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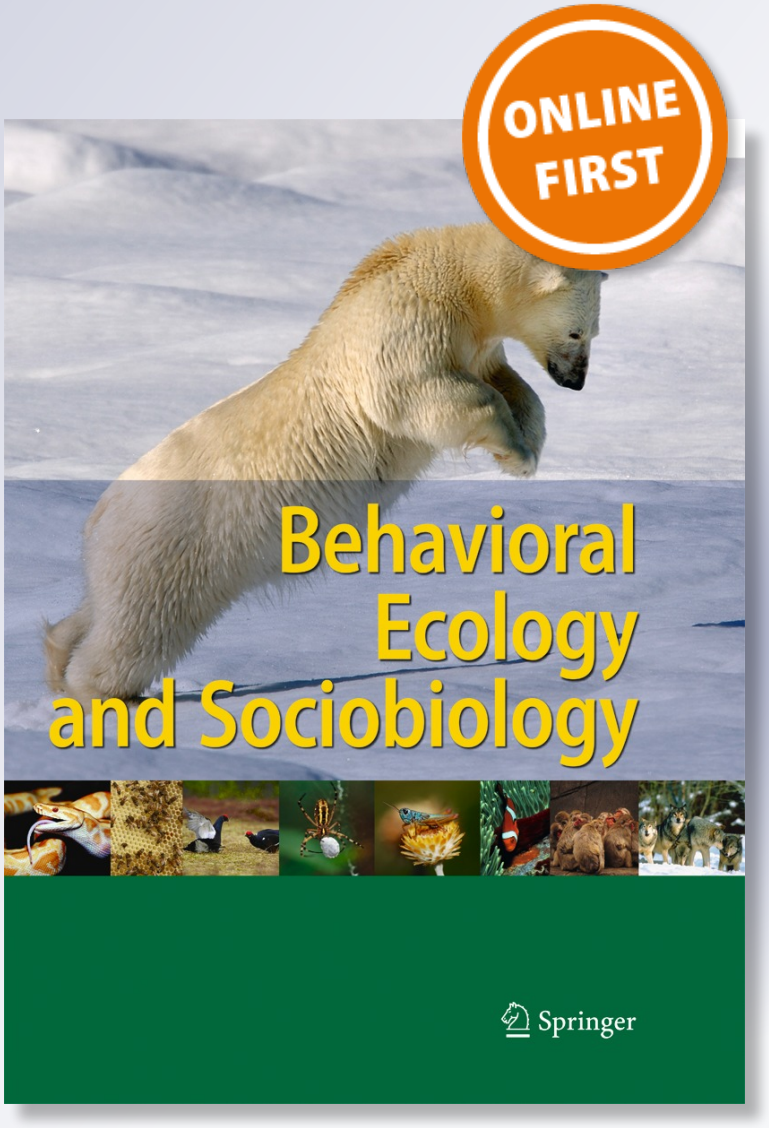
Behavioral Ecology and Sociobiology

ISSN 0340-5443

Behav Ecol Sociobiol
DOI 10.1007/s00265-013-1681-8



Springer

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ORIGINAL PAPER

Phaeomelanin- and carotenoid-based pigmentation reflect oxidative status in two populations of the yellow warbler (*Setophaga petechia*)

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Received: 19 September 2013 / Revised: 24 December 2013 / Accepted: 31 December 2013

Abstract Carotenoid- and phaeomelanin-based sexual pigmentation may signal a capacity to maintain oxidative balance and viability. However, diverse empirical results leave the association between pigmentation and oxidative stress (OS) unclear. We assessed the hypothesis that population-specific levels of oxidative challenge, or strategies for managing OS, affect relationships between sexual pigmentation and OS. Specifically, intense oxidative challenge in migratory, temperate breeding birds might enhance correlations between pigmentation and OS relative to allied tropical breeders, since quality-based differences in OS may arise only under intense oxidative challenge. Alternatively, in temperate breeders with intense within-season reproductive effort, high-quality birds may invest in reproduction over oxidative balance, dampening negative correlations between pigmentation and OS. To assess these alternatives, we compared prenesting relationships between pigmentation and OS in a migratory, Californian population of yellow warblers (*Setophaga petechia brewsteri*) and in a resident, Mexican population (*Setophaga petechia bryanti*, "mangrove warblers"). Yellow warblers displayed higher OS than mangrove warblers. However, year of capture and sex had bigger influences on correlations between pigmentation and OS than population. Males with more intense melanin pigmentation had lower OS among mangrove warblers and yellow warblers captured in 2011, but not among yellow warblers captured in 2012. In females only, lower OS levels were associated with more colorful carotenoid pigmentation. Results suggest that both phaeomelanin- and carotenoid-based pigmentation have the potential to correlate with OS levels, but that the signaling potential of pigmentation may shift with inter-annual variation in environmental conditions and display sex-specific dynamics.

Keywords Oxidative stress · Oxidative challenge · Sexual pigmentation · Life history · *Setophaga petechia*

Communicated by K. McGraw

Electronic supplementary material The online version of this article (doi:10.1007/s00265-013-1681-8) contains supplementary material, which is available to authorized users.

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Introduction

Viability costs of sexual ornamentation are required to enforce reliable signaling of individual quality (Grafen 1990). Oxidative stress (OS) is one physiological mechanism that may generate a viability cost of ornamentation. OS arises when production of pro-oxidants by metabolic processes or immune reactions overwhelms antioxidant defenses and damages biomolecules, and has critical effects on organismal fitness, ultimately leading to senescence and morbidity (Alonso-Alvarez et al. 2004a, b; Costantini and Verhulst 2009; Monaghan et al. 2009). Expression of diverse varieties of ornamentation may increase oxidative challenges by elevating metabolism and energetically taxing aggressive interactions (Garratt and Brooks 2012). However, pigment-based sexual coloration may be especially associated with oxidative status, because several types of pigmentation are generated from molecules with antioxidant functions. Carotenoids act as micromolecular antioxidants, as well as underlying sexual

Published online: 23 January 2014

coloration (von Schantz et al. 1999; Alonso-Alvarez et al. 2008; Catoni et al. 2008). Further, phaeomelanin-based pigmentation is manufactured directly from cysteine, which is also essential to the critical thiol-based antioxidant glutathione (Galván and Solano 2009). Thus, oxidative stress may inhibit pigment production, and intense pigmentation may indicate the capacity to maintain oxidative balance.

However, recent studies have found positive, negative, and null relationships between levels of OS and sexual pigmentation, leaving the relationship between OS and sexual pigmentation far from solidly established (Alonso-Alvarez et al. 2004b; Isaksson and Andersson 2008; Alonso-Alvarez and Galván 2011; Simons et al. 2012). One potential explanation for null relationships between pigment-based sexual signals and OS is that the molecules underlying pigmentation (e.g., carotenoids and melanins) do not play essential roles in antioxidant economy (Isaksson and Andersson 2008; Costantini and Møller 2008). However, another potential explanation for a diversity of relationships between sexual pigmentation and OS is that population level differences in oxidative challenges, or in the balance of tradeoffs between OS and reproductive effort, alter the relationship between sexual pigmentation and OS. Indeed, Galván and Møller (2013) propose that phaeomelanin-based pigmentation may more reliably indicate oxidative status under conditions of high OS, when the tradeoff between phaeomelanogenesis and glutathione should be more pronounced, and the same can be argued for carotenoid pigmentation. Indeed, male great tits (*Parus major*) with colorful carotenoid pigmentation displayed higher antioxidant capacity than other birds when investing in experimentally increased paternal effort, but not under normal conditions, when oxidative challenge is less intense (Losdat et al. 2011). Moreover, in addition to levels of oxidative challenge, how individuals balance tradeoffs between reproductive effort and maintenance of oxidative status may alter relationships between sexual pigmentation and OS. For example, in zebra finch (*Taeniopygia guttata*), lower oxidative stress correlates with more colorful carotenoid-based beak pigmentation in young birds, but not in old birds, because old birds terminally invest in pigmentation and reproduction over oxidative status (Cote et al. 2010). However, no study has considered whether population level differences in oxidative challenges, or in the balance of life history tradeoffs, affect the relationship between OS and either carotenoid or phaeomelanin pigmentation. Thus, we investigated this question in the context of a temperate versus tropical breeding population of a single species.

Previous research suggests that levels of oxidative challenge are higher in migratory, temperate breeding species of passerine birds compared to resident tropical species that are phylogenetically allied (Wiersma et al. 2004, 2007; Cohen et al. 2008; Williams et al. 2010). Migratory behavior may reduce oxidative challenges compared to species that are resident within highly seasonal environments, by allowing species to avoid energetically challenging environmental conditions (Galván et al. 2012). However, migration itself has high energetic and oxidative costs (Wikelski et al. 2003; Costantini et al. 2007). Thus, species that migrate are expected to face higher oxidative challenges than resident species from relatively stable environments. Further, migratory, temperate breeding birds often invest more reproductive effort per season than tropical species (Wiersma et al. 2007), and sometimes breed in more energetically challenging environments (e.g., arctic and alpine areas), both of which elevate oxidative challenges. Indeed, past studies have documented higher plasma antioxidant levels, higher muscle mass, and higher basal metabolic rates in temperate birds breeding in seasonal environments compared to allied tropical species (Wiersma et al. 2007; Cohen et al. 2008; Williams et al. 2010). These physiological differences may support higher and more concentrated per-season reproductive effort in temperate breeders, and lead to higher oxidative challenge and OS.

Thus, we predicted that OS levels should be higher in migratory temperate bird populations compared to allied tropical populations, and made two contrasting predictions regarding how the relationship between sexual pigmentation and OS might vary between these populations. First, assuming that sexual pigmentation is reduced in birds with a poor capacity to maintain oxidative balance (von Schantz et al. 1999; Alonso-Alvarez et al. 2007, 2008; Alonso-Alvarez and Galván. 2011), we predicted that intense oxidative challenge might enhance negative relationships between OS and sexual pigmentation during breeding in temperate birds relative to allied tropical counterparts. On the other hand, in populations with intense within-season reproductive effort, high-quality individuals might benefit by investing in reproduction over oxidative status and longevity (Wiersma et al. 2004, 2007; Lindström et al. 2009). In this case, negative correlations between OS and sexual pigmentation might be dampened in temperate compared to tropical populations. Indeed, in some species, males invest in reproductive effort over viability, but in longer-lived species with slower reproduction this may be less likely to occur (Hunt et al. 2004; Badyaev and Duckworth 2003; Cote et al. 2010).

To make a first assessment of how population level differences might modify the relationship between carotenoid- and phaeomelanin-based pigmentation and OS, we used a population comparison in the yellow warbler (*Setophaga petechia*), a species that displays both resident tropical populations and migratory populations of temperate breeders (Salgado-Ortiz et al. 2008). Specifically, we compared OS levels and correlations with sexual pigmentation between a migratory population of yellow warblers breeding in California (*Setophaga petechia brewsteri*; YEWA) and a resident population of "mangrove warblers" (*Setophaga petechia bryanti*; MANGWA) breeding in Yucatán, Mexico. Mangrove and

yellow warblers share the same basic pattern of pigmentation, though mangrove warblers display a melanic hood that yellow warblers lack (Fig. 1). Mangrove warblers in Yucatán display lower preseason reproductive rates and higher survivorship than California yellow warblers, and do not migrate (Salgado-Ortiz et al. 2008; A. Grunst, unpublished data; Electronic supplementary material (ESM) Table S1). Thus, we expected lower OS in mangrove warblers than yellow warblers. We first asked whether mangrove warblers and yellow warblers, or the two sexes, display different OS levels. Second, we asked whether correlations between sexual pigmentation and OS (or other condition metrics) differ between the populations, sexes, or types of pigmentation.

Materials and methods

Study system and field methods

In 2011 and 2012, we collected data on OS and sexual pigmentation in yellow warblers breeding along riparian corridors at the University of California's Sierra Nevada Aquatic Research Laboratory (SNARL) and in the adjacent Inyo National Forest. SNARL is located in Mono County, CA, USA on the eastern slope of the Sierra Nevada (37°36′51″N/ 118°49′ 47″W), at an elevation of ~2,100 m. The exact migration distance of our Californian study population is unknown, but lies between 2,000 and 6,000 km, based on the known winter distribution of northern yellow warblers (Lowther et al. 1999). Yellow warblers at SNARL face a short breeding season with nesting activity generally confined to the month of June. Further, environmental conditions at SNARL are physiologically challenging, with high altitude limiting oxygen supply, temperatures early in the season often near freezing at night, and storm systems causing early season snow. Yellow warblers at SNARL may also have greater opportunities for high within-season reproductive success than mangrove warblers, with larger clutch sizes and lower nest predation rates than those documented for mangrove warblers (Salgado-Ortiz et al. 2008; ASG, unpublished data; ESM Table S1).

Fig. 1 Male mangrove warbler (**a**) and *yellow* warbler (**b**). Both subspecies show carotenoid-based yellow coloration and phaeomelanin-based chestnut coloration, but mangrove warblers display a melanic hood that yellow warblers lack. Chemical analyses of the composition and concentration of pigmentation in feathers have not been performed in the yellow warbler. Thus, inferences about pigment composition and concentration are based on reflectance spectrum shape (see ESM Figure S1). See online version for color photographs

To obtain data on OS and pigmentation in mangrove warblers, we collected data in 2012 in a mangrove warbler population that resides in mangrove habitat near the Celestún Biosphere Reserve (20°51.5′N 90°24′W), Yucatán, Mexico. At Celestún, mangrove warbler nests are initiated beginning in May or late April (Salgado-Ortiz et al. 2008). In addition to the life history differences between yellow and mangrove warblers discussed above, mangrove warblers breeding at Celestún do not migrate and experience a more stable environment than yellow warblers at SNARL, with higher, less variable temperatures and elevations near sea level. Further, mangrove warblers at Celestún experience a slightly longer breeding season than yellow warblers at SNARL, with recorded first nesting attempts ranging between April 20 and June 20 (Salgado-Ortiz et al. 2008).

To capture mangrove and yellow warblers during a standardized life cycle stage, we captured mangrove warblers during mid-April 2012 and yellow warblers in early-mid May 2011 to 2012 after the prebreeding molt but prenesting. We lured warblers into mist nets using conspecific song playback and a conspecific decoy. We banded yellow warblers with a US Geological Survey (USGS) aluminum band and an additional combination of three colored leg bands and mangrove warblers with color bands only. We immediately removed birds from nets upon capture, and immediately released birds after collection of data. We obtained data from 40 male and 20 female yellow warblers in 2011 and from 36 males and 8 females in 2012. In 2012, we collected data on 52 male and 8 female mangrove warblers. However, sample sizes differ somewhat between statistical tests due to missing data points, as indicated in the results section.

At time of capture, we obtained small blood samples (see below) and took standard body measurements. Specifically, we measured unflattened wing chord (±1.0 mm), mass (±0.1 g), and tarsus length (±0.01 mm). To calculate an index of body condition, we later obtained the residuals of a mass on body size (wing chord) regression (Schulte-Hostedde et al. 2005). Further, we calculated residual wing chord as the residuals of a wing chord on tarsus length regression. Residual wing chord reflects body condition at postbreeding molt, since birds in better condition at molt tend to grow longer, more durable feathers (Harper 1999). We also aged all birds as second year (in their first breeding season) or after second year (in subsequent breeding seasons) based on molt limits and tail feather shape.

The University of California, Riverside's Animal Care and Use Committee (Protocol A-20100003E) approved all field protocols. A USGS bird banding subpermit (23035-G), a California state collecting permit (SC11060), a federal migratory bird collecting permit (MB22669A-0), and a special use permit through the Inyo National Forest (MLD100007P) authorized sample collection in California. A collection permit issued to J. Salgado-Ortiz by the Secretaria de Medio Ambiente y Recursos Naturales (00637/12) authorized collection of samples in Mexico.

Sampling blood and assaying OS

To assess oxidative status, we obtained ~70–100 μL blood samples via brachial venipuncture within 10 min of capturing birds. We stored blood samples on ice in the field. Upon returning to the field station, we centrifuged blood samples to separate cell fraction and plasma, and then froze samples pending transported to the University of California, Riverside (UCR).

At UCR, we stored blood samples for 3 to 5 months at −30 °C before performing assays. We stored yellow warbler samples collected in 2011 and mangrove warbler samples for 5 months and 2012 yellow warbler samples for 3 months. However, assay results for 2011 and 2012 yellow warblers did not differ significantly. Thus, variable storage times did not affect results.

We measured total antioxidant capacity (TAC) of the plasma using the OXY-adsorbent assay kit commercially available through Diacron International (and distributed by Innovatics Laboratories in the USA). We measured the concentration of reactive oxygen metabolites (ROMs) in the plasma using Diacron International's d-ROMs assay kit (Costantini and Dell'Omo 2006; Costantini et al. 2007). We performed assays using a Spectra Max Plus 96-well plate reader, capable of temperature control. In 2012, we randomly distributed yellow warbler and mangrove warbler samples across the wells of the 96-well plate. We ran samples in duplicate in both assays.

We performed the OXY-adsorbent assay using the manufacturer protocol, with the exception that we reduced plasma volume from 5 to 2 μL. In preliminary trials, we compared results obtained when using 5 versus 2 μL of plasma and obtained similar results. We diluted plasma 1:100 with distilled water (2 μL plasma in 198 μL water). We then generated a standard curve consisting of solutions capable of neutralizing 0, 115, 230, and 460 mM of hypochloric acid (HOCl), a generic antioxidant. We added 200 μL of HOCl and 5 μL of diluted plasma or standard to the microplate, and performed a pre-read of the plate to control for variation in sample absorbance. After a 5-min incubation at 37 °C, we added 2 μL of a chromogenic solution, mixed thoroughly, and immediately read absorbance at 505 nm. We report results in terms of millimolar of HOCl neutralized. Intra-assay coefficients of variation were calculated from the two replicates via the formula, $\frac{\sum \frac{SD}{mean}}{N} \times 100$, and averaged 5.0 % in 2011 and 8.3 % in 2012.

To perform the d-ROMs assay, we again followed manufacture instructions, but reduced plasma volume from 5 to 2 μL. Again, in preliminary trials, results were similar when using 5 versus 2 μL of plasma. We generated a standard curve consisting of 0, 1.32, 2.64, and 5.29 mM H_2O_2 (peroxide, the most common reactive oxygen metabolite). We then added 200 μL of a buffer solution and 2 μL of plasma to the microplate, and performed a pre-read of the plate to control for variation in plasma absorbance. Following the pre-read, we added 2 μL of chromogenic solution. We incubated the plate for 45 min at 37 °C, after which absorbance was read at 505 nm. Intra-assay coefficients of variation were 12.0 % in 2011 and 9.0 % in 2012. We report results in millimolar H_2O_2 equivalents. We calculated oxidative stress (OS) as: $\frac{\text{mM ROMs}}{\text{mM HOCl neutralized}} \times 1,000$ (Costantini et al. 2007), where millimolar HOCl neutralized was derived from the OXY-adsorbent assay, and was our measure of antioxidant capacity. We performed preparation for assays on ice to avoid oxidation of samples.

Measurement of sexual pigmentation

Upon capture of birds, we obtained digital photographs and feather samples to allow quantification of carotenoid- and phaeomelanin-based pigmentation. To measure area of phaeomelanic ventral streaking, we placed a 1 cm grid adjacent to the breast and used an Olympus Stylus 800 digital camera to take multiple photographs (two to four) from the front and both sides. We took all photographs outdoors in full shadow to maximize contrast between yellow carotenoid and red phaeomelanin color. We later used the color thresholding function in *ImageJ* to extract percent coverage of melanin-based pigmentation from photographs (Parker et al. 2003). We determined melanin coverage in a 2 cm × 1.5 cm rectangle centered at the top of the breast and in a 2 cm × 1 cm rectangle centered on the side of the bird at the top of the wing. We extracted percentages from two photographs from both the front and side of each bird, and averaged across pictures and body regions to obtain a final measure of melanin coverage.

We collected five yellow (carotenoid bearing) feathers from nonadjacent breast regions of all birds. In addition, from yellow warblers, we collected five breast feathers bearing melanic streaking, and from mangrove warblers we collected five crown feathers colored with melanin. We subsequently arranged feather samples on a black felt background (with zero reflectance) to mimic natural feather alignment. We used an USB4000 spectrometer with a xenon light source (range, 200–1100 nm; Ocean Optics Inc., Dunedin, FL, USA) to

obtain reflectance readings from feathers. We averaged five reflectance spectra from each patch of feathers, with readings spanning from 300 to 700 nm (across the avian visual range). We enclosed the probe of the spectrometer in a black rubber sheath to exclude ambient light, held the probe perpendicular to the sample, and slightly repositioned the probe between each reading.

To characterize reflectance spectra, we used colorimetric measurements of brightness (reflectance), saturation (chroma or spectral purity), and hue (spectral location). Carotenoid pigmentation displays a bimodal reflectance spectrum, with peaks of reflectance for both ultraviolet and yellow (or red) light, and high absorbance of blue-green light. Thus, to characterize the carotenoid reflectance spectrum, we calculated carotenoid saturation (chroma), blue saturation, ultraviolet saturation, average reflectance, and lambda 50 (Table 1) (Andersson and Prager 2006; Parker et al. 2003; Hegyi et al. 2007a). Lambda 50 is the wavelength at which reflectance is halfway between its minimum and maximum value, and is a measurement of hue. Previous studies suggest that as the concentration of yellow carotenoids in feathers increases, hue increases (shifts towards orange), average reflectance decreases, and ultraviolet and carotenoid saturation increases (Andersson and Prager 2006). Melanin-based pigmentation displays a simpler reflectance spectrum, with reflectance steadily increasing across the visible wavelengths. Thus, to characterize phaeomelanin-based spectra, we calculated red (melanin) saturation, average reflectance, and lambda 50 (Table 1). Previous studies indicate that more concentrated melanin pigmentation is less reflective, with lambda 50 shifted towards longer wavelengths and higher melanin saturation (McGraw et al. 2004, 2005). Since colorimetric variables were highly correlated, we performed a principal component analysis to derive single factors (PC1) descriptive of variance in reflectance spectra (Parker et al. 2003; Safran and McGraw 2004; Andersson and Prager 2006; Montgomerie 2006; Table 2). We refer to these factors (PC1) as "carotenoid saturation" and "melanin saturation" due to high positive loadings of these variables on PC1. For examples of reflectance spectra and histograms showing the distribution of colorimetric variables entered into principal components analyses, see ESM Figs. S1–S2.

Statistical analysis

We performed statistical analyses using R 2.15.2 (R Core Team 2012). ROM and OS levels were not normally distributed in our dataset. Thus, we used Wilcoxon sum rank tests to assess whether ROM or OS levels differed between the sexes or age classes across both populations combined. We then used a Kruskal–Wallis rank sum test to assess whether OS or ROM levels differed between populations (mangrove warblers, 2011 yellow warblers, and 2012 yellow warblers), and pairwise Wilcoxon sum rank tests with a Holm correction for multiple comparisons to assess which population categories differed significantly. TAC levels showed normal distribution and equality of variance between the groups, so we used ANOVA with Tukey's HSD post hoc tests to compare TAC between the populations. We used Levene's test to assess whether populations showed different variances in the three oxidative status metrics. We captured eight yellow warbler individuals in both 2011 and 2012. Thus, when comparing oxidative status variables between populations, we removed duplicate observations on individuals from the dataset. However, duplicate observations on individuals were retained when performing the linear mixed effects models used to assess relationships between pigmentation and oxidative

Table 1 Formulas for calculating colorimetric variables used to describe the shape of carotenoid- and phaeomelanin-based reflectance spectra

Variable	Formula	Interpretation
Carotenoid saturation (chroma)	$\frac{\text{median R700}-\text{median R450}}{\text{median R700}}$	Saturation of coloration in the yellow wavelengths
UV saturation	$\frac{\text{median R350}-\text{median R450}}{\text{median R350}}$	Saturation of coloration around the UV peak of lutein reflectance
Blue saturation	$\frac{\sum R435-\sum R500}{\sum R300-\sum R700}$	Saturation of coloration in blue wavelengths (low for lutein)
Red (melanin) saturation	$\frac{\sum R605-\sum R700}{\sum R300-\sum R700}$	Saturation of coloration in the red wavelengths
Average reflectance	Average(R300−R700)	Reflectance averaged across all wavelengths
Lambda 50 %	λ_{R50}, where, $R50 = \frac{R734+R450}{2}$	A measurement of spectral location, or hue

For carotenoid saturation, we calculated median R700 nm between 505 and 710 nm, because for yellow warbler, reflectance tended to rise towards maximum by 505 nm. We calculated median R450 nm between 400 and 500 nm, where reflectance was at a minimum for the lutein reflectance curve. Due to the bimodal nature of the carotenoid reflectance spectrum, when calculating carotenoid and UV saturation we used the median wavelength of maximum reflectance, rather than total reflectance (ΣR300−R700), as the denominator of the saturation (chroma) equation. Thus, carotenoid saturation characterizes the reflectance peak in the visual wavelengths (around 700 nm) relative to the reflectance minimum (around 450 nm), whereas UV saturation characterizes the reflectance peak in the UV region (around 350 nm) relative to the reflectance minimum

R percent reflectance at the following wavelength in nanometer. λ wavelength in nanometer

Table 2 Results from principal components analyses on colorimetric variables. Loadings on variables and proportion of variance explained

	Carotenoid PC1	Melanin PC1
UV chroma	0.48	–
Carotenoid chroma	0.51	–
Blue chroma	−0.52	–
Total reflectance	−0.06	−0.57
Lambda 50	0.46	0.59
Red chroma	–	0.55
Eigenvalue	3.61	2.27
Proportion of variance	0.72	0.75

stress, which are described below. We indicate how many birds were duplicated in each analysis in the results section.

We used linear mixed effects models (LMM) fit with reduced maximum likelihood to explore if the relationship between TAC and ROM levels differed between populations, with individual identity entered as a random effect. We predicted the natural log of ROM levels from the interaction between TAC level and population, with TAC level centered to ensure accuracy of main effects. We used Satterthwaite approximations of degrees of freedom for final *F* tests (package lmerTest in R).

To analyze if oxidative stress level predicts sexual pigmentation differently across populations, we again employed linear mixed effects models with individual identity as a random effect. In males, we predicted coloration variables (melanin saturation, melanin coverage, and carotenoid saturation) from the interaction between population (mangrove warbler, 2011 yellow warbler, and 2012 yellow warbler) and oxidative stress, with age initially included as a covariate. After performing the linear mixed effects models, we used simple linear models (LM) to investigate whether oxidative stress predicted pigmentation variables within population categories. For analyses involving male pigmentation, we eliminated two second-year mangrove warblers from the dataset, because these birds displayed drastically lower levels of pigmentation than other males. Removing these individuals did not qualitatively alter results. For females, we analyzed only whether carotenoid saturation can be predicted from oxidative stress level, and used a linear model rather than a mixed effects model, since few females were sampled twice (we eliminated duplicate observations).

Finally, in males only, we used linear mixed effects models to investigate whether pigmentation variables could be predicted from the interaction between population and the two other metrics of condition obtained (residual wing chord and residual mass, in separate models). We corrected residual wing chord for age before entry into the model, since second-year birds retain wing feathers grown during the nestling stage, which are shorter than those of after second-year birds.

Results

Population differences in OS, ROMs, and TAC

In a dataset containing the populations combined, the sexes did not differ in OS, ROM, or TAC levels (Table 3). OS and ROM levels also did not differ significantly between age classes, but older birds had lower TAC (Table 3). Thus, we tested for a population effect on OS and ROMs in the sexes and age classes combined. When testing the population effect on TAC, we confirmed that relationships existed when the dataset was restricted to after-second-year birds, as well as in the entire dataset.

Within a dataset with duplicate observations removed, the populations differed significantly in OS (Kruskal–Wallis test: $\chi^2_2=14.03$, $p<0.001$), ROM ($\chi^2_2=19.81$, $p<0.001$), and TAC levels (ANOVA: $F_{2,\ 152}=4.93$, $p=0.008$). OS and ROM levels were higher in yellow warblers captured in both 2011 and 2012 compared to mangrove warblers, whereas yellow warblers captured in the two different years did not differ in either OS or ROM levels (Fig. 2; Table 3). Yellow warblers captured in 2011 also had higher TAC than mangrove warblers, but TAC did not differ between yellow warblers captured in 2012 and mangrove warblers, or between the two yellow warbler groups (Fig. 2; Table 3). Further, yellow warblers displayed higher variance in OS than mangrove warblers (Levene's test: $F_{2,\ 140}=8.80$, $p=0.001$). In pairwise comparisons, variance in OS was higher in both 2011 ($F_{1,\ 101}=15.23$, $p<0.001$) and 2012 ($F_{1,\ 93}=10.54$, $p=0.001$) yellow warblers compared to mangrove warblers, but did not differ between the yellow warbler groups ($F_{1,\ 86}=0.66$, $p=0.41$). Variance in TAC did not differ between populations (Levene's test: $F_{2,\ 159}=0.29$, $p=0.74$), and results regarding TAC levels were equivalent when restricting the analysis to after-second-year birds only. Further, TAC was positively associated with ROM levels across populations (LMM: $F_{2,\ 140}=42.44$, $\beta=0.12\pm0.01$, $p<0.001$, $N=143$ captures, 136 birds), with no indication that population and TAC interacted to predict ROM level (LMM: $F_{1,\ 138}=0.91$, $\beta=0.04\pm0.04$, $p=0.34$).

Sexual pigmentation and OS

Males with lower OS displayed higher melanin saturation, and melanin saturation also depended on an interaction between melanin saturation and population (see Table 4 for final model). However, the relationship between OS and melanin saturation did not differ consistently between mangrove warblers and yellow warblers caught in the two different years. Rather, the relationship between melanin saturation and OS was less negative in yellow warblers captured in 2012 than among mangrove warblers, whereas mangrove warblers and yellow warblers captured in 2011 did not differ in this relationship (see treatment contrasts in Table 4). Indeed, linear models

Table 3 Differences in oxidative stress (OS, ROMs/TAC×1000), reactive oxygen metabolites (ROMs, millimolar H_2O_2), and total antioxidant capacity (TAC, millimolar HOCl neutralized) between the sexes, age classes, and populations

	OS			ROMs (mM)			TAC (mM)		
	N	Mean±SE	*p*[a]	*N*	Mean±SE	*p*	*N*	Mean±SE	*p*
Sex									
Males	119	5.92±0.34	0.61	120	1.69±0.11	0.72	127	275.83±5.25	0.32
Females	24	5.56±0.69		26	1.70±0.28		35	279.59±10.42	
Age									
ASY birds	111	5.78±0.36	0.20	113	1.63±0.12	0.06	127	271.28±5.20	0.03
SY birds	32	6.16±0.57		33	1.89±0.20		35	296.07±10.06	
Population									
YEWA 2011	42	6.60±0.61	0.003[b]	42	2.05±0.22	<0.001	54	294.86±8.10	0.005
			0.97			0.53			0.22
YEWA 2012	39	6.58±0.60	0.005[c]	41	1.97±0.23	0.001	41	274.9±9.63	0.45
			0.97			0.53			0.22
MANGWA	55	4.47±0.30	0.003[d]	55	1.15±0.08	<0.001	60	261.0±6.80	0.005
			0.005			0.001			0.45

Means and sample sizes for population comparisons reported from a dataset with duplicate observations eliminated. Sample sizes differed slightly between analyses because we were unable to obtain results from both the d-ROM and OXY assays for some birds

ASY after second year, *SY* second year, *YEWA* yellow warbler, *MANGWA* mangrove warbler

[a] *p* values from Wilcoxon sum rank tests for OS and ROM levels, and *t* tests or Tukey's HSD tests for TAC

[b] YEWA 2011 contrasted to MANGWA, then YEWA 2012

[c] YEWA 2012 contrasted to MANGWA, then YEWA 2011

[d] MANGWA contrasted to YEWA 2011, then YEWA 2012

performed within population categories supported a negative relationship between melanin saturation and OS in 2011 yellow warblers (p=0.007) and mangrove warblers (p=0.02), but not in 2012 yellow warblers (p=0.87; Fig. 3; Table 5). Male melanin coverage and carotenoid saturation were unrelated to OS levels, irrespective of population (Tables 4 and 5). Further, age did not have a significant effect on male melanin saturation (LMM: $F_{1,\ 108}$=1.07, β=−0.34±0.32) or carotenoid saturation ($F_{1,\ 106}$=0.43, β=−0.11±0.17, p=0.51), so was dropped from these models. However, younger males had lower melanin coverage ($F_{1,\ 67}$=8.37, β=−3.16±1.09, p=0.005; Table 4), so we retained age in the model predicting melanin coverage.

In contrast to males, females with higher OS levels displayed lower carotenoid saturation (LM: $F_{1,\ 22}$=5.39, p=0.02; Fig. 4), although sample size was small. Population (β=−0.17±0.65, p=0.78) and age (β=−0.71±0.51, p=0.18) did not significantly correlate with female carotenoid saturation, when included as covariates in the model with OS levels.

Sexual pigmentation and other metrics of condition

Male warblers with longer residual wing chords displayed higher coverage of melanin (LMM: $F_{1,\ 85}$=6.76, β=0.77±0.28, p=0.007, N=127 observations, 121 birds, 50

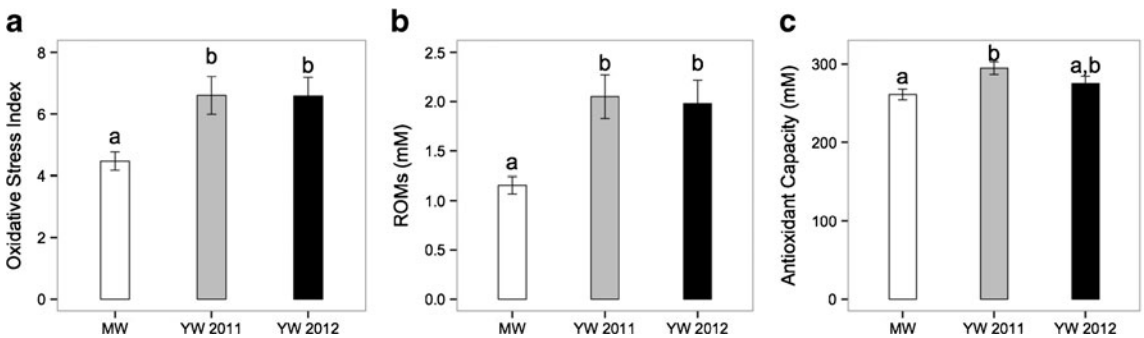

Fig. 2 Differences in oxidative stress (millimolar ROMs/millimolar TAC×1000) (**a**), ROM levels (**b**), and antioxidant capacity (**c**) between mangrove warblers (MW), yellow warblers captured in 2011 (YW 2011), and yellow warblers captured in 2012 (YW 2012). *Error bars* represent standard error. *Different letters above bars* indicate that these categories differ at the α=0.05 significance threshold

Table 4 Linear mixed effects models predicting male pigmentation variables from the interaction between oxidative stress and population

		Estimate (β±SE)	p (>t)[a]	F	$d.f.$	p (>F)
Melanin saturation[b]						
Intercept		0.41±0.20	0.04	–	–	–
Population	YEWA 11	−1.42±0.29	<0.001	15.68	2, 109	<0.001
	YEWA 12	0.04±0.29	0.88			
OS		−0.78±0.28		9.06	1, 109	0.003
OS×population	YEWA 11	0.37±0.32	0.26	3.22	2, 109	0.04
	YEWA 12	0.83±0.34	0.01			
Melanin coverage[c]						
Intercept		10.73±0.77	<0.001	–	–	–
Population	YEWA 11	6.76±1.22	<0.001	16.63	2, 78	<0.001
	YEWA 12	5.20±1.05	<0.001			
OS		−1.27±1.09		1.18	1, 89	0.28
OS×population	YEWA 11	1.46±1.19	0.22	0.76	2, 56	0.47
	YEWA 12	1.01±1.22	0.40			
Age		−3.16±1.09		8.37	1, 67	0.005
Carotenoid saturation[b]						
Intercept		1.33±0.10	<0.001	–	–	–
Population	YEWA 11	−0.36±0.16	0.02	3.19	2, 102	0.04
	YEWA 12	−0.02±0.16	0.89			
OS		0.19±0.15		0.29	1, 87	0.21
OS×population	YEWA 11	−0.35±0.18	0.06	2.43	2, 107	0.09
	YEWA 12	−0.11±0.19	0.54			

[a] p values for treatment contrasts from LMM, YEWA categories contrasted to MANGWA. Degrees of freedom for the t tests are the same as for the F test denominator

[b] N=115 observations, 108 birds, 34 YEWA from 2011, 34 YEWA from 2012, 47 MANGWA

[c] N=117 observations, 110 birds, 35 YEWA from 2011, 34 YEWA from 2012, 48 MANGWA

MANGWA, 76 YEWA), when controlling for population ($F_{1,\ 121}$=34.99, β=5.21±0.88, p<0.001) and age effects ($F_{1,\ 29}$=6.76, β=−2.33±0.89, p=0.01), with no indication that residual wing chord related differently to melanin coverage in the two populations ($F_{1,\ 117}$=0.12, β=−0.21±0.62, p=0.72, for the interaction term). Statistics reported are from a model simplified to code warblers only by population, rather than also by year. Indeed, in both populations separately, males with greater coverage of melanin had longer residual wing chords in both mangrove (LM: $F_{1,\ 48}$=3.95, β=0.92±0.46, p=0.05) and yellow warblers (LM: $F_{1,\ 45}$=6.07, β=0.69±0.35, p=0.05, controlling for age). The other pigmentation variables were not related to residual wing chord (p>0.06 in all cases, ESM Table S2),

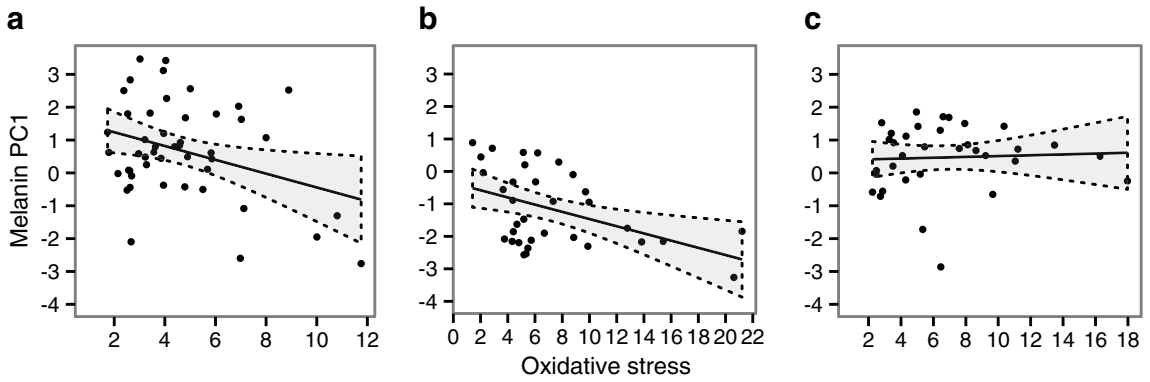

Fig. 3 Linear regressions of the relationship between melanin chroma and oxidative stress within population categories. Males lower oxidative stress had higher melanin saturation (PC1) in mangrove warblers (p=0.02) (**a**) and *yellow* warblers captured in 2011 (p=0.007) (**b**), but not in yellow warblers captured in 2012 (p=0.87) (**c**). *Shaded regions* represent 95 % confidence intervals

Table 5 Linear models predicting male pigmentation variables from oxidative stress within population categories

	Estimate (β±SE)	R^2	F	$d.f.$	p (>F)
Melanin saturation					
MANGWA	−0.21±0.08	0.09	5.69	1, 45	0.02
YEWA 11	−0.11±0.03	0.18	8.24	1, 32	0.007
YEWA 12	0.01±0.04	<0.001	0.07	1, 32	0.78
Melanin coverage					
MANGWA	−0.33±0.25	0.01	1.68	1, 46	0.20
YEWA 11	−0.05±0.21	<0.001	0.05	1, 33	0.81
YEWA 12	−0.14±0.19	<0.001	0.53	1, 32	0.46
Carotenoid saturation					
MANGWA	0.05±0.04	0.02	2.32	1, 45	0.13
YEWA 11	−0.04±0.03	0.03	2.05	1, 32	0.16
YEWA 12	0.02±0.02	<0.001	0.77	1, 32	0.38

and none of the pigmentation variables varied with male body condition (residual mass), irrespective of population (p>0.06 in all cases, ESM Table S3).

Discussion

Our results suggest that differences in oxidative stress co-occur with shifts in life history and environment across a temperate to tropical gradient (Wiersma et al. 2007; Cohen et al. 2008; Williams et al. 2010). As predicted based on life history and habitat differences (Salgado-Ortiz et al. 2008), yellow warblers displayed higher OS than mangrove warblers. Further, yellow warblers also displayed higher variance in OS than mangrove warblers, suggesting that more variation in OS levels may arise where oxidative challenges are intense, with potential implications for sexual signaling. Yellow warblers appeared to upregulate TAC to offset higher pro-oxidant production. However, higher plasma TAC in yellow warblers might also reflect compensation for low levels of alternative antioxidants, such as intracellular glutathione. For instance, great tits (*P. major*) compensate for the low glutathione levels associated with eumelanin (rather than phaeomelanin) production by increasing alternative plasma antioxidants (Galván and Alonso-Alvarez 2009). A more comprehensive assessment of different antioxidants would be needed to assess this possibility. In any case, mangrove warblers displayed lower OS indices due to lower ROM levels than yellow warblers, not due to higher TAC (Costantini and Verhulst 2009).

The mechanism by which lower pro-oxidant (ROM) production in mangrove warblers is accomplished is unclear, but may involve lower territorial effort (Studd and Robertson 1988; Goymann et al. 2004), nonmigratory habit (Costantini et al. 2007), and fewer environmental challenges (e.g., food limitation and cold temperatures early in the breeding season). In addition, mangrove warblers maintain long-term pair bonds (JS-O, unpublished data). Thus, in mangrove warblers, competition for females may be less intense than in yellow warblers, at least following initial pair formation. On the other hand, mangrove warblers display habitat saturation and intense competition for high quality territories (JS-O, unpublished data), suggesting that differences in intermale competition may not be the primary determinant of the differences in prenesting OS levels (see also Horton et al. 2010).

Regardless of mechanism, the fact that yellow warblers and mangrove warblers display different means and variances in OS and differences in life history strategy (Salgado-Ortiz et al. 2008), could affect pigment-based signaling of OS and viability in the two populations (Losdat et al. 2011; Vergara et al. 2011a, b). However, we found no evidence that pigment-based signaling of OS or other condition metrics differed with population. Further, we found no evidence that highly pigmented birds invest in pigmentation and reproductive effort at the expense of oxidative status, as might occur given

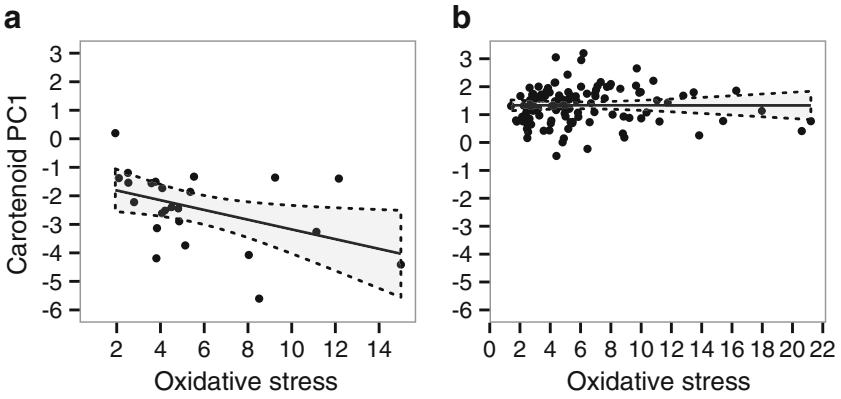

Fig. 4 Relationship between carotenoid saturation (PC1) and oxidative stress within the two sexes. Females (**a**) with lower oxidative stress had higher carotenoid saturation (LM: $F_{1,\ 22}$=5.39, β=−0.17±0.07, R^2=0.16, p=0.02; N=13 YEWA 2011, 6 YEWA 2012, 5 MANGWA), but there was no relationship between carotenoid saturation and oxidative stress among males (**b**) (LMM: $F_{1,\ 87}$=0.29, β=0.19±0.15, p=0.21). *Shaded regions* represent 95 % confidence intervals

prioritization of current reproduction over survival (Cote et al. 2010). Rather, in male warblers, melanin saturation correlated negatively with OS on a year- rather than population-dependent basis. Moreover, across years and populations, male melanin coverage and carotenoid saturation were unassociated with OS, and melanin coverage positively correlated with residual wing chord.

One potential reason that a relationship between phaeomelanin-based pigmentation and good oxidative status may persist in mangrove warblers, despite low levels of oxidative challenge, is expanded expression of phaeomelanin pigmentation in mangrove warblers, in the form of a melanic hood (Fig. 1). Galván and Møller (2013) suggested that phaeomelanin-based pigmentation might serve as a more effective sexual signal of individual quality under conditions of high OS, when the tradeoff between phaeomelanogenesis and glutathione is acute. However, under low oxidative challenge, expanded expression of phaeomelanin pigmentation could raise production costs, thus maintaining the utility of phaeomelanin-based signaling of oxidative status. On the other hand, the extent to which mangrove warblers express more phaeomelanin pigmentation than yellow warblers is unclear, since yellow warblers display more phaeomelanin-based breast streaking (Fig. 1; Table 4).

Further, the expected relationship between expression of phaeomelanin-based sexual pigmentation and level of oxidative challenge is not straightforward. For nonsexually selected pigmentation, less phaeomelanogenesis is expected under high oxidative challenge, due to the cost of reduced glutathione levels (Galván and Alonso-Alvarez 2011; Galván et al. 2011; Roulin et al. 2011). In contrast, sexual selection could more strongly favor phaeomelanin-based ornaments under high oxidative challenge, if phaeomelanin more effectively signals OS under these conditions (Galván and Møller 2013). Thus, if melanin pigmentation is sexually selected in *S. petechia*, one might expect greater melanism in yellow than mangrove warblers, but the opposite is observed with respect to the melanic hood (Fig. 1). A potential explanation is that the melanic hood is not sexually selected. In yellow warblers, melanin pigmentation has been linked to extrapair paternity, suggesting a sexual signaling function (Yezerinac and Weatherhead 1997), but the function of the melanic hood seen in mangrove warblers is unclear. However, natural as well as sexual selection acts on sexual ornaments and balance between the selective forces determines elaboration of ornaments in an often-complex fashion (Kokko et al. 2002).

Instead of population differences, inter-annual environmental variation experienced by yellow warblers appeared to cause shifts in the relationship between pigmentation and OS. Differences in oxidative challenges early in the breeding season did not seem to cause this year effect. Despite milder conditions at our California study site in 2012 (ASG, unpublished data), 2012 and 2011 yellow warblers did not differ in OS levels. As an alternative, environmental conditions during molt might have caused pigmentation to correlate differently with prenesting OS in the 2 years (Hegyi et al. 2007b; Scordato et al. 2012). Indeed, mean melanin saturation was higher in 2012 than 2011. Favorable winter conditions can increase mean expression of sexual pigmentation in birds (Hegyi et al. 2007b; Vitousek et al. 2012) and may reduce the relationship between individual quality and ornamentation, if variance in body condition at molt decreases (Scordato et al. 2012). The winter of 2011 to 2012 featured La Niña conditions, which are associated with wetter weather in the Neotropics and have been shown to improve condition in wintering warblers (Sillett et al. 2000). However, we lack direct knowledge of winter conditions experienced, and how climatically driven shifts in ornament expression affect correlations with individual quality remains unclear (Saino et al. 2004; Hegyi et al. 2007b; Scordato et al. 2012).

In addition to year effects, our results further suggest that sex may affect pigment-based signaling of OS, despite no difference in prenesting OS levels between the sexes. Specifically, male warblers with higher OS had lower melanin saturation, but carotenoid saturation was unrelated to OS in males. However, female warblers with higher OS levels had lower carotenoid saturation. Intersex differences in sexual signaling strategy may explain the divergence in how carotenoid pigmentation correlates with OS in the two sexes. For instance, in males but not females, some individuals may invest in carotenoid pigmentation at the expense of OS to achieve within-season reproductive gains (Cote et al. 2010). Alternatively, intersex differences in oxidative challenge or carotenoid availability during molt could cause shifts in how sexual pigmentation varies with oxidative balance during other life cycle stages. Indeed, in some Neotropical migrant warblers, females occupy suboptimal wintering territories (Marra and Holmes 2001), which may influence OS levels (van de Crommenacker et al. 2011).

The fact that phaeomelanin pigmentation reflected OS in males, whereas carotenoid pigmentation reflected OS in females, suggests that correlations can arise between different types of pigmentation and oxidative status even within different sexes of the same species. Thus, our findings are in opposition to the suggestion that carotenoid pigmentation is more strongly linked to condition metrics and oxidative status than melanin-based pigmentation (von Schantz et al. 1999), and rather supports a connection between melanin pigmentation and oxidative physiology (Galván and Solano 2009). A number of studies have reported that carotenoid pigmentation negatively correlates with OS (Simons et al. 2012), though contrary results have also been reported (Isaksson and Andersson 2008). Far fewer studies have reported a relationship between phaeomelanin pigmentation and OS. Roulin et al. (2011) found that barn owl (*Tyto alba*) nestlings with more phaeomelanin pigmentation are less resistant to OS, in

seeming contradiction to our results. However, in barn owls, phaeomelanism is associated with a distinct morph, which may be adapted to enviroments characterized by low OS. In contrast, in barn swallows (*Hirundo rustica*), phaeomelanin pigmentation is positively related to viability and fitness, suggesting higher individual quality and higher resistance to OS, in accordance with our results (Galván and Møller 2013).

In conclusion, our study provides important insights into sexual signaling of OS by carotenoid- and phaeomelanin-based pigmentation. Whether pigmentation correlated with oxidative status did not vary with population-level differences in oxidative challenges and life history, but rather varied on an inter-annual basis and with sex. Results suggest that both carotenoid- and melanin-based pigmentation have the capacity to convey information about good oxidative status (von Schantz et al. 1999; Galván and Solano 2009), with no indication that highly pigmented birds invested in sexual signaling or other elements of reproductive effort at the expense of oxidative status.

Acknowledgments We thank the Sierra Nevada Aquatic Research Laboratory (SNARL) and the Celestún field station of Ducks Unlimited Mexico (DUMAC) for providing facilities and access to field sites. Stephen J. Myers provided invaluable support by sponsoring the bird-banding subpermit (United States Geological Survey) essential to avian research in California. Jesús Vargas-Soriano (University of Campeche) provided critical field assistance, and David Costantini provided helpful advice on performing laboratory assays. Thanks also to Marlene Zuk, Daphne Fairbairn, Derek Roff and two anonymous reviewers for comments that aided in improving the manuscript. A National Science Foundation Doctoral Dissertation Improvement Grant to JTR and ASG, Sigma Xi, the University of California Natural Reserve System (Mildred E. Mathias Research Grant), and the Valentine Eastern Sierra Reserve provided funding.

Ethical standards and conflicts of interest We conducted all procedures described in this manuscript in accordance with rigorous ethical and legal standards, and the University of California, Riverside's institutional Animal Care and Use Committee approved all methods. The authors declare that they have no conflict of interest.

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