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ABSTRACT

Avian malaria is a vector-borne disease whose effects on reproductive success and life history strategies are poorly understood. Avian malaria likely has consequences on carotenoid metabolism and color display in many species of birds. Carotenoid deposition in feathers and the associated yellow, orange, and red feather colorations are often used to evaluate individual quality. We analyzed feathers and tissues from specimens of a woodpecker species, *Sphyrapicus ruber,* collected from California and Oregon in 2012. This species is known to contract avian malaria and has several carotenoid-based plumage patches on its body. Infected individuals had a higher percentage of red, carotenoid-based plumage on the breast region but a lower concentration of carotenoids deposited in feathers, compared to indidivuals not infected with avian malaria. We suggest that avian malaria influences carotenoid metabolism and that the type of carotenoid being deposited in feathers is more important, biologically, than overall carotenoid concentrations. With the vectors of avian malaria experiencing range changes, our work helps shed light on how avian malaria may affect avian life strategies.

Keywords: malaria, carotenoids, disease, plumages, *Sphyrapicus*

INTRODUCTION

Avian malaria is a widespread disease caused by a diverse subset of protozoan blood parasites (Ricklefs and Fallon 2002, Bensch et al. 2004), many of which are protozoans in the genus *Plasmodium* (LaPointe et al. 2012). Avian malaria has been intensively studied in a variety of bird populations worldwide for over a century (Garamszegi 2011), making avian malaria an excellent study system for investigating avian disease ecology. Malaria pathogens are commonly transferred via mosquito vectors, belonging to the genus *Culex* (Reeves et al. 1954, Valkiūnas 2005). Mosquitos are non-discriminate blood feeders, making the transfer of avian malaria to multiple species and individuals in an area likely (Hellgren et al. 2009), causing the disease to have a widespread distribution. As the earth's surface air temperature continues to rise, there will be significant impacts on the biology of *Culex* mosquitos and *Plasmodium* protozoa. There will likely be a shift in the range of avian malaria vectors northward and upward in elevation as the temperatures in northern latitudes increase (Garamszegi 2011). Additionally, there is evidence that *Plasmodium* protozoa will benefit directly from increases in ambient temperature as the

incubation phase within a host is temperature sensitive (Patz and Olson 2006, Paaijmans et al. 2009).

Many of the vibrant red, yellow, and orange colorations seen in numerous animal ornamental displays are carotenoid-based (Weaver et al. 2018). Carotenoid pigments are unique in that they cannot be synthesized *de novo* by animals, and therefore must be obtained directly from the diet (Völker 1938, Goodwin 1984). This indicates that carotenoid-based color displays are directly linked to an individual's foraging capabilities (McGraw 2002). An animal in proper nutritional condition will produce a brighter, possibly more extensive, color display than a counterpart that is nutritionally stressed (Hill 2000). Carotenoid color displays have been found to play a role in female mate choice in several species of fish and birds (Hill 1999), likely due to the conveyance of an individual's quality (Hill 2002). In addition to the visual displays of health, carotenoids also are critical for immunostimulation, vitamin A synthesis, and the uptake of free radicals (Britton 1995, Chew and Park 2002). The dual role of carotenoids in both immunocompetence and plumage coloration forms the basis for the resource allocation trade-off hypothesis (Navara and Hill 2003). This hypothesis states that because carotenoids are key components in both color display and immune function, only individuals with an abundance of carotenoids, due to good health status, may utilize carotenoids for both general body maintenance and full ornamental display. Whether the carotenoids are immediately deposited as pigments upon consumption or undergo further metabolic conversion and then deposited in plumage plays a strong role in whether there is a relationship between an individual's overall quality and plumage coloration (Weaver et al. 2018).

It is well-documented that parasitic infections suppress deposition of carotenoids into plumage as decorative coloration (Thompson et al 1997, Zuk et al. 1998, Brawner et al. 2000, Hill 2000, Mcgraw and Hill 2000). However, exlcuding direct mortality, the impacts of avian malaria on a host's overall reproductive success and fitness is poorly understood (Lachish et al. 2011). In this study we sought to evaluate the resource allocation hypothesis by comparing the differences in carotenoid pigment concentration and physical amounts of red plumage coloration in a species of woodpeckers, *Sphyrapicus ruber,* infected with avian malaria.

METHODS

Genomic Sampling, Sequencing, and Genetic Ancestry

We studied patterns of malaria infection in a hybrid zone between *Sphyrapicus ruber* and *S. nuchalis*. For this study we used 65 vouchered *S. ruber* specimens collected from California and Oregon that are housed at the UWYMV. These specimens were collected as part of a larger study on patterns of introgression in sapsuckers in 2012 (Billerman *et al*. in review). The genomic data we used for this study was generated in Billerman *et al*. (in review). Briefly, we: 1) extracted total genomic data from pectoralis muscle using a DNeasy tissue extraction kit (Qiagen, Valencia, California), 2) sent the extracted DNA to the Cornell Institute for Genomic Diversity (Elshire *et al*. 2011), where samples were digested with PstI and then prepared for Genotyping-by-Sequencing (GBS), and 3) processed the GBS sequence reads as in White *et al*. (2013) on the TASSEL pipeline (Bradbury *et al*. 2007, Lynch *et al*. 2009). For sequence filtering and SNP calling parameters, see Billerman *et al*. (in review). We calculated a genomic hybrid index using 13,832 SNPs that passed our filtering parameters (Billerman *et al*. in review). Briefly, we calculated the genomic hybrid index using the program STRUCTURE version 2.3.1

(Pritchard *et al*. 2000), which calculates admixture proportions using a model of Bayesian inference. We ran 3 chains for 500000 generations following a 10000-generation burn-in. To ensure that allopatric populations were scaled to 0 and 1, which made interpreting patterns of introgression in hybrids more feasible, we assumed a *k* of 2, using the prior population information parameter to set allopatric individuals at q-values of 0 and 1, respectively.

Parasitic Infection Detection

Using the same extracted DNAs as above, we used a nested PCR assay (Waldenström et al., 2004) to screen for avian malaria infections through the amplification of cytochrome b (cyt b) of the blood parasite genera Haemoproteus and Plasmodium. All PCRs were performed in 10 µl volumes with 6.85 µl dH2O, 1 µl (~20 ng) genomic DNA, 0.6 µl MgCl2 (2.5mM), 1.0 µl 10x PCR buffer, 0.25 µl dNTPs (10 mM for each dNTP), 0.1 µl of each primer (10 mM), and 0.1 µl AmpliTaq (5U/µl; Applied Biosystems, Foster City, California). The thermocycler reaction profile was as follows: initial denaturation at 95 C for 3 min, followed by 20 cycles (first PCR) or 35 cycles (second PCR) of 96 C for 30 sec, 49 C for 30 sec, and 72 C for 45 sec, and a final elongation step at 72 C for 10 min. The first PCR utilized primers HAEMNF (5'CATATATTAAGAGAATTATGGAG-3') and HAEMNR2 (5'AGAGGTGTAGCATATCTATCTAC-3'). The second PCR was utilized primers HAEMF (5'-ATGGTGCTTTCGATATATGCATG-3') and HAEMR2 (5'-GCATTATCTGGATGTGATAATGGT-3'); (Jones et al. 2013). The first set of primers amplified a conserved region of avian malaria cyt b, which borders the fragment amplified with the second pair of primers primer during the final PCR reaction. This final PCR amplified 524-bp of avian malaria parasite cyt b. For the final PCR, 1.0 µl of the initial PCR product (using first set of primers) was used as template in 10 µl volume reactions with the primers HAEMF and HAEMR2. Identical reagents, proportions, and thermocycler profile was used for the second PCR as in the initial PCR, with the exception that 35 cycles instead of 20 cycles being used during amplification. Using a 2% agarose gel, final PCR products were then screened for parasite cyt b. Each individual underwent PCR and gel electrophoresis 3 times within the same PCR run, to confirm the presence or absence of infection.

Plumage Analysis

We quantified phenotypes using digital photographs of the specimens we used in this study. We characterized the phenotype based on a single character, the amount of red in the plumage of individuals. All specimens were photographed under standard light conditions with calibrated equipment (McKay *et al*. 2013, Billerman *et al*. in review); photographs archived on the Arctos database (http://arctos.database.museum) linked to museum specimens housed at the UWYMV were used to calculate the phenotype of individuals. RAW photo files were converted into 16-bit TIFF format for analysis. We took 4 measurements from each specimen: 1) proportion of the length of red on the breast, 2) proportion of the length of red on the crown, 3) percent of red on the breast, and 4) percent of red on the face. For the first two measurements, we used the profile pictures of specimens. Briefly, we used IMAGEJ (Schneider *et al*. 2012) to calculate the first two measures from the profile pictures of each specimen. We measured the length of the red on the breast and on the crown and calculated the relative proportion of these lengths using the total

length of the specimen from the base of the bill to the vent. All length measurements were repeated 10 times, and we took the mean to account to variation in measures (Billerman *et al*. in review.). We used R (R Core Development Team 2015) to calculate the percent of red in selected patches on the breast and face of each specimen. We selected a standardized plumage patch on the breast and face of each specimen as in Billerman *et al*. (in review). From each standardized patch, we calculated the percent of pixels in each selection that were "red." We defined red here as pixels that had red channel values in RGB colorspace that were less than 60, and greater than 1.5 times the value of the green channel. We used the 'rtiff' (Kort 2015) and 'rgl' (Adler and Murdoch 2016) packages to calculate the RGB values of each pixel and to select red pixels (Billerman *et al*. in review).

Feather collection and carotenoid analysis

Five to seven red feathers were plucked from a standardized location on the throat of each specimen and stored in sealed envelopes in the dark until carotenoid analysis. To remove surface lipids, feathers were washed in ethanol and hexane, sequentially, then blotted dry and weighed to the nearest 0.01 mg. The distal red portion of feathers were trimmed, re-weighed to the nearest 0.01 mg, and placed in a 1.8 mL microcentrifuge tube for carotenoid extraction.

Carotenoids were extracted from the red portion of feathers by modifying previously reported methods (Stradi et al. 1995, McGraw et al. 2002). Briefly, feathers were placed in 500 µL acidified pyridine, the tube headspace was capped with N_2 gas, and the sample was incubated at 95 °C for 1h. After allowing the samples to cool to room temperature, the pyridine was aspirated pyridine from the feathers and placed into a new tube, and a second pyridine extraction was performed under the same conditions. After each incubation, 250 µL ddH_20, 250 µL *tert*-butyl methyl ether, then 250 µL hexane were added to the pyridine solution. The tube was then shook vigorously for 1 min to transfer the carotenoid fraction from the pyridine to the upper organic phase. Adding the solvents in this order produced a pronounced meniscus and phase separation without the need to vortex. The process was repeated with the second pyridine extraction and combined the upper colored phase from the first and second extraction containing the carotenoids in a new tube and evaporated it to dryness under vacuum at 30 °C.

To measure total carotenoid content, each sample was resuspended in 240 µL ethanol, aliquoted 120 µL in a 96-well plate in duplicate and absorbance was measured at 450 nm using a microplate spectrophotometer (BioTek, Winooski, VT). Total carotenoid content of the sample was calculated by reference to the absorbance from a calibration curve of known concentrations of canthaxanthin measured under the same conditions. Total carotenoid content measured spectrophotometrically correlated strongly with calculations based on HPLC analysis *(*Pearson's $r = 0.951$, supplementary note 1). We report total carotenoid content as µg mg $^{-1}$ red feather mass.

Data Analysis

To evaluate whether infections rates of avian malaria are influenced by sex, we performed a Pearson's Chi square test with a Yates' continuity correction. We used two-tailed, paired sample t-tests to assess significance (α=0.05 for all tests) between infected versus uninfected individuals and red facial percentages, red breast percentages, and total feather carotenoid concentration. We used linear models to evaluate the interactions between carotenoid concentrations and the

percentage of red breast plumage in conjunction with infection status. Another linear model was used to test the interactions between carotenoid concentration and red breast plumage as a function of infection status. We conducted all data analysis in Program R (R Core Team 2016).

RESULTS

We analyzed 65 individuals for which we had both plumage and infection data. A total of 26 individuals (40%) were infected with avian malaria across 5 distinct populations in California and Oregon, USA. Between males and females there were no significant differences in infection rates (X-squared < 0.001, df = 1, P = 0.977).

Individuals infected with avian malaria had a significantly higher percentage of red plumage on the breast than individuals not infected with avian malaria (t = 2.654, df = 51.794, P = 0.011, Figure 1). Conversely, there was no significant difference in the percentage of red plumage on the face between infected and uninfected individuals (t = - 0.074, df = 48.867, P = 0.941, Figure 2). Individuals infected with avian malaria had significantly lower concentrations of carotenoids found in feathers compared to uninfected individuals (t = -2.397, df = 55.165, P = 0.02, Figure 3). A basic linear model positing carotenoid concentration and percentage of red breast plumage as a function of infection status was found to be significant (R^2 = 0.129, df = 53, P = 0.016).

DISCUSSION

The impacts of avian malaria on metabolic processes and sexual selection are not clear, our data helps better predict how avian malaria may influence the life strategies of *Sphyrapicus ruber* and other bird species. Our data suggest that the percentage of carotenoid pigmentation in the facial area of *S. ruber* is not as heavily influenced by infection status (Figure 1) as the pigmentation percentages of the breast feathers (Figure 2). This may suggest that the extend of coloration on the breast is a stronger signal of individual quality than the extent of red coloration in the facial region. Individuals with avian malaria had a greater percentage of red coloration on the breast region, which contradicts the resource allocation trade-off hypothesis (Navara and Hill 2003). Weaver et al. (2018) shows that individual quality, and therefore mate choice, is impacted by whether carotenoid coloration is converted or not. With mating and raising young a priority for short-lived species, there is the potential that indiviudal *S. ruber* are prioritizing mating over combatting infection. With red coloration being a signal of quality, a greater extent of red coloration on the breast may be an adaptive stategy in response to avian malaria in this bird species.

We determined that the total concentrations of carotenoid pigments deposited in feathers was significantly less in individuals infected with avian malaria (Figure 3). This indicates that carotenoid deposition and perhaps the conversion of individual carotenoid pigment types (Weaver et al. 2018) are being impacted by an individual's infections status. Our interaction model also suggests an interaction between carotenoid concentrations being deposited in feathers and the physical coloration and extent of that coloration on breast feathers, as a function of infection status (Figure 4). How the interactions of disease, coloration, and carotenoid metabolism intersect is still a point of interest that merits further research. As *Culex* moquitos and avian malaria continue to expand latitudinally and elevationally, how this disease changes the life strategies and reproductive biology of birds will become more relevant in the future.

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FIGURES

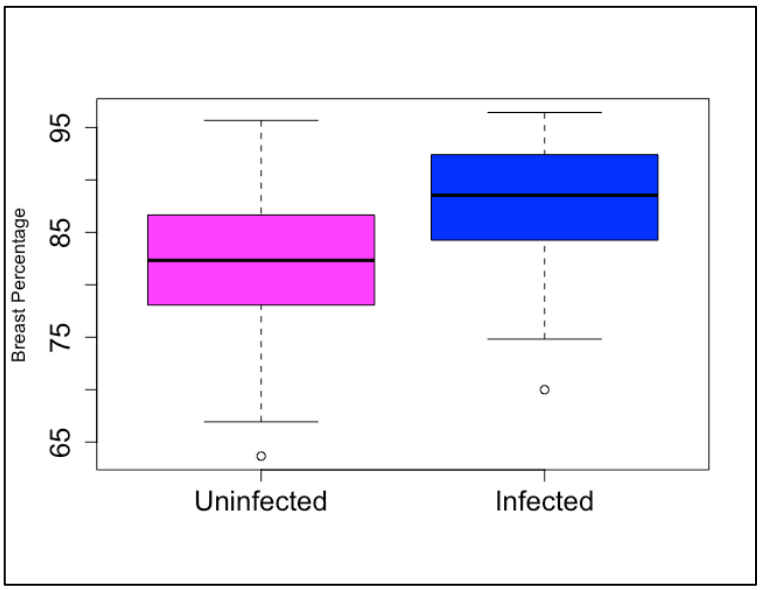

Figure 1: Box and whisker plot showing significant differences in the mean percentage of red plumage on the breast of *S. ruber* specimens, collected from California and Oregon in 2012, between those infected with avian malaria and those uninfected.

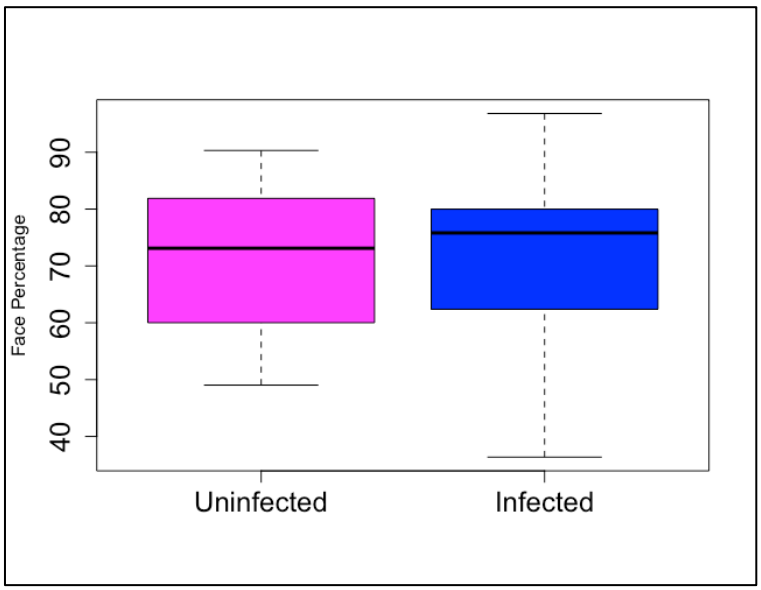

Figure 2: Box and whisker plot showing insignificant differences in the mean percentage of red plumage on the breast of *S. ruber* specimens, collected from California and Oregon in 2012, between those infected with avian malaria and those uninfected.

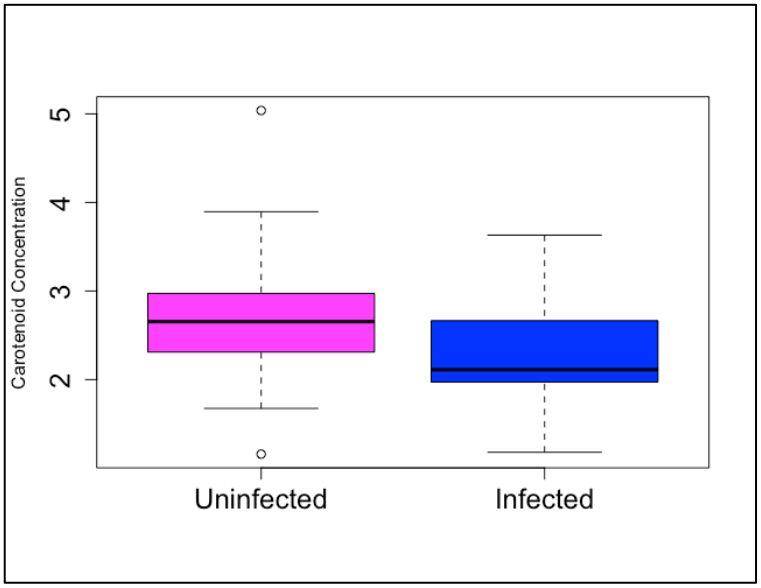

Figure 3: Box and whisker plot showing significant differences in the mean carotenoid concentration of feathers sampled from *S. ruber* specimens, collected from California and Oregon in 2012, between those infected with avian malaria and those uninfected.

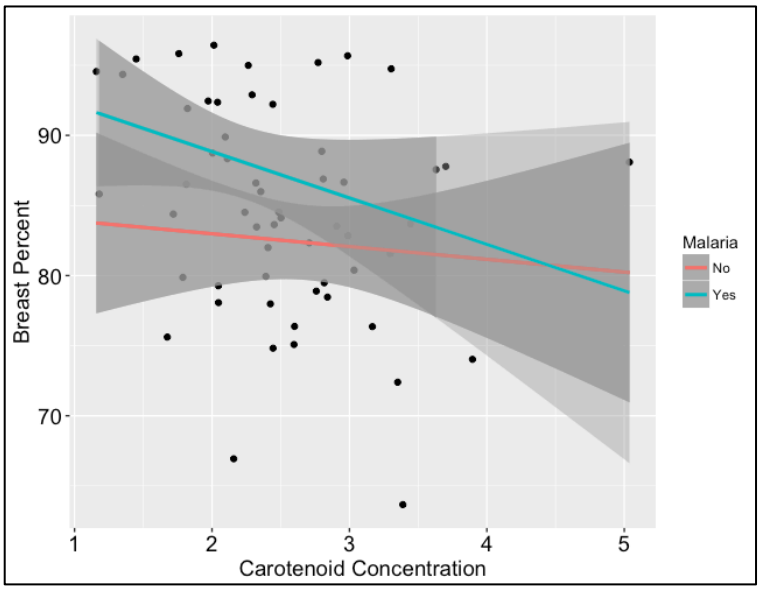

Figure 4: Linear regression depicting the interactions between percentage of red plumage on the breast and carotenoid concentration as a function of infection status in *S. ruber* specimens collected from California and Oregon in 2012.