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RESEARCH ARTICLE

Season, anthocyanin supplementation, and flight training have mixed effects on the antioxidant system of migratory European Starlings

Abigail E. Frawley,[1,*] Kristen J. DeMoranville,[1] Katherine M. Carbeck,[2] Lisa Trost,[3] Amadeusz Bryła,[4] Maciej Dzialo,[4] Edyta T. Sadowska,[4] Ulf Bauchinger,[4,5] Barbara J. Pierce,[6] and Scott R. McWilliams[1]

[1] Department of Natural Resources Science, University of Rhode Island, Kingston, Rhode Island, USA
[2] Department of Forest and Conservation Sciences, University of British Columbia, Vancouver, British Columbia, Canada
[3] Department for Behavioural Neurobiology, Max Planck Institute for Ornithology, Seewiesen, Germany
[4] Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland
[5] Nencki Institute of Experimental Biology PAS, Warszawa, Poland
[6] Department of Biology, Sacred Heart University, Fairfield, Connecticut, USA
* Corresponding author: aefrawley@uri.edu

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ABSTRACT

Migratory birds engage in 2 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 fed them either a diet supplemented with dietary anthocyanins or a diet without added anthocyanins. We flew females in a wind tunnel for 15 days during fall and spring migration seasons and measured over time oxidative lipid damage (d-ROMs) and 3 components of the antioxidant system: nonenzymatic antioxidant capacity (OXY), uric acid, and glutathione peroxidase (GPx) activity. Prior to flight training, OXY and oxidative damage were lower in females during spring compared with fall, and females fed a low-antioxidant diet had consistently higher circulating uric acid. GPx activity decreased more in spring immediately after a long-duration flight. Females fed a high-antioxidant diet had a greater decrease in OXY after the 15-day flight training. Flight-trained females had higher 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 nonenzymatic 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 similar flights in fall and spring likely represent distinct oxidative challenges in the life history of female birds.

Keywords: anthocyanin, dietary antioxidants, female fall and spring migration, flight training, 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 wind tunnel.
- 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.

La estación, la suplementación con antocianinas y el entrenamiento de vuelo tienen efectos mixtos sobre el sistema antioxidante de los individuos migratorios de *Sturnus vulgaris*

RESUMEN

Las aves migratorias realizan dos veces al año vuelos de resistencia entre las áreas reproductivas de verano y las áreas no reproductivas de invernada, y estos vuelos de resistencia probablemente ocasionan costos oxidativos.

Estos costos pueden variar entre la migración de otoño y la de primavera, especialmente en las hembras que deben prepararse para criar y poner huevos en la primavera. En este trabajo, estudiamos un ave migratoria con el objetivo de evaluar las hipótesis propuestas sobre cómo los componentes claves del sistema antioxidante de las hembras varían en respuesta el entrenamiento de vuelo en otoño y primavera y a la suplementación de la dieta con antioxidantes. Criamos a mano hembras de *Sturnus vulgaris* y las alimentamos ya sea con una dieta suplementada con antocianinas o una dieta sin antocianinas agregadas. Hicimos volar a las hembras en un túnel de viento por 15 días durante las estaciones migratorias de otoño y primavera, y medimos a lo largo del tiempo el daño oxidativo (d-ROMs) y tres componentes del sistema antioxidante: capacidad antioxidante no enzimática (OXY), ácido úrico y actividad del peróxido de glutatión (GPx). Antes del entrenamiento de vuelo, la capacidad antioxidante no enzimática y el daño oxidativo fueron más bajos en las hembras durante la primavera que en otoño, y las hembras alimentadas con una dieta baja en antioxidantes tuvieron consistentemente ácido úrico circulante más alto. La actividad del GPx disminuyó más en primavera, inmediatamente después de un vuelo de larga duración. Las hembras alimentadas con una dieta alta en antioxidantes tuvieron una mayor disminución en la capacidad antioxidante no enzimática luego del día 15 del entrenamiento de vuelo. Las hembras entrenadas en vuelo tuvieron ácido úrico circulante más alto que las hembras no entrenadas, inmediatamente después del vuelo de más larga duración, y menor actividad del GPx luego del día 15 del entrenamiento de vuelo. En resumen, las hembras regularon los antioxidantes endógenos enzimáticos y no enzimáticos en primavera, y las hembras alimentadas con una dieta con menos antioxidantes parecen compensar aumentando el ácido úrico circulante. Nuestros hallazgos enfatizan el importante rol de los antioxidantes de la dieta para las aves durante la migración, y que vuelos similares en otoño y primavera probablemente representan desafíos oxidativos distintos en la historia de vida de las aves hembras.

Palabras clave: antioxidantes dietarios, antocianina, daño oxidativo, entrenamiento de vuelo, migración de otoño y primavera de la hembra

INTRODUCTION

The antioxidant system of vertebrates is multifaceted, consisting of exogenous and endogenous as well as enzymatic and nonenzymatic 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 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 with 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 vs. sedentary birds (Skrip et al. 2016). Eikenaar et al. (2017) also demonstrated that migratory Common Blackbirds (*Turdus merula*) had higher nonenzymatic antioxidant capacity (OXY) 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 30× 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 the 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 migrations 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 tradeoff or carryover 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 that 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 2 weeks, and season affected 2 measures of nonenzymatic 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 nonenzymatic 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 to 2 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 with fall migration, and, therefore, oxidative damage is lower during spring migration. This hypothesis was evaluated in birds prior to 2 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 diets. This hypothesis was also evaluated in birds prior to 2 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 2 weeks of flying.

METHODS

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 wind tunnel as demonstrated by many recent studies (Carter et al. 2020, Casagrande et al. 2020, McWilliams et al. 2020).

Female European Starling chicks (n = 115) were collected from nest boxes located in Upper Bavaria, South Germany (47.9667°N, 11.2167°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 × 4 × 2 m) 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).

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[a]	1.37	3.19
Glucose[b]	16.84	39.19
Casein[c]	8.21	19.12
Cellulose[d]	2.14	4.97
Canola oil[e]	5.18	12.05
Sunflower oil[f]	3.03	7.06
Ground mealworms	2.65	6.16
Elderberry powder[g]	0.18	0.42
Salt mixture[h]	2.05	4.78
Amino acid mix[i]	1.15	2.68
Vitamin mix[j]	0.16	0.38
Water	57.04	

[a] Agar, Ombilab-laborenzentrum, GmbH, Bremen, Germany.
[b] Glucose, VWR International GmbH, Darmstadt, Germany.
[c] Casein, Affymetrix UK Ltd., High Wycombe, UK.
[d] Alphacel, MP Biomedicals, Solon, Ohio, USA.
[e] Canola oil, Jedwards International, Inc., Braintree, Massachusetts, USA.
[f] Sunflower oil, Jedwards International, Inc., Braintree, Massachusetts, USA.
[g] Standardized Elderberry extract powder, Artemis International, Inc., Fort Wayne, Indiana, USA; contains 6.5% anthocyanins; amount added was 0.04% wet mass from September 21 to October 5, then 0.18 % thereafter.
[h] Brigg's salt mix, MP Biomedicals, Solon, Ohio, USA.
[i] Amino acid mix, Sigma-Aldrich, St. Louis, Missouri, USA.
[j] AIN vitamin mix, MP Biomedicals, Solon, Ohio, USA. Includes vitamin E.

Experimental Design, Experimental Diets, and Flight Training

During August 2016, female starlings were allocated to 2 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 September 21, 2016, we randomly assigned all hand-raised starlings to be fed 1 of the 2 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, Indiana, 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^{-1} (or ~17 berries day^{-1}); the low-antioxidant diet had no anthocyanin added. Each diet had identical macronutrient content (19% fat, 6% protein, and 63% carbohydrates; 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 4 to 6 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 1 of the 8 cohorts (4 high-antioxidant cohorts and 4 low-antioxidant cohorts), whereas reproductive females were assigned to 1 of the 4 cohorts (2 high-antioxidant cohorts and 2 low-antioxidant cohorts). Trained and untrained reproductive females were distributed evenly across all 4 cohorts.

Every 3 to 6 days starting on September 26, 2016, a new cohort was taken from their outdoor aviary and moved to indoor cages (1.5 × 2.5 × 2.5 m) in the wind tunnel facility. This allowed each cohort to be housed together and habituated over 4 days to fly directly into the wind tunnel from their indoor cage without being handled and to acclimate to the wind tunnel conditions. This 4-day pretraining regime was followed by a 15-day flight training regime. Such wind tunnel 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^{-1} wind speed, 15°C ambient temperature, and 75% humidity. The duration of flight increased over the course of the regimen: 20–30 min on days 1–4, 60 min on days 5 and 7, 30 min on day 6, 90 min on day 8, rest on day 9, 2–3 hr on days 10 and 11, rest on day 12, 60 min on day 13, 30 min on day 14, and then up to 6 hr of voluntary flight on day 15 (average flight time ~3 hr). 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.

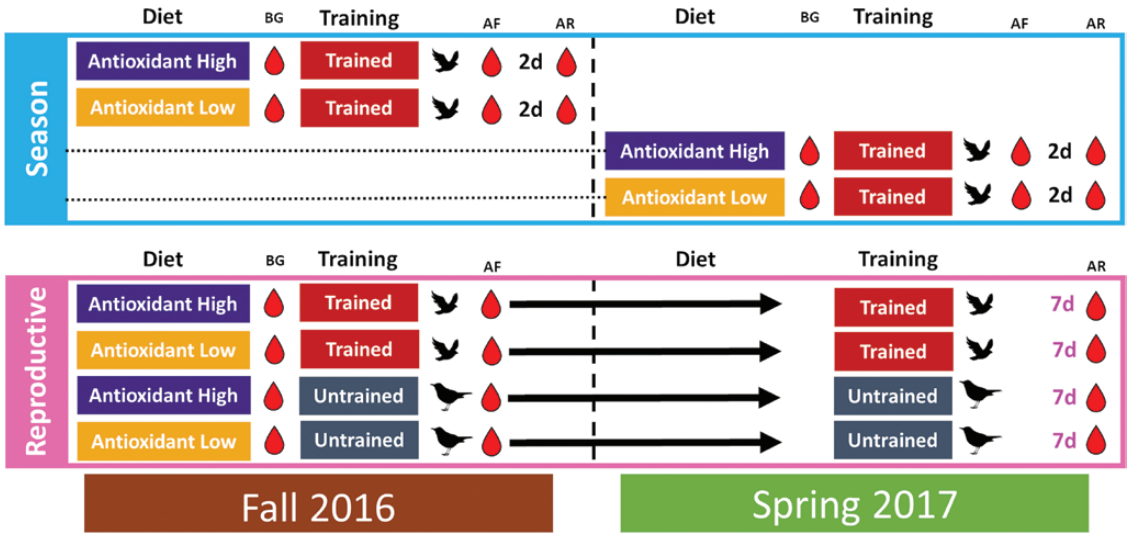

FIGURE 1. Experimental groups used to test hypotheses about the effect of dietary antioxidants, flight training, and season (fall and spring) on key components of the antioxidant system in female migratory European Starlings. Season females (top panel) were flown during either fall 2016 or spring 2017 and then sacrificed at the end of their 15-day flight training plus 2 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 [~1,040 µL]; AF, after-flight sample taken immediately after longest flight on day 15 of flight training [~1,120 µL]; AR, after-recovery blood sample taken 2 days after the longest flight [~1,040 µL]). 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.

Blood Sampling and Antioxidant Analyses

Blood was sampled the day each cohort was established in the wind tunnel aviaries to obtain a background blood sample ("BG", Figure 1). Blood was then drawn immediately after the 6-hr day 15 flight ("AF", Figure 1) and after a 2-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. Other studies in both wild and captive birds have established that the components of the antioxidant system that we measured recover quickly after exercise or during stopover (within ~24 hr); therefore, we assumed that 2 days would be ample time for the antioxidant system to recover in our trained birds (Skrip et al. 2015, Cooper-Mullin et al. 2019, McWilliams et al. 2020). 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 5 min 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 OXY, 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, Vermont, USA). The d-ROMs and OXY-adsorbent assays were conducted at the MPIO using plasma, whereas GPx and uric acid were assayed at the University of Rhode Island (Kingston, Rhode Island, 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 OXY. Uric acid is a nonenzymatic antioxidant that is not measured by the OXY-adsorbent test and so was, therefore, measured separately in plasma using a colorimetric assay (Teco Diagnostics, Anaheim, California, 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, Michigan,

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 the final analyses. Outliers were identified using Tukey's method and removed from the dataset. The final sample size for each analysis represents the number of remaining individuals after outliers and samples with coefficient of variation above 15% were removed.

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 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 (Supplementary Material 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) (Supplementary Material 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 (Supplementary Material 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 (AIC). 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 OXY prior to flight training. All results reported are from type III analysis of variance (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 (ie. 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 AIC (Supplementary Material Table S1).

Hypothesis 1: Season Effect

Some components of the antioxidant system are upregulated more in spring compared with fall, reducing oxidative damage during spring.

Prior to flight-training. Background levels (prior to any flight training) of circulating OXY were significantly higher in fall (n = 21) compared with spring (n = 29; $F_{3,46}$ = 9.73, P = 0.003; Figure 2A). 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 with 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 ($F_{2,18}$ = 6.52, P = 0.02; no diet effect: $F_{2,18}$ = 0.04, P = 0.85; Figure 3A) but not in the spring ($F_{2,26}$ = 1.37, P = 0.25; no diet effect: $F_{2,26}$ = 0.05, P = 0.83; Figure 3B). In contrast, GPx activity significantly decreased with date in both the fall ($F_{2,16}$ = 5.13, P = 0.04; no diet effect: $F_{2,16}$ = 0.39, P = 0.54; Figure 3C) and the spring ($F_{2,24}$ = 5.54, P = 0.03; no diet effect: $F_{2,24}$ = 0.60, P = 0.45; Figure 3D).

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,

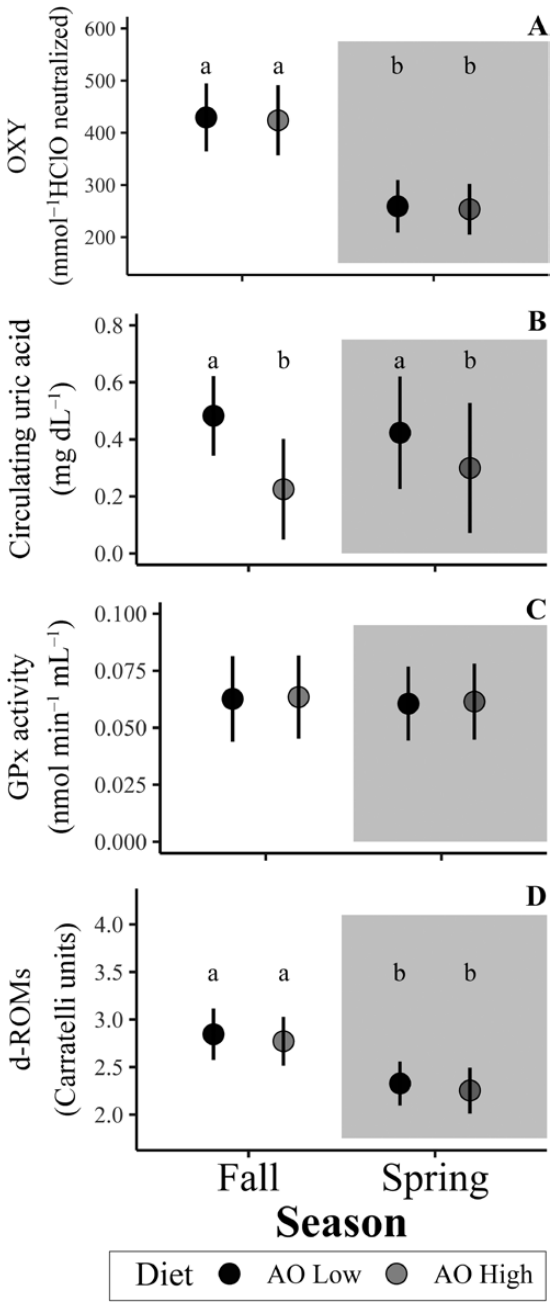

FIGURE 2. Least square means and 95% confidence intervals of antioxidant measures prior to flight training. (A) Background OXY in plasma of females differed between seasons but not diets. (B) Background circulating uric acid in plasma of females differed between diets but not seasons. (C) Background enzymatic activity (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).

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).

Immediately after long-duration flight. There was a significant diet × season interaction for circulating OXY, such that individuals fed the low-antioxidant diet had slightly decreased circulating OXY immediately after the final fall flight (n = 8) and increased 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 × 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 greater in spring (n = 14) compared with 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).

Hypothesis 2: Diet Effect

Females fed fewer dietary antioxidants upregulate some components of the antioxidant system and so prevent oxidative damage.

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- and 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- and 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- and n = 19 low-antioxidant diet; $F_{5,27}$ = 0.22, P = 0.64), uric acid (n = 14 high- and n = 16 low-antioxidant diet; $F_{2,27}$ = 0.30, P = 0.59), and GPx activity (n = 11 high- and 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- and n = 19 low-antioxidant diet; $F_{4,27}$ = 0.02, P = 0.90).

After 15-day flight training regime. For females flown either in fall or in spring (Figure 1, season birds), there was no significant difference between background and post-training levels of OXY for either diet (n = 16 high- and n = 19 low-antioxidant diet; $F_{4,30}$ = 2.59, P = 0.12). Circulating uric acid (n = 7 high- and n = 11 low-antioxidant diet; $F_{2,15}$ = 0.98, P = 0.34) and GPx activity (n = 13 high- and 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

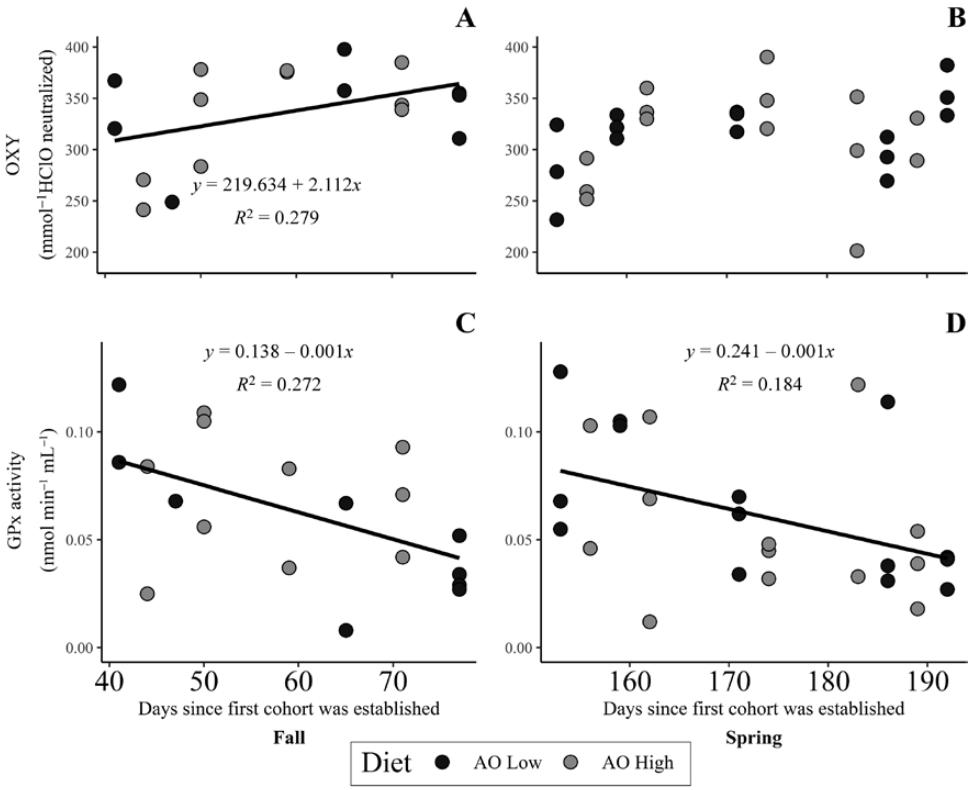

FIGURE 3. Antioxidant measures through time in females prior to flight training. OXY significantly increased with date in the fall (A) for females fed either diet but not in the spring (B). Background enzymatic activity (GPx) in red blood cells significantly decreased with date in the fall (C) and spring (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.

did not differ between diets (n = 18 high- and 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 with 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- and 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- and n = 18 low-antioxidant diet; Figure 5D; $F_{2,25}$ = 0.1.92, P = 0.18).

Immediately after long-duration flight. There was a significant diet * 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- and n = 13 low-antioxidant diet; Figure 4B; $F_{3,20}$ = 0.23, P = 0.64) and GPx activity (n = 12 high- and 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- and 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- and n = 17 low-antioxidant diet; Figure 5E; $F_{2,27}$ = 1.03, P = 0.32). Trained birds (n = 13) had significantly higher levels of circulating uric acid immediately after their flight compared with 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- and n = 13 low-antioxidant diet; Figure 5F, $F_{2,24}$ = 0.63, P = 0.43). GPx activity did not differ between

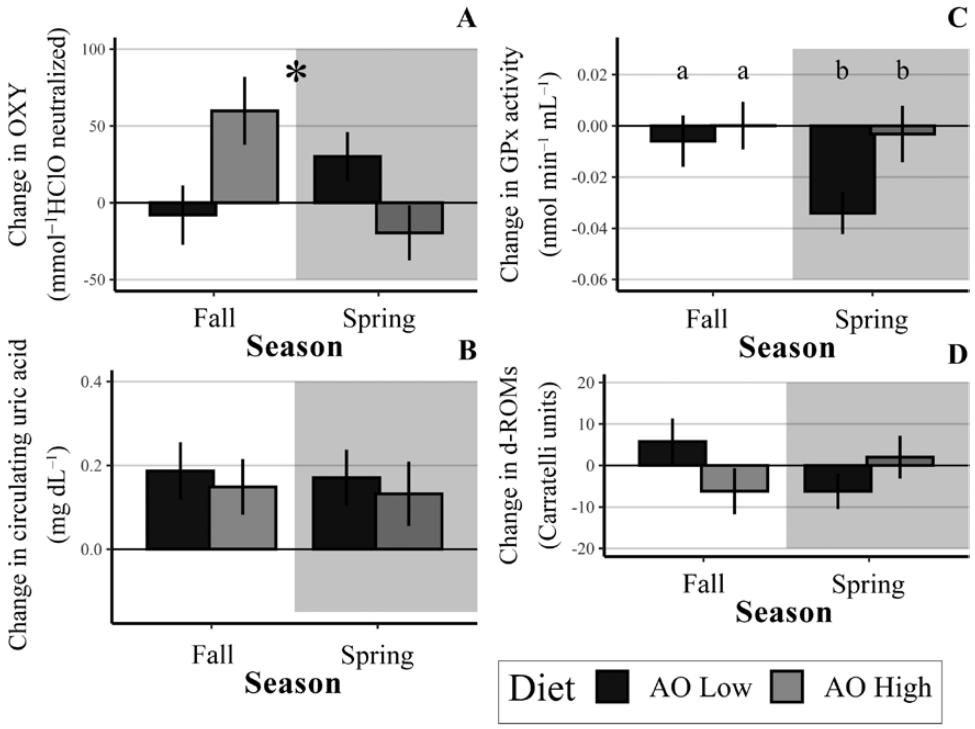

FIGURE 4. Least square means (± SE) of acute changes in antioxidant response of females immediately after a long-duration flight (AR timepoint – AF timepoint; see Figure 1). (A) Acute change in OXY in plasma of females decreased between fall and spring for birds fed the high-antioxidant diet, whereas birds fed the low-antioxidant diet had lower OXY in fall compared with spring (Diet * Season interaction denoted by asterisk [*]; 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 (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).

trained (n = 16) and untrained birds (n = 14; Figure 5G; $F_{2,27}$ = 0.08, P = 0.77) or diets (n = 13 high- and 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- and n = 17 low-antioxidant diet; Figure 5H; $F_{3,24}$ = 2.47, P = 0.13).

DISCUSSION

Seasonal Changes Prior to Flight Training

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 with 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 lipid 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, nonenzymatic oxidative capacity was lower in spring compared with 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 OXY that may indicate a coordinated and compensatory antioxidant

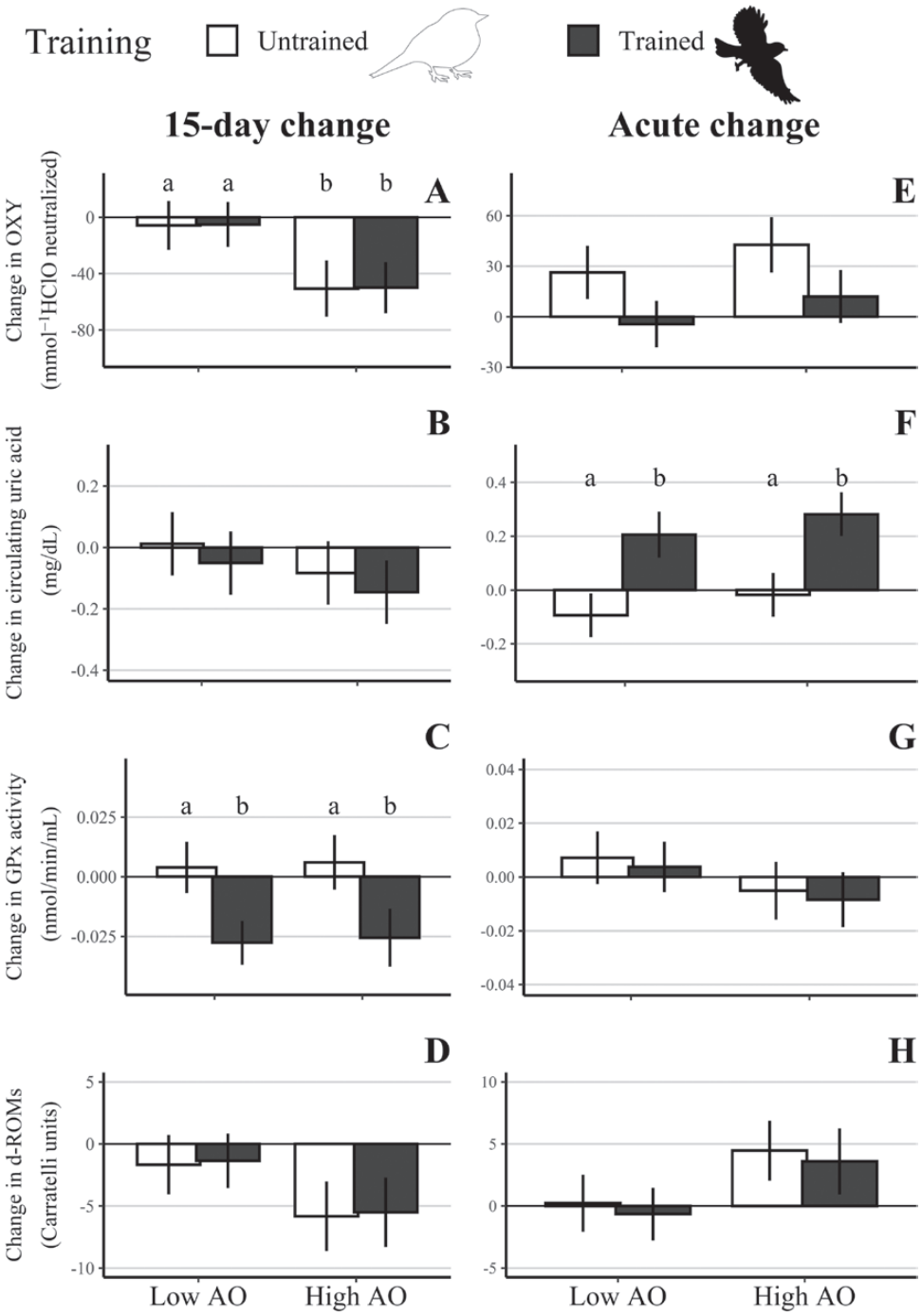

FIGURE 5. Least square means (± SE) of changes in antioxidant response in trained vs. untrained females (A–D) after a 15-day flight training regime in the fall (AR timepoint – BG timepoint; see Figure 1, reproductive females only) and (E–H) immediately after the trained females' longest-duration flight in the fall (AR timepoint – AF timepoint; see Figure 1, reproductive females only). (A) Change in 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 (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 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 higher in flight-trained vs. untrained females fed both diets. (G) Acute change in enzymatic activity (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).

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 6 weeks of flight training. As fall and spring progress, females may switch to relying on nonenzymatic 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 nonenzymatic 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

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 OXY (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 with control birds.

We predicted that antioxidant supplementation would increase OXY, 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 OXY did not differ between diets that were supplemented with antioxidants. As OXY measures both dietary antioxidants and nonenzymatic 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

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,D). The acute decrease in GPx activity over the longest flight was significantly greater in spring than fall, indicating that GPx was utilized more during the spring flight (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 nonenzymatic 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

Contrary to hypothesis 2b, the 15-day flight training produced no consistent changes in total OXY, 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 OXY during the 15-day flight training period regardless of if they were trained or untrained, whereas females on the low-antioxidant diet maintained OXY over the course of training (Figure 5A). These results indicate that females on the high-antioxidant diet utilized nonenzymatic 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). DeMoranville et al. (2020) similarly found that flight-trained birds had lower enzymatic antioxidant activity than untrained birds in liver and pectoralis tissue, though the genetic expression of GPx, catalase (CAT), and superoxide dismutase (SOD) was upregulated in these organs. 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 Long-Duration Flight

Diet affected enzymatic components of the antioxidant system during the longest-duration flight. GPx activity declined after flight more for females during spring, and there was a trend for females fed the low-antioxidant diet to have a greater net change in GPx activity 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 decrease in GPx activity immediately after flight, 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 and 5F). However, we did observe a substantial increase in uric acid in response to the longest-duration flight in flight-trained vs. 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 with 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 OXY, ferric-reducing ability of plasma and Trolox equivalent antioxidant capacity, 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 2 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 2 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 decreased enzymatic antioxidant activity immediately after the longest-duration flight (Figure 4C) in spring compared with fall, possibly to reduce the potential negative carryover 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) and had a greater decrease in enzymatic antioxidant activity immediately after the longest-duration flight compared with females fed a high-antioxidant diet (Figure 4C). Contrary to hypothesis 2, baseline nonenzymatic 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.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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

Data depository: Analyses reported in this article can be reproduced using the data provided by Frawley et al. (2021).

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