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RESEARCH ARTICLE

Flight training and dietary antioxidants have mixed effects on the oxidative status of multiple tissues in a female migratory songbird

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ABSTRACT

Birds, like other vertebrates, rely on a robust antioxidant system to protect themselves against oxidative imbalance caused by energy-intensive activities such as flying. Such oxidative challenges may be especially acute for females during spring migration, as they must pay the oxidative costs of flight while preparing for reproduction; however, little previous work has examined how the antioxidant system of female spring migrants responds to dietary antioxidants and the oxidative challenges of regular flying. We fed two diets to female European starlings, one supplemented with a dietary antioxidant and one without, and then flew them daily in a windtunnel for 2 weeks during the autumn and spring migration periods. We measured the activity of enzymatic antioxidants (glutathione peroxidase, superoxide dismutase and catalase), non-enzymatic antioxidant capacity (ORAC) and markers of oxidative damage (protein carbonyls and lipid hydroperoxides) in four tissues: pectoralis, leg muscle, liver and heart. Dietary antioxidants affected enzymatic antioxidant activity and lipid damage in the heart, non-enzymatic antioxidant capacity in the pectoralis, and protein damage in leg muscle. In general, birds not fed the antioxidant supplement appeared to incur increased oxidative damage while upregulating non-enzymatic and enzymatic antioxidant activity, though these effects were strongly tissue specific. We also found trends for diet×training interactions for enzymatic antioxidant activity in the heart and leg muscle. Flight training may condition the antioxidant system of females to dynamically respond to oxidative challenges, and females during spring migration may shift antioxidant allocation to reduce oxidative damage.

KEY WORDS: Anthocyanin, Spring migration, Enzymatic antioxidants, Non-enzymatic antioxidants, Oxidative damage

INTRODUCTION

Energy-intensive activities, such as endurance exercise, increase metabolic rate and the production of reactive species (Bailey et al., 2007, 2015). Reactive species can scavenge electrons from biologically important molecules such as proteins, lipids and DNA (Nikolaidis et al., 2012; Powers and Jackson, 2008), and can cause oxidative damage that negatively impacts an animal's fitness (Jenni-Eiermann et al., 2014; Smarsh and Williams, 2016; Sun et al., 2010). As a consequence of these oxidative costs of intense activity, vertebrates have evolved a robust antioxidant system 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). Vertebrates may also consume dietary antioxidants to supplement the endogenous antioxidant system during exercise (Beaulieu and Schaefer, 2014). Metabolic demands may differ across tissues that are more or less involved in energy-intensive activities, therefore incurring different oxidative costs across tissues. However, no previous studies have compared the extent to which key components of the antioxidant system of wild vertebrates respond to exercise and access to dietary antioxidants across multiple tissues.

Some of the most remarkable endurance athletes on Earth include migratory birds, which engage in endurance exercise in the form of seasonal flights between wintering and breeding grounds. During flight, birds may operate at 6–8 times their basal metabolic rate (Lindström et al., 1999; Schmidt-Wellenburg et al., 2007), which likely increases production of reactive species (Costantini, 2014; Jenni-Eiermann et al., 2014). Migratory birds rely primarily on fat stores to fuel migration, and reactive species can cause cascading degradation of fatty acids (Guglielmo, 2018; Jenni-Eiermann et al., 2002; McWilliams et al., 2004). 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 energetic challenges. European robins (*Erithacus rubecula*) caught during migratory flight had higher circulating enzymatic antioxidant activity than resting birds (Jenni-Eiermann et al., 2014). Similarly, flight-trained zebra finches (*Taeniopygia guttata*) had elevated enzymatic antioxidant activity immediately after 1 h of flight (Cooper-Mullin et al., 2019). Dietary antioxidants also play an important role in supporting the endogenous antioxidant system and quenching reactive species. Dietary antioxidant supplementation has been demonstrated to improve flight performance in zebra finches (Blount and Matheson, 2006) and budgerigars (*Melopsittacus undulates*: Arnold et al., 2010), and songbirds during autumn migration preferentially consume high-antioxidant fruits (Beaulieu and Schaefer, 2014; Bolser et al., 2013). However, it remains to be determined how exogenous and endogenous components of the antioxidant system interact to protect against reactive species and oxidative damage associated with flight. Furthermore, how multiple components of the antioxidant system flexibly interact to respond to seasonal changes in activity, competing

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Received 7 July 2021; Accepted 6 October 2021

energy and nutrient demands, and the availability of dietary antioxidants is inadequately understood.

The functional importance of components of the vertebrate antioxidant system may change over the course of seasons as individuals must satisfy the energy demands of events such as migration and reproduction while managing potential trade-offs between themselves and their offspring (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 as they must pay the oxidative costs of migratory flights while also preparing for egg production and laying. Females with a more robust antioxidant system or access to dietary antioxidants may be able to avoid oxidative damage associated with repeated flights and subsequently allocate more antioxidants to eggs (Royle et al., 2003; Skrip et al., 2016). Previous work has shown that investing antioxidants into eggs improves hatching and fledging success, enhances immune function and improves growth rates (Berthouly et al., 2007, 2008; Marri and Richner, 2014; Norte et al., 2010; Saino et al., 2003). However, less is known about the trade-offs females must make between their own antioxidant protection and provisioning antioxidants for eggs, and the ways in which oxidative damage from spring migration can carry over into the reproductive period. Previous work in zebra finches has shown that exercised females deposit lower amounts of lipid-soluble antioxidants into eggs than females that were not exercised, suggesting that flight had a carryover effect on reproduction (Skrip et al., 2016).

To date, most studies that have examined the avian antioxidant system have done so using markers in plasma and red blood cells, which allows for repeated measures over time (i.e. Cooper-Mullin et al., 2019; Costantini et al., 2008; Larcombe et al., 2008). Fewer studies have examined tissue-level differences in the antioxidant response in birds, and those that have tend to examine one or two organs (DeMoranville et al., 2020; Dick and Guglielmo, 2019; Møller et al., 2010). Key organs involved in migration – pectoralis, heart and liver – have different capacities to store lipid-soluble antioxidants and produce differing ratios of antioxidant enzymes (Cooper-Mullin and McWilliams, 2016). Furthermore, skeletal muscles involved in exercise produce uric acid, an endogenous antioxidant, as a result of protein catabolism (Guglielmo et al., 2001; Tsahar et al., 2006), and flight muscles likely produce more reactive species than muscles that are not involved in flight (i.e. leg muscles). Examining a suite of organs is vital to understanding the differing roles of components of the antioxidant system, and how different systems respond to accumulating oxidative damage.

The objective of our study was to determine how the availability of dietary water-soluble antioxidants, 2 weeks of daily flying during the spring migration period, and exposure to breeding cues 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 oxidative damage to protein and lipid molecules in four tissues (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 1 – 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 with birds that consume lower amounts of dietary antioxidants. Furthermore, if daily flying poses an additional oxidative challenge and females prioritize investing antioxidants into eggs during the spring migration period (hypothesis 2 – diet×training interaction effect), then flight-trained birds fed lower amounts of dietary antioxidants will have substantially lower non-enzymatic antioxidant capacity and increased enzymatic antioxidant activity and oxidative damage compared with 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 the most comprehensive studies to date detailing how dietary antioxidants and exercise affect key components of the antioxidant system in a suite of organs in a migratory songbird.

MATERIALS AND METHODS

Starling husbandry

Migratory European starlings (*Sturnus vulgaris* Linnaeus 1758) from southern Germany were used for this study. This population consists of medium-distance diurnal 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. Starlings, like many songbirds, complete their migration from wintering to breeding grounds over many days of flying and stopover (Feare, 1984; Newton, 2006). Male starlings usually arrive at breeding areas before females, and pair formation occurs quickly after the arrival of females and typically precedes egg laying by less than 1 week (Eens et al., 1994; Gwinner, 1997). European starlings are social and curious, and numerous recent studies have demonstrated that they can quickly learn to fly in small groups in a windtunnel (Carter et al., 2020; Casagrande et al., 2020; Nebel et al., 2012; McWilliams et al., 2020).

Female European starling chicks ca. 1 week old (n=92) were collected from a colony located in Upper Bavaria, South Germany (47°58′N, 11°13′E) during April and May in 2015 (n=63) and 2016 (n=29). 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 (3×4×2 m). Fledglings 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). 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).

Experimental groups and antioxidant diets

During August 2016, females were separated into two overarching groups (Fig. 1). ‘Season’ females (n=55) were flight-trained in either autumn 2016 or spring 2017 and killed by decapitation at the end of their 15 day flight training regime to assess the seasonal differences (autumn versus spring). ‘Reproductive’ females (n=30) included two subgroups, one that underwent the 15 day flight training each season (‘trained’, n=16) and a sedentary control subgroup that did not undergo the flight training regime each season (‘untrained’, n=14), so that we could assess the direct effect of flight training (trained versus untrained). After this 15 day training or sedentary period in spring, Reproductive females were transferred to

Journal of Experimental Biology (2021) 224, jeb243158. doi:10.1242/jeb.243158

Table 1. Composition of semi-synthetic high-antioxidant diet fed to female European starlings during autumn 2016 and spring 2017

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	

Half of the females (n=43) were fed the high-antioxidant diet with added elderberry; the remaining females (n=43) were fed a low-antioxidant diet with the exact same composition excluding elderberry.
[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.; [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 September to 5 October, then 0.18% thereafter); [8]Brigg's salt mix, MP Biomedicals; [9]Amino acid mix, Sigma-Aldrich, St Louis, MO, USA; [10]AIN vitamin mix, MP Biomedicals (includes vitamin E).

outdoor breeding aviaries that contained breeding cues and male starlings (see details in 'Organ collection and antioxidant analyses', below) and killed after 6 days in these breeding aviaries. Unfortunately, samples from the Season groups from early autumn 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 tissue 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 autumn and spring is provided in Frawley et al. (2021a). Females in the autumn Season group were hand-raised in 2016, whereas females in the spring Season and the Reproductive groups were hand-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).

Starting on 21 September 2016, all hand-raised starlings were randomly assigned to one of two semi-synthetic, high-polyunsaturated fat diet groups (Table 1, Fig. 1). Each diet had identical macronutrient content (19% fat, 6% protein, 63% carbohydrates) that simulates a high-lipid fruit diet (Johnson et al., 1985; Smith et al., 2007), 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 (AO high) had 119 mg kg^{-1} (wet mass) added anthocyanin (Standardized Elderberry 6.5% Powder, Artemis International, Inc., Fort Wayne, IN, USA), whereas the low-antioxidant diet (AO low) 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 primary molt score (Jenni and Winkler, 2020) of each bird and assigned individuals to cohorts of 4–6 females each based on most to least advanced molt. Each cohort was 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 autumn 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 indoor aviaries (1.5×2.5×2.5 m) in the windtunnel facility every 3–6 days. The cohorts from the Reproductive group consisted of 3–4 flight-trained females and 1–2 untrained females from the same diet group. While in the windtunnel facility, females were exposed to a natural photoperiod as a result of the presence of large windows and additional supplemental lights (Osram LUMILUX T8 58 W/865). Over a 4 day pre-training regime, each cohort in turn was habituated to fly directly into the windtunnel from their indoor cage without being handled and was 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 m s^{-1}

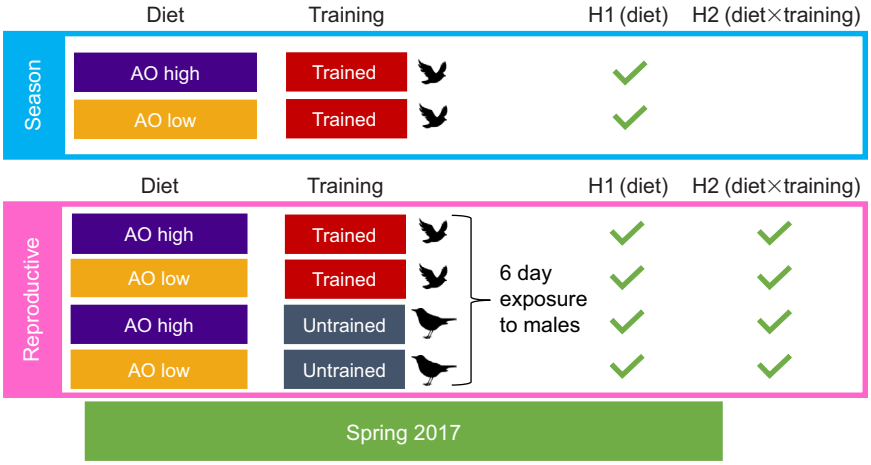

Fig. 1. Experimental groups used to test hypotheses about the effect of dietary antioxidants (hypothesis 1) and diet×flight training (hypothesis 2) on key components of the antioxidant system in female European starlings. 'Season' females (top, n=55) were flown during spring 2017 and data were obtained at the end of their 15 day flight training plus 2 days of 'recovery' from the last flight. 'Reproductive' females (bottom, n=30) were flown in both autumn 2016 and spring 2017 and data were obtained in the spring after a 6 day exposure to male starlings as part of a companion study (Carbeck et al., 2018). Bird icons represent whether the bird was trained (flying icon, n=16) or untrained (standing icon, n=14). Green ticks indicate which group was used to test each hypothesis (hypothesis 1: diet hypothesis; or hypothesis 2: diet×training hypothesis). AO high, high-antioxidant diet; AO low, low-antioxidant diet.

wind speed, 15°C ambient temperature and 75% humidity. The duration of flight increased over the course of flight training: 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 h on days 10 and 11, rest on day 12, 60 min on day 13, 30 min on day 14, and up to 6 h 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., 2020; 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. Thus, over the 14 day training schedule in the windtunnel, excluding the longest flight on day 15, the flight-trained group was exposed to an additional 600–700 km of continuous flapping flight at 15 m s^{-1} compared with the untrained birds. Some birds were dropped from flight training because of poor performance (i.e. frequent landing or refusal to fly), 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 2 days, after which they were euthanized by decapitation and tissues were sampled as per Reproductive females (see below). This 2 day recovery period ensured that any differences in antioxidant enzyme activity, non-enzymatic antioxidant capacity and oxidative damage to protein and lipid molecules in females could be attributed to the flight training and were not confounded by the acute effect(s) of the longest flight. Skrip et al. (2015) and Eikenaar et al. (2020) found that garden warblers and northern wheatears, respectively, were able to recover from oxidative imbalance within 2–3 days during stopover. Flight-trained and untrained females from the Reproductive group were transferred to outdoor breeding aviaries that contained male starlings and breeding cues (i.e. nest boxes, nest materials and herbs) in order to stimulate reproductive readiness (Carbeck et al., 2018). Females were killed after 6 days in these breeding aviaries, and the liver, pectoralis, gastrocnemius and heart were immediately dissected and weighed. During dissection, ovaries were visually inspected to determine reproductive status. Both Season and Reproductive females were observed to have visible follicles, and Reproductive females had larger and more developed follicles than Season females. All tissues (n=272 tissue samples, 4 per individual) were placed on dry ice and stored at −80°C within 2 h of dissection 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 autumn 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 (autumn: 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 per individual). Occasionally, a few tissue samples did not produce usable results, so we report the exact sample sizes for each tissue and treatment comparison (see Results).

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. All tissues were initially homogenized in chilled 0.1 mol l^{-1} phosphate buffer. For the homogenate aliquots used in the enzymatic antioxidant and protein damage assays, 2.8 mmol l^{-1} EDTA was added as a preservative, resulting in a final concentration of 0.5 mol l^{-1} phosphate buffer and 0.1 mmol l^{-1} EDTA. The homogenate aliquots used in the lipid damage and oxygen radical absorbance capacity (ORAC) assays did not have added EDTA, resulting in a final concentration of 0.5 mol l^{-1} 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 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 peroxyl and hydroxyl radicals using the fluorescent algal pigment phycoerythrin and serves as a measure of total non-enzymatic antioxidant capacity. Commercially available microplate assay kits were used to determine circulating enzymatic antioxidant activity, lipid damage and protein damage. The activity of enzymatic antioxidants glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) was measured using colorimetric assays from Cayman Chemical Company (Ann Arbor, MI, USA). Oxidative damage was measured using the lipid hydroperoxide (LPO) and protein carbonyl assays from Cayman Chemical Company. The LPO 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 (diet×training interaction effect) using only the Reproductive females (Fig. 1). The loss of most of the autumn Season group samples as a consequence of thawing (see above) restricted our testing of diet effects (hypothesis 1) to females during only spring, although the results from the small number of samples (n=4 individuals per diet) from the autumn Season group are provided in Table S1 for comparison. Furthermore, we tested hypothesis 1 (diet effect) separately for Season and Reproductive females in spring given that only the latter group were exposed to breeding cues (i.e. nest boxes, nest materials and herbs) and male starlings. All analyses were performed in R version 4.0.3 (http://www.R-project.org/). Each measure was log-transformed prior to analysis to reduce the skew of measurements and thus help to meet the assumptions of the inferential statistics. Linear mixed models were selected for analysis based on their ability to account for repeated measurements from the same individual (Detry and Ma, 2016). For hypothesis 1, linear mixed models (lmer package, https://cran.r-project.org/package=lme4) were fitted to infer the effects of diet (high or low antioxidant) on enzymatic antioxidant activity (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC) and oxidative damage (protein carbonyls and LPO). An organ×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 death were evaluated as covariates. For hypothesis 2,

linear mixed models (lmer package, https://cran.r-project.org/package=lme4) were fitted to infer the interactions and effects of diet (high or low antioxidant) and training (trained or untrained) on enzymatic antioxidant activity (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC) and oxidative damage (protein carbonyls and LPO). A three-way organ×diet×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 death were evaluated as covariates. Total flight training time was not assessed as a covariate in this model because of the inclusion of the untrained birds. Welch's *t*-tests were used to determine whether 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 (https://cran.r-project.org/package=emmeans) was used to estimate least-squares means and assess contrasts for the final models. A Bonferroni correction was applied for measures of enzymatic antioxidants to account for repeated measures, such that we considered $P \leq 0.017$ to be significant for enzymatic antioxidants, and $P<0.05$ to be significant for all other measures.

RESULTS

We report the main effects of diet (hypothesis 1) separately for Season and Reproductive females because only the latter were exposed to breeding cues and male starlings during spring. We report the diet×training interaction (hypothesis 2) 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 removed these as covariates when they were uninformative and not strongly preferred by Akaike's information criterion.

Hypothesis 1 – dietary antioxidant effect in Season females during spring

Antioxidant enzymes in flight-trained Season females

For Season females, GPx activity was significantly higher in heart tissue (n=14 AO high, n=17 AO low; Fig. 2D; $F_{8,136}$=7.00, P=0.010) for females fed the AO low diet. GPx activity did not differ between diets for the pectoralis (n=16 AO high, n=21 AO low; Fig. 2A; $F_{8,136}$=0.35, P=0.557), leg muscle (n=16 AO high, n=21 AO low; Fig. 2B; $F_{8,136}$=0.05, P=0.816) or liver (n=16 AO high, n=21 AO low; Fig. 2C; $F_{8,136}$=2.56, P=0.113). CAT activity did not differ between diets in the pectoralis (n=15 AO high, n=21 AO low; Fig. 2E; $F_{8,130}$=0.88, P=0.352), leg muscle (n=16 AO high, n=20 AO low; Fig. 2F; $F_{8,130}$=2.51, P=0.117), liver (n=16 AO high, n=17 AO low; Fig. 2G; $F_{8,130}$=0.26, P=0.613) or heart (n=14 AO high, n=19 AO low; Fig. 2H; $F_{8,130}$=0.46, P=0.502). SOD activity did not differ between diets in the pectoralis (n=16 AO high, n=21 AO low; Fig. 2I; $F_{9,136}$=0.15, P=0.702), leg muscle (n=16 AO high, n=21 AO low; Fig. 2J; $F_{9,136}$=0.47, P=0.497), liver (n=16 AO high, n=21 AO low; Fig. 2K; $F_{9,136}$=0.001, P=0.982) or heart (n=14 AO high, n=20 AO low; Fig. 2L; $F_{9,136}$=0.31, P=0.577).

Non-enzymatic antioxidant capacity in flight-trained Season females

There was a trend in the pectoralis for non-enzymatic antioxidant capacity against peroxyl radicals to be higher for females fed the AO low diet (n=15 AO high, n=20 AO low; Fig. 3A; $F_{8,136}$=2.73, P=0.102). Non-enzymatic antioxidant capacity against peroxyl radicals did not differ between diets for leg muscle (n=16 AO high, n=20 AO low; Fig. 3B; $F_{8,136}$=0.17, P=0.679), liver (n=16 AO high, n=21 AO low; Fig. 3C; $F_{8,136}$=0.28, P=0.600) or heart (n=16 AO high, n=21 AO low; Fig. 3D; $F_{8,136}$=0.74, P=0.393). Non-enzymatic antioxidant capacity against hydroxyl radicals did not differ between diets for the pectoralis (n=15 AO high, n=20 AO low; Fig. 3E; $F_{8,136}$=0.17, P=0.686), leg muscle (n=16 AO high, n=20 AO low; Fig. 3F; $F_{8,136}$=2.14, P=0.147), liver (n=16 AO high, n=21 AO low; Fig. 3G; $F_{8,136}$=0.02, P=0.895) or heart (n=16 AO high, n=21 AO low; Fig. 3F; $F_{8,136}$=0.64, P=0.426).

Damage markers in flight-trained Season females

Females fed the high-antioxidant diet trended toward having higher concentrations of LPOs in heart tissue (n=16 AO high, n=21 AO low; Fig. 4D; $F_{8,121}$=3.67, P=0.059), and had significantly lower concentrations of protein carbonyls in leg muscle (n=14 AO high, n=17 AO low; Fig. 4F; $F_{8,123}$=6.27, P=0.014). LPO did not differ between diets in the pectoralis (n=13 AO high, n=17 AO low; Fig. 4A; $F_{8,121}$=1.38, P=0.243), leg muscle (n=15 AO high, n=15 AO low; Fig. 4B; $F_{8,121} \leq 0.001$, P=0.984) or liver (n=15 AO high, n=20 AO low; Fig. 4C; $F_{8,121}$=0.22, P=0.641). Protein carbonyls were similar between the pectoralis (n=15 AO high, n=18 AO low; Fig. 4E; $F_{8,123}$=0.11, P=0.746), liver (n=14 AO high, n=21 AO low; Fig. 4G; $F_{8,123}$=0.29, P=0.595) and heart (n=15 AO high, n=20 AO low; Fig. 4H; $F_{8,123}$=0.28, P=0.599).

Hypothesis 2 – dietary antioxidant×flight training interaction for Reproduction females during spring

Antioxidant enzymes in trained and untrained Reproductive females

There was a trend in heart tissue for untrained females to have higher GPx activity when fed the AO low diet (trained AO high n=7, AO low n=8; untrained AO high n=7, AO low n=6; Fig. 5D; interaction $F_{9,107}$=3.20, P=0.076), and untrained females had consistently higher GPx than trained females (training effect $F_{9,107}$=13.59, $P \leq 0.001$; diet effect $F_{9,107}$=1.59, P=0.210). There were no significant diet×training interactions for GPx in the pectoralis (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; Fig. 5A; $F_{9,107}$=0.57, P=0.452), leg muscle (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; Fig. 5B; $F_{9,107}$=0.01, P=0.920) or liver (trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=6; Fig. 5C; $F_{9,107}$=0.21, P=0.649), nor any diet or training main effects ($P>0.150$ in all cases). In leg muscle, there was a trend for trained females to have higher CAT activity than untrained females fed the AO low diet, but lower CAT activity than untrained females fed the AO high diet (trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=7; Fig. 5F; interaction $F_{15,95}$=4.45, P=0.037; training effect $F_{15,95}$=2.06, P=0.154; diet effect $F_{15,95}$=1.14, P=0.288). The liver exhibited an inverse trend such that trained females had lower CAT activity than untrained females fed the AO low diet, and higher CAT activity than untrained females fed the AO high diet (trained AO high n=7, AO low n=7; untrained AO high n=6, AO low n=7; Fig. 5G; interaction $F_{15,95}$=3.22, P=0.075; training effect $F_{15,95}$=0.26, P=0.612; diet effect $F_{15,95}$=1.02, P=0.313). There were no significant diet×training interactions for CAT activity in the pectoralis (trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=7; Fig. 5E; $F_{15,95}$=0.30, P=0.587) or the heart (trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=5; Fig. 5H; $F_{15,95}$=0.10, P=0.758), nor any diet or training main effects ($P>0.141$ in all cases). There was no significant diet×training interaction for SOD activity in

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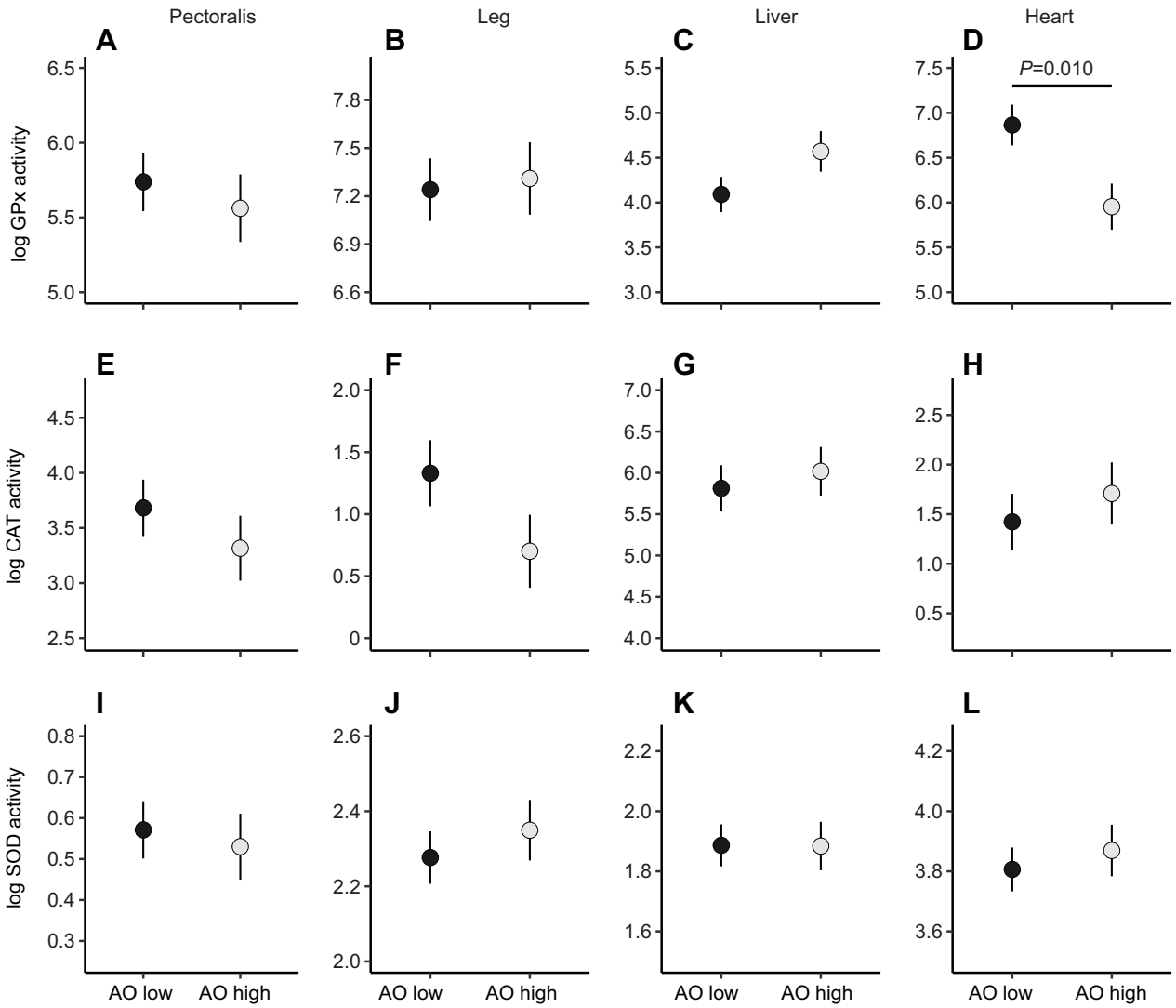

Fig. 2. Least square means (±s.e.m.) of enzymatic antioxidant activity in pectoralis, leg muscle, liver and heart in flight-trained Season females fed the AO high (grey) or AO low (black) diet during spring. Solid bars over groups denote a significant difference. A Bonferroni correction was applied for measures of enzymatic antioxidant activity, such that we considered $P \leq 0.017$ to be significant. Glutathione peroxidase (GPx) activity (nmol min^{-1} ml^{-1}) did not differ between diets for the pectoralis (A, n=16 AO high, n=21 AO low; P=0.557), leg muscle (B, n=16 AO high, n=21 AO low; P=0.816) or liver (C, n=16 AO high, n=21 AO low; P=0.113), but was significantly higher in the heart (D, n=14 AO high, n=17 AO low; P=0.010) for females fed the AO low diet. Catalase (CAT) activity (nmol min^{-1} ml^{-1}) did not differ between diets in the pectoralis (E, n=15 AO high, n=21 AO low; P=0.352), leg muscle (F, n=16 AO high, n=20 AO low; P=0.117), liver (G, n=16 AO high, n=17 AO low; P=0.613) or heart (H, n=14 AO high, n=19 AO low; P=0.502). Superoxide dismutase (SOD) activity (U ml^{-1}) did not differ between diets for the pectoralis (I, n=16 AO high, n=21 AO low; P=0.702), leg muscle (J, n=16 AO high, n=21 AO low; P=0.497) liver (K, n=16 AO high, n=21 AO low; P=0.982) or heart (L, n=14 AO high, n=20 AO low; P=0.577).

the pectoralis (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; Fig. 5I; $F_{15,108}$=0.32, P=0.571), leg muscle (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; Fig. 5J; $F_{15,108}$=0.13, P=0.717), liver (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; Fig. 5K; $F_{15,108}$=0.004, P=0.949) or heart (trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=6; Fig. 5L; $F_{15,108}$=0.002, P=0.964), nor any diet or training main effects (P>0.110 in all cases). There was a trend for a training effect for the liver, such that untrained females had higher SOD activity than trained females (Fig. 5K; $F_{15,108}$=3.17, P=0.077), but no diet effect ($F_{15,108}$=0.17, P=0.678).

Non-enzymatic antioxidant capacity in trained and untrained Reproductive females

There were no diet×training interactions for non-enzymatic antioxidant capacity against peroxyls in the pectoralis (trained AO high n=6, AO low n=8; untrained AO high n=7, AO low n=7; $F_{15,102}$=0.19, P=0.662), leg muscle (trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=7; $F_{15,102}$=0.69, P=0.410), liver (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; $F_{15,102}$=0.08, P=0.773) or heart (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; $F_{15,102}$=0.24, P=0.629), nor any diet or training effects (P>0.145 in all cases). There were no interactions between diet×training regime for non-enzymatic antioxidant capacity against hydroxyls in the pectoralis (trained AO high n=6, AO low n=8; untrained AO high n=7, AO low n=7; $F_{16,101}$=0.04, P=0.843), leg muscle (trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=7; $F_{16,101}$=0.71, P=0.401), liver (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; $F_{16,101}$=0.24, P=0.628) or heart (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; $F_{16,101}$=2.37, P=0.126), nor any diet or training effects (P>0.468 in all cases).

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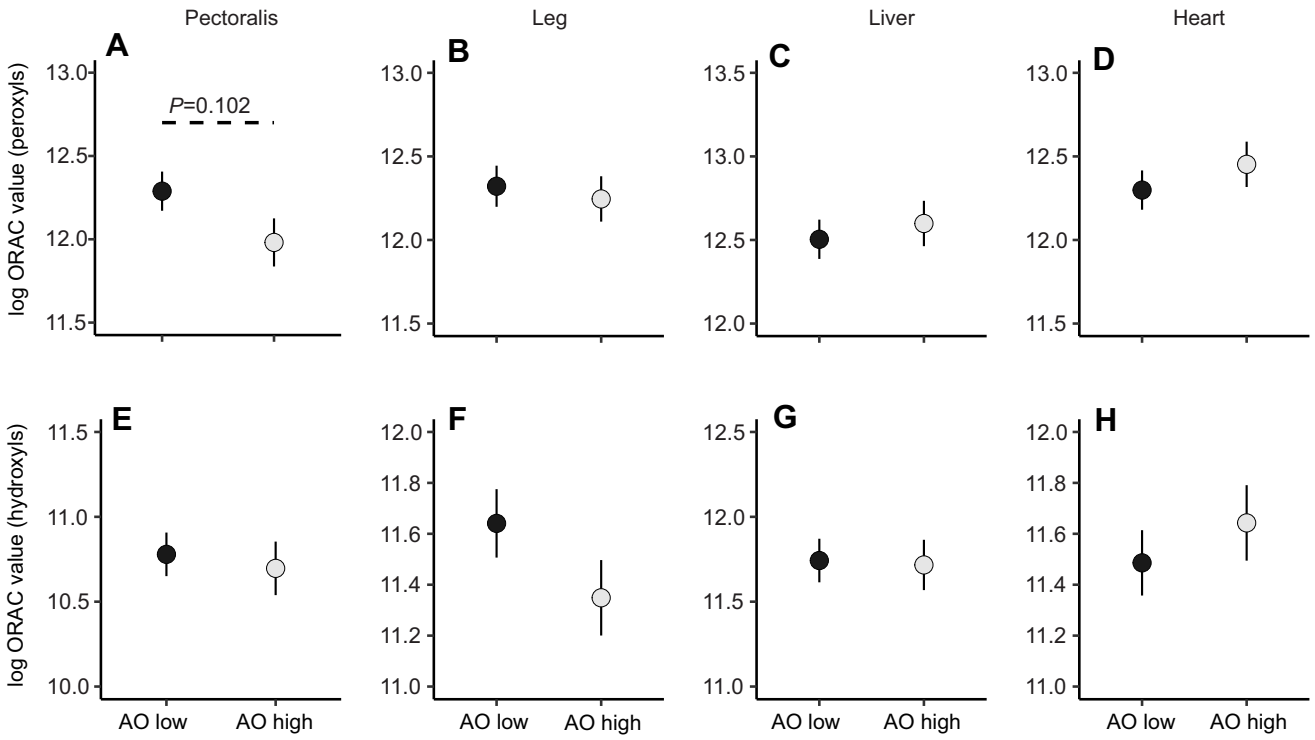

Fig. 3. Least square means (±s.e.m.) of non-enzymatic antioxidant capacity against peroxyl and hydroxyl radicals in pectoralis, leg muscle, liver and heart for flight-trained Season females fed the AO high (grey) or AO low (black) diet during spring. Dashed bars over groups denote a trend. There was a trend in the pectoralis (A, n=15 AO high, n=20 AO low; P=0.102) for non-enzymatic antioxidant capacity (ORAC) against peroxyl radicals to be higher in the pectoralis for birds fed the AO low diet. Non-enzymatic antioxidant capacity against peroxyl radicals did not differ between diets in the leg muscle (B, n=16 AO high, n=20 AO low; P=0.679), liver (C, n=16 AO high, n=21 AO low; P=0.600) or heart (D, n=16 AO high, n=21 AO low; P=0.393). Non-enzymatic antioxidant capacity against hydroxyl radicals did not differ between diets in the pectoralis (E, n=15 AO high, n=20 AO low; P=0.686), leg muscle (F, n=16 AO high, n=20 AO low; P=0.147), liver (G, n=16 AO high, n=21 AO low; P=0.895) or heart (H, n=16 AO high, n=21 AO low; P=0.426).

Damage markers in trained and untrained Reproductive females

There were no interactions between diet×training treatments for LPO concentrations in the pectoralis (trained AO high n=4, AO low n=9; untrained AO high n=7, AO low n=6; $F_{15,90}$=0.62, P=0.433), leg muscle (trained AO high n=7, AO low n=5; untrained AO high n=7, AO low n=4; $F_{15,90}$=0.27, P=0.601), liver (trained AO high n=7, AO low n=8; untrained AO high n=6, AO low n=5; $F_{15,90}$=0.09, P=0.762) or heart (trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; $F_{15,90}$=0.01, P=0.905), nor any diet or training effects (P>0.403 in all cases). There were no significant interactions for protein carbonyl concentrations in the pectoralis (trained AO high n=6, AO low n=8; untrained AO high n=6, AO low n=5; $F_{16,86}$=0.17, P=0.681), leg muscle (trained AO high n=6, AO low n=7; untrained AO high n=6, AO low n=7; $F_{16,86}$=0.45, P=0.502), liver (trained AO high n=5, AO low n=9; untrained AO high n=6, AO low n=7; $F_{16,86}$=0.81, P=0.370) or heart (trained AO high n=6, AO low n=6; untrained AO high n=6, AO low n=6; $F_{16,86}$=0.98, P=0.323), nor any diet or training effects in the pectoralis, leg muscle or liver (P>0.504 in all cases). For heart tissue, females fed the AO high diet had higher concentrations of protein carbonyls ($F_{16,86}$=7.60, P=0.007).

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

Here, we compare the main effect of dietary antioxidants for Season females (reported above) with that for flight-trained Reproductive females, which were sampled after a 6 day exposure to males. The significantly higher GPx activity in heart tissue for Season females fed the AO low diet (Fig. 2D) was not apparent in flight-trained Reproductive females (n=7 high, n=8 low; Fig. 5D; $F_{8,136}$=0.01, P=0.905). For flight-trained Reproductive females, there was a trend for higher CAT activity in leg tissue (n=7 high, n=9 low; Fig. 5F; $F_{3,57}$=3.82, P=0.055) for females fed the low-antioxidant diet. There was a trend for CAT activity in liver tissue to be affected by dietary antioxidants in flight-trained Reproductive females (n=7 each; Fig. 5G; $F_{3,57}$=2.78, P=0.100), although, unlike in leg tissue, females fed more dietary antioxidant had higher CAT activity. The few significant effects of dietary antioxidants on non-enzymatic antioxidant capacity (Fig. 3) and oxidative damage (Fig. 4) in Season females were not apparent in flight-trained Reproductive females (Table S2).

DISCUSSION

In this study, we measured multiple markers of antioxidant capacity and oxidative damage in four tissues. We found mixed, tissue-specific support for our two proposed hypotheses. For example, dietary antioxidant supplementation (hypothesis 1) affected the activity of one of three antioxidant enzymes, one of two non-enzymatic antioxidant capacity measures, and one measure of damage. Flight training and diet together (hypothesis 2) affected the activity of 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 and their implications.

Hypothesis 1 – dietary antioxidant effect

If the availability of dietary antioxidants reduces the need to invest in the endogenous antioxidant system, then we predicted that females

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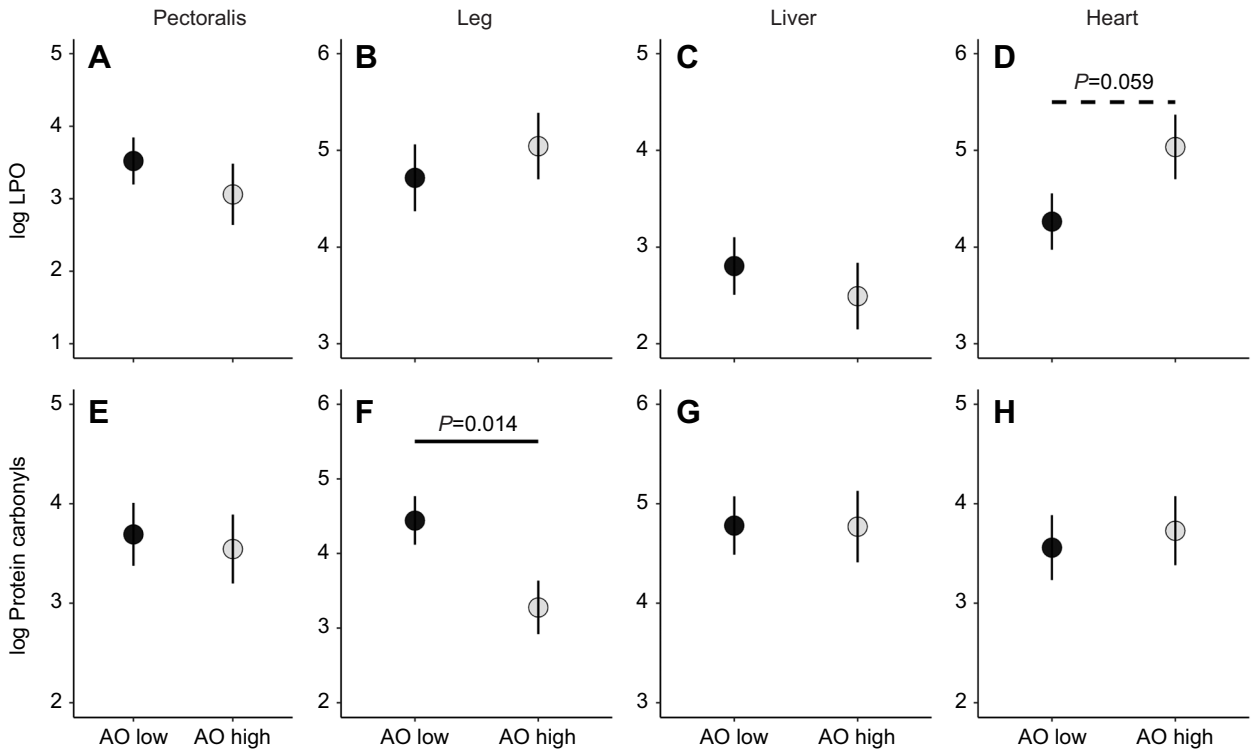

Fig. 4. Least square means (±s.e.m.) of oxidative damage to lipids and proteins in pectoralis, leg muscle, liver and heart for flight-trained Season females fed the AO high (grey) or AO low (black) diet during spring. Solid bars over groups denote a significant difference, whereas dashed bars denote a trend. Lipid hydroperoxides (LPO; nmol) did not differ between diets in the pectoralis (A, n=13 AO high, n=17 AO low; P=0.243), leg muscle (B, n=15 AO high, n=15 AO low; P=0.984) or liver (C, n=15 AO high, n=20 AO low; P=0.641). There was a trend for LPO to be higher in the heart (D, n=16 AO high, n=21 AO low; P=0.059) for females fed the AO high diet. Protein carbonyls (nmol ml^{-1}) did not differ between diets in the pectoralis (E, n=15 AO high, n=18 AO low; P=0.746), liver (G, n=14 AO high, n=21 AO low; P=0.595) or heart (H, n=15 AO high, n=20 AO low; P=0.599), but were significantly lower in the leg muscle (F, n=14 AO high, n=17 AO low; P=0.014) for females fed the AO high diet.

fed the AO high diet would have higher non-enzymatic antioxidant capacity, lower enzymatic antioxidant activity and lower oxidative damage than females fed the AO low diet. We found mixed support for this hypothesis. In support of hypothesis 1, GPx activity was lower in the heart for Season females in spring fed more dietary antioxidants, but not in any other organs (Fig. 2D). CAT and SOD activity were not affected by anthocyanin supplementation for Season females. To date, studies examining the effects of antioxidant supplementation on antioxidant enzymes in both avian and mammalian models have also found mixed results. The expression of CAT genes was higher in the liver of European starlings fed a diet that was not supplemented with anthocyanin compared with that in birds fed a diet supplemented with anthocyanin, though starlings fed the supplemented diet had higher expression of CAT and SOD1 genes in the pectoralis (DeMoranville et al., 2020). Broiler chickens fed a diet supplemented with an algae-based antioxidant had higher GPx, SOD and CAT activity in the pectoralis than birds fed a control diet (Delles et al., 2014). Anthocyanins have also been demonstrated to increase GPX activity in rat liver cells (Shih et al., 2007). In contrast, mice fed a diet supplemented with vitamin C had decreased GPx, SOD and CAT activity in liver tissue compared with individuals fed a control diet (Selman et al., 2006). These results indicate that dietary antioxidants may react with enzymatic antioxidants in differing ways depending on the enzyme's location within the body, and our results suggest that anthocyanin supplementation may decrease the activity of an enzymatic antioxidant in a major organ used in exercise. However, further analysis of 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 AO low diet (Fig. 3E). These results indicate that females fed a lower level of dietary antioxidants may upregulate non-enzymatic defenses, and that supplementation with water-soluble antioxidants may not contribute proportionally to overall non-enzymatic antioxidant capacity as we predicted. Uric acid is one antioxidant molecule that has been demonstrated to quench 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. Long-duration flights are associated with increased circulating uric acid in many bird species (Gannes et al., 2001; Gerson and Guglielmo, 2011; Jenni-Eiermann and Jenni, 1991). In a companion study using the same groups of female starlings, we found that females fed the AO low diet had higher baseline circulating uric acid in plasma than females fed the AO high diet (Frawley et al., 2021a). Females fed a lower level of 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 more dietary antioxidants had lower indices of protein damage in the leg muscle (Fig. 4F), although not in the other three tissues sampled. In contrast, females

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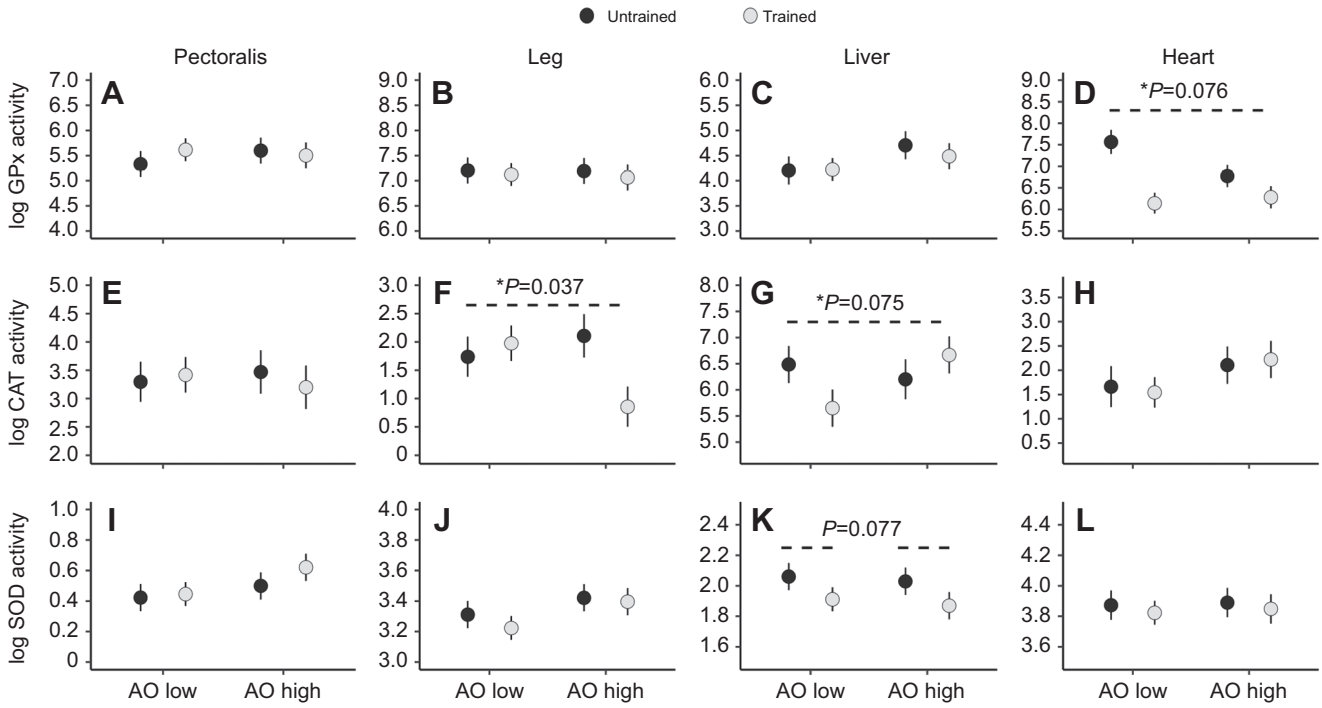

Fig. 5. Least square means (±s.e.m.) of enzymatic antioxidant activity in pectoralis, leg muscle, liver and heart with/without training for Reproductive females fed the AO high or AO low diet during spring. Solid bars over groups denote a significant difference, whereas dashed bars denote a trend. Asterisks and a bar covering all four groups indicate a diet×training interaction. A Bonferroni correction was applied for measures of enzymatic antioxidant activity, such that we considered $P \leq 0.017$ to be significant. GPx activity (nmol min^{-1} ml^{-1}) did not differ with training in the pectoralis (A, trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; P=0.452), leg muscle (B, trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; P=0.920) or liver (C, trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=6; P=0.649). There was a diet×training interaction trend for GPx in the heart (D, trained AO high n=7, AO low n=8; untrained AO high n=7, AO low n=6; P=0.076), such that untrained females fed the AO low diet trended toward having high GPx activity. CAT activity (nmol min^{-1} ml^{-1}) did not differ with training in the pectoralis (E, trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=7; P=0.587) or heart (H, trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=5; P=0.758). There was a diet×training interaction trend for CAT activity in leg muscle (F, trained AO high n=7, AO low n=9; untrained AO high n=6, AO low n=7; P=0.037), such that trained females had higher CAT activity than untrained females on the AO low diet, but lower CAT activity than untrained females on the AO high diet. There was a diet×training interaction for CAT in the liver (G, trained AO high n=7, AO low n=7; untrained AO high n=6, AO low n=7; P=0.075), such that trained females had lower CAT activity than untrained females on the AO low diet, but higher CAT activity than untrained females on the AO high diet. SOD activity (U ml^{-1}) did not differ with training in the pectoralis (I, trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; P=0.571), leg muscle (J, trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; P=0.717) or heart (L, trained AO high n=7, AO low n=9; untrained AO high n=7, AO low n=7; P=0.949), and there was a trend for untrained birds to have higher SOD activity than trained birds in the liver (K, trained AO high n=6, AO low n=9; untrained AO high n=6, AO low n=6; P=0.077).

fed more dietary antioxidants tended to have higher concentrations of LPO in the heart only (Fig. 4D). Leg muscle is not used in flight, and our result suggests that antioxidant supplementation can decrease baseline oxidative damage when tissues are less challenged during exercise. However, the trends seen in the heart, an organ heavily involved in exercise, and the lack of differences seen in the pectoralis and liver indicate that the effects of antioxidant supplementation are not directly related only to the extent of physiological challenge for a given tissue. Though few analyses have been done on oxidative damage in multiple tissues from birds and mammals, dietary antioxidant supplementation typically reduced markers of oxidative damage in plasma and a few other tissues. Budgerigars fed antioxidant-supplemented diets had lower circulating DNA damage and malondialdehyde (MDA), an indicator of lipid peroxidation, than birds fed a low-antioxidant diet (Larcombe et al., 2008). In contrast, blue tit nestlings (*Cyanistes caeruleus*) fed diets supplemented with either carotenoids or vitamin E, two lipid-soluble antioxidants, had similar levels of plasma oxidative damage to those of 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 with those 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 with 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). Although we would expect lipid peroxidation to be lower in all organs for females fed the AO high diet, oxidative damage to both protein and lipids did not significantly differ between the AO high and AO low diets in the pectoralis or liver. One possible explanation may be that the data were collected from females in spring, when free-living birds would be preparing to breed, and therefore when females may prioritize the reduction of oxidative damage regardless of diet. Depositing antioxidants into eggs has been demonstrated to be beneficial for offspring (Marri and 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 lower levels of antioxidants into their eggs (Giordano et al., 2015). As part of a companion study (Frawley et al., 2021a), we found that females in the spring had lower circulating oxidative damage prior to flight training than females in the autumn. Viewed together, these results suggest that females may shift antioxidant allocation to reduce oxidative damage in spring, regardless of the availability of dietary antioxidants.

Surprisingly, dietary anthocyanin supplementation had no effect on enzymatic antioxidant activity, non-enzymatic antioxidant capacity or oxidative damage in the liver. The liver primarily stores fat-soluble antioxidants, which may explain why it was less affected by water-soluble anthocyanins (Galván et al., 2012; Møller et al., 2010). Further studies should explore whether supplementing females with a fat-soluble antioxidant affects enzymatic antioxidant activity, oxidative damage and non-enzymatic antioxidant capacity in the liver.

Hypothesis 2 – dietary antioxidant×flight training interaction

If daily flying poses an additional oxidative challenge for females attempting to conserve antioxidants during spring migration, then we predicted that flight-trained females fed a lower level of dietary antioxidants would have lower non-enzymatic antioxidant capacity, higher enzymatic antioxidant activity and higher oxidative damage than flight-trained females fed more dietary antioxidants. We found diet×training interaction trends for two enzymatic antioxidants across three organs that provide mixed support for this hypothesis. Untrained females had significantly higher GPx activity in the heart than trained females, and this difference was most pronounced for females fed lower levels of dietary antioxidant (Fig. 5D). This result is consistent with the hypothesis that enzymatic antioxidants partially compensate when dietary antioxidants are scarce, although we found that this partial compensation does not occur when females are flight trained. Studies in mammals have 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 and Clarkson, 2003). One study found that racehorses fed a dietary antioxidant supplement had increased circulating GPx activity after 12 weeks of training compared with baseline levels (de Moffarts et al., 2005). In contrast, we found a trend such that female starlings fed more dietary antioxidants had lower CAT activity in leg muscle after 2 weeks of flight training compared with untrained starlings as well as females fed lower levels of dietary antioxidants regardless of training (Fig. 5F). There was a trend for starlings fed more dietary antioxidants to have higher CAT activity in liver after 2 weeks of flight training compared with untrained starlings, whereas the opposite effect of flight training occurred when females were fed lower levels of dietary antioxidants (Fig. 5G). The pattern seen in leg muscle indicates that anthocyanin supplementation may have allowed trained females to decrease their reliance on CAT and complements the change in GPx seen in the heart for untrained females. The trend seen in the liver, however, may indicate that anthocyanin supplementation improves 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 with rats that received only one of these treatments (Marsh et al., 2006). Flight-trained starlings, in contrast, had consistently lower enzymatic antioxidant activity compared with untrained birds (DeMoranville et al., 2020). These contrasting results may point to important differences in the antioxidant response of mammals and birds and indicate that the effects of dietary antioxidant supplementation and training on antioxidant enzymes are usually tissue specific.

There was a trend for SOD to be higher in the liver of untrained females (Fig. 5K) 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 liver of trained birds to protect the fatty acids necessary to fuel flight (McWilliams et al., 2004). This trend may suggest that flight training allows females to decrease their reliance on enzymatic antioxidants. For example, GPx and SOD production has been suggested to be costly because of the necessity for cofactors that are also used in amino acid synthesis and signaling pathways (Halliwell and Gutteridge, 2015; Zwolak and Zaporowska, 2012). Enzymatic antioxidants may be used primarily for acute challenges (Cooper-Mullin et al., 2019; Jenni-Eiermann et al., 2014), and training may result in a decreased reliance on enzymatic antioxidants over time. 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 acute changes in the pectoralis after flight may prove informative. Viewed together, these results emphasize that dietary antioxidants do not affect enzymatic antioxidant activity in a simple reciprocal way. Furthermore, different sites of enzyme activity 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 pathways involved in the regulation of enzymatic antioxidants.

Oxidative damage and non-enzymatic antioxidant capacity did not differ between trained and untrained birds, contrary to hypothesis 2. Studies examining oxidative damage indicate that flight incurs an oxidative cost. Free-living European robins caught during nocturnal migratory flights had elevated levels of protein damage compared with 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). The mechanism by which training can reduce oxidative damage remains understudied in birds, although studies of exercising mammals find an upregulation of the endogenous antioxidant system with training (e.g. Gul et al., 2006; Oztasan et al., 2004) whereas we found no such upregulation in flight-trained female starlings.

Our experiment is one of the most comprehensive examinations of tissue-specific differences in the antioxidant system of wild vertebrates to date and provides valuable insight into interactions between the endogenous and exogenous components of the antioxidant system, along with the effects of exercise on the antioxidant system. Dietary antioxidants did not simply reduce the need to upregulate enzymatic antioxidants, nor did they simply prevent oxidative damage. However, females appeared to defend against oxidative damage during spring migration in that we found consistently low oxidative damage even with exercise. Importantly, the response of the antioxidant system to dietary antioxidant supplementation and repeated exercise appears to be strongly tissue specific, but we found few effects of dietary antioxidants and exercise in the pectoralis, the primary flight muscle. Flight training may condition the antioxidant system of females to dynamically respond to oxidative challenges, though further study of tissue-specific responses is needed.

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 (MPIO) animal care staff for handling much of the animal husbandry responsibilities, and to Susan Forrest and Aleksandra Małochleb for helping to 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 USA. We also thank two anonymous reviewers for their thoughtful feedback on the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: U.B., B.P., S.R.M.; Methodology: U.B., B.P., S.R.M.; Validation: U.B., B.P., S.R.M.; Formal analysis: A.E.F.; Investigation: A.E.F., K.J.D., K.M.C., L.T., A.B., M.D., E.T.S., U.B., B.P., S.R.M.; Resources: U.B., B.P., S.R.M.; Data curation: A.E.F.; Writing - original draft: A.E.F., S.R.M.; Writing - review & editing: A.E.F., K.J.D., K.M.C., L.T., A.B., M.D., E.T.S., U.B., S.R.M.; Visualization: A.E.F.; Supervision: U.B., B.P., S.R.M.; Project administration: U.B., B.P., S.R.M.; Funding acquisition: U.B., B.P., S.R.M.

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

Data availability
Data are available from the Dryad digital repository (Frawley et al., 2021b): doi:10.5061/dryad.pnvx0k6mq

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Table S1. Effects of dietary antioxidants (high or low antioxidant diet, AH or AL, respectively) on the activity of enzymatic antioxidants (GPx, SOD, CAT), non-enzymatic antioxidant capacity (ORAC), and markers of oxidative damage (protein carbonyls and lipid hydroperoxides) in three different tissues (pectoralis, liver, and heart) in Season females sampled in fall. These fall samples from Season females were not included in the testing of the main effect of Diet (Hypothesis H1) because too many tissue samples thawed in transit from Germany to the USA (see methods for details). 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 ± s.e.m.	d.f.	*F*	p	ΔAIC_c
GPx	Pectoralis	AH: 4.96 ± 0.33 AL: 5.12 ± 0.29	7, 17	0.13	0.717	14.2
	Liver	AH: 3.23 ± 0.38 AL: 3.83 ± 0.33		1.40	0.244	
	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	0.999	16.01
	Liver	AH: 5.82 ± 0.44 AL: 6.02 ± 0.39		0.11	0.741	
	Heart	AH: 1.72 ± 0.52 AL: 1.98 ± 0.39		0.16	0.690	
SOD	Pectoralis	AH: 0.52 ± 0.38 AL: 0.45 ± 0.34	8, 19	0.02	0.892	8.75
	Liver	AH: 1.39 ± 0.38 AL: 1.60 ± 0.34		0.16	0.693	
	Heart	AH: 2.70 ± 0.44 AL: 3.63 ± 0.34		2.64	0.112	
Protein carbonyl	Pectoralis	AH: 3.63 ± 0.71 AL: 4.43 ± 0.53	7, 19	0.81	0.374	17.97
	Liver	AH: 4.80 ± 0.60 AL: 4.52 ± 0.53		0.13	0.723	
	Heart	AH: 4.28 ± 0.60		2.90	0.097	

		AL: 2.82 ± 0.61				
Lipid hydroperoxides	Pectoralis	AH: 3.21 ± 0.61 AL: 4.24 ± 0.53	7, 17	1.64	0.209	19.74
	Liver	AH: 3.47 ± 0.52 AL: 4.16 ± 0.62		0.74	0.395	
	Heart	AH: 5.11 ± 0.52 AL: 5.14 ± 0.46		0.001	0.971	
ORAC (peroxyls)	Pectoralis	AH: 12.5 ± 0.33 AL: 12.3 ± 0.30	7, 23	0.13	0.716	9.21
	Liver	AH: 13.0 ± 0.33 AL: 12.8 ± 0.30		0.22	0.642	
	Heart	AH: 12.4 ± 0.33 AL: 11.6 ± 0.30		2.88	0.098	
ORAC (hydroxyls)	Pectoralis	AH: 11.1 ± 0.23 AL: 10.8 ± 0.21	8, 22	0.001	0.980	9.49
	Liver	AH: 11.7 ± 0.23 AL: 11.6 ± 0.21		0.14	0.715	
	Heart	AH: 12.0 ± 0.23 AL: 11.4 ± 0.21		0.82	0.369	

Table S2. Effects of dietary antioxidants (high or low antioxidant diet, AH or AL, respectively) on oxidative damage (protein carbonyls and lipid hydroperoxides) and antioxidant capacity (ORAC) in four different tissues (pectoralis, leg, liver, and heart) in flight-trained Reproductive females in spring. 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. ΔAICc is the difference between the final model used and the global model with all covariates included. Statistical analyses for enzymatic antioxidants are reported in the main text.

AO Measure	Organ	Estimated mean ± s.e.m.	d.f.	*F*	p	ΔAIC_c
Protein carbonyl	Pectoralis	AH: 3.68 ± 0.48 AL: 3.72 ± 0.56	4, 49	0.003	0.955	6.24
	Leg	AH: 3.81 ± 0.57 AL: 4.21 ± 0.52		0.27	0.608	
	Liver	AH: 4.50 ± 0.62 AL: 5.09 ± 0.46		0.59	0.445	
	Heart	AH: 4.09 ± 0.56 AL: 3.13 ± 0.57		1.46	0.231	
Lipid hydroperoxides	Pectoralis	AH: 3.75 ± 0.64 AL: 3.57 ± 0.41	3, 53	0.06	0.808	8.67
	Leg	AH: 5.55 ± 0.47 AL: 4.92 ± 0.57		0.73	0.397	
	Liver	AH: 1.93 ± 0.47 AL: 2.10 ± 0.44		0.07	0.794	
	Heart	AH: 5.01 ± 0.47 AL: 4.74 ± 0.41		0.20	0.660	
ORAC (peroxyls)	Pectoralis	AH: 12.2 ± 0.23 AL: 11.9 ± 0.20	3, 59	1.31	0.257	8.16
	Leg	AH: 12.2 ± 0.22 AL: 12.4 ± 0.19		0.97	0.330	
	Liver	AH: 15.5 ± 0.22 AL: 12.5 ± 0.19		0.01	0.938	
	Heart	AH: 12.1 ± 0.22 AL: 12.2 ± 0.19		0.06	0.802	
ORAC (hydroxyls)	Pectoralis	AH: 10.7 ± 0.18	4, 58	0.05	0.817	5.06

	AL: 10.8 ± 0.16		
Leg	AH: 11.2 ± 0.17	2.58	0.113
	AL: 11.5 ± 0.15		
Liver	AH: 11.5 ± 0.17	0.04	0.847
	AL: 11.5 ± 0.15		
Heart	AH: 11.3 ± 0.17	0.68	0.413
	AL: 11.5 ± 0.15		