Acute flight and flight training deplete non-enzymatic antioxidant capacity and protect against oxidative damage in a migratory songbird, but dietary antioxidants and fat quality have little effect on oxidative status

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Abstract

Ecologically-relevant factors such as exercise and diet quality can directly influence how multifaceted physiological systems work; however, little is known about how such factors directly and interactively affect key components of the antioxidant system in multiple tissues of migratory songbirds. We tested 3 main hypotheses across three tissues in European Starlings fed diets with more or less antioxidants (anthocyanins) and long-chain polyunsaturated fats (18:2n6) while being flight-trained in a wind tunnel. Stimulatory effect of flight: Flight-training stimulated the antioxidant system in that a) plasma oxidative damage was reduced during a given acute flight, and b) antioxidant capacity and oxidative damage in plasma and tissues of flight-trained birds were similar to that of untrained birds. Flight-trained birds that expended more energy per unit time (kJ/min) during their longest, final flight decreased the non-enzymatic component of their antioxidant system the most during the final flight. Dietary antioxidant effect: Flight-trained birds that consumed more dietary anthocyanins had similar antioxidant capacity in liver and flight-muscle compared to untrained birds, and oxidative damage was prevented in the flight-muscle and reduced in the liver of flight-trained birds compared to untrained birds. Dietary fat quality effect: Contrary to our predictions, dietary 18:2n-6 did not influence oxidative status even after flight training. We found limited evidence that circulating and tissue-level oxidative capacity and damage were tightly regulated in flight-trained starlings, in contrast to the precise regulation on gene expression and enzyme activity that were observed in companion studies. In sum, the antioxidant system of songbirds flexibly responded to changes in availability of dietary antioxidants as well as increased flight time and effort, and such condition-dependent, individual-level, tissue-specific responses to the oxidative costs of long-duration flights apparently requires recovery periods for maintaining oxidative balance during migration.

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Keywords migration, antioxidants, antioxidant capacity, dietary fat quality, flight training, oxidative damage

Introduction

The challenges of maintaining oxidative balance for migratory birds

During energetically-challenging periods, all aerobic organisms must contend with an increased production of reactive species (RS) by either neutralizing the RS with antioxidants to minimize resulting oxidative damage, or damage must be restored using repair mechanisms (Halliwell and Gutteridge 2007, Costantini 2014, 2019). Long-distance flight increases metabolism (Swanson 2010, Corder and Schaeffer 2015, DeMoranville et al. 2019, Bishop, C.M., Guglielmo 2022) and poses a potential oxidative challenge for flying animals (McWilliams et al. 2021, McWilliams, S.R., Ramenofsky, M., Pierce 2022). For example, Nathusius' bats *Pipistrellus nathusii* that were captured and sampled during a migratory flight had higher circulating oxidative damage markers compared to individuals that were captured and rested for 18-24 hours (Costantini et al. 2018). Similarly, circulating protein damage and the antioxidant enzyme glutathione peroxidase (GPx) were higher in European robins *Erithacus rubecula* during a nocturnal migratory flight compared to resting individuals caught during the day, indicating that damage to muscle occurs with flight and the antioxidant system can respond rapidly (Jenni-Eiermann et al. 2014). These acute effects of flight were also demonstrated in captive Yellow-rumped warblers (*Setophaga coronata coronate)* , as protein carbonyls and superoxide dismutase (SOD) activity were higher in the pectoralis muscle immediately after a flight in a wind tunnel compared to individuals at rest (Dick and Guglielmo 2019). Similarly, flight-trained zebra finches *Taeniopygia guttata* had increased circulating oxidative damage compared to untrained sedentary individuals (Skrip et al. 2016). These studies together demonstrate that migratory bats and birds respond to such oxidative challenges by increasing antioxidant enzyme activities, depleting or augmenting non-enzymatic antioxidant capacity, and in these cases increasing oxidative damage. They also reveal that too little is known about tissue-specific oxidative status of migratory birds (Dick and Guglielmo 2019) and the extent to which an individual's circulating oxidative status reflects the oxidative state of muscles and organs at a given time (Costantini 2019, Frawley et al. 2021a). What remains to be determined for migratory songbirds is how the oxidative status of different tissues and plasma responds to both flight training and ecologically-relevant differences in diet quality (i.e. antioxidants and fat composition).

Certain dietary fats challenge the antioxidant system - Birds rely primarily on fatty acids to fuel flight (Guglielmo, 2018; McWilliams, Guglielmo, Pierce, & Klaassen, 2004), and several species of migratory songbirds increase the amounts of unsaturated fats and especially long-chain polyunsaturated fats (PUFAs) in their diet, fat stores and in circulation during migration compared to non-migration periods (Pierce et al. 2004, Pierce and McWilliams 2005, Price et al. 2008, Smith and McWilliams 2010, Jensen et al. 2020,

McWilliams, S.R., Ramenofsky, M., Pierce 2022). The potential benefits of consuming certain long-chain PUFA (e.g., 18:2n-6 PUFA) including enhanced efficiency of energy metabolism during exercise and reduced flight costs (Pierce and McWilliams 2014, Guglielmo 2018), are also associated with potential oxidative costs because PUFA are highly susceptible to oxidative damage (Halliwell and Gutteridge 2007, Skrip and McWilliams 2016, McWilliams et al. 2020). The resulting lipid radicals often cause a self-perpetuating chain reaction damaging nearby PUFAs and other molecules (Halliwell and Gutteridge 2007, Skrip and McWilliams 2016, Cooper-Mullin et al. 2019). Birds preferentially consuming 18:2n-6 PUFA during migration to enhance their metabolism (Pierce and McWilliams 2014) likely require an augmented antioxidant system to protect against the oxidative challenge that such PUFAs pose (McWilliams et al. 2021).

Dietary antioxidants augment the antioxidant system - Many songbird species select fruits that are high in antioxidants and fat during fall (post-breeding) migration, suggesting that antioxidant consumption may be important to protect against oxidative damage during this life history stage (Alan and McWilliams 2013, Bolser et al. 2013). Water-soluble antioxidants such as anthocyanins are preferentially consumed by certain songbird species during fall migration and in cafeteria-style choice experiments (Schaefer et al. 2008, Alan et al. 2013, Bolser et al. 2013). Anthocyanins are potent antioxidants (Halliwell and Gutteridge 2007) that are exclusively stable at acidic pH levels (i.e. they degrade extensively in less than an hour at pH 7.4) and are more likely to remain intact and absorbed in the 2 orders of magnitude more acidic stomachs of songbirds (pH 2) relative to mammals (pH 4.4) (Dangles and Fenger 2018). Anthocyanins can directly or indirectly affect the antioxidant system and also have been shown to protect birds from an immune challenge (Catoni et al. 2008) and protect against some of the metabolic costs associated with flight training (Casagrande et al. 2020) as well as courtship (Carbeck et al. 2018) and reproduction in spring (Frawley et al. 2021b). These energy savings associated with consuming anthocyanins may allow birds to invest in increasing enzymatic or non-enzymatic antioxidants to prevent damage (McWilliams et al. 2020). Non-enzymatic antioxidant capacity increases with fat stores in songbirds on stopovers during migration (Costantini et al. 2007, Skrip et al. 2015) and is greatest during the pre-migratory fueling stage in shorebirds (Gutiérrez et al. 2019). Thus, hyperphagia associated with preparation for migration in birds may augment their antioxidant system by increasing fat-soluble antioxidants stored within newly accumulated fat stores or by increasing water-soluble antioxidant metabolites continuously released by gut microbes (Dogan Comert and Gokman 2017).

How does flight training, dietary fat, and dietary anthocyanins affect the oxidative status of the plasma and metabolic tissues in a migratory songbird?

To better understand the multifaceted antioxidant system of birds, we conducted a factorial experiment that manipulated three ecologically relevant factors (i.e. flight training, dietary fat, dietary antioxidants) to determine their effects on equivalent measures of lipid damage and antioxidant capacity in the plasma, liver, and flight-muscle of a migratory songbird. We tested the following hypotheses:

1. *Stimulatory effect of flight* (H1): Acute flight and flight-training stimulate the non-enzymatic antioxidant system and protect against oxidative damage in the plasma, liver, and pectoralis.
2. *Acute-effect of a long flight* : plasma antioxidant capacity decreases during a given long-duration flight and so enables circulating oxidative damage levels to remain low.
3. *Long-term effect of flight-training* : a bird's plasma non-enzymatic antioxidant capacity (OXY) increases and lipid damage (d-ROMs) decreases over the course of several weeks of daily flight training.
4. *Flight-training effect across multiple tissues* : compared to untrained birds, flying regularly over several weeks increases non-enzymatic antioxidant capacity (Oxygen radical absorbance capacity, ORAC in flight muscle and liver; OXY in plasma) and decreases lipid damage (Lipid hydroperoxides, LPO, in flight muscle and liver; d-ROMs in plasma) in a consistent manner across tissues.
5. *Dietary fat effect* (H2): migratory songbirds fed diets composed of more 18:2n-6 PUFA are more susceptible to oxidative damage and thus preventatively increase antioxidant capacity in the plasma, liver, and flight-muscle so as to maintain low levels of lipid damage compared to birds fed diets with less PUFA.
6. *Dietary antioxidant effect* (H3): migratory songbirds fed dietary anthocyanins have increased non-

enzymatic antioxidant capacity, and lower levels of lipid damage in all three tissues (i.e., liver, muscle, plasma) compared to birds not fed anthocyanins.

We also examined whether these three ecologically relevant factors (flying, fat quality of diet, dietary antioxidants) interacted to affect key components of the antioxidant system.

Methods

Experimental Design

Omnivorous migratory songbirds undergo endurance flights biannually and many species switch to eating mostly berries that are rich in fats and antioxidants during their fall (post-breeding) migration (Alan et al. 2013, Bolser et al. 2013); thus, they are an ideal natural system to study how the endogenous antioxidant system responds to flight training, dietary antioxidants, and dietary fat. We used European Starlings (*Sturnus vulgaris*) as representative songbirds for this study because they are abundant in the New World and Old World, are omnivorous and acclimate well to captivity and new diets, and have been successfully trained and flown in wind tunnels in other studies (Nebel et al. 2012, Hall et al. 2014, Casagrande et al. 2020, McWilliams et al. 2020). Hatch year European starlings were caught at a dairy farm 20 km north of the Advanced Facility for Avian Research (AFAR), University of Western Ontario, London, Ontario prior to fall migration in 2015 (August 19-23). Starlings from this southern Canada wild population are considered to be partial migrants as inferred by banding records (Cabe 1993). Starlings were housed in one of four large indoor aviaries at AFAR (two 2.4m x 3.7m x 3.1m and two 2.4m x 2.3m x 3.5m). On August 24th we measured morphological characteristics, body mass, and molt score (0 – 5; (Ginn and Melville 1983)) for each individual. Birds were then randomly sorted into four groups (Figure 1) with roughly equal distributions of body size and molt score. We maintained aviaries at 21°C on a natural light cycle from capture until the start of the experiment on September 21st when we fixed the light schedule at 11:13 L:D (day length on this date in London, Ontario). Upon capture and until the start of the experiment each week we weighed and inspected all birds to assess their health. All birds were cared for under animal care protocols for University of Western Ontario (2010-216) and the University of Rhode Island (AN11-12-009).

Figure 1.

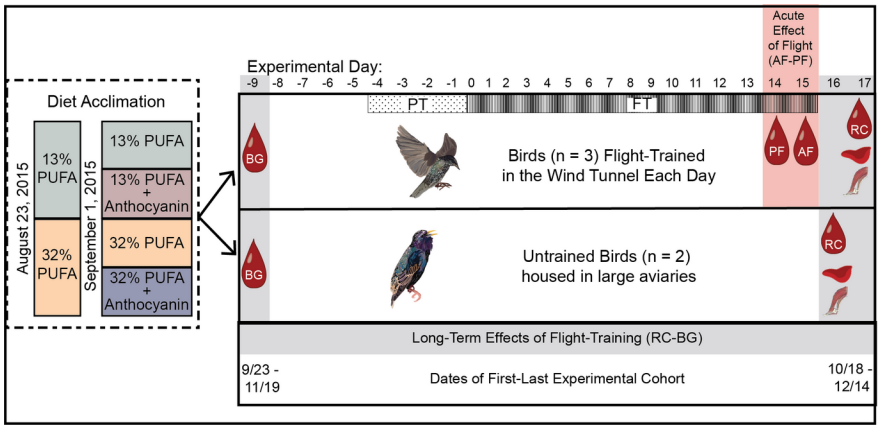


Figure 1. Experiment timeline included an initial acclimation to diets containing either 13% 18:2n-6 or 32% 18:2n-6 and then the addition of an antioxidant supplement to the diets of half the birds on 1 September to produce the 2 X 2 factorial diet manipulation with four diet groups: 13% 18:2n-6, anthocyanin unsupplemented (N = 23), 13% 18:2n-6, anthocyanin supplemented (N = 23), 32% 18:2n-6, anthocyanin unsupplemented (N = 21), and 32% 18:2n-6, anthocyanin supplemented (N = 20). After at least one month of acclimation to one of these four diets, birds were assigned to one of 20 cohorts each comprised of 3 flight-trained birds and 2 untrained birds from each diet treatment (5 cohorts per treatment). On September 23rd,

and continuing every three days thereafter, a cohort began the four days of pre-training (PT) followed by fifteen days of flight training (FT). The 25 day flight-training schedule ended with birds performing a long duration flight (pink shaded) followed by two recovery days (gray shaded). Blood sampling timepoints are indicated by the blood droplets and include for all birds a Background (BG) blood sample and a Recovery (RC) blood sample, and for flight-trained birds a Pre-flight (PF) and After flight (AF) blood sample taken just before and after the long duration flight. Liver and pectoralis samples were taken from all birds on the final day. The acute effects of flight were assessed by comparing the change in plasma OXY and d-ROM at the AF and PF time points. The long-term effects of flight-training on the antioxidant system were assessed two ways: (a) by comparing the change in plasma OXY and d-ROMs at the RC and BG time points, and (b) by comparing the oxidative status in three tissues (blood, liver, pectoralis) of flight-trained and untrained birds at the RC time point.

Experimental Diets

Birds had *ad libitum* access to one of two semi-synthetic diets that had the same macronutrient content as a lipid-rich fruit diet (41% carbohydrate, 13% protein, 30% fat) and differed only in fatty acid composition. We manipulated the proportions of canola, sunflower, and palm oil so that the diets were either high (32%) or low (13%) in 18:2n-6 PUFA (linoleic acid) which was primarily traded off with 16:0 (palmitic acid). Thus, our experimental design requires us to attribute any observed dietary fat effects to both 18:2n-6 and 16:0 content. However, our interpretations focus on the potential effects of 18:2n-6 due to its demonstrated importance in metabolic signaling (Forman et al. 1997, Kennedy et al. 2007, Hamilton et al. 2018, Dick and Guglielmo 2019, Price et al. 2022). The complete list of diet ingredients and amounts have been previously published (citation redacted for initial review). Starlings in two aviaries received a 13% 18:2n-6 diet and two others received a 32% 18:2n-6 diet. The two diets have been shown to produce reliable differences in tissue fatty acid composition of starlings (citation redacted for initial review). On September 1, after we were confident birds were well acclimated to the fat quality in the semisynthetic diets, we began adding a supplementary water-soluble antioxidant, anthocyanin (elderberry powder; Artemis International, Fort Wayne, IN) to the diets of birds in one 13% 18:2n-6 aviary and one 32% 18:2n-6 aviary, producing a 2 X 2 factorial diet manipulation with four diet groups (Figure 1): 13% 18:2n-6, anthocyanin unsupplemented (N = 23), 13% 18:2n-6, anthocyanin supplemented (N = 23), 32% 18:2n-6, anthocyanin unsupplemented (N = 21), and 32% 18:2n-6, anthocyanin supplemented (N = 20). We chose the anthocyanin concentration used by researchers studying the effects of anthocyanin supplementation on food choice and immunocompetence in European blackcaps, *Sylvia atricapilla,* (Catoni et al. 2008, Schaefer et al. 2008). The anthocyanin supplement was equal to eating 2.8 mg per day which is equal to consuming 17 berries per day based on an average daily synthetic diet consumption of ca. 35 wet g day^{-1} (as observed in food intake trials in this study).

Experimental Timeline

On September 21st we randomly assigned groups of 5 starlings to each of twenty cohorts (Figure 1) although this cohort selection was stratified by extent of Pre-basic I molt (Ginn and Melville 1983) – birds furthest along in molt were flight-trained earliest. This stratified random sampling of individuals ensured that all birds had completed their flight feather molt prior to flight training. One cohort from each of the four diet groups were tested sequentially over 10 days although the sampling order of the diet groups was randomly assigned so a diet group was not consistently sampled first or last. Thereafter another set of cohorts from each of the four diet groups were tested and so forth until the 20 cohorts of starlings had been tested by early December.

On September 23rd, and continuing every three days, the 5 individuals from each selected cohort were removed from their aviaries, and we randomly assigned 2 birds as untrained birds and 3 birds as flight-trained birds. Each selected cohort was initially placed in individual cages (0.6m x 0.5m x 0.5m) for two days (days -9 and -8) to measure food intake and another two days (days -7 and -5) to measure basal and peak metabolic rates (citation redacted for initial review). On day -5 we returned the two untrained birds to their original aviary and moved the 3 flight-trained birds to a 0.8m x 1.5m x 2m flight aviary.

Each cohort was blood sampled at consistent time points throughout the 25-26 day experimental period (Figure 1: Background, BG; Pre-Flight, PF; After-Flight, AF; Recovery, RC). Birds were fasted for at least 1 hour before all blood sampling time points and were bled within 30 minutes of capture. Blood samples were taken from the brachial vein, and within 10 min of blood sampling the plasma was separated from the red blood cells following centrifugation at 11,000 *g* for 10 minutes (Damon/IEC Division, IEC MB centrifuge, micro hematocrit). Plasma was stored at -80°C until OXY-adsorbent test and d-ROM analyses. A blood sample was obtained 9 days prior to the start of flight-training at 8:00 hr in the morning to obtain background oxidative measurements (BG) for each individual. Measuring indices of circulating oxidative status is important since an unbalanced oxidative state in the plasma would indicate damage to crucial molecules transported by the plasma (e.g. fatty acids, hemoglobin, cytokines) to muscles and organs.

Flight Training

In order to assess the impact of diet and endurance flight on the endogenous antioxidant system, three flight-trained birds were flown in a wind tunnel for four days of pre-training followed by fifteen days of flight training. Such a flight training regime has demonstrated success at eliciting long-duration flights in starlings (Engel et al. 2006). The wind tunnel was set to 12 m/s windspeed, 15°C, and 70% humidity, and birds were fasted for 1 hr prior to all flights. Pre-training (PT; days -4 to -1) consisted of allowing training birds to fly between their flight cage and the wind tunnel followed by 20 minutes of habituation time per day in the wind tunnel with a perch. These initial four 'pre-training' days were not included in the reported overall training time, since birds could rest when needed. Starlings in the flight-training group then participated in a fifteen-day training regimen (FT) that consisted of increasing periods of flight (20 min – 180 min) in the wind tunnel as follows: days 1-4, 20 min each day; day 5-6, 30 min each day; day 7, 60 min; day 8, 90 min; day 9, 30 min; day 10, 120 min; day 11, 180 min; day 12, rest day; day 13, 60 min; and day 14, 30 min. This flight training culminated in a flight on day 15 that lasted as long as birds would voluntarily fly, up to 6 hrs. To determine fuel use during flight, body condition (fat and lean masses) was measured using a quantitative magnetic resonance machine (QMR; Echo Medical Systems, Houston, TX) immediately before and after the final flight. Energy expenditure during the flight was estimated by multiplying the mass of fat and lean tissue lost during flight by their respective energy densities, adding them, and dividing by fight duration (full methods and results reported previously, citation redacted for initial review). The final flight was on average 193 +/-71 (SD) min and the maximum was 360 min. In order to test the acute effects of flight, we blood sampled flight-trained birds on the morning of day 14 at 8:00 hr before their 30 min flight (Pre-flight, PF), this is at the same time that they would have been blood sampled before their longest flight on day 15. We sampled birds the morning prior to their long-flight to avoid excess stress associated with handling immediately prior to their long-flight. Since all experimental conditions were the same among the two days, we assume that this blood sample reflects the state of the flight-trained birds before a flight. An after-flight (AF) blood sample was taken immediately after the experimental flight (day 15) ranging from 11:00-14:00 hr. Flight-trained birds were returned to their flight cages for two days to recover from their last flight. At 1400hr – 1500hr on days 16 and 17 the untrained and trained birds, respectively, in each cohort were blood sampled for the final Recovery sample (RC). Birds were euthanized by cervical dislocation while under isofluorane anesthesia and the liver and pectoralis muscle samples were collected and immediately weighed. All tissues were flash frozen in liquid nitrogen and stored at -80°C until analysis. This sampling design allowed us to compare oxidative status in the liver and pectoralis of untrained (control) birds and flight-trained birds that had recovered (for 48 hrs) from their longest flight on day 15. The liver is a crucial food processing organ, especially for exercising birds that rely on fat to fuel flight (Guglielmo, 2018; Scott R. McWilliams, Guglielmo, Pierce, & Klaassen, 2004), and the pectoralis is the major skeletal muscle used to power flight (Biewener, 2011). Thus, these two metabolic tissues relied on by flying birds likely have high antioxidant capacities to protect against oxidative damage, yet it remains unknown how the oxidative status of both these tissues along with that of plasma respond to flight training, dietary fats, and dietary antioxidants or how these different classes of antioxidants work together to protect individuals against oxidative damage.

Non-enzymatic antioxidant capacity and oxidative damage

OXY Adsorbent test- OXY was measured in the plasma (concentration unit: mmol l^{-1} of HClO neutralized; Diacron International, Grosseto, Italy). OXY directly measures the ability of a plasma sample to quench the oxidant hypochlorous acid. In addition to directly reacting with biological molecules, hypochlorous acid can form more deleterious hydroxyl radicals (Candeias et al. 1993). OXY provides an index of non-enzymatic antioxidant capacity (e.g. dietary and non-dietary antioxidants), without being complicated by inclusion of uric acid (Alan and McWilliams, 2013; Cooper-Mullin et al. 2019; Costantini, 2011; Skrip and McWilliams, 2016).

Oxygen radical absorbance capacity- In preparation for the measurement of ORAC and LPO, approximately 250 mg of liver or pectoralis was homogenized on ice in 9 volumes of 0.1 M phosphate buffer, pH 7 with 3 x 10 sec pulses of a high-speed stainless-steel homogenizer (Tissue Master 125, Omni International, Kennesaw GA USA). Homogenate was centrifuged at 10,000 g for 10 minutes at 4degC (Beckman Coulter Allegra 21R), and the supernatant was aliquoted to 2 separate tubes to conduct the two separate assays (ORAC, LPO). Supernatant was immediately frozen at -80degC until the time of the assays (5-8 months after homogenization). We estimated antioxidant capacity against two of the more damaging forms of ROS that readily react with lipids (Halliwell and Gutteridge 2007), peroxyl and hydroxyl radicals (concentration unit: arbitrary units per gram of tissue, a.u.g^{-1}), by using a microplate-based version of the competitive ORAC assay (Cao and Prior 1999) following (Jimenez et al. 2020). When in vitro production of the radicals exceeds the antioxidant capacity of the tissue, these ROS modify the algal pigment phycoerythrin (545 nm/575 nm) and decrease its fluorescence. Peroxyl radicals were generated by 320 mmol l^{-1}2,2'-azobis (2-amidinopropane) dihydrochloride, and hydroxyl radicals were generated in separate plates by adding 0.25 µl per well of 10 mmol l^{-1} CuSO4 and 0.667 mol l^{-1}ascorbate mixture. ORAC values for peroxyl and hydroxyl radicals were determined by integrating the area under the fluorescence decay curve.

Uric acid -Uric acid is a by-product of protein catabolism that acts as an antioxidant (Halliwell and Gutteridge 2007). Methods and results for this metabolite were reported previously (citation redacted for initial review). Briefly, we assayed uric acid concentration (concentration unit: mmol/L) using an absorbance endpoint assay adapted for small volumes (TECO Diagnostics, Anaheim, CA) on 96-well plates in duplicate.

Lipid oxidative damage d-ROMs- Oxidative damage in the plasma was measured using the d-ROMs test (concentration unit: mmol l^{-1} H_2O_2 equivalents; Diacron International). This test works by first decreasing the pH of the plasma to release metal ions from proteins to cleave circulating ROMs through incubation with a solution of 0.01 mol l^{-1} acetic acid/sodium acetate buffer. The subsequent products react with a chromogen (N,N-diethyl-p-phenylenediamine) which has a color intensity that is proportional to the concentration of reactive oxygen metabolites (ROMs) in the plasma and was measured at 505 nm (Costantini et al. 2007, Costantini 2016, Cooper-Mullin et al. 2019). ROMs measured in this test are primarily hydroperoxides, and in plasma are primarily produced when reactive species interact with lipids (Davies 2016, Ito et al. 2017).

Lipid hydroperoxides- Oxidative damage in the liver and pectoralis was measured using the LPO test (concentration unit: hydroperoxide concentration µM; Cayman Chemical, Ann Arbor, MI). This assay provides a general measure of lipid peroxidation by directly measuring hydroperoxides compared to assays that measure the byproducts of specific fatty acid peroxidation (e.g. MDA, 4-HNE). Hydroperoxides in the sample were extracted using a Chloroform and methanol method. Hydroperoxides in the extraction react with ferrous ions detected by thiocyanate ion chromogen and was read at 500 nm in a glass 96-well plate.

Plasma or tissue from each individual was measured in triplicate for LPO and in duplicate for all other assays (OXY, peroxyl and hydroxyl radical absorbance capacity, d-ROMs); all coefficient of variations were under 10%, and replicates were averaged prior to statistical analyses.

Statistics

Model selection

We used R (v3.5.3; R Core Team, 2019, Vienna Austria) for all analyses. We constructed either linear mixed effects models using the *lme4* package or linear models using *stats* package to test our four main hypotheses.

Final best-fit models were selected using an AIC selection criterion, ΔAIC [?] 3. The only exception was when ΔAIC was within 3 for the models for liver and pectoralis lipid damage including cohort as a random factor compared with excluding it, and in these cases, we decided to control for cohort.

Linear Mixed Effects Models

Linear mixed effects models including bird identification number and cohort number as random factors were used to analyze the longitudinal plasma data to test the hypotheses (H1a) that acute flight decreases oxidative capacity during flight and enables oxidative damage levels to remain low, and (H1b) that regularly flying increases antioxidant capacity and oxidative damage. Linear mixed models that controlled for experimental cohort as a random factor were also used to test the hypothesis that flight-training (H1c), dietary fat (H2), and dietary antioxidants (H3) influenced Δ during training of plasma lipid damage and lipid damage in the liver and pectoralis. We conducted pairwise comparisons using least square means to determine the differences in oxidative status between specific timepoints.

Linear Models

Linear models were constructed to test the hypothesis that flight training (H1), dietary fat (H2), and dietary antioxidants (H3) influenced antioxidant capacity and lipid damage in the plasma at discrete timepoints (i.e., BG, PF, AF, RC), over the course of the experiment (calculated as RC-BG), during an acute flight (calculated as AF-PF), and in the liver and pectoralis at Recovery sampling. We used a global model without interaction terms that best matched this hypothesis and included possible explanatory covariates (i.e., cohort, sex, and wing chord), however, these covariates were not included in the global linear models.

To test the hypothesis that flight-training, dietary fat, and dietary antioxidants had an interactive effect on oxidative status, we compared our global models to models including a 3-way interaction between dietary fat, antioxidants, and training treatment. These models also tested the 2-way interactions between covariates. The models with the 3-way interactions were not among the best fit models, thus we report results for only the main effects.

To determine if energy expenditure during flight influenced antioxidant capacity or oxidative damage, we included energy consumed (kJ/min) during the acute flight as a fixed effect in linear models that compared both plasma oxidative status measured After-flight and the change in these measures during the acute flight (calculated as AF-PF) among diet groups across timepoints. The best fit models for AF oxidative status included energy expenditure, whereas those for change in oxidative status during the acute flight included energy expenditure x diet interaction term.

Results

Stimulatory effect of flight training across time: Acute-effect of a long flight (H1a)

Regular bouts of acute flight influenced OXY and d-ROMs in the plasma of flight-trained birds (i.e., comparison of BG, PF, AF, and RC blood samples; Fig. 1). Plasma d-ROMs was highest after two weeks of daily flight training compared to all other timepoints (Fig. 2; BG vs PF: T_{49} = -3.042, P = 0.0027, PF vs RC: T_{49}= 3.085, P = 0.0023). Immediately after an on average 193 +/-71 min flight the next day, plasma d-ROMs had substantially decreased relative to PF (Fig. 2A, Table S1; PF vs AF: T_{49} = 3.85, P = 0.0002). In contrast, plasma OXY after two weeks of daily flight (PF) was substantially lower than BG and recovery levels two days later (Fig. 2B, Table S1; BG vs PF: T_{49} = 6.058, P < 0.0001, PF vs RC: T_{49} = -6.113, P < 0.0001). Similar to d-ROMs, immediately after a long-duration flight the next day, plasma OXY further decreased relative to PF (Fig. 2B, Table S1; PF vs AF: T_{49} = 2.330, P = 0.021), and then returned to baseline levels at RC (BG vs AF: T_{49} = 8.993, P < 0.0001, AF vs RC: T_{49} = -8.544, P < 0.0001, BG vs RC: T_{49} = 0.0694, P since plasma antioxidant capacity decreased during a given long-duration flight and levels of circulating oxidative damage remained lower than or at baseline levels.

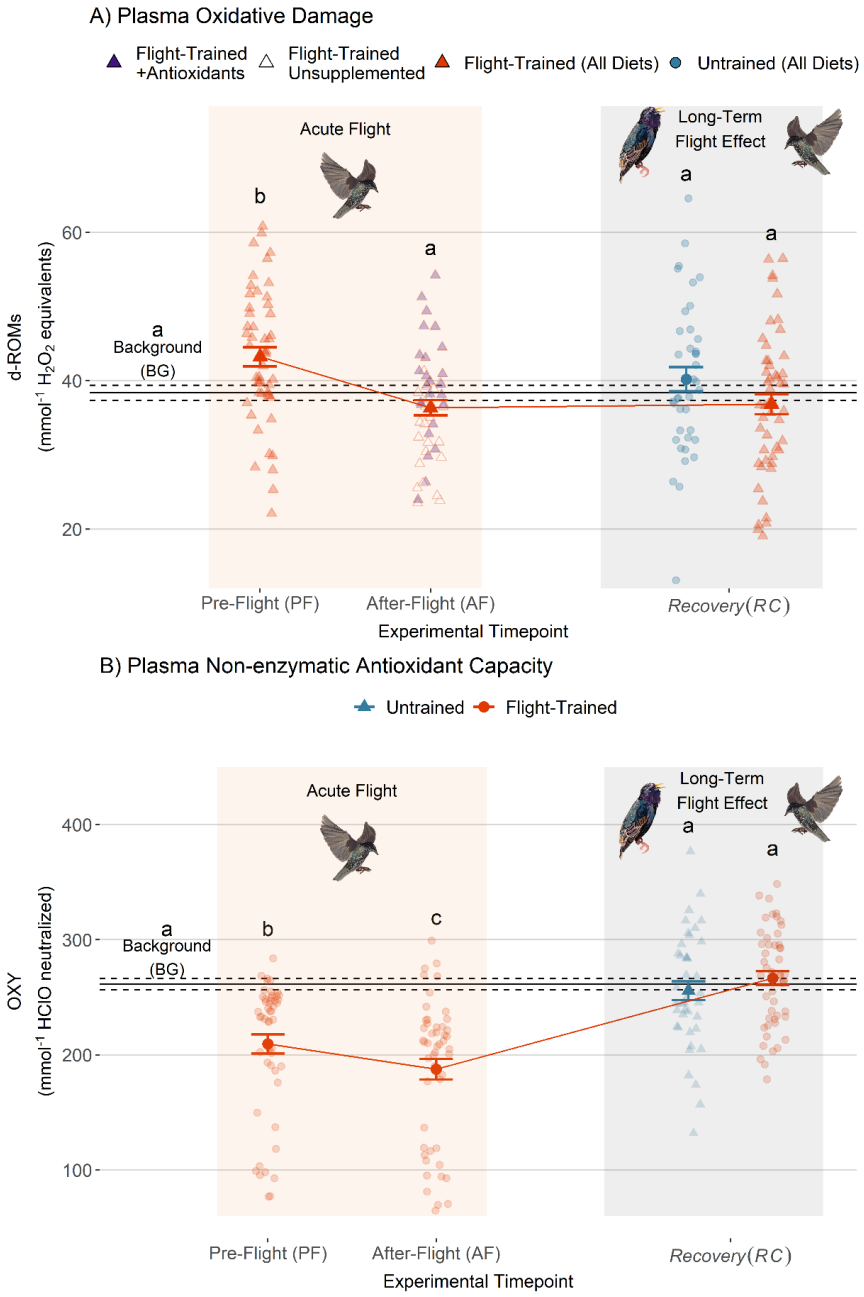


Figure 2

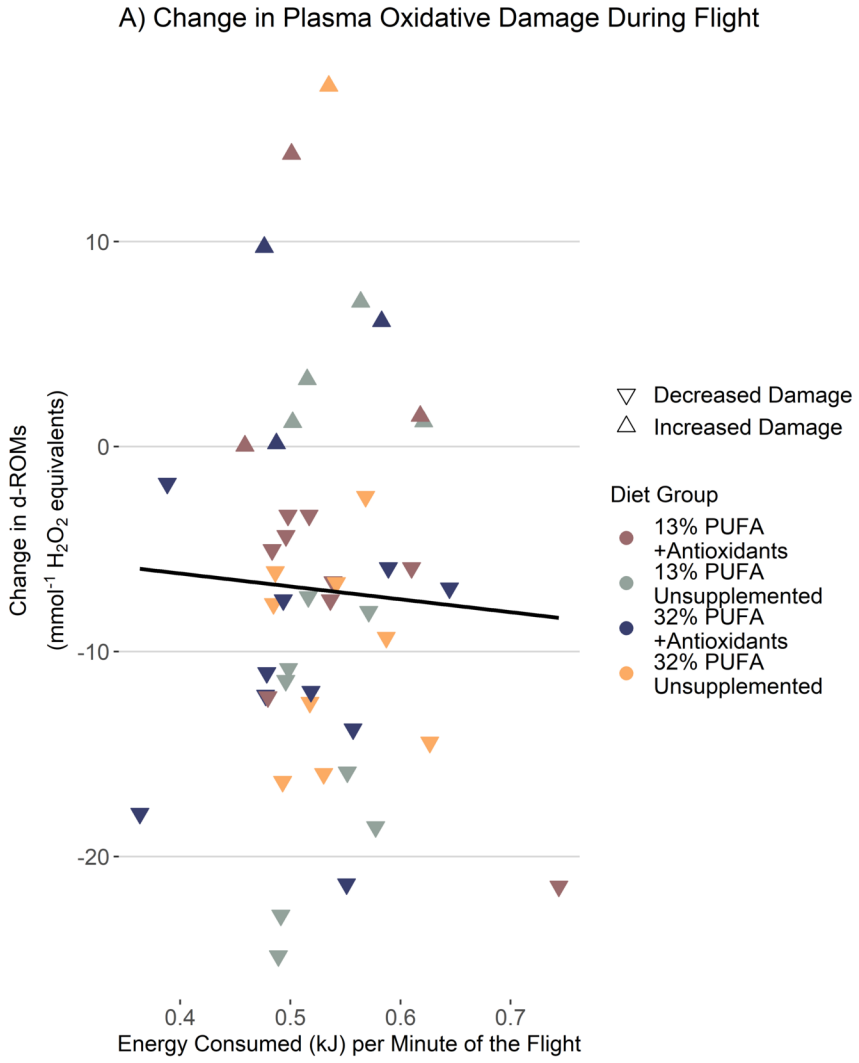
Figure 2. Changes in **A).** oxidative damage (d-ROMs) and**B).** non-enzymatic antioxidant capacity (OXY) in the plasma (means ± standard error) in relation to flight-training. Background (BG) levels of d-ROMs and OXY (solid horizontal line ± standard error, dotted lines) were not significantly different for flight-trained and untrained birds (see text) and so were combined. Dietary antioxidants significantly affected only plasma d-ROMs measured after flight (AF) so we discriminate between the two diets (filled or open triangles) for only this time point. Different lower-case letters for the 4 timepoints (BG, PF, AF, RC) for d-ROMs or OXY denote significant pairwise comparisons (Table S1).

Energy expenditure during flight implicated in regulating OXY depletion but not d-ROMs

Rate of energy expended during the flight (kJ/min) was not related to levels of plasma oxidative damage (R^2=0.12, Table S3; Fig. 3A) but was related to the decrease in non-enzymatic antioxidant capacity during the acute flight (R^2= 0.39, Table S1; Fig. 3B). Only a quarter of birds experienced increased d-ROM levels during an acute flight suggesting that most birds employed a successful antioxidant strategy to avoid the accumulation of d-ROMs at all energy expenditures (Fig. 3A, note direction of arrow; S1C, note labels indicating d-ROM levels). Energy consumption during the flight did not explain AF levels of d-ROMs or the change in plasma d-ROMs during the flight (AF-PF; Fig. 3A, Table S3; AF: T_{46} = -2.018, P = 0.050, Δ During Flight d-ROMs: T_{46} = -1.485, P = 0.149). In contrast, OXY was lowest in birds that consumed more energy during flight (Table S3; AF: T_{46} = -3.133, P = 0.003), and Δ During Flight OXY decreased to the greatest extent in birds expending more energy (Fig. 3B, Table S3; Δ During Flight OXY: T_{46}= -2.821, P = 0.007). This relationship was primarily driven by 7 individuals that decreased OXY by an average of -128 mmol l^{-1} of HClO neutralized (Fig. 3B and Fig. S1C), well below the mean decrease (-22.7 $\pm$ 10.4 mmol l^{-1} of HClO neutralized). These birds expended energy in the upper 50% range (>0.51 kJ/min was consumed by the upper 50% of individuals) (Fig. 3B and Fig. S1C), although other individuals that expended similar amounts of energy during flight did not have such extreme decreases in OXY. Instead, 5 of these birds increased uric acid by an average of 1.4 mmol, well above the mean increase (0.5 +- 0.06 mmol); whereas the remaining birds did not change these circulating measures (OXY, uric acid) from the averages.

Stimulatory effect of flight training across time: Long-term effect of flight-training (H1b)

Circulating oxidative status was similar between untrained and flight-trained birds (Fig. 2A, Table S1; Training: d-ROMs, T_{279} = -0.400, P = 0.690, OXY, T_{279} = 0.623, P = 0.534) at the start and end of the experiment (BG vs RC: d-ROMs, T_{90} = 0.054, P = 0.957, OXY, T_{90}= -0.069, P = 0.945) suggesting that 15 days of flight training did not have substantial long-term effects on plasma oxidative status. However, the change in plasma d-ROMs over time (i.e., the difference from RC to BG) decreased more in flight-trained compared to untrained birds (Table S2; Δ During Training d-ROMs, T_{89} = -2.025, P = 0.046); we detected no such differences in change in OXY over time within individuals in relation to flight training (Δ During Training OXY, T_{89} = 0.982, P = 0.329). These results provide partial evidence for H1b (Long-term effects of flying) as d-ROMs decreased over the course of several weeks of flying within flight-trained birds compared to untrained birds.



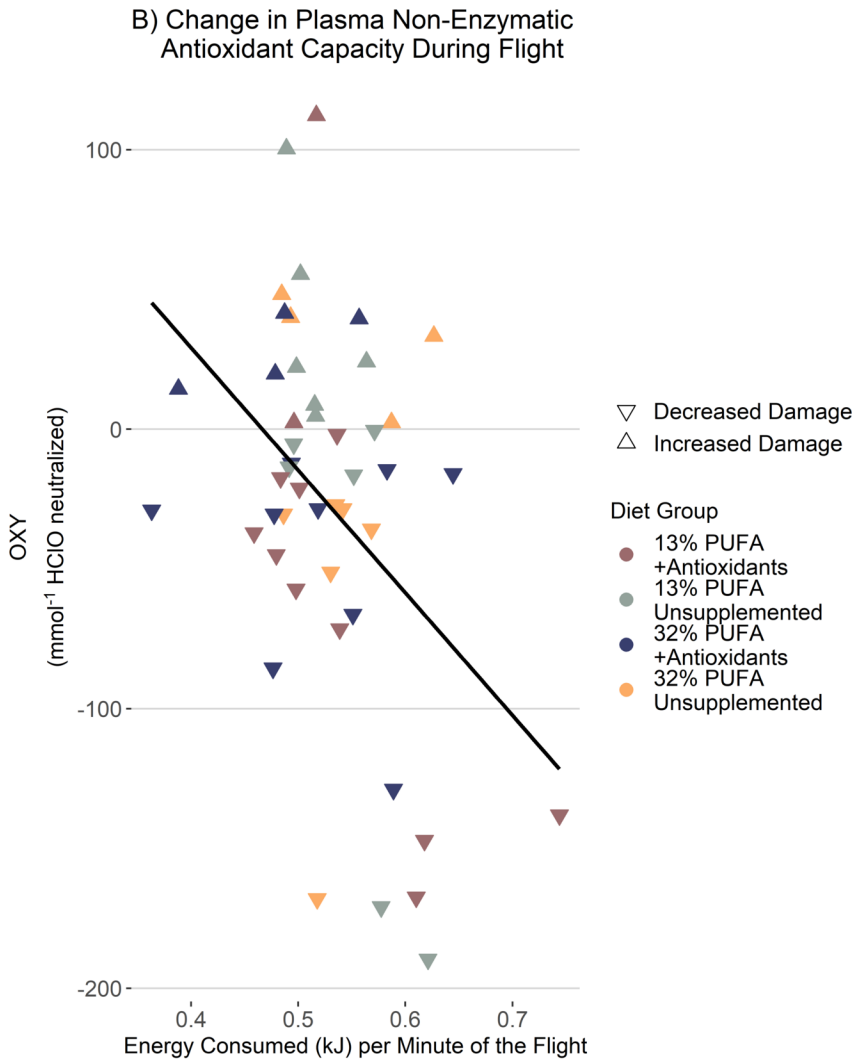


Figure 3.

Figure 3. The change in A). oxidative damage (d-ROMs) and B). non-enzymatic antioxidant capacity (OXY) in the plasma (means ± standard error) (AF-PF) related to the energy consumed during the flight (KJ/min). Birds generally decreased (inverted triangle) d-ROMs and OXY during flight, however, some birds increased (triangle) these measures. The individual variation in change in d-ROMs was not explained by energy consumed during flight. Whereas, birds that expended more energy had larger decreases in OXY however, this was driven by 7 birds that decreased OXY significantly more than the mean and consumed

Stimulatory effect of flight-training across multiple tissues (H1c)

Lipid hydroperoxide concentration in the liver was lowest in flight-trained birds compared to untrained birds at RC (Fig. 4A; $T_{93} = 1.253$, $P < 0.001$). There were no differences in lipid hydroperoxide concentration in the pectoralis (Fig. 4A; $T_{95} = 1.000$, $P = 0.108$) or in plasma d-ROM levels (Table S2; $T_{88} = 2.120$, $P = 0.107$) at RC between flight-trained and untrained birds. Antioxidant capacity was also not affected by 15 days of flight training in the plasma (Table S2; RC, $T_{89} = 1.102$, $P = 0.274$), liver (Fig. 4B, Table S4; Hydroxyl, $T_{92} = -0.744$, $P = 0.459$, Peroxyl, $T_{92} = -1.501$, $P = 0.137$), or pectoralis (Fig. 4B, Table S4; Hydroxyl, $T_{92} = -1.939$, $P = 0.056$, Peroxyl, $T_{96} = 0.683$, $P = 0.496$). These lipid damage results provide

evidence for the stimulatory effect of flight-training (H1c) in the liver but not in the pectoralis or plasma, whereas there is no evidence for such a stimulatory effect on antioxidant capacity.

Dietary fat (H2) and antioxidant (H3) effects on oxidative status

There were no effects of diet fat quality or dietary antioxidants on oxidative status of the liver or pectoralis (Table S4), rather the effect of diet was limited to plasma d-ROMs and associated with acute flight (Fig. 2A, Table S2). Contrary to hypothesis 3, d-ROM levels immediately after acute flight were lower in birds consuming diets unsupplemented with anthocyanins compared to birds supplemented with antioxidants (Table S2; 13% PUFA, Unsupplemented, T_{46} = -2.161, P = 0.036, 32% PUFA, Unsupplemented, T_{46} = -2.594, P = 0.013, 32% PUFA, T_{46} = -1.440, P = 0.586), whereas anthocyanin supplementation did not affect OXY levels during acute flight (Table S2; 13% PUFA, Unsupplemented, T_{46} = 0.492, P = 0.625, 32% PUFA, Unsupplemented, T_{46} = -0.214, P = 0.832, 32% PUFA Supplemented, T_{46} = -0.813, P = 0.421). These results provide no support for H2 or H3 for all three tissues with the exception that dietary antioxidants influenced plasma lipid damage levels after flight.

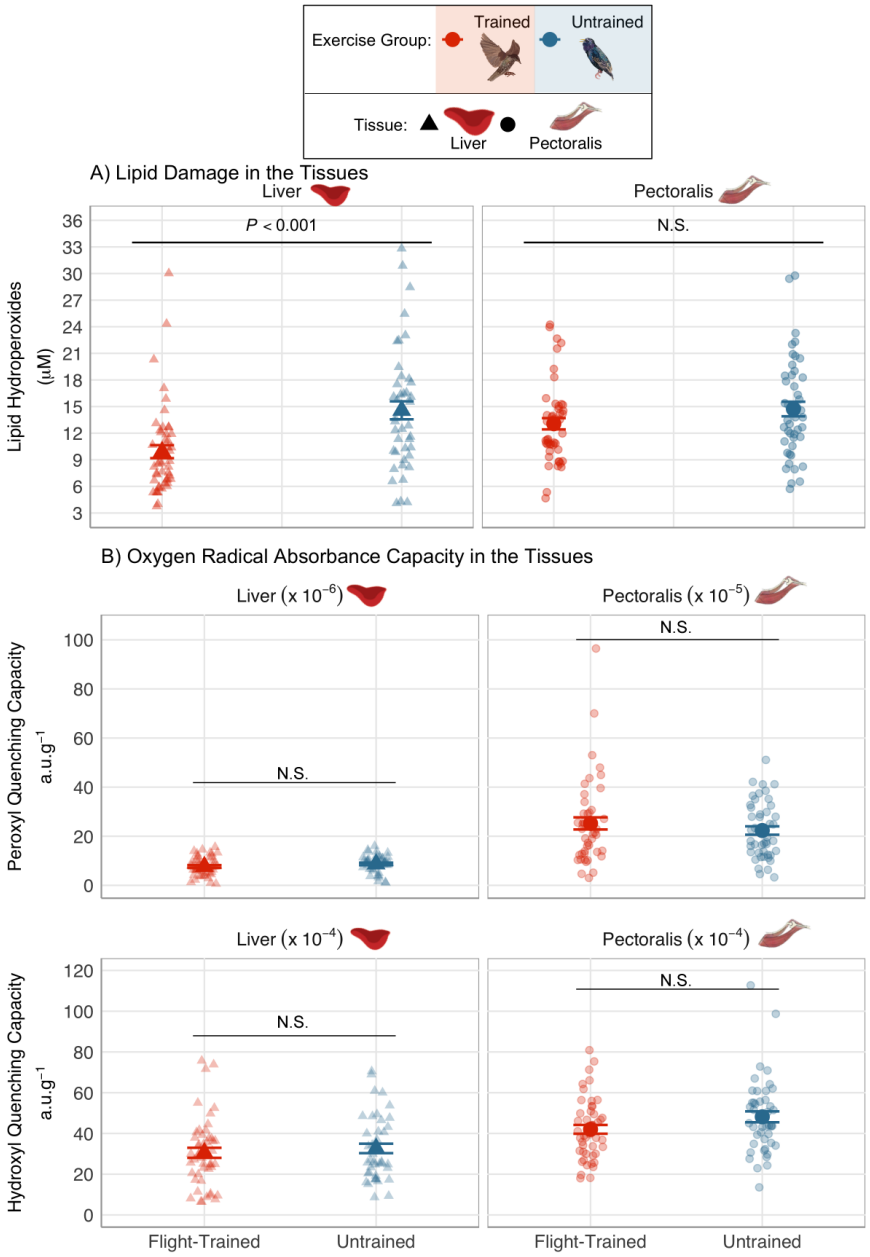


Figure 4.

Figure 4. A). Lipid hydroperoxide concentration and**B).** Oxygen radical absorbance capacity (ORAC) (means ± standard error; Table S4) in the liver and pectoralis muscle of European starlings that were or were not flown in the wind tunnel for 15 days, Flight-trained (N=49) or Untrained (N=40), respectively. Flight-trained birds had lower levels of lipid hydroperoxides in the liver compared to untrained birds but there were no differences in the pectoralis related to flight training. There were no significant differences in hydroxyl or peroxyl scavenging capacities between flight-trained and untrained birds in the liver or pectoralis. Asterisks correspond to significance levels: *p<0.1, **p<0.05 , ***p<0.01

Discussion

Acute- and long-term flight stimulated antioxidant protection (H1)

We found evidence that repeated bouts of flight initiated a hormetic response that activated the antioxidant system to protect against the accumulation of oxidative damage, consistent with H1. This concept of hormesis, the mild exposure to reactive species and subsequent activation of protective and repair mechanisms, has been demonstrated mainly in the skeletal muscle of humans (McArdle et al. 2002, Rattan 2008). In the present study, the immediate, acute effects of a flight included a reduction in both non-enzymatic antioxidant capacity as well as oxidative damage (PF-AF; Fig. 2A, B), consistent with the acute-effects of flight hypothesis (H1a). The coincident reduction in both antioxidant capacity and oxidative damage suggests that birds effectively avoided oxidative damage caused by short-term exercise at least in part by using the non-enzymatic component of their antioxidant system. Similarly, birds exposed to 15 days of flight-training and two days of recovery were able to decrease circulating damage to lipids (RC-BG; Fig. 2A) while untrained birds were not (long-term flight effect, H1b). The apparent over-compensation of the antioxidant system to enable a decrease in oxidative damage during a given flight (H1a) seems novel to this study, as some flight-training studies report no change in markers of circulating damage after an acute flight (Skrip et al. 2016, Cooper-Mullin et al. 2019). Most studies report increases in markers of circulating oxidative damage shortly after a migratory flight in free-living birds (Jenni-Eiermann et al. 2014, Costantini et al. 2018), after a 200 km experimental flight in homing pigeons (Costantini et al. 2008), or in the pectoralis after an experimental flight in a wind tunnel (Dick and Guglielmo 2019). We found that the reduction in both non-enzymatic antioxidant capacity as well as oxidative damage was more apparent in birds that expended more energy per unit time during their longest flight while flying at a prescribed pace (Fig. 3). Perhaps flying birds that expend less energy per unit time are less metabolically challenged, and thus do not need to deplete OXY as much to prevent oxidative damage. This conclusion is similar in concept to (Dick and Guglielmo 2019) who found less pectoralis damage in Yellow-rumped warblers with lower flight energy expenditures.

Consistent with the flight effects proposed in H1b, regular daily flying depleted non-enzymatic antioxidant capacity with an associated decrease in oxidative damage immediately after a flight; although both non-enzymatic antioxidant capacity and oxidative damage returned to baseline levels after 48 hrs of rest (without wind tunnel flying). These results are in accordance with flight-trained zebra finches that also decreased OXY during 2-hrs of acute flight and then increased OXY after birds had a reprieve from regular flying (Cooper-Mullin et al. 2019). This consistent and relatively rapid (no more than a few days) depletion and recovery of non-enzymatic antioxidant capacity suggests that stopovers during migration may be important in allowing birds to maintain their oxidative status over the course of the entire migration (McWilliams et al. 2021). In support of this, plasma d-ROMs decreased with increasing stopover duration (0-8 nights) in Garden warblers*Sylvia borin* , and plasma non-enzymatic antioxidant capacity increased with fat stores accumulated at a stopover site in Blackpoll Warblers *Setophaga striata* and Red-eyed Vireos *Vireo olivaceus* (Skrip et al. 2015). Importantly, after 15 days of exercise training, European starlings exposed to an acute flight further depleted their non-enzymatic antioxidant capacity without an associated increase in oxidative damage. This provides additional evidence that birds during migration seem able to rapidly adjust their antioxidant system to maintain overall low levels of circulating damage.

We also found evidence in the liver and pectoralis that flight training activated the antioxidant system so that after several weeks of daily flying the non-enzymatic antioxidant capacity was maintained at levels similar to those of untrained birds, and oxidative damage was prevented in all tissues (H1c). Repeated bouts of flight apparently protected birds against lipid damage in the liver and the pectoralis (Frawley et al. 2021a), as lipid hydroperoxide concentration was lower or similar in the liver and pectoralis, respectively, of flight-trained birds compared to untrained birds. In our companion study (citation redacted for initial review), the gene expression of *CAT* , *SOD2* , and*GPX1* were upregulated in the liver of flight-trained birds, while only *SOD2* was upregulated in the pectoralis, which likely explains how lower liver damage levels in flight-trained birds was achieved. This enzymatic upregulation combined with lower damage in the liver suggests that the liver is preferentially protected compared to the pectoralis, perhaps to preserve its crucial role in processing

fatty acids during flight or to protect those fatty acids from degradation. There were also no differences detected in plasma OXY or liver or pectoralis hydroxyl and peroxyl scavenging capacities between flight-trained and untrained birds after 48 hrs of rest; thus birds were able to maintain constant long-term levels of antioxidant capacity when energetically challenged and avoid increases in oxidative damage. Although long-term antioxidant capacity in the tissues was unchanged, it is possible that flight-trained birds were able to prevent the accumulation of oxidative damage by utilizing non-enzymatic antioxidant capacity in tissues during flight, as also shown for OXY in the plasma, and by increasing the gene expression of enzymatic antioxidants (citation redacted for initial review).

Dietary fat quality did not affect oxidative status (H2)

Our study does not provide evidence to support H2 that migratory songbirds fed diets composed of more 18:2n-6 are more susceptible to oxidative damage and thus must increase antioxidant capacity compared to when fed diets with less 18:2n-6. This hypothesis was informed by the biochemistry and oxidative susceptibility of PUFA (Halliwell and Gutteridge 2007, Skrip and McWilliams 2016) and the demonstrated responsiveness of the antioxidant system in migratory birds (Jenni-Eiermann et al. 2014, Skrip et al. 2015, Dick and Guglielmo 2019). Additionally, 18:2n-6 has been shown to stimulate antioxidant enzymes in fish (Li et al. 2013, Zengin and Yilmaz 2016) but not in rats (Tou et al. 2011). Dietary long-chain n-3 or n-6 PUFA did not affect oxidative damage or enzymatic antioxidants in the pectoralis of Yellow-rumped warblers (Dick and Guglielmo 2019), and a companion study (citation redacted for initial review) demonstrated that antioxidant gene expression and enzyme activities (GPx, SOD, CAT) did not increase in these same starlings consuming more 18:2n-6. The lack of change in oxidative parameters among diets suggest that dietary 18:2n-6 composition did not oxidatively challenge birds even after flight training. It is possible that a bird's antioxidant system is equipped to combat reactive species associated with consuming any amount of PUFA which would explain the lack of differences among diet groups. For example, White-throated sparrows (*Zonotrichia albicollis*) fed diets with more 18:2n-3 and 18:2n-6 PUFA had higher levels of d-ROMs, but similar ratios of oxidative damage: antioxidant capacity (Alan and McWilliams 2013), and Common blackbirds (*Turdus merula*) caught during migration had higher circulating total n-3 and n-6 PUFAs and had higher non-enzymatic antioxidant capacity and similar damage levels compared to resident birds (Eikenaar et al. 2017, Jensen et al. 2020). Alternatively, Common blackbirds may instead mediate total unsaturation levels in their diet and hence fat stores to minimize the oxidative challenge of consuming more PUFA (Jensen et al. 2020) rather than relying on their antioxidant system for protection. A companion study revealed that increased 18:2n-6 elevated the eicosanoid hormone prostacyclin which mediates potential downstream effects on energetics and aerobic performance, and this could be another alternative explanation for the lack of an effect of dietary PUFA on oxidative parameters (citation redacted for initial review). In sum, lipid peroxidation likely remains a relevant challenge for migratory songbirds that rely on 18:2n-6 and other fats to fuel migratory flights (Pierce et al. 2004, Pierce and McWilliams 2005, Price et al. 2008, Smith and McWilliams 2010), and birds seem able to modulate their antioxidant system in response to the oxidative challenges fats pose and thereby successfully protect against damage. Whether or not these adjustments to the antioxidant system have metabolic tradeoffs or tradeoffs with immunity remains unknown (Costantini 2019). Thus, future studies that compare the effect of different amounts of dietary 18:2n-6 on a migratory bird's physiological status, (e.g. oxidative, metabolic, and immune statuses) will better elucidate the direct effects of dietary 18:2n-6 on the potential tradeoffs among physiological systems.

Dietary antioxidants increased plasma lipid damage after flight (H3)

We also did not find support for H3 that migratory songbirds fed dietary anthocyanins have increased non-enzymatic antioxidant capacity, and lower levels of lipid damage in all three tissues compared to songbirds not fed anthocyanins. Anthocyanin supplementation did not affect non-enzymatic antioxidant capacity in the plasma or the two metabolic tissues. The same dietary anthocyanin concentration positively affected immune function in European blackcaps (Catoni et al. 2008, Schaefer et al. 2008), attenuated the cortisone response to flight in European starlings (Casagrande et al. 2020), increased testosterone levels and breeding behaviors in male European starlings (Carbeck et al. 2018), and affected enzymatic antioxidant activity and

lipid damage in the heart, non-enzymatic antioxidant capacity in the pectoralis, and protein damage in leg muscle (Frawley et al. 2021a). Thus, we were surprised to find that the same concentration of anthocyanins did not directly affect antioxidant capacity as measured in this study. Perhaps when dietary anthocyanins are available they are used in a tissue-specific manner for these other functions (immunity, metabolic protection) rather than directly for reactive species mitigation which then may allow any energy-cost savings to be invested in other aspects of the antioxidant system including, for example, enzymatic antioxidants or glutathione (the precursor to GPx) that are not detected in the OXY measurement. There is considerable cross-talk among inflammatory, immune, metabolic, and antioxidant pathways so that simultaneously measuring these multiple pathways seems necessary to understand potential trade-offs in response to dietary antioxidants (Costantini 2019).

Contrary to hypothesis 3, we found that d-ROM levels immediately after acute flight were lower in birds consuming diets without anthocyanins compared to birds supplemented with antioxidants. There are two potential explanations for this result. First, dietary anthocyanins prevented the accumulation of reactive species used to stimulate the endogenous antioxidant system, resulting in more oxidative damage. This preventative effect of consuming daily antioxidants (vitamin E and C in this case) decreased the expression of antioxidant enzymes and transcription factors in the skeletal muscle of humans (Ristow et al. 2009, Merry and Ristow 2016). Second, birds not consuming anthocyanins were more oxidatively challenged during flight which required them to upregulate endogenous antioxidants in red blood cells to reduce circulating damage. The circulating enzymatic antioxidant system can respond rapidly; for example, GPx activity increases in European robins during nocturnal migration and in zebra finches during experimentally-imposed 2-hr daily flights (Jenni-Eiermann et al. 2014, Cooper-Mullin et al. 2019), respectively. Given that water-soluble antioxidants such as anthocyanins are likely utilized as they are metabolized rather than stored for later use, as are lipid-soluble dietary antioxidants (Halliwell and Gutteridge 2007), perhaps this short half-life may be why we detected an antioxidant effect in the plasma in direct response to an acute oxidative challenge rather than in plasma or tissues after recovery from flight. We provide evidence that dietary anthocyanins influenced oxidative damage after an energetic challenge, yet to untangle the mechanistic underpinnings of this relationship, future studies that measure multiple antioxidant classes and evaluate the crosstalk among various physiological pathways are required.

Relevance and significance

The multifaceted antioxidant system consists of numerous components that collectively provide individuals with a tool-box of 'antioxidant strategies' they can use to protect against oxidative damage (Halliwell and Gutteridge 2007, Costantini 2014). Migratory birds can use a combination of non-enzymatic antioxidants gained from their diets and a suite of endogenous antioxidant enzymes and sacrificial molecules to protect against excess reactive species produced by relevant oxidative challenges, including repeated bouts of flight and high-PUFA diets (Skrip and McWilliams 2016, McWilliams et al. 2021). This study demonstrated the ability of a songbird to use OXY during an acute flight and to recover OXY levels within 2 days of rest (Fig. 2), revealing the recovery timeline of antioxidant capacity for the first time (McWilliams et al. 2021). We also found that such rapid adjustments to the antioxidant system allowed birds to maintain overall low levels of circulating damage, although the extent was tissue-dependent. Researchers should remain mindful of the tissue-dependency of antioxidant capacity and oxidative damage demonstrated here and elsewhere (Frawley et al. 2021a) and that future studies that assess oxidative status select biologically relevant tissues to measure that directly relate to the hypotheses of interest.

Individual variation in the ability to utilize antioxidants must exist for phenotypic flexibility of the antioxidant system to persist (Piersma and Van Gils 2011) and we found evidence of such individual-level, condition-dependent modulation of OXY and uric acid during long-duration flight (Fig. S1). A subset of birds that expended energy in the upper 50% range (>0.51 kJ/min) seemed to either deplete OXY or increase levels of uric acid, a potent antioxidant (Fig. 3B and Fig. S1C), whereas the remaining birds did not change these circulating measures (OXY, uric acid) from the averages. Studies, such as those done with mosquitofish (Loughland and Seebacher 2020), that elucidate the antioxidant strategies used by individuals

to maintain their oxidative balance under oxidatively challenging conditions and its effects on performance (e.g. temperature acclimation, running speed, breeding success, flight efficiency) would be particularly revealing for migratory songbirds.

The study described here was part of a larger integrated research project that also compared metabolic and antioxidant gene expression and enzyme activities in multiple tissues within the same individuals (citations redacted for review). This allows us to compare how flight training, dietary antioxidants, and dietary PUFA affect the antioxidant system across the traditional scales of the Central Dogma (DNA, to RNA, to protein). All measures except oxidative status in tissues responded strongly to flight training. In the plasma, proteins are subject to posttranslational modifications and so better able to optimal to detect individual strategies to combat oxidative damage in response to flight exercise. In contrast, diet effects on oxidative status were most evident in tissues (i.e., relative gene expression and antioxidant assays) compared to functional assays in the plasma (i.e., markers for antioxidant capacity and oxidative damage) perhaps because the former is more tightly regulated. Our studies also suggest that plasma oxidative capacity is not a good predictor of oxidative damage in tissues, whereas antioxidant and metabolic gene expression and antioxidant enzyme activities are significant predictors of oxidative damage in the pectoralis and liver (citation redacted for initial review). Thus, future studies must carefully consider the appropriate scale of measurement(s) to best reveal how the antioxidant system of wild animals responds to ecologically-relevant oxidative challenges such as migration.

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