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THE EFFECTS OF ANTIOXIDANT SUPPLEMENTATION, MIGRATORY
SEASON, AND FLIGHT TRAINING ON KEY COMPONENTS OF THE
ANTIOXIDANT SYSTEM OF MIGRATORY EUROPEAN STARLINGS

(STURNUS VULGARIS)

BY

ABIGAIL E. FRAWLEY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2020

MASTER OF SCIENCE THESIS

OF

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2020

ABSTRACT

Migratory birds engage in two periods of endurance flight as they travel from summer breeding to overwintering grounds, and these flights likely increase the production of reactive species that can degrade biologically important molecules. Fall and spring likely represent different oxidative challenges, as many females prepare to breed shortly after spring migration, and antioxidant-rich fruits are plentiful in fall but not spring. I examined the effects of season, anthocyanin supplementation, and flight training on the antioxidant system of female European starlings (*Sturnus vulgaris*) to determine how each of these factors effects enzymatic antioxidant activity, non-enzymatic antioxidant capacity, and levels of oxidative damage. We fed females a diet that was either supplemented with dietary anthocyanin or not supplemented. We then flew females each day in a windtunnel for two weeks in both fall and spring, concluding with a long-duration flight on day 15. This study is one of the most comprehensive examinations of the antioxidant system of a migratory bird to date.

In Chapter I, I compared birds flown in fall or spring to study seasonal differences, the effects of anthocyanin supplementation, and the impact of flight training on the antioxidant system. I analyzed red blood cells and plasma to assess circulating uric acid, circulating non-enzymatic antioxidant capacity (OXY), glutathione peroxidase (GPx) activity, and levels of lipid oxidation (d-ROMs). We blood-sampled females at three timepoints: prior to flight training, immediately after the longest-duration flight on day 15, and after a two-day recovery period. Enzymatic antioxidant capacity and oxidative damage were lower in spring compared to fall.

Circulating uric acid was higher in females fed the low antioxidant diet compared to the high antioxidant diet, and females fed the high antioxidant diet had a greater decrease in non-enzymatic antioxidant capacity after flight training than females fed the low antioxidant diet. Flight training had acute and chronic effects on one measure of non-enzymatic antioxidant capacity and GPx activity.

In Chapter II, I compared birds flown in the spring to determine how flight training and dietary anthocyanin supplementation impact the antioxidant system. I analyzed tissue from two muscles (leg and pectoralis), heart, and liver to assess three enzymatic antioxidants (catalase (CAT), superoxide dismutase (SOD), and GPx), two measures of oxidative damage (protein carbonyls and lipid peroxides), and one measure of non-enzymatic antioxidant capacity (ORAC). The effects of dietary antioxidants and training were tissue specific. Dietary antioxidant supplementation decreased GPx activity in the heart, decreased non-enzymatic antioxidant capacity in the pectoralis, and increased protein damage in the leg. Flight training affected SOD activity in the liver, and we found significant diet x training interactions for enzymatic antioxidants in the liver, leg, and heart.

The results reported in Chapters I and II suggest that flight training may help to condition the antioxidant system and so allow birds to prevent oxidative damage. Dietary anthocyanin had mixed effects on enzymatic antioxidants, and both complemented and inhibited the activity of antioxidant enzymes. Furthermore, females appear to prioritize reducing oxidative damage during spring migration. In general, similar flights during fall and spring represent unique oxidative challenges for female

birds, and dietary antioxidant supplementation can both complement and attenuate key components of the antioxidant system.

ACKNOWLEDGMENTS

There are many people who helped shape and support this thesis, and I am indebted to each of them. First, I would like to thank my advisor, Dr. Scott McWilliams, for his support, good humor, and patience. You have challenged me to become a better and more creative scientist, and you always helped me see the forest for the trees. Thank you for helping me navigate graduate school, teaching me how to balance my responsibilities, and guiding me through the publishing process.

I would also like to thank my committee members, Dr. Barbara Pierce and Dr. Navindra Seeram. Thank you, Barb, for helping coordinate shipping the samples from Germany and for your invaluable feedback on my manuscripts. Navindra, thank you for your valuable insight into antioxidant analyses and for our discussions about how best to study dietary antioxidant consumption in birds. I would also like to thank Dr. Kathleen Melanson for serving as my defense chair.

Each member of the McWilliams lab assisted this project in a unique way. Clara, Wales, and Kristen, thank you all for assisting me with lab analyses, troubleshooting assays, and figuring out the best way to analyze these datasets. Clay, thank you for always being a sounding board when I got overwhelmed. Colby, thank you for always giving me a reason to laugh. Erin, thank you for being the other Dungeons and Dragons enthusiast in the lab and for your encouragement. Martina, thank you for your boundless positivity and your good advice. Kris and Tori, it has been a pleasure getting to know you this semester and I wish you all the best at URI. Finally, I would like to thank Liam Corcoran for his help with lab work. Liam, you are one of the

hardest workers I know, and I appreciate all of those long hours you spent running ORAC plates.

I also have to thank numerous members of the greater URI community. Without Deb Bourassa, this project would not have been possible. Thank you for helping me with ordering, paperwork, grant management, and a thousand other things. Thank you as well to Dr. Michael Starkey for never letting me lose sight of the end goal.

I would also like to thank all of the people at the Max Planck Institute for Ornithology for making this project possible. Thank you to all of the animal care staff, including Susan Forrest and Aleksandra Małochleb. Thank you as well to Maude Baldwin, Glenn Cockburn, and the entire Baldwin lab for providing dewars to transport samples to the US, and for providing access to their lab and equipment at MPIO.

I owe a great deal to my family. Mom, Dad, Emily, Brendan, and Caitlin, thank you for always being interested in my work and for supporting me. Dad, thanks for teaching me how to be a “bird nerd”.

Finally, I have to thank Kevin Kane. Thank you for being my best friend, my Number One, and an endless well of support. I couldn’t have done this without you.

PREFACE

This thesis was prepared using the manuscript format guidelines established by the University of Rhode Island Graduate School. Manuscript I is titled “Season, anthocyanin supplementation, and flight training have mixed effects on the antioxidant system of migratory European starlings (*Sturnus vulgaris*)” and was submitted to *The Auk*, where it is currently under review. Manuscript II is titled “Flight training and diet have mixed effects on antioxidant enzymes, non-enzymatic antioxidant capacity, and oxidative damage in female European starlings (*Sturnus vulgaris*)” and is formatted for submission for *Journal of Experimental Biology*.

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CHAPTER 1

Season, anthocyanin supplementation, and flight training have mixed effects on the antioxidant system of migratory European starlings (*Sturnus vulgaris*)

by

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ABSTRACT

Migratory birds engage in two periods of endurance flight annually as they travel between summer breeding and overwintering grounds, and such endurance flights likely incur oxidative costs. These costs may differ between fall and spring migration, especially for females who must prepare for breeding and egg laying in spring. The objective of this study of a migratory bird was to test proposed hypotheses about how key components of the female's antioxidant system differ in response to flight training in the fall and spring and to dietary antioxidant supplementation. We hand-raised female European starlings (*Sturnus vulgaris*) and then fed them either a diet supplemented with dietary anthocyanins or a diet without added anthocyanins. We then flew females in a windtunnel for 15 days during fall and spring migration seasons and measured over time oxidative damage (d-ROMs) and three components of the antioxidant system—non-enzymatic antioxidant capacity (OXY), uric acid, and glutathione peroxidase (GPx) activity. Prior to flight training, non-enzymatic antioxidant capacity and oxidative damage were lower in females during spring compared to fall, and females fed a low-antioxidant diet had consistently higher circulating uric acid. GPx was higher in spring immediately after a long-duration flight. Females fed a high-antioxidant diet had a greater decrease in non-enzymatic antioxidant capacity after the 15-day flight training. Flight-trained females had lower circulating uric acid than untrained females immediately after the longest-duration flight, and decreased GPx activity after the 15-day flight training. In sum, females upregulated enzymatic and non-enzymatic endogenous antioxidants in spring, and females fed a diet with less antioxidants appear to compensate by increasing

circulating uric acid. Our findings emphasize the important role of dietary antioxidants for birds during migration, and that similar flights in fall and spring likely represent distinct oxidative challenges in the life history of female birds.

Keywords:

anthocyanin, dietary antioxidants, flight training, female fall and spring migration, oxidative damage

Lay summary:

- Migratory birds incur oxidative costs during their long endurance flights and these costs may differ between fall and spring migration, especially for females who must prepare for breeding and egg laying in spring.
- We fed female European Starlings diets with different quantities of anthocyanin, a dietary antioxidant, and then measured during both fall and spring migration periods how their antioxidant system responded to diet and two weeks of daily flight in a windtunnel.
- Females upregulated some key components of their endogenous antioxidant system in spring and had lower oxidative damage than females in fall, and females fed less dietary antioxidants appear to have compensated by increasing circulating uric acid.
- Similar flights in fall and spring likely represent distinct oxidative challenges for female songbirds, and dietary antioxidants play an important role in modulating the antioxidant system.

INTRODUCTION

The antioxidant system of vertebrates is multifaceted, consisting of exogenous and endogenous as well as enzymatic and non-enzymatic antioxidants (Costantini 2014). The mechanisms by which different aspects of the antioxidant system interact are complex, especially in birds (reviewed in Costantini 2014 and Cooper-Mullin and McWilliams 2016), in part because the antioxidant system has been shown to be influenced by a suite of factors including diet, exercise, and seasonal cycles (Costantini et al. 2008, Larcombe et al. 2008, Cohen et al. 2009, Skrip et al. 2016, Cooper-Mullin et al. 2019). Migratory birds provide a unique opportunity to study the antioxidant system, as they undergo annual migratory flights and rely on polyunsaturated fatty acids, which are particularly vulnerable to oxidative damage, to fuel flight (Jenni and Jenni-Eiermann 1998, McWilliams et al. 2004, Price 2010, Guglielmo 2018).

Several studies have demonstrated that the antioxidant system responds to dietary antioxidants and exercise, and that oxidative damage may increase following flight. For example, free-living European robins (*Erithacus rubecula*) caught during nocturnal migratory flight had elevated markers of oxidative damage and higher circulating levels of glutathione peroxidase (GPx), an enzymatic antioxidant, compared to resting robins (Jenni-Eiermann et al. 2014). Similarly, a study in captive zebra finches (*Taeniopygia guttata*) demonstrated that oxidative damage was higher in exercised birds versus sedentary birds (Skrip et al. 2016). Eikenaar et al. (2017) also demonstrated that migratory common blackbirds (*Turdus merula*) had higher non-enzymatic antioxidant capacity than resident blackbirds. Dietary antioxidants

attenuated the stress responses and bolstered the immune response in blackcaps (*Sylvia atricapilla*) and European starlings (Catoni et al. 2008, Casagrande et al. 2020). Some dietary antioxidants, including anthocyanin, play important roles in modulating lipid and glucose metabolism in mammals (Piovezana et al. 2019). Furthermore, birds preferentially consume high-antioxidant foods during fall migration (Bolser et al. 2013, Beaulieu and Schaefer 2014). What remains to be demonstrated is how key components of this multifaceted antioxidant system respond to both exercise and antioxidant supplementation for birds during the migration period.

Migrations between wintering and breeding grounds are challenging periods in the annual cycle of migratory birds, as individuals must navigate unfamiliar terrain and contend with the physiological challenges of often repeated long-duration flights and recovery at stopover sites. These challenges include operating at 30x their basal metabolic rate while flying (Tatner and Bryant 1986, Nudds and Bryant 2000, Hedenström et al. 2009), rapidly building fat stores to fuel their journeys (Jenni and Jenni-Eiermann 1998, McWilliams et al. 2004, Guglielmo 2010), upregulating key components of the energy delivery system (Guglielmo 2010), and satisfying water and protein requirements (Klaassen 1996, Bauchinger and Biebach 2001, Gerson and Guglielmo 2011). Endurance exercise and the catabolism of fats also have the potential to increase generation of reactive species, which can damage biologically important molecules (Costantini et al. 2007, 2008, Jenni-Eiermann et al. 2014, Skrip et al. 2015). Left unchecked by antioxidant defenses, reactive species can degrade DNA, damage cells and tissues, and negatively impact an organism's fitness through accumulating oxidative damage (Niess and Simon 2007, Halliwell and Gutteridge

2015). Birds can ameliorate these oxidative challenges associated with migration by maintaining a robust antioxidant system and consuming dietary antioxidants, although to date we know relatively little about the dynamics of the antioxidant system for birds during fall and spring migration and the role of dietary antioxidants in preventing damage (Costantini 2008, Cooper-Mullin and McWilliams 2016).

Fall and spring migration in the northern hemisphere likely present distinct oxidative challenges for migratory birds, as antioxidant-rich fruits are plentiful in fall but not in spring (Parrish 1997, Bolser et al. 2013, Smith et al. 2015), and adults prepare to breed shortly after spring migration (Ramenofsky and Wingfield 2006, Legagneux et al. 2012). It has been established that depositing antioxidants into eggs is beneficial and leads to higher hatching and fledging rates for offspring (McGraw et al. 2005, Norte et al. 2010). Female birds may face an oxidative trade-off or carry-over effect if they must balance preventing oxidative damage during migration with their future reproductive success (Skrip et al. 2016). Specifically, females may prioritize depositing antioxidants into eggs rather than investing them in their own antioxidant defenses. Thus, studying both migratory seasons is vital for understanding the distinct physiological challenges individuals may face during each period of the annual cycle and how they ameliorate such challenges.

The objective of our experimental study was to determine how dietary antioxidant supplementation, daily flying for two weeks, and season affected two measures of non-enzymatic antioxidant capacity, oxidative damage to lipids, and a representative antioxidant enzyme in female migratory birds. We chose these measures in order to assess how antioxidant enzymes and non-enzymatic antioxidants

potentially interact and differ in response to an oxidative challenge. This design also allowed us to determine if flight training, season, and antioxidant supplementation affect the amount of oxidative damage done to lipids, a potentially important byproduct of using fats as the primary fuel while flying. Specifically, we evaluated the following hypotheses regarding how season (fall vs. spring migration) and dietary anthocyanin supplementation affected the response of the antioxidant system in migratory female European starlings (*Sturnus vulgaris*) to two weeks of repeated daily flying and a final long-duration flight. Hypothesis H1 (Season Effect): Key components of the antioxidant system are upregulated during spring compared to fall migration, and therefore oxidative damage is lower during spring migration. This hypothesis was evaluated in birds prior to two weeks of flight-training (H1a), after flight-training (H1b), and after an ultimate long-duration flight (H1c). Hypothesis H2 (Diet Effect): Key components of the antioxidant system are upregulated more when birds are not supplied with dietary anthocyanin, and therefore oxidative damage is similar between a high- and low-antioxidant diet. This hypothesis was also evaluated in birds prior to two weeks of flight-training (H2a), after flight-training (H2b), and after an ultimate long-duration flight (H2c). We also evaluated whether diet and season interacted to affect key components of the antioxidant system and the levels of oxidative damage associated with flight-training or long-duration flights. Furthermore, we directly evaluated the effect of flight training by including a subset of birds that were not flight trained. This is the first study to examine the main effects of season and dietary antioxidant supplementation and their potential interactions on the

regulation of multiple components of the antioxidant system of a migratory bird exposed to two weeks of flying.

METHODS

I. Female Starling Husbandry and Experimental Diets

European starlings from southern Germany were used for this study because individuals from this population are medium-distance migrants that leave for wintering grounds in October and November and return to their Bavarian breeding grounds in April (Bairlein et al. 2014). Migratory distances for this population vary, with some individuals overwintering in the Euro-Mediterranean region and others in northwest Africa (Bairlein et al. 2014). European starlings are also quite social and curious, and quickly learn to successfully fly together in a given windtunnel as demonstrated by many recent studies (Carter et al. 2020, Casagrande et al. 2020, McWilliams et al. 2021).

Female European starling chicks ($n = 115$) were collected from nest boxes located in Upper Bavaria, South Germany ($47^{\circ}58' \text{ N}$, $11^{\circ}13' \text{ E}$) during April and May in 2015 ($n = 72$) and 2016 ($n = 43$). Chicks were hand raised at the Max Planck Institute for Ornithology (MPIO), Seewiesen, Germany and hand-fed a high-protein diet that consisted of wax worms, beef heart, insect larvae, a vitamin mixture, and calcium carbonate powder until they were able to feed independently after ~35 days. Birds were then moved to outdoor aviaries (3 x 4 x 2 meters) and fed *ad libitum* a standard MPIO maintenance diet of fruits, vegetables, and live mealworms until the experiment began in August 2016. During August 2016, all birds were gradually

switched over several weeks from the MPIO diet to a semi-synthetic diet high in polyunsaturated fat (Table 1).

II. Experimental Design, Experimental Diets, and Flight Training

During August 2016, female starlings were allocated to two different groups (Figure 1): (1) “Season” females (n = 55) were flown in either fall 2016 or spring 2017 and sacrificed at the end of their 15-day flight training, whereas (2) “Reproductive” females (n = 30) included a flight-trained group (“trained”, n = 16) and a sedentary control group (“untrained”, n = 14) that underwent their respective treatments in both fall 2016 and spring 2017 with an overwintering period in an outdoor aviary. Females in the Season group that were flown only in fall were raised in 2016, whereas females from the Season group flown only in spring were raised in 2015. All Reproductive females were raised in 2015. Older females were chosen for the spring Season and Reproductive birds to increase the chance that they would be in breeding condition at the time of flight training, both to better represent natural conditions in the spring and for a companion study (Carbeck et al. 2018).

Starting on 21 September 2016, we randomly assigned all hand-raised starlings to be fed one of two semi-synthetic high polyunsaturated diets that differed only in anthocyanin content (Table 1). We chose to supplement diets with anthocyanin, a water-soluble antioxidant, because anthocyanins are common in many fruits that are consumed by wild birds (Schaefer et al. 2008, Bolser et al. 2013) and because free-living birds preferentially consume fruits with more anthocyanin during fall migration (Bolser et al. 2013). The high antioxidant diet (Table 1) had 119 mg kg⁻¹ (wet

weight) added anthocyanin (Standardized Elderberry 6.5% Powder; Artemis International, Inc., Fort Wayne, IN, USA) so that a bird such as a starling that ate 35 g total each day would consume the equivalent of 4.2 mg anthocyanin/day (or about 17 berries per day); the low antioxidant diet had no anthocyanin added. Each diet had identical macronutrient content (19% fat, 6% protein, 63% carbohydrate; Table 1). After assignment to diet (high antioxidant or low antioxidant) and experimental group (Season: fall vs. spring; Reproductive: trained vs. untrained), we measured molt score (Jenni and Winkler 2020) of all female starlings and then assigned females to cohorts of four to six individuals based on descending order of molt score (from most to least advanced in molt) so that the early cohorts had completed their molt prior to the start of flight training in fall 2016. Season females were assigned to one of eight cohorts (four high antioxidant cohorts, four low antioxidant cohorts), whereas Reproductive females were assigned to one of four cohorts (two high antioxidant cohorts, two low antioxidant cohorts). Trained and untrained Reproductive females were distributed evenly across all four cohorts.

Every three to six days starting on 26 September 2016, a new cohort was taken from their outdoor aviary and moved to indoor cages (1.5 x 2.5 x 2.5 meters) in the windtunnel facility. This allowed each cohort to be housed together and habituated over four days to fly directly into the windtunnel from their indoor cage without being handled, and to acclimate to the windtunnel conditions. This four-day pre-training regime was followed by a 15-day flight training regime. Such windtunnel flight training has demonstrated success at eliciting long-duration flights that simulate migratory flights (Engel et al. 2006). Flight training consisted of continuous periods of

flying at 15 m/s wind speed, 15°C ambient temperature, and 75% humidity. The duration of flight increased over the course of the regimen: 20-30 minutes on days 1-4, 60 minutes on days 5 and 7, 30 minutes on day 6, 90 minutes on day 8, rest on day 9, 2-3 hours on days 10 and 11, rest on day 12, 60 minutes on day 13, 30 minutes on day 14, and then up to 6 hours of voluntary flight on day 15 (average flight time ~3 hours). Reproductive birds assigned to the untrained group remained in their respective aviaries and were short-term fasted each day while trained birds underwent flight training. The same protocol was used across all groups and cohorts.

III. Blood Sampling and Antioxidant Analyses

Blood was sampled the day each cohort was established in the windtunnel aviaries to obtain a background blood sample (“BG”, Figure 1). Blood was then drawn immediately after the six-hour Day 15 flight (“AF”, Figure 1) and after a two-day post-flight recovery period (“AR”, Figure 1). The design of this blood sampling over time allowed us to determine how the antioxidant measures changed over the 15-day flight training (i.e., the difference between BG and AR measures) and during the long-duration flight on day 15 (i.e., the difference between AF and AR measures), and how these changes were affected by diet treatment and season. We did not sample blood immediately before the longest-duration flight to avoid negatively affecting this final flight. We assumed that the post-recovery measures (AR) reflected oxidative status immediately before the longest-duration flight, and therefore used this measure to analyze the changes over the longest-duration flight. The only exception to this pattern was the Reproductive birds, which were not blood sampled in the spring in order to

minimize stress and facilitate breeding. In all cases, blood was drawn from the brachial vein after puncture with a 17G needle and collected via a heparinized capillary tube. Blood was then kept on ice, transferred to a 0.5 mL microcentrifuge tube, and then centrifuged for five minutes at 10,000 rpm to separate plasma and red blood cells. After separation plasma and red blood cells were stored at -80°C until laboratory analyses.

Commercially available microplate assay kits were used to determine non-enzymatic antioxidant capacity, circulating levels of endogenous antioxidants, and oxidative damage. All assays were conducted using 96-well microplates and a BioTek Powerwave 340 (BioTek Instruments, Inc., Winooski, VT, USA). The d-ROMs and OXY-adsorbent assays were conducted at the Max Planck Institute for Ornithology using plasma, whereas GPx and uric acid were assayed at the University of Rhode Island (Kingston, RI, USA) using red blood cells and plasma, respectively. The OXY-adsorbent test (Diacron International, Grosseto, Italy) measures a sample's ability to neutralize oxidizing hypochlorous acid molecules and therefore serves as an estimate of circulating non-enzymatic antioxidant capacity. Uric acid is a non-enzymatic antioxidant that is not measured by the OXY-adsorbent test, so was therefore measured separately in plasma using a colorimetric assay (Teco Diagnostics, Anaheim, CA, USA). Uric acid is a product of protein catabolism and acts as a sacrificial antioxidant molecule (Tsahar et al. 2006). Glutathione peroxidase (Cayman Chemical Company, Ann Arbor, MI, USA) served as the representative enzymatic antioxidant in red blood cells to facilitate comparisons with previous studies. The d-ROMs test (Diacron International, Grosseto, Italy) measures circulating free radicals

derived from hydroperoxides, which are indicative of lipid oxidation and serves as a measure of oxidative damage.

The OXY, d-ROMs, and uric acid tests were run in duplicate. The GPx test was run in triplicate. Only individuals with a coefficient of variation $< 15\%$ were included in final analyses. Outliers were identified using Tukey's method and removed from the dataset. Final sample size for each analysis represents the number of remaining individuals after outliers and samples with coefficient of variation above 15% were removed.

IV. Statistical Analyses

Hypothesis 1 (Season Effects) was tested using only the birds flown in either fall or spring (Season birds) because the other group (Reproductive birds) was not blood sampled at the same times in the spring (Figure 1). Hypothesis 2 (Diet Effects) was tested using both groups of birds. All statistical analyses were performed in R version 4.0.0 (R Core Team 2020). Welch's t-tests were used to determine if there were any significant differences in mass or flight times between seasons or diets. For the Season birds, linear models were fit to infer the effects and interactions of season (fall or spring) and diet (high or low antioxidant) on measures of oxidative capacity (OXY), uric acid, GPx, and oxidative damage (d-ROMs), the final after log transformation (Table S1). Regression analyses for trends over date were performed for OXY and GPx measures prior to flight training; these analyses were performed within seasons. For the Reproductive birds sampled in the fall only, linear models were fit to infer the effects and interactions of flight training (trained or untrained) and

diet (high or low antioxidant) on measures of oxidative capacity (OXY), uric acid, GPx, and oxidative damage (d-ROMs) (Table S1). For models assessing background levels (hypotheses 1a and 2a), body mass and day since the first cohort was established were evaluated as covariates. For models assessing changes in measurements (hypotheses 1b, 1c, 2b, and 2c), final body mass and total flight time of the longest-duration flight were evaluated as covariates for Season birds, and final body mass and date since the first cohort was established were evaluated as covariates for Reproductive birds. Uninformative covariates were removed when possible to simplify models (Table S1). Residual plots were visually inspected and did not reveal noticeable deviation from homoscedasticity or normality. Final model(s) for evaluating each hypothesis were selected using Akaike's information criterion. Least-squares means were estimated for each model using the emmeans package in R (Lenth 2020). Pearson's correlations and linear regression were used to assess the relationship between GPx activity and non-enzymatic antioxidant capacity prior to flight-training. All results reported are from type III ANOVA for each linear model.

RESULTS

We report below the results for the main effects of diet and season except in the one case where we detected a significant interaction between diet and season (i.e., for acute changes in OXY during the longest flights). We also detected no significant differences between seasons or diets in female body mass over time or flight times (both over the 15-day training and the duration of the longest flight on Day 15). As such, body mass, number of days since the first cohort was established, and duration

of the longest flight were removed as covariates in models where they were uninformative and not strongly preferred using Akaike's information criterion (Table S1).

1. Hypothesis 1 (Season Effect)— some components of the antioxidant system are upregulated more in spring compared to fall, reducing oxidative damage during spring

1a. Prior to flight-training

Background levels (prior to any flight training) of circulating OXY were significantly higher in fall ($n = 21$) compared to spring ($n = 29$; Figure 2A; $F_{3,46} = 9.73$, $p = 0.003$). No seasonal change was apparent for circulating uric acid ($n = 11$ fall, $n = 7$ spring; Figure 2B; $F_{4,15} = 0.003$, $p = 0.96$) or GPx activity ($n = 19$ fall, $n = 27$ spring; Figure 2C; $F_{2,43} = 0.04$, $p = 0.84$). As predicted by hypothesis 1, oxidative damage (d-ROMs) was significantly lower in spring ($n = 29$) compared to fall ($n = 21$; Figure 2D; $F_{3,46} = 12.51$, $p = 0.001$). Uric acid (fall $F_{2,10} = 0.36$, $p = 0.56$; spring $F_{2,4} = 0.43$, $p = 0.55$) and oxidative damage (fall $F_{2,18} = 0.56$, $p = 0.46$; spring $F_{2,26} = 0.32$, $p = 0.58$) remained consistent across dates in the fall and spring. Circulating OXY concentrations increased significantly with date in the fall (Figure 3A; $F_{2,18} = 6.52$, $p = 0.02$; no diet effect: $F_{2,18} = 0.04$, $p = 0.85$), but not in the spring (Figure 3B; $F_{2,26} = 1.37$, $p = 0.25$; no diet effect: $F_{2,26} = 0.05$, $p = 0.83$). In contrast, GPx activity significantly decreased with date in both the fall (Figure 3C; $F_{2,16} = 5.13$, $p = 0.04$; no diet effect: $F_{2,16} = 0.39$, $p = 0.54$) and the spring (Figure 3D; $F_{2,24} = 5.54$, $p = 0.03$; no diet effect: $F_{2,24} = 0.60$, $p = 0.45$).

1b. After 15-day flight-training regime

After the 15-day flight training (BG timepoint subtracted from AR timepoint; Figure 1), we detected no differences between seasons in circulating OXY ($n = 15$ fall, $n = 20$ spring; $F_{4,30} = 0.40$, $p = 0.53$), circulating uric acid ($n = 11$ fall, $n = 7$ spring; $F_{2,15} = 0.5$, $p = 0.82$), or GPx activity ($n = 13$ fall, $n = 15$ spring; $F_{2,25} = 1.13$, $p = 0.30$). Changes in oxidative damage during training also did not differ between fall and spring ($n = 16$ fall, $n = 19$ spring; $F_{3,31} = 0.40$, $p = 0.53$).

1c. Immediately after long-duration flight

There was a significant diet x season interaction for circulating OXY, such that individuals fed the low-antioxidant diet had slightly increased circulating OXY immediately after the final fall flight ($n = 8$) and decreased circulating OXY immediately after the final spring flight ($n = 12$), whereas individuals fed the high-antioxidant diet showed the opposite trend ($n = 7$ fall, $n = 9$ spring; Figure 4A; diet x season interaction; $F_{4,30} = 9.72$, $p = 0.004$). The acute change in circulating uric acid did not differ between seasons ($n = 13$ fall, $n = 11$ spring; Figure 4B; $F_{3,20} = 0.04$, $p = 0.85$). The acute change in circulating GPx activity was significantly higher in spring ($n = 14$) compared to fall ($n = 13$; Figure 4C; $F_{3,23} = 4.69$, $p = 0.04$). Changes in oxidative damage after flight did not differ between seasons ($n = 15$ fall, $n = 20$ spring; Figure 4D; $F_{4,29} = 2.77$, $p = 0.11$).

2. Hypothesis 2 (Diet Effect)—females fed fewer dietary antioxidants

upregulate some components of the antioxidant system and so prevent oxidative damage

2a. Prior to flight training

For females flown either in fall or spring (Figure 1, “Season” birds), background levels (prior to flight-training) of circulating OXY were similar for females fed a high- (n = 25) or low-antioxidant diet (n = 25; Figure 2A; $F_{3,46} = 0.18$, $p = 0.67$). As predicted by hypothesis 2, circulating uric acid was significantly higher in females fed a low antioxidant diet (n = 8 high-, n = 12 low-antioxidant diet; Figure 2B; $F_{4,15} = 4.84$, $p = 0.04$), whereas GPx activity did not differ between diets (n = 17 high-, n = 20 low-antioxidant diet; Figure 2C; $F_{2,34} = 0.01$, $p = 0.93$). As predicted, oxidative damage was the same for females fed each diet (n = 25 both diets; Figure 2D; $F_{3,46} = 0.8$, $p = 0.60$).

For females flown in fall and spring but blood sampled only in fall, (Figure 1, “Reproductive” birds), fall background levels of circulating OXY (n = 13 high-, n = 19 low-antioxidant diet; $F_{5,27} = 0.22$, $p = 0.64$), uric acid (n = 14 high-, n = 16 low-antioxidant diet; $F_{2,27} = 0.30$, $p = 0.59$), and GPx activity (n = 11 high-, n = 16 low-antioxidant diet; $F_{2,24} = 2.53$, $p = 0.13$) were similar for females fed a high- or low-antioxidant diet. Oxidative damage was also consistent across diets (n = 13 high-, n = 19 low-antioxidant diet; $F_{4,27} = 0.02$, $p = 0.90$).

2b. After 15-day flight training regime

For females flown either in fall or spring (Figure 1, “Season” birds), there was no significant difference between background and post-training levels of OXY for either diet (n = 16 high-, n = 19 low-antioxidant diet; $F_{4,30} = 2.59$, $p = 0.12$). Circulating uric acid (n = 7 high-, n = 11 low-antioxidant diet; $F_{2,15} = 0.98$, $p = 0.34$) and GPx activity (n = 13 high-, n = 15 low-antioxidant diet; $F_{2,25} = 0.56$, $p = 0.46$) in females fed either diet also did not change during flight training. Changes in oxidative

damage during training also did not differ between diets ($n = 18$ high-, $n = 17$ low-antioxidant diet; $F_{3,31} = 0.09$, $p = 0.76$).

For the trained and untrained group of females sampled in the fall (Figure 1, “Reproductive” birds), individuals on the high antioxidant diet ($n = 12$) had a greater net negative change in circulating OXY than individuals on the low antioxidant diet ($n = 18$) during the 15-day flight training regime (Figure 5A; $F_{2,27} = 4.70$, $p = 0.04$), though this effect did not differ between trained ($n = 17$) and untrained birds ($n = 13$; Figure 5A; $F_{2,27} = 0.001$, $p = 0.97$). Changes in circulating uric acid were consistent for trained and untrained females ($n = 14$ trained and untrained; Figure 5B; $F_{2,25} = 0.28$, $p = 0.60$) and for birds fed each diet ($n = 14$ both diets; Figure 5B; $F_{2,25} = 0.63$, $p = 0.43$). Trained females ($n = 12$) had a significant decrease in GPx activity after 15 days of flight training compared to the untrained females ($n = 11$; Figure 5C, $F_{2,20} = 6.43$, $p = 0.02$), although changes in GPx activity did not differ between diets ($n = 9$ high-, $n = 15$ low-antioxidant diet; Figure 5C; $F_{2,21} = 0.028$, $p = 0.870$). Changes in oxidative damage did not significantly differ between trained and untrained females ($n = 15$ trained, $n = 13$ untrained; Figure 5D; $F_{2,25} = 0.01$, $p = 0.91$) or between females fed each diet ($n = 10$ high-, $n = 18$ low-antioxidant diet; Figure 5D; $F_{2,25} = 0.1.92$, $p = 0.18$).

2c. Immediately after long-duration flight

There was a significant diet x season interaction for circulating OXY, such that individuals on the high and low antioxidant diets showed inverse trends between seasons as described above (H1c, Figure 4A). The acute change in uric acid ($n = 11$ high-, $n = 13$ low-antioxidant diet; Figure 4B; $F_{3,20} = 0.23$, $p = 0.64$) and GPx activity

(n = 12 high-, n = 15 low-antioxidant diet; Figure 4C; $F_{3,23} = 0.20$, $p = 0.66$) during the longest duration flight did not differ between diets. Changes in oxidative damage during the longest duration flight also did not differ between diets (n = 16 high-, n = 19 low-antioxidant diet; Figure 4D; $F_{4,29} = 2.33$, $p = 0.14$).

For the trained and untrained females sampled in the fall, OXY did not differ between trained (n = 17) and untrained birds (n = 13; Figure 5E; $F_{2,27} = 2.73$, $p = 0.12$), and there were no differences between diets (n = 13 high-, n = 17 low-antioxidant diet; Figure 5E; $F_{2,27} = 1.03$, $p = 0.32$). Trained birds (n = 13) had significantly lower levels of circulating uric acid immediately after their flight compared to untrained birds (n = 14; Figure 5F; $F_{2,24} = 9.88$, $p = 0.004$), and this acute change in uric acid was consistent between diets (n = 14 high-, n = 13 low-antioxidant diet; Figure 5F, $F_{2,24} = 0.63$, $p = 0.43$). GPx activity did not differ between trained (n = 16) and untrained birds (n = 14; Figure 5G; $F_{2,27} = 0.08$, $p = 0.77$) or diets (n = 13 high-, n = 17 low-antioxidant diet; Figure 5G; $F_{2,27} = 1.11$, $p = 0.30$). Acute changes in oxidative damage did not significantly differ between training status (n = 14 trained, n = 14 untrained; Figure 5H; $F_{3,24} = 0.09$, $p = 0.76$) or diets (n = 11 high-, n = 17 low-antioxidant diet; Figure 5H; $F_{3,24} = 2.47$, $p = 0.13$).

DISCUSSION

Seasonal changes prior to flight training (H1a)

Our results provide partial support for the hypothesis (H1a) that key components of the antioxidant system are upregulated prior to flight training more in spring compared to fall, and thus oxidative damage is reduced in spring. We found

evidence for the latter but not the former. In support of hypothesis 1, oxidative damage (d-ROMs) was lower in spring females (Figure 2D). We predicted that spring would present a greater oxidative challenge for female migratory birds, as the pacing of spring migration is quicker than fall (Morris and Glasgow 2001, Nilsson et al. 2013), and females must contend with carry-over effects that can affect reproduction (Bauchinger et al. 2009, Harrison et al. 2011, Legagneux et al. 2012). Females may prioritize reducing oxidative damage in spring if the oxidative cost of breeding is high, as demonstrated in both free-living barn swallows (Costantini et al. 2014) and captive zebra finches (Wiersma et al. 2004). Furthermore, Giordano et al. (2015) demonstrated that female great tits (*Parus major*) with higher levels of oxidative damage during breeding deposited fewer antioxidants into eggs. Therefore, reducing oxidative damage prior to spring migration may help females prepare for the breeding and egg-laying season.

Contrary to hypothesis 1a, non-enzymatic oxidative capacity (OXY) was lower in spring compared to fall (Figure 2A). Circulating uric acid (Figure 2B) and enzymatic antioxidant activity (Figure 2C) did not increase in spring as might occur to compensate for a reduction in oxidative capacity in spring. However, we found within-season patterns for enzymatic antioxidant activity and non-enzymatic antioxidant capacity that may indicate a coordinated and compensatory antioxidant response. Specifically, GPx activity significantly decreased as each season progressed, while there was a trend for OXY concentrations to increase as each season progressed (Figure 3). Cooper-Mullin et al. (2019) demonstrated a similar coordinated response in zebra finches undergoing six weeks of flight training. As fall and spring progress,

females may switch to relying on non-enzymatic antioxidants to prepare for a costly migratory event, only upregulating GPx in response to acute oxidative challenges (Figure 4C). The overall decrease in OXY during spring may indicate that females are utilizing more non-enzymatic antioxidants than in the fall to prepare for breeding, as oxidative damage was also lower in spring. Antioxidant uptake may also differ between seasons, possibly due to seasonal diet changes and differences in the availability of dietary antioxidants. Directly measuring the antioxidant uptake from the experimental diets would have helped to inform these results. The use of labeled antioxidant molecules along with direct measures of water-soluble and lipid-soluble antioxidants may also help inform differences in antioxidant usage and uptake in females.

Diet effects prior to flight training (H2a)

Our results for birds prior to flight training provide some support for our second hypothesis (H2a) that females fed fewer dietary antioxidants would upregulate key components of the endogenous antioxidant system to prevent oxidative damage. Prior to flight training, females fed less anthocyanin increased circulating uric acid in both fall and spring (Figure 2B) while non-enzymatic antioxidant capacity (Figure 2A) and GPx activity (Figure 2C) did not change. Uric acid is a powerful antioxidant and a natural byproduct of protein catabolism (Battley et al. 2000), and these results suggest that it may be the first to be upregulated when birds lack access to dietary antioxidants. Oxidative damage was identical across diets prior to flight training (Figure 2D), possibly indicating that uric acid may play an adequately compensatory role in the absence of the anthocyanin supplement to prevent oxidative damage.

Eikenaar et al. (2016) similarly found that northern wheatears (*Oenanthe oenanthe*) faced with an oxidative challenge (refueling) had elevated circulating uric acid but similar oxidative damage as compared to control birds.

We predicted that antioxidant supplementation would increase non-enzymatic antioxidant capacity, as demonstrated by Beaulieu and Schaefer's (2014) study on Gouldian finches which found an increase in OXY in birds fed a diet with more polyphenols. However, our results more closely resemble those of Skrip et al.'s (2016) study on female zebra finches, in that non-enzymatic antioxidant capacity did not differ between diets that were supplemented with antioxidants. As OXY measures both dietary antioxidants and non-enzymatic endogenous antioxidants (excluding uric acid), we hypothesize that females fed more dietary antioxidants decreased their reliance on endogenous antioxidants. This is supported by our findings that females on the high antioxidant-diet had lower uric acid than females on the low-antioxidant diet (Figure 2B). These results suggest that dietary antioxidants may promote protein sparing in females, which can help preserve muscle mass and valuable stores of water found in protein (Bauchinger and Biebach 1998, Jenni-Eiermann and Jenni 1998, Gerson and Guglielmo 2011). Protein sparing—and therefore dietary antioxidants—may be especially valuable for spring migratory females that are preparing to breed, when protein stores are necessary for egg formation.

One limitation of our study design is that we did not measure how much of the anthocyanin acquired in their semisynthetic diet was absorbed by the females. It is therefore possible that individual birds had differing amounts of circulating

anthocyanins, and that these individual differences influenced our findings. Further study is needed on how birds metabolize and allocate dietary antioxidants.

Seasonal differences in how females respond to repeated flying (H1b, H1c)

We found no evidence that the response of the antioxidant system to the 15-day flight training regime changes between seasons (H1b), and we found mixed support for hypothesis 1 when considering the acute response of birds to the longest duration flight (H1c). OXY changed with season although the direction of change depended on diet (Figure 4A). Change in uric acid and oxidative damage did not differ between spring and fall (Figure 4B, 4D). The acute increase in GPx activity over the longest flight was significantly higher in spring than fall (Figure 4C). Raja-aho et al. (2012) demonstrated a similar trend of GPx upregulation during spring and summer, with circulating levels lowest in fall. Spring females may favor the use of enzymatic antioxidants when responding to an acute oxidative challenge in order to preserve stores of non-enzymatic antioxidants that are valuable for protection during breeding and provisioning to eggs (Costantini 2010, Skrip et al. 2016). This hypothesis is further corroborated by the lower circulating oxidative capacity that we observed in spring females prior to flight training (Figure 2A). These results may also be the product of consistent seasonal differences in the availability of dietary antioxidants accessible to wild migratory birds. Antioxidant rich fruits are abundant during fall migration and generally absent in spring (Parrish 1997, Alan et al. 2013) and so migratory birds may have evolved a greater reliance on enzymatic antioxidants in the spring when dietary antioxidants may be scarcer.

Diet effects on how females respond to repeated flying over 15 days (H2b)

Contrary to hypothesis 2b, the 15-day flight training produced no consistent changes in total non-enzymatic antioxidant capacity, uric acid, GPx activity and oxidative damage between birds fed the high or low antioxidant diets. However, we were able to detect an effect of diet on the antioxidant system of the “Reproductive” females (Figure 1) during fall. Contrary to hypothesis 2b, females fed the high-antioxidant diet decreased non-enzymatic antioxidant capacity during the 15-day flight training period regardless of if they were trained or untrained, whereas females on the low antioxidant diet maintained non-enzymatic antioxidant capacity over the course of training (Figure 5A). These results indicate that females on the high-antioxidant diet utilized non-enzymatic antioxidants more than females on the low-antioxidant diet during the 15-day flight training, although oxidative damage was similar between diets (Figure 5D) and we did not detect a compensatory change in uric acid (Figure 5B) or GPx (Figure 5C) for females fed the low-antioxidant diet. Studies that fed dietary antioxidants to birds demonstrate enhancements in metabolic performance, suggesting that dietary antioxidants may serve additional functions to preventing damage. A complementary study conducted using the same group of female starlings found that females fed a high-antioxidant diet had lower corticosterone after flight than birds fed a low-antioxidant diet, indicating that anthocyanins assist in activating catabolism-inducing pathways during flight (Casagrande et al. 2020). Furthermore, zebra finches fed a diet supplemented with carotenoids and budgerigars (*Melopsittacus undulates*) fed a diet supplemented with an antioxidant mixture had faster flight escape times than non-supplemented individuals (Blount and Matheson 2006, Arnold et al. 2010). Viewed altogether, these results indicate that dietary anthocyanins may be allocated to

metabolic pathways rather than used primarily as antioxidant defenses, therefore sparing other biologically important molecules.

In contrast, enzymatic antioxidant activity was affected by endurance exercise but not diet. Specifically, GPx activity decreased across the 15-day flight training for trained but not untrained birds, and these differences were consistent across diets (Figure 5C). The acute rise and fall of GPx upregulation in response to exercise found by Jenni-Eiermann et al. (2014) and Cooper-Mullin et al. (2019) indicate that GPx production may be costly due to the necessary selenium cofactors, which are also utilized for immune function and amino acid synthesis (Zwolak and Zaporowska 2012), and therefore saved for immediate oxidative challenges. The decrease in GPx activity seen only in trained birds indicates that females may exhaust the ability to upregulate GPx during the span of flight training, perhaps due to the depletion of GPx cofactors. When endogenous defenses are depleted, dietary antioxidants, especially the rapidly-assimilated hydrophilic antioxidants commonly found in fruits, may act to compensate and prevent oxidative damage (Bolser et al. 2013).

Diet effects on how females acutely respond to a longest duration flight (H2c)

Diet affected enzymatic components of the antioxidant system during the longest duration flight. GPx activity increased more for females during spring and there was a trend for females fed the low-antioxidant diet to increase GPx activity to a larger extent during spring (Figure 4C). None of the other components of the antioxidant system were affected by diet. Studies in zebra finches and European robins have established that GPx regulation responds quickly to acute challenges and can serve as a “first line of defense” to reduce oxidative damage (Jenni-Eiermann et al.

2014, Cooper-Mullin et al. 2019). The potential diet-mediated increase in GPx activity, especially during spring, suggests that dietary antioxidants may play an important role in mitigating acute oxidative stressors when they are available. Somewhat paradoxically, studies of free-living birds in New England have established that migrating birds preferentially consume fruits high in antioxidants in the fall, further suggesting the importance of dietary antioxidants when faced with an acute oxidative challenge (Alan et al. 2013, Bolser et al. 2013), although such fruits are much less available during spring. Our results suggest that dietary antioxidants may be preferentially used to combat reactive species, and that endogenous antioxidants like GPx are used when dietary antioxidants are absent or cannot fully meet the oxidative challenge.

The same potential effects of dietary antioxidants on GPx activity were not evident in trained and untrained females fed low- or high-antioxidant diets during fall. Specifically, oxidative capacity, GPx activity, and oxidative damage during the longest duration flight did not differ between diets or depend on flight-training (Figure 5). One explanation for this is the season in which the trained and untrained birds were blood sampled. Dietary antioxidants had a greater effect on spring birds in the first group (“Season”, Figure 1), possibly due to preparations for breeding. If females do not prioritize reducing oxidative damage in the fall as they may in the spring (Figure 2D), then dietary antioxidants or flight-training may not have a substantial effect on endogenous components of the antioxidant system.

Although females fed less dietary antioxidants increased circulating uric acid, a potent antioxidant, prior to flight training (Figure 2B), we did not observe a diet-

dependent change in uric acid with flight training (Figure 5B), or in response to the longest duration flight (Figures 4B, 5F). However, we did observe a substantial increase in uric acid in response to the longest duration flight in flight trained versus untrained females (Figure 5F). Uric acid is a byproduct of protein catabolism, which is increased during flight (Battley et al. 2000), and has been demonstrated to reduce lipid peroxidation in birds (Stinefelt et al. 2005). Increases in circulating uric acid after long-duration flights have been demonstrated in many bird species (Jenni-Eiermann and Jenni 1991, Gannes et al. 2001, Jenni-Eiermann et al. 2002). Furthermore, wheatears exposed to an oxidative challenge (refueling) had elevated uric acid but similar oxidative damage as compared to control birds fed ad libitum (Eikenaar et al. 2016). These results indicate that flying birds may utilize one of the natural byproducts of exercise to combat reactive species and prevent oxidative damage. Two common measures of non-enzymatic antioxidant capacity, ferric reducing ability of plasma (FRAP) and Trolox equivalent antioxidant capacity (TEAC), have been demonstrated to correlate strongly with circulating uric acid in plasma (Cohen et al. 2009, Cohen and McGraw 2009, Costantini 2011, Eikenaar et al. 2016). When viewed in tandem with the dietary effect on uric acid prior to flight-training (Figure 2B), the strong correlation between two measures of circulating antioxidant capacity and uric acid emphasizes the importance of uric acid as an antioxidant molecule for flying birds.

Conclusions

Our experiment is the first to examine the additive effects of season, diet, and exercise on the antioxidant system of a flight-trained female bird. Our experiment

provides unique insight into how females regulate the antioxidant system during two unique migratory periods, and how dietary antioxidant supplementation affects the response of antioxidant molecules. We found mixed support for each of our hypotheses. Consistent with hypothesis 1, we found that females reduced baseline oxidative damage (Figure 2D) and increased enzymatic antioxidant activity immediately after the longest duration flight (Figure 4C) in spring compared to fall, possibly to reduce the potential negative carry-over effects from spring migration to breeding. However, we did not detect any seasonal differences in baseline enzymatic antioxidant activity, or in levels of damage and circulating uric acid immediately after the longest-duration flight. Consistent with hypothesis 2, females fed a low-antioxidant diet had higher circulating levels of uric acid prior to flight training (Figure 2B). Females fed a low-antioxidant diet had increased enzymatic antioxidant activity immediately after the longest duration flight compared to females fed a high-antioxidant diet (Figure 4C). Contrary to hypothesis 2, baseline non-enzymatic oxidative capacity and enzymatic activity did not differ between diets. In summary, dietary antioxidants may be preferentially used to combat reactive species, and endogenously produced antioxidants and enzymes may be upregulated during parts of the annual cycle when dietary antioxidants may be scarce. In sum, the antioxidant system is a plastic and dynamic system that is responsive to ecological factors such as diet and season.

ACKNOWLEDGEMENTS

We thank Clara Cooper-Mullin for guidance on antioxidant assays and statistical analyses. We are especially grateful to the Max Planck Institute for

Ornithology animal care staff for handling much of the animal husbandry responsibilities, and to Susan Forrest and Aleksandra Małochleb for helping raise the starlings. Special thanks are extended to Maude Baldwin and the entire Baldwin lab, especially point-person Glenn Cockburn, for providing access to their lab and equipment at MPIO to conduct assays and for providing us with dewars to transport samples to the US.

Funding statement: The experiment was supported by a grant from the National Science Foundation to S.R.M. and B.J.P. (IOS-1354187); by OPUS grant from National Science Foundation (NCN), Poland, to U.B. (UMO-2015/19/B/NZ8/ 01394) and DS grant from the Institute of Environmental Sciences, Jagiellonian University to U.B. (DS/WBINOZ/INOS/757). A.E.F. was supported in part by NSF (U.S.A.) and in part by the University of Rhode Island and the College of Environment and Life Sciences.

Ethics statement: All the experimental protocols were approved by the University of Rhode Island IACUC (Protocol #AN08-02-014) and the Government of Upper Bavaria, Germany (AZ 55.2-1-54-2532-216-2014).

Author contributions: A.E.F. and K.J.D. carried out laboratory analyses. A.E.F. performed statistical analyses and with S.R.M. drafted the manuscript. S.R.M., B.J.P. and U.B. designed the experiment and with A.E.F. formulated the hypotheses. K.J.M.,

K.M.C., L.T., B.P., A.B., M.D., E.T.S. and U.B. conducted the experiment. All authors edited and approved the manuscript.

Data depository: The datasets supporting this article have been uploaded as part of the electronic supplementary material and will be available from the Dryad Digital Repository upon publication.

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TABLES

Table 1. Composition of semi-synthetic high-antioxidant diet fed to female European starlings during the fall 2016 and spring 2017 experiments. Half of the females (n = 43) were fed the high- antioxidant diet with added anthocyanin; other females (n = 43) were fed a low- antioxidant diet with the exact same composition but without added anthocyanin.

Ingredients	% wet mass	% dry mass
Agar ¹	1.37	3.19
Glucose ²	16.84	39.19
Casein ³	8.21	19.12
Cellulose ⁴	2.14	4.97
Canola oil ⁵	5.18	12.05
Sunflower oil ⁶	3.03	7.06
Ground mealworms	2.65	6.16
Elderberry powder ⁷	0.18	0.42
Salt mixture ⁸	2.05	4.78
Amino acid mix ⁹	1.15	2.68
Vitamin mix ¹⁰	0.16	0.38
Water	57.04	

¹ Agar, Ombilab-laborenzentrum, GmbH, Bremen, Germany

² Glucose, VWR International GmbH, Darmstadt, Germany

³ Casein, Affymetrix UK Ltd., High Wycombe, UK

⁴ Alphacel, MP Biomedicals, Solon, OH, USA

⁵ Canola oil, Jedwards International, Inc., Braintree, MA, USA

⁶ Sunflower oil, Jedwards International, Inc., Braintree, MA, USA

⁷ Standardized Elderberry extract powder, Artemis international, Inc, Fort Wayne, IN, USA; contains 6.5% anthocyanins; amount added was 0.04 % wet mass from 21 Sept to 5 Oct, then 0.18 % thereafter

⁸ Brigg's salt mix, MP Biomedicals, Solon, OH, USA

⁹ Amino acid mix, Sigma-Aldrich, St. Louis, MO, USA

¹⁰ AIN vitamin mix, MP Biomedicals, Solon, OH, USA. Includes vitamin E.

Supplemental Material Table S1. Final models for all antioxidant measures and hypotheses. Variables indicates the main effects considered and any interaction between main effects. Covariates are significant predictors for each measure; uninformative covariates were removed from the models. Final models for each hypothesis were selected using Akaike’s information criterion; ΔAIC_c listed is between final and global models. See main text for reporting of statistics for each model.

Model		Main effects, interactions in final model	Covariates	ΔAIC_c
<i>Group 1 “Season” females (timepoint) measure</i>				
(background)	OXY	season, diet	date	0.61
(background)	Uric acid	season, diet	N/A	2.17
(background)	GPx	season, diet	N/A	1.07
(background)	d-ROMs	season, diet	mass	0.65
(background, over fall)	OXY	diet, date	N/A	
(background, over fall)	GPx	diet, date	N/A	
(background, over spring)	OXY	diet, date	N/A	
(background, over spring)	GPx	diet, date	N/A	
(15-day flight training)	OXY	season, diet, season * diet	mass	5.57
(15-day flight training)	Uric acid	season, diet	N/A	5.35
(15-day flight training)	GPx	season, diet	N/A	3.48
(15-day flight training)	d-ROMs	season, diet	mass	2.37
(acute)	OXY	season, diet, season * diet	mass	12.89
(acute)	Uric acid	season, diet	flight time	5.61
(acute)	GPx	season, diet, season * diet	N/A	9.05
(acute)	d-ROMs	season, diet, season * diet	mass	3.20
<i>Group 2 “Reproductive” females (timepoint) measure</i>				
(background)	OXY	training, diet	mass, date	Global used
(background)	Uric acid	training, diet	N/A	5.73
(background)	GPx	training, diet	N/A	6.22
(background)	d-ROMs	training, diet	mass, date	Global used
(after recovery – background)	OXY	training, diet	date	3.04
(after recovery – background)	Uric acid	training, diet	N/A	4.61
(after recovery – background)	GPx	training, diet	N/A	6.66
(after recovery – background)	d-ROMs	training, diet	N/A	4.14
(acute)	OXY	training, diet	N/A	4.21
(acute)	Uric acid	training, diet	N/A	6.97
(acute)	GPx	training, diet	N/A	4.68
(acute)	d-ROMs	training, diet	mass	2.92

FIGURES

Figure 1. Experimental groups used to test hypotheses about the effect of dietary antioxidants, flight training, and season (fall, spring) on key components of the antioxidant system in female migratory European Starlings. “Season” females (top panel) were flown during either fall 2016 or spring 2017 and then sacrificed at the end of their 15-day flight training plus two days of ‘recovery’ from the last long-duration flight. “Reproductive” females (bottom panel) were flown in both fall 2016 and spring 2017 and then sacrificed in the spring after a 7-day exposure to male starlings as part of a companion study (Carbeck et al. 2018). This experiment utilized samples from both fall and spring season birds and fall reproduction birds, but excluded spring reproduction birds due to their unbalanced sampling design. Blood droplets represent when blood sampling occurred (BG = background sample prior to flight training (ca. 1040 ul), AF = after-flight sample taken immediately after longest flight on day 15 of flight training (ca. 1120 ul), AR = after-recovery blood sample taken two days after the longest flight (ca. 1040 ul)). Bird icons represent whether the bird was trained

(flying icon) or untrained (standing icon). Numbers ('#d') represent the number of days between the day 15 longest flight and final blood sample.

Figure 2. Least square means and 95% confidence intervals of antioxidant measures prior to flight training. **(A)** Background non-enzymatic antioxidant capacity (OXY) in plasma of females differed between seasons but not diets. **(B)** Background circulating

uric acid in plasma of females differed between diets but not seasons. **(C)** Background enzymatic activity (glutathione peroxidase, GPx) in red blood cells of females did not differ between seasons and diets. **(D)** Background oxidative lipid damage (d-ROMs) in plasma of females differed between seasons but not diets. Points that share letters for a given antioxidant measure are not significantly different; no letters are included for antioxidant measures with no significant differences (see text).

Figure 3. Antioxidant measures through time in females prior to flight training. Non-enzymatic antioxidant capacity (OXY) significantly increased with date in the fall (**A**) for females fed either diet, but not in the spring (**B**). Background enzymatic activity (glutathione peroxidase, GPx) in red blood cells significantly decreased with date in the fall (**C**) and spring (**D**) for females fed either diet. Lines represent statistically significant least squares linear regression fit to data (see text). Days were counted from the day each group began the experimental antioxidant diets.

Figure 4. Least square means ($\pm$ SE) of acute changes in antioxidant response of females immediately after a long-duration flight (AR timepoint – AF timepoint, see Figure 1). **(A)** Acute change in non-enzymatic antioxidant capacity (OXY) in plasma of females increased between fall and spring for birds fed the high-antioxidant diet whereas birds fed the low-antioxidant diet had higher OXY in fall compared to spring (Diet x Season interaction denoted by *, see text). **(B)** Acute change in circulating uric acid in plasma of females did not differ between seasons and diets. **(C)** Acute change in enzymatic activity (glutathione peroxidase, GPx) in red blood cells of females differed between seasons but not diets. **(D)** Acute change in oxidative lipid damage (d-ROMs) in plasma of females did not differ between seasons and diets. Bars that share letters for a given antioxidant measure are not significantly different; no letters are included for antioxidant measures with no significant differences (see text).

Figure 5. Least square means ($\pm$ SE) of changes in antioxidant response in trained versus untrained females (**A-D**) after a 15-day flight training regime in the fall (AR timepoint – BG timepoint; see Figure 1, “Reproductive” females only) and (**E-H**)

immediately after the trained females' longest-duration flight in the fall (AR timepoint – AF timepoint; see Figure 1, “Reproductive” females only). **(A)** Change in non-enzymatic antioxidant capacity (OXY) in plasma of trained and untrained females differed between diets but not flight training. **(B)** Change in circulating uric acid in plasma of trained and untrained females did not differ between diets or with flight training. **(C)** Change in enzymatic activity (glutathione peroxidase, GPx) in red blood cells of trained and untrained females did not differ between diets but differed with flight training. **(D)** Change in oxidative lipid damage (d-ROMs) in plasma of trained and untrained females did not differ between diets or flight training. **(E)** Acute change in non-enzymatic antioxidant capacity (OXY) in plasma of trained and untrained females did not differ between diets or flight training. **(F)** After the long duration flight, circulating uric acid levels were lower in flight-trained versus untrained females fed both diets. **(G)** Acute change in enzymatic activity (glutathione peroxidase, GPx) in red blood cells of flight-trained and untrained females did not differ between diets. **(H)** Acute change in oxidative lipid damage (d-ROMs) in plasma of flight-trained and untrained females did not differ between diets. Bars that share letters for a given antioxidant measure are not significantly different; no letters are included for antioxidant measures with no significant differences (see text).

CHAPTER 2

Flight training and diet have mixed effects on antioxidant enzymes, non-enzymatic antioxidant capacity, and oxidative damage in female European starlings (*Sturnus vulgaris*)

by

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ABSTRACT

Energy-intensive activities such as flying can increase the production of reactive species and birds, like other vertebrates, rely on a robust antioxidant system to protect themselves against oxidative damage. The challenges for female birds are especially acute during spring migration since they must pay the oxidative costs of migratory flights while also preparing for reproduction; however, no previous work has compared how the antioxidant system of female migrants in spring responds to the oxidative challenges of regular flying and dietary antioxidant supplementation. We fed two diets to female European starlings, one supplemented with anthocyanin, a dietary antioxidant, and one without, and then flew them daily in a windtunnel for two weeks during the fall and spring migration periods. We then measured the activity of enzymatic antioxidants (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC), and markers of oxidative damage (protein carbonyls and lipid hydroperoxides) in the pectoralis, leg, liver, and heart. Dietary antioxidants affected GPx activity and lipid damage in the heart, as well as non-enzymatic antioxidant capacity in the pectoralis and protein damage in leg muscle. We found a significant diet x training interaction for CAT activity in the leg, and strong trends for diet x training interactions for GPx activity in the heart and CAT activity in the liver. Flight-training may condition the antioxidant system to respond to an oxidative challenge, and individuals fed less antioxidants appear to incur increased oxidative damage while upregulating non-enzymatic and enzymatic antioxidant activity, though these effects were strongly tissue-specific.

Keywords: anthocyanin, flight training, enzymatic antioxidants, oxidative damage

INTRODUCTION

Energy-intensive activities, such as endurance exercise, increase metabolic rate and have the potential to produce reactive species that can scavenge electrons from biologically important molecules such as proteins, lipids, and DNA and negatively impact an animal's fitness (Halliwell and Gutteridge, 2015; Nikolaidis et al., 2012; Powers and Jackson, 2008), thereby causing oxidative damage (Costantini et al., 2008; Jenni-Eiermann et al., 2014; Smarsh and Williams, 2016; Sun et al., 2010). As a consequence of these potential oxidative costs of activity, vertebrates rely on a robust antioxidant system to protect themselves against oxidative damage (Beaulieu and Schaefer, 2014; Halliwell and Gutteridge, 2015) that can flexibly respond to changes in energy demand and the associated oxidative costs (Cooper-Mullin et al., 2019, Halliwell and Gutteridge, 2015, Jenni-Eiermann et al., 2014). How the multiple components of the antioxidant system of wild vertebrates flexibly respond to seasonal changes in activity, competing energy and nutrient demands, and availability of dietary antioxidants is inadequately understood (Cooper-Mullin & McWilliams, 2016; Skrip & McWilliams, 2016).

Some of the most remarkable endurance athletes on earth include migratory birds. Migratory birds routinely engage in endurance exercise in the form of seasonal flights between wintering and breeding grounds, during which they may operate at 6-8X their basal metabolic rate (Lindström et al., 1999; Schmidt-Wellenburg et al., 2007; Wikelski et al., 2003). These flights have the potential to increase the production of reactive species (Costantini 2014; Costantini et al., 2008; Jenni-Eiermann et al., 2014). Furthermore, migratory birds rely primarily on fat stores to

fuel migration, and reactive species can cause cascading degradation of fatty acids (Costantini et al., 2007; Guglielmo, 2018; Jenni-Eiermann et al., 2002; McWilliams et al., 2004; Skrip et al., 2015). Like other vertebrates, birds can ameliorate oxidative challenges using a multifaceted antioxidant system that consists of exogenous dietary antioxidants and endogenous enzymatic and non-enzymatic antioxidants (Costantini 2014, Cooper-Mullin and McWilliams 2016). The antioxidant system of birds has been demonstrated to be quite flexible in that it responds quickly to challenges. For example, European robins (*Erithacus rubecula*) caught during migratory flight had higher circulating glutathione peroxidase (GPx) compared to resting birds (Jenni-Eiermann et al. 2014), and flight-trained zebra finches (*Taeniopygia guttata*) had elevated GPx activity immediately after one hour of flight (Cooper-Mullin et al. 2019). Dietary antioxidant supplementation has also been demonstrated to improve flight performance in zebra finches (Blount and Matheson, 2006) and budgerigars (*Melopsittacus undulates*, Arnold et al., 2010), and songbirds during fall migration preferentially consume high-antioxidant fruits (Beaulieu and Schaefer, 2014; Bolser et al., 2013). However, it remains to be determined how the exogenous and endogenous components of the antioxidant system of birds interact to protect against reactive species and oxidative damage associated with regular flying.

The functional importance of key components of the antioxidant system of vertebrates may change over the course of seasons as individuals must satisfy the energy and nutrient demands of important events such as reproduction (Costantini, 2008; Wiersma et al., 2004). Spring migration in birds provides a unique opportunity to study how key components of the antioxidant system respond to potentially

competing oxidative challenges, as adults typically prepare to breed shortly after completing their migratory flights (Legagneux et al., 2012; Ramenofsky and Wingfield, 2006). The challenges for female birds may be especially acute during spring migration since they must pay the oxidative costs of migratory flights while also preparing for egg production and laying (Skrip et al., 2015, 2016). Females with a more robust antioxidant system during spring migration may be able to avoid oxidative damage associated with repeated flights and then subsequently allocate more antioxidants to eggs (Royle et al., 2003; Skrip et al., 2016). Previous work has shown that such investment of antioxidants by females into eggs results in improved hatching and fledging success, enhanced immune function, improved growth rates, and increased sex appeal in mature males (Berthouly et al., 2007, 2008; Marri & Richner, 2014; McGraw et al., 2005; Norte et al., 2010; Saino et al., 2003).

The objective of our study was to determine how the availability of dietary anthocyanins and two weeks of daily flying during the spring migration period affected key components of the antioxidant system in multiple tissues of a female songbird. We compared three measures of enzymatic antioxidant activity, one measure of non-enzymatic antioxidant capacity, and measures of oxidative damage to protein and lipid molecules in four tissues (i.e., pectoralis and leg muscle, liver, and heart). If the availability of dietary antioxidants reduces the need to invest in the endogenous component of the antioxidant system (Hypothesis H1 - Diet effect), then birds that consume more dietary antioxidants will have higher non-enzymatic antioxidant capacity and reduced enzymatic antioxidant activity and oxidative damage compared to birds that consume less dietary antioxidants. Furthermore, if regular (daily) flying

poses an additional oxidative challenge and females during the spring migration period conserve dietary antioxidants for investment into eggs (Hypothesis H2 -Diet x Training interaction effect), then flight-trained birds fed less dietary antioxidants will have substantially lower non-enzymatic antioxidant capacity and increased enzymatic antioxidant activity and oxidative damage compared to flight-trained birds fed more dietary antioxidants and untrained birds fed either diet. We also expected that the functional differences between tissues would affect their response to dietary antioxidant supplementation and flight training. This is one of most comprehensive studies to date detailing how dietary antioxidants and exercise affect antioxidant enzyme activity, non-enzymatic antioxidant capacity, and damage to biologically important molecules in a suite of organs in a migratory bird.

METHODS

Starling husbandry

Migratory European starlings from southern Germany were used for this study. This population consists of medium-distance migrants that depart for wintering grounds in October and November and return to their breeding grounds in April (Bairlein et al., 2014). Migratory distances for this population vary, as some individuals overwinter in northwest Africa while others overwinter in the Euro-Mediterranean region. European starlings are social and curious, and numerous recent studies have demonstrated that they can quickly learn to fly in small groups in a given windtunnel (Carter et al., 2020; Casagrande et al., 2020; Nebel et al., 2012, McWilliams et al., 2021).

Female European starling chicks ca. one week old ($n = 115$) were collected from a colony located in Upper Bavaria, South Germany ($47^{\circ}58' \text{ N}$, $11^{\circ}13' \text{ E}$) during April and May in 2015 ($n = 72$) and 2016 ($n = 43$). Chicks were hand-raised at the Max Planck Institute for Ornithology (MPIO) in Seewiesen, Germany and fed a diet of beef heart, insect larvae, wax worms (*Galleria mellonella*), a vitamin mixture, and calcium carbonate powder. Chicks were hand-fed until they were able to feed independently after ca. 35 days, after which they were moved to outdoor aviaries (3 x 4 x 2 meters). Birds were then fed *ad libitum* a standard MPIO maintenance diet of fruits, vegetables, and live mealworms until August 2016, when they were gradually switched from the maintenance diet to a semi-synthetic diet high in polyunsaturated fat (Table 1).

Experimental groups and antioxidant diets

During August 2016, females were separated into two overarching groups. “Season” females ($n = 55$) were flown in either fall 2016 or spring 2017 and sacrificed at the end of their 15-day flight training regime. “Reproductive” females ($n = 30$) were flown in both fall 2016 and spring 2017, and were further divided into a group that underwent the 15-day flight training each season (“trained”, $n = 16$) and a sedentary control group that did not undergo the flight training regime (“untrained”, $n = 14$). Females in the fall Season group were hand-raised in 2016, whereas females in the spring Season and the Reproductive groups were raised in 2015. Older females were selected for the spring Season and Reproductive birds so that they would be in breeding condition by spring 2017, both to simulate natural conditions during spring

migration and for a companion study (Carbeck et al., 2018). The purpose of including these two groups was to assess both seasonal differences (fall vs. spring) and flight training differences (trained vs. untrained) among females. Unfortunately, samples from the Season groups from early fall 2016 ($n = 8$ individuals, 32 tissue samples) were delayed in transit from MPIO to the University of Rhode Island, causing the samples to thaw and so not be viable. Therefore, we no longer had adequate samples for a seasonal comparison, and instead focused this study on the antioxidant system during spring. A comparison of circulating antioxidants and oxidative damage during fall and spring is provided in Chapter 1 of Frawley 2020.

Starting on September 21, 2016, all hand-raised starlings were randomly assigned to one of two semi-synthetic, high-polyunsaturated fat diet groups (Table 1). Each diet had identical macronutrient content (19% fat, 6% protein, 63% carbohydrates), but differed in the amount of anthocyanin, a water-soluble antioxidant, that they contained. We chose to supplement the experimental diets with anthocyanin because free-living birds preferentially consume fruits that are high in anthocyanin (Bolser et al., 2013) and many fruits eaten by migrating songbirds contain this type of purple-red antioxidant (Bolser et al., 2013, Catoni et al., 2008). The high antioxidant diet had 119 mg kg^{-1} (wet weight) added anthocyanin (Standardized Elderberry 6.5% Powder; Artemis International, Inc., Fort Wayne, IN, USA), whereas the low antioxidant diet had no added anthocyanin. If starlings ate about 35 g of this high antioxidant diet each day, then they would consume the equivalent of 4.2 mg anthocyanin per day (or about 17 berries per day).

After each bird was assigned to a diet and experimental group, we measured molt score (Jenni and Winkler, 2020) of each bird and assigned individuals to cohorts of four to six females each based on most to least advanced molt which were then sequentially flight trained over the course of each season. This ensured that all females had completed their molt prior to the start of flight training in fall 2016. Season females were assigned to one of eight cohorts (four high antioxidant diet cohorts, four low antioxidant diet cohorts), whereas Reproductive females were assigned to one of four cohorts (two high antioxidant cohorts, two low antioxidant cohorts) with trained and untrained females distributed evenly across the four cohorts.

Flight training

Starting on 26 September 2016, a new cohort was moved to cages (1.5 x 2.5 x 2.5 meters) in the windtunnel facility every three to six days. Over a four-day pre-training regime, each cohort in turn was habituated to fly directly into the windtunnel from their indoor cage without being handled and were acclimatized to the windtunnel conditions. Pre-training was then followed by a 15-day flight training regime. Flight training consisted of continuous periods of flying at 15 ms⁻¹ wind speed, 15°C ambient temperature, and 75% humidity. The duration of flight increased over the course of flight training: 20-30 minutes on days 1-4, 60 minutes on days 5 and 7, 30 minutes on day 6, 90 minutes on day 8, rest on day 9, 2-3 hours on days 10 and 11, rest on day 12, 60 minutes on day 13, 30 minutes on day 14, and up to 6 hours of voluntary flight on day 15. Such flight training in a windtunnel has demonstrated success at eliciting long-duration flights that simulate migratory flights (Engel et al., 2006, McWilliams et al.,

2021, Nebel et al., 2012). Reproductive birds assigned to the untrained group remained in their respective indoor aviaries and were fasted while trained birds in the same cohort flew in the adjacent windtunnel. Some birds were dropped from flight training due to poor performance (i.e. frequent land), and were not used in the final analyses (n = 20 individuals).

Organ collection and antioxidant analyses

After the longest-duration flight on day 15 of the flight training, Season females remained in their aviaries for two days, after which they were killed with an isoflurane overdose and decapitated. This two-day recovery period ensured that any differences in antioxidant enzyme activities, non-enzymatic antioxidant capacity, and oxidative damage to protein and lipid molecules in females could be attributed to the flight training and was not confounded by the acute effect(s) of the longest flight. Reproductive females remained in their aviaries for seven days after the longest-duration flight so that they could be paired with a male starling and allowed to reproduce prior to sacrifice (Carbeck et al., 2018). The liver, pectoralis, gastrocnemius, and heart were immediately dissected and weighed. All tissues (n = 272 tissue samples, 4/individual) were snap frozen with dry ice and stored at -80 °C until homogenization. Tissues were transported from MPIO to the University of Rhode Island in -80 °C dewars in multiple batches. Unfortunately, one of these shipments which contained samples from the Season groups from early fall 2016 (n = 8 individuals, 32 tissue samples) was delayed in transit, causing the samples to thaw and so not be viable. Thus, the final sample size for both the Season group (Fall: n = 4

individuals AO High, n = 5 individuals AO low; Spring: n = 9 individuals AO High, n = 12 individuals AO low) and Reproductive group (n = 7 individuals Flight-trained, AO High; n = 9 individuals Flight-trained, AO Low; n = 7 individuals Untrained, AO High; n = 7 individuals Untrained, AO Low) was 240 samples from 60 individuals (4 samples/individual). Occasionally a few tissue samples did not produce usable results, so we report in the Results section the exact sample sizes for each tissue and treatment comparison.

All assays were conducted using 96-well microplates and a BioTek Powerwave 340 plate reader (BioTek Instruments, Inc., Winooski, VT, USA) at the University of Rhode Island (Kingston, RI, USA). All tissues were initially homogenized in chilled 0.1M phosphate buffer. For the homogenate aliquots used in the enzymatic antioxidant and protein damage assays, 2.8 mM EDTA was added as a preservative, resulting in a final concentration of 0.5 M phosphate buffer and 0.1 mM EDTA. The homogenate aliquots used in the lipid damage and ORAC assays did not have added EDTA, resulting in a final concentration of 0.5 M phosphate buffer. Tissue homogenates were stored at -80 °C until analysis. Tissue homogenates from the heart, liver, pectoralis, and leg muscle were analyzed separately for each female. Non-enzymatic antioxidant capacity was measured using the oxygen radical absorbance capacity (ORAC) test adapted for microplates (Cao and Prior, 1999; Jimenez et al., 2019; Prior and Cao, 1999). The ORAC assay measures a sample's ability to quench peroxy and hydroxyl radicals using the fluorescent algal pigment phycoerythrin and serves as a measure to total non-enzymatic antioxidant capacity. Commercially available microplate assay kits were used to determine circulating enzymatic

antioxidant activity, lipid damage, and protein damage. The enzymatic antioxidants glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) were measured using colorimetric assays from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA). Oxidative damage was measured using the lipid hydroperoxide (LPO) and protein carbonyl assays from Cayman Chemical. The lipid hydroperoxide assay measures the concentration of ferric ions produced by redox reactions between reactive hydroperoxide species and ferrous ions. The protein carbonyl assay measures the concentration of protein-hydrazone produced by reactions between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls, a marker of protein oxidative damage.

The GPx assay was run in triplicate; all other assays were run in duplicate. Only individuals with a coefficient of variation < 15% were included in final analyses. Final sample sizes represent the number of remaining individuals after samples with coefficients of variation >15% were removed.

Statistics

Given the experimental design included trained and untrained control females for the Reproductive groups but not the Season groups, we tested Hypothesis 1 (Diet effect) using the Season and Reproductive females, whereas we tested Hypothesis 2 (Training x Diet interaction effect) using only the Reproductive females. The loss of most of the fall Season group samples due to thawing (see above) restricted our testing of Diet effects (H1) to females during only spring, although the results from the small

number of samples ($n = 4$ individuals per diet) from the fall Season group are provided in the supplemental materials for comparison (Table S1). All analyses were performed in R version 4.0.3 (R Core Team, 2020). Each measure was log-transformed prior to analysis. For hypothesis 1, linear mixed models were fit to infer the effects of diet (high or low antioxidant) on enzymatic antioxidants (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC), and oxidative damage (protein carbonyls and lipid hydroperoxides). An organ x diet interaction was included to account for differences in organ response to diet. Bird ID was used as a random factor to account for the repeated measures (four organs) taken from each individual. Day since the first cohort was established, total flight training time, and mass at time of sacrifice were evaluated as covariates. For hypothesis 2, linear mixed models were fit to infer the interactions and effects of diet (high or low antioxidant) and training (trained or untrained) on enzymatic antioxidants (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC), and oxidative damage (protein carbonyls and lipid hydroperoxides). A three-way organ x diet x training interaction was included to account for differences in organ response to diet and flight training and to assess interactions between diet and training. Bird ID was used as a random factor to account for the repeated measures (four organs) for each individual. Day since the first cohort was established and mass at time of sacrifice were evaluated as covariates. Welch's t-tests were used to determine if there were significant differences in mass or flight time between training status or diet. Final models for evaluating each hypothesis were selected using Akaike's information criterion adjusted for small sample size and the significance of covariates. The residual plots for each final model were visually inspected and did not

show noticeable deviation from normality or homoscedasticity. The emmeans package (Lenth, 2020) was used to estimate least-squares means and assess contrasts for the final models.

RESULTS

We report the main effects of diet (H1) for Season and Reproductive females. We report the diet x flight-training interaction (H2) for Reproductive females, along with the main effects of dietary antioxidants and flight-training. We found no significant differences in final mass or total flight time between diet and flight-training groups, and therefore we removed these as covariates when they were uninformative and not strongly preferred by Akaike's information criterion to simplify the linear models.

Hypothesis 1—dietary antioxidant effect in Season females

Antioxidant enzymes in flight-trained Season females fed dietary antioxidants

For Season females, glutathione peroxidase (GPx) activity was significantly higher in heart tissue ($n = 14$ high, $n = 17$ low; Figure 1D; $F_{8,136} = 7.00$, $p = 0.01$) for females fed the low antioxidant diet. GPx activity did not differ between diets for the pectoralis ($n = 16$ high, $n = 21$ low; Figure 1A; $F_{8,136} = 0.35$, $p = 0.56$), leg muscle ($n = 16$ high, $n = 21$ low; Figure 1B; $F_{8,136} = 0.05$, $p = 0.82$), or liver ($n = 16$ high, $n = 21$ low; Figure 1C; $F_{8,136} = 2.56$, $p = 0.13$). Catalase (CAT) activity did not differ between diets in the pectoralis ($n = 15$ high, $n = 21$ low; Figure 1E; $F_{8,130} = 0.88$, $p = 0.35$), leg muscle ($n = 16$ high, $n = 20$ low; Figure 1F; $F_{8,130} = 2.51$, $p = 0.12$), liver (n

= 16 high, n = 17 low; Figure 1G; $F_{8,130} = 0.26$, $p = 0.61$), or heart (n = 14 high, n = 19 low; Figure 1H; $F_{8,130} = 0.46$, $p = 0.50$). Superoxide dismutase (SOD) activity did not differ between diets in the pectoralis (n = 16 high, n = 21 low; Figure 1I; $F_{9,136} = 0.15$, $p = 0.70$), leg muscle (n = 16 high, n = 21 low; Figure 1J; $F_{9,136} = 0.47$, $p = 0.50$), liver (n = 16 high, n = 21 low; Figure 1K; $F_{9,136} = 0.001$, $p = 0.98$), or heart (n = 14 high, n = 20 low; Figure 1L; $F_{9,136} = 0.31$, $p = 0.58$).

Non-enzymatic antioxidant capacity in flight-trained Season females (ORAC) fed dietary antioxidants

There was a trend in the pectoralis for non-enzymatic antioxidant capacity against peroxy radicals to be higher for females fed the low antioxidant diet (n = 15 high, n = 20 low; Figure 2A; $F_{8,136} = 2.73$, $p = 0.10$). Non-enzymatic antioxidant capacity against peroxy radicals did not differ between diets for leg muscle (n = 16 high, n = 20 low; Figure 2B; $F_{8,136} = 0.17$, $p = 0.68$), liver (n = 16 high, n = 21 low; Figure 2C; $F_{8,136} = 0.28$, $p = 0.60$), or heart (n = 16 high, n = 21 low; Figure 2D; $F_{8,136} = 0.74$, $p = 0.39$). Non-enzymatic antioxidant capacity against hydroxyl radicals did not differ between diets for the pectoralis (n = 15 high, n = 20 low; Figure 2E; $F_{8,136} = 0.17$, $p = 0.69$), leg muscle (n = 16 high, n = 20 low; Figure 2F; $F_{8,136} = 2.14$, $p = 0.15$), liver (n = 16 high, n = 21 low; Figure 2G; $F_{8,136} = 0.02$, $p = 0.89$), or heart (n = 16 high, n = 21 low; Figure 2H; $F_{8,136} = 0.64$, $p = 0.43$).

Damage markers in flight-trained Season females fed dietary antioxidants

Females fed the high antioxidant diet trended toward having higher concentrations of lipid peroxides in heart tissue (n = 16 high, n = 21 low; Figure 3D; $F_{8,121} = 3.67$, $p = 0.06$), and had significantly lower concentrations of protein carbonyls in leg muscle (n = 14 high, n = 17 low; Figure 3F; $F_{8,123} = 6.27$, $p = 0.01$). Lipid peroxide concentrations did not differ between diets in the pectoralis (n = 13 high, n = 17 low; Figure 3A; $F_{8,121} = 1.38$, $p = 0.24$), leg muscle (n = 15 high, n = 15 low; Figure 3B; $F_{8,121} = <0.001$, $p = 0.98$), or liver (n = 15 high, n = 20 low; Figure 3C; $F_{8,121} = 0.22$, $p = 0.64$). Concentrations of protein carbonyls were similar between the pectoralis (n = 15 high, n = 18 low; Figure 3E; $F_{8,123} = 0.11$, $p = 0.75$), liver (n = 14 high, n = 21 low; Figure 3G; $F_{8,123} = 0.29$, $p = 0.59$), and heart (n = 15 high, n = 20 low; Figure 3H; $F_{8,123} = 0.28$, $p = 0.60$).

Hypothesis 2—dietary antioxidant x flight-training interaction for Reproduction females

Antioxidant enzymes in trained and untrained Reproductive females fed dietary antioxidants

For heart tissue, untrained females trended toward having higher GPx activity when fed the low antioxidant diet (Figure 4D; interaction $F_{9,107} = 3.20$, $p = 0.08$), and untrained females had consistently higher GPx activity than trained females (training effect $F_{9,107} = 13.59$, $p = <0.001$; diet effect $F_{9,107} = 1.59$, $p = 0.21$). There were no significant diet x training interactions for GPx activity in the pectoralis (Figure 4A; $F_{9,107} = 0.57$, $p = 0.45$), leg muscle (Figure 4B; $F_{9,107} = 0.01$, $p = 0.92$), or liver (Figure

4C; $F_{9,107} = 0.21$, $p = 0.65$) nor any diet or training main effects ($p > 0.15$ in all cases). In leg muscle, trained females had higher catalase activity than untrained females fed the low antioxidant diet, but lower catalase activity than untrained females fed the high antioxidant diet (Figure 4F; interaction $F_{15,95} = 4.45$, $p = 0.04$; training effect $F_{15,95} = 2.06$, $p = 0.15$; diet effect $F_{15,95} = 1.14$, $p = 0.29$). The liver exhibited an inverse trend such that trained females had lower catalase activity than untrained females fed the low antioxidant diet, and higher catalase activity than untrained females fed the high antioxidant diet (Figure 4G; interaction $F_{15,95} = 3.22$, $p = 0.08$; training effect $F_{15,95} = 0.26$, $p = 0.61$; diet effect $F_{15,95} = 1.02$, $p = 0.31$). There were no significant diet x training interactions in catalase activity in muscle from the pectoralis (Figure 4E; $F_{15,95} = 0.30$, $p = 0.59$) or the heart (Figure 4H; $F_{15,95} = 0.10$, $p = 0.76$) nor any diet or training main effects ($p > 0.14$ in all cases). There was no significant diet x training interaction for SOD concentration in the pectoralis ($F_{15,108} = 0.32$, $p = 0.57$), leg muscle (Figure 4J; $F_{15,108} = 0.13$, $p = 0.72$), liver (Figure 4K; $F_{15,108} = 0.004$, $p = 0.95$), or heart (Figure 4L; $F_{15,108} = 0.002$, $p = 0.96$) nor any diet or training main effects ($p > 0.11$ in all cases). There was a trend for a significant training effect for the liver, such that untrained females had higher SOD concentration than trained females (Figure 4K; $F_{15,108} = 3.17$, $p = 0.08$), but no significant diet effect ($F_{15,108} = 0.17$, $p = 0.68$).

Non-enzymatic antioxidant capacity in trained and untrained Reproductive females (ORAC) fed dietary antioxidants

There were no diet x training interactions for non-enzymatic antioxidant capacity against peroxy radicals in the pectoralis ($F_{15,102} = 0.19$, $p = 0.66$), leg muscle ($F_{15,102} = 0.69$, $p = 0.41$), liver ($F_{15,102} = 0.08$, $p = 0.77$), or heart ($F_{15,102} = 0.24$, $p = 0.63$) nor any diet or training main effects ($p > 0.15$ in all cases). There were no interactions between diet x training regime for non-enzymatic antioxidant capacity against hydroxyl radicals in the pectoralis ($F_{16,101} = 0.04$, $p = 0.84$), leg muscle ($F_{16,101} = 0.71$, $p = 0.40$), liver ($F_{16,101} = 0.24$, $p = 0.63$), or heart ($F_{16,101} = 2.37$, $p = 0.13$) nor any diet or training main effects ($p > 0.47$ in all cases).

Damage markers in trained and untrained Reproductive females fed dietary antioxidants

There were no interactions between diet x training treatments for lipid peroxide concentrations in the pectoralis ($F_{15,90} = 0.62$, $p = 0.43$), leg muscle ($F_{15,90} = 0.27$, $p = 0.60$), liver ($F_{15,90} = 0.09$, $p = 0.76$), or heart ($F_{15,90} = 0.01$, $p = 0.90$) nor any diet or training main effects ($p > 0.40$ in all cases). There were also no significant interactions for protein carbonyl concentrations in the pectoralis ($F_{16,86} = 0.17$, $p = 0.68$), leg muscle ($F_{16,86} = 0.45$, $p = 0.50$), liver ($F_{16,86} = 0.81$, $p = 0.37$), or heart ($F_{16,86} = 0.98$, $p = 0.32$) nor any diet or training main effects in the pectoralis, leg muscle, or liver ($p > 0.50$). For heart tissue, females fed the high antioxidant diet had higher concentrations of protein carbonyls ($F_{16,86} = 7.60$, $p = 0.01$).

Revisiting Hypothesis 1 - dietary antioxidant effect for flight-trained Season and Reproductive females

Females in both the Season and Reproductive groups were fed the same two diets, one supplemented with dietary antioxidants and the other not, although the Season females were sampled two days after their longest flight on Day 15 of flight training whereas Reproductive females were sampled seven days later after exposure to males. Here we compare the main effect of dietary antioxidants for Season females (reported above) to Reproductive females that were also flight-trained (the main effects of dietary antioxidants on both flight-trained and untrained Reproductive females are reported above). The significantly higher glutathione peroxidase (GPx) activity in heart tissue for Season females fed the lower antioxidant diet (Fig. 1D) was not apparent in flight-trained Reproductive females ($n = 7$ high, $n = 8$ low; Figure 5D; $F_{8,136} = 0.01$, $p = 0.90$). For flight-trained Reproductive females, CAT activity was significantly higher in leg tissue ($n = 7$ high, $n = 9$ low; Figure 5F; $F_{3,57} = 3.82$, $p = 0.05$) for females fed the low antioxidant diet, whereas a similar trend ($p = 0.12$) was apparent for Season females (Fig. 1F). There was a trend for CAT activity in liver tissue to be affected by dietary antioxidants in flight-trained Reproductive females ($n = 7$ each; Figure 5G; $F_{3,57} = 2.78$, $p = 0.10$), although, unlike in leg tissue, females fed more dietary antioxidant had higher CAT activity. No such trend was apparent in liver tissue of Season females. The few significant effects of dietary antioxidants on non-enzymatic antioxidant capacity (Fig. 2) and oxidative damage (Fig. 3) in Season females were not apparent in flight-trained Reproductive females. (Fig. S1, Table S2).

DISCUSSION

Our study is one of the most comprehensive examinations of the effects of dietary antioxidant supplementation and exercise on the avian antioxidant system to date. We found mixed support for each of our two hypotheses that was strongly tissue-specific. For example, dietary antioxidant supplementation (hypothesis 1) affected one of the three antioxidant enzymes (GPx) and one of the two non-enzymatic antioxidant capacity measures (ORAC hydroxyls) but in each case in only one of four tissues (pectoralis or heart). Flight training and diet together (hypothesis 2) affected all three antioxidant enzymes but only in one or two tissues and never in pectoralis, the primary flight muscle, and had no effect on oxidative damage or non-enzymatic antioxidant capacity. Below we discuss these results in detail and their implications.

Hypothesis 1—dietary antioxidant effect

We predicted that females fed the high antioxidant diet would have higher non-enzymatic antioxidant capacity, and lower enzymatic antioxidant activity and oxidative damage than females fed the low-antioxidant diet. We found mixed support for this hypothesis. In support of hypothesis 1, GPx activity was lower in the heart for Season females fed more dietary antioxidants, but not for any other organs (Figure 1D). CAT activity and SOD concentration were not affected by anthocyanin supplementation in the Season females. To date, studies examining the effects of antioxidant supplementation on antioxidant enzymes in domestic chickens and captive-bred rodents have found mixed results. For example, broiler chickens fed a diet supplemented with a commercially available algae-based antioxidant had higher GPx, SOD, and CAT activity in the pectoralis than birds fed a control diet (Delles et

al., 2014), indicating that antioxidant supplementation may have promoted enzyme activity. In contrast, mice fed a diet supplemented with vitamin C had decreased GPx, SOD, and CAT activity in liver tissue compared to individuals fed a control diet (Selman et al., 2006). Anthocyanins have been demonstrated to increase the activity of glutathione-related enzymes, including GPx, in rat liver cells (Shih et al., 2007). It is plausible that dietary antioxidants react with enzymatic antioxidants in differing ways depending on the enzyme's cofactors and location within the body and depending on the antioxidant's solubility in fat or water. Our result suggests that anthocyanin supplementation may allow female birds to decrease their reliance on costly enzymatic antioxidants in a major organ used in exercise. However, further analysis into the chemical pathways of enzymatic antioxidants is needed to better understand ways in which dietary anthocyanin may complement or regulate the function of enzymatic antioxidants in different tissues.

Contrary to hypothesis 1, non-enzymatic antioxidant capacity against hydroxyl radicals was higher in pectoralis muscle for females fed the low antioxidant diet (Figure 2F). These results indicate that females fed less dietary antioxidants may upregulate non-enzymatic defenses, and that supplementation with water-soluble antioxidants may not contribute to overall non-enzymatic antioxidant capacity in tissue as we predicted. Uric acid is one antioxidant molecule that has been demonstrated to quench hydroxyl radicals and prevent lipid peroxidation, and is a natural byproduct of protein catabolism, which is elevated in flight muscles such as the pectoralis (Battley et al., 2000, Stinefelt et al., 2005). Previous studies have suggested that uric acid may be one of the first antioxidant molecules to be

upregulated when dietary antioxidants are not available or when birds face an oxidative challenge. For example, Northern wheatears (*Oenanthe oenanthe*) faced with the oxidative challenge of refueling had higher circulating uric acid but similar oxidative damage compared to control birds (Eikenaar et al., 2016). In a companion study using the same groups of female starlings, we found that females fed the low-antioxidant diet had higher baseline circulating uric acid in plasma than females fed the high-antioxidant diet (Frawley et al., in press). Furthermore, long-duration flights have been demonstrated to increase circulating uric acid in many bird species (Gannes et al., 2001, Gerson & Guglielmo, 2011, Jenni-Eiermann & Jenni, 1991). Females fed less dietary antioxidants may rely on uric acid produced through protein catabolism to increase non-enzymatic antioxidant capacity (Tsahar et al., 2006).

In support of hypothesis 1, females fed less dietary antioxidants had higher indices of protein damage in leg (Figure 3F) but not the other three tissues sampled, indicating higher protein catabolism. However, females fed more dietary antioxidants tended to have higher concentrations of lipid hydroperoxides, but in only the heart (Figure 3D) and not the other three tissues. Though few analyses have been done on oxidative damage in tissue from birds, plasma studies have indicated that antioxidant supplementation lowers circulating markers of oxidative damage. Budgerigars fed diets supplemented with antioxidants had lower DNA damage and circulating malondialdehyde (MDA), an indicator of lipid peroxidation, in plasma than birds fed the low antioxidant diet (Larcombe et al., 2008). In contrast, blue tit nestlings (*Cyanistes caeruleus*) fed diets supplemented with either carotenoids or vitamin E had similar levels of oxidative damage in plasma compared to nestlings on a control diet

(Larcombe et al., 2010). Heifers fed a diet supplemented with vitamin E had lower oxidative damage in the liver compared to heifers fed a control diet (Bouwstra et al., 2008), and female rats injected with selenium and vitamin E had lower oxidative damage in liver and renal tissue compared to control females (Nazıroğlu et al., 2004). Anthocyanin is a water-soluble antioxidant, though it has been demonstrated to play a role in fat metabolism and can lower rates of lipid peroxidation in mammals (Ramirez-Tortosa et al., 2001, Tsuda et al., 1996). Therefore, we would expect lipid peroxidation to be lower in all organs for females fed the high antioxidant diet. However, oxidative damage to both protein and lipids did not significantly differ between the high and low antioxidant diets in the pectoralis or liver. This may be due to the fact that the females were sacrificed in spring, when free-living birds would be preparing to breed and therefore may be prioritizing the reduction of oxidative damage regardless of diet. Studies on free-living barn swallows (Costantini et al., 2014) and captive zebra finches (Wiersma et al., 2004) have demonstrated that the oxidative cost of breeding is high for females. Furthermore, depositing antioxidants into eggs has been demonstrated to be beneficial for offspring (Marri & Richner, 2014, McGraw et al., 2005, Norte et al., 2010), and female great tits (*Parus major*) with higher levels of oxidative damage during breeding deposited fewer antioxidants into their eggs (Giordano et al., 2015). As part of a companion study, we blood-sampled Season females prior to flight-training in the fall and spring (Frawley Chapter 1, 2020). Females in the spring had lower circulating oxidative damage prior to flight training than females in the fall. Viewed altogether, these results suggest that females may shift

antioxidant allocation to reduce oxidative damage in spring, regardless of the availability of dietary antioxidants.

Dietary anthocyanin supplementation had no effect on enzymatic antioxidants, non-enzymatic antioxidant capacity, or oxidative damage to lipids and proteins in the liver. The liver is primarily used to store fat-soluble antioxidants, and so perhaps this is why the liver was less affected by water-soluble anthocyanins (Galván et al., 2012, Møller et al., 2010). Supplementing females with a fat-soluble antioxidant such as vitamin E may help to determine if antioxidant supplementation effects enzymatic antioxidants, oxidative damage, and non-enzymatic antioxidant capacity in the liver.

Hypothesis 2—diet and training interactions

We predicted that daily flying would pose an additional oxidative challenge for females conserving antioxidants during spring migration, and that flight-trained female fed less dietary antioxidants would have lower non-enzymatic antioxidant capacity and higher enzymatic antioxidant activity and oxidative damage than flight-trained females fed more dietary antioxidants. We found diet x training interactions for two enzymatic antioxidants, CAT and GPx, across three organs. We also found significant training effect for SOD concentrations in the liver. Untrained females had significantly higher GPx activity in the heart than trained females, and this difference was most pronounced for females fed less dietary antioxidant (Figure 4D). This result is consistent with the hypothesis that enzymatic antioxidants at least partially compensate when dietary antioxidants are scarce, although we show that this partial compensation does not occur when females are flight-trained. Studies in mammals have generally established an opposing trend, such that antioxidant supplementation

generally increases enzymatic antioxidant activity in red blood cells, especially for GPx and SOD (Tauler et al., 2006, Urso & Clarkson, 2003). For example, a study in racehorses found that horses fed a dietary antioxidant supplement had increased circulating GPx activity after 12 weeks of training compared to baseline levels, and higher GPx activity than trained control horses (de Moffarts et al., 2005). In contrast, we found that female starlings fed more dietary antioxidants had lower CAT activity in leg muscle after two weeks of flight-training compared to untrained starlings as well as females fed less dietary antioxidants regardless of training (Figure 4F). In contrast, female starlings fed more dietary antioxidants had higher CAT activity in liver after two weeks of flight-training compared to untrained starlings, whereas the opposite effect of flight-training occurred when females were fed less dietary antioxidants (Figure 4G). The pattern seen in leg muscle indicates that anthocyanin supplementation may have allowed trained females to decrease reliance on CAT and complements the change in GPx seen in the heart for untrained females. The pattern seen in the liver, however, indicates that anthocyanin supplementation may improve CAT activity in this organ. Rats that underwent exercise training and were fed a diet supplemented with vitamin E had elevated GPx and CAT activity after 14 weeks compared to rats that either underwent training or were fed a diet supplemented with vitamin E (Marsh et al., 2006).

SOD activity was higher in only the liver of untrained females (Figure 4K) and not the other three tissues sampled. The liver is vital to fatty acid metabolism, and therefore we expected to see higher enzymatic antioxidant activity in the livers of trained birds to protect the fatty acids necessary to fuel flight (McWilliams et al.,

2004). These results suggest that flight training may allow females to decrease their reliance on enzymatic antioxidants. For example, GPx production has been suggested to be costly due to the necessity of selenium cofactors that are also used in amino acid synthesis and the immune response (Zwolak and Zaporowska, 2012). The three varieties of SOD rely on manganese, iron, or copper and zinc as cofactors, and these metals are also used in respiratory and signaling pathways (Halliwell and Gutteridge, 2015). Enzymatic antioxidants may be used primarily for acute challenges such as bouts of flight (Cooper-Mullin et al., 2019, Jenni-Eiermann et al., 2014), and training may result in a decreased reliance on enzymatic antioxidants. Given that the pectoralis is the primary muscle involved in flight and a major site of fatty acid uptake (McWilliams et al., 2004), we expected to see differences in antioxidant capacity and enzymatic antioxidant activity between trained and untrained females. However, fatty acids stored within muscle are used to fuel flight for short durations (McWilliams et al., 2004); therefore, examining the acute changes in the pectoralis after flight may prove more informative.

Viewed altogether, these results emphasize that dietary antioxidants do not affect enzymatic antioxidants in a universal way. Furthermore, different sites of enzyme activity—muscle versus the liver, for example—may exhibit different responses to dietary antioxidants and training depending on their metabolic role in fueling flight. Further study is needed to better understand the ways in which migratory birds assimilate dietary antioxidants into the endogenous antioxidant system, and the pathways involved in the regulation of enzymatic antioxidants.

Oxidative damage and non-enzymatic antioxidant capacity did not differ between trained and untrained birds. Studies examining oxidative damage associated with a given flight indicate that flight does incur an oxidative cost. Free-living European robins (*Erithacus rubecula*) caught during nocturnal migratory flights had elevated levels of protein damage compared to resting birds (Jenni-Eiermann et al., 2014), and homing pigeons (*Columba livia*) had elevated oxidative damage and lower non-enzymatic antioxidant capacity after a long-distance flight (Costantini et al., 2008). However, studies in budgerigars and rats indicate that training may help to attenuate oxidative damage (Larcombe et al., 2010, Oztasan et al., 2004). Our study further supports the benefits of training, as trained birds had similar oxidative damage and non-enzymatic antioxidant capacity after their 15-day flight training regime and 2-day recovery period compared to untrained birds. Thus, any oxidative damage associated with a given long-duration flight seems repaired during the recovery period.

It is important to acknowledge that free-living migrants experience challenges that our captive birds did not, and that these ecological factors may drive changes in the redox state. For this study, we hand-raised nestlings from a wild population and therefore would expect them to have the genotype of free-living migrants. We also exposed the females to a natural photoperiod. However, migratory birds spend much of migration at stopover sites, foraging for food and refueling for the next leg of their journey. Stopover has been estimated to incur double the energy cost of migratory flight and may represent an important oxidative challenge to migrants (Wikelski et al., 2003). Regardless, our study provides valuable insight into the physiology of wild migrants, as it demonstrates the effect of antioxidant supplementation using a dietary

antioxidant that birds consume in the wild, and the effect of repeated flights such as those experienced by many wild migrants. Comparing oxidative status during stopover and migratory flight may reveal if flight, ecological factors such as stopover periods, or a mixture of both factors help mold the oxidative status of migrants and will better inform future studies in both captive and free-living populations.

ACKNOWLEDGEMENTS

We thank Liam Corcoran for assistance in running the ORAC and Bradford assays. We are especially grateful to the Max Planck Institute for Ornithology animal care staff for handling much of the animal husbandry responsibilities, and to Susan Forrest and Aleksandra Małochleb for helping raise the starlings. Special thanks are extended to Maude Baldwin and the entire Baldwin lab, especially Glenn Cockburn, for providing access to their lab and equipment at MPIO to conduct assays and for providing us with dewars to transport samples to the US.

Funding statement: The experiment was supported by a grant from the National Science Foundation to S.R.M. and B.J.P. (IOS-1354187); by OPUS grant from National Science Foundation (NCN), Poland, to U.B. (UMO-2015/19/B/NZ8/ 01394) and DS grant from the Institute of Environmental Sciences, Jagiellonian University to U.B. (DS/WBINOZ/INOS/757). A.E.F. was supported in part by NSF (U.S.A.) and in part by the University of Rhode Island and the College of Environment and Life Sciences.

Ethics statement: All the experimental protocols were approved by the University of Rhode Island IACUC (Protocol #AN08-02-014) and the Government of Upper Bavaria, Germany (AZ 55.2-1-54-2532-216-2014).

Author contributions: A.E.F. carried out laboratory analyses. A.E.F. performed statistical analyses and with S.R.M. drafted the manuscript. S.R.M., B.J.P. and U.B. designed the experiment and with A.E.F. formulated the hypotheses. K.J.M., K.M.C., L.T., B.P., A.B., M.D., E.T.S. and U.B. conducted the experiment. All authors edited and approved the manuscript.

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TABLES

Table 1. Composition of semi-synthetic high-antioxidant diet fed to female European starlings during the fall 2016 and spring 2017 experiments. Half of the females (n = 43) were fed the high- antioxidant diet with added anthocyanin; other females (n = 43) were fed a low- antioxidant diet with the exact same composition but without added anthocyanin.

Ingredients	% wet mass	% dry mass
Agar ¹	1.37	3.19
Glucose ²	16.84	39.19
Casein ³	8.21	19.12
Cellulose ⁴	2.14	4.97
Canola oil ⁵	5.18	12.05
Sunflower oil ⁶	3.03	7.06
Ground mealworms	2.65	6.16
Elderberry powder ⁷	0.18	0.42
Salt mixture ⁸	2.05	4.78
Amino acid mix ⁹	1.15	2.68
Vitamin mix ¹⁰	0.16	0.38
Water	57.04	

¹ Agar, Ombilab-laborenzentrum, GmbH, Bremen, Germany

² Glucose, VWR International GmbH, Darmstadt, Germany

³ Casein, Affymetrix UK Ltd., High Wycombe, UK

⁴ Alphacel, MP Biomedicals, Solon, OH, USA

⁵ Canola oil, Jedwards International, Inc., Braintree, MA, USA

⁶ Sunflower oil, Jedwards International, Inc., Braintree, MA, USA

⁷ Standardized Elderberry extract powder, Artemis international, Inc, Fort Wayne, IN, USA; contains 6.5% anthocyanins; amount added was 0.04 % wet mass from 21 Sept to 5 Oct, then 0.18 % thereafter

⁸ Brigg's salt mix, MP Biomedicals, Solon, OH, USA

⁹ Amino acid mix, Sigma-Aldrich, St. Louis, MO, USA

¹⁰ AIN vitamin mix, MP Biomedicals, Solon, OH, USA. Includes vitamin E.

Supplemental Table 1. Statistical analyses for fall diet comparison. This comparison was not included in the main text due to small sample size. Estimated means and standard errors listed were calculated using estimated marginal means. Linear mixed models were fit for all analyses using bird ID as a random factor. ΔAIC_c is the difference between the final model used and the global model with all covariates included. Estimates for the leg are not included because there were not enough observations for each diet to include in the model.

AO Measure	Organ	Estimated mean $\pm$ S.E.	d.f.	F	p
GPx	Pectoralis	AH: 4.96 ± 0.33 AL: 5.12 ± 0.29	7, 17	0.13	0.72
	Liver	AH: 3.23 ± 0.38 AL: 3.83 ± 0.33		1.40	0.24
	Heart	AH: 4.95 ± 0.38 AL: 7.28 ± 0.33		20.95	0.0001
CAT	Pectoralis	AH: 3.00 ± 0.44 AL: 3.00 ± 0.39	7, 20	0	1
	Liver	AH: 5.82 ± 0.44 AL: 6.02 ± 0.39		0.11	0.74
	Heart	AH: 1.72 ± 0.52 AL: 1.98 ± 0.39		0.16	0.69
SOD	Pectoralis	AH: 0.52 ± 0.38 AL: 0.45 ± 0.34	8, 19	0.02	0.89
	Liver	AH: 1.39 ± 0.38 AL: 1.60 ± 0.34		0.16	0.69
	Heart	AH: 2.70 ± 0.44 AL: 3.63 ± 0.34		2.64	0.11
Protein carbonyl	Pectoralis	AH: 3.63 ± 0.71 AL: 4.43 ± 0.53	7, 19	0.81	0.37
	Liver	AH: 4.80 ± 0.60 AL: 4.52 ± 0.53		0.13	0.72
	Heart	AH: 4.28 ± 0.60 AL: 2.82 ± 0.61		2.90	0.10
Lipid hydroperoxides	Pectoralis	AH: 3.21 ± 0.61 AL: 4.24 ± 0.53	7, 17	1.64	0.21
	Liver	AH: 3.47 ± 0.52 AL: 4.16 ± 0.62		0.74	0.40
	Heart	AH: 5.11 ± 0.52 AL: 5.14 ± 0.46		0.001	0.97
ORAC (peroxyls)	Pectoralis	AH: 12.5 ± 0.33 AL: 12.3 ± 0.30	7, 23	0.13	0.72
	Liver	AH: 13.0 ± 0.33 AL: 12.8 ± 0.30		0.22	0.64
	Heart	AH: 12.4 ± 0.33 AL: 11.6 ± 0.30		2.88	0.10
ORAC (hydroxyls)	Pectoralis	AH: 11.1 ± 0.23 AL: 10.8 ± 0.21	8, 22	0.001	0.98
	Liver	AH: 11.7 ± 0.23 AL: 11.6 ± 0.21		0.14	0.72
	Heart	AH: 12.0 ± 0.23 AL: 11.4 ± 0.21		0.82	0.37

Supplemental Table 2. Statistical analyses for oxidative damage and non-enzymatic antioxidant capacity for diet comparison in flight-trained Reproductive birds. Linear mixed models were fit for all analyses using bird ID as a random factor. ΔAICc is the difference between the final model used and the global model with all covariates included.

AO Measure	Organ	Sample Size	d.f.	<i>F</i>	<i>p</i>
Protein carbonyl	Pectoralis	AH: 6 AL: 8	4, 49	0.003	0.96
	Leg	AH: 6 AL: 7		0.27	0.61
	Liver	AH: 5 AL: 9		0.59	0.44
	Heart	AH: 6 AL: 6		1.46	0.23
Lipid hydroperoxides	Pectoralis	AH: 4 AL: 9	3, 53	0.06	0.81
	Leg	AH: 7 AL: 5		0.73	0.40
	Liver	AH: 7 AL: 8		0.07	0.79
	Heart	AH: 7 AL: 9		0.20	0.66
ORAC (peroxyls)	Pectoralis	AH: 6 AL: 8	3, 59	1.31	0.26
	Leg	AH: 7 AL: 9		0.97	0.33
	Liver	AH: 7 AL: 9		0.01	0.94
	Heart	AH: 7 AL: 9		0.06	0.80
ORAC (hydroxyls)	Pectoralis	AH: 6 AL: 8	4, 58	0.05	0.82
	Leg	AH: 7 AL: 9		2.58	0.11
	Liver	AH: 7 AL: 9		0.04	0.85
	Heart	AH: 7 AL: 9		0.68	0.41

FIGURES

Figure 1. Least square means ($\pm$ SE) of enzymatic antioxidants in pectoralis, leg, liver, and heart tissue by diet in Season females. Solid lines over groups denote a significant difference. Glutathione peroxidase activity did not differ between diets for the pectoralis (A), leg (B), liver (C), but was significantly higher in the heart (D) for females fed the low antioxidant diet. Catalase activity did not differ between diets in the pectoralis (E), leg (F), liver (G), or heart (H). Superoxide dismutase activity did not differ between diets for the pectoralis (I), leg (J), or liver (K), or heart (L).

Figure 2. Least square means ($\pm$ SE) of non-enzymatic antioxidant capacity against peroxy and hydroxyl radicals in pectoralis, leg, liver, and heart tissue by diet for Season females. Dashed lines over groups denote a trend. There was a trend in the pectoralis (A) for non-enzymatic antioxidant capacity against peroxy radicals to be higher in the pectoralis for birds fed the low antioxidant diet. Non-enzymatic antioxidant capacity against peroxy radicals did not differ between diets in the leg (B), liver (C), or heart (D). Non-enzymatic antioxidant capacity against hydroxyl radicals did not differ between diets in the pectoralis (E), leg (F), liver (G) or heart (H).

Figure 3. Least square means ($\pm$ SE) of oxidative damage to lipids and proteins in pectoralis, leg, liver, and heart tissue by diet for Season females. Solid lines over groups denote a significant difference, whereas dashed lines denote a trend. Lipid hydroperoxides did not differ between diets in the pectoralis (A), leg (B), or liver (C). There was a trend for lipid hydroperoxides to be higher in the heart (D) for females fed the high antioxidant diet. Protein carbonyls did not differ between diets in the pectoralis (E). Protein carbonyls were significantly lower in the leg (F) muscle for females fed the high antioxidant diet. Protein carbonyls did not differ between diets in the liver (G) or heart (H).

Figure 4. Least square means ($\pm$ SE) of enzymatic antioxidants in pectoralis, leg, liver, and heart tissue by training and diet for Reproductive females. Solid lines over groups denote a significant difference, whereas dashed lines denote a trend. * and a line covering all four groups indicates a diet x training interaction. Glutathione peroxidase activity did not differ with training in the pectoralis (A), leg (B), or liver (C). There was a diet x training interaction for GPx in the heart (D), such that untrained females fed the low antioxidant diet trended toward having high GPx activity. Catalase activity did not differ with training in the pectoralis (E). There was a diet x training interaction for CAT in leg muscle (F), such that trained females had higher CAT activity than untrained females on the low antioxidant diet, but lower CAT activity than untrained females on the high antioxidant diet. There was a diet x training interaction for CAT in the liver (G), such that trained females had lower CAT activity than untrained females on the low antioxidant diet, but higher CAT activity

than untrained females on the high antioxidant diet. Catalase activity did not differ with training in the heart (**H**). Superoxide dismutase activity did not differ with training in the pectoralis (**I**), leg (**J**), liver (**K**), or heart (**L**).

Figure 5. Least square means ($\pm$ SE) of enzymatic antioxidants in pectoralis, leg, liver, and heart tissue by diet for flight-trained Reproductive females. Solid lines over groups denote a significant difference, whereas dashed lines denote a trend. Glutathione peroxidase activity did not differ between diets for the pectoralis (A), leg (B), liver (C), or heart (D). Catalase activity did not differ between diets in the pectoralis (E) or heart (H), but was significant higher for females fed the low antioxidant diet in the leg (F). There was a trend for catalase activity to be lower for females fed the low antioxidant diet in the liver (G). Superoxide dismutase activity did not differ between diets for the pectoralis (I), leg (J), or liver (K), or heart (L).