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Plumage Ornaments Signal Male Physiological Quality in Common Yellowthroats

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by

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ABSTRACT

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by

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Elaborate ornaments are thought to honestly signal quality to potential mates. These ornaments may signal a variety of physiological processes that affect health and fitness. I examined the relationship between ornaments and physiological quality in a bird, the common yellowthroat (*Geothlypis trichas*). Male common yellowthroats have two plumage ornaments, a black (eumelanin-based) mask and a yellow (carotenoid-based) bib. Males with larger masks are preferred by females for both extra-pair and social mates. I found that both the mask and the bib of male common yellowthroats honestly signal their ability to resist oxidative stress. Males with larger masks and more colorful bibs also produce a greater amount of corticosterone, a hormone that releases stored energy and induces adaptive behavioral changes, during a short-term stress response. This suggests that these ornaments signal how well males cope with stressful situations. In contrast, neither the mask or the bib signal the infection intensity of haemosporidian parasites across males in the population. However, haemosporidian infection intensity was not related to overwinter survival or body mass, suggesting that these parasites may not be very costly. Together, these results suggest that both melanin- and carotenoid-based plumage ornaments honestly signal male physiological quality in common yellowthroats.

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Chapter I. Oxidative stress is related to both melanin- and carotenoid-based ornaments in the common yellowthroat

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Abstract

Male ornaments are hypothesized to signal the ability of males to produce an effective immune response without extensive oxidative stress and damage to DNA.

We examined this hypothesis in male common yellowthroats (*Geothlypis trichas*), which have two ornaments, a black (eumelanin-based) facial mask and a yellow (carotenoid-based) bib. In our study population, only the black mask is sexually selected. As predicted by the oxidative stress hypothesis, males with larger black masks were more resistant to oxidative stress, as measured by an in vitro assay of the resistance of erythrocytes to haemolysis by free radicals. Furthermore, males with larger masks also tended to have lower levels of glutathione, which was predicted because glutathione inhibits eumelanin production. In contrast, mask size was not related to absolute levels of oxidative stress measured in the plasma. Although the yellow bib is not under sexual selection in our population, males with larger bibs and feathers with greater carotenoid chroma had lower levels of oxidative stress. The oxidative stress hypothesis was first proposed for carotenoid-based ornaments. However, our results suggest that, even in the same individuals, carotenoid and eumelanin-based plumage ornaments may both signal the ability of males to resist or manage oxidative stress.

INTRODUCTION

Elaborate male ornaments, such as the colorful plumage of many species of birds, are used by females to choose mates (Andersson 1994), potentially because they signal mates that are healthier or more vigorous as a consequence of a superior immune system (Hamilton & Zuk 1982; von Schantz et al. 1999). An efficient immune response will result in greater disease resistance and also less oxidative stress (Costantini & Møller 2009), which is an imbalance in the ratio of harmful pro-oxidants to protective antioxidants (Sies 1991; Monaghan, Metcalfe & Torres 2009). This imbalance can lead to damage to important macromolecules, which could inhibit the expression of elaborate male ornaments. Thus, male ornaments may signal the ability of males to mount an efficient immune response and avoid oxidative stress (von Schantz et al. 1999).

Most tests of this hypothesis have focused on carotenoid-based ornaments (e.g. Pike et al. 2007; Pérez-Rodríguez, Mougeot & Alonso-Alvarez 2010; Simons, Cohen & Verhulst 2012), because carotenoids can be used both as antioxidants and pigments (McGraw 2006a), and, thus, ornament expression may reflect a trade-off with oxidative stress (von Schantz et al. 1999). However, fewer studies have examined black ornaments, which are pigmented with eumelanin. Eumelanin synthesis can also be negatively affected by oxidative stress (Moreno & Møller 2006; Galván & Alonso-Alvarez 2008; reviewed in Metcalfe & Alonso-Alvarez 2010), and, thus, black ornaments may also reflect a trade-off with oxidative stress. In this study, we examined the role of ornaments in signaling resistance to oxidative stress in a species with both carotenoid- and eumelanin-based plumage ornaments.

Producing eumelanin ornaments may increase oxidative stress in several ways. First, melanocytes that are actively producing eumelanin have high levels of reactive oxygen species (ROS; a type of pro-oxidant; Jenkins & Grossman 2013), which in turn can inhibit melanogenesis (Schallreuter et al. 2008) and lead to melanocyte apoptosis and senescence (reviewed in Meierjohann 2014). Secondly, glutathione (GSH), an important intracellular antioxidant (Wu et al. 2004), inhibits the production of eumelanin in addition to combating pro-oxidants (Prota 1992; Fig. 3; Galván & Alonso-Alvarez 2008). Therefore, to produce eumelanin ornaments, GSH levels must be low, which potentially leads to more pro-oxidants (Galván & Alonso-Alvarez 2008). Thus, individuals with large eumelanin ornaments may be advertising their ability to both produce a large ornament and manage the resulting increase in pro-oxidants through a strong antioxidant system (Moreno & Møller 2006; Metcalfe & Alonso-Alvarez 2010) or more effective DNA repair (Song et al. 2009).

The expression of carotenoid-based ornaments may also be associated with oxidative stress through several mechanisms. Carotenoids pigment many brightly colored ornaments (e.g. reds, yellows, oranges) and in contrast to melanins, which are produced endogenously (McGraw 2006b), vertebrates must acquire carotenoids from plants and invertebrates in the diet (McGraw 2006a). Carotenoids can be used as antioxidants (McGraw 2006a) and, therefore, individuals potentially face a trade-off between using carotenoids to prevent oxidative stress and using them to pigment ornaments (Olson & Owens 1998; von Schantz et al. 1999). Although recent evidence has suggested that carotenoids may not be a major part of antioxidant defenses (reviewed in Costantini & Møller 2008; Simons, Cohen & Verhulst 2012), it is possible

that carotenoid ornaments signal the ability of an individual to protect carotenoids from oxidation (Hartley & Kennedy 2004). Indeed, several studies have found that the size or brightness of carotenoid-based ornaments is negatively related to oxidative stress or damage (Mougeot et al. 2009; Pérez-Rodríguez, Mougeot & Alonso-Alvarez 2010) or positively related to other antioxidants (Pérez, Lores & Velando 2008; Trigo & Mota 2015). For example, in a New York (NY) population of common yellowthroats (*Geothlypis trichas*), females prefer males with larger and more colorful yellow bibs (Freeman-Gallant et al. 2010), and brighter bibs are associated with lower oxidative DNA damage (Freeman-Gallant et al. 2011). This suggests that carotenoid ornaments honestly signal resistance to oxidative stress.

In this study, we examined whether both eumelanin and carotenoid-based male plumage ornaments honestly signal the ability of males to resist oxidative stress in a Wisconsin (WI) population of common yellowthroats in which the eumelanin ornament is sexually selected. Males in both NY and WI have a black (eumelanin-based) facial mask and a carotenoid-based yellow bib. Despite similar plumage in both populations, females prefer males with larger black masks in WI and larger, more colorful bibs in NY (Dunn et al. 2008, 2010; Freeman-Gallant et al. 2010). There is no evidence for sexual selection on the yellow bib of males in WI (Pedersen, Dunn & Whittingham 2006). In each population, the ornament preferred by females (black mask in WI and yellow bib in NY) is positively correlated with male antibody production (IgG), body condition, survival (Dunn et al. 2010), and genetic variation at the major histocompatibility complex (MHC; Dunn et al. 2012; Whittingham et al. 2015), an important component of the adaptive immune response (Janeway et al. 2005).

Our aim in this study was to determine whether the size of the sexually selected black mask signals the ability of males in WI to resist oxidative stress. Although there are several possible reasons to expect this relationship, von Schantz et al. (1999) predicted that a more diverse MHC will produce a more effective immune defense that will reduce sickness and the production of pro-oxidants. Consistent with this hypothesis, the color and size of the bib are related to MHC variation (Whittingham et al. 2015) and resistance to oxidative damage (Freeman-Gallant et al. 2011) in the NY population. In WI, the sexually selected trait (mask size) is also related to MHC variation, but the relationship with oxidative stress is unknown. Based on our previous results, we predict *a priori* that mask size will be a more reliable signal of oxidative stress in the WI population than bib size and color. In this study, we test this prediction with several commonly used estimates of oxidative stress (see Methods) both immediately after capture and after experimentally inducing an immune response, which provided a standardized measure of resistance to oxidative stress.

METHODS

General Methods and Study Area

We studied common yellowthroats during May–June 2013 and 2014 at the University of Wisconsin-Milwaukee Field Station in Saukville, WI (43°23′N, 88°01′W). Males were caught in mist nets and individually marked with a USFWS band and a unique combination of colored leg bands. After capture, we measured body mass (to nearest 0.1 g) and tarsus length (to the nearest 0.1 mm). To measure mask and bib size, we took pictures of the head (both sides) and breast and traced each ornament in ImageJ

(http://imagej.nih.gov/ij/) after scaling the image to a 1-cm^2 grid in each picture. Bib color was estimated from reflectance spectrometer measurements (USB2000; Ocean Optics, Dunedin, FL, USA) of five feathers that were plucked from the center of the bib and overlapped on a black background (see Dunn et al. 2008, 2010 for more details). Color variables included yellow brightness (average reflectance from 550 to 625 nm) and carotenoid chroma [Ccar; (R at 700 nm – R at 450 nm)/ R at 700 nm]. Ccar is an estimate of saturation due to carotenoid concentration in the feather and is, therefore, positively related to feather carotenoid concentration, while brightness is negatively related to carotenoid concentration (Andersson & Prager 2006). Thus, as predicted, we found that carotenoid chroma was negatively correlated with yellow brightness (Fig. S1.1). Carotenoid chroma was also negatively related to bib size and positively related to mask size, but no other ornament measurements were correlated (Fig. S1.1). We examined bib size and the components of bib color separately because they are independently correlated with different aspects of male quality in NY (Freeman-Gallant et al. 2010, 2011; Taff et al. 2012; Taff & Freeman-Gallant 2014). We captured all males at our study site in 2012–2014. Males usually return to their territories or an adjacent territory each year, so we considered returning males to be experienced breeders and newly captured males to be inexperienced in 2013 and 2014. Breeding experience is likely related to age and is correlated with mask size in our population (Thusius et al. 2001).

We determined the resistance of each individual to oxidative stress using an in vitro assay of the resistance of erythrocytes to haemolysis by free radicals (Kit Radicaux Libres, KRL assay). For this assay, we collected 10 µL of whole blood, which was

immediately placed in 365 µL of KRL buffer (158 mM Na^+, 144 mM Cl^-, 6 mM K^+, 24 mM HCO_3^-, 2 mM $Ca2^+$, 340 mOsm, pH 7·4; Alonso-Alvarez et al. 2004) and stored on ice until the KRL assay was performed (4–10 h later). We performed the KRL assay with reagents prepared in our laboratory. To estimate oxidative stress, we measured the ratio of pro-oxidants (reactive oxygen metabolites, ROMs) to antioxidants (total antioxidant capacity, TAC) in the plasma (Costantini et al. 2006). We also quantified the level of the antioxidant GSH in erythrocytes. For analysis of ROMs, TAC and GSH, we collected a small blood sample (10–70 µL) from the brachial vein in heparinized capillary tubes and stored samples on ice packs in the field (maximum of 1–4 h). After collection, we centrifuged blood samples at 10 000 g for 5 min to collect plasma and erythrocyte samples, which were immediately placed on dry ice for transport (1–3 h) until they could be stored at −80 and −20 °C, respectively, for up to 10 months. We collected samples for the KRL assay in 2014 and the ROMs, TAC and GSH assays in 2013.

Resistance to Oxidative Stress (KRL Assay)

The KRL assay measures resistance to oxidative stress as the time for 50% of erythrocytes in a sample to haemolyse after being exposed to a free radical initiator [2, 2′-Azobis (2-methylpropionamidine) dihydrochloride, AAPH]. We performed the assay by adding 136 µL of AAPH solution (150 mm) to 40 µL of whole blood diluted in KRL buffer in a 96-well plate. Optical density of each well was measured at 540 nm every 10 min for 150 min using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). We left plates on the plate reader between reads, which was set to shake continuously at a constant temperature of 40 °C. We ran each sample in quadruplicate, and

repeatability was 0.85. Our analyses examined the mean time to 50% haemolysis from all successful replicates and the number of replicates (some failed to reach the 50% haemolysis point).

Reactive Oxygen Metabolites

Measuring pro-oxidants, such as reactive oxygen species, is difficult as these chemicals are short-lived (Monaghan, Metcalfe & Torres 2009). Therefore, to estimate pro-oxidants, we used the dROMs kit from Diacron International (Philadelphia, PA, USA) to measure hydroperoxides, which originate from damage caused to macromolecules (lipids, proteins, DNA) by pro-oxidants (Alberti et al. 2000). The dROMs kit provides an alkyl-substituted aromatic amine, which produces a photometrically quantifiable derivative after being oxidized by free radicals produced by ROMs in the sample. After thawing plasma, we centrifuged each sample and added 1 µL of each sample to a different well in a 96-well plate. We also added 1 µL of a standard and a blank (H_2O) to separate wells. After adding acidic buffer (200 µL) and alkyl-amine (2 µL) supplied by the manufacturer to each well, we incubated the plate at 37 °C for 90 min and then took a single reading at 505 nm using a microplate reader. We calculated total ROMs according to the manufacturer's instructions and report ROMs in Carratelli Units (CARR U) where 1 CARR U is the equivalent of 0.08 mg H_2O_2/dL. We ran all standards, samples and blanks in duplicate, and between-plate repeatability for this assay was 0.92 (n = 34), while within-plate repeatability was 0.93 (n = 102).

Total Antioxidant Capacity

We measured total antioxidant capacity (TAC) in plasma using a kit (Antioxidant Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) that measures the capacity of antioxidants

in a sample to inhibit the oxidation of ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulphonate)] by metmyoglobin. Plasma samples were diluted 1:20 with buffer supplied by the manufacturer and run with a Trolox standard in duplicate on 96-well plates according to the manufacturer's instructions. Absorbance at 750 nm was measured after 5 min of shaking on a microplate reader (Molecular Devices) and converted to mm Trolox equivalents, which was our estimate of TAC. Between-plate repeatability for this assay was 0.78 (n = 46), and within-plate repeatability for this assay was 0.91 (n = 122). This method of estimating TAC does not control for uric acid, which is produced during amino acid catabolism and is also a component of antioxidants. Thus, high levels of antioxidants could indicate greater catabolism rather than a greater ability to produce protective antioxidants (Cohen, Klasing & Ricklefs 2007). However, TAC and uric acid levels are correlated in many species (r = 0.79; Cohen, Klasing & Ricklefs 2007), and for the purposes of our study, we assumed that the effect of uric acid on TAC was not related to the size or color of male ornaments.

Glutathione

We used a GSH assay kit (Cayman Chemical) to measure total GSH in erythrocytes. This assay utilizes DTNB [5,5′-dithio-bis-2-(nitrobenzoic acid)], which reacts with the sulfhydryl group of GSH to produce TNB (5-thio-2-nitrobenzoic acid), for which absorbance can be measured at 405 nm. We lysed erythrocytes and deproteinated these lysates on the day of collection and stored samples at −20 °C. Prior to each assay, we added 0.5 µL of a 4 m solution of triethanolamine (TEAM) to 10 µL of lysate and then diluted each sample 1:50 with MES buffer provided by the manufacturer. Samples were run in duplicate on a 96-well plate, and absorbance was measured for 30

min at 5-min intervals on a microplate reader. To determine total GSH (μm) in samples, we performed calculations according to the manufacturer's instructions for the kinetic method. Between-plate repeatability for this assay was 0.87 (n = 192), and within-plate repeatability was 0.96 (n = 110).

Experimental Challenge with lipopolysaccharide

We also experimentally tested the relationship between ornament expression and the ability of males to resist oxidative stress during an immune response by injecting experimental males with lipopolysaccharide (LPS). LPS is a membrane component of gram-negative bacteria that induces an innate immune response and subsequent oxidative stress (Torres & Velando 2007; van de Crommenacker et al. 2010). We injected experimental males (n = 33) with 0.01 mg of LPS (Lipopolysaccharides from Escherichia coli 055:B5, L4005; SIGMA, St. Louis, MO, USA) dissolved in 50 μL of saline and control males (n = 16) with 50 μL of saline (0.9% sodium chloride, 0.308 mOsmol/mL; pH of 4.5–7.0).

Intramuscular injections were administered in the center of the right pectoral muscle after the skin was swabbed with ethanol and allowed to air dry. After injection, we transported males in a cloth bag to an outdoor aviary located at the field station (see Tarof, Dunn & Whittingham 2005 for aviary details). Each compartment in the aviary contained bushes and ground cover (straw) to approximate natural conditions. We provided each male with food (meal worms and fly larvae) and water ad libitum. We took a second blood sample from the other brachial vein 22 ± 2 h after the initial injection and then released males back onto their own territories. We quantified ROMs,

TAC and GSH before and after the injection of LPS to determine changes in oxidative stress.

Behavioral Observations

To better understand how the behavior of males changes during an immune response, we quantified foraging and flying activity after injection, as decreased activity has been observed in previous studies that used a similar dosage of LPS to stimulate an immune response (Lee, Martin & Wikelski 2005; Burness et al. 2010; Lopes et al. 2012). Four hours after injection, we observed each male (both LPS and saline injected) for 30 min to estimate changes in activity. We scored the number of flights, number of visits to food dish, and time spent foraging or perched (sitting) for each male.

Data Analyses

We predicted that males with larger masks would have the following: (i) greater resistance to oxidative stress, (ii) lower GSH and (iii) lower oxidative stress than males with smaller masks. Furthermore, we predicted that these relationships would be stronger for the mask than the bib (size and color) because the mask is the sexually selected trait in WI.

We tested these predictions in general linear models that included capture date, mask size, bib size, bib yellow brightness, carotenoid chroma, tarsus length, mass and breeding experience as predictors. We conducted all analyses in JMP 11 Pro (SAS Institute, Inc., Cary, NC, USA). We did not have data for all variables from all birds, so to test the robustness of these full models to changes in sample size as well as potential correlations between variables (multicollinearity), we also removed non-significant ($P > 0.15$) and correlated predictors from the full model to produce a reduced model.

Multicollinearity between predictor variables was checked with variance inflation factors (VIF) and separate bivariate correlations. Variables with relatively high VIF (>2.5, Neter et al. 1996) were removed in the reduced models. We did not perform corrections for multiple tests because our conclusions are based on a priori predictions tested in the full models and involve assays of different components of the blood (whole blood: KRL, plasma: ROMs/TAC, intracellular: GSH) that are not correlated. There were no significant correlations between GSH and ROMs/TAC ($r = 0.10$, $n = 31$, $P = 0.608$) or ROMs and TAC ($r = 0.25$, $n = 34$, $P = 0.161$). Few individuals had measures of both KRL and GSH ($n = 6$) or KRL and ROMs/TAC ($n = 2$) so we could not examine correlations between those variables. Note that the data set for the KRL analysis is independent of the other analyses because these birds were primarily measured in a different year. Bivariate correlations between measures of oxidative stress and ornaments are in Table S1.1.

Assuming that LPS stimulated the immune system and increased oxidative stress, we predicted that males with larger masks would have the following: (i) a smaller increase in oxidative stress and (ii) a larger decrease in GSH after LPS injection. For the analysis of experimental birds, we analysed change in GSH or ROM/TAC with a variable indicating treatment group (LPS or saline injected) and the same predictors as the unmanipulated birds mentioned above. Interactions between ornaments and treatment were tested in separate analyses because of small sample sizes.

RESULTS

Oxidative Stress and Mask Size

As predicted, the size of the black (eumelanin) mask was related to the ability of males to avoid oxidative stress. Overall, males with larger black masks had greater resistance to oxidative stress (KRL assay; Table 1.1; Fig. 1.1) and in the reduced model also tended to have lower levels of GSH after controlling for capture date, breeding experience and body mass (Table 1.2). However, mask size was not related to oxidative stress (ROMs/TAC) in the plasma in either the full or reduced model (Table 1.3).

Among the experimental birds (both LPS and saline injected), males with larger masks had a greater decrease in GSH than males with smaller masks, although this was only significant in the reduced model (Table 1.4). Mask size was not related to change in oxidative stress after injection (Table 1.5). Overall, there was no evidence that GSH or oxidative stress levels differed between the LPS and saline groups (Tables 1.4 and 1.5), or that it had differential effects on males with masks of different size. There were no significant interactions between mask size and treatment when tested in separate models that included capture date and breeding experience (GSH: $F_{1,16}$ = 0.39, P = 0.31; ROMs/TAC: $F_{1,6}$ = 0.08, P = 0.79).

Oxidative Stress and Bib Size and Color

Bib size and color were also related to several measures of oxidative stress. Overall, males with larger yellow bibs had lower oxidative stress in the plasma (ROMs/TAC, Table 1.3), but bib size was not associated with resistance of erythrocytes to an oxidative challenge (KRL assay, Table 1.1) or to GSH levels in erythrocytes (Table 1.2). Bib size was a significant predictor of resistance to oxidative stress in the full model, but this relationship was not significant in the reduced model with a larger sample size (Table 1.1). Males with brighter yellow bibs had more GSH (Table 1.2) and males with

greater carotenoid chroma had lower oxidative stress in plasma, after controlling for capture date and breeding experience (Table 1.3).

In the LPS experiment, males with larger bibs had a larger increase in oxidative stress (ROMs/TAC) than males with smaller bibs, but this was only found in the reduced model (Table 1.5). Bib size was not related to the change in GSH (Table 1.4). Bib color was also not related to change in GSH (Table 1.4) or change in oxidative stress (Table 1.5) after injection. There were no interactions between ornaments and treatment for either change in GSH or change in ROMs/TAC ($P > 0.37$). These interactions were tested one ornament at a time in separate models because of low sample sizes (12–22; Tables 1.4 and 1.5). Many of our experimental males were caught away from our main study area and, thus, their breeding experience was not known, which led to their exclusion from the analyses presented in Tables 1.4 and 1.5. Breeding experience has a strong effect on GSH and ROMs/TAC among unmanipulated birds (Tables 1.2 and 1.3; unlike KRL), so we considered it important to also include breeding experience in the analysis of the LPS experiment.

Capture Date and Breeding Experience

Oxidative stress and GSH were also influenced by day of capture within the breeding season and male breeding experience. Overall, males captured later in the breeding season had greater resistance to oxidative stress in erythrocytes (Table 1.1), lower oxidative stress in plasma (Table 1.3) and lower GSH (Table 1.2) than males captured earlier in the season. Experienced breeders had higher GSH (Table 1.2) and greater oxidative stress at capture (Table 1.3), but experience was not related to resistance to oxidative stress (Table 1.1). In the LPS experiment, males captured later in the breeding

season had a larger increase in oxidative stress after injection than males captured earlier in the season (Table 1.5); however, capture date was not related to change in GSH after injection (Table 1.4). Breeding experience also affected the results of the LPS experiment, as experienced males had a greater decrease in oxidative stress after injection (Table 1.5) than inexperienced breeders. However, breeding experience did not affect change in GSH after injection (Table 1.4).

LPS Challenge

Changes in GSH (Table 1.4) and oxidative stress (ROMs/TAC; Table 1.5) during the 22 ± 2 h males were held in the aviary were not related to treatment (i.e. LPS or saline injection). Treatment with LPS also did not have a significant effect on male behavior in the aviary, nor was behavior related to ornamentation (Table S1.2). However, males that weighed more at the start of the LPS experiment made more flights, and males captured later in the season spent less time foraging (Table S1.2).

Discussion

In this study, we examined the relationship between several measures of oxidative stress and a eumelanin-based ornament that is an honest indicator of several aspects of male immune function and health. As we predicted, males with larger black (eumelanin) masks had greater resistance to oxidative stress (in an in vitro challenge to whole blood; KRL assay, Table 1.6), even though they tended to have lower levels of GSH, an intracellular antioxidant (Table 1.2). However, contrary to our initial prediction, the mask was not the only trait related to measures of oxidative stress. Males with larger bibs and greater carotenoid chroma in their bib feathers also had lower oxidative

stress in some assays (e.g. ROMs/TAC in the plasma; Table 1.6). These results suggest that both eumelanin and carotenoid coloration can honestly signal oxidative stress in the same species. However, each plumage trait (mask and bib) was related to measurements of oxidative stress in different blood components (i.e. whole blood or plasma), which might help to explain the maintenance of multiple ornaments (Møller & Pomiankowski 1993).

Eumelanin Coloration and Oxidative Stress

Consistent with our previous results showing that males with a larger mask have higher antibody levels (Dunn et al. 2010), greater MHC variation and better survival (Dunn et al. 2012), this study suggests that mask size is an honest indicator of red blood cell resistance to oxidative stress. Thus, larger eumelanin ornaments may be signaling both stronger immunity and greater oxidative balance, as hypothesized by von Schantz et al. (1999). However, we know very little about the underlying mechanisms for this relationship, so it is also possible that mask size is only related indirectly to oxidative stress. For example, males with larger masks are dominant over males with smaller masks (Tarof, Dunn & Whittingham 2005) and, thus, they may have greater access to food or other resources that improve oxidative balance. It is also not clear what specific aspects of oxidative balance might be signaled by the mask, because other measures of oxidative stress (ROMs/TAC in the plasma) were only related to bib size and chroma (Table 1.3). Resistance to oxidative stress (KRL assay) is an index of antioxidants in both the plasma and the cell (Blache & Prost 1992), as well as previous oxidative damage to lipid membranes (Brzezińska-Ślebodzińska 2001; Alonso-Alvarez et al. 2007); in contrast, the ratio of ROMs to TAC measures the pro- and antioxidants only in

the plasma. It is possible that in our population, resistance of red blood cells to pro-oxidants is more closely related to male health than the ratio of ROMs/TAC. Alternatively, each measure of oxidative stress may affect male health equally and mask size may only be an honest signal of some aspects of oxidative stress. Until we know more about how each measure of oxidative stress is related to male quality, it may be necessary to measure several components of resistance to oxidative stress or balance to find links with ornament expression.

The relationship between producing eumelanin and managing oxidative balance is also not straightforward because a key intracellular antioxidant, GSH, inhibits eumelanin synthesis (Galván & Alonso-Alvarez 2008). Thus, individuals producing large eumelanin ornaments may have to decrease GSH, which could increase oxidative stress, unless they can increase other antioxidants to compensate (Galván & Alonso-Alvarez 2008). For example, in great tits (*Parus major*), nestlings produced larger black ornaments and increased plasma antioxidants after an experimental reduction in GSH (Galván & Alonso-Alvarez 2008). However, a recent experiment in house sparrows (*Passer domesticus*) suggested that the relationship between GSH and eumelanin bib size is also influenced by male quality and social environment, such that even under favorable experimental conditions, low-quality males cannot 'cheat' and produce a large black bib (Galván et al. 2015). In common yellowthroats, males with larger masks tended to have lower GSH at initial capture and had a larger decrease in GSH after being held 22 h in an aviary. However, neither levels of GSH ($r^2 = 0.01$, $F_{1,29} = 0.27$, $P = 0.608$) nor mask size (Table 1.3) were related to oxidative stress (ROMs/TAC) in the plasma. Instead, males with larger masks had greater resistance to oxidative stress

(KRL assay), suggesting that there might be other mechanisms to resist oxidative stress when levels of GSH are low.

Carotenoid Coloration and Oxidative Stress

Carotenoid coloration is predicted to honestly signal oxidative stress (von Schantz et al. 1999), and experimental studies have shown that reduced oxidative stress is associated with larger (comb size; Mougeot et al. 2009) or brighter (foot color, Torres & Velando 2007; eye ring color, Pérez-Rodríguez, Mougeot & Alonso-Alvarez 2010) carotenoid-based skin ornaments. In contrast, experimentally increased oxidative stress did not affect the production of carotenoid-based plumage in greenfinches (*Carduelis chloris*; Hõrak et al. 2010) or great tits (*Parus major*; Isaksson & Andersson 2008), suggesting that the relationship between carotenoid ornamentation and oxidative stress may be more direct in skin than plumage ornaments. Nevertheless, in common yellowthroats, a carotenoid-based plumage ornament (bib brightness and chroma) appears to be a reliable signal of less oxidative damage to DNA in NY (Comet Assay; Freeman-Gallant et al. 2011) and lower oxidative stress in WI (ROMs/TAC, this study).

If both the mask and bib signal some aspects of lower oxidative stress in WI, then why is the mask and not the bib sexually selected in the WI population? One reason may be that the mask is more informative about overall male quality. The bib was not related to other components of male quality such as antibody production, MHC variation and survival in WI (Dunn et al. 2010, 2012). Therefore, in WI the mask may signal more aspects of male health and quality than the bib. Mask size is also more variable in WI than bib size, which may make it an easier signal for females to use to discriminate among males (Dunn et al. 2008). It is also possible that the relationship

between the bib and measures of oxidative stress is simply a by-product of condition, and, as a consequence, healthy birds in good condition have low oxidative stress and can produce both large and colorful masks and bibs. In this case, we might not necessarily expect carotenoid-based plumage to be a target of sexual selection. Indeed, there was a negative relationship between oxidative stress and carotenoid chroma of the yellow breast feathers in female, but not male, yellow warblers (*Dendroica petechial*; Grunst et al. 2014). This suggests that carotenoid plumage does not necessarily have to be an ornament under sexual selection to honestly signal measures of oxidative stress.

The relationship between ornament expression and oxidative stress may also be influenced by several other factors such as sex (Grunst et al. 2014) and age (Cote et al. 2010). Controlling for time within the breeding season and male breeding experience is also potentially important because reproductive activities can increase oxidative stress (e.g. Alonso-Alvarez et al. 2004, 2006; Costantini 2008; Losdat et al. 2011). In our study, males captured later in the breeding season had greater resistance to oxidative stress and lower plasma oxidative stress, despite having lower GSH. We do not know whether the males sampled later in the season were still breeding, so it would be valuable to know how oxidative balance changes within individuals over the season. Experienced males had higher GSH, but contrary to expectation, they also had greater oxidative stress (ROMs/TAC).

LPS Experiment

Injection of LPS did not appear to increase oxidative stress in our study. This was surprising as other studies (using a similar dosage) have shown that LPS induces changes in oxidative stress (Torres & Velando 2007; van de Crommenacker et al. 2010)

and behavior (Lee, Martin & Wikelski 2005; Burness et al. 2010; Lopes et al. 2012). It is possible that the strength of immune responses may be adjusted to the oxidative balance of an individual and not result in oxidative stress (Cram et al. 2015). Nonetheless, in common yellowthroats, there were some changes in oxidative stress during captivity that were related to ornaments, as males with larger masks and bibs had a larger decrease in GSH and a larger increase in plasma oxidative stress (ROMs/TAC), respectively. The increase in oxidative stress for males with larger bibs (Table 1.5) was surprising because it was opposite of the pattern among unmanipulated birds (Table 1.3). Although the exact cause of these changes is unknown, it is possible more ornamented males experienced greater physiological stress (i.e. a greater increase in the stress hormone corticosterone) in the aviary, which could have led to a reduction in antioxidants (GSH) and an increase in plasma oxidative stress (Costantini, Fanfani & Dell'Omo 2008; Stier et al. 2009; Costantini, Marasco & Møller 2011). Greater plasma oxidative stress may also have resulted from higher levels of physical activity in the aviary (Costantini 2008); however, we did not find any differences in physical activity between more or less ornamented males based on our behavioral observations (Table S1.1).

This is one of a handful of studies to examine oxidative stress in relation to both carotenoid and eumelanin-based ornaments in the same species (Galván & Alonso-Alvarez 2009; Hõrak et al. 2010; Alonso-Alvarez & Galván 2011; Freeman-Gallant et al. 2011). The size of the black mask and color and size of the yellow bib were related to different components of oxidative balance in our population. Interestingly, in the New York population, where the bib is sexually selected, it was a better indicator of

resistance to oxidative damage than the size of the mask (Freeman-Gallant et al. 2011) and also correlated with greater MHC variation (Whittingham et al. 2015). Thus, even in the same species, different ornaments can potentially signal similar aspects of male health and vigor in different populations.

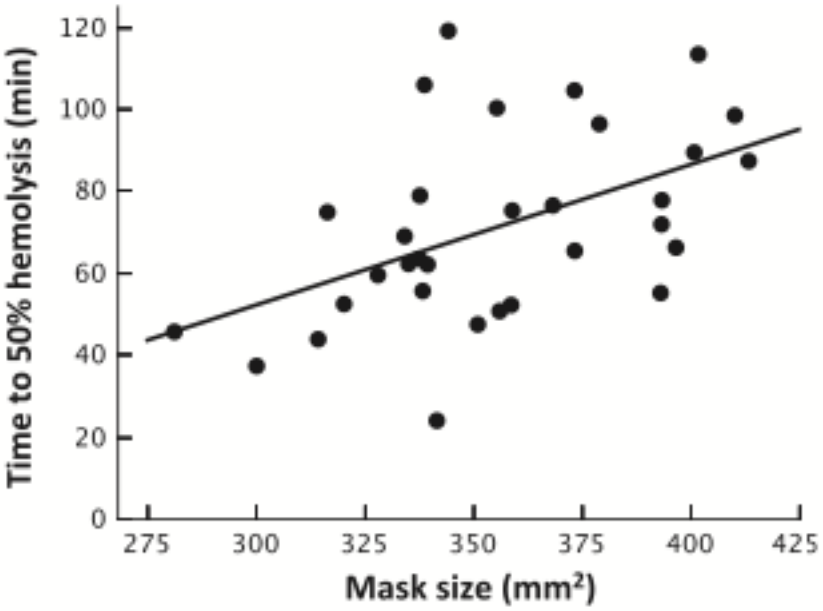

Fig. 1.1. Relationship between mask size and resistance to oxidative stress (KRL assay; $F_{1,8} = 14.61$, $P = 0.005$). Regression line is from the reduced model in Table 1.1.

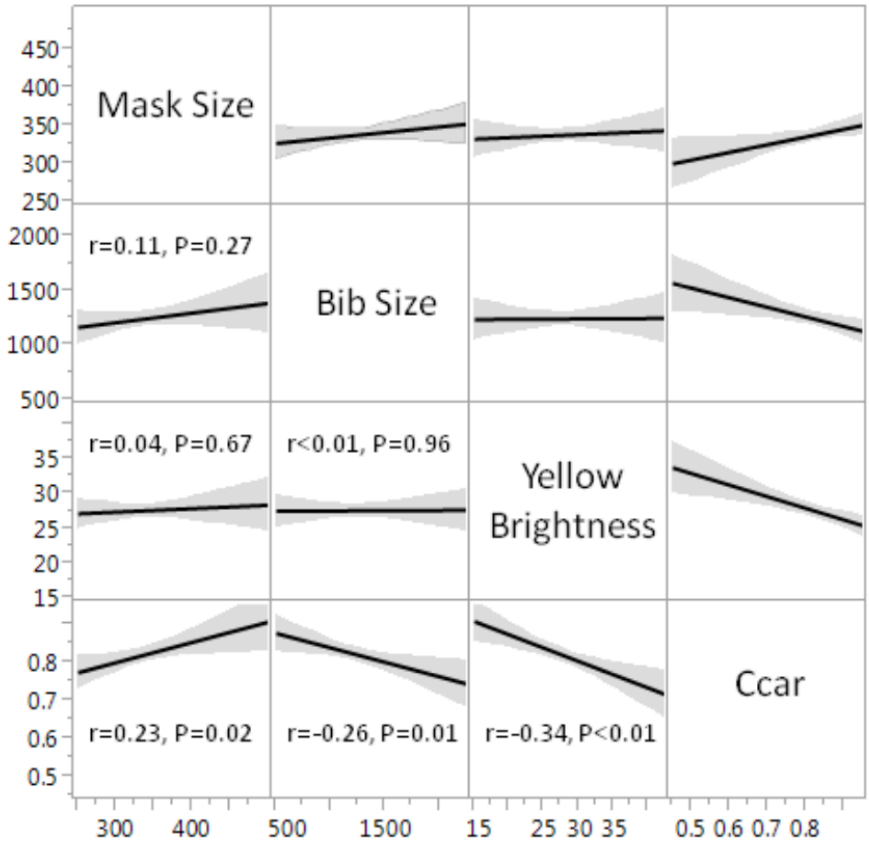

Fig. S1.1. Correlations between morphological traits of male common yellowthroats. Data are from 2013 and 2014 and sample sizes are 95 -96 for each correlation.

Table 1.1. Resistance to oxidative stress (KRL, time to 50% hemolysis of red blood cells) in relation to plumage ornaments, capture date, and breeding experience of male common yellowthroats. Full model R^2= 0.83 ($F_{9,8}$= 4.2, *P*= 0.028). Reduced model R^2 = 0.48 ($F_{5,26}$= 4.85, *P*= 0.003). Sample sizes were 18 and 32 for the full and reduced models, respectively.

Predictor	Estimate	SE	t	*P*
Full Model				
Intercept	112.4	272.46	0.41	0.691
Capture date	3.1	0.76	4.05	**0.004**
Mask size	0.7	0.2	3.82	**0.005**
Bib size	-0.1	0.04	-2.34	**0.047**
Yellow brightness	-2.2	1.75	-1.25	0.248
Carotenoid chroma	-131.9	110.29	-1.20	0.266
Tarsus length	24.5	17.13	1.43	0.190
Mass	-51.9	27.07	-1.92	0.092
Breeding experience	7.6	6.4	1.20	0.266
Number of replicates	-54.4	16.26	-3.35	**0.010**
Reduced Model				
Intercept	61.4	106.98	0.57	0.571
Capture date	1.7	0.58	3.09	**0.005**
Mask size	0.3	0.12	2.87	**0.008**
Bib size	<-0.1	0.02	-0.13	0.897
Mass	-9.2	10.14	-0.90	0.375
Number of replicates	-42.0	10.42	-4.03	**0.000**

Table 1.2. Glutathione (GSH) in relation to plumage ornaments, capture date, and breeding experience of male common yellowthroats. Full model R^2= 0.49 ($F_{8,14}$= 1.69, *P*= 0.186). Reduced model R^2= 0.47 ($F_{5,17}$= 3.01, *P*= 0.04). Sample sizes were 23 for both full and reduced models.

Predictor	Estimate	SE	t	*P*
Full Model				
Intercept	-1298.2	757.12	-1.71	0.109
Capture date	-6.3	2.58	-2.42	**0.029**
Mask size	-1.2	0.76	-1.64	0.124
Bib size	-0.0	0.06	-0.22	0.828
Yellow brightness	12.5	5.22	2.40	**0.031**
Carotenoid chroma	98.8	264.80	0.37	0.715
Tarsus length	17.9	27.82	0.64	0.530
Mass	140.4	55.75	2.52	**0.025**
Breeding experience	-59.5	20.33	2.93	**0.011**
Reduced Model				
Intercept	-909.5	468.50	-1.94	0.069
Capture date	-5.7	2.20	-2.59	**0.019**
Mask size	-1.1	0.57	-1.95	0.069
Yellow brightness	11.7	4.23	2.77	**0.013**
Mass	139.8	48.87	2.86	**0.011**
Breeding experience	56.5	18.35	3.08	**0.007**

Table 1.3. Oxidative stress (ROMs/TAC) in relation to capture date, plumage ornaments and breeding experience of male common yellowthroats. Full model R^2= 0.88 ($F_{8,8}$= 7.12, *P*= 0.006). Reduced model R^2= 0.85 ($F_{4,13}$= 17.83, *P*= <0.0001). Sample sizes were 17 and 18 for the full and reduced models, respectively.

Predictor	Estimate	SE	t	*P*
Full Model				
Intercept	133.3	97.55	1.37	0.209
Capture date	-1.1	0.33	-3.45	**0.009**
Mask size	0.0	0.09	-0.31	0.766
Bib size	0.0	0.01	-3.89	**0.005**
Yellow brightness	-0.2	0.73	-0.34	0.745
Carotenoid chroma	-124.8	38.46	-3.25	**0.012**
Tarsus length	1.6	3.82	0.43	0.681
Mass	6.5	8.57	0.76	0.471
Breeding experience	9.4	3.60	2.6	**0.031**
Reduced Model				
Intercept	219.1	27.11	8.08	**0.000**
Capture date	-1.0	0.21	-4.87	**0.000**
Bib size	-0.03	0.01	-4.93	**0.000**
Carotenoid chroma	-138.4	29.82	-4.64	**0.001**
Breeding experience	7.7	2.80	2.75	**0.017**

Table 1.4. Change in glutathione (GSH) in relation to capture date, plumage ornaments and breeding experience of male common yellowthroats after LPS or saline injection and 22 ± 2 h in an aviary. Full model R^2= 0.66 ($F_{9,9}$= 1.92, *P*= 0.174). Reduced model R^2= 0.44 ($F_{4,17}$= 3.32, *P*= 0.035). Sample sizes were 19 and 22 for the full and reduced models, respectively.

Predictor	Estimate	SE	t	*P*
Full Model				
Intercept	135.2	968.48	0.14	0.892
Capture date	5.6	2.98	1.88	0.093
Mask size	-1.8	1.08	-1.70	0.123
Bib size	0.0	0.07	0.67	0.520
Yellow brightness	-2.3	5.81	-0.40	0.699
Carotenoid chroma	-117.0	329.95	-0.35	0.731
Tarsus length	59.7	35.67	1.67	0.129
Mass	-86.5	69.75	-1.24	0.246
Experimental group	18.8	21.61	0.87	0.407
Breeding experience	-41.0	25.80	-1.59	0.146
Reduced Model				
Intercept	-291.3	513.44	-0.57	0.578
Capture Date	2.5	1.84	1.34	0.198
Mask size	-2.0	0.84	-2.39	**0.029**
Tarsus length	39.9	30.63	1.30	0.210
Breeding Experience	-17.0	20.66	-0.82	0.422

Table 1.5. Change in oxidative stress (ROMs/TAC) in relation to capture date, plumage ornaments and breeding experience of male common yellowthroats following LPS or saline injection and 22 ± 2 h in an aviary. Full model R^2 = 0.90 ($F_{9,2}$=2.11, *P* = 0.362). Reduced model R^2 = 0.75 ($F_{4,7}$=5.26, *P* = 0.028). Sample sizes were 12 for both the full and reduced models.

Predictor	Estimate	SE	t	*P*
Full Model				
Intercept	-394.4	390.69	-1.01	0.419
Capture date	1.4	1.07	1.3	0.322
Mask size	0.3	0.24	1.14	0.373
Bib size	0.0	0.05	0.83	0.493
Yellow brightness	-2.6	1.61	-1.62	0.247
Carotenoid chroma	173.4	183.77	0.94	0.445
Tarsus length	-1.6	12.98	-0.13	0.912
Mass	15.8	21.33	0.74	0.537
Experimental group	3.8	11.58	0.33	0.773
Breeding experience	-11.4	15.96	-0.71	0.550
Reduced Model				
Intercept	-244.4	82.01	-2.98	**0.021**
Capture date	1.8	0.43	4.09	**0.005**
Bib size	0.1	0.02	2.53	**0.039**
Carotenoid chroma	133.3	73.11	1.82	0.111
Breeding experience	-14.3	6.22	-2.3	0.055

Table 1.6. Summary of observed relationships between ornaments and measures of oxidative stress for unmanipulated birds. Positive and negative correlations are indicated by + and --, respectively. Non-significant correlations are indicated by 0. Results are based on consistent relationships in both the full and reduced models. Note that GSH is an antioxidant, so a positive relationship indicates that birds with brighter bibs had more of this antioxidant. Results from the LPS experiment are not included as no relationships were significant in both full and reduced models.

Oxidative stress	Mask Size	Bib Size	Yellow Brightness	Ccar
KRL	+	0	0	0
GSH	0	0	+	0
ROMs/TAC	0	--	0	--

Table S 1.1. Correlations between morphological traits and measures of oxidative balance. Note that sample sizes differ because GSH and ROMs/TAC were primarily measured in 2013 and KRL was measured in 2014. Thus, the data are mostly independent; only six birds were measured for both GSH and KRL and only two were measured for both ROMs/TAC and KRL. Data are from birds prior to any manipulation (e.g., LPS or saline injection).

Variable	By Variable	r	N	Lower 95%	Upper 95%	*P*
ROMs/TAC	Mask_size	-0.487	18	-0.777	-0.026	0.041
ROMs/TAC_	Bib size	-0.226	18	-0.627	0.270	0.368
ROMs/TAC_	Bib yellow	-0.325	18	-0.687	0.168	0.189
ROMs/TAC_	Ccar	-0.584	18	-0.826	-0.160	0.011
GSH	Mask_size	0.014	23	-0.400	0.424	0.948
GSH	Bib size	-0.102	23	-0.494	0.324	0.643
GSH	Bib yellow	0.179	23	-0.252	0.550	0.415
GSH	Ccar	-0.059	23	-0.460	0.362	0.790
KRL	Mask size	0.279	31	-0.083	0.577	0.128
KRL	Bib size	0.066	31	-0.295	0.411	0.725
KRL	Bib yellow	0.203	31	-0.163	0.520	0.273
KRL	Ccar	-0.241	31	-0.548	0.124	0.192

Table S 1.2. Behavior of birds in an aviary 4 h after injection with LPS or saline (treatment group). Full models for time foraging R^2= 0.22 ($F_{8,35}$=1.20, *P* = 0.325). Full model for time sitting R^2= 0.04 ($F_{8,35}$= 0.18, *P* =0.992). Full model for flights R^2= 0.25 ($F_{8,35}$= 1.44, *P* =0.216). Full model for visits to food dish R^2=0.19 ($F_{8,35}$= 1.00, *P* =0.452).

		Estimate	SE	t	*P*
Time foraging (sec)					
Full model					
	Intercept	355.1	1808.30	0.20	0.846
	Capture date	-10.0	4.76	-2.09	**0.044**
	Experimental group	-34.2	54.18	-0.63	0.532
	Mask size	-1.9	1.81	-1.03	0.311
	Bib size	0.1	0.17	0.36	0.719
	Yellow brightness	9.3	11.74	0.79	0.434
	Carotenoid chroma	225.2	533.10	0.42	0.675
	Tarsus length	104.3	87.62	1.19	0.242
	Mass	-159.2	112.85	-1.41	0.167
Reduced model					
	Intercept	657.2	147.82	4.45	**0.000**
	Capture date	-7.5	3.82	-1.97	**0.055**
Time sitting (sec)	Intercept	161.4	3312.55	0.05	0.961
	Capture date	-0.4	8.72	-0.04	0.967
	Experimental group	11.3	99.24	0.11	0.910
	Mask size	0.7	3.31	0.20	0.842
	Bib size	0.2	0.31	0.52	0.605
	Yellow brightness	16.6	21.51	0.77	0.445
	Carotenoid chroma	16.6	976.57	0.02	0.987
	Tarsus length	-3.3	160.50	-0.02	0.984
	Mass	-42.4	206.73	-0.20	0.839
Flights					
Full model					
	Intercept	-471.6	434.97	-1.08	0.286
	Capture date	1.1	1.14	0.92	0.363
	Experimental group	-1.8	13.03	-0.14	0.889
	Mask size	0.1	0.43	0.21	0.837
	Bib size	-0.1	0.04	-1.18	0.246
	Yellow brightness	-1.4	2.83	-0.49	0.624
	Carotenoid chroma	94.5	128.23	0.74	0.466
	Tarsus length	-7.1	21.08	-0.34	0.738
	Mass	62.6	27.15	2.31	**0.027**
Reduced model					
	Intercept	-578.9	238.378	-2.43	**0.020**
	Bib size	-0.1	0.03	-1.82	0.076
	Mass	70.7	23.28	3.04	**0.004**
Visits to food dish					
Full model					
	Intercept	-2.7	10.38	-0.26	0.799
	Capture date	-0.0	0.03	-1.13	0.265
	Experimental group	0.3	0.31	0.81	0.426
	Mask size	-0.0	0.01	-1.21	0.235
	Bib size	0.00	0.00	0.42	0.679
	Yellow brightness	0.1	0.07	0.94	0.352
	Carotenoid chroma	-1.6	3.06	-0.52	0.605
	Tarsus length	0.7	0.50	1.44	0.159
	Mass	-0.6	0.65	-0.98	0.335
Reduced model					
	Intercept	4.3	1.91	2.22	**0.031**
	Carotenoid chroma	-3.8	2.43	-1.57	0.124

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Chapter II. The relationship between blood parasites and ornamentation depends on the level of analysis in the common yellowthroat

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Abstract

The Hamilton–Zuk hypothesis predicts that ornament expression is a signal of the ability of individuals to resist parasite infection. Thus, across a population (i.e. between-individuals) more ornamented individuals should have lower levels of parasitism. Numerous studies have tested this prediction and the results are mixed. One reason for these conflicting results may be that many studies have examined this relationship at the between-individual level, which may be affected by confounding factors such as selective mortality. Using within-subject centering we examined the relationship between male ornamentation and avian blood parasites at both the between- and within-individual levels. These relationships focus on differences in genetically-based resistance to parasites and the trade-off in resource allocation between parasite resistance and ornament expression within an individual, respectively. We studied male common yellowthroats (*Geothlypis trichas*), which have two plumage ornaments, a yellow, carotenoid-based bib (throat and chest) and a black, melanin-based facial mask. Surprisingly, within-individuals, an increase in parasitism between years was associated with an increase in mask size and, potentially, greater concentration of carotenoids in the yellow feathers. This suggests that males may be able to tolerate an increase in parasitism and still increase ornament expression. In contrast, ornamentation was not related to parasitism at the between-individual level. Thus, our study revealed relationships between ornaments and parasitism at the within- individual level that were not present at the between-individual level. Our results highlight the importance of examining both within- and between-individual relationships as correlations between

variables, such as ornaments and parasites, may depend on the level of analysis (i.e. within- or between-individuals).

INTRODUCTION

Male ornaments are thought to honestly advertise male quality to females (Andersson 1994). However, there are many different genetic and physiological aspects of quality that may be selected for by females through ornaments. Hamilton and Zuk (1982) hypothesized that male ornaments may honestly advertise genetic resistance to parasites and, thus, females that mate with more ornamented males will obtain superior genes for parasite resistance for their offspring. This hypothesis assumes that ornament production and parasitic infection are both costly, and thus, within individuals there will be a trade-off between allocating resources to immune response or ornament expression. If these assumptions hold, then males in a population with lower levels of parasitism are predicted to have more elaborate ornaments than males with higher levels of parasitism (Hamilton and Zuk 1982). Numerous studies have tested this hypothesis by measuring ornamentation and parasitism across males in a population – a between-individual comparison (Martin and Johnsen 2007, Setchell et al. 2009, Vergara et al. 2012, Molnár et al. 2013, Merrill et al. 2015). However, the results of these studies have been mixed, with negative (Höglund et al. 1992, Doucet and Montgomerie 2003), positive (Korpimäki et al. 1995, Trigo and Mota 2016) and no (Dias et al. 2016) relationship reported between parasites and plumage ornaments in various species of birds. These mixed results may be a consequence of confounding factors that could either produce or obscure correlations between parasite resistance and plumage ornamentation between individuals.

Indeed, in analyses between individuals, selective mortality could result in either positive or negative relationships between parasitism and resistance alleles, depending

on the virulence of parasites (Fig. 1; Westerdahl 2007). In the case of mildly deleterious parasites (Fig. 2.1a), we might predict a negative relationship between parasitism and resistance, because there are no individuals with high resistance and high parasitism. In the case of potentially lethal parasites (Fig. 2.1b), however, low resistance individuals that are highly parasitized are more likely to die before they are sampled (i.e. selective mortality). This can produce a positive relationship between resistance and parasitism because there are both low resistance individuals that are, by chance, never infected, and individuals with high resistance that can tolerate these virulent parasites. If more resistant individuals produce larger ornaments (Fig. 2.1c), then there should be a negative relationship between ornamentation and parasitism when parasites are mild (Fig. 2.1d) and a positive relationship when parasites are more virulent (Fig. 2.1e). In this latter case (Fig. 2.1e), more resistant individuals can better tolerate a given parasite load, and, thus, they have relatively more resources to produce elaborate ornaments (Getty 2002).

Most studies of ornaments and parasitism have focused on between-individual analyses, but the correlation between ornaments and parasitism is influenced by variation at both the between- (population) and within-individual levels (Downs and Dochtermann 2014). Between-individual effects are driven by differences in genetically-based resistance, while within-individual effects are driven by the trade-off between resources for ornamentation and resistance to parasites. Under the Hamilton and Zuk hypothesis, the intraspecific relationship between ornaments and parasitism is predicted to be negative at both the between- and within-individual levels. However, these relationships are not necessarily the same. For example, as noted above, the between-

individual relationship could be positive or negative depending on the virulence of the parasite, even when parasitism is costly to individuals (i.e. the within-individual relationship is negative). Thus, understanding how parasites and ornamentation are related requires analyses at both the within- and between- individual levels. Although the problem of conflating levels of analysis has been widely acknowledged in evolutionary theory (Reznick et al. 2000, van de Pol and Wright 2009), it has only recently been discussed in ecoimmunology (Downs and Dochtermann 2014), and, to our knowledge, no study has partitioned within- and between-individual effects in analyses of the Hamilton and Zuk hypothesis.

In this study of common yellowthroats (*Geothlypis trichas*), we analyzed both the between- and within-individual relationship between plumage ornamentation and haemosporidian parasites. Parasitism by haemosporidians negatively affects the expression of plumage ornaments in some bird species (Doucet and Montgomerie 2003, del Cerro et al. 2010), but not all (Dias et al. 2016, Purves et al. 2016). Male common yellowthroats have two plumage ornaments, a black facial mask that is melanin based and a yellow bib (throat and chest) that is carotenoid based. In our study population, females prefer social and extra-pair mates with larger black masks (Thusius et al. 2001, Tarof et al. 2005, Pedersen et al. 2006); however, there is no evidence that the size or color of the yellow bib is sexually selected (Pedersen et al. 2006). Despite the difference in selection on these two ornaments, both the mask and the bib are honest indicators of some aspects of male quality in this population. Males with both larger masks and bibs are more resistant to oxidative stress (Henschen et al. 2016). In addition, males with larger masks produce more antibodies (IgG) and are more likely to

survive overwinter than small-masked males (Dunn et al. 2010). Finally, there is also evidence to suggest that males with larger masks may be more resistant to parasites because they have greater genetic variation at the major histocompatibility complex (MHC; Dunn et al. 2013), which is an important component of the adaptive immune system (Klein 1986). Despite this, a previous study found no relationship between haemosporidian parasites and male ornaments at the between-individual level in common yellowthroats (Dunn et al. 2013). Here we test both the between- and within-individual prediction that ornament expression will be negatively related to parasitism. Males molt and regrow plumage ornaments once a year at the end of the breeding season. Therefore, we measured infection intensity and ornamentation of individuals over two breeding seasons, which provided the repeated measures necessary to test the within-individual relationship.

METHODS

General methods

We captured male common yellowthroats during the breeding season (May–Aug) at the Univ. of Wisconsin-Milwaukee Field Station in Saukville, WI (43°23′N, 88°01′W) in 2003–2004 and 2012–2014. When possible, males were captured in two consecutive years (i.e. 2003–2004, 2012– 2013, 2013–2014). During the first capture, we marked each individual with a USFWS metal leg band and three colored plastic leg bands in a unique combination. During every capture we measured tarsus length, wing chord (±0.1 mm) and body mass (±0.1 g), took video images to measure mask and bib size, plucked

four feathers from the breast to measure color, and took a blood sample for parasite analysis (see below).

To measure the size of the mask and bib, we took a video of each side of the head and the bib with males held against a 1 cm^2 grid. We used still frames from these videos to determine the size of the mask and bib in ImageJ (< http://imagej. nih.gov/ij/>). Each image was scaled to the grid and then ornaments were traced to determine the area (Thusius et al. 2001). To measure the color of the yellow bib, we plucked four feathers from the center of the bib, overlapped them on a black matte background, and measured color using a spectrometer (USB2000, Ocean Optics, Dunedin, FL). For each male, we measured UV brightness (average R from 320–400 nm), yellow brightness (average R from 550–625 nm), yellow saturation (sum of R from 550–625 nm/total R), and carotenoid chroma (Ccar; (R 700–450 nm)/R 700 nm; Andersson and Prager 2006). These color variables are affected by carotenoid concentration, specifically lutein (Andersson and Prager 2006), which is the main carotenoid found in the yellow plumage of common yellowthroats (McGraw et al. 2003).

We collected a small (~70 ml) blood sample from the brachial vein in a heparinized capillary tube. A drop was used to make a blood smear to determine haemosporidian intensity and the remainder was centrifuged to measure hematocrit (% red blood cells). Packed cells were stored in Queen's Lysis Buffer (QLB; Seutin et al. 1991) at 4°C and DNA was extracted from these samples to determine presence and identity of haemosporidian parasites (Plasmodium spp. and Haemoproteus spp.) in the blood.

Detection and identification of haemosporidian parasites

Blood smears

We determined the intensity of haemosporidian infection using blood smears. Smears were prepared in the field using a small drop of blood, allowed to air dry, and then fixed in methanol for 30 s. Similar to previous studies of avian blood parasites (Giammarino et al. 2007, Levin et al. 2013) we stained slides using a Dip Quick Stain kit (Jorgensen Labora- tories, Loveland, CO), which is similar to the Wright–Giesma method. We then mounted a coverslip on each slide using Permount adhesive (Thermo Fisher Scientific, Waltham, MA). For each individual, we scanned approximately 4000 red blood cells under 1000X oil immersion magnification to estimate the intensity of intracellular haemosporidian parasites. We quantified intensity of infection as the number of cells infected per 4000 (Godfrey et al. 1987).

Nested PCR

To determine the presence of parasites, we extracted DNA from whole blood using a GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA). The genomic DNA extracted from these samples included DNA from both bird and parasites. We determined infection status (i.e. presence or absence of haemosporidian parasites) using a nested PCR (Hellgren et al. 2004) which amplifies a 520 bp fragment of a conserved region of the cytochrome b gene of Plasmodium and Haemoproteus spp. The first PCR included 1 ml of DNA (~25 ng of genomic DNA), 1.6 mM of forward primer (HaemNF1), 1.6 mM of reverse primer (HaemNR3), 1.5 mM of $MgCl_2$, 5 ml of 5X GoTaq Flexi buffer, 200 mM dNTPs, and 1 U GoTaq DNA polymerase in a total reaction volume of 25 ml. The second PCR used 1 ul of product from the first PCR and the HaemF/HaemR2 (Bensch et al. 2000) primers with the same

reagents and concentrations as the first PCR. We used the same thermal profiles as Hellgren et al. (2004) for both PCRs. For each PCR, we included a positive control from an individual known to be infected with haemosporidian parasites and a negative control. To determine if parasite DNA amplified in the sample we ran each PCR product on a 2% agarose gel. We considered samples positive for infection if they had a fragment around 520 bp. For negative samples (i.e. had no fragment at 520 bp) the nested PCR reaction was repeated two more times with the amount of DNA doubled each time. Only samples with three negative PCRs were considered negative for haemosporidian blood parasites.

Identification of parasite lineages

We identified the lineage of parasites by sequencing the PCR product (the parasite cytochrome b gene) of positive individuals in both directions. Sequencing was performed at the University of Chicago Cancer Research Center DNA Sequencing Facility. We used Geneious 7.1.9 software (Biomatters, <www.geneious.com/>, Kearse et al. 2012) to align forward and reverse sequences and the BLAST tool on the NCBI website to compare our cytochrome b sequences with those previously characterized by Pagenkopp et al. (2008). All of our parasites were Plasmodium (see Results) and the numbering of their lineages follows Pagenkopp et al. (2008).

Survival and breeding experience

We banded all males in the study area in 2003 and 2012 and all new males in 2004 and 2013–2014. We determined putative overwinter survival and breeding experience by surveying the territory and the surrounding area (a minimum of three times May–June) where males were captured the previous year. Males typically return to the same or a

nearby territory as the one they occupied the previous breeding season (Taff et al. 2013). Thus, newly banded males in 2004 and 2013–2014 were considered inexperienced breeders and males that did not return to the study area were assumed to not have survived.

Feather growth rate

Parasitism may also affect the condition of birds and, hence, the rate at which they grow new feathers (Coon et al. 2016). To measure feather growth rate, we plucked the third tail feather (R3), counting inward from the outermost right tail feather, and estimated feather growth rate by measuring the average growth bar width on each tail feather. Growth bars are alternating dark and light markings on the feather vane, perpendicular to the shaft (see Fig. 1 in Gienapp and Merilä 2010), and are typically found on wing and tail primaries. A single set of dark and light bars represents 24 h of feather growth. Thus, wider growth bars indicate more feather growth during a 24 h period (Michener and Michener 1938). In addition, growth bar width is positively correlated with nutritional state (Grubb 1989, 1991) and ornament expression (Murphy and Pham 2012). To measure growth bars, we mounted each tail feather on a block of foam covered in black paper and pushed a thin metal pin through the paper at the beginning and end of five growth bars (Hill and Montgomerie 1994, Frasz et al. 2014). We measured the width of the first five growth bars that were visible from the distal end of the feather (Saino et al. 2013) using digital calipers (±0.01 mm). Feather growth rate was expressed as the average width of these five growth bars.

Data analysis

Using nested PCR, we measured presence of parasites for 172 males. For 128 of these 172 males, we determined infection intensity with microscopic examination of blood smears. We had repeated data on infection intensity (in two consecutive breeding seasons) for 28 of the 128 males. Of these 28, we measured mask size for all males, bib size for 27 males, and bib color for 17 males. Finally we measured feather growth rate in 30 individuals, hematocrit in 107 individuals, and survival in 82 individuals. As a consequence, sample sizes differ across statistical models (Table 2.1–2.5). Degrees of freedom for each model are based on the number of individuals, rather than the number of observations. We included all available individuals (including uninfected males) in our models. All statistical analyses were performed in JMP 12 (SAS Inst.).

We used within-subject centering to examine the relationships between plumage ornamentation and infection intensity both between- and within-individuals (van de Pol and Wright 2009). To examine whether infection intensity predicts plumage ornamentation across individuals we compared the mean infection intensity for each individual (between-individual slope, $ß_b$). To examine how changes in plumage ornamentation within-individuals were related to variation in infection intensity we examined the mean- centered infection intensity for each individual (within-individual slope, $ß_w$). Mean-centered infection intensities were calculated by finding the average infection intensity for each individual and subtracting this number from each measure of infection intensity for that individual. Our mixed models included measurements of plumage ornament size or color as the dependent variable and the mean infection intensity and mean-centered infection intensity as predictors. To determine if the between-individual slope ($ß_b$) was significantly different from the within-individual slope

($ß_w$), we used mixed models that included mean infection intensity and infection intensity as predictors. In these models, the mean infection intensity term tests for a difference between the within- and between-individual slopes ($ß_b – ß_w$) and the infection intensity term tests for a significant negative or positive slope within-individuals ($ß_w$). Individual identity was included in both models as a random factor. We ran separate models for bib size and the components of bib color because these traits are independently related to different aspects of male quality (Freeman-Gallant et al. 2010, 2011, Taff et al. 2012, Taff and Freeman-Gallant 2014, Henschen et al. 2016), although some components of these ornaments are correlated with each other.

To determine if feather growth rate or hematocrit were related to infection presence or intensity we used general linear models (GLM) that included hematocrit or feather growth rate as the dependent variable and year, date of capture, tarsus length, body mass, breeding experience and either presence of infection or infection intensity as predictors. We also used within-subject centering to determine if there was a within-individual relationship between infection intensity and hematocrit. Lastly, we performed nominal logistic regressions to determine if survival (yes/no) was related to the presence or intensity of parasite infection. These models included survival as the dependent variable and year, date of capture, tarsus length, body mass, breeding experience and either presence of infection or infection intensity as predictors.

RESULTS

Presence, intensity, and lineage of haemosporidian parasites

We determined the presence of haemosporidian parasites for 172 individuals using a nested PCR and found that 83% of individuals were positive for these parasites. Based on our analysis of blood smears, 82 of 128 (64%) individuals were infected with haemosporidian parasites. Both methods (n = 120) gave the same result for 72% of individuals (11 negative, 75 positive), but differed for the other 34 individuals (4 were positive only with the blood smear method and 30 were positive only with the PCR method). Infection intensity (number of cells infected per 4000) ranged from 0 to 3.1% of cells (mean = 0.26%). We sequenced the cytochrome b gene of haemosporidian parasites for 117 males with a positive PCR product. All parasites shared 100% sequence identity with five previously characterized lineages of the Plasmodium genus, except for three samples that varied at one or two bases. Most (70%; 82/117) individuals were infected with lineage 6a (Genbank: EU328172) as described by Pagenkopp et al. (2008). The next most common lineages were 4b (21/117; EU328173) and 6b (11/117; EU328171). Lineages 1 (EU328168) and 5 (EU328175) were the least common, detected in one and two individuals, respectively. There was no evidence of multiple infections. The corresponding sequences in the Malavi database (< http://mbio-serv2.mbioekol.lu.se/Malavi/index.html >) are: 1 = TABI08, 4b = SEINOV01, 5 = BT7, 6a = GEOTRI01 and 6b = BAEBIC02.

Haemosporidian infection and ornamentation

Between-individuals

At the between-individual level males with greater bib UV brightness tended to have fewer infected red blood cells than males with lower bib UV brightness ($ß_b$; Table 2.1).

No other measures of ornamentation were related to infection intensity at the between-individual level ($ß_b$; Table 2.1).

Within-individuals

When individuals were measured over two breeding seasons, infection intensity was related to several measures of plumage ornamentation within-individuals. Males that had an increase in infection intensity from one breeding season to the next also had an increase in mask size ($ß_w$, Fig. 2.2, Table 2.1), and they tended to have an increase in bib saturation ($ß_w$, Table 2.1). In contrast, an increase in infection intensity between years was related to a decrease in UV brightness (Table 2.1). No other measures of plumage ornamentation (i.e. bib size, yellow brightness, or carotenoid chroma) were related to changes in infection intensity within-individuals (Table 2.1). For mask size, the between-individual slope ($ß_b$) tended to differ ($p = 0.08$) from the within-individual slope, but there was no difference for any of the other plumage traits ($ß_b – ß_w$; Table 2.2).

Feather growth rate, hematocrit and survival

Infection intensity was not related to hematocrit either between- ($p>0.10$; Table 2.3) or within-individuals ($p>0.58$; Table 2.4). In between-individual analyses, neither the presence nor the intensity of haemosporidian infection was related to feather growth rate (average growth bar width; $p>0.66$; Table 2.5) or survival (all $p>0.85$; Table 2.6).

DISCUSSION

In common yellowthroats, we found differences in the relationship between haemosporidian infection and plumage ornamentation at the within- and between-individual levels. At the between-individual level (i.e. when we compared individuals

across the population) the size and yellow coloration of plumage ornaments (black mask and yellow bib) was not related to the intensity of blood parasite infection. Thus, our results do not support the between-individual pre-diction of the Hamilton–Zuk hypothesis; i.e. males with the lowest parasitism will have the most elaborate ornaments. However, plumage ornaments (i.e. mask size and bib UV brightness) were related to infection intensity at the within- individual level. Here, we discuss potential reasons why there are within-individual, but not between-individual, relationships between ornaments and parasitism.

The lack of a between-individual relationship between parasites and ornaments was surprising, because there is some evidence that male common yellowthroats with larger black masks have better immunity (e.g. greater diversity of MHC class II alleles) than males with smaller masks (Dunn et al. 2013). However, there are a number of factors that can obscure the predicted relationship at the between-individual level and lead to discrepancies between results of the two types of analyses. In particular, between-individual studies may include males of different ages, and age can independently affect both parasitism and the expression of ornaments.

For example, older individuals are potentially more likely to be infected by virtue of their longer exposure (longer lifetime), and they are more likely to be resistant by virtue of their survival to an older age (Fig. 2.1). Older males may also express a more elaborate ornament because it is favored by sexual selection (Kokko 1997), or because they have more experience, which improves their ability to acquire resources for ornament production (Daunt et al. 2007, Zimmer et al. 2011). Between-individual relationships may also be masked because individual parasitism can change between

seasons and years (Hegemann et al. 2012), but static ornaments, such as plumage, only reflect levels of parasitism at the time they were produced and not necessarily when the parasites were sampled. This may be a widespread problem in studies of plumage ornaments as parasitism is often measured during the breeding season, but ornaments are often produced during the previous spring or autumn molt. For example, in house finches, plumage redness was not related to mite load before molt (i.e. between-individuals), but males with fewer mites had a greater increase in plumage redness after molt (i.e. within-individuals; Thompson et al. 1997). In this study the between-individual relationship may be obscured because we measured both ornamentation and infection intensity during the summer breeding season (May–Aug), although the plumage ornaments of common yellowthroats are produced during the autumn molt.

Although the Hamilton–Zuk hypothesis predicts a negative within-individual relationship between ornamentation and parasitism, male common yellowthroats actually showed the opposite relationship, an increase in both mask size and parasitism between years. An age-related increase in parasite tolerance could produce this positive within-individual relationship. For example, males could gain access to more resources as they age if they obtain better territories or learn to forage more efficiently (Daunt et al. 2007, Zimmer et al. 2011). Thus, older males might have more energy or nutrients available to both increase ornamentation and tolerate an increase in parasitism (Morehouse 2014). Another possibility is that haemosporidian infection is not very costly. In our study, the infection intensity (i.e. percent of red blood cells infected) of all but one individual was less than 2%, which suggests that these are chronic, rather

than acute, infections (Valkiūnas 2004). In addition, parasites did not affect survival, hematocrit, or feather growth rate, further suggesting that these parasites were not costly. In this case, there may be no direct relationship between ornamentation and parasitism; they each increase with age for different reasons. For example, ornament expression may increase if ornaments are sexually selected indicators of age (Kokko 1997, Brooks and Kemp 2001, Marini et al. 2015), and parasitism may increase with age simply due to increased exposure to infection. In common yellowthroats mask size is a sexually selected trait through both within- and extra-pair mate choice, and it also increases with age (Thusius et al. 2001). Thus, the benefits of increased mating success for older males with larger masks likely outweighs the apparently minimal costs imposed by a low-level infection. Finally, it is also possible that older males invest more resources in reproduction (i.e. ornament expression) at the expense of self-maintenance (i.e. immune response; Williams 1966, Velando et al. 2006).

Similar to melanin-based ornaments, an increase in parasitism is predicted to negatively affect the expression of carotenoid-based ornaments. In particular, parasitism is thought to decrease the availability of carotenoids for ornamentation, as more carotenoids will be needed for regulating oxidative stress induced by parasites (von Schantz et al. 1999, Mougeot et al. 2010). In addition, carotenoids can influence the immune response itself (Lozano 1994, Blount et al. 2003, Aguilera and Amat 2007), as they can stimulate both the humoral and the cell-mediated immune response (reviewed by Chew and Park 2004). In our study, we found that UV brightness was negatively related to parasitism at the within-individual level, while bib saturation tended to be positively related to parasitism. These seem like opposing relationships, but it is

important to note that carotenoid (lutein) concentration in feathers affects UV brightness and saturation in opposite ways – as carotenoid concentration increases, saturation increases, while UV brightness decreases (Andersson and Prager 2006). Thus, this suggests that the concentration of carotenoids in the yellow bib feathers is actually related positively to parasitism. This is consistent with the positive within-individual relationship between mask size and parasitism. In yellowthroats, these relationships may be due to age-related factors, such as increased foraging ability, and, thus, experiments may be necessary to determine any causal relationships between pigments (carotenoids and melanins) and parasitism.

Overall, we found that more elaborate plumage ornaments did not signal lower haemosporidian infection in male common yellowthroats at the between-individual level, and, at the within-individual level, mask size was positively related to greater infection intensity. Carotenoid concentration in the breast feathers, as indexed by UV brightness and saturation, was also related to greater infection intensity. These relationships were only evident in within-individual analyses, which highlights the importance of this type of analysis for understanding physiological tradeoffs that underlie population-wide patterns.

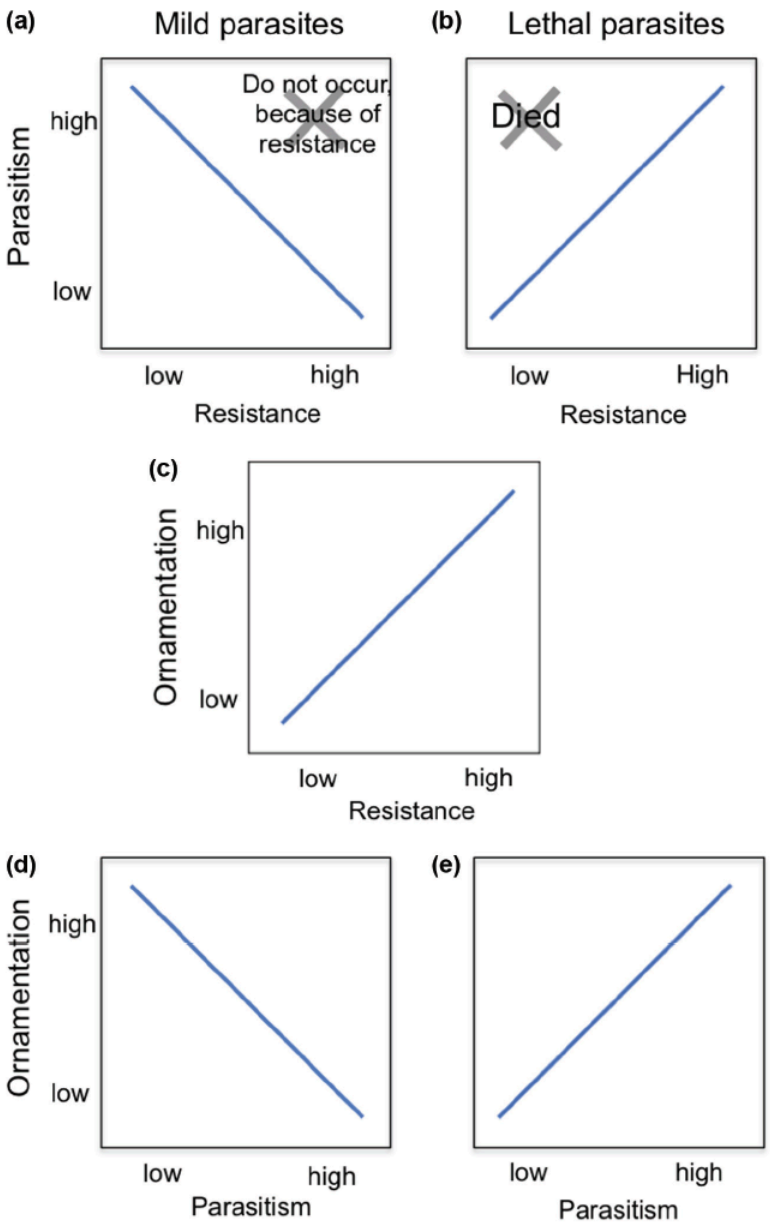

Fig. 2.1. Schematic diagram of predicted relationships between parasitism and resistance (top row) in cases of mild (a) and lethal (b) parasites (based on Fig. 2 in Westerdahl, 2007). In the case of mild parasites (a), there is a predicted negative relationship between parasitism and resistance because there are no individuals with high resistance and high parasitism. In the case of lethal parasites, there is a predicted positive relationship because low resistance individuals that are highly parasitized die before they are sampled (i.e., selective mortality). Thus, assuming that more resistant individuals produce larger ornaments (c) there is a predicted negative relationship between ornamentation and parasitism when parasites are mild (d) and a predicted positive relationship when parasites are lethal (e).

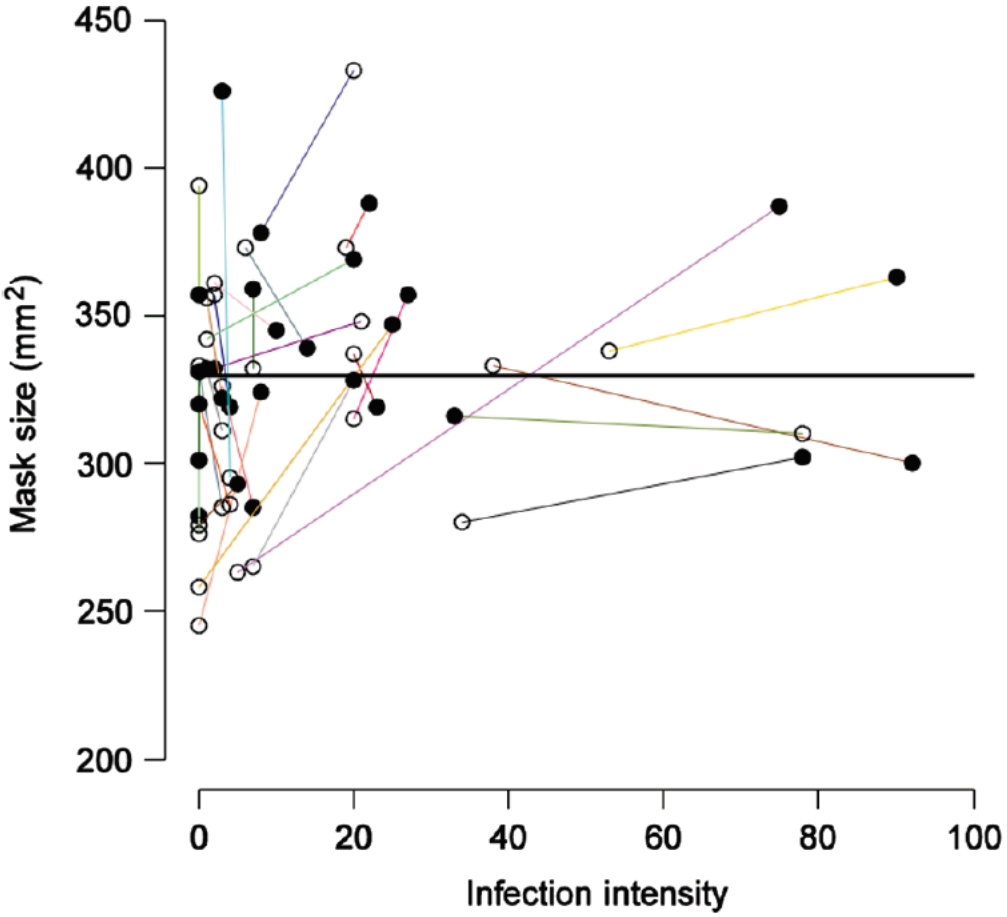

Fig. 2.2. Relationship between mask size (mm^2) and intensity of infection by haemosporidia (number of cells infected). Thin lines represent within-individual slopes and each color represents a different individual. The thick line represents the between-individual slope. Open circles are the first year of capture and closed circles are the second year of capture.

Table 2.1. Between-individual (β_B) and within-individual (β_W) relationships between plumage ornaments and intensity of haemosporidian infection. The within-individual relationship was based on changes between breeding seasons in the same individual.

	Term	Estimate	SE	*t*	df	*P*
Mask size	Intercept	329.60	7.66	43.04		<0.01
	Mean infection intensity (β_B)	-0.03	0.29	0.11	1,25	0.92
	Centered mean infection intensity (β_W)	0.85	0.39	2.15	1,25	**0.04**
Bib size	Intercept	1055.38	52.68	20.04		<0.01
	Mean infection intensity (β_B)	1.39	1.96	0.71	1,24	0.49
	Centered mean infection intensity (β_W)	-1.37	3.28	0.42	1,24	0.68
UV brightness	Intercept	21.40	1.18	18.12		<0.01
	Mean infection intensity (β_B)	-0.07	0.04	1.90	1,14	0.08
	Centered mean infection intensity (β_W)	-0.11	0.05	2.31	1,14	**0.03**
Yellow brightness	Intercept	30.20	1.06	28.45		<0.01
	Mean infection intensity (β_B)	-0.05	0.03	1.44	1,14	0.17
	Centered mean infection intensity (β_W)	-0.09	0.05	1.64	1,14	0.12
Saturation	Intercept	0.27	0.01	49.11		<0.01
	Mean infection intensity (β_B)	<0.01	<0.01	1.40	1,14	0.18
	Centered mean infection intensity (β_W)	<0.01	<0.01	2.02	1,14	0.06
Carotenoid chroma	Intercept	0.78	0.02	35.68		<0.01
	Mean infection intensity (β_B)	<0.01	<0.01	0.76	1,14	0.46
	Centered mean infection intensity (β_W)	<0.01	<0.01	1.70	1,14	0.11

Table 2.2. Difference between the slopes for between- and within-individual relationships (β_B - β_W) of haemosporidia parasite infection intensity (number of cells infected) with mask size, bib UV brightness, and bib saturation.

	Term	Estimate	SE	*t*	df	*P*
Mask size	Intercept	329.60	7.66	43.04		<0.01
	Mean infection intensity (β_B - β_W)	-0.88	0.49	1.80	1,25	0.08
	Infection intensity (β_W)	0.85	0.39	2.15	1,25	**0.04**
UV brightness	Intercept	21.40	1.18	18.12		<0.01
	Mean infection intensity (β_B - β_W)	0.04	0.06	0.71	1,14	0.48
	Infection intensity (β_W)	-0.11	0.05	2.31	1,14	**0.03**
Saturation	Intercept	0.27	0.01	49.11		<0.01
	Mean infection intensity (β_B - β_W)	<-0.01	<0.01	0.39	1,14	0.70
	Infection intensity (β_W)	<0.01	<0.01	2.02	1,14	0.06

Table 2.3. Relationship between hematocrit and the presence or absence of haemosporidia parasites (based on PCR), or haemosporidia infection intensity (number of cells infected). Year was included as a categorical variable in this model.

	Term	Estimate	SE	*t*	*P*
Hematocrit	Intercept	52.66	19.2	2.74	0.01
	Year[2004-2003]	0.12	1.46	0.08	0.94
	Year[2005-2004]	2.50	2.49	1.00	0.32
	Year[2006-2005]	-2.84	2.81	1.01	0.31
	Year[2013-2006]	0.62	1.99	0.31	0.76
	Year[2014-2013]	0.17	1.73	0.10	0.92
	Capture date	-0.12	0.03	3.69	**<0.01**
	Breeding experience	0.22	0.59	0.38	0.71
	Tarsus length	-0.21	0.92	0.22	0.82
	Body mass	0.58	1.11	0.52	0.60
	Infection presence	1.24	0.75	1.65	0.10
	R^2= 0.19, $F_{10,97}$= 2.21, *P*= 0.02				
Hematocrit	Intercept	52.89	20.79	2.54	0.01
	Year[2004-2003]	-0.86	1.52	0.57	0.57
	Year[2013-2004]	1.50	1.81	0.83	0.41
	Year[2014-2013]	-0.48	1.98	0.24	0.81
	Capture date	-0.12	0.03	3.71	**<0.01**
	Breeding experience	0.62	0.67	0.92	0.36
	Tarsus length	0.09	1.01	0.09	0.93
	Body mass	-0.14	1.23	0.12	0.91
	Infection intensity	<0.01	0.03	0.17	0.87
	R^2= 0.25, $F_{8,69}$= 2.85, *P*= 0.01				

Table 2.4. Between-individual (β_B) and within-individual (β_W) relationships between haemosporidian infection and hematocrit. The within-individual relationship was based on changes in hematocrit and infection intensity in individuals over two separate breeding seasons.

	Term	Estimate	SE	*t*	df	*P*
Hematocrit	Intercept	45.64	1.59	28.63		<0.01
	Mean infection intensity (β_B)	0.15	0.11	1.37	1,11	0.20
	Centered mean infection intensity (β_W)	0.04	0.08	0.56	1,11	0.58

Table 2.5. Relationship between tail feather growth rate (average growth bar width) and the presence or absence of haemosporidia parasites (based on PCR), or haemosporidia parasite infection intensity (number of cells infected).

	Term	Estimate	SE	*t*	*P*
Feather growth rate	Intercept	-1.35	2.39	0.57	0.58
	Year	-0.09	0.11	0.79	0.44
	Capture date	<-0.01	0.01	0.08	0.94
	Tarsus length	0.04	0.10	0.39	0.70
	Body mass	0.31	0.18	1.68	0.11
	Breeding experience	-0.05	0.06	0.75	0.46
	Infection presence	-0.03	0.07	0.44	0.66
	R^2= 0.16, $F_{6,24}$= 0.76, P= 0.61				
Feather growth rate	Intercept	-0.66	2.66	0.25	0.81
	Year	0.11	0.13	0.85	0.41
	Capture date	<-0.01	0.01	0.63	0.54
	Tarsus length	<-0.01	0.11	0.01	0.99
	Body mass	0.33	0.19	1.73	0.10
	Breeding experience	-0.02	0.07	0.31	0.76
	Infection intensity	<-0.01	<0.01	0.33	0.75
	R^2= 0.19, $F_{6,16}$= 0.63, P= 0.70				

Table 2.6. Relationship between overwinter survival (in 2004, 2013-2015) and the presence or absence of haemosporidia parasites (based on PCR), or haemosporidia infection intensity (number of cells infected).

		Estimate	SE	ChiSquare	*P*
Survival	Intercept	8.99	10.17	0.78	0.38
	Year[2004-2003]	1.64	0.70	5.50	**0.02**
	Year[2005-2004]	-1.65	0.92	3.20	0.07
	Year[2013-2005]	0.02	1.03	<0.01	0.99
	Year[2014-2013]	0.47	0.90	0.28	0.60
	Capture date	-0.02	0.02	1.31	0.25
	Tarsus length	0.09	0.49	0.04	0.85
	Body mass	-0.98	0.63	2.39	0.12
	Breeding experience	-0.12	0.34	0.14	0.71
	Infection presence	0.07	0.36	0.03	0.85
	R^2= 0.11, $ChiSqr_{9,73}$= 12.63, *P*= 0.18				
Survival	Intercept	10.41	12.67	0.68	0.41
	year[2004-2003]	1.87	0.87	4.62	**0.03**
	year[2013-2004]	-3.71	1.35	7.5	**0.01**
	year[2014-2013]	3.70	1.77	4.37	**0.04**
	Capture date	-0.01	0.02	0.13	0.72
	Tarsus length	0.74	0.64	1.3	0.25
	Body mass	-2.41	0.91	7.09	**0.01**
	Breeding experience	-0.21	0.40	0.29	0.59
	Infection intensity	<0.01	0.03	0.03	0.86
	R^2= 0.28, $ChiSqr_{8,52}$= 23.43, *P*= <0.01				

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Chapter III. Male stress response is related to ornamentation but not resistance to oxidative stress in a warbler

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Abstract

Ornaments are thought to honestly signal individual quality to potential mates. Individual quality may include the ability to cope with stress through the production of glucocorticoids (GCs), such as corticosterone (CORT), which help to redirect resources from growth or reproduction to survival during an acute stress response. However, elevated levels of CORT may also increase oxidative stress and reduce immune function. Thus, an important question is whether high quality individuals, with more elaborate ornaments, signal their ability to produce a strong stress response and mitigate some of the negative effects of doing so through higher resistance to oxidative stress. We tested whether ornamentation and resistance to oxidative stress were related to the increase in CORT during an acute stress response in a warbler, the common yellowthroat (*Geothlypis trichas*). Males in this species have two plumage ornaments, a black (eumelanin-based) facial mask and a yellow (carotenoid-based) bib. We measured the increase in CORT in response to the stress of capture and handling. Males with more elaborate ornaments (larger masks and more colorful bibs) had a greater increase in CORT during an acute stress response. However, the increase in CORT was not related to resistance to oxidative stress. These results suggest that both melanin- and carotenoid-based plumage ornaments can signal the ability of a male to cope with stressors through a greater increase in CORT. Thus, the association between ornamentation and stress induced CORT is not likely due to a mechanism specific to a particular color (melanin or carotenoid), but instead may result from more general interactions between CORT and health or condition.

INTRODUCTION

Females often use elaborate male ornaments to choose mates because these ornaments are honest indicators of male quality (Andersson, 1994). In particular, ornaments may advertise high quality individuals that are able to cope with and maintain homeostasis during stressful environmental situations (Hill, 2011). A short-term (acute) stress response (Cockrem, 2007) helps individuals to cope with stressful situations by releasing stored energy and inducing changes in behavior, such as escape responses and increased foraging (Wingfield et al., 1998; Sapolsky, Romero, & Munck 2000). These effects are carried out mainly by glucocorticoids, such as corticosterone (CORT, the main stress hormone in birds), which increase sharply when individuals encounter predators or competitors (Sapolsky et al., 2000). Thus, individuals with more elaborate ornaments may be advertising their ability to cope with stressful situations through a large increase in CORT during stress responses.

However, there are examples of both negative and positive relationships between ornaments and CORT production (Moore, Shuker, & Dougherty 2016) possibly because both CORT and ornament production can be influenced by other important physiological processes. For example, in barn owls (*Tyto alba*; Almasi, Jenni, Jenni-Eiermann, & Roulin 2010; Almasi, Roulin, & Jenni 2013) and Atlantic salmon (*Salmo salar*; Kittilsen et al., 2009; Kittilsen, Johansen, Braastad, & Overli 2012), individuals with more eumelanin-based (black) coloration had lower stress-induced CORT levels than individuals with less eumelanin coloration. Eumelanin is produced when α-melanocyte stimulating hormone (α-MSH) binds to the melanocortin 1 receptor (MC1R); however, α-MSH also binds to the melanocortin 4 receptor (MC4R), which inhibits the

production of CORT (Ducrest, Keller, & Roulin 2008; Almasi et al., 2010). Thus, a negative relationship between stress-induced CORT and eumelanin-based coloration might occur because these individuals have higher levels of α-MSH (at least during the development of the coloration). CORT also influences other physiological processes and can lead to increased oxidative stress (Lin, Decuypere, & Buyse 2004; Costantini, Marasco, & Møller 2011; reviewed by Spiers, Chen, Sernia, & Lavidis 2015), decreased immune function (Berger et al., 2005), or both (Stier et al. 2009). Despite these costs, positive relationships have been reported between stress-induced CORT and ornaments in house sparrows (*Passer domesticus*; melanin-based badge size; Lendvai & Chastel, 2010) and king penguins (*Aptenodytes patagonicus*; carotenoid-based beak color; Viblanc et al., 2016). Thus, in some cases, positive relationships between ornaments and stress-induced CORT may indicate that individuals are able to cope with stressful situations, as well as the associated negative effects of high CORT levels.

Individuals with elaborate ornaments may avoid some negative effects of high CORT levels if they are more resistant to CORT-induced oxidative stress. Oxidants are a natural byproduct of cellular respiration (Murphy, 2009) and can potentially damage nucleic acids, lipids, and proteins (Sies, 1991; Monaghan, Metcalfe, & Torres 2009), leading to decreased ornament expression (von Schantz, Bensch, Grahn, Hasselquist, & Wittzell 1999). However, the level of oxidative damage will depend on the ratio of protective antioxidants to harmful pro-oxidants, such as reactive oxygen species ([ROS], Sies, 1991; Monaghan et al., 2009). Experimentally administered antioxidants protect against CORT-induced oxidative stress (Ohtsuka, Kojima, Ohtani, & Hayashi 1998; Eid, Ohtsuka, & Hayashi 2003; Ghadrdoost et al., 2011), which suggests that a

greater endogenous antioxidant capacity may also protect individuals from oxidative stress during an acute stress response.

In this study, we tested the hypotheses that: 1) ornaments signal the ability of males to mount a strong acute stress response, and 2) this response to stress is related to the ability of an individual to resist oxidative stress. We studied male common yellowthroats (*Geothlypis trichas*), which have two plumage ornaments, a black, eumelanin-based facial mask and a yellow, carotenoid-based bib. In our study population, females prefer males with larger black masks for both social and extra-pair mates (Thusius, Peterson, Dunn, & Whittingham 2001; Tarof, Dunn, & Whittingham 2005; Pedersen, Dunn, & Whittingham 2006), while the yellow bib is not under intra- or intersexual selection (Pedersen et al., 2006). However, both the mask and bib advertise some aspects of male quality. Males with larger masks produce more antibodies, have a higher rate of overwinter survival (Dunn, Garvin, Whittingham, Freeman-Gallant, & Hasselquist 2010), and have greater immune genetic variation at the major histocompatibility complex (MHC; Dunn, Bollmer, Freeman-Gallant, & Whittingham 2013), while males with larger and more colorful bibs have a more favorable ratio of pro-oxidants to antioxidants (Henschen, Whittingham, & Dunn 2016). To test our first hypothesis, we measured the increase in CORT after 30 min. of capture and handling, which is known to induce an acute stress response (Wingfield, Smith, & Farner 1982; Wingfield, Vleck, & Moore 1992). We predicted that mask size, as well as bib size and color would signal the magnitude of a stress-induced increase in CORT. Our previous work has also shown that males with larger black masks are more resistant to oxidative stress, as measured by the KRL assay (Henschen et al., 2016), which measures both

intracellular and extracellular antioxidants (Blache & Prost, 1992), as well as previous oxidative damage (Brzezińska-Ślebodzińska, 2001). Our second hypothesis predicts that individuals with greater resistance to oxidative stress will also have a greater increase in CORT after 30 min. than individuals that are less resistant to oxidative stress. To our knowledge, this is the first study to determine if both melanin- and carotenoid-based ornaments in the same species are related to CORT levels during an acute stress response.

METHODS

General methods

We captured male common yellowthroats during the breeding season (14 May- 22 June) at the University of Wisconsin- Milwaukee Field Station in Saukville, Wisconsin (43°23'N, 88°01'W) in 2014. Males were captured in mist-nets using song playback. We watched the mist-nets continuously to quantify the time from capture in the net until blood sampling for corticosterone. All males had a blood sample taken from the left brachial vein within three minutes of capture in a mist-net (Romero & Romero, 2002; Romero & Reed, 2005). We used this blood sample to determine baseline CORT levels and measure resistance to oxidative stress (KRL Assay). All males were banded with a USFWS leg band and a unique combination of three colored leg bands. We then took video images of plumage ornaments to measure black mask and yellow bib size, and collected four feathers from the center of the bib to measure bib color with a spectrophotometer (see below). To estimate body size, we measured tarsus length to the nearest 0.1 mm. After processing (which typically took less than 30 minutes), males

were placed in a cloth bag until a total of 30 minutes had elapsed since capture in the net. At 30 minutes, we took a second blood sample from the right brachial vein to measure the increase in CORT levels due to capture stress (Wingfield et al., 1982; Wingfield et al., 1992). To determine if ambient temperature affected baseline or stress-induced levels of CORT, we obtained temperature data every 30 min from an automated weather station located in the middle of our study area. Temperature at the time of capture was estimated using the closest 30 min reading from the weather station.

Plumage ornamentation

To measure the size of the black mask and the yellow bib, we made video recordings of males while holding them against a 1 cm^2 grid in three standardized positions to record both sides of the head and the bib. From the videos, we made still images of both sides of the head and bib for each individual, imported them into ImageJ (http://imagej.nih.gov/ij/), standardized each picture to the 1 cm^2 grid, and measured the area of the mask (left and right side) and bib (Thusius et al., 2001). The same individual (AEH) completed all mask and bib measurements and repeatability for these methods is high ($r > 0.98$; Tarof et al., 2005). To measure bib color, we overlapped the four bib feathers and mounted them on black matte paper. We then measured yellow brightness (average R from 550-625 nm) and carotenoid chroma (Ccar; (R 700-450 nm)/ R 700 nm; Andersson & Prager, 2006) using a reflectance spectrophotometer (USB2000; Ocean Optics, Dunedin, FL, USA; Dunn, Whittingham, Freeman-Gallant, & DeCoste 2008). Bib yellow brightness was negatively correlated with carotenoid chroma of the bib ($r = -0.40$, $P = < 0.01$) and positively, although not significantly, correlated with mask

size (r = 0.25, *P* = 0.07). No other measures of plumage ornamentation were correlated with each other (all r < 0.12 all *P* > 0.42).

Corticosterone assays

We quantified baseline and stress-induced corticosterone in plasma using a Corticosterone ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY). Plasma components and the steroid displacement buffer (SDB; provided in the kit) can interfere with the accuracy of this assay (Wada, Hahn, & Breuner 2007). Thus, plasma from each new species must be optimized for this assay. We optimized plasma from the common yellowthroat using the method described in Wada et al. 2007. Briefly, we stripped the endogenous corticosterone from a sample of plasma pooled from several individuals using a solution containing 1% activated charcoal and 0.1% Dextran. We then spiked the stripped plasma with corticosterone to a concentration of 500 pg/mL. We assayed this sample under several dilution conditions (1:20, 1:40, 1:60, 1:80, and 1:100) and with several concentrations (0%, 1%, 1.5%, and 2%) of SDB to estimate a dilution and SBD concentration that did not interfere with the accuracy of the assay. For common yellowthroat plasma, we used a 1:80 dilution with a 1% concentration of SDB and ran all assays according to manufacturer instructions, including standards and a blank in each plate. We assayed all standards and samples in triplicate. Plates were read at 405 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Within plate repeatability was 0.99 and between plate repeatability was 0.98. Overall there were 49 males with both a baseline and stress-induced sample, which were run next to each other on the same plate. Two additional males had a baseline but no stress-induced sample.

Resistance to oxidative stress (KRL assay)

We measured resistance to oxidative stress with the KRL (Kit Radicaux Libres) assay adapted for birds by Alonso-Alvarez et al. (2004a,b). The KRL assay measures resistance to oxidative stress as the time for 50% of erythrocytes in a sample to haemolyse after being exposed to a free radical initiator [2, 2 -Azobis (2-methylpropionamidine) dihydrochloride, AAPH]. We performed this assay as described in Alonso-Alvarez, Bertrand, Faivre, & Sorci (2007) and Henschen et al. (2016). In the field, we diluted samples of whole blood with KRL buffer (prepared as described in Alonso-Alvarez et al. 2007). We then incubated samples with AAPH solution in a 96 well plate. Using a microplate reader (Molecular Devices, Sunnyvale, CA, USA), we measured the optical density of each well every 10 min for 150 min at 540 nm and 40 °C. We ran samples in quadruplicate the same day they were collected. Repeatability for this assay was 0.85.

Data analysis

We calculated the increase in CORT by subtracting baseline CORT from stress-induced (30 min.) CORT. The increase in CORT was not related to ambient temperature ($b = -0.30 \pm 1.64$, $t_{48} = 0.18$, $P = 0.86$), time of capture ($b = -0.05 \pm 0.04$, $t_{48} = 1.34$, $P = 0.19$), or date of capture ($b = -1.15 \pm 0.63$, $t_{48} = 1.82$, $P = 0.08$) in bivariate models (Table S3.1). Therefore, these variables were not included in further analyses. Size of the black mask and yellow bib were not related to body size, as indexed by tarsus length (Table S3.2), so we also did not include tarsus length in further analyses.

The relationship between the stress-induced increase in CORT and ornamentation was first tested using bivariate models with increase in CORT as the

dependent variable and ornament (i.e., size of the black mask or yellow bib, bib yellow brightness, or bib carotenoid chroma) as the independent variable (n= 49 males). To test our second prediction, that individuals with greater resistance to oxidative stress would have a greater increase in CORT, we used general linear models with the increase in CORT as the dependent variable and the ornaments, resistance to oxidative stress (KRL), and the interaction between ornaments and KRL as the predictors. We expected the interaction to be significant if our prediction was correct. Stress-induced CORT (i.e., the level of CORT at 30 min.) was strongly correlated with the increase in CORT (r = 0.94, P = <0.01) and our results remained unchanged if we analyzed stress-induced CORT rather than the increase in CORT as the dependent variable in our models.

Baseline CORT and the increase in CORT are likely distinct traits (Stedman, Hallinger, Winkler, & Vitousek 2017), so we treated them as separate variables in our analyses. In bivariate models, baseline CORT was negatively related to ambient temperature at the time of capture and date of capture in the season (capture date ranged from 14 May to 22 June; median = 30 May). However, baseline CORT was not related to time of capture during the day, any measure of ornamentation, or oxidative stress (Table S3.3) and was only weakly correlated with the increase in CORT (r = 0.18, P = 0.21). Therefore, we did not include measures of baseline CORT in further analyses.

Two individuals had unusually high baseline CORT and one individual had an unusually high increase in CORT (i.e., greater than three standard deviations from the mean). However, the exclusion of these individuals from our analyses did not qualitatively

change our results. We present the results from the full data set here. We performed all analyses using JMP Pro 13 (SAS Institute, Inc.).

RESULTS

CORT and Ornamentation

The average stress-induced increase in CORT was 115.19 ± 5.85 ng/mL and varied from 58.92 to 239.18 ng/mL. Males with larger black masks had a greater increase in CORT than males with smaller masks (Table 3.1; Fig. 3.1). Increase in CORT was also positively associated with the carotenoid chroma of the yellow bib (Table 3.1), and the slopes were similar for both the mask size (standardized b = 13.37 ± 5.63, t_{48} = 2.37, P = 0.02) and bib chroma (standardized b = 15.20 ± 5.46, t_{48} = 2.78, P = 0.01) regressions. There were no significant relationships between increase in CORT and bib size or yellow brightness of the bib feathers (Table 3.1; Fig. 3.1).

CORT and Oxidative Stress

For 23 of the 49 males in this study we also had data on resistance to oxidative stress (KRL). In general linear models, resistance to oxidative stress did not affect the relationships between ornaments and the increase in CORT, as there were no significant interactions between the ornaments and resistance to oxidative stress (Table 3.2). The increase in CORT was also not related to resistance to oxidative stress in a bivariate model (R^2 = 0.04, b = 0.28 ± 0.30, t_{22} = 0.92, P = 0.37). In contrast to our previous results (Henschen et al., 2016), the relationship between mask size and resistance to oxidative stress was not significant (b = 0.03 ± 0.14, t_{22} = 0.20, P = 0.84;

Table 3.3), probably due to the smaller sample size in our current study. Resistance to oxidative stress (KRL) was also not related to bib size or color (Table 3.3).

DISCUSSION

Ornaments may signal the ability of an individual to cope with a variety of stressors. In our study, male common yellowthroats with larger black (melanin-based) masks and more colorful yellow (carotenoid-based) bibs had a greater increase in CORT during an acute stress response. However, the relationship between the increase in CORT and ornaments was not affected by the ability of an individual to resist oxidative stress. Thus, while our results indicate that both melanin- and carotenoid-based ornaments are related to a greater acute stress response (increase in CORT), an individual's resistance to oxidative stress (as assayed by KRL) does not influence the relationships between CORT and ornaments.

Although melanin-based ornaments can be positively (Lendvai & Chastel, 2010; this study) or negatively (Almasi et al., 2010, 2013; Kittilsen et al., 2009, 2012) related to stress induced levels of CORT in several species (Table 3.4), the mechanisms driving these relationships are not clear. A negative relationship between melanin-based ornamentation and the increase in CORT may be influenced by the hormone α-MSH, which initiates eumelanin production and also inhibits the release of CORT (Ducrest et al., 2008; Almasi et al., 2010). However, α-MSH and CORT can vary seasonally (McGuire, Calisi, & Bentley 2010) so this negative relationship may only exist during moult, when plumage ornaments are being produced, but not during the breeding season when many studies, including ours, are conducted. This would explain how

eumelanin coloration could be positively related to CORT in male house sparrows (Lendvai & Chastel, 2010) and common yellowthroats studied during the breeding season. CORT levels may also be linked to melanin coloration through social interactions. Dominant individuals have greater levels of CORT during an acute stress response in several species, including mountain chickadees (*Poecile gambeli*; Pravosudov, Mendoza, & Clayton 2003), mallards (*Anas platyrhynchos*), and pintails (*Anas acuta*; Poisbleau, Fritz, Guillon, & Chastel 2005). In common yellowthroats, males with larger black masks are dominant over males with smaller masks (Tarof et al., 2005) and, thus, social interactions may also influence the positive relationship between the melanin-based black mask and increase in CORT.

We found that carotenoid-based plumage ornaments can also be positively related to CORT levels during an acute stress response. Unlike melanins, carotenoids cannot be synthesized by vertebrates and instead must be obtained through the diet (McGraw 2006). High levels of CORT increase the availability of carotenoids for ornamentation by releasing stored carotenoids (McGraw, Lee, & Lewin 2011), which could result in a positive relationship between carotenoid-based ornamentation and stress induced increase in CORT. Although this mechanism can affect skin or beak color (e.g., king penguins; Viblanc et al., 2016) at any time, it will only affect plumage ornamentation during molt. Nevertheless, in male common yellowthroats there was a positive relationship between both melanin- and carotenoid-based plumage ornaments and acute levels of stress-induced CORT, suggesting that pigment-specific mechanisms are not likely to explain these relationships.

There are many general mechanisms that could link stress induced increases in CORT with different types of ornaments. For example, more ornamented males may produce the strongest stress responses because they can afford or avoid the negative effects of high CORT (e.g., oxidative stress). There is no evidence in this study or in barn owls (Roulin, Almasi, Meichtry-Stier, & Jenni 2011; Almasi et al. 2013) that individuals that are more resistant to oxidative stress have higher levels of stress-induced CORT. However, different indices of oxidative stress are not necessarily related to ornamentation (Viblanc et al., 2016; Henschen et al., 2016) or CORT (Costantini, Fanfani, & Dell'Omo 2008) in the same way. Thus, it is possible that another indicator of oxidative stress might be related to ornaments and CORT. It is also possible that highly ornamented males have other ways to cope with the negative effects of high CORT levels. For example, by returning to baseline CORT levels faster after a stress response (Almasi et al., 2010) or coping with the increased energetic demands of high CORT levels more easily. In support of this latter idea, individuals in other species of birds with access to more food (Heath & Dufty, 1998; Marra and Holberton, 1998; Kitaysky, Wingfield, & Piatt, 1999), greater fat stores (Jenni, Jenni-Eiermann, Spina, & Schwabl 2000), or better body condition (Blas, Baos, Bortolotti, Marchant, & Hiraldo, 2005) have higher levels of CORT, or a quicker increase in CORT, during an acute stress response.

CORT produced during an acute stress response may also affect the expression of ornaments, for example, through the effects CORT has on mitochondrial function (Hill, 2014). Increased CORT production will increase oxidative phosphorylation in the mitochondria (Manoli et al., 2007), potentially providing the energy necessary to produce elaborate ornaments. Mitochondrial function also affects other processes that

can, in turn, affect the expression of melanin- and carotenoid-based ornaments, including immune responses (Arnoult, Soares, Tattoli, and Girardin 2011) and the production of reactive oxygen species (Murphy, 2009). Studies that measure ornamentation and stress-induced CORT levels during moult (when plumage ornaments are being produced) will be necessary to determine if CORT levels affect ornament expression by regulating other physiological processes.

Overall, our study suggests that ornaments may signal the ability of individuals to cope with environmental stressors by producing a large increase in CORT during an acute stress response. The positive relationships between the increase in CORT and both melanin- and carotenoid-based ornaments suggest that general, rather than pigment specific, mechanisms produce these relationships. Thus, future studies of the relationship between ornamentation and stress-induced CORT levels may benefit from measuring a variety of other indices of health or condition that might help mediate high levels of CORT.

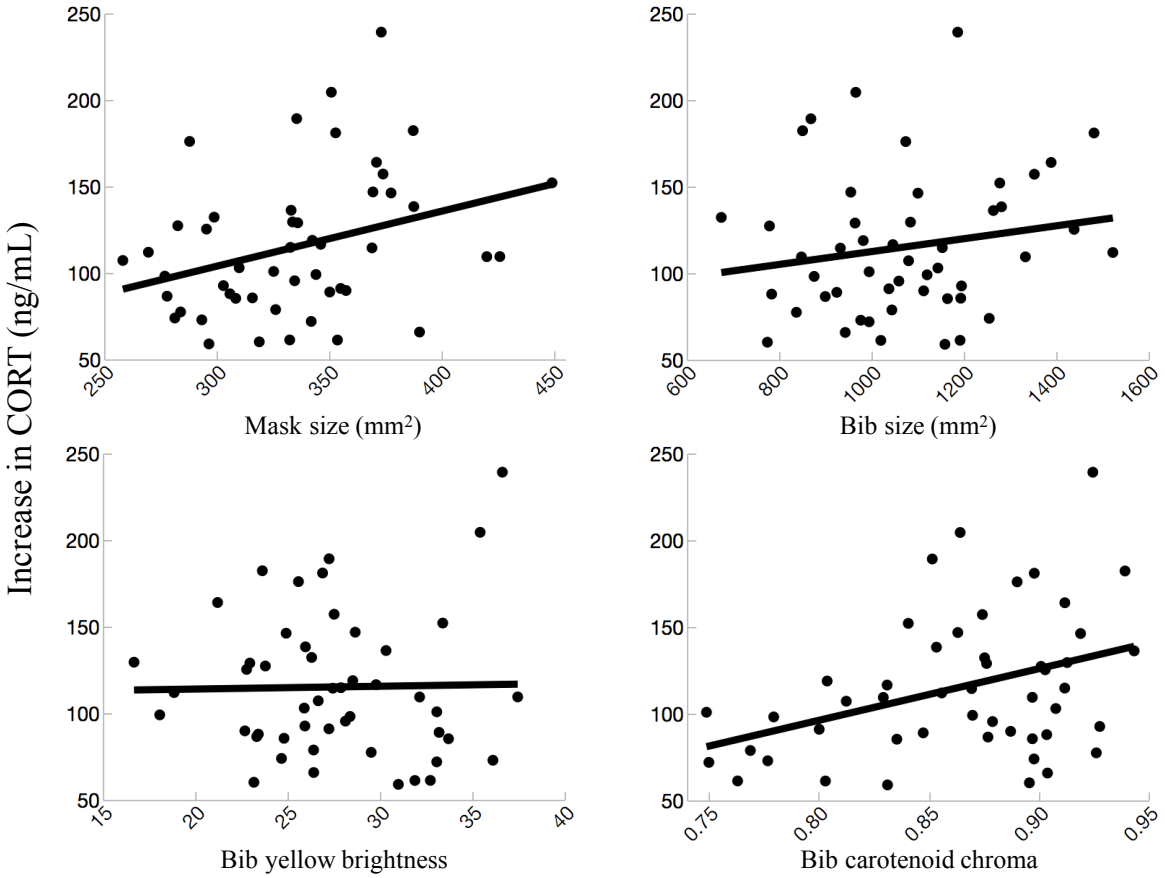

Fig. 3.1. Bivariate relationships between the stress-induced increase in CORT and ornaments. Increase in CORT is positively related to both mask size (b = 0.32 ± 0.13, t_{48} = 2.37, *P* = 0.02) and bib carotenoid chroma (b = 297.54 ± 106.84, t_{48} = 2.78, *P* = 0.01) but not bib size (b = 0.04 ± 0.03, t_{48} =1.23, *P* = 0.23) or bib yellow brightness (b = 0.16 ± 1.25, t_{48} = 0.13, *P* = 0.90).

Table 3.1. Relationships between the stress-induced increase in CORT and ornaments in common yellowthroats (four separate bivariate analyses).

Predictors	R^2	N	Estimate	SE	t	*P*
Intercept	0.11	49	8.30	45.35	0.18	0.86
Mask size			0.32	0.13	2.37	**0.02**
Intercept	0.03	49	75.28	33.05	2.28	0.03
Bib size			0.04	0.03	1.23	0.23
Intercept	<0.01	49	110.72	35.01	3.16	<0.01
Bib yellow brightness			0.16	1.25	0.13	0.90
Intercept	0.14	49	-141.86	92.47	1.53	0.13
Bib carotenoid chroma			297.54	106.84	2.78	**0.01**

Table 3.2. Effects on the stress-induced increase in CORT of ornaments, resistance to oxidative stress (KRL), and the interaction between ornaments and resistance to oxidative stress (four separate GLMs).

Predictors	R^2	N	Estimate	SE	t	*P*
Intercept	0.26	23	-46.92	67.09	0.70	0.49
KRL			0.22	0.35	0.64	0.53
Mask size			0.44	0.22	2.03	0.06
Mask size * KRL			<0.01	0.01	0.12	0.91
Intercept	0.15	23	40.59	45.36	0.90	0.38
KRL			0.24	0.32	0.74	0.47
Bib size			0.05	0.04	1.47	0.16
Bib size * KRL			<0.01	<0.01	0.28	0.78
Intercept	0.16	23	91.49	48.70	1.88	0.08
KRL			0.46	0.32	1.45	0.16
Bib yellow brightness			-0.32	1.63	0.19	0.85
Bib yellow brightness * KRL			-0.12	0.08	1.59	0.13
Intercept	0.10	23	-11.37	140.17	0.08	0.94
KRL			0.36	0.31	1.14	0.27
Bib carotenoid chroma			117.04	156.97	0.75	0.47
Bib carotenoid chroma * KRL			4.95	7.41	0.67	0.51

Table 3.3. Bivariate relationships between resistance to oxidative stress (KRL) and plumage ornaments.

Predictors	R^2	N	Estimate	SE	t	*P*
Intercept	<0.01	23	52.47	47.02	1.12	0.28
Mask size			0.03	0.14	0.20	0.84
Intercept	<0.01	23	62.10	28.28	2.20	0.04
Bib size			<-0.01	0.03	0.01	0.99
Intercept	<0.01	23	63.45	31.21	2.03	0.05
Bib yellow brightness			-0.07	1.18	0.06	0.96
Intercept	0.02	23	121.23	94.65	1.28	0.21
Bib carotenoid chroma			-68.35	108.56	0.63	0.54

Table 3.4. Examples of studies that examined the relationship between baseline or stress-induced levels of glucocorticoid (GC) hormones and ornamentation. Relationships between GCs and ornamentation are indicated as follows: 0 indicates none, + indicates positive, and a – indicates negative relationship.

Hormone Measurement	Species	Ornament Type	Relationship	Study
Baseline GCs	nestling barn owls (*Tyto alba*)	eumelanin-based plumage	0	**Almasi et al. 2010**
	nestling barn owls (*Tyto alba*)	pheomelanin-based plumage	0	**Almasi et al. 2010**
	barn owls (*Tyto alba*)	eumelanin-based plumage	0	**Almasi et al. 2013**
	king penguins (*Aptenodytes patagonicus*)	carotenoid-based beak coloration (UV hue)	0	**Viblanc et al. 2016**
	nestling barn owls (*Tyto alba*)	pheomelanin-based plumage	+	**Almasi et al. 2008**
	common lizards (*Lacerta vivipara*)	carotenoid-based skin ornament (red hue)	+	**Fitze et al. 2009**
	sand lizards (*Lacerta agilis*)	green badge	+	**Lindsay et al. 2016**
Stress-induced GCs	nestling barn owls (*Tyto alba*)	pheomelanin-based plumage	0	**Almasi et al. 2010**
	nestling barn owls (*Tyto alba*)	eumelanin-based plumage	-	**Almasi et al. 2010**
	barn owls (*Tyto alba*)	eumelanin-based plumage	-	**Almasi et al. 2013**
	Atlantic salmon (*Salmo salar*)	eumelanin-based skin color	-	**Kittilsen et al. 2009**
	Atlantic salmon (*Salmo salar*)	eumelanin-based skin color	-	**Kittilsen et al. 2012**
	house sparrows (*Passer domesticus*)	eumelanin-based plumage	+	**Lendvai and Chastel 2010**
	king penguins (*Aptenodytes patagonicus*)	carotenoid-based beak coloration (UV hue)	+	**Viblanc et al. 2016**

Table S3.1. Bivariate relationships between the stress-induced increase in CORT and temperature at capture, time of capture and capture date.

	Predictors	R^2	N	Estimate	SE	t	*P*
Increase in CORT	Intercept	<0.01	49	120.72	30.74	3.93	<0.01
	Temp at capture			-0.30	1.64	0.18	0.86
Increase in CORT	Intercept	0.04	49	160.03	33.94	4.71	<0.01
	Time of capture			-0.05	0.04	1.34	0.19
Increase in CORT	Intercept	0.07	49	151.72	20.91	7.26	<0.01
	Capture date			-1.15	0.63	1.82	0.08

Table S3.2. Relationships between tarsus length and mask or bib size.

	Predictors	R^2	N	Estimate	SE	t	*P*
Mask size	Intercept	0.01	48	515.13	236.68	2.18	0.03
	Tarsus length			-8.80	11.60	0.76	0.45
Bib size	Intercept	0.02	48	-28.68	1174.65	0.02	0.98
	Tarsus length			53.70	57.58	0.93	0.36

Table S3.3. Bivariate relationships between baseline CORT and capture date, time of capture, temperature at capture, ornamentation, and resistance to oxidative stress (KRL assay).

	Predictors	N	Estimate	SE	t	*P*
Baseline CORT	Intercept	51	57.21	13.03	4.39	<0.01
	Temp at capture		-1.68	0.70	2.41	**0.02**
Baseline CORT	Intercept	51	30.72	15.42	1.99	0.05
	Time of capture		-0.01	0.02	0.28	0.78
Baseline CORT	Intercept	51	46.52	8.93	5.21	<0.01
	Capture date		-0.64	0.27	2.35	**0.02**
Baseline CORT	Intercept	51	-1.61	21.06	0.08	0.94
	Total mask		0.08	0.06	1.34	0.19
Baseline CORT	Intercept	51	24.56	14.75	1.66	0.10
	Bib size		<0.01	0.01	0.13	0.90
Baseline CORT	Intercept	51	43.82	15.37	2.85	0.01
	Yellow brightness		-0.63	0.55	1.15	0.26
Baseline CORT	Intercept	51	-7.73	44.74	0.17	0.86
	Carotenoid chroma		39.49	51.64	0.76	0.45
Baseline CORT	Intercept	23	28.35	10.44	2.72	0.01
	KRL		-0.06	0.16	0.39	0.70

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Wingfield, J.C., Smith, J.P. & Farner, D.S. (1982) Endocrine Responses of White-Crowned Sparrows to Environmental Stress. *The Condor,* 84, 399-409.

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Amberleigh Henschen

Curriculum Vitae

EDUCATION/TRAINING

Ph.D. Candidate, Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin.

Anticipated Graduation Date: May 2018

Advisors: Dr. Peter Dunn and Dr. Linda Whittingham

Dissertation title: Plumage ornaments signal male physiological quality in common yellowthroats

B.S. in Biology, *magna cum laude*, Creighton University, Omaha, Nebraska.

ACADEMIC APPOINTMENTS

2011-Present	Teaching Assistant, University of Wisconsin- Milwaukee.
	Lab instructor for: Introduction to Zoology, Introduction to Biological Sciences, Birds of Wisconsin, Elements of Biology
2011	Teaching Assistant, Introduction to Biology, Creighton University.

CURRENT RESEARCH INTERESTS

Evolution, ecology, and animal behavior. Specific projects include determining the relationships between parasitism, stress, gene expression and male ornamentation and characterizing the immune gene diversity of small populations. I use a combination of genetic techniques, physiological assays, and field studies to address these questions.

RELEVANT SKILLS

- Extensive laboratory experience, including expertise in:
  - Molecular genetics techniques, including RNA and DNA extraction
  - Physiological assays, including ELISA and colorimetric assays
  - Parasite quantification using blood smears
- Data analysis experience:
  - Transcriptomic (RNAseq) data analysis
  - Large data sets and general linear models
  - Within-subject centering
- Field experience, including avian capture and sampling techniques
- Strong oral and written communication skills

PUBLICATIONS

2018 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. Accepted. Male stress response is related to ornamentation but not resistance to oxidative stress in a warbler. *Functional Ecology*.

2017 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2017. The relationship between blood parasites and ornamentation depends on the level of analysis in the common yellowthroat. *Journal of Avian Biology* DOI: 10.1111/jav.01418.

2016 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2016. Oxidative stress is related to both melanin- and carotenoid-based ornaments in the common yellowthroat. *Functional Ecology* 30:749-758.

2014 Bateson, Z.W., P.O. Dunn, S.D. Hull, **A.E. Henschen**, J.A. Johnson, and L.A. Whittingham. 2014. Genetic restoration of a threatened population of greater prairie-chickens. *Biological Conservation* 174:12-19.

CURRENT PROJECTS

Henschen, A.E., L.A. Whittingham, and P.O. Dunn. Differences in gene expression in developing ornamental and non-ornamental feathers. Anticipated manuscript submission: Fall 2018.

Schneider, R.L., **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. Physiological differences between resident and migratory populations of the common yellowthroat. Anticipated manuscript submission: Fall 2018.

GRANTS

2017 University of Wisconsin- Milwaukee, Clifford H. Mortimer Award, $1,500.

2016 National Science Foundation, Doctoral Dissertation Improvement Grant, “Functional Genomics of Ornament Production in a Warbler”, $18,359.

2015 University of Wisconsin- Milwaukee, Louise Neitge Mather Award, $1,200.

2014 University of Wisconsin- Milwaukee, Ruth Walker Memorial Research Award, $1,500.

2013 The American Ornithologists' Union, Margaret Morse Nice Research Award: "Immune genes and male ornaments in the Common Yellowthroat", $2,450.

2013 Animal Behavior Society, Student Research Grant: "Immune genes and male ornaments in the Common Yellowthroat", $1,500.

2013 Wisconsin Society for Ornithology, WSO Grant: "Immune genes and male ornaments in the Common Yellowthroat", $500.

PRESENTATIONS

2018 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2018. Resistance to oxidative stress mediates the acute stress response in common yellowthroats. Talk. Annual meeting of the Society for Integrative and Comparative Biology, San Francisco, California.

2017 **Henschen, A.E.** 2017. What do male ornaments signal to female birds? Department of Biological Sciences Colloquium, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin.

2017 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2017. Do plumage ornaments signal how individuals respond to stress? Talk. Annual meeting of the Society for Integrative and Comparative Biology, New Orleans, Louisiana.

2016 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2016. Parasitism and ornamentation: a within-individual study in the common yellowthroat. Talk. Annual meeting of the Animal Behavior Society, Columbia, Missouri.

2016 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2016. Parasitism and ornamentation: a within-individual study in the common yellowthroat. Talk. Annual meeting of the Society for Integrative and Comparative Biology, Portland, Oregon.

2015 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2015. Oxidative stress is related to a melanin-based ornament in the common yellowthroat. Biological Sciences Research Symposium, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin.

2015 **Henschen, A.E.**, L.A. Whittingham, and P.O. Dunn. 2015. Oxidative stress, immune response, and male ornaments in the common yellowthroat. Society for Integrative and Comparative Biology Annual Meeting, West Palm Beach, Florida.

2011 **Henschen, A.E.** and S. Cho. 2011. Evolution of the hominoid alpha-amylase gene family. Poster presentation. Annual meeting of the Society for the Study of Evolution, Norman, Oklahoma.

2011 **Henschen, A.E.** and S. Cho. 2011. Evolution of the hominoid alpha amylase gene family. Poster presentation. Department of Biology Research Colloquium, Creighton University, Omaha, Nebraska.

HONORS AND AWARDS

2011-2017 Chancellor's Award, UW- Milwaukee, Milwaukee, Wisconsin.

2017 Graduate Student Travel Award, UW- Milwaukee, Milwaukee, Wisconsin.

2015 Best Graduate Student Presentation, UW-Milwaukee Biological Sciences Research Symposium, Milwaukee, Wisconsin.

2015 Graduate Student Travel Award, UW- Milwaukee, Milwaukee, Wisconsin.

PROFESSIONAL DEVELOPMENT & SERVICE

2018 Mentor, Society for Integrative and Comparative Biology Broadening Participation Mentorship Program.

2017-2018 Peer reviewer, Journal of Animal Ecology and Behavioral Ecology.

2015-2017 Coordinator for the Behavioral and Molecular Ecology Journal Club, UW-Milwaukee.

2017 Judge for the Biological Sciences Undergraduate Research Symposium, UW- Milwaukee.

2017 Judge for the Undergraduate Research Symposium, UW- Milwaukee.

2014 Judge for the Badger State Science & Engineering Fair.

2013 Completed the National Science Foundation's Responsible Conduct of Research Training.

INVITED LECTURES

2016 Guest Lecture on Cnidarians. BioSci 100, Survey of Zoology. UW-Milwaukee Department of Biological Sciences, Milwaukee, Wisconsin.

2015 Guest Lecture on Oxidative Stress in Birds. BioSci 523, Evolution and Ecology of Birds. UW- Milwaukee Department of Biological Sciences, Milwaukee, Wisconsin.

PUBLIC OUTREACH

2017 Lecture given by **A.E. Henschen**. "If these feathers could talk: What feather samples can tell researchers about wild birds", Brew City Birding Festival, Urban Ecology Center, Milwaukee, Wisconsin.

2016 Joint lecture given by **A.E. Henschen**, R.L. Schneider, Z.W. Bateson, and P.O. Dunn. "50 years of bird research at UW- Milwaukee", Wisconsin Society for Ornithology Annual Convention, Racine, Wisconsin.

2015 **Henschen, A.E.**, L. A. Whittingham, and P. O. Dunn. 2015. Do male ornaments advertise parasitic infection intensity in the common yellowthroat? Talk. Public Research Symposium, Riveredge Nature Center, Saukville, Wisconsin.

2014 Research featured in "Discoveries Abound in UWM's Natural Classrooms", University of Wisconsin- Milwaukee Alumni Magazine.

2013 **Henschen, A.E.**, L. A. Whittingham, and P. O. Dunn. 2013. Male ornaments and oxidative stress in the Common Yellowthroat. Talk. Public Research Symposium, Riveredge Nature Center, Saukville, Wisconsin.

PROFESSIONAL MEMBERSHIPS

Society for Integrative and Comparative Biology

Animal Behavior Society

Society for the Study of Evolution

Phi Sigma Biological Honors Society