006$). MG-infected birds circulated significantly less 3-hydroxy-echinenone than non-symptomatic birds (Fig. 1c). As expected, males (who develop extensive red plumage) also circulated significantly more 3-hydroxy-echinenone than females (who only occasionally develop hints of red plumage on the rump; $F_{1,55} = 32.86$, $p < 0.0001$; Fig. 1c). The MG presence ∗ sex interaction was significant as well ($F_{1,55} = 5.64$, $p = 0.02$), such that the magnitude of disease-related change in 3-hydroxy-echinenone concentration was statistically significant in males but not in females.

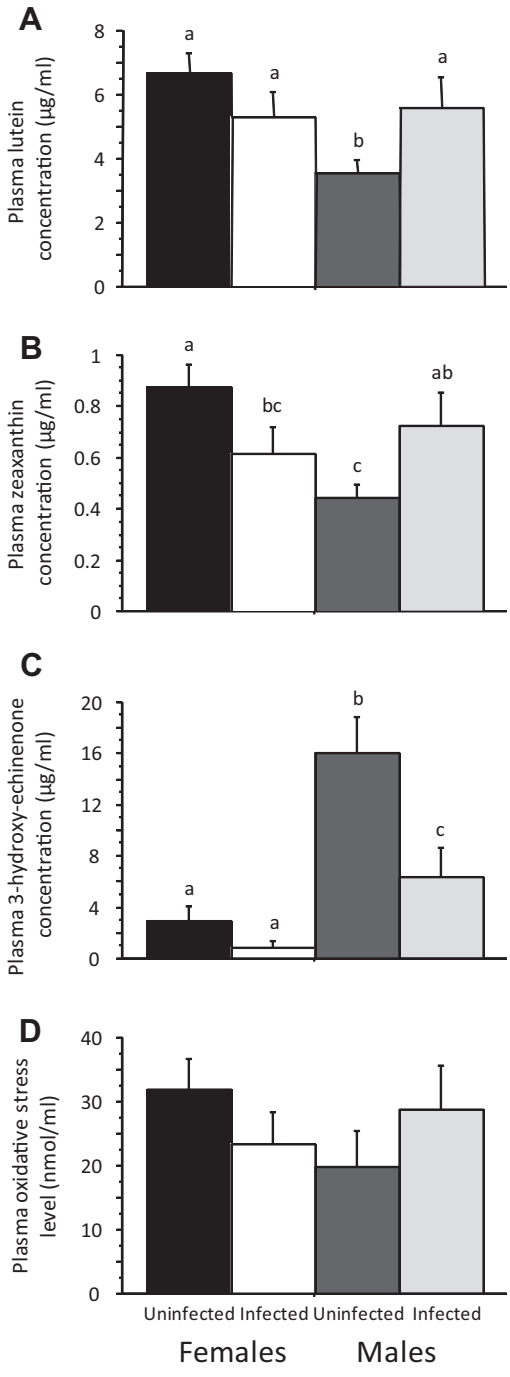

Fig. 1. Plasma variables as a function of MG infection status in male and female house finches during molt: (A) lutein concentration, (B) zeaxanthin concentration, (C) 3-hydroxy-echinenone concentration, and (D) oxidative stress levels. Means + standard errors are shown. Unshared letters denote significant differences between individual groups (Tukey–Kramer HSD post hoc tests, $p < 0.05$); see text for main statistical results.

We found no significant effects of MG presence ($F_{1,38} = 0.001$, $p = 0.98$), sex, ($F_{1,38} = 0.36$, $p = 0.55$) or their interaction ($F_{1,38} = 2.38$, $p = 0.13$) on plasma oxidative stress levels (Fig. 1d). We also failed to find significant effects of MG presence, sex, or their interaction on any of the retinal carotenoid types (all $p > 0.08$; Fig. 2).

Correlations between plasma and retinal measurements

Because retinal carotenoid and oxidative stress levels were independent of disease state and sex (as above), we pooled all birds for these analyses. As expected [37], we found significant positive correlations among the concentrations of the retinal carotenoid types (all $r > 0.46$, all $p < 0.012$). Retinal zeaxanthin concentration was significant positively correlated with plasma oxidative stress levels (Fig. 3a; $p > 0.64$ for other retinal carotenoids). Because there were significant effects of sex/MG presence in the above analyses of plasma carotenoids, we analyzed correlations with these variables separately for each sex and MG class. Plasma lutein and zeaxanthin concentrations were always positively correlated (all $r > 0.78$, all $p < 0.002$), but both were uncoupled from circulating 3-hydroxy-echinenone levels (all $p > 0.15$). In uninfected females only, plasma lutein concentration was significantly negatively correlated with plasma oxidative stress (Fig. 3b; the relationship for plasma zeaxanthin was nearly significant: $r = -0.55$, $p = 0.053$; all other $p > 0.40$). Also, in uninfected males only, we found that birds circulating higher concentrations of 3-hydroxy-echinenone had higher levels of plasma oxidative stress (Fig. 3c; all other $p > 0.57$).

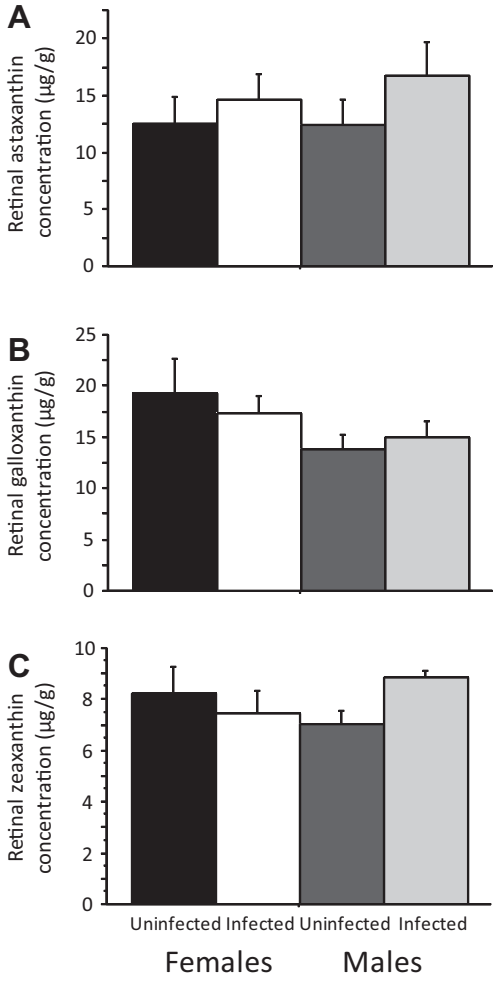

Fig. 2. Retinal carotenoids as a function of MG infection status in male and female house finches during molt: (A) astaxanthin concentration, (B) galloxanthin concentration, and (C) zeaxanthin concentration. Means + standard errors are shown. Unshared letters denote significant differences between individual groups (Tukey–Kramer HSD post hoc tests, $p < 0.05$); see text for main statistical results.

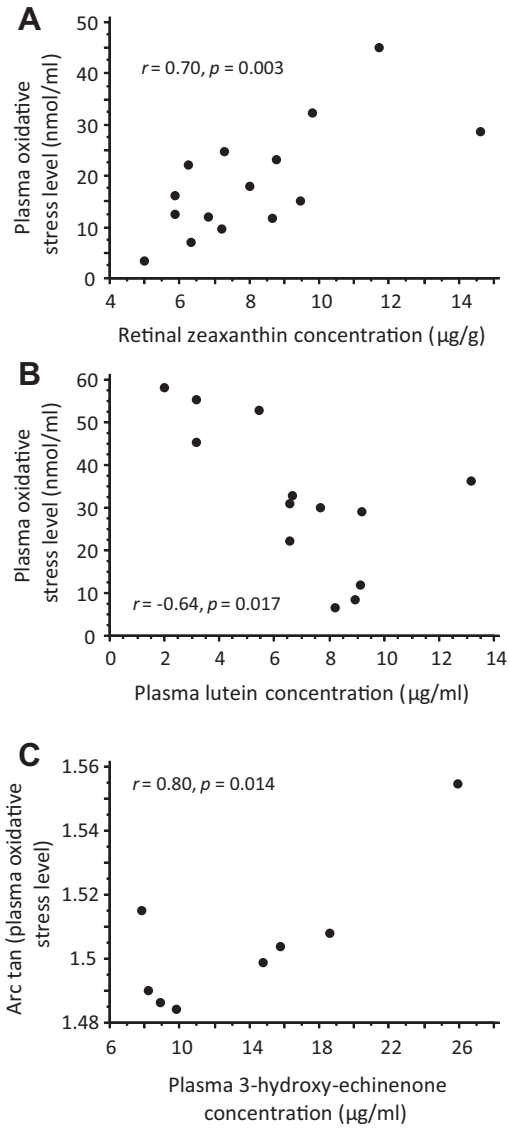

Fig. 3. Scatterplots showing the correlations between plasma oxidative stress levels and various carotenoid metrics: (A) retinal zeaxanthin concentration, (B) plasma lutein concentration, and (C) plasma 3-hydroxy-echinenone concentration. The relationship in (C) is not significant if we exclude the upper-right point ($r = 0.34$, $p = 0.48$).

Correlations between severity of MG infection and plasma carotenoids, plasma oxidative stress, and retinal carotenoids

Among infected birds only, we found no significant correlations between MG severity and either plasma oxidative stress or any of our measures of plasma or retinal carotenoid concentration; this was true both for when we ran separate analyses for males and females (all $p > 0.2$; data not shown) and when we pooled data from the sexes into a single analysis (Table 1).

Discussion

We investigated relationships between incidence and severity of an infectious eye disease, oxidative stress, and carotenoid pools

Table 1
Correlations (Fisher's *r*-to-*z* test) between severity of MG infection (as measured by the mean infection score of both eyes) and our metrics of plasma oxidative stress and plasma and retinal carotenoids.

Predictor	Response	*r*	*n*	*p*
MG severity	Plasma oxidative stress (nmol/ml)	−0.13	17	0.64
	Plasma lutein concentration (μg/ml)	−0.014	25	0.95
	Plasma zeaxanthin concentration (μg/ml)	0.02	25	0.95
	Plasma 3-hydroxy-echinenone concentration (μg/ml)	−0.14	25	0.50
	Retinal astaxanthin concentration (μg/g)	0.08	17	0.77
	Retinal galloxanthin concentration (μg/g)	−0.01	17	0.98
	Retinal zeaxanthin concentration (μg/g)	−0.15	17	0.59

in plasma and retina of a colorful songbird. House finches with mycoplasmal conjunctivitis circulated significantly lower concentrations of a key ketocarotenoid for molting into red plumage. No carotenoids in retina, and no other carotenoids in plasma, related significantly to MG incidence or severity. Oxidative stress (OS) also was unassociated with disease parameters, though we uncovered some interesting relationships (both positive and negative) between OS and body carotenoid concentrations. Despite considerable emerging ecological interest in oxidative balance [50], immunology [51], and infection dynamics [52], to our knowledge this is only the second study to examine correlations between carotenoids, oxidative stress, and infectious disease in a wild animal (see [53] for a review in humans). In the other, Mougeot et al. [39] found that experimental reductions in parasite (nematode) load led to greater decreases in oxidative stress, and greater increases in antioxidant concentrations, for more heavily parasitized male red grouse (*Lagopus lagopus scoticus*).

Several diseases have previously been found to impair expression of carotenoid color signals in animals [54–56], including MG [33,57] and three other pathogens/parasites in house finches (coccidia: [15,57]; poxvirus: [58,59]; feather mites: [58]). However, aside from studies of "pale-bird syndrome" in chickens [60], little attention has been paid to tracking pigmentary mechanisms underlying disease-related declines in carotenoid coloration [21]. Here we monitored carotenoid circulation during molt in house finches and revealed an association between disease status and levels of a pigment specific to developing sexually attractive red plumage coloration. House finches acquire red plumage by depositing red ketocarotenoids like 3-hydroxy-echinenone (3HE) into feathers, which they metabolize from dietary precursors [34,35]. Compared to birds showing no MG symptoms, we detected a severe decrease – by 61% in males and by 70% in females, on average – in 3HE circulation for birds infected with MG. Our results thus illuminate a key stage in the pigment-processing pathway whereby disease presence can constrain carotenoid pigmentation [33].

The fact that no other plasma carotenoid concentrations differed significantly between MG-infected and–uninfected birds suggests a unique cost of ketocarotenoid processing in this system. For some parasites (e.g. coccidians that inhabit the gut lining and disrupt nutrient, especially lipid, uptake; [61]), it is mechanistically clear how infection can alter carotenoid color expression via nutrient absorption. However, for mycoplasmal infections of the eye conjunctiva and respiratory tract [62], there is a less obvious link between pathogen action and carotenoid physiology. There are several possible, non-mutually-exclusive mechanisms to explain a link between ketocarotenoids and MG infection:

(1) MG may have altered pigment transport mechanisms specific to this particular, metabolically produced carotenoid. Lipoproteins that bind and transport carotenoids in the body have been suggested to be limiting agents for carotenoid circulation and coloration in birds [63,64], but never for specific carotenoids relative to others (e.g. see covariation of different circulating carotenoids in [64]). Given the similar polarity and solubility of the plasma hydroxy- and ketocarotenoids under study here, yet their differential covariation with MG infection status, this seems like the least likely explanation for our observed results and would require unprecedented partitioning of carotenoids in lipoprotein fractions [65].
(2) MG may have altered the performance of carotenoid-metabolizing enzymes (e.g. ketolases). Models of carotenoid signal evolution predict high differential costs of developing elaborate plumage when animals metabolize dietary pigments [66,67], compared to when birds deposit dietary forms directly into feathers (e.g. as occurs with dietary xanthophylls and the production of sexually disfavored yellow plumage in male *H. mexicanus*; [34]). It is suspected that these enzymes are highly sensitive to stress (e.g. nutritional, cage confinement; [67–69]), such that in captivity finches will only grow yellow feathers on a natural diet and are unable to manufacture red ketocarotenoids [27]. However, at present, no such carotenoid-metabolizing enzymes (e.g. in liver, feather, beak) have been identified [70–72].
(3) Ketocarotenoids may act as valuable antioxidants/immunostimulants, and the MG-related decline in plasma 3HE represented a physiological allocation to boost immunoresponsiveness. Hill et al. [33] proposed this as a mechanism to explain prior observed effects of mycoplasmosis on finches, but at that time there was little evidence for carotenoid immunomodulation in wild birds [73]. Since, differential immunomodulatory roles for different carotenoids in birds have been elucidated [24], and several studies using even non-infectious challenges [17,74] have noted rapid carotenoid declines from circulation associated with the immunological perturbation. No information is available currently on the relative immunological potency of 3HE versus. other carotenoids in avian systems, but based on the suite of possible costs and benefits of these metabolically derived integumentary colorants it is due time to directly pursue these digestive, molecular, enzymatic, and immunological pathways in a colorful bird model like the house finch.

Unlike in plasma, we failed to detect any significant relationships between MG incidence/severity and retinal carotenoid accumulation. This was somewhat unexpected, given the local, direct effects of MG infections on eye tissue [62] and given our prior experimental work showing that retinal carotenoid levels fall during prolonged immune challenges in house finches [75]. Moreover, though 3HE is absent from retina, another ketocarotenoid (astaxanthin) is metabolized there [37]. A possible explanation for this result is the shorter time-course of MG infection relative to that needed to perturb retinal-carotenoid accumulation. In our prior work on finch retinas, we found that it took 2 months to (a) detect significant changes in retinal carotenoids as a function of dietary carotenoid supplementation [22] and immune challenge [75]. By comparison, MG progression in finches can be rapid, on the order of a few weeks, with most extreme symptoms and death occurring at an average of 35–58 days since exposure, depending on the year and population [41]. Because most of the birds we studied showed only moderate MG symptoms (mean MG eye score of infected birds = 2.23), it is possible that they were in the early stages of infection, and thus there may not have been sufficient time to observe modifications to retinal carotenoid accumulation. This is compared to the few days over which circulating carotenoids can respond to immune challenges [74,76]. Because avian retinal

carotenoids are central to eye photoprotection [77] and visual tuning [23,78], selection may favor these animals to comparatively prioritize the production/retention of oil-droplet carotenoids over other body-carotenoid pools in the face of environmental/physiological challenges [75].

We also failed to find a link between disease incidence/severity and OS levels in plasma. Elevations in reactive oxygen species are commonly observed during pathogen/parasite infestations [79], including for MG infections in gray partridge (*Perdix perdix*; [80]); the evidence in partridge was even uncovered using our same assay of lipid peroxidation (the TBARS test). It is possible that mild infections in our study birds – typical now after the intense epidemic selection event starting in 1995 in Alabama [13] – failed to yield the same OS outcomes as the intense outbreak in naïve, farmed partridge (100% and 60% morbidity and mortality, respectively; [80]). However, we did uncover some significant associations (some positive, some negative) between OS in plasma and carotenoid accumulation in molting finches, which speak directly to the complexities that have been uncovered in recent years on relationships between carotenoids and OS in birds [39,81,82]. First, we found high retinal zeaxanthin accumulation to be correlated with high plasma OS. This was unexpected, but zeaxanthin is a precursor for the formation of most other retinal carotenoids [83], such that higher levels of this compound in retina may reveal a less metabolically active (i.e. oxidatively damaged) state. Yet we detected the same positive correlation between plasma OS and plasma 3HE levels, but only in uninfected males, and one might expect this relationship if a *more* metabolically active state (i.e. ketocarotenoid conversion) is costly (as above) and generates reactive oxygen species. Finally, in uninfected females, we found that higher circulating xanthophyll levels were associated with lower plasma OS, which are the predicted results if carotenoids serve an antioxidant role. However, because both carotenoids [84] and reactive oxygen species can be both helpful (e.g. signaling molecules; [85]) and harmful (e.g. toxic in high quantities; [86]), we must very cautiously approach all of these results, especially when based on somewhat small sample sizes and when trying to interpret causal relationships from correlational results. We still await a model *in vivo* system for addressing physiologically relevant actions and balances of carotenoids and ROS in a colorful wild animal.

In summary, we have uncovered special pathogen-sensitivity of a carotenoid pigment that is critical for the production of a classic condition-dependent, sexually selected trait. For decades, understanding how elaborate signals reveal disease state has been central to research on the evolution of ornamental traits [87], and it is exciting that we are now positioned to address molecule-centered interactions between pathogens/parasites and the production of exaggerated characteristics. Further infusion of genetic, enzymatic, and biochemical data will solidify carotenoid color systems as model targets for the study of signal control, function, and evolution.

Conflict of interest

KJM, MG, GEH, and MBT have nothing to declare.

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References

[1] S.H. Wilson, W.A. Suk, Biomarkers of Environmentally Associated Disease: Technologies, Concepts, and Perspectives, CRC Press, 2002.
[2] T.L. Fry, M.R. Dunbar, Proc. 12th Wildl. Damage Management Conference, Paper 759, 2007.
[3] C.A. Bradley, S. Altizer, Trends Ecol. Evol. 22 (2007) 95–102.
[4] J.K. Tyczkowski, J.L. Schaeffer, P.B. Hamilton, Poult. Sci. 70 (1991) 2275–2279.
[5] C.M. Lee, A.C. Boileau, T.W. Boileau, A.W. Williams, K.S. Swanson, K.A. Heintz, J.W. Erdman Jr., J. Nutr. 129 (1999) 2271–2277.
[6] A.P. Møller, C. Biard, J.D. Blount, D.C. Houston, P. Ninni, N. Saino, P.F. Surai, Avian Poult. Biol. Rev. 11 (2000) 137–159.
[7] J.A. Mares-Perlman, A.E. Millen, T.L. Ficek, S.E. Hankinson, J. Nutr. 132 (2002) 518S–524S.
[8] S.T. Mayne, J. Nutr. 133 (2003) 933S–940S.
[9] F. Mougeot, L. Perez-Rodriguez, N. Sumozas, J. Terraube, J. Avian Biol. 40 (2009) 67–74.
[10] K.J. McGraw, Mechanics of carotenoid-based coloration, in: G.E. Hill, K.J. McGraw (Eds.), Harvard University Press, Cambridge, MA, 2006, pp. 177–242.
[11] E.A. Koutsos, C.C. Calvert, K.C. Klasing, Comp. Biochem. Physiol. A 135 (2003) 635–646.
[12] G.E. Hill, K.L. Farmer, Naturwissenschaften 92 (2005) 30–34.
[13] P.M. Nolan, G.E. Hill, A.M. Stoehr, Proc. R. Soc. Lond. B 265 (1998) 961–965.
[14] H. van Oort, R.D. Dawson, Funct. Ecol. 19 (2005) 822–827.
[15] W.R. Brawner III, G.E. Hill, C.A. Sundermann, The Auk 117 (2000) 952–963.
[16] P. Horak, M. Zilmer, L. Saks, I. Ots, U. Karu, K. Zilmer, J. Exp. Biol. 209 (2006) 4329–4338.
[17] L. Perez-Rodriguez, F. Mougeot, C. Alonso-Alvarez, J. Blas, J. Vinuela, G.R. Bortolotti, J. Exp. Biol. 211 (2008) 2155–2161.
[18] P.L. Pap, C.I. Vagasi, G.A. Czirjak, A. Titilincu, A. Pintea, Z. Barta, J. Exp. Biol. 212 (2009) 3228–3235.
[19] P. Horak, I. Ots, H. Vellau, C. Spottiswoode, A.P. Møller, Oecologia 126 (2001) 166–173.
[20] R.D. Dawson, G.R. Bortolotti, Naturwissenschaften 93 (2006) 597–602.
[21] J. Martinez-Padilla, F. Mougeot, L. Perez-Rodriguez, G.R. Bortolotti, Biol. Lett. 3 (2007) 161–164.
[22] M.B. Toomey, K.J. McGraw, Arch. Biochem. Biophys. 504 (2010) 161–168.
[23] M.B. Toomey, K.J. McGraw, PLoS One 6 (2011) e21653.
[24] P.S. Fitze, B. Tschirren, J. Gasparini, H. Richner, Am. Nat. 169 (2007) S137–S144.
[25] S.A. Paiva, R.M. Russell, J. Am. Coll. Nutr. 18 (1999) 426–433.
[26] D.M. Snodderly, Am. J. Clin. Nutr. 62 (1995) 1448S–1461S.
[27] G.E. Hill, A Red Bird in a Brown Bag: The Function and Evolution of Colorful Plumage in the House Finch, Oxford University Press, 2002.
[28] P.M. Nolan, S.R. Roberts, G.E. Hill, Avian Dis. 48 (2004) 879–885.
[29] C. Bonneaud, S.L. Balenger, A.F. Russell, J. Zhang, G.E. Hill, S.V. Edwards, Proc. Natl. Acad. Sci. USA 108 (2011) 7866–7871.
[30] D.M. Hawley, R.C. Fleischer, PLoS One 7 (2012) e30222.
[31] J.L. Grodio, D.H. Ley, K.A. Schat, D.M. Hawley, Vet. Immunol. Immunopathol. 154 (2013) 129–137.
[32] D.M. Hawley, K.V. Sydenstricker, G.V. Kollias, A.A. Dhondt, Biol. Lett. 1 (2005) 326–329.
[33] G.E. Hill, K.L. Farmer, M.L. Beck, J. Exp. Biol. 207 (2004) 2095–2099.
[34] C.Y. Inouye, G.E. Hill, R.D. Stradi, R. Montgomerie, The Auk 118 (2001) 900–915.
[35] K.J. McGraw, P.M. Nolan, O.L. Crino, Funct. Ecol. 20 (2006) 678–688.
[36] P.R. Hosseini, A.A. Dhondt, A. Dobson, Proc. R. Soc. Lond. B 271 (2004) 2569–2577.
[37] M.B. Toomey, K.J. McGraw, Funct. Ecol. 23 (2009) 321–329.
[38] C.L. Martin, Ophthalmic Disease in Veterinary Medicine, CRC Press, 2009.
[39] F. Mougeot, J. Martinez-Padilla, J.D. Blount, L. Perez-Rodriguez, L.M.I. Webster, S.B. Piertney, J. Exp. Biol. 213 (2010) 400–407.
[40] P.C. Allen, Poult. Sci. 76 (1997) 814–821.
[41] K.L. Farmer, G.E. Hill, S.R. Roberts, J. Wildl. Dis. 38 (2002) 282–286.
[42] M.B. Toomey, K.J. McGraw, Invest. Ophthalmol. Vis. Sci. 48 (2007) 3976–3982.
[43] C. Isaksson, J. Sturve, B.C. Almroth, S. Andersson, Environ. Res. 109 (2009) 46–50.
[44] K.J. McGraw, E.A. Tourville, M.W. Butler, Behav. Ecol. Sociobiol. 62 (2008) 1991–2002.
[45] C. Alonso-Alvarez, L. Pérez-Rodríguez, J.T. García, J. Viñuela, R. Mateo, Physiol. Biochem. Zool. 83 (2010) 110–118.
[46] A. Hunnisett, S. Davies, J. McLaren-Howard, P. Gravett, M. Finn, D. Gueret-Wardle, Biol. Trace Elem. Res. 47 (1995) 125–132.
[47] J.K. Callaway, P.M. Beart, B. Jarrott, J. Pharmacol. Toxicol. Methods 39 (1998) 155–162.
[48] K.D. Oakes, G.J. Van Der Kraak, Aquat. Toxicol. 63 (2003) 447–463.
[49] B.C. Almroth, J. Sturve, A. Berglund, L. Forlin, Aquat. Toxicol. 73 (2005) 171–180.
[50] K.J. McGraw, A.A. Cohen, D. Costantini, P. Horak, Funct. Ecol. 24 (2010) 947–949.
[51] L.B. Martin, D.M. Hawley, D.R. Ardia, Funct. Ecol. 25 (2011) 1–4.

[52] D.M. Hawley, S. Altizer, Funct. Ecol. 25 (2011) 48–60.
[53] B.M. Winklhofer-Roob, E. Rock, J. Ribalta, D.H. Shmerling, J.M. Roob, Mol. Aspects Med. 24 (2003) 391–402.
[54] A.E. Houde, A.J. Torio, Behav. Ecol. 3 (1992) 346–351.
[55] I. Folstad, A.M. Hope, A. Karter, A. Skorping, Oikos 69 (1994) 511–515.
[56] G.E. Hill, Environmental regulation of ornamental coloration, in: G.E. Hill, K.J. McGraw (Eds.), Harvard University Press, Cambridge, MA, 2006, pp. 507–560.
[57] G.E. Hill, W.R. Brawner III, Proc. R. Soc. Lond. B 265 (1998) 1105–1109.
[58] C.W. Thompson, N. Hillgarth, M. Leu, H.E. McClure, Am. Nat. 149 (1997) 270–294.
[59] S.N. Zahn, S.I. Rothstein, The Auk 116 (1999) 35–44.
[60] J.K. Tyczkowski, P.B. Hamilton, M.D. Ruff, Poult. Sci. 70 (1991) 2074–2081.
[61] C. Adams, Br. J. Nutr. 75 (1996) 867–873.
[62] D.H. Ley, J.E. Berkhoff, J.M. McLaren, Avian Dis. 40 (1996) 480–483.
[63] K.J. McGraw, G.E. Hill, R.S. Parker, Anim. Behav. 69 (2005) 653–660.
[64] K.J. McGraw, R.S. Parker, Physiol. Behav. 87 (2006) 103–108.
[65] E. Trams, Comp. Biochem. Physiol. 28 (1969) 1177–1184.
[66] G.E. Hill, Ethol. Ecol. Evol. 8 (1996) 157–175.
[67] G.E. Hill, J.D. Johnson, Am. Nat. 180 (2012) E127–E150.
[68] H. Weber, J. Ornithol. 102 (1961) 158–163.
[69] J. Hudon, The Auk 111 (1994) 218–221.
[70] A.H. Brush, FASEB J. 4 (1990) 2969–2977.
[71] K.J. McGraw, J. Avian Biol. 35 (2004) 471–476.
[72] K.J. McGraw, Naturwisssenschaften 96 (2009) 987–988.
[73] K.J. Navara, G.E. Hill, Behav. Ecol. 14 (2003) 909–916.
[74] C. Biard, C. Hardy, S. Motreuil, J. Morneau, J. Exp. Biol. 212 (2009) 1336–1343.
[75] M.B. Toomey, M.W. Butler, K.J. McGraw, J. Exp. Biol. 213 (2010) 1709–1716.
[76] K.J. McGraw, K.C. Klasing, The Auk 123 (2006) 1161–1171.
[77] L.R. Thomson, Y. Toyoda, A. Langner, F.C. Delori, K.M. Garnett, N. Craft, C.R. Nichols, K.M. Cheng, C.K. Dorey, Invest. Ophthalmol. Vis. Sci. 43 (2002) 3538–3549.
[78] B. Knott, M.L. Berg, E.R. Morgan, K.L. Buchanan, J.K. Bowmaker, A.T. Bennett, Proc. R. Soc. Lond. B 277 (2010) 953–962.
[79] N.V. Georgieva, V. Koinarski, V. Gadjeva, Vet. J. 172 (2006) 488–492.
[80] F. Vitula, L. Peckova, H. Bandouchova, M. Pohanka, L. Novotny, D. Jira, J. Kral, K. Ondracek, J. Osickova, D. Zendulkova, K. Rosenbergova, F. Treml, J. Pikula, BMC Vet. Res. 7 (2011) 34.
[81] D. Costantini, A.P. Møller, Funct. Ecol. 22 (2008) 367–370.
[82] P. Horak, E. Sild, U. Soomets, T. Sepp, K. Kilk, J. Exp. Biol. 213 (2010) 2225–2233.
[83] P. Bhosale, B. Serban, D.Y. Zhao, P.S. Bernstein, Biochemistry 46 (2007) 9050–9057.
[84] V. Olson, I.P.F. Owens, Trends Ecol. Evol. 13 (1998) 510–514.
[85] B. D'Autreaux, M.B. Toledano, Nat. Rev. Mol. Cell Biol. 8 (2007) 813–824.
[86] K.A. Huggins, K.J. Navara, M.T. Mendonca, G.E. Hill, Naturwissenschaften 97 (2010) 637–644.
[87] W.D. Hamilton, M. Zuk, Science 218 (1982) 384–387.