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Carotenoid-based sexual ornaments are hypothesized to be reliable signals of male quality, based on an allocation tradeoff between the use of carotenoids as pigments and their use in antioxidant defense against reactive oxygen species (ROS). Carotenoids appear to be poor antioxidants *in vivo*, however, and it is not clear whether variation in ornament expression is correlated with measures of oxidative stress (OXS) under natural conditions. We used single cell gel electrophoresis to assay oxidative damage to erythrocyte DNA in the common yellowthroat (*Geothlypis trichas*), a sexually dichromatic warbler in which sexual selection favors components of the males' UV-yellow "bib." We found that the level of DNA damage sustained by males predicted their over-winter survivorship and was reflected in the quality of their plumage. Males with brighter yellow bibs showed lower levels of DNA damage, both during the year the plumage was sampled (such that yellow brightness signaled current OXS) and during the previous year (such that yellow brightness signaled past OXS). We suggest that carotenoid-based ornaments can convey information about OXS to prospective mates and that further work exploring the proximate mechanism(s) linking OXS to colouration is warranted.

Keywords: oxidation handicap hypothesis, good genes, epigamic signaling

1. INTRODUCTION

Over a decade ago, von Schantz *et al.* (1999) proposed that a simple allocation tradeoff between the signaling and antioxidant functions of carotenoids may underlie good-genes sexual selection for colourful traits. Assuming that carotenoids are both limiting and an important component of an individual's total antioxidant defense, and recognizing that oxidative damage to DNA and other cell constituents can have negative consequences at the level of the whole organism, von

Schantz *et al.* (1999) hypothesized that the deposition of carotenoids in inert, ornamental structures imposes an oxidative handicap on males, preventing poor quality individuals from efficiently coping with the reactive oxygen species (ROS) produced during aerobic metabolism and immune activation. By preferring colourful males, females choose individuals that can most afford to divert carotenoids away from antioxidant defense and towards display; that is, they select healthy males in a favorable oxidative state.

The idea that ROS mediate sexual selection on colourful traits is compelling, in part because ROS may lie at the nexus of critical life history decisions in animals. For example, investment in reproductive activities generates ROS yet this investment may come at the expense of defense and repair mechanisms, yielding a cost of reproduction in the form of oxidative stress (OXS) that decreases survivorship, increases the rate of senescence, or both (Monaghan *et al.* 2009). Since pathways regulating ROS appear to be heritable (Kim *et al.* 2010), ornaments revealing OXS are potential targets of good-genes sexual selection.

Despite significant recent attention, ROS-mediated sexual selection remains controversial. For example, experimental manipulation of carotenoid supply often has little direct effect on ROS, antioxidant capacity, or measures of oxidative damage, leading to the general conclusion that carotenoids are poor antioxidants *in vivo* (Costantini & Møller 2008). However, supplementation with other antioxidants appears to free (or protect) carotenoids for eventual incorporation into ornaments (Pike *et al.* 2007), suggesting that colouration can signal total antioxidant capacity. Several recent studies have demonstrated changes in ornamentation with increased OXS (Torres & Velando 2007; Alonso-Alvarez *et al.* 2009; Mougeot *et al.* 2009), but there are exceptions (Isaksson & Andersson 2008).

Here, we use single-cell gel electrophoresis (SCGE) of erythrocytes to measure oxidative damage to DNA and relate this damage to male ornamentation in the common yellowthroat. Male common yellowthroats posses a carotenoid-based, UV-yellow "bib" (figure 1a) that is a condition-dependent signal of quality preferred by females in our population (Dunn *et al.* 2008; Freeman-Gallant *et al.* 2010).

2. MATERIAL AND METHODS

We studied common yellowthroats nesting along power line and riparian corridors in Saratoga County, New York from 2005-2009. Males were captured in mist-nests soon after arrival and filmed in standardized posture using digital video. We quantified the size (area) of the mask and bib using ImageJ, as described in Freeman-Gallant *et al.* (2010). Although our analyses focus on the bib, we included the melanin-based mask because the mask is both condition-dependent and a target of female choice in some populations (Dunn *et al.* 2010). Colourimetrics were obtained using UV-vis spectrometry (Ocean Optics 2000; Dunedin, FL) performed in the laboratory on feather samples collected at random from the center of each male's bib. We quantified yellow brightness, carotenoid chroma (C_{car}), and UV saturation (Table 1; see Freeman-Gallant *et al.* (2010) for details). C_{car} provides a measure of yellow saturation that is positively correlated with feather carotenoid concentration in some species (Isaksson & Andersson 2008).

In 2008-2009, we performed single cell gel electrophoresis (SCGE) on erythrocytes to quantify oxidative damage to DNA. At the time of capture, we diluted 50 μL of whole blood from the brachial vein in 1.0 mL of ice-cold buffer (10% DMSO, 90% Newborn Bovine Serum) and stored samples on ice until cryopreservation at -80°C. After thawing at 37°C for 2 min, erythrocytes were pelleted, washed in 1X PBS, and then mixed with low melting point agarose to

achieve a final suspension of 10 cells/μL. Two 75μL gels were poured onto a Trevigen (Gaithersburg, MD) CometSlide and subjected to SCGE in 1X TBE for 10 min at 35 V after first lysing cells and then denaturing DNA in alkaline solution (200 mM NaOH, 1 mM EDTA). Following SCGE, gels were washed in 70% ethanol and air dried. To visualize DNA, slides were stained with SYBR Green and digitally imaged at 25X. Comets representing erythrocyte nuclear DNA (in the "head") and any DNA degraded through single and double-strand breaks (in the "tail") were analyzed using Comet Score v1.5 (figure 1b). Percent DNA in the tail was averaged over all 204±53 (s.d.) comets scored for each male and square-root arcsine transformed prior to analysis. Average %DNA in the tail was repeatable across the two gels examined for each male (ANOVA, $F_{67,68}$=9.8, p<0.0001; repeatability=0.82; Lessells & Boag 1987). Early validation work in our laboratories showed no effect of cryopreservation on DNA damage (difference in mean damage between fresh and cryopreserved cells <1%; paired t-test; t=1.07, d.f.=13, p=0.31). We use DNA damage as an index of OXS because it combines both free radical production and attack as well as antioxidant and repair mechanisms. See Collins (2009) for discussion of SCGE and the comet assay as a measure of DNA damage caused by ROS and other sources.

In 2008, we obtained information on OXS and ornamentation for 17 males new to our study sites ("inexperienced males") and 16 males with a prior history of breeding ("experienced males"). In 2009, we studied 17 inexperienced males and 19 experienced males. We examined the relationship between DNA damage and ornamentation separately for inexperienced and experienced males because patterns of selection and condition-dependence are different in the two experience classes (Freeman-Gallant *et al.* 2010) and there are significant experience-by-ornament interactions in analyses of OXS pooling over males (ANCOVA, R^2=0.34, p<0.01,

n=48; experience*bib size, experience*yellow brightness, both $p<0.04$). To avoid pseudoreplication, we used the most recent data for males that were present in both years. Sample sizes vary where incomplete data forced the exclusion of some males.

3. RESULTS

OXS increased significantly with sampling date within a season, but there is no evidence that OXS increased with male age, either in cross-sectional analysis comparing inexperienced, first-time breeders (at our study sites) with experienced males (ANCOVA with year as a fixed effect, *n*=50; date: $F_{1,46}=11.5$, $p=0.001$; experience: $F_{1,46}=0.01$, $p=0.93$), or in longitudinal comparisons of OXS across successive seasons for 19 returning birds (paired $t=1.1$, d.f.=18, $p=0.31$). However, OXS was a significant predictor of survivorship from 2008 to 2009 (multiple logistic regression controlling for sampling date; OXS effect: Wald $\chi^2=5.1$, *n*=33, $p=0.02$; figure 1c). OXS was also reflected in the yellow colouration of the bib at the time of sampling. In multiple regressions of mask and bib traits on OXS, increasing OXS was associated with reduced yellow brightness (experienced males) and carotenoid chroma (inexperienced males) but not with mask size or bib UV colouration (Table 2). Bib yellow brightness (in the year *n*) also revealed the level of OXS experienced by males the preceding year (in the year *n-1*), when the plumage was obtained by males during molt (Table 2).

4. DISCUSSION

We found that elements of a carotenoid-based plumage ornament reflect OXS, as measured by SCGE and the comet assay. Experienced males with brighter yellow plumage and inexperienced males with greater carotenoid chroma showed reduced OXS which, in turn, was linked to greater

overwinter survivorship in our population. We have previously shown that sexual selection favors increased carotenoid chroma among inexperienced (but not experienced) males and that, at the population level, males with greater yellow brightness achieve higher mating success. By selecting brighter bibs, females mate with older (Freeman-Gallant *et al.* 2010) and healthier males (Dunn *et al.* 2010) who also show reduced OXS (this study).

Although our results suggest an important role for ROS in sexual signaling, OXS need not be revealed to prospective mates via direct, carotenoid-allocation tradeoffs. For example, if androgens are both pro-oxidants and important for the exaggeration of secondary sexual traits, an oxidative cost to ornament development may produce a relationship between OXS and the extent of exaggeration even if carotenoids are themselves irrelevant to ROS surveillance (Alonso-Alvarez *et al.* 2007). Additionally, the pro-oxidant consequences of infection coupled with a role for carotenoids in immune stimulation (McGraw & Ardia 2003) may generate complex associations between OXS, health, and ornamentation that do not necessarily rely on an antioxidant function for carotenoids. Attention to such indirect linkages has already proven fruitful (Mougeot *et al.* 2009; Alonso-Alvarez *et al.* 2009).

Different aspects of yellow colouration revealed more OXS in inexperienced versus experienced males. In addition, increasing bib size was correlated with DNA damage only among inexperienced males. To the extent that inexperienced males new to our study areas were younger than males with a known breeding history, these results suggest that the proximate mechanisms linking OXS to male ornamentation may change with male age. Age-related changes in sexual signaling have been found in other systems (Badyaev & Duckworth 2003) but they are rarely studied in the context of ROS-mediated sexual selection (but see Torres &

Velando 2007; Alonso-Alvarez *et al.* 2009; Cote *et al.* 2010), perhaps contributing to the mixed results in the literature.

To the best of our knowledge, this is the first study to relate DNA damage to sexual signaling in a vertebrate. Recent tests of ROS-mediated sexual selection measure the extent of lipid peroxidation when quantifying OXS, but ROS clearly affect other cell constituents (Monaghan *et al.* 2009). DNA damage caused by ROS may be particularly important, as this damage contributes to high rates of telomere shortening, abnormal patterns of gene regulation, and the etiology of many diseases (Halliwell & Gutteridge 2007). Indeed, the significance of DNA damage for proper cell function is revealed by the existence of a large number of complex damage recognition and repair pathways (Halliwell & Gutteridge 2007). Our results suggest that oxidative damage to DNA should be considered when assessing the information-content of ornaments and ROS-related costs of sexual signaling.

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Table 1. Colourimetrics based on reflectance spectrometry.

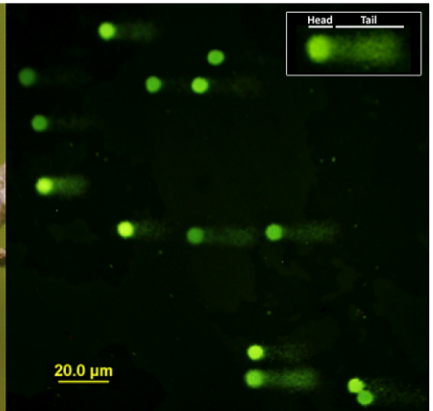
Yellow Brightness	average reflectance (R) across 550-625 nm	Avg ($R_{550\text{-}625}$)
UV Saturation	proportion of reflectance (R) across 320-700 nm attributed to reflectance in the UV (320-400 nm)	$\sum (R_{320\text{-}400}) / \sum (R_{320\text{-}700})$
Carotenoid Chroma	relative extent to which yellow reflectance (at R_{700}) exceeds blue-green reflectance (at R_{450})	$(R_{700} - R_{450}) / R_{700}$

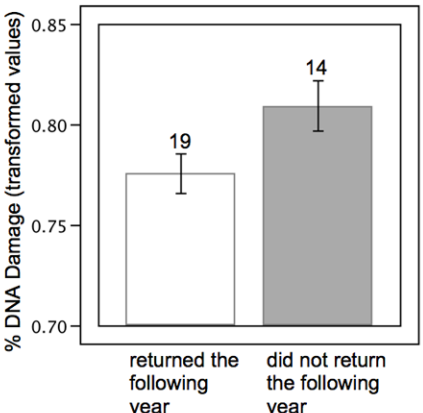
Table 2. Relationship between male ornamentation and oxidative stress (OXS) measured as %DNA damage to erythrocytes using SCGE. Ornaments were measured in the year (*n*) and OXS in the year (*n*) and (*n-1*). *F*-statistics are for multiple linear regressions.

	oxidative stress in the year *n:*						**oxidative stress in the year (*n-1*):**		
	inexperienced males*			experienced males†			experienced males§		
Overall Model	R^2=0.29, p=0.02			R^2=0.44, p=0.01			R^2=0.41, p=0.05		
	Effect	*F*	*P*	Effect	*F*	*P*	Effect	*F*	*P*
Bib Size	**0.02**	**4.7**	**0.04**		2.6	0.13		0.0	0.61
Bib Yellow Brightness		1.6	0.22	**-0.04**	**6.7**	**0.02**	**-0.03**	**5.4**	**0.04**
Bib Carotenoid Chroma	**-0.02**	**4.2**	**0.05**		1.4	0.26		1.9	0.19
Bib UV Saturation		1.2	0.27		0.3	0.57		0.0	0.99
Mask Size		0.2	0.70		0.6	0.45		0.9	0.38
Sampling Date	**0.03**	**10.1**	**0.004**		3.2	0.09	**0.03**	**9.2**	**0.01**

*n=33 males from 2008 and 2009; †n=23 males from 2008 and 2009 (using most recent data for each male to avoid pseudoreplication); §n=18 males from 2009; effect sizes are for data that have been standardized to a mean of zero with unit variance.

(*a*) (*b*) (*c*)


Head
Tail
20.0 µm


% DNA Damage (transformed values)
0.85
0.80
0.75
0.70
19
14
returned the following year
did not return the following year