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ORIGINAL PAPER

Effects of carotenoid supplementation and oxidative challenges on physiological parameters and carotenoid-based coloration in an urbanization context

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Abstract Worldwide urbanization continues to present new selection pressures on organisms. Carotenoid pigmentation of animals provides an ideal study system for identifying the source and significance of urban impacts because it is an environmentally derived trait and carotenoid molecules have widespread physiological, phenotypic, and fitness functions. Prior work indicates that in some bird species, urban individuals display less colorful carotenoid ornaments than rural birds. However, few studies have experimentally identified the causal factors that drive such a pattern of reduced "sexiness in the city". We performed two common-garden experiments with house finches, in which we manipulated carotenoid access and exposure to oxidative stress to understand how urban and desert birds respond to these drivers of carotenoid utilization. Urban finches were less colorful than desert birds at capture, but we found no differences between urban and desert finches in how carotenoid provisioning or oxidative stress affected plumage coloration. The only notable site differences in our experiments were that (a) the oxidative challenge caused a larger mass loss in urban compared to desert birds (experiment 1), (b) urban birds circulated higher levels of carotenoids than desert birds after receiving the same diet for 4 months (experiment 2), suggesting that, compared to desert birds, urban finches can better assimilate carotenoids from food or do not deplete as many carotenoids for use in free-radical scavenging. Overall, our results fail to reveal key carotenoid-specific physiological differences in urban and desert finches, and instead implicate other ecophysiological factors that drive urban/desert differences in carotenoid ornamentation.

Keywords Urbanization · Carotenoids · House finch · Oxidative stress · Plumage coloration

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Mathieu Giraudeau and Afton Chavez have participated equally in this study.

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Introduction

Recent anthropogenic activity has had a range of environmental consequences due to, among other things, rapid urbanization. Such consequences include higher temperature, elevated levels of carbon dioxide and other pollutants (Shocat et al. 2006), and reduced habitat availability for wildlife (Marzluff 2001). These effects challenge the ability of organisms to persist in urban environments and have served as selection pressures on animals and plants that have led to changes in morphology, physiology, behavior, and genetics (Shocat et al. 2006). For example, the physiological stress response is lower and timing of reproduction is advanced in urban blackbirds (*Turdus merula*) from Europe compared to rural ones (Partecke et al. 2004, 2006).

One means by which to assess the impact of these human-induced environmental changes on organisms is to track the expression of condition-dependent signals like songs or colors

(Hill 1995; Hõrak et al. 2000). Carotenoid-based color is a classic example of a condition-dependent signal of sexual attractiveness and competitive ability because animals must acquire carotenoid pigments from foods (i.e., a limiting resource) and because these pigments are also used to boost health, as antioxidants and immunomodulators (Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004). Thus, animals displaying the most intense coloration are those in the best nutritional and health condition or oxidative status (i.e., imbalance between the levels of reactive oxygen species and the status of the antioxidant machinery) and hence can afford to devote substantial amounts of pigment to their plumage or integument (Møller et al. 2000; Peters et al. 2004; McGraw 2006).

There is evidence that carotenoid-based plumage color in great tits (*Parus major*; Eeva et al. 1998; Hõrak et al. 2000, 2001; Isaksson et al. 2005) and house finches (*Haemorhous mexicanus*, see "Results" section) is paler in animals living in cities than in those from natural surroundings, suggesting that carotenoid-based plumage coloration can be used to monitor environmental quality. To date, however, most studies on this subject have been observational, and the abiotic or biotic mechanism(s) driving urban–rural differences in plumage coloration are unclear.

There are several possible proximate explanations for why carotenoid-based plumage color differs between urban and rural environments. Differences in the dietary availability of carotenoids (e.g., types and amounts in insects, seeds, etc.) between urban and rural environments may explain urban decreases in plumage coloration (Slagsvold and Lifjeld 1985; Eeva et al. 1998, 2005; Isaksson et al. 2007). Alternatively, animals in cities may be exposed to more disease or oxidative stress (Schilderman et al. 1997; Isaksson et al. 2005) and, due to their role as immunomodulators and antioxidants (Edge et al. 1997; von Schantz et al. 1999; Møller et al. 2000), carotenoids in the body may be drained and thus comparatively less available to urban birds for use in ornate coloration. At present, however, there is considerable debate over the potency of carotenoids as antioxidants in birds (Alonso-Alvarez et al. 2004; Isaksson et al. 2005, 2007; Tummeleht et al. 2006; Costantini et al. 2007; Costantini and Møller 2008). Thirdly, exposure to urban pollutants may affect carotenoid-based coloration through an alteration of the endocrine system and especially on steroid hormones (Bortolotti et al. 2003). A fourth possibility is that plumage color differences between urban and rural birds could arise from a differential ability to physiologically assimilate carotenoids from food (e.g., uptake, storage, conversion of precursors to those deposited into feathers; Blount et al. 2003; Koutsos et al. 2003; McGraw and Parker 2006).

To begin to isolate causal factors behind this geographic variation in color, we performed two common-garden experiments (experiment 1 in winter and experiment 2 during the late summer/early fall) in which we exposed urban- and desert-caught male house finches (*H. mexicanus*) to variation in carotenoid availability [supplementation with zeaxanthin (experiment 1) and canthaxanthin (experiment 2)] and oxidative stress (paraquat treatment) under controlled captive conditions. Our specific objective was to determine if and/or how differential carotenoid intake and use abilities (i.e., in oxidative challenges) explains observed urban ecological patterns of carotenoid color expression. House finches are a model system for studying sexual selection and condition-dependent color signaling (Hill 2002), with males showing striking plumage variation from brilliant red through drab yellow, and females preferring to mate with the reddest males (including in our Arizona population; Toomey and McGraw 2012). House finches inhabit both natural desert and urban environments in their native range (southwestern United States), and desert house finches have redder plumage than those living around humans in our study population in the Phoenix metropolitan area (Hasegawa et al. 2014; also see below). We predicted that if natural, local environmental features (e.g., diet) drive geographic variation in carotenoid status, we would see few differences in carotenoid circulation, oxidative stress susceptibility, and plumage coloration between captive urban and desert birds fed standardized diets. However, if genetic, developmental, or physiological variation is behind observed site differences in carotenoid status, then we expected desert birds to show superior carotenoid circulation and/or resistance to oxidative stress in our experiments. As in our prior study of carotenoids, oxidative stress, and immunity in house finches (McGraw et al. 2011; Giraudeau et al. 2013), we ran this experiment two times, in November/December (outside of the breeding or molt periods) and August (during molt), to assess whether or not there are special seasonally restricted differential carotenoid intake and use abilities between urban and rural birds.

Methods

Experimental procedure

Experiment 1—winter

We captured 80 male house finches from 2 to 13 November 2009 using basket and Potter traps baited with sunflower seeds; trapping sessions lasted from 0700 to 1100 h each morning. Urban birds were trapped on the campus of Arizona State University (ASU) in Tempe, AZ (33°25′N, 112°55′W), which is an area that has had considerable human presence (current city population size >160,000) for at least 50 years (Fig. 1). Desert (which we use interchangeably with "rural" throughout) birds were captured at South Mountain Park/Preserve (SMP, 33°21′N, 112°4′W), which consists of

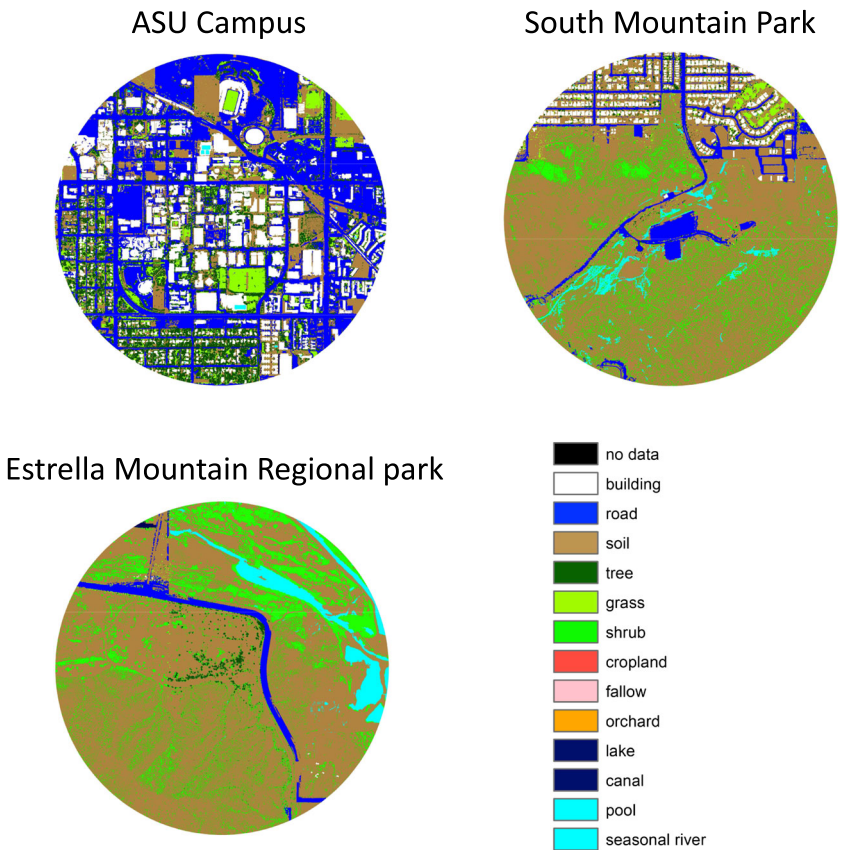

Fig. 1 Land Use Land Cover (LULC) within the 1-km radius around each of our trapping sites

over 16,000 acres of undisturbed Sonoran desert habitat located 11.5 km south of the large urban center of Phoenix, AZ and 21.5 km from our urban capture site (Fig. 1, see Giraudeau et al. 2014 for a detailed description of the sites). We are confident that these represent distinct sites and samples of adult birds because, in a larger project involving the trapping of approximately 2000 adult house finches during an 18-month period in the Phoenix metropolitan area along a gradient of urbanization (with some sites separated by less than 3 km), we never trapped the same bird in two different sites. Thus, we believe that the adult home range is limited to <3 km.

At capture, each bird was leg banded with a numbered United States Fish and Wildlife Service metal tag for individual identification. We determined body mass with a digital scale (to the nearest 0.1 g) and tarsus length (to the nearest 0.1 mm) and drew a sample of blood (~80 μL) from the alar vein into heparinized microcapillary tubes. Plasma samples were stored at −80 °C until laboratory analyses were performed (see below).

Wild-caught finches were randomly divided into four groups (20 birds for each treatment group, comprised of ten desert and ten urban finches) that each received a different carotenoid and/or oxidative stress treatment for a period of 4 weeks (24 November–21 December 2009): control (CO; nothing added to drinking water), carotenoid-supplemented (CA), paraquat-supplemented (PA), or both carotenoid- and paraquat-supplemented (BO). To the drinking water of CA finches, we added 0.756 g L^{-1} of Optisharp™ zeaxanthin beadlets (DSM Nutritional Products, Belvidere, NJ). Zeaxanthin is a common and concentrated xanthophyll carotenoid found in finch food, plasma, and yellow feathers and among other body sites (e.g., liver, retina; McGraw et al. 2006; Toomey and McGraw 2009). We chose this carotenoid dose based on a pilot study (MBT and KJM, unpublished data) so that we could elevate plasma carotenoid levels over the course of a month to the high end of the physiological range of wild birds. To the drinking water of PA finches, we added 0.1 g L^{-1} methyl viologen dichloride (i.e., paraquat dichloride; Sigma-Aldrich Corporation, St. Louis, MO). Paraquat is an herbicide that has been commonly used to induce oxidative stress in animals (Galvani et al. 2000; Salmon et al. 2001; Wang et al. 2001; Isaksson and Andersson 2008; Almroth et al. 2010; Meitern et al. 2013) and may have other physiological side effects (e.g., gastro-intestinal, renal—Berny 2007; on insulin—Kimura et al. 2010). Isaksson and Andersson (2008) previously used this chemical at a dose of 0.09 g L^{-1} in a study of oxidative stress in great tits. We chose a slightly higher dosage (0.1 g L^{-1}) to account for the fact that, on average, house finches (17–22 g) are slightly larger than great tits (16–20 g). This concentration was tested on three pilot finches for 8 days, who showed no declines in activity level

or body mass. Finally, to the water of BO birds, we added both zeaxanthin and paraquat at the aforementioned concentrations. All of the water treatments were kept refrigerated in plastic gallon jugs, and any solution was discarded and renewed no later than 1 week after preparation.

We measured body mass and drew blood from captive finches as above at the beginning (day 1), middle (day 15), and end (day 28) of the experiment. We were also interested to test if the paraquat treatment influenced water intake (since the paraquat treatment was provided through the drinking water), food intake, and activity because a potential effect of the paraquat treatment on these behaviors may influence our results on body mass and oxidative stress levels. From days 12–27 of the experiment, we videotaped each bird for one randomly selected afternoon hour (range of observation times of day=1500–1800 h). Later we scored these for time spent eating from the food dish (time spent with seed in beak or chewing) and drinking water (time spent with the beak in the water cup, all in seconds), and the number of hops per minute (hops on the same perch or from the first to the second perch of the cage were counted). Ideally, we would have gathered continuous feeding and behavioral data, but because this was not the focus of the study, we only took a snapshot of individual activities during the experiment.

Experiment 2—fall molt

We again used basket traps to capture 44 adult male house finches from 28 June to 29 July 2010. Urban birds were again trapped on the ASU campus in Tempe, AZ, but for this experiment, desert birds were captured at Estrella Mountain Regional Park (33°25′N, 112°25′ W), which consists of 19,840 acres of desert habitat located 53 km southwest of ASU (Fig. 1). We used a different desert site in this experiment because the trapping rate was really low in July 2010 at South Mountain; the Estrella Mountain site provided the added advantage of sampling birds from an even more distant, rural area than South Mountain. As above, each bird was banded and weighed at capture. Tarsus length was measured and a blood sample was collected and stored at −80 °C until laboratory analyses were performed.

Because of our smaller sample size in this study compared to experiment 1, we limited the experiment to two treatment groups: carotenoid-supplemented (CA, 11 desert and 12 urban birds) and carotenoid- and paraquat-supplemented (BO, nine desert and 12 urban birds). We avoided using carotenoid-unsupplemented groups in this study because it was run during the molt period and we wanted to provide sufficient carotenoids (above those low levels found in seed provided in captivity; McGraw et al. 2001) for all birds to become colorful. Thus, this second experiment allows us to examine the potential detrimental effects of paraquat treatment in urban and desert birds that receive carotenoid supplementation. Treatments were administered for a period of 15 weeks (27 July–10 November 2010, spanning the full molt period), and to better control dosage in this experiment we hand-fed birds known amounts of each substance, using a plastic syringe and a feeding needle that was inserted into the bill, twice per week. Carotenoid-supplemented birds received 0.4 mL of water containing canthaxanthin (70 μg), while carotenoid- and paraquat-supplemented birds received 0.4 mL of water containing both this carotenoid amount and paraquat (0.5 μg). We chose a slightly lower dosage of paraquat than in the first experiment knowing that house finches in this study would receive the treatment for 3 months, compared to 4 weeks in experiment 1. In addition, we decided to supplement birds with canthaxanthin during molt instead of zeaxanthin because canthaxanthin is a component of the sexually attractive red coloration of house finch plumage (Inouye et al. 2001; by comparison, zeaxanthin is a yellow plumage colorant). We measured body mass and drew blood from captive finches as above every month, starting on the first day of the experiment. At the end of the experiment, birds were euthanized by rapid decapitation and liver samples were collected and frozen at −80 °C for later carotenoid analyses (see more below).

Housing and husbandry

All birds were held in captivity in an animal-approved indoor room on the ASU campus. Finches were housed individually in wire cages (35.5 cm tall×27.5 cm wide×20 cm deep) and received an ad libitum diet of black oil sunflower seeds (which have low carotenoid content; McGraw et al. 2001) and water. Room temperature was held constant at 24 °C and lighting conditions were set to the outdoor day/night cycle. Throughout the study, food was replenished daily and water was replaced every other day to ensure freshness.

Plumage coloration

Plumage coloration was quantified at the start and end of the second experiment using digital photography, following standard published methods for this species (Oh and Badyaev 2006; Giraudeau et al. 2012) and others (e.g., McGraw et al. 2003). Because house finch plumage does not reflect significantly in the UV (McGraw and Hill 2000), techniques that rely on visible light are sufficient to capture variation in carotenoid-based coloration. Using a Canon PowerShot SD1200S (Lake Success, NY), we took two separate photographs of the head, breast, and rump of each bird against a gray-board, using identical distance from camera to object, shutter, exposure, and flash settings for each photograph and including a color/size standard in each photo to control for any slight variations in object illumination. Ambient lighting was kept constant by photographing finches inside a room with no external light. Digital images

(JPEG, 3648×2736 pixels) were imported into Adobe Photoshop to extract hue values for each of the three body regions photographed. Values for the two pictures of each bird were averaged for statistical analyses (repeatability=0.99, Lessells and Boag 1987) to obtain an overall plumage hue score for each bird. Because Photoshop assigns hue values around a 360° color wheel, with red set at 0, lower hue scores denote redder birds.

Carotenoids and vitamin E in plasma and tissues

We chose to measure plasma levels of vitamin E, in addition to carotenoids, because of its similar lipid solubility to carotenoids and because of its positive effects on carotenoid coloration in another bird species (yellow-legged gull, *Larus michahellis*; Pérez-Rodriguez et al. 2008). For carotenoid and vitamin E analyses, we vortexed 20 μL thawed plasma with 200 μL ethanol in a 1.5-mL Eppendorf snap-cap tube and then added 200 μL 1:1 hexane/*tert*-butyl methyl ether (MTBE). The mixture was then vortexed briefly again and centrifuged for 3 min at 10,000 rpm, at which time we transferred the supernatant to a 1.5-mL screw-cap Eppendorf tube and evaporated it to dryness under a stream of nitrogen. We resuspended the residue in 200 μL HPLC mobile phase (methanol/acetonitrile/dichloromethane, 46:46:8, *v*/*v*/*v*) and injected 50 μL into a Waters Alliance autosampler HPLC (Waters Corporation, Milford, MA) fitted with a C30, 5 μm Waters Carotenoid column (250×4.6 mm inner diameter; Waters Corporation) and a column heater (set at 30 °C). A gradient solvent system was run as previously described (McGraw et al. 2006), and carotenoids were identified and quantified by comparing their retention times and absorbance spectra to external purified standards. In our field samples, we detected a more complex and variable mixture of carotenoids (e.g., lutein, zeaxanthin, β-cryptoxanthin, 3-hydroxy-echinenone) than in our experiments, when birds circulated only lutein and zeaxanthin in experiment 1 (which were highly positively intercorrelated within samples; data not shown) as well as the canthaxanthin we supplemented in experiment 2.

For liver carotenoid analyses in experiment 2, we weighed a thawed, excised portion of the tissue to the nearest 0.0001 g, ground the tissue in the presence of 1:1 hexane/MTBE using a ball grinder (Retsch MM200), and followed the chemical extraction procedures described in Butler and McGraw (2010) prior to HPLC analyses (as above).

Oxidative stress analysis

Similar to previous studies of songbirds (Pérez-Rodriguez et al. 2008; Isaksson et al. 2009; Alonso-Alvarez et al. 2010; Giraudeau et al. 2013) and other animals (e.g., Hunnisett et al. 1995; Callaway et al. 1998; Oakes and Van Der Kraak 2003; Almroth et al. 2005), we measured thiobarbituric acid-reactive 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 mainly measuring levels of lipid peroxidation (even if other products such as the sialic acid present in glycoproteins may lead to an overestimation of the lipid peroxidation Devasagayam et al. 2003), which is 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^{-1} and are expressed in nanomoles per milliliter of MDA equivalents.

Statistics

We used ANOVAs to test or the effects of treatment(s), geographic location, and their interactions before the start of the experiment. We used general linear mixed models to test for the effects of treatment(s), geographic location, time, and their interactions on all of our variables. We have also included the individual identity as a random factor in all of these models. We reduced each full model stepwise by excluding the variable with the highest P value in each step until only P <0.05 predictors remained. Oxidative stress data were log transformed to achieve normality; no transformation could normalize plasma carotenoid, hue, liver carotenoid, and behavioral data, so we rankerized them (Conover and Iman 1981) before analyses. Data were analyzed using STATISTICA 6.0 software (Statsoft, Tulsa, OK, USA), SYSTAT 12, and R 3.01.

Results

Experiment 1

At capture Urban house finches had significantly higher hue scores (i.e., were less red; $F_{1,73}$=19.60, P<0.0001), higher body mass ($F_{1,80}$=5.40, P=0.02), and higher concentrations

of plasma zeaxanthin (Z=−3.49, P=0.0005) and β-cryptoxanthin (Z=−2.91, P=0.004) than desert birds. Desert males had a higher concentration of plasma 3-hydroxy-echinenone (the main red plumage carotenoid in house finches; Inouye et al. 2001) than urban males (Z=3.17, P=0.02), but similar levels of lutein (P>0.5). Urban birds had higher levels of oxidative stress (OS) than desert birds (Fig. 2, $F_{1,52}$=6.36, P=0.01). Urban and desert finches had similar tarsus lengths ($F_{1,74}$=2.71, P=0.104).

Plasmatic levels of carotenoids During the experiment, total carotenoid levels differed by site (significant site×time interaction), with urban birds having higher levels of carotenoids (Table 1, Fig. 2a). As expected, our carotenoid (CA) treatment significantly increased total levels of plasma carotenoids during the experiment (Table 1, Fig. 2a). The carotenoid treatment equally affected birds from the two sites, but the paraquat treatment differentially impacted desert and urban birds (Table 1, Fig. 2a). Desert birds circulated lower levels of carotenoids compared to urban birds when they were given paraquat+carotenoids whereas that was not the case in any other groups (Table 1, Fig. 2a). Finally, we did not find any significant interaction effects of the two treatments (paraquat and carotenoid) on total plasma carotenoid concentrations (Fig. 2a; see online supplementary material and Supplementary material Fig. 1 for the plasma zeaxanthin results).

Body mass At the start of the experiment, body mass did not differ between urban and desert birds ($F_{1,73}$=0.76, P=0.39) nor among treatments groups ($F_{3,73}$=0.45, P=0.72). Body mass significantly decreased over time in our experiment (Table 1, Fig. 2b), though these changes were very small in magnitude [e.g., CO birds lost only 0.1 g and CA birds lost 0.3 g (CA); on average, this is less than 2 % of total body mass]. Body mass was not influenced by site or the carotenoid treatment but was affected by the paraquat treatment (Table 1, Fig. 2b). Birds that received paraquat lost more mass than those from the other treatment groups (Table 1, Fig. 2b); the interactions between site and the two treatments were not significant. Finally, the time × site × treatment carotenoid interaction was significant. Within the PA group, urban birds lost significantly more mass than desert birds ($F_{2,32}$=7.88, P=0.0016), while in the BO group, desert birds tended to lose more mass than urban birds ($F_{2,32}$=3.15, P=0.057). Lost more mass than desert birds when they received Paraquat while the opposite pattern emerged when birds received the Paraquat+Carotenoid treatment (Table 1).

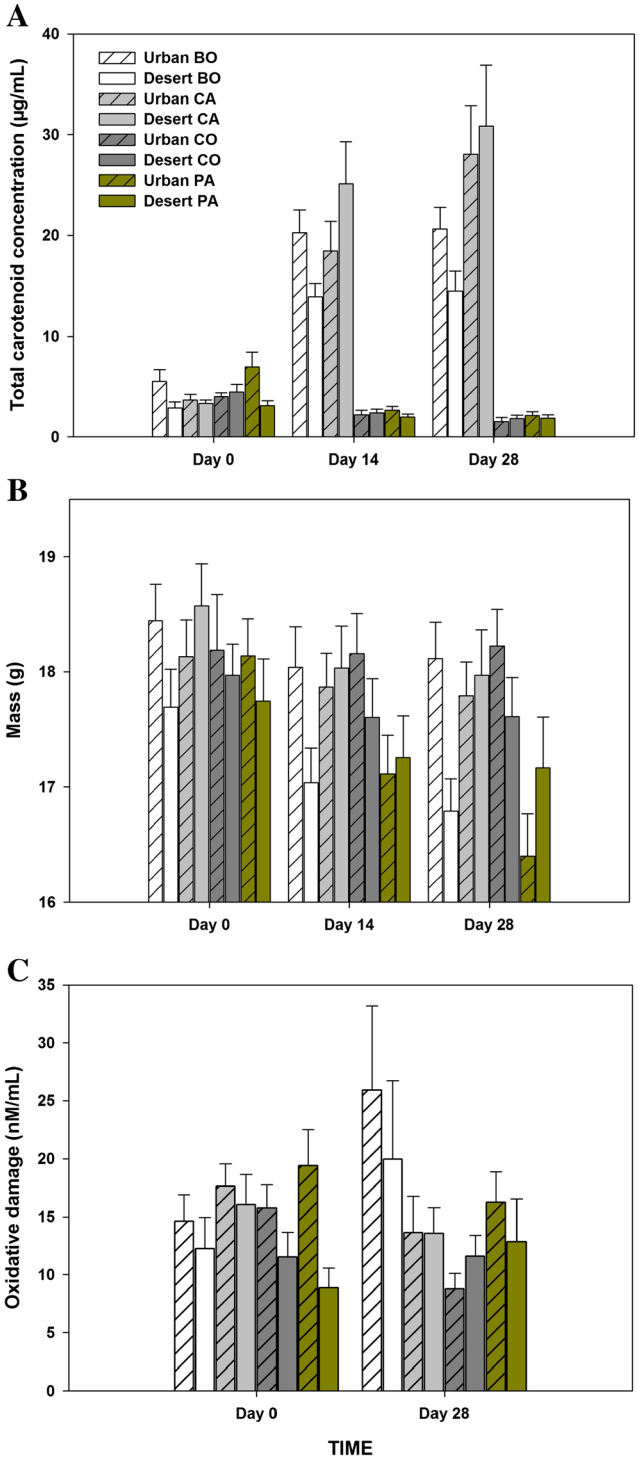

Fig. 2 Effects of paraquat (PA) and carotenoid (CA) administration on **a** plasma carotenoid concentrations, **b** body mass, and **c** oxidative damages levels during the first experiment (*BO* carotenoid+paraquat-supplemented group, *CA* carotenoid-supplemented group, *CO* control group, *PA* paraquat-supplemented group). Results are presented with means (+SE)

Oxidative stress The paraquat treatment significantly increased OS levels from beginning to end of the experiment in finches that received the paraquat+carotenoids, while these levels stayed constant in the other treatment groups, including the treatment group that received only paraquat (PA) (Table 1, Fig. 2c). We found no effect of

Table 1 Results of statistical tests examining the effects of geographic location, carotenoid, and paraquat supplementation on various physiological, behavioral, and morphological variables in house finches during two experiments (experiment 1=winter experiment; experiment 2=molting experiment)

Dependent variables	Factors		Initial model		Reduced model	
		df	*F*	*P*	*F*	*P*
Experiment 1						
	Plasma carotenoid levels					
	Time	2,209	**34.77**	**<0.0001**	**36.07**	**<0.0001**
	Site	1,209	2.80	0.10	2.91	0.09
	Carotenoid treatment	1,209	**16.82**	**<0.0001**	**17.47**	**<0.0001**
	Paraquat treatment	1,209	0.0062	0.94	0.065	0.93
	Time×site	2,209	**6.18**	**0.002**	**6.42**	**0.002**
	Time×carotenoid treatment	2,209	**221.70**	**<0.0001**	**230.48**	**<0.0001**
	Time×paraquat treatment	2,209	0.95	0.91		
	Carotenoid×paraquat treatment	1,209	0.51	0.60		
	Site×paraquat treatment	1,209	**4.36**	**0.04**	**4.53**	**0.04**
	Site×carotenoid treatment	1,209	0.11	0.74		
	Time×site×paraquat treatment	2,209	1.19	0.31		
	Time×site×carotenoid treatment	2,209	0.0092	0.99		
	Time×carotenoid×paraquat treatment	2,209	0.23	0.87		
	Site×carotenoid×paraquat treatment	1,209	0.17	0.68		
	Time×site×carotenoid×paraquat treatment	2,209	0.75	0.48		
	Body mass					
	Time	2,209	**38.80**	**<0.0001**	**38.80**	**<0.0001**
	Site	1,209	1.52	0.22	1.52	0.22
	Carotenoid treatment	1,209	0.52	0.47	0.52	0.47
	Paraquat treatment	1,209	**6.76**	**0.01**	**6.76**	**0.01**
	Time×site	2,209	1.11	0.33		
	Time×carotenoid treatment	2,209	1.44	0.24	1.35	0.26
	Time×paraquat treatment	2,209	**5.90**	**0.003**	**6.10**	**0.003**
	Carotenoid×paraquat treatment	1,209	0.35	0.55		
	Site×paraquat treatment	1,209	0.26	0.61		
	Site×carotenoid treatment	1,209	0.09	0.77		
	Time×site×paraquat treatment	2,209	0.56	0.57		
	Time×site×carotenoid treatment	2,209	**4.66**	**0.01**	**2.88**	**0.02**
	Time×carotenoid×paraquat treatment	2,209	2.88	0.06	**2.94**	**0.06**
	Site×carotenoid×paraquat treatment	1,209	**5.12**	**0.03**	1.45	0.23
	Time×site×carotenoid×paraquat treatment	2,209	**3.06**	**0.05**	1.78	0.13
	Eating behavior					
	Site	1,66	0.01	0.92		
	Carotenoid treatment	1,66	0.13	0.71		
	Paraquat treatment	1,66	1.40	0.24		
	Carotenoid×paraquat treatment	1,66	0.21	0.65		
	Site×carotenoid treatment	1,66	0.49	0.49		
	Site×paraquat treatment	1,66	0.73	0.40		
	Site×carotenoid×paraquat treatment	1,66	0.69	0.41		
	Drinking behavior					
	Site	1,66	0.45	0.50		
	Carotenoid treatment	1,66	2.81	0.10		
	Paraquat treatment	1,66	0.05	0.81		
	Carotenoid×paraquat treatment	1,66	0.77	0.38		

Table 1 (continued)

Dependent variables	Factors		Initial model		Reduced model	
		df	*F*	*P*	*F*	*P*
	Site×carotenoid treatment	1,66	0.35	0.56		
	Site×paraquat treatment	1,66	1.22	0.27		
	Site×carotenoid×paraquat treatment	1,66	0.41	0.53		
	Activity					
	Site	1,66	1.86	0.18	1.75	0.19
	Carotenoid treatment	1,66	0.23	0.64	0.26	0.61
	Paraquat treatment	1,66	3.39	0.07	3.58	0.06
	Carotenoid×paraquat treatment	1,66	3.92	0.06	3.85	0.052
	Site×carotenoid treatment	1,66	0.34	0.56		
	Site×paraquat treatment	1,66	1.40	0.24		
	Site×carotenoid×paraquat treatment	1,66	0.19	0.66		
	Oxidative damage					
	Time	1,113	0.43	0.52	0.41	0.52
	Site	1,113	**5.03**	**0.03**	**5.14**	**0.03**
	Carotenoid treatment	1,113	2.43	0.13		
	Paraquat treatment	1,113	0.37	0.55	0.37	0.54
	Time×site	1,113	0.47	0.23		
	Time×carotenoid treatment	1,113	1.23	0.27		
	Time×paraquat treatment	1,113	**6.31**	**0.01**	**5.36**	**0.02**
	Carotenoid×paraquat treatment	1,113	1.14	0.71		
	Site×paraquat treatment	1,113	0.80	0.37		
	Site×carotenoid treatment	1,113	0.39	0.53		
	Time×site×paraquat treatment	1,113	1.56	0.22		
	Time×site×carotenoid treatment	1,113	2.60	0.11		
	Time×carotenoid×paraquat treatment	1,113	1.26	0.27		
	Site×carotenoid×paraquat treatment	1,113	0.005	0.94		
	Time×site×carotenoid×paraquat treatment	1,113	0.05	0.82		
Experiment 2						
	Body mass					
	Site	1,297	2.91	0.10	2.94	0.09
	Time	7,297	**6.42**	**<0.0001**	**6.66**	**<0.0001**
	Paraquat treatment	1,297	0.77	0.38		
	Time×site	7,297	0.32	0.94		
	Time×paraquat treatment	7,297	0.43	0.88		
	Site×paraquat treatment	1,297	0.01	0.99		
	Time×site×paraquat treatment	7,297	0.49	0.84		
	Plasma carotenoid levels					
	Site	1,178	0.02	0.87	0.03	0.87
	Time	4,178	**15.39**	**<0.0001**	**15.37**	**<0.0001**
	Paraquat treatment	1,178	2.88	0.10	2.81	0.10
	Time×site	4,178	18.30	**<0.0001**	**17.79**	**<0.0001**
	Time×paraquat treatment	4,178	0.78	0.54		
	Site×paraquat treatment	1,178	1.86	0.18		
	Time×site×paraquat treatment	4,178	0.83	0.51		
	Carotenoid titers in liver					
	Site	1,34	3.39	0.07	3.43	0.07
	Paraquat treatment	1,34	0.40	0.53	0.58	0.45

Table 1 (continued)

Dependent variables	Factors		Initial model		Reduced model	
		df	*F*	*P*	*F*	*P*
	Site×paraquat treatment	1,34	0.55	0.47		
	Oxidative damage					
	Site	1,143	0.54	0.47	0.54	0.47
	Time	5,143	**4.91**	**<0.001**	**5.04**	**<0.001**
	Paraquat treatment	1,143	0.06	0.81	0.06	0.81
	Time×site	5,143	0.73	0.60		
	Time×paraquat treatment	5,143	1.88	0.10	**1.83**	0.09
	Site×paraquat treatment	1,143	**5.83**	**0.02**	**5.83**	**0.02**
	Time×site×paraquat treatment	5,143	0.62	0.68		
	Plumage hue					
	Site	1,64	1.66	0.21	1.63	0.21
	Time	1,64	**223.76**	**<0.0001**	**235.81**	**<0.0001**
	Paraquat treatment	1,64	1.23	0.28	1.20	0.28
	Time×site	1,64	0.52	0.48		
	Time×paraquat treatment	1,64	0.35	0.56		
	Site×paraquat treatment	1,64	0.52	0.48		
	Time×site×paraquat treatment	1,64	0.12	0.73		

Values in bold are statistically significant ($P<0.05$)

time or the carotenoid treatment on OS change during the experiment (Table 1). We found a significant effect of site on oxidative stress levels (due to the difference of oxidative stress levels at the start of the experiment), but we did not find a significant effect of the site on the change of oxidative stress levels during the experiment (time×site interaction). All interactions involving site and diet treatments were not significant (Table 1).

Behavior Time spent drinking and eating were not influenced by site, carotenoid treatment, paraquat treatment, or any interactions (Table 1). These results show that the four groups of birds that received the paraquat treatment (PA and BO in urban and desert birds) have drunk comparable amounts of paraquat during this experiment. In addition, these results show that potential effects of our treatments on body mass, oxidative stress, or circulating carotenoid levels were not due to an effect of the paraquat treatment on food/water intake. However, these results should be considered very cautiously since a post hoc power analysis (alpha level of 0.05) revealed that the power of detecting an effect of the paraquat treatment on the time spent eating and drinking were 0.2 and 0.11, respectively.

The number of hops per minute was not influenced by site, carotenoid treatment, and paraquat treatment. However, among the birds that received carotenoid, the number of hops tended to be reduced for those who also received paraquat compared to the birds that did not receive paraquat, while no effect of the paraquat treatment was found in birds that have not received the carotenoid treatment (Table 1).

Experiment 2

At capture Urban and desert house finches had similar hue scores ($F_{1,41}=2.66$, $P=0.11$). Desert birds had a higher body mass at the time of capture than urban birds ($F_{1,41}=5.66$, $P=0.02$). Urban and desert finches had similar tarsus lengths ($F_{1,41}=0.65$, $P=0.42$) and plasma vitamin E levels ($t=-1.6$, $P=0.12$). At capture, urban finches had lower concentrations of zeaxanthin ($t=4.3$, $P=0.0001$) and lutein ($t=-2.4$, $P=0.02$) but similar levels of β-cryptoxanthin ($Z=-0.61$, $P=0.54$) and β-carotene ($Z=-0.93$, $P=0.35$).

Plasmatic levels of carotenoids Total carotenoid concentrations differed by site at the beginning of the experiment ($F_{1,40}=21.40$, $P<0.0001$), with desert finches having a higher plasma carotenoid concentration than urban birds. However, total carotenoid levels did not differ among treatment groups prior to the experiment ($F_{1,40}<0.0001$, $P=0.98$; treatment×site: $F_{1,40}<0.0001$, $P=0.99$). In addition, lutein and zeaxanthin levels did not differ among treatment groups prior to the experiment (lutein: treatment: $F_{1,40}=0.12$, $P=0.73$; treatment×location: $F_{1,40}=0.20$, $P=0.66$; zeaxanthin: treatment: $F_{1,40}=0.29$, $P=0.59$; treatment×location: $F_{1,40}=0.24$, $P=0.53$).

During the experiment, total carotenoid levels differed by site (significant site×time interaction) but not treatment (Table 1, Fig. 3a). Plasma levels of carotenoids decreased in desert birds during the first month of the experiment, while these levels increased in urban birds. This difference is explained by the fact that desert birds started the experiment with higher levels of carotenoids. Then, at the end of the experiment, carotenoid levels increased for all the birds, but this increase was higher in urban birds (Table 1, Fig. 3a). The interaction between site and treatment did not influence total carotenoids levels during the experiment (Table 1; see supplementary material and the supplementary material Fig. 2 for the canthaxanthin results).

Carotenoid titers in liver Total liver carotenoid concentration was not significantly affected by treatment, but urban birds (in the BO group) tended to have higher liver carotenoid levels than desert birds (Table 1, Fig. 3b).

Body mass Desert birds had higher body mass at the start of the experiment than did urban birds ($F_{1,40}$=8.65, P=0.005), but birds from the different treatment groups did not differ in mass ($F_{1,40}$=0.1, P=0.75). Body mass significantly decreased over time during the experiment, but did not differ by site or treatment group (Table 1, Fig. 3c).

Oxidative stress Oxidative damage levels did not differ by site, but tended to be affected by the paraquat treatment during the experiment (paraquat treatment×time interaction, Table 1, Fig. 3d). Oxidative stress increased during the first month of experiment for CA birds and then decreased during the rest of the experiment, whereas these levels stayed constant during the entire experiment for BO birds. In addition, desert birds that received the paraquat treatment had higher levels of oxidative damage than birds that only received carotenoid (in July and October), which was not the case for urban birds (Table 1, Fig. 3d).

Carotenoid-based coloration Similar to numerous studies where house finches molt in captivity on a generally low-carotenoid base diet (Hill et al. 2004; Giraudeau et al. 2013), we found that ornamental plumage was less colorful at the end of the experiment than at the beginning. We found no effect of site, paraquat treatment, or the interaction between these two factors on carotenoid coloration developed during our experiment (Table 1, Fig. 3e).

Discussion

To better isolate possible carotenoid-specific mechanisms and differences in urban and desert house finches, we conducted two common-garden experiments in which we provided supplemental dietary carotenoid access and performed an oxidative challenge to determine if, under standardized environmental conditions, urban and desert birds exhibit physiological differences in carotenoid uptake or use abilities. During experiment 2, we showed that urban birds circulated higher levels of carotenoids and tended to have higher levels of carotenoids in the liver than deserts birds (when exposed to Paraquat) after 4 months on the same diet, suggesting that urban birds are better able to extract carotenoids (canthaxanthin) from food or do not use as many carotenoids as desert birds for pigmentation, antioxidant action, or immunomodulation. We also showed during our second experiment that when urban and desert birds are supplemented with carotenoids, they develop similar plumage color. Taken together, these results suggest that there are no substantial urban/desert differences in carotenoid assimilation that could translate into the site-related plumage color differences we observe locally in the wild (i.e., desert birds are redder).

We also showed that, compared to urban birds at capture, desert birds had higher body mass during molt but lower mass during winter. Similarly, desert finches circulated a lower concentration of zeaxanthin and β-cryptoxanthin (but a higher concentration of 3-hydroxy-echinenone and similar concentration of lutein) in winter but had higher levels of zeaxanthin and lutein during molt compared to urban birds. Thus, it seems that desert birds are more colorful than urban birds because of higher body condition and superior ability to either locate carotenoid-rich foods or assimilate those carotenoids from the food during the molt period. These results are in accordance with a recent study showing that rural populations have higher concentrations of vitamin E and carotenoids in their liver than urban populations in several bird species (Møller et al. 2010). Our results on body mass and plasma carotenoids levels in winter were more unexpected. The dietary availability of zeaxanthin and β-cryptoxanthin may decrease in desert environment in winter while the vegetation present in city parks or private gardens may provide a higher availability of these two carotenoids in urban environments. Finally, it should be noted that the differences of body mass between urban and desert finches at capture disappeared (before the start of the first experiment and after 2 weeks during the second experiment) when the birds were feeding on the same food in captivity, strongly suggesting that none of our results were influenced by these differences in mass at capture.

We also used an oxidative challenge to probe the antioxidant value/use of carotenoids in urban and desert house finches. Here, patterns of body mass loss during the first experiment revealed one of the few experimental differences we uncovered between sites, namely that PA treatment induced more of a mass loss in urban than in desert birds. Because we failed to find a significant increase in oxidative stress in PA birds during this experiment, it is difficult to identify the mechanism behind this difference of mass loss in this treatment group. One possibility is that the urban PA birds may

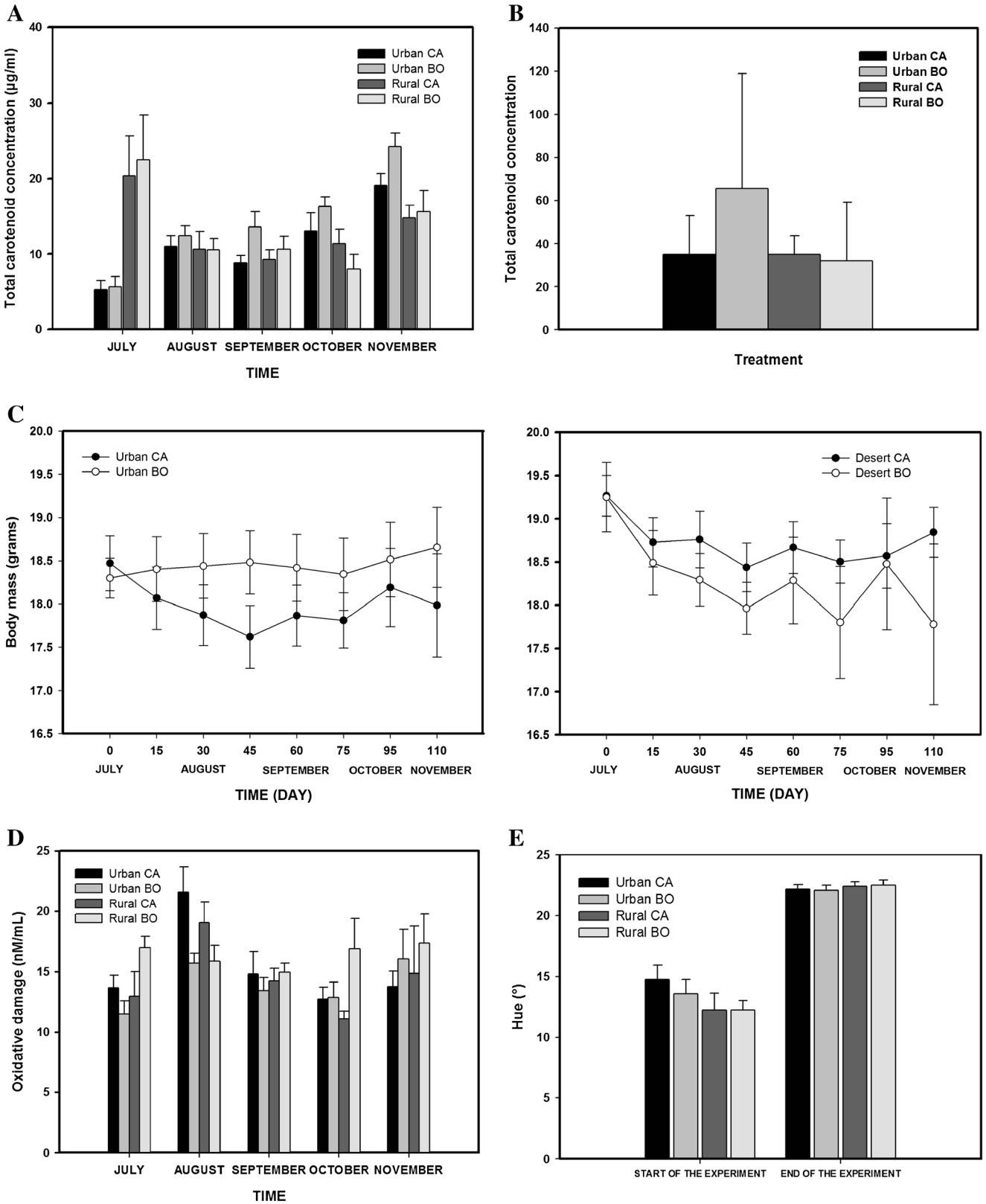

Fig. 3 Effects of paraquat (PA) and carotenoid (CA) administration on **a** plasma carotenoid concentrations, **b** liver carotenoid concentrations, **c** body mass, **d** oxidative damages levels in plasma, and **e** breast plumage coloration in urban and desert birds during the second experiment (*BO* carotenoid+paraquat-supplemented group, *CA* carotenoid-supplemented group). Results are presented with means (+SE)

have been more oxidatively stressed in ways not probed by the TBARS assay. Another hypothesis is that paraquat treatment may have had physiological effects other than increasing oxidative stress, such as on intestinal absorption (Berny 2007). However, BO birds did not lose more mass than CO birds, suggesting that carotenoid supplementation potentially helped birds avoid any deleterious effect of paraquat treatment on intestinal absorption.

Our experiment also generally speaks to possible relationships between carotenoids and oxidative stress that are currently debated in the avian literature (Hartley and Kennedy 2004; Alonso-Alvarez et al. 2004; Isaksson et al. 2005, 2007; Tummeleht et al. 2006; Costantini et al. 2007; Costantini and Møller 2008; Alonso-Alvarez and Galvan 2011). First, the fact that carotenoid levels were consistently higher in CA birds compared to BO birds during the first experiment suggests that carotenoids may generally be serving as antioxidants (hence drained in the system) in animals subjected to oxidative stress. This is one of the few experimental examples of this phenomenon in a wild-caught bird (von Schantz et al. 1999; Møller et al. 2000; Krinsky 2001; Surai 2002; Alonso-Alvarez et al. 2004). However, we cannot rule out the idea that paraquat may have reduced carotenoid absorption efficiency. The fact that we failed to detect a difference in plasma carotenoid levels between CA and BO birds during the second experiment when we gave them canthaxanthin suggests that the phenomenon observed during the first experiment may have a seasonal- and/or carotenoid-specific component.

Juxtaposed with this result, we found that oxidative stress levels were significantly increased only by the BO treatment and not by the PA treatment during the first experiment. It is unclear why the BO treatment would increase oxidative stress more than PA and not have a similar or increased effect as PA treatment on body mass. At first glance, one might presume that PA birds showed decreased food or water intake but our video monitoring data revealed that there were no significant differences in time spent eating or drinking across treatment groups. In addition, during the second experiment, we found that oxidative stress increased during the first month of experiment for CA birds and then decreased during the rest of the experiment, whereas these levels stayed constant during the entire experiment for BO birds. One possible idea behind these contrasting results during both experiments is that carotenoids concurrently serve as both antioxidants and pro-oxidants, rescuing some oxidative damage but also serving as degraded by-products that might also exert direct free-radical damage. The antioxidant and pro-oxidant actions of carotenoids have been touted elsewhere (Young and Lowe 2001; El-Agamey et al. 2004; Siems et al. 2005; Costantini and Møller 2008), and clearly what is now needed in the avian literature is a good index of carotenoid pro-oxidant activity to better identify its series of oxidant effects or more importantly its most dominant effect.

To the best of our knowledge, the idea that carotenoid-based color reflects individual antioxidant status (von Schantz et al. 1999) has been experimentally tested only two times in animals (Isaksson et al. 2007; Alonso-Alvarez and Galvan 2011). Isaksson et al. (2007) failed to detect any effect of an oxidative challenge induced by the administration of paraquat in the drinking water on carotenoid-based color of great tits. However, Alonso-Alvarez and Galvan (2011) found that red-legged partridges (*Alectoris rufa*) exposed to diquat during development developed paler carotenoid-based ornaments. In our molt experiment, we did not find any effect of paraquat administration on carotenoid-based coloration, consistent with the results of Isaksson et al. (2007). As proposed by Alonso-Alvarez and Galvan (2011), these contrasting results on the effect of an oxidative challenge on carotenoid-based coloration may be explained by the use of different methodology (adult vs. growing animals, diquat vs. paraquat) or by species- or tissue-specific (plumage vs. bare parts) responses to oxidative challenges. We now need to repeat similar experiments in free-ranging animals in order to assess how an oxidative challenge would affect animal coloration in the field (i.e., with natural molt conditions and carotenoid intake).

In summary, we uncovered little evidence that urban and desert birds differ in key carotenoid-accumulation or oxidative-stress physiological parameters that could explain geographic variation in carotenoid-based coloration observed in the wild. Thus, our results strongly suggest that local environmental factors (e.g., food availability, plant diversity and abundance) drive urban/desert differences in carotenoid ornamentation in house finches. Finally, we see this complex carotenoid system in house finches as one that is ideal for examining the spectrum of antioxidant and prooxidant actions and benefits in wild animals—something that has largely been addressed under artificial/lab settings or in domesticated animals to date.

Ethical Standards The study was approved by the Arizona State University Institutional Animal Care and Use Committee, and it complies with the current laws of the USA.

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