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THE FREQUENT FLYER'S GUIDE TO MIGRATION: HOW
SONGBIRD METABOLISM AND OXIDATIVE STATUS
RESPOND TO ENDURANCE FLIGHT
AND DIET QUALITY
BY
KRISTEN J. DEMORANVILLE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
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UNIVERSITY OF RHODE ISLAND

2020

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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UNIVERSITY OF RHODE ISLAND
2020

ABSTRACT

Migratory songbirds are among the best high-performance endurance athletes on the planet and during their annual peregrinations they are particularly vulnerable to oxidative damage because they maintain relatively high metabolic rates at rest, they are able to fly for long durations while operating at 9 times their resting metabolic rate, and they rely on fats to fuel this exercise which increases reactive oxygen species (ROS) formation. The concept of evolutionary economic design of physiological systems considers such phenotypic flexibility in metabolic and antioxidant responses adaptive in that it allows birds to match the capacity of key physiological systems to prevailing demands and then modulate this capacity as demands change and so provide adequate but not excessive spare capacity at any given time. In addition to energetic demands associated with flying, this phenotypic flexibility is also modulated by environmental factors including diet. During migration, many songbirds consume a berry-heavy diet that is both rich in certain polyunsaturated fat that are known to provide energy savings at a longer-term oxidative cost, and rich in specific water-soluble antioxidants that protect against these metabolic costs. What remains unknown is how regular flight and diet (i.e. fat and antioxidants) influence molecular pathways involved in fat metabolism and antioxidant protection to modulate upper levels of phenotypic flexibility (e.g. metabolic rates, antioxidant defenses) in migratory songbirds. Our study investigated how these relevant ecological factors affected key metabolic and antioxidant transcription factors and their target genes (Chapters 1 and 2, respectively) to modulate the antioxidant defense system (Chapter 3). Molecular and phenotypic flexibility driven by flight and

diet would allow songbirds living in shifting environments to track environmental change, whether natural or anthropogenic.

We employed a rigorous, ecologically-relevant experimental design to determine how metabolic (Chapter 1) and endogenous antioxidant (Chapter 2) pathways and oxidative balance (Chapter 3) in songbirds during the migration period responded to 2 weeks of flight training in a wind tunnel, as well as a factorial combination of dietary polyunsaturated fatty acids (18:2n-6 PUFA) and water-soluble antioxidants (anthocyanins). European starlings (*Sturnus vulgaris*) were fed diets composed of either a high or low 18:2n-6 PUFA and supplemented with or without anthocyanins, and half of these birds were flight-trained in a wind-tunnel while the rest were untrained. In Chapters 1 and 2, we tested specific hypotheses related to the molecular flexibility in the flight-muscle and liver that occurs in response to flight training, dietary 18:2n-6 PUFA, and dietary anthocyanins. In Chapter 1 we measured the expression of 7-10 key genes involved in fat metabolism in flight-muscle and liver, and in Chapter 2 we measured 8 key antioxidant genes in these same two tissues. In Chapter 3, we tested specific hypotheses related to the phenotypic flexibility of the antioxidant defense system in response to flight training, 18:2n-6 PUFA, and dietary anthocyanins. We measured antioxidant capacity and oxidative damage levels in the flight-muscle, liver, and plasma of flight-trained and untrained birds over the course of the flight training and during and acute flight.

The causal network analyses that we conducted as part of these studies suggest that transcription factors implicated in regulating metabolism (PPARs) and antioxidants (NRF2, PPARs) in mammals also regulate the selected genes involved in fat metabolism

(Chapter 1) and antioxidant protection (Chapter 2) in migratory songbirds. Furthermore, flight training modulated the causal network between metabolic genes involved in fat metabolism more than did diet (Chapter 1). In addition, our studies revealed that the energetic challenge posed by daily flights modulated metabolic and antioxidant gene expression profiles and oxidative damage levels in a tissue-dependent manner. For example, we found that flight training increased the expression of half of the metabolic genes measured in the pectoralis but not in the liver (Chapter 1), and that flight training increased 40% of the antioxidant genes measured in the liver but not in the pectoralis (Chapter 2). In addition, after two weeks of flight-training, birds maintained antioxidant capacity and oxidative damage levels similar to untrained birds in the pectoralis and plasma and reduced damage in the liver (Chapter 3). These tissue-specific differences in response to flight training may be related to functional differences between tissues as well as fundamental differences in their turnover rates. Furthermore, we demonstrated in Ch. 3 that the variation between individuals in the rate of energy expenditure (kJ/min) during longer (ca. 3-hr) flights was related to the extent to which birds modulated their circulating non-enzymatic antioxidants (OXY, uric acid) during the flight. Taken together, these results suggest that songbirds modulate their metabolic and antioxidant pathways in a tissue-specific manner when faced with an energetic challenge, and they employ condition-dependent antioxidant strategies that depend on the degree of that challenge.

Nutrient content of the diets (i.e. dietary fat and antioxidants) had a more selective effect on metabolism and oxidative status in our study compared to flight training. In Chapter 1, we found that birds fed more 18:2n-6 PUFA (and thereby less 16:0) increased

the expression of only two genes, one in the pectoralis that is involved in the hydrolysis of circulating triglycerides (LPL, dependent on flight training) and the expression of a metabolic transcription factor (PPAR α) in the liver. In contrast, expression of a gene involved in fat transport across the muscle membrane was higher in birds fed less 18:2n-6 PUFA. In addition, birds that consumed more dietary anthocyanins increased the expression of two antioxidant enzyme genes in the pectoralis (CAT, SOD1; Chapter 2) and had higher circulating levels of oxidative damage immediately after an acute flight (Chapter 3). Thus, dietary anthocyanins have a tissue-dependent stimulatory or inhibitory effect on the antioxidant system. Considered together, these results suggest that dietary fats have a selective signaling role in muscle and liver metabolism and that dietary anthocyanins have multiple roles that depend largely on the metabolic state of both the organism and their various tissues.

Birds during migration undergo regular, often daily flights interrupted by often longer periods at stopover sites as they travel between breeding and wintering areas. Migrating songbirds are quite selective in what they eat, and these diet choices directly affect their supply of nutrients and energy. Our experiment has revealed that molecular-level metabolism is modulated by flight training and dietary fat quality and that molecular-level antioxidant pathways are modulated by flight training and dietary anthocyanins in a migratory songbird. The implication is that these same environmental factors (i.e., flying, dietary fat quality and antioxidants) will affect the migratory performance of birds in the wild. Flight- and diet-mediated molecular flexibility in the endogenous antioxidant system seems to regulate upper level phenotypic flexibility in the antioxidant defense system, as acute flight stimulates the antioxidant system and protects

birds from the accumulation of oxidative damage. Our study demonstrates that metabolic and antioxidant flexibility in songbirds is driven by energetic demands and ecological factors associated with migration, yet how the timing and seasonal availability of these factors initiate and maintain these types of molecular and physiological phenotypes remains unknown. Thus, migratory birds possess the ability to track and respond to environmental change relatively quickly (within 2 weeks), yet it is unknown whether birds can adjust their physiology at rates that match the rapidly and unpredictably shifting environments experienced during the Anthropocene.

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I kindly thank my committee members Barbara Pierce, Hollie Putnam, and Navindra Seeram for being powerful role models and for thoughtfully and constructively criticizing my science.

I wouldn't have been able to finish this degree without Clara Cooper-Mullin acting as my friend and colleague. There aren't nearly enough pages to thank her for her constant advice, figure wizardry skills (take *that*, Harry Potter), and her contagious laugh. Many thanks to the rest of my lab friends for being there for me, especially those who worked alongside me making gourmet bird food and sitting in dark wind tunnels: Wales Carter, Keara Bohannon, Luke Douglas, Katherine Carbeck, Olivia Fatica, Liam Corcoran, Clay Graham, Steve Brenner, Dustin Meattey, Juliet Lamb, Megan Skrip, Martina Muller, Erin Harrington, Abby Frawley, Colby Slezak.

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PREFACE

This dissertation is using the Manuscript Format. Each of the three chapters are formatted to their specified target journals. Chapter 1 has been accepted in the *American Journal of Physiology*, Chapter 2 has been submitted to *PLOS Biology*, and Chapter 3 will be submitted to *Functional Ecology*.

TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS	vi
PREFACE.....	viii
TABLE OF CONTENTS	ix
LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER 1	1
ABSTRACT.....	2
INTRODUCTION	4
METHODS	9
RESULTS	18
DISCUSSION	21
PERSPECIVES AND SIGNIFICANCE	32
REFERENCES	35
CHAPTER 2.....	68
ABSTRACT.....	69
INTRODUCTION	71
METHODS	77
RESULTS	86
DISCUSSION	91
RELEVANCE AND SIGNIFICANCE	98
REFERENCES	101

CHAPTER 3.....	137
ABSTRACT.....	138
INTRODUCTION	139
METHODS	144
RESULTS	155
DISCUSSION	160
RELEVANCE AND SIGNIFICANCE	167
REFERENCES	170

LIST OF TABLES

TABLE	PAGE
Table 1.1 Key symbols used in manuscript 1.	53
Table 1.2 Piecewise-structural equation models for gene expression in the pectoralis..	54
Table 1.3. Linear model results for pectoralis metabolic gene expression	55
Table 1.4. Linear model results for liver metabolic gene expression	56
Table 2.1. Key symbols used in manuscript 2	116
Table 2.2 Linear model results for liver antioxidant gene expression.	117
Table 2.3 Linear model results for pectoralis antioxidant gene expression.....	118
Table 2.4. Linear model results for antioxidant enzyme activities	119
Table 2.5. Main effect of experimental cohort on antioxidant enzyme activities.....	120
Table 3.1. Linear model results for antioxidant indices and oxidative damage.....	179

LIST OF FIGURES

FIGURE	PAGE
Figure 1.1. Relative metabolic gene expression in the pectoralis muscle.	57
Figure 1.2. Relative metabolic gene expression in the liver.	58
Figure 1.3. Relative LPL and CD36 expression by dietary fat	59
Figure 1.4. The causal structure to explain the non-hierarchical gene expression of PPAR pathway	61
Figure 2.1. Relative antioxidant gene expression in the liver and pectoralis by flight-training.	121
Figure 2.2. Antioxidant enzyme activities by flight-training.	123
Figure 2.3. Relative antioxidant gene expression in the liver and pectoralis by dietary antioxidants	125
Figure 2.4. The causal structure to explain the hierarchical gene expression of antioxidant pathways in the liver	127
Figure 2.5. The causal structure to explain the hierarchical gene expression of antioxidant pathways in the pectoralis	129
Figure 3.1. Experimental timeline schematic.	180
Figure 3.2. Time course changes plasma in oxidative status.	182
Figure 3.3. Change in oxidative status during acute flight	184
Figure 3.4. Oxidative status in the liver and flight-muscle.....	186

CHAPTER 1

FLIGHT TRAINING IN A MIGRATORY BIRD DRIVES METABOLIC GENE EXPRESSION IN THE FLIGHT MUSCLE BUT NOT LIVER, AND DIETARY FAT QUALITY INFLUENCES SELECT GENES

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In press

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ABSTRACT

Training and diet are hypothesized to directly stimulate key molecular pathways that mediate animal performance, and flight-training, dietary fats, and dietary antioxidants are likely important in modulating molecular metabolism in migratory birds. This study experimentally investigated how long-distance flight-training as well as diet composition, affected the expression of key metabolic genes in the pectoralis muscle and the liver of European starlings (*Sturnus vulgaris*, N=95). Starlings were fed diets composed of either a high or low polyunsaturated fatty acid (PUFA; 18:2n-6) and supplemented with or without a water-soluble antioxidant, and half of these birds were flight-trained in a wind-tunnel while the rest were untrained. We measured the expression of 7 (liver) or 10 (pectoralis) key metabolic genes in flight-trained and untrained birds. Fifty percent of genes involved in mitochondrial metabolism and fat utilization were upregulated by flight-training in the pectoralis ($P<0.05$), whereas flight-training increased the expression of only one gene responsible for fatty acid hydrolysis (LPL) in the liver ($P=0.04$). Dietary PUFA influenced the gene expression of LPL and fat transporter CD36 in the pectoralis and one metabolic transcription factor (PPAR α) in the liver, whereas dietary antioxidants had no effect on the metabolic genes measured in this study. Flight-training initiated a simpler causal network between PGC-1 coactivators, PPARs, and metabolic genes involved in mitochondrial metabolism and fat storage in the pectoralis. Molecular metabolism is modulated by flight-training and dietary fat quality in a migratory songbird indicating that these environmental factors will affect the migratory performance of birds in the wild.

Keywords

metabolic gene expression, peroxisome proliferator-activated nuclear receptors, flight training, dietary fat quality, antioxidants

Introduction

Coordinated metabolic increases are required for endurance exercise

Feats of endurance are common throughout the animal kingdom (77, 81, 93, 116), and these energetically demanding feats are associated with increased metabolic capacity that can be achieved through exercise training (24, 121) or are initiated endogenously by time of year (31, 98, 125). Migrations that require intense intermittent flight have the highest energetic costs compared to aquatic and terrestrial migrations (45, 116) - for example, long-distance flight requires birds to operate at 9 times above their basal metabolic rates (12) whereas elite human athletes riding in the Tour de France only operate at 4.3 times their basal metabolic rates (49).

Physiological alterations that increase energetic capacity during migration in birds include increased oxidative enzyme activities (4, 46) and fatty acid transport and oxidation in the pectoralis (69), hypertrophy/hyperplasia of the pectoralis to provide increased power for sustained flight (26, 31, 66, 70, 91), and thus enhanced basal and summit metabolic rates (22, 26, 117). The liver, an organ important in fuel processing, is nearly double the size during migration compared to wintering periods in select songbird species and liver fatty acid synthesis increases during pre-migratory and migratory periods (45). Recent mostly correlative studies demonstrate that pathways linked to fatty acid metabolism (28) including genes responsible for regulating metabolism (PPAR γ , PPAR α), and key genes responsible for fat transport (FABPpm, CD36, H-FABP) and fat oxidation (ATGL, LPL, MCAD) (21, 26, 71, 129) are coincidentally upregulated during migration (120). Although such metabolic adjustments suggest a possible coordinated upregulation on both molecular and biochemical (e.g. enzymatic) levels in birds during

migration, the direct effect of flight training and key aspects of diet (e.g., fat quality, antioxidants) on the expression of metabolic genes and regulatory factors remains largely unexplored.

PPARs regulate metabolism in a wide variety of organisms

Peroxisome proliferator-activated nuclear receptors (PPARs) are transcription factors that act as key regulators of fatty acid oxidation (102), glucose catabolism (63), enzymatic antioxidants (59), and the production of reactive oxygen species (129) and so may be important for birds which primarily rely on fat for fuel and must contend with production of lipid peroxides (20, 112). There are three PPAR isoforms, PPAR γ , PPAR δ , and PPAR α , that are all activated by fatty acid ligands. The binding of fatty acid ligands to PPAR binding sites recruits coactivators (e.g PGC-1 α , PGC-1 β) to bind to the PPAR complex and increases PPAR activity to initiate the transcription of specific metabolic genes involved in lipid transport (fatty acid binding protein, FABPpm; fatty acid transport protein 1, FATp1; fatty acid translocase, CD36), lipolysis (lipoprotein lipase, LPL; perilipins, Plin), mitochondrial fatty acid oxidation and respiration (medium-chain acyl-CoA dehydrogenase, MCAD; citrate synthase, CS), and mitochondrial proton carrying (UCP) (7, 10, 35, 62, 101, 123). Mammalian studies that directly manipulate PPAR expression show that these receptors are crucial for maintaining metabolic homeostasis (123), as dysregulation of these receptors leads to metabolic disorders including insulin resistance, glucose intolerance, and type 2 diabetes (7, 101, 123). The roles of these transcription factors and their metabolic genes are likely preserved in wildlife since PPAR sequences are highly conserved across humans, mice, bats, ground squirrels, fish, birds. In addition, PPARs respond to fatty acid ligands and drug agonists

and antagonists in vitro in Atlantic salmon, *Salmo salar* (105), and Grey catbirds, *Dumatella carolensis* (48) and in vivo in Syrian hamsters, *Mesocricetus auratus* (17) and Bobwhite quail, *Colinus virginianus* (80). Although PPARs have been shown to respond to certain dietary polyunsaturated fatty acids in Atlantic salmon (56), to the migration season in Grey catbirds (26), and during hibernation in Syrian hamsters (17), the specific roles of PPARs in regulating metabolism in birds during migration remain largely unknown.

Fat ligands and exercise stimulate PPARs

PPAR ligands include mono- and poly-unsaturated fatty acids (MUFA, PUFA) and fatty acid metabolites, eicosanoids (39, 54, 60), and a variety of fatty acids that have been shown to activate PPAR isoforms in mammals, Atlantic salmon, and Grey catbirds (48, 54, 56, 60). In Gray catbird myocytes the fatty acids 16:0, 18:0, 18:1n-9 MUFA, 18:2n-6 PUFA activated PPAR α , only 18:1n-9 MUFA significantly activated PPAR δ , and only 20:5n-3 PUFA activated PPAR γ in kidney cells (48). In the pectoralis of another migratory songbird, PPAR δ expression and oxidative enzyme activities were higher in the pectoralis of Yellow-rumped warblers containing relatively less 22:6n-3 PUFA in their muscle phospholipids, fat stores, and circulating lipids suggesting that MUFAs and shorter chain PUFAs are important for regulating relative mRNA abundance of PPARs (29). Specifically, 18:2n-6 PUFA is considered a strong candidate for stimulating metabolism in songbirds that preferentially consume this fatty acid during migration due to its properties as a high-affinity ligand for PPARs (48), the greater ability to produce eicosanoids (PPAR ligands) from higher concentrations of dietary 18:2n-6 PUFA (6), and on the enhanced energetic efficiency demonstrated by animals consuming dietary 18:2n-6

PUFA (14, 56, 73, 122). Exercise may also indirectly stimulate PPAR activity by increasing circulating ligands and by increasing the activities of PPAR transcriptional coactivators, like PGC-1 α (3, 119). An eight-week cycling training regime in humans demonstrated that exercise training increased plasma PPAR ligands, PPAR γ activity, and PPAR target gene expression (CD36, LXR α , ABCA1) within three hours post-exercise (119). Similarly, the PGC-1 coactivators increased two-fold within 18 hours of a single bout of swimming exercise in rats, although the effect of PGC-1 expression was not examined alongside PPARs in this study (3). PPAR expression is not as responsive to a single bout of exercise compared to its PGC-1 coactivator (115), suggesting that PPAR transcriptional activity may have a greater response time (more than 30 minutes post-exercise), or PPAR upregulation may require repeated bouts of exercise that amounts to an endurance training regime. Consistent with this, four weeks of regular exercise increased the mRNA expression of PGC-1 α , PGC-1 β , PPAR γ , and antioxidant enzyme target genes in human skeletal muscle (103). It remains unknown how repeated bouts of flight, like that experienced by birds during migration, affects PPARs and their metabolic genes, nor how dietary fat quality influences this metabolic response to endurance flight training.

Antioxidants attenuate PPAR activity

Metabolic increases are required to meet the energetic demands of exercise but increases in aerobic respiration rates can be costly in terms of an increased production of reactive oxygen species (ROS), unstable oxygen-containing molecules that can damage other cellular components. Exercising organisms can upregulate their antioxidant system to combat these reactive molecules and thereby avoid damage to tissues and substrates

(20, 112). Interestingly, PPAR γ and PPAR δ regulate antioxidant enzyme gene expression, and an upregulation of these PPAR isoforms cause increases in the antioxidant enzyme activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) in mammalian cardiac tissue (59) and in human skeletal muscle (103). PPAR pathways are less activated in skeletal muscle when humans are consuming daily antioxidants, vitamin E and vitamin C (76, 103), suggesting that the upregulation of these endogenous enzymes are not necessary when organisms increase their antioxidant capacity by consuming dietary antioxidants. Certain songbird species select fruits high in antioxidants during migration, suggesting that antioxidant consumption may be important to protect against oxidative damage during this life history stage in these species (1, 11, 113). Although there is evidence in mammals that PPARs regulate antioxidant enzymes and respond to dietary antioxidants, the extent to which PPARs are affected by antioxidants in birds is unknown.

How do dietary fat, flight training, and dietary antioxidants affect metabolic gene expression?

The goal of this study was to identify how the expression of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes respond to diet and flight training in an omnivorous migratory songbird that opportunistically feeds on fruit, the European starling (*Sturnus vulgaris*). Migratory songbirds are an ideal natural system to study how metabolism on a molecular level responds to endurance training, dietary fat, and dietary antioxidants since they undergo endurance flights biannually and switch to diets heavy in berries that are rich in fats and antioxidants during their fall migration (1, 11). This study experimentally investigated how flight training in a wind tunnel as well as

dietary fats (18:2n-6 PUFA) and antioxidant composition (water-soluble anthocyanins), affected the expression of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes in the pectoralis muscle and the liver. This study tested the following four hypotheses: 1 (Flying effect). Flight training increases metabolic capacity in migratory songbirds, and thereby increases the expression of (a) PPAR coactivators, including PGC-1 α and PGC-1 β , and (b) these metabolic increases related to flight-training are associated with a coordinated upregulation of PPARs and (c) their metabolic genes (genes that code for fatty acid transporters, fatty acid oxidation, and oxidative enzymes). 2 (Dietary fat effect). Migratory songbirds fed diets composed of more 18:2n-6 PUFA have increased expression levels of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes compared to when fed diets with less 18:2n-6 PUFA. 3 (Flight x dietary fat interaction). Flight training and dietary 18:2n-6 PUFA further stimulate expression levels of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes in migratory songbirds. 4 (Dietary antioxidant effect). Migratory songbirds not fed dietary antioxidants have increased expression levels of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes compared to birds supplemented with dietary antioxidants.

Methods

Experimental Design

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, before fall migration, between 19-23 August 2015. The experiment was conducted between August and December 2015 as part of a larger study examining the

exercise physiology of migratory songbirds. Starlings from this wild population are considered partially migratory as inferred by banding records (13). 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; (42)) for each individual. Birds were then randomly sorted into four groups with roughly equal distributions of body size and molt score. We maintained aviaries at 21°C on a natural light cycle from capture and 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). Samples brought from Canada to the U.S.A. were authorized by USDA (Import permit 129139).

Experimental Diets

Birds had *ad libitum* access to one of two semi-synthetic diets that had the same macronutrient content as fruit and differed only in fatty acid composition. The macronutrient composition of the two semisynthetic diets (41% carbohydrate, 13% protein, 30% fat) simulates a natural high-lipid fruit diet (51, 113) and the primary fatty acids in the diet (>90% 16:0, 18:1, and 18:2) are also the most common fatty acids in natural fruits (89) and in songbirds that eat fruits during their migration (88, 89). 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 (29, 39, 48, 55). The complete list of diet ingredients and amounts have been published previously (Table 1 in (14)). Starlings in two aviaries received a 13% 18:2n-6 diet and two others received a 32% 18:2n-6 diet (hereafter referred to as 13% PUFA and 32% PUFA, respectively). As expected, the two diets produced reliable differences in tissue fatty acid composition of starlings (Fig. 5 in (14)). On September 1, we began adding a supplementary water-soluble antioxidant, anthocyanin (elderberry powder; Artemis International, Fort Wayne, IN) to the diets of one 13% PUFA aviary and one 32% PUFA aviary, producing a 2 X 2 factorial diet manipulation with four diet groups: 13% PUFA low anthocyanin (N = 23), 13% PUFA high anthocyanin (N = 23), 32% PUFA low anthocyanin (N = 21), and 32% PUFA high anthocyanin (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* (16, 107). The anthocyanin supplement was equal to eating 2.8 mg per day which is equal to approximately 17 berries per day based on an average daily synthetic diet consumption of 35 wet g day⁻¹ (as observed in food intake trials in this study).

Experimental Timeline

On September 21st we randomly assigned the starlings to twenty cohorts (5 cohorts per diet group) of 5 individuals each in descending order of molt score (i.e., from most to least advanced in molt). On September 23rd, and continuing every three days thereafter (Complete timeline in Appendix A: Fig. A1 in (14)), 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 placed in individual cages (0.6m x 0.5m x 0.5m) for two days (days -9 and -8 relative to flight training) to measure food intake and another two days (days -7 and -5) to measure basal and peak metabolic rates (results in (14)). On day -5 we returned the two untrained birds to their original aviary and moved the 3-4 flight-trained birds to a 0.8m x 1.5m x 2m flight aviary.

Flight Training

In order to assess the impact of diet and endurance flight on molecular metabolism, three flight-trained birds followed a training regime in a climatic wind tunnel designed for birds that consisted of four days of pre-training and fifteen days of flight training, which has demonstrated success at eliciting long-duration flights (37). 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 (days -4 to -1) included training birds to fly between their flight cage and the wind tunnel and 20 minutes of habituation time per day in the wind tunnel with a perch to take short voluntary flights. These initial four ‘pre-training’ days were not included in the reported overall training time. Flight-trained starlings then participated in a fifteen-day training regimen 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. The final flight was on average 193 min $\pm$ 71 and the maximum was 360

min. At 1400hr – 1500hr on days 16 and 17 the untrained and flight-trained birds, respectively, were euthanized by cervical dislocation while under isofluorane anesthesia and the pectoralis muscle and liver tissue 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 metabolic gene expression in the pectoralis and liver of untrained (control) birds and flight-trained birds that had recovered (for 48 hrs) from their longest flight on day 15.

Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed to quantify relative expression of key metabolic genes, PGCs, and PPARs. Total RNA was extracted from pectoralis muscle and liver (25-30 mg) using RNeasy® Fibrous Mini Kit (QIAGEN®, Germantown, MD, USA) following kit instructions including the recommended DNase treatment step, but without the proteinase K digestion step for the liver. RNA concentrations and quality were verified using a NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). RNA (0.5 µg) was reverse transcribed using the SuperScript IV First-strand Synthesis System Kit (Thermofisher, Burlington, ON, CA), and cDNA was used as a template for qPCR. Each 17.5 µl PCR reaction mixture was comprised of 1:15 diluted cDNA template, 400 nM gene-specific primers and The Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (Thermofisher, Burlington, ON, CA). The temperature cycles for each PCR reaction were as follows: 2 min at 50°C, 2 min at 95°C, 45 cycles of 95°C for 15 s and a primer-specific optimal temperature (62–68°C) for 1 min. Each PCR run was completed with a melt curve analysis to confirm the presence of a single PCR product and amplification efficiency

verified for every primer pair. The gene expression levels were derived from a standard curve generated for each primer set. Primer sequences were derived using NCBI's BLASTN v2.10.0 program and searching the European starling's genome database (Sturnus_vulgaris-1.0 reference Annotation Release 100) for predicted genes. Primers were designed so that at least one primer was exon spanning. Primers in our study met the following criteria: amplification of a single product indicated by a single peak in the melting curve analysis and efficiency of amplification between 90 and 107%. In all cases, cycle threshold (Ct) values ranged from 18 to 29, except for LPL in the liver which was detected between 27 and 31, PPAR γ which was detected between 30 and 32, and PGC-1 β which was detected in the 32–33 range. Primer sequences and Genbank accession numbers are shown in Table S1 (Supplementary materials available at: <https://figshare.com/s/a82358309c98b7db58b5>). Gene selection for the pectoralis and liver was based on tissue-specific expression patterns and functional roles demonstrated in the medical metabolic literature (7, 62, 123) and their biological relevance in migratory birds (21, 26, 46, 48, 71).

Transcript expression levels were normalized to the reference gene β -actin, which codes for the beta actin gene responsible for the structure and motility of cells, and is highly conserved across tissues and avian species (8, 23, 71, 87, 128). β -actin genes are commonly used as a reference gene in migratory songbirds (e.g. in White-throated sparrows (71) and European starlings (8, 23, 87)), and was among the two most stable reference genes in all tissues tested in yellow-feathered broilers (128). β -actin did not vary across the 8 diet and training treatments in the pectoralis ($F_{8,80}=1.68$, $P=0.12$) or liver ($F_{8,78}=0.94$, $P=0.49$) or over the course of the experiment in the pectoralis (Julian

date: estimate $\pm$ standard error 0.02 ± 0.02 , $P=0.30$) or liver (Julian date: estimate $\pm$ standard error 0.01 ± 0.02 , $P=0.67$). Transcript expression normalized to β -actin was used for causal pathway analyses. Normalized transcript expression relative to the 13% PUFA, low antioxidant, untrained reference group was used for all linear models.

Statistics

Linear Models

We used R (v3.5.3; R Core Team, 2019, Vienna Austria) for all analyses. Linear models were constructed to test the hypothesis that dietary fat, dietary antioxidants, and flight training influenced the gene expression of PPAR transcription factors, PCG1 coactivators, and their metabolic genes. We used a global model without interaction terms that best matched this hypothesis and included possible explanatory covariates (i.e. Julian date, sex, and wing chord). Non-significant explanatory covariates were removed from the final models (sex and wing chord). Julian date was the only covariate retained in the models for LPL in the pectoralis and liver and CD36 in the liver. To test the hypothesis that dietary fat, dietary antioxidants, and flight training had an interactive effect on gene expression, we compared our global models to models including a 3-way interaction between dietary fat, antioxidants, and training treatment. For all genes in all tissues, with the exception of LPL in the pectoralis, the global models without interaction terms were considered the best fit models determined by the lowest Akaike Information Criterion (AIC) score. The Δ AIC score was at least 4-5 lower for the global models compared to the alternative model containing interaction terms, and the Δ AIC score was 3 lower for the LPL model containing interaction terms compared to the global model.

Piecewise Structural Equation Modelling

To test the hypothesis that PPAR coactivators and PPAR transcription factors regulate metabolic gene expression, we conducted a unidirectional path analysis that tests the causal relationships among regulatory genes (PGC-1 coactivators and PPARs) and between regulatory genes and downstream metabolic genes (CD36, MCAD, CS, LPL, Plin2, avUCP). Path analyses, otherwise known as structural equation models (SEM), can be applied to any complex system, including ecological and physiological ones, to explicitly test scientifically informed cause and effect relationships; these *a priori* relationships are evaluated using a graphical network framework (reviewed extensively in (43, 108)). SEMs have been used to understand trophic interactions, microbial communities, evolutionary processes (43), and how risk factors contribute to disease (36, 58). This approach has been suggested but rarely used for physiological ecology studies (19) that utilize the flexible network architecture of SEMs to infer cause-effect relationships among complex physiological processes. We conducted a path analysis separately for flight-trained and untrained birds to determine if the PPAR regulatory involvement was more evident in flight-trained birds (Hypothesis 1). We did not control for diet in our models since we found that the effects of dietary fat and antioxidants were relatively weak and uncoordinated among genes (see Results). We did not conduct a path analysis for the liver since differences in gene expression levels in this tissue were also uncoordinated, and we detected only a few differences in gene expression (see Results).

Piecewise structural equation modeling (PSEM; Table 2) was used to test two alternative hypotheses to determine if PPARs and PPAR coactivators control metabolic gene expression in pectoralis of flight-trained and untrained European starlings. Causal

Model 1 uses PSEM to determine the casual relationship(s) for the expression of the PPAR coactivators on the expression of PPAR transcription factors (Table 2; Equation 1), and to test the causal relationship(s) for the PPAR transcription factors on the downstream metabolic genes (Table 2; Equation 2). PGC-1 coactivators are not essential for PPAR transcription, but they bind directly with PPARs to induce their transcriptional activity. Some studies report the coexpression of PGC-1 coactivators and PPARs which potentially indicates that coactivation occurs (104, 119). Alternatively, the expression of PGC-1 coactivators may be linked to PPAR activity which we did not measure. Causal Model 2 tests the hypothesis that both PGC-1 coactivator expression, perhaps by causing an increase in PPAR activity, and PPAR expression cause the expression of the key target metabolic genes (Table 2; Equations 4 and 5). Additionally, PGC-1 coactivators may be acting independently of PPARs (Table 2; Equation 6) since PGC-1 α and PGC-1 β also act as coactivators for other transcription factors that regulate mitochondrial metabolism (nuclear respiratory factors, NRFs and estrogen-related nuclear receptors, ERRs) and associate with other metabolic pathways (HNF-4, FOXO1, MEF-2) (38, 61). The PSEM R package (64) calculates linear regression coefficients for each specified causal relationship (Table 2; Equations 1-2, 4-5). The PSEM goodness-of-fit is obtained using tests of directed separation ('dsep'). These tests evaluate the assumption that the specific causal structure reflects the data (64, 108). This is accomplished by testing the unspecified relationships in the model that are assumed to be independent because they are biologically and mechanistically insignificant (Table 2; Equations 3, 6). The log of the null probabilities for these independent claims are summed and multiplied by negative 2, to calculate a Fisher's C-statistic (Table 1; Equation 7). The claims are

considered independent (the null hypothesis) if the associated p-value is greater than $\alpha=0.05$, and we can reject the alternative hypothesis that the claims are correlated. The PSEM package calculates the Fisher's C-statistic using individual linear models. However, PGC-1 α and PGC-1 β are co-correlated, so to determine the joint effect of the coactivators we used R and Equation 7 to calculate the simplified independence claims using p-values extracted from the simplified linear models (Table 1; Equations 3,6).

Results

Flight training affects gene expression more in pectoralis than liver

Flight training increased the wet mass of both the pectoralis (Table 3; $T_{95} = 4.650$, $P < 0.0001$) and the liver (Table 4; $T_{95} = 9.667$, $P < 0.0001$) yet only drove metabolic gene expression in the pectoralis. Flight training consistently influenced metabolic gene expression in the pectoralis (70% of measured genes: 50% upregulated and 20% downregulated; Fig. 1) but affected only LPL in the liver (Fig. 2). Genes involved in lipid utilization (CD36, MCAD) and PGC-1 coactivators in the pectoralis were expressed to the greatest extent in flight-trained European starlings (Fig. 1, Table 3). For example, gene expression in pectoralis of flight-trained starlings relative to untrained birds was greatest for genes involved in lipid transport (CD36, $T_{88} = 3.144$, $P = 0.0023$) and lipid oxidation (MCAD, $T_{89} = 2.542$, $P = 0.0128$). The expression of the oxidative enzyme, citrate synthase, was greatest in pectoralis of flight-trained birds, but this trend was not significant despite the similar effect size of 0.226 likely due to greater variability compared to CD36 and MCAD expression ($T_{88} = 1.597$, $P = 0.114$). Flight training also stimulated the expression of PPAR coactivators in pectoralis (PGC-1 α , $T_{89} = 2.066$, $P = 0.0419$; PGC-1 β , $T_{89} = 2.562$, $P = 0.0122$), but in contrast to our predictions, expression

patterns of the PPAR transcription factors and their coactivators were not well coordinated in response to flight training. For example, the expression of the transcription factor PPAR δ was relatively lower in pectoralis of flight-trained birds ($T_{88} = -2.268$, $P = 0.0259$), and PPAR α gene expression was unchanged with training ($T_{89} = -0.363$, $P = 0.717$). Two of the measured metabolic genes were expressed lower in pectoralis of flight-trained birds compared to untrained birds: Plin2, a gene involved in lipid storage, and an ATP uncoupling protein (Plin2, $T_{89} = -2.286$, $P = 0.0247$; avUCP, $T_{89} = -2.240$, $P = 0.0277$). Metabolic gene expression in the liver was not influenced by flight training except LPL expression was greater in flight-trained birds (Fig. 2, Table 4, $T_{86} = 2.136$, $P = 0.0357$). Flight training did not influence the expression of metabolic genes in the liver (CD36 $T_{86} = 1.158$, $P = 0.25$; Plin2, $T_{87} = -0.205$, $P = 0.838$; FATp1, $T_{87} = 0.108$, $P = 0.914$; FABPpm, $T_{87} = 1.497$, $P = 0.138$) or the expression of PPAR α and PPAR γ ($T_{87} = 1.517$, $P = 0.1331$ and $T_{87} = 1.432$, $P = 0.156$ respectively).

Dietary fat quality affects gene expression

Dietary fat quality had a less coordinated influence on metabolic genes in the pectoralis and liver (Fig. 3) compared to flight training. For example, 32% PUFA diets positively influenced the expression of LPL in the pectoralis of flight-trained birds (Table 3 shows nonsignificant main effects; Fig. 3, flight training X diet interaction: $T_{88} = -2.286$, $P = 0.0247$), and positively influenced the expression of PPAR α in the liver (Fig. 3, Table 4; $T_{87} = 2.363$, $P = 0.0205$). In contrast, 32% PUFA negatively influenced CD36 expression in the pectoralis which was relatively greater in starlings consuming the 13% PUFA diet (Fig. 3, Table 3; $T_{88} = -2.290$, $P = 0.0245$). Antioxidant supplementation did not affect gene expression in the pectoralis (Table 3) or liver (Table 4). Overall time on

the experimental diets or the progression of the fall migratory season may have influenced a few genes since we observed a positive effect of Julian date on LPL expression in the pectoralis ($T_{87} = -2.286$, $P = 0.0247$), and a negative effect of date on LPL and CD36 in the liver (LPL, $T_{86} = -1.717$, $P = 0.0899$; CD36, $T_{87} = -2.369$, $P = 0.02019$).

Path Analysis- how the expression of PPAR transcription factors, coactivators, and metabolic genes interact in flight-trained and untrained birds

In order to test hypothesis 1 that flight-training increases (a) PPAR coactivator expression and thereby increases (b) PPAR expression and (c) the expression of their metabolic genes we constructed Causal Model 1 (Table S5, S6). There was no support for Causal Model 1 in flight-trained starlings (Fisher's $C = 61.97$, $df=24$, $P < 0.001$) or in untrained starlings (Fisher's $C = 75.12$, $df=24$, $P < 0.001$). The poor goodness of fit was due to significant relationships among the proposed independent claims, specifically between PGC-1 α and MCAD and CS in trained birds (Independent claims; Table S4) and PGC-1 α and LPL, CD36, MCAD, and CS in the untrained birds (Independent claims; Table S5). Thus, the expression of PPAR coactivators seems to not be causally linked to the expression of PPAR α or PPAR δ in trained (Causal relationships; Table S5) or untrained birds (Causal relationships; Table S6).

Causal Model 2 that tested the alternative hypothesis that both PPAR coactivators and PPAR transcription factors jointly influenced metabolic gene expression was well supported for both flight-trained starlings (Fig. 4; Fisher's $C = 10.39$, $df=8$, $P = 0.24$) and untrained starlings (Fig. 4; Fisher's $C = 5.17$, $df=8$, $P = 0.74$). The regression coefficient associated with each causal relationship (Fig. 4) indicates the extent of change in the

number of transcripts of the metabolic genes (CD36, MCAD, CS, LPL, Plin2, avUCP) for each one-transcript change in the PPAR and PGC-1 coactivator genes (PPAR α , PPAR δ , PGC-1 α , PGC-1 β). For example, for flight-trained birds (Fig. 4A) one of the strongest causal relationships showed that a one-transcript change in PPAR α resulted in a 0.91 transcript change in the Plin2 gene. In general, the flight-trained birds had a more simplified, focused network of causation (Fig. 4A) relative to the untrained birds (Fig. 4B). For example, there were fewer causal relationships between PPAR δ and metabolic genes (33% in flight-trained birds and 83% in untrained birds) and one fewer causal relationship between PGC-1 α and metabolic genes in flight-trained compared to untrained birds (Fig. 4). However, the causal role between PGC-1 α and metabolic genes shifted between flight-trained and untrained birds (Fig. 4). Specifically, there was a causal relationship between PGC-1 α and CS only in trained birds, and between PGC-1 α and LPL, and PGC-1 α and CD36 only in untrained birds (Causal relationships; Table S4 and S5). The only retained causal relationship between flight-trained and untrained birds was between PGC-1 α and MCAD, and PPAR δ and MCAD (Causal relationships; Tables S4 and S5). Interestingly, flight-training produced stronger causal relationships between PPAR α and Plin2, and PPAR δ and Plin2 (Fig. 4A; Causal relationships in Table S4).

Discussion

Most studies to date that characterize the metabolic roles of specific genes and the molecular regulatory factors that control their expression are focused on understanding metabolic dysregulation patterns in humans that occur due to chronic diseases, like fatty liver disease, diabetes, and metabolic syndrome (63, 85, 102). These medically-focused studies of model organisms informed our experimental design and selection of key

metabolic genes, although their relevance to our study of wild-caught migratory birds is limited because their approaches (i.e. using pharmacological activation or inactivation of metabolic regulatory factors (47, 82, 97, 102) or use of metabolic gene-deficient mice (knock-out mice) (47, 63, 79, 82, 85)) were much less ecologically relevant than ours. Additionally, many studies demonstrate that fatty acids are ligands for PPAR metabolic regulators (39, 48, 54, 60), but the few studies that manipulate dietary fat quality use medical model systems that increase dietary fat quantity to reflect obesity (18, 40, 86), utilize transgenic mice (18), or concurrently inject mice with heparin to stimulate LPL activity and induce free fatty acid release (40). Integrative studies that mechanistically link how ecologically-relevant environmental factors (e.g., diet quality, exercise) affect metabolic gene expression and the molecular regulation of this gene expression are considered incipient. Most such studies focus on organisms that rely on lipid metabolism (e.g., hibernating mammals, migrating salmon, and migrating birds (17, 21, 26, 33, 34, 52, 53, 83)) and many demonstrate that PPAR transcription factors respond to changes in seasonal metabolic demands (17, 33, 34, 52, 53, 83). For example, genes involved in lipid catabolism, lipid anabolism, and PPAR α were upregulated in Syrian hamsters during pre-hibernation and hibernation, and pharmacological activation of PPAR δ and PPAR γ increased the expression of lipid metabolic genes (17). Additionally, PPARs respond to ecologically relevant dietary fatty acid manipulations. For example, dietary 18:2n-6 PUFA increased PPAR expression in vivo in four species of fish (2, 55, 56, 65) and in broiler chickens (100). In contrast, dietary 20:5n-3 and 22:6n-3 did not affect PPAR expression in the pectoralis of Bobwhite quail (80). These studies demonstrate that seasonal metabolic demands or dietary fatty acids influence PPARs and select metabolic

genes in ecological systems. However, studies have yet to simultaneously manipulate energetic demands over a sustained period of time and manipulate dietary fat, thus it remains unclear how these environmental factors initiate PPAR pathways in wild animals.

The goal of our study was to determine how repeated bouts of flight and differences in certain diet components (i.e. fats and antioxidants) affect the expression of PPARs, PGC-1 coactivators, and key metabolic genes in the pectoralis muscle and the liver of a migratory songbird. We found support for hypothesis 1 (Flying effect), flight training increased the expression of (a) PPAR coactivators, including PGC-1 α and PGC-1 β , and (b) these metabolic increases with flight-training were associated with an upregulation of metabolic genes (genes that code for fatty acid transporters, fatty acid oxidation, and oxidative enzymes), but not PPARs. Therefore, there was no support for a simple coordinated upregulation of cofactors, PPARs, and metabolic genes, but rather causal gene networks were dependent on flight training and the associated increase in energy demands. There was some evidence to support hypothesis 2 (Dietary fat effect); specifically, we found that migratory songbirds fed diets composed of more 18:2n-6 PUFA had increased expression levels of metabolic transcription factors CD36 and LPL in the pectoralis but not PPAR transcription factors or PGC-1 coactivators. In support of hypothesis 3 (flight X dietary PUFA interaction), dietary 18:2n-6 PUFA and flight training additively increased expression of one metabolic gene (LPL), but did not affect the upstream PPAR metabolic transcription factors or PGC-1 coactivators. We found no evidence to support hypothesis 4 (Dietary antioxidant effect), as antioxidants did not

affect expression levels of PPAR metabolic transcription factors, PGC-1 coactivators, or key metabolic genes.

Tissue-specific differences in patterns of gene expression in response to flight-training

We found strong support for hypothesis 1 (Flying effect), that flight training stimulated the expression of metabolic genes, but for only one of the two tissues (i.e., pectoralis but not liver), despite the observed size increases in *both* the pectoralis and liver in flight-trained birds consistent with studies demonstrating larger pectoralis and livers during migration (21, 26, 31, 66, 70, 91). These results are in accordance with exercise physiology studies that demonstrate the upregulation of PPAR pathways in human skeletal muscle following exercise training (38, 104) whereas this is the first study to our knowledge that investigates how PPAR pathways in the liver of any vertebrate respond to exercise. In the pectoralis, genes involved in fatty acid transport and oxidation (CD36, MCAD) were upregulated after the 15-day flight training regime. Given that CD36 transports fatty acids from the sarcolemma across the muscle membrane (72) and MCAD dehydrogenates fatty acids during beta oxidation in the mitochondria (101), their upregulation in response to flight training suggests that exercising birds may require a greater influx of fatty acids into the muscle and a higher rate of beta oxidation. This reliance on fat utilization is in accordance with the observed increase in fatty acid metabolism in birds during migration (21, 26, 71, 129). In the liver, LPL was the only gene upregulated by flight training, and LPL is the rate limiting step for the hydrolysis of circulating triglycerides bound to lipoproteins (e.g. VLDL, portomicrons) and the subsequent uptake of fatty acids by muscles and organs (57, 127). Circulating triglycerides decreased in flight-trained birds immediately after an acute flight (14)

similar to four other species experimentally exercised (reviewed in (45)), suggesting a series of repeated bouts of flight may increase liver LPL activity.

In accordance with the preference for fat transport and oxidation in flight-trained birds, the expression of genes responsible for *initiating* fat storage and mitochondrial uncoupling from ATP production to heat dissipation were lowest in the pectoralis of flight-trained birds. Plin2 promotes lipid accumulation in lipid droplets, whereas the disassociation between Plin2 and lipases promotes lipolysis (78), and the lower expression levels of Plin2 in flight-trained birds indicates a preference away from fat storage to lipolysis. Intramuscular fat stores only provide 10% of the fuel used by muscle while the rest is derived from circulation (45), thus Plin2 transcription may be prioritized in adipose tissue rather than the flight muscle. Consistent with this, Plin2 and Plin3 were upregulated in adipose tissue of Syrian hamsters during hibernation likely to promote fat storage for use during torpor-associated fasting (17). PPAR δ promotes the expression of muscle Plin (10) and PPAR δ was also expressed to the lowest extent in flight-trained birds. The regulatory role of PPAR δ on Plin2 is also supported by Causal Model 2 in flight-trained birds but not untrained birds (Fig. 4) indicating that the avoidance of fat storage is prioritized by birds during flight training.

There is no consensus on the functions of avUCP, but favored hypotheses propose roles in reducing the amount of reactive species production via uncoupled respiration in the presence of reactive superoxide (25, 32, 118). In this study, avUCP was expressed at lower levels in the pectoralis of flight-trained birds compared to untrained birds suggesting that regular flight-training may maintain a level of reactive species required for signaling and increasing muscle metabolism (103, 111) or that energetically

demanding flight training requires constant ATP production rather than an uncoupling of ATP production. PPAR δ regulates UCPs in skeletal muscle, as demonstrated by a constitutively active or pharmacologically activated PPAR δ (35), but PPAR δ only significantly regulated avUCP in untrained birds (Fig. 4). Mitochondrial uncoupling in the pectoralis seems to be avoided in flight-trained birds suggested by the low expression levels of avUCP, but there is no evidence that avUCP expression is mediated by PPARs.

Contrary to predictions, PPAR expression in the pectoralis or liver was not coordinately upregulated with PGC-1 coactivators or metabolic genes responsible for fat transport and oxidation (Causal Model 1; Tables S5,S6), as PPAR α expression was unchanged between flight training groups in the pectoralis and liver and PPAR γ expression was unchanged in the liver. The distinct gene expression profiles for PGC-1 cofactors and metabolic genes for the two tissues may be driven by two alternative mechanisms: 1) The liver is responsible for many aspects of digestion and also functions as an endocrine and exocrine gland (94, 126) - these multiple functions may require the associated metabolic genes to be expressed at consistently high levels that already meet the demands associated with flight training. In contrast, the pectoralis may require an upregulation of metabolic genes to match the higher demands of flight training (21, 26, 71, 129). 2) The tissue-specific turnover rates of proteins in the liver are two times faster than the protein turnover rates of the pectoralis in migratory birds (5), and this may have allowed the liver to advance from a 'metabolic state' that prioritizes the expression of genes involved in fat metabolism to a 'repair and recovery state' that prioritizes the expression of antioxidant and pro-inflammatory genes (94, 124) within two days after the longest flight. The relatively slower turnover of the pectoralis may mean it remained in a

metabolic state that prioritizes the expression of genes involved in fat metabolism. Taken together, our study demonstrates that the expression of PGC-1 cofactors and select metabolic genes in the pectoralis, but not in the liver, are stimulated by increased energetic demands posed by flight-training suggesting that this tissue-specific molecular metabolic flexibility is required for wild birds during migration.

Flight training simplifies and strengthens gene expression relationships between PPAR α , PPAR δ , PGC-1 α , and key metabolic genes

The piecewise structural equation modeling (PSEM) allowed us to evaluate the metabolic regulatory roles of PGC-1 coactivators and PPARs in the pectoralis of European starlings in response to flight-training. We found no evidence for PGC-1 and PPAR coexpression and subsequent regulation of the selected metabolic genes (Causal model 1; Tables S5, S6). There are two possible explanations for the lack of causal relationships between the expression of PGC-1 coactivators and PPARs: 1) PGC-1 coactivators are not essential for PPAR transcription, but they bind directly with PPARs to induce their transcriptional activity (38, 61); thus, increased PGC-1 coactivator expression may have directly increased PPAR activity (without changes in its transcription), which we did not measure in this study. 2) PGC-1 α and PGC-1 β also coactivate unmeasured transcription factors responsible for mitochondrial energy metabolism including nuclear respiratory factors (NRFs) or estrogen-related nuclear receptors (ERRs), or associate with pathways involved in gluconeogenesis, glucose transport, and lipogenesis (HNF-4, FOXO1, MEF-2) (38, 61); thus, it is possible that their association with other transcription factors better explains metabolic gene expression in this study.

We found that causal relationships between PGC-1 coactivators and PPARs on metabolic gene expression depended on flight-training (Causal model 2; Fig. 4). Flight-trained and untrained birds maintained significant relationships between PGC-1 α and MCAD, and PPAR δ and MCAD suggesting that there is a constant regulatory requirement for fatty acid oxidation in the pectoralis muscle. The untrained birds flew within room-sized aviaries, thus their metabolically active flight-muscles likely still required MCAD fatty acid oxidation. The other primary relationship in the causal network for flight-trained birds involved Plin2. The significant relationship between PPAR α and Plin2, and PPAR δ and Plin2 in flight-trained but not untrained birds indicates that such flight training initiates the PPAR-mediated prevention of lipid storage as indicated by their coordinated decreased expression in flight-trained birds (Fig. 1).

In general, the flight-trained birds had a simplified and stronger network of causation relative to untrained birds (Fig. 4). For example in untrained birds, PPAR δ strongly regulated five of the six measured metabolic genes while PGC-1 α strongly regulated three of these same metabolic genes. In flight-trained birds, PGC-1 α and PPAR regulation was simplified to metabolic genes involved in mitochondrial metabolism (MCAD, CS) and lipid storage (Plin2). In contrast, in the absence of flight, PPARs and PGC-1 α were more general regulators of metabolic genes involved in fatty acid transport (CD36), fatty acid hydrolysis (LPL) proton uncoupling (avUCP), mitochondrial metabolism (MCAD, CS), but not lipid storage (Plin2). These results are consistent with studies that describe PPARs and PGC-1 coactivators as ‘master regulators of metabolism’ (35, 62, 123). Our study suggests that the regulatory roles of PPAR δ and PGC-1 α are maintained in untrained European starlings during the migration season like

in mammals (35). However, we found that PPAR transcription factors did not provide a complete explanation for metabolic gene expression, suggesting transcription factors other than PPARs (e.g. NRFs, ERRs) may regulate important aspects of metabolism during flight. To best understand which molecular mechanisms are responsible for regulating metabolic gene expression, transcriptomic-wide studies are required to determine which genes are differentially expressed and to identify causal networks based on known relationships among the differentially expressed genes (50).

Dietary fat quality and antioxidants weakly affect metabolic gene expression

We found some evidence in support of hypothesis 2 (Dietary fat effect), that dietary 18:2n-6 PUFA affects the expression of PPAR α and select metabolic genes (CD36, LPL), but not PPAR δ or PGC-1 coactivators, demonstrating the selective signaling role of dietary fat in muscle and liver metabolism. We found that birds fed more 18:2n-6 PUFA (and thereby less 16:0) increased the expression of only two genes, one in the pectoralis that is involved in the hydrolysis of circulating triglycerides (LPL, dependent on flight training) and PPAR α expression in the liver. In contrast, expression of a gene involved in fat transport across the muscle membrane was higher in 13% PUFA groups (independent of training). Thus, the support for hypothesis 2 was tissue-specific and expression patterns in response to dietary fat were not consistent across PPARs, PGC-1 coactivators, and metabolic genes. We predicted that 32% PUFA would consistently increase metabolic gene expression among all components based on the high affinity for 18:2n-6 PUFA as a ligand for PPAR α , PPAR δ , and PPAR γ (48), the greater ability to produce eicosanoids (PPAR ligands) from higher concentrations of dietary 18:2n-6 PUFA (6), and on the enhanced energetic efficiency demonstrated by

animals consuming dietary PUFA (14, 56, 73, 122). PUFA are preferentially mobilized from bird adipocytes and more rapidly oxidized by bird pectoralis (44, 89, 95, 96). Consistent with this hypothesis, 18:2n-6 increased the expression of PPAR α in the liver and LPL in the pectoralis; however, this occurred only in flight-trained birds and not untrained birds fed the same diet. This suggests something more complex than simple selective mobilization and oxidation of PUFA is at work as described below. In contrast, the higher proportion of dietary 16:0 increased CD36 expression in the pectoralis. Perhaps an excess of 16:0 stimulates fat transport and fuel delivery to the muscle to avoid 16:0 accumulation and lipotoxicity in the liver (109). Although dietary fat quality did not consistently modulate entire metabolic pathways in either tissue, we have shown that consumption of 18:2n-6 and 16:0 during migration can directly affect expression of select metabolic genes (i.e., PPAR α , LPL, CD36) and thus adjust metabolism in migrating songbirds.

Instead of a conditional stimulatory effect of flight training and dietary 18:2n-6 PUFA as proposed by hypothesis 3 (Flight x dietary fat effect), we found that expression of only *one* metabolic gene (LPL) in the pectoralis increased with flight training when birds were fed the 32% PUFA diet but not when fed the 13% PUFA diet (Fig. 3A). LPL is a critical determinant of the uptake of triglyceride into tissues (57, 127). It is well established that regular exercise training stimulates LPL activity in humans (57, 67, 84) and inactivity after training decreases muscle LPL activity (9, 57, 110). Several studies have established that migratory season alone does not affect LPL expression (41, 74, 99, 106). Consistent with this interaction hypothesis, the only study that manipulated both dietary fat and flight in a migratory bird demonstrated that both ecological factors

modulated PPAR expression (30) although LPL expression was not measured in this study. Specifically, Dick and Guglielmo (2020) found that PPAR δ expression in the pectoralis was highest in Yellow-rumped warblers immediately after completing a three hour flight compared to unflown birds, and PPAR γ expression was lowest in flown birds consuming MUFA and n-3 PUFA compared to unflown birds (30). To our knowledge, no previous studies have demonstrated LPL activity or expression is affected by an interactive effect of dietary fat and regular repeated exercise. Dietary 18:2n-6 PUFA and 16:0 (compared to 18:1n-9) delivered by chylomicron remnants to cultured hepatocytes stimulated TG-VLDL packaging (68). In the present study, flight-trained birds would benefit from increased LPL activity if liver TG-VLDL packaging and secretion was increased by dietary 18:2n-6 for transport to the pectoralis. Whether or not dietary fat types have differential effects on VLDL secretion remains unstudied in migratory birds.

We found no effect of dietary antioxidants on the expression of PGC-1 coactivators, PPAR metabolic transcription factors, or key metabolic genes as proposed (hypothesis 4, Dietary antioxidant effect). Humans supplemented with a mixture of fat soluble (vitamin E) and water soluble (vitamin C) antioxidants had an attenuated insulin response and lower PGC-1 and PPAR expression in muscle after 4 weeks of training suggesting that antioxidant supplementation blocked an adaptive increase in muscle metabolism in response to exercise (103). Thus, we expected that migratory songbirds not fed dietary antioxidants would increase expression of PPAR metabolic transcription factors, PGC-1 coactivators, and key metabolic genes compared to birds supplemented with dietary antioxidants. We chose to supplement birds with a certain amount of water-soluble antioxidant rather than a fat soluble antioxidant because anthocyanins are widely

consumed by songbirds during migration (1, 11, 16, 107), and such diet supplementation positively affected immune function in European blackcaps, *Sylvia atricapilla* (16, 107) and attenuated the cortisone response to flight in European starlings (15). It is possible that the differences in bioavailability of water- and fat-soluble antioxidants may produce differences in gene expression levels, or that a decreased insulin response does not inhibit a metabolic response to training in birds. Migratory songbirds that consume fruit likely rely on the protective properties of dietary antioxidants (20, 112) and future studies should determine if dietary antioxidants such as anthocyanins directly influence the expression of antioxidant enzymes.

Perspectives and Significance

Exercise, dietary fat, and dietary antioxidants can influence PPAR transcription factors and their downstream metabolic genes in other organisms (30, 56, 104), yet this is the first study that investigates all three simultaneously in an ecologically-relevant avian system. Our study confirms that repeated bouts of flight and dietary fat, but less so dietary antioxidants, stimulate the downstream expression of select metabolic genes in a tissue-specific manner suggesting that these environmental factors selectively increase the transcriptional activity of PPARs in starlings. In general, tissues may respond differently to the same environmental factors because of fundamental differences in their functional role (e.g., muscles involved in flight) or their protein turnover rates which then creates variation in the time course of response. The potential regulatory role of PPARs on select metabolic genes in the pectoralis of migratory songbirds, consistent with previous studies (26, 30), depended on extent of energetic demands. The concept of evolutionary economic design of physiological systems considers such phenotypic

flexibility in metabolic response adaptive (27, 49) in that it allows birds and other animals to match the capacity of key physiological systems to prevailing demands and then modulate this capacity as demands change and so provide adequate but not excessive spare capacity at any given time (92). This spare capacity and phenotypic flexibility allows animals living in changing environments to track environmental change, whether natural or anthropogenic (75). Birds during migration are selective in what they eat (1, 11, 88, 90, 95, 107, 114), and these diet choices directly affect the available supply of nutrients and energy but also, as we have shown, can affect the regulation of select metabolic genes (LPL, CD36, PPAR α). Future studies that utilize transcriptomic analyses to characterize metabolic responses to ecological factors and that continue to examine metabolism on multiple levels of biological organization will help elucidate how an organism's ecology impacts key aspects of their physiology and behavior.

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No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

SRM and BJP conceived and designed the experiment. KJD and WAC and SRM refined the methodology and conducted the experiment. KJD analyzed the data. KJD and SRM interpreted the results and primarily wrote the manuscript, and WAC and BJP provided editorial advice to the manuscript.

References

1. **Alan ARR, McWilliams SR, McGraw KJ.** The importance of antioxidants for avian fruit selection during autumn migration. *Wilson J Ornithol* 125: 513–525, 2013.
2. **Ayisi CL, Zhao JL, Hua XM, Apraku A.** Replacing fish oil with palm oil: effects on mRNA expression of fatty acid transport genes and signalling factors related to lipid metabolism in Nile tilapia (*Oreochromis niloticus*). *Aquac Nutr* 24: 1822–1833, 2018.
3. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO.** Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J* 16: 1879–1886, 2002.
4. **Banerjee S, Mohini Chaturvedi C.** Migratory preparation associated alterations in pectoralis muscle biochemistry and proteome in Palearctic–Indian emberizid migratory finch, red-headed bunting, *Emberiza bruniceps*. *Comp Biochem Physiol - Part D Genomics Proteomics* 17: 9–25, 2016.
5. **Bauchinger U, McWilliams SR.** Extent of phenotypic flexibility during long-distance flight is determined by tissue- specific turnover rates: a new hypothesis. *J Avian Biol* 41: 603–608, 2010.
6. **Bell J, Sargent J, Raynard R.** Effects of increasing dietary linoleic acid on phospholipid fatty acid composition and eicosanoid production in leucocytes and gill cells of Atlantic salmon (*Salmo salar*). *Prostaglandins Leukot Essent Fat Acids* 45: 197–206, 1992.
7. **Bensinger SJ, Tontonoz P.** Integration of metabolism and inflammation by lipid-

- activated nuclear receptors. *Nature* 454: 470–477, 2008.
8. **Bernard DJ, Bentley GE, Balthazart J, Turek FW, Ball GF.** Androgen receptor, estrogen receptor α , and estrogen receptor β show distinct patterns of expression in forebrain song control nuclei of European starlings. *Endocrinology* 140: 4633–4643, 1999.
 9. **Bey L, Hamilton MT.** Suppression of skeletal muscle lipoprotein lipase activity during physical inactivity: A molecular reason to maintain daily low-intensity activity. *J Physiol* 551: 673–682, 2003.
 10. **Bindesbøll C, Berg O, Arntsen B, Nebb HI, Dalen KT.** Fatty acids regulate perilipin5 in muscle by activating PPAR δ . *J Lipid Res* 54: 1949–1963, 2013.
 11. **Bolser JA, Alan RR, Smith AD, Li L, Seeram NP, McWilliams SR.** Birds select fruits with more anthocyanins and phenolic compounds during autumn migration. *Wilson J Ornithol* 125: 97–108, 2013.
 12. **Butler PJ.** The physiological basis of bird flight. *Philos Trans R Soc B Biol Sci* 371: 1–11, 2016.
 13. **Cabe P.** European starling (*Sturnus vulgaris*). In: A.F. Poole & F.B. Gill (Eds.). Ithica, NY: Cornell Lab of Ornithology, 1993.
 14. **Carter WA, DeMoranville KJ, Pierce BJ, McWilliams SR.** The effects of dietary linoleic acid and hydrophilic antioxidants on basal, peak, and sustained metabolism in flight-trained European starlings. *Ecol Evol* 10: 1552–1566, 2020.
 15. **Casagrande S, DeMoranville KJ, Trost L, Pierce BJ, Bryla A, Dzialo M, Sadowska ET, Bauchinger U, McWilliams SR.** Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird. *Proc*

Natl Acad Sci B 18: 1–8, 2020.

16. **Catoni C, Martin Schaefer H, Peters A.** Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird. *Funct Ecol* 22: 649–654, 2008.
17. **Chayama Y, Ando L, Sato Y, Shigenobu S, Anegawa D, Fujimoto T, Taii H, Tamura Y, Miura M, Yamaguchi Y.** Molecular basis of white adipose tissue remodeling that precedes and coincides with hibernation in the Syrian hamster, a food-storing hibernator. *Front Physiol* 9: 1–16, 2019.
18. **Ciana P, Biserni A, Tatangelo L, Tiveron C, Sciarroni AF, Ottobrini L, Maggi A.** A novel peroxisome proliferator-activated receptor responsive element-luciferase reporter mouse reveals gender specificity of peroxisome proliferator-activated receptor activity in liver. *Mol Endocrinol* 21: 388–400, 2007.
19. **Cohen AA, Martin LB, Wingfield JC, McWilliams SR, Dunne JA.** Physiological regulatory networks: ecological roles and evolutionary constraints. *Trends Ecol Evol* 27: 428–435, 2012.
20. **Cooper-Mullin C, McWilliams SR.** The role of the antioxidant system during intense endurance exercise: lessons from migrating birds. *J Exp Biol* 219: 3684–3695, 2016.
21. **Corder KR, DeMoranville KJ, Russell DE, Huss JM, Schaeffer PJ.** Annual life-stage regulation of lipid metabolism and storage and association with PPARs in a migrant species: the gray catbird (*Dumetella carolinensis*). *J Exp Biol* 219: 3391–3398, 2016.
22. **Corder KR, Schaeffer PJ.** Summit metabolic rate exhibits phenotypic flexibility

- with migration, but not latitude in a neotropical migrant, *Parkesia noveboracensis*. *J Ornithol* 156: 547–550, 2015.
23. **Cordes M, Stevenson S, Driessen T, Eisinger B, Ritters L.** Sexually-motivated song is predicted by androgen-and opioid-related gene expression in the medial preoptic nucleus of male European starlings (*Sturnus vulgaris*). *Behav Brain Res* 278: 12–20, 2016.
 24. **Coyle EF.** Physiological determinants of endurance exercise performance. *J Sci Sport Med* 2: 181–189, 1999.
 25. **Criscuolo F, Gonzalez-Barroso MDM, Bouillaud F, Ricquier D, Miroux B, Sorci G.** Mitochondrial uncoupling proteins: new perspectives for evolutionary ecologists. *Am Nat* 166: 686–699, 2005.
 26. **DeMoranville KJ, Corder KR, Hamilton A, Russell DE, Huss JM, Schaeffer PJ.** PPAR expression, muscle size and metabolic rates across the gray catbird's annual cycle are greatest in preparation for fall migration. *J Exp Biol* 222: 1–11, 2019.
 27. **Diamond J, Hammond K.** The matches, achieved by natural selection, between biological capacities and their natural loads. *Experientia* 48: 551–557, 1992.
 28. **Dick MF.** The long haul: Migratory flight preparation and performance in songbirds [Online]. Western University: 2017. <https://ir.lib.uwo.ca/etd/4520>.
 29. **Dick MF, Guglielmo CG.** Flight muscle protein damage during endurance flight is related to energy expenditure but not dietary polyunsaturated fatty acids in a migratory bird. *J Exp Biol* 222: 1–9, 2019.
 30. **Dick MF, Guglielmo CG.** Physical activity and inactivity dietary polyunsaturated

- fatty acids influence flight muscle oxidative capacity but not endurance flight performance in a migratory songbird. *Am J Physiol* 316: 362–375, 2019.
31. **Dietz MW, Piersma T, Dekinga A.** Body-building without power training: endogenously regulated pectoral muscle hypertrophy in confined shorebirds. *J Exp Biol* 202: 2831–2837, 1999.
 32. **Echtay KS, Brand MD.** 4-Hydroxy-2-nonenal and uncoupling proteins: an approach for regulation of mitochondrial ROS production. *Redox Rep* 12: 26–29, 2007.
 33. **Eddy SF, Morin PJ, Storey KB.** Cloning and expression of PPAR γ and PGC-1 α from the hibernating ground squirrel, *Spermophilus tridecemlineatus*. *Mol Cell Biochem* 269: 175–182, 2005.
 34. **Eddy SF, Storey KB.** Differential expression of Akt , PPAR γ , and PGC-1 during hibernation in bats. *Biochem Cell Biol* 81: 269–274, 2003.
 35. **Ehrenborg E, Krook A.** Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor δ . *Pharmacol Rev* 61: 373–393, 2009.
 36. **Eldridge RC, Goodman M, Bostick RM, Fedirko V, Gross M, Thyagarajan B, Flanders WD.** A novel application of structural equation modeling estimates the association between oxidative stress and colorectal adenoma. *Cancer Prev Res* 11: 52–58, 2018.
 37. **Engel S, Biebach H, Visser GH.** Metabolic costs of avian flight in relation to flight velocity: a study in Rose coloured starlings (*Sturnus roseus*, Linnaeus). *J Comp Physiol B Biochem Syst Environ Physiol* 176: 415–427, 2006.

38. **Finck BN, Kelly DP.** Review series PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 116: 615–622, 2006.
39. **Forman BM, Chen J, Evans RM.** Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proc Natl Acad Sci United States Am* 94: 4312–4317, 1997.
40. **Garcia-Roves P, Huss JM, Han D-H, Hancock CR, Iglesias-Gutierrez E, Chen M, Holloszy JO.** Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. *Proc Natl Acad Sci U S A* 104: 10709–10713, 2007.
41. **George JC, Vallyathan N V.** Lipase and succinic dehydrogenase activity of the particulate fractions of the breast muscle homogenate of the migratory starling *Sturnus roseus* in the pre-migratory and post-migratory periods. *J Cell Comp Physiol* 63: 381–391, 1964.
42. **Ginn H, Melville D.** *Moult in birds*. Thetford, UK: British Trust for Ornithology, 1983.
43. **Grace JB, Schoolmaster DR, Guntenspergen GR, Little AM, Mitchell BR, Miller KM, Schweiger EW.** Guidelines for a graph-theoretic implementation of structural equation modeling. *Ecosphere* 3: art73, 2012.
44. **Guglielmo CG.** Move that fatty acid: fuel selection and transport in migratory birds and bats. *Integr Comp Biol* 50: 336–345, 2010.
45. **Guglielmo CG.** Obese super athletes: fat-fueled migration in birds and bats. *J Exp Biol* 221: 1–16, 2018.
46. **Guglielmo CG, Haunerland NH, Hochachka PW, Williams TD.** Seasonal

- dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a migratory shorebird. *Am J Physiol - Regul Integr Comp Physiol* 282: 1405–1413, 2002.
47. **Haemmerle G, Moustafa T, Woelkart G, Buttner S, Schmidt A, van de Weijer T, Hesselink M, Jaeger D, Keinesberger PC, Zierler K, Schreiber R, Eichmann T, Kolb D, Kotzbeck P, Schweiger M, Kumari M, Eder S, Schoiswohl G, Wongsiriroj N, Pollak NM, Radner FP, Preiss-Landl K, Kolbe T, Rulicke T, Pieske B, Trauner M, Lass A, Zimmermann R, Hoefler G, Cinti S, Kershaw EE, Schauwen P, Madeo F, Mayer B, Zechner R.** ATGL-mediated fat catabolism regulates cardiac mitochondrial function via PPAR- α and PGC-1. *Nat Med* 17: 1076–1085, 2011.
 48. **Hamilton A, Ly J, Robinson JR, Corder KR, DeMoranville KJ, Schaeffer PJ, Huss JM.** Conserved transcriptional activity and ligand responsiveness of avian PPARs: potential role in regulating lipid metabolism in migratory birds. *Gen Comp Endocrinol* 268: 110–120, 2018.
 49. **Hammond KA, Diamond J.** Maximal sustained energy budgets in humans and animals. *Nature* 386: 457–462, 1997.
 50. **Jax E, Wink M, Kraus RH.** Avian transcriptomics: opportunities and challenges. *J Ornithol* 159: 599–629, 2018.
 51. **Johnson RA, Willson MF, Thompson JN, Bertin RI.** Nutritional values of wild fruits and consumption by migrant frugivorous birds. *Ecology* 66: 819–827, 1985.
 52. **Kabine M, El Kebbaj Z, Oaxaca-Castillo D, Clémencet MC, El Kebbaj MS, Latruffe N, Cherkaoui-Malki M.** Peroxisome proliferator-activated receptors as

regulators of lipid metabolism; tissue differential expression in adipose tissues during cold acclimatization and hibernation of jerboa (*Jaculus orientalis*).

Biochimie 86: 763–770, 2004.

53. **Kebbaj Z El, Andreoletti P, Mountassif D, Kabine M, Schohn H, Dauça M, Latruffe N, Kebbaj MS El, Cherkaoui-Malki M.** Differential regulation of peroxisome proliferator-activated receptor (PPAR)- α 1 and truncated PPAR α 2 as an adaptive response to fasting in the control of hepatic peroxisomal fatty acid β -oxidation in the hibernating mammal. *Endocrinology* 150: 1192–1201, 2009.
54. **Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W.** Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci* 90: 2160–2164, 1993.
55. **Kennedy SR, Bickerdike R, Berge RK, Porter AR, Tocher DR.** Influence of dietary conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on growth, lipid composition and key enzymes of fatty acid oxidation in liver and muscle of Atlantic cod (*Gadus morhua* L.). *Aquaculture* 264: 372–382, 2007.
56. **Kennedy SR, Leaver MJ, Campbell PJ, Zheng X, Dick JR, Tocher DR.** Influence of dietary oil content and conjugated linoleic acid (CLA) on lipid metabolism enzyme activities and gene expression in tissues of Atlantic salmon (*Salmo Salar* L.). *Lipids* 41: 423–426, 2006.
57. **Kersten S.** Physiological regulation of lipoprotein lipase. *Biochim Biophys Acta - Mol Cell Biol Lipids* 1841: 919–933, 2014.
58. **Khodarahmi M, Asghari-Jafarabadi M, Farhangi MA.** A structural equation

- modeling approach for the association of a healthy eating index with metabolic syndrome and cardio-metabolic risk factors among obese individuals. *PLoS One* 14: 1–20, 2019.
59. **Kim T, Yang Q.** Peroxisome-proliferator-activated receptors regulate redox signaling in the cardiovascular system. *World J Cardiol* 5: 164–174, 2013.
 60. **Kliwer SA, Sundseth SS, Jones SA, Brown PJ, Wisely BG, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM.** Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ . *Proc Natl Acad Sci U S A* 94: 4318–4323, 1997.
 61. **Knutti D, Kralli A.** PGC-1, a versatile coactivator. *Trends Endocrinol Metab* 12: 360–365, 2001.
 62. **Lamichane S, Dahal Lamichane B, Kwon S-M.** Pivotal roles of peroxisome proliferator-activated receptors (PPARs) and their signal cascade for cellular and whole-body energy homeostasis. *Int J Mol Sci* 19: 1–14, 2018.
 63. **Lee CH, Olson P, Hevener A, Mehl I, Chong L-W, Olefsky JM, Gonzalez FJ, Ham J, Kang H, Peters JM, Evans RM.** PPAR δ regulates glucose metabolism and insulin sensitivity. *Proc Natl Acad Sci U S A* 103: 3444–3449, 2006.
 64. **Lefcheck J.** *Piecewise Structural Equation Modeling Package 'piecewiseSEM.'* 2019.
 65. **Li Y, Zhao Y, Zhang Y, Liang X, Zhang Y, Gao J.** Growth performance, fatty acid composition, peroxisome proliferator-activated receptors gene expressions antioxidant abilities of blunt snout bream, *Megalobrama amblycephala*, fingerlings fed different dietary oil sources. *J World Aquac Soc* 46: 395–408, 2015.

66. **Lindström Å, Kvist A, Piersma T, Dekinga A, Dietz MW.** Avian pectoral muscle size rapidly tracks body mass changes during flight, fasting and fuelling. *J Exp Biol* 203: 913–919, 2000.
67. **Lithell H, Örlander J, Schele R, Sjodin B, Karlsson J.** Changes in lipoprotein-lipase activity and lipid stores in human skeletal muscle with prolonged heavy exercise. *Acta Physiol Scand* 107: 257–261, 1979.
68. **López-Soldado I, Avella M, Botham KM.** Differential influence of different dietary fatty acids on very low-density lipoprotein secretion when delivered to hepatocytes in chylomicron remnants. *Metabolism* 58: 186–195, 2009.
69. **Lundgren BO, Kiessling K-H.** Seasonal variation in catabolic enzyme activities in breast muscle of some migratory birds. *Oecologia* 66: 468–471, 1985.
70. **Marsh RL.** Adaptations of the Gray catbird (*Dumatella carolinensis*) to long-distance migration: flight muscle hypertrophy associated with elevated body mass. *Physiol Zool* 57: 105–117, 1984.
71. **McFarlan JT, Bonen A, Guglielmo CG.** Seasonal upregulation of fatty acid transporters in flight muscles of migratory white-throated sparrows (*Zonotrichia albicollis*). *J Exp Biol* 212: 2934–2940, 2009.
72. **McFarlan JT, Yoshida Y, Jain SS, Han XX, Snook LA, Lally J, Smith BK, Glatz JF, Luiken JJ, Sayer RA, Tupling AR, Chabowski A, Holloway GP, Bonen A.** In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation. *J Biol Chem* 287: 23502–23516, 2012.
73. **McKenzie D, Higgs D, Dosanjh B, Deacon G, Randall D.** Dietary fatty acid

- composition influences swimming performance in Atlantic salmon (*Salmo salar*) in seawater. *Fish Physiol Biochem* 19: 111–122, 1998.
74. **McWilliams SR, Guglielmo C, Pierce B, Klaassen M.** Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective. *Source J Avian Biol* 35: 377–393, 2004.
 75. **McWilliams SR, Karasov WH.** Spare capacity and phenotypic flexibility in the digestive system of a migratory bird: defining the limits of animal design. *Proc R Soc B Biol Sci* 281, 2014.
 76. **Merry TL, Ristow M.** Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training? *J Physiol* 594: 5135–5147, 2016.
 77. **Miller KM, Schulze AD, Ginther N, Li S, Patterson DA, Farrell AP, Hinch SG.** Salmon spawning migration: metabolic shifts and environmental triggers. *Comp Biochem Physiol - Part D Genomics Proteomics* 4: 75–89, 2009.
 78. **Morales PE, Bucarey JL, Espinosa A.** Muscle lipid metabolism: role of lipid droplets and perilipins. *J Diabetes Res* 2017: 1–10, 2017.
 79. **Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, Winegar DA, Christopher Corton J, Lynis Dohm G, Kraus WE.** Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) α knock-out mice. *J Biol Chem* 277: 26089–26097, 2002.
 80. **Nagahuedi S, Popesku JT, Trudeau VL, Weber J-M.** Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary n-3 fatty acids on muscle membranes and PPAR expression. *J Exp Biol* 212: 1106–1114, 2009.

81. **Naidoo R, Chase M, Beytell P, Du Preez P, Landen K, Stuart-Hill G, Taylor R.** A newly discovered wildlife migration in Namibia and Botswana is the longest in Africa. *Oryx* 50: 138–146, 2016.
82. **Narkar VA, Downes M, Yu RT, Embler E, Wang Y-X, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ, Evans RM.** AMPK and PPAR δ agonists are exercise mimetics. *Cell* 134: 405–415, 2008.
83. **Nelson CJ, Otis JP, Carey H V.** A role for nuclear receptors in mammalian hibernation. *J Physiol* 9: 1863–1870, 2009.
84. **Nikkilä EA, Taskinen MR, Rehunen S, Härkönen M.** Lipoprotein lipase activity in adipose tissue and skeletal muscle of runners: relation to serum lipoproteins. *Metabolism* 27: 1661–1671, 1978.
85. **Pandey NR, Benkirane K, Amiri F, Schiffrin EL.** Effects of PPAR- γ knock-down and hyperglycemia on insulin signaling in vascular smooth muscle cells from hypertensive rats. *J Cardiovasc Pharmacol* 49: 346–354, 2007.
86. **Pérez-Schindler J, Kanhere A, Edwards L, Allwood JW, Dunn WB, Schenk S, Philp A.** Exercise and high-fat feeding remodel transcript-metabolite interactive networks in mouse skeletal muscle. *Sci Rep* 7: 1–11, 2017.
87. **Perfito N, Guardado D, Williams TD, Bentley GE.** Social cues regulate reciprocal switching of hypothalamic Dio2/Dio3 and the transition into final follicle maturation in European starlings (*Sturnus vulgaris*). *Endocrinology* 156: 694–706, 2015.
88. **Pierce BJ, McWilliams SR.** Seasonal changes in composition of lipid stores in migratory birds: causes and consequences. *Condor* 107: 269–279, 2005.

89. **Pierce BJ, McWilliams SR.** The fat of the matter: how dietary fatty acids can affect exercise performance. *Integr Comp Biol* 54: 903–912, 2014.
90. **Pierce BJ, McWilliams SR, Place AR, Huguenin MA.** Diet preferences for specific fatty acids and their effect on composition of fat reserves in migratory Red-eyed Vireos (*Vireo olivaceus*). *Comp Biochem Physiol - A Mol Integr Physiol* 138: 503–514, 2004.
91. **Piersma T.** Phenotypic flexibility during migration: optimization of organ size contingent on the risks and rewards of fueling and flight. *J Avian Biol* 29: 511–520, 1998.
92. **Piersma T, Van Gils JA.** *The flexible phenotype: a body-centred integration of ecology, physiology, and behaviour*. New York, NY: Oxford University Press, 2011.
93. **Pilliod DS, Peterson CR, Ritson PI.** Seasonal migration of Columbia spotted frogs (*Rana luteiventris*) among complementary resources in a high mountain basin. *Can J Zool* 80: 1849–1862, 2002.
94. **Pillon Barcelos R, Freire Royes LF, Gonzalez-Gallego J, Bresciani G.** Oxidative stress and inflammation: liver responses and adaptations to acute and regular exercise. *Free Radic Res* 51: 222–236, 2017.
95. **Price ER, Krokfors A, Guglielmo CG.** Selective mobilization of fatty acids from adipose tissue in migratory birds. *J Exp Biol* 211: 29–34, 2008.
96. **Price ER, Staples JF, Milligan CL, Guglielmo CG.** Carnitine palmitoyl transferase activity and whole muscle oxidation rates vary with fatty acid substrate in avian flight muscles. *J Comp Physiol B Biochem Syst Environ Physiol* 181:

565–573, 2011.

97. **Rakhshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, De Groot PJ, Müller M, Kersten S.** Comprehensive analysis of PPAR α -dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Res* 2007: 1–13, 2007.
98. **Ramenofsky M, Cornelius JM, Helm B.** Physiological and behavioral responses of migrants to environmental cues. *J Ornithol* 153: 181–191, 2012.
99. **Ramenofsky M, Savard R, Greenwood MRC.** Seasonal and diel transitions in physiology and behavior in the migratory dark-eyed junco. *Comp Biochem Physiol - A Mol Integr Physiol* 122: 385–397, 1999.
100. **Ramiah SK, Meng GY, Ebrahimi M.** Upregulation of peroxisome proliferator-activated receptors and liver fatty acid binding protein in hepatic cells of broiler chicken supplemented with conjugated linoleic acids. *Ital J Anim Sci* 14: 416–422, 2015.
101. **Reddy JK, Hashimoto T.** Peroxisomal β -oxidation and peroxisome proliferator-activated receptor α : an adaptive metabolic system. *Annu Rev Nutr* 21: 193–230, 2001.
102. **Riserus U, Sprecher D, Johnson T, Olson E, Hirschberg S, Liu A, Fang Z, Hedge P, Richards D, Sarov-Blat L, Strum JC, Basu S, Cheeseman J, Fielding BA, Humphreys SM, Danoff T, Moore NR, Murgatroyd P, O’Rahilly S, Sutton P, Willson T, Hassall D, Frayn KN, Karpe F.** Activation of peroxisome proliferator-activated receptor PPAR δ promotes reversal of multiple metabolic abnormalities, reduces oxidative stress, and increases fatty acid oxidation in

- moderately obese men. *Diabetes* 57: 332–339, 2008.
103. **Ristow M, Zarse K, Oberbach A, Klötting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, Blüher M.** Antioxidants prevent health-promoting effects of physical exercise in humans. *PNAS* 2: 2–7, 2009.
 104. **Ristow M, Zarse K, Oberbach A, Klötting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, Blüher M.** Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A* 106: 8665–8670, 2009.
 105. **Ruyter B, Anderson O, Dehli A, Ostlund Farrants A-K, Gjoen T, Thomassen MS.** Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochim Biophys Acta* 1348: 331–338, 1997.
 106. **Savard R, Ramenofsky M, Greenwood MRC.** A north-temperate migratory bird: a model for the fate of lipids during exercise of long duration. *Can J Physiol Pharmacol* 69: 1443–1447, 1991.
 107. **Schaefer H, McGraw K, Catoni C.** Birds use fruit colour as honest signal of dietary antioxidant rewards. *Funct Ecol* 22: 303–310, 2008.
 108. **Shipley B.** Cause and correlation in biology: A user's guide to path analysis and structural equations and causal inference with R. 2nd ed. Cambridge: Cambridge University Press, 2016.
 109. **Silbernagel G, Kovarova M, Cegan A, Machann J, Schick F, Lehmann R, Häring HU, Stefan N, Schleicher E, Fritsche A, Peter A.** High hepatic SCD1 activity is associated with low liver fat content in healthy subjects under a

- lipogenic diet. *J Clin Endocrinol Metab* 97: 2288–2292, 2012.
110. **Simsolo RB, Ong JM, Kern PA.** The regulation of adipose tissue and muscle lipoprotein lipase in runners by detraining. *J Clin Invest* 92: 2124–2130, 1993.
 111. **Sindler AL, Reyes R, Chen B, Ghosh P, Gurovich AN, Kang LS, Cardounel AJ, Delp MD, Muller-Delp JM.** Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles. *J Appl Physiol* 114: 681–693, 2013.
 112. **Skrip MM, McWilliams SR.** Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists. *J F Ornithol* 87: 1–20, 2016.
 113. **Smith SB, McPherson KH, Backer JM, Pierce BJ, Podlesak DW, McWilliams SR.** Fruit quality and consumption by songbirds during autumn migration. *Wilson J Ornithol* 119: 419–428, 2007.
 114. **Smith SB, McWilliams SR.** Patterns of fuel use and storage in migrating passerines in relation to fruit resources at autumn stopover sites. *Auk* 127: 108–118, 2010.
 115. **Spangenburg EE, Brown DA, Johnson MS, Moore RL.** Alterations in peroxisome proliferator-activated receptor mRNA expression in skeletal muscle after acute and repeated bouts of exercise. *Mol Cell Biochem* 332: 225–231, 2009.
 116. **Suarez RK.** Energy metabolism during insect flight: biochemical design and physiological performance. *Physiol Biochem Zool* 73: 765–771, 2000.
 117. **Swanson DL.** Seasonal metabolic variation in birds: functional and mechanistic correlates. In: *Current Ornithology*, p. 131–189.
 118. **Talbot DA, Hanuise N, Rey B, Rouanet J-L, Duchamp C, Brand MD.**

- Superoxide activates a GDP-sensitive proton conductance in skeletal muscle mitochondria from king penguin (*Aptenodytes patagonicus*). *Biochem Biophys Res Commun* 312: 983–988, 2003.
119. **Thomas AW, Davies NA, Moir H, Watkeys L, Ruffino JS, Isa SA, Butcher LR, Hughes MG, Morris K, Webb R.** Exercise-associated generation of PPAR γ ligands activates PPAR γ signaling events and upregulates genes related to lipid metabolism. *J Appl Physiol* 112: 806–815, 2012.
 120. **Vogel C, Marcotte EM.** Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* 13: 227–232, 2012.
 121. **Vogt M, Puntschart A, Howald H, Mueller B, Mannhart C, Gfeller-Tuescher L, Mullis P, Hoppeler H.** Effects of dietary fat on muscle substrates, metabolism, and performance in athletes. *Med Sci Sports Exerc* 35: 952–960, 2003.
 122. **Wagner G, Balfry S, Higgs D, Lall S, Farrell A.** Dietary fatty acid composition affects the repeat swimming performance of Atlantic salmon in seawater. *Comp Biochem Physiol - A Mol Integr Physiol* 137: 567–576, 2004.
 123. **Wang Y-X.** PPARs: diverse regulators in energy metabolism and metabolic diseases. *Cell Res* 20: 124–137, 2010.
 124. **Wilson DO, Johnson P.** Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. *J Appl Physiol* 88: 1791–1796, 2000.
 125. **Wingfield JC.** Flexibility in annual cycles of birds: implications for endocrine control mechanisms. *J Ornithol* 146: 291–304, 2005.
 126. **Zaefarian F, Abdollahi MR, Cowieson A, Ravindran V.** Avian liver: the forgotten organ. *Animals* 9: 1–23, 2019.

127. **Zechner R, Zimmermann R, Eichmann TO, Kohlwein SD, Haemmerle G, Lass A.** Fat signals-lipases and lipolysis in lipid metabolism and signaling. *Cell Metab.* (2012). doi: 10.1016/j.cmet.2011.12.018.
128. **Zhang J, Gao Y-Y, Huang Y-Q, Fan Q, Lu X-T, Wang C-K.** Selection of housekeeping genes for quantitative gene expression analysis in Yellow-feathered broilers. *Ital J Anim Sci* 17: 540–546, 2018.
129. **Zhang Y, King MO, Harmon E, Eyster K, Swanson DL.** Migration-induced variation of fatty acid transporters and cellular metabolic intensity in passerine birds. *J Comp Physiol B* 185: 797–810, 2015.

TABLES

Table 1.1. List of key symbols and abbreviations used throughout the manuscript

List of symbols and abbreviations	
avUCP	avian uncoupling protein
β -actin	beta actin
CD36	fatty acid translocase
CS	citrate synthase
ERR	estrogen-related receptor
FABPpm	plasma membrane fatty acid binding protein
FATP1	fatty acid transport protein 1
LPL	lipoprotein lipase
MCAD	medium-chain acyl-CoA dehydrogenase
MUFA	monounsaturated fatty acid
NR	nuclear receptor
NRF	nuclear respiratory factor
PGC-1	peroxisome proliferator-activated receptor gamma coactivator
PLIN2	perilipin 2
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PSEM	piecewise structural equation modeling
PUFA	polyunsaturated fatty acid
qRT-PCR	reverse transcription quantitative PCR
RXR	9-cis-retinoic acid receptor

Table 1.2. Piecewise-structural equation models for the causal and independent relationships for gene expression in the pectoralis implied by Causal Structure 1 (explained in text) and Causal Structure 2 (Figure 4a, Figure 4b). Models were conducted separately for flight-trained and untrained birds.

Equations for Causal Structures for gene expression in the pectoralis for flight-trained and untrained birds:

Relationships for Causal Structure 1.		
<i>Eq. #, Type of Relationship</i>	<i>Linear Model</i>	<i>$Y_i =$</i>
1. Causal	$Y_i = \beta_0 + \beta_1 PGC - 1\alpha + \beta_2 PGC - 1\beta$	{PPAR α , PPAR δ }
2. Causal	$Y_i = \beta_0 + \beta_1 PPAR\alpha + \beta_2 PPAR\delta$	{CD36, MCAD, CS, LPL, Plin2, avUCP}
3. Independent	$Y_i = \beta_0 + \beta_1 PGC - 1\alpha + \beta_2 PGC - \beta$	{CD36, MCAD, CS, LPL, Plin2, avUCP}
Relationships for Causal Structure 2.		
<i>Eq. #, Type of Relationship</i>	<i>Linear Model</i>	<i>$Y_i =$</i>
4. Causal	$Y_i = \beta_0 + \beta_1 PPAR\alpha + \beta_2 PPAR\delta$	{CD36, MCAD, CS, LPL, Plin2, avUCP}
5. Causal	$Y_i = \beta_0 + \beta_1 PGC - 1\alpha + \beta_2 PGC - 1\beta$	{CD36, MCAD, CS, LPL, Plin2, avUCP}
6. Independent	$Y_i = \beta_0 + \beta_1 PGC - 1\alpha + \beta_2 PGC - 1\beta$	{PPAR α , PPAR δ }
Goodness of Fit, Fisher's C statistic for Causal Structures 1 and 2:		
7. Fisher's C-Statistic	$C = -2 \sum_{i=1}^k \ln(p_i)$	C-statistic is χ^2 distributed with 2k degrees of freedom, where k is the number of independent claims

Table 1.3. Linear model results for pectoralis wet mass and metabolic gene expression in the pectoralis in relation to flight-training, dietary antioxidant, and dietary PUFA. The intercept is the 13% PUFA, Low antioxidant, Untrained Group (13L, U). Data are reported as estimates (standard error) for each gene and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$. Interactions or Julian Date were not retained in any models except for LPL^a.

<i>Covariate</i>	<i>Dependent variable:</i>										
	Mass (g)	LPL ^a	CD36	MCAD	CS	Plin2	avUCP	PGC-1 α	PGC-1 β	PPAR δ	PPAR α
Training (Flight-trained)	1.080*** (0.232)	-0.248 (0.211)	0.288*** (0.092)	0.247** (0.097)	0.226 (0.141)	-0.154** (0.072)	-0.201** (0.090)	0.321** (0.155)	0.662** (0.258)	-0.296** (0.130)	-0.019 (0.051)
Antioxidant (High)	-0.003 (0.232)	-0.182 (0.225)	-0.079 (0.091)	0.002 (0.097)	0.173 (0.140)	-0.095 (0.072)	-0.022 (0.089)	0.040 (0.154)	-0.038 (0.257)	-0.014 (0.130)	-0.021 (0.051)
PUFA (32% High)	-0.064 (0.232)	-0.281 (0.225)	-0.209** (0.091)	-0.154 (0.097)	-0.153 (0.140)	-0.099 (0.072)	-0.095 (0.089)	-0.138 (0.155)	-0.144 (0.257)	0.047 (0.130)	0.022 (0.051)
Intercept (13L, U)	13.12*** (0.24)	-1.737* (0.993)	0.986*** (0.093)	0.956*** (0.099)	0.880*** (0.143)	0.951*** (0.074)	0.933*** (0.091)	0.893*** (0.158)	1.207*** (0.263)	0.900*** (0.132)	1.008*** (0.052)
Observations	96	88	88	89	88	89	89	89	89	88	89
R ²	0.193	0.229	0.161	0.098	0.061	0.087	0.067	0.058	0.076	0.060	0.006
Adjusted R ²	0.166	0.151	0.131	0.066	0.027	0.055	0.034	0.025	0.043	0.026	-0.029
Residual Std. Error	1.134 (df=92)	0.502 (df = 79)	0.428 (df = 84)	0.456 (df = 85)	0.658 (df = 84)	0.339 (df = 85)	0.420 (df = 85)	0.728 (df = 85)	1.211 (df = 85)	0.609 (df = 84)	0.239 (df = 85)
F Statistic	7.319*** (df= 3; 92)	2.940*** (df = 8; 79)	5.360*** (df = 3; 84)	3.085** (df = 3; 85)	1.804 (df = 3; 84)	2.699* (df = 3; 85)	2.030 (df = 3; 85)	1.751 (df = 3; 85)	2.331* (df = 3; 85)	1.773 (df = 3; 84)	0.168 (df = 3; 85)

^aFor LPL expression, there were no significant main effects, but there was a significant interaction between training and PUFA (**0.750****, 0.304) but not for training and antioxidant (0.500, 0.302), antioxidant and PUFA (-0.022, 0.318), or for training, antioxidant, and PUFA (-0.780*, 0.430). Julian Date was only a significant covariate for LPL (**0.009*****, 0.003) but was not retained for any other genes in the pectoralis.

Table 1.4. Linear model results for liver wet mass and metabolic gene expression in the liver in relation to flight-training, dietary antioxidant, and dietary PUFA. The intercept is the 13% PUFA, Low antioxidant, Untrained Group (13L, U). Data are reported as estimates (standard error) for each gene and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$. Interactions were not retained in any models, but Julian Date was retained in CD36 and LPL models^a.

<i>Covariate</i>	<i>Dependent variable:</i>							
	Mass (g)	LPL ^a	CD36 ^a	Plin2	FATp1	FABPpm	PPAR γ	PPAR α
Training (Flight-trained)	0.355*** (0.037)	0.532** (0.249)	0.108 (0.094)	-0.035 (0.172)	0.008 (0.074)	0.119 (0.079)	0.219 (0.153)	0.055 (0.036)
Antioxidant (High)	0.034 (0.037)	0.032 (0.251)	-0.037 (0.094)	-0.169 (0.171)	-0.033 (0.073)	-0.037 (0.079)	0.153 (0.152)	-0.041 (0.036)
PUFA (32% High)	-0.002 (0.037)	0.378 (0.248)	-0.013 (0.093)	0.011 (0.171)	-0.011 (0.073)	0.025 (0.079)	-0.108 (0.152)	0.085** (0.036)
Intercept (13L, U)	1.295*** (0.038)	4.679** (2.271)	2.92*** (0.856)	1.035*** (0.173)	0.940*** (0.074)	0.907*** (0.080)	1.099*** (0.154)	1.036*** (0.037)
Observations	95	86	87	87	87	87	87	87
R ²	0.510	0.107	0.084	0.012	0.003	0.029	0.043	0.098
Adjusted R ²	0.494	0.063	-0.040	-0.023	-0.033	-0.006	0.008	0.066
Residual Std. Error	0.179 (df=91)	1.146 (df = 81)	0.433 (df = 82)	0.796 (df = 83)	0.341 (df = 83)	0.366 (df = 83)	0.708 (df = 83)	0.168 (df = 83)
F Statistic	31.6*** (df= 3; 91)	2.432* (df = 4; 81)	1.89 (df = 4; 82)	0.349 (df = 3; 83)	0.079 (df = 3; 83)	0.837 (df = 3; 83)	1.235 (df = 3; 83)	3.022** (df = 3; 83)

^aJulian Date was retained for CD36 (**-0.006****, 0.003) and LPL (-0.012*, 0.007) models, but was not retained for any other genes in the liver.

FIGURES

Figure 1.1. Relative gene expression (estimated marginal means from the linear mixed models; Table 2) in the 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. Genes involved in fat utilization (CD36, MCAD) and PPAR cofactors (PGC-1a, PGC-1b) were expressed to the greatest extent in flight-trained birds, whereas Plin2, avUCP, and PPARδ were expressed to the greatest extent in untrained birds. CS and PPARα were not influenced by flight training. The upper and lower lines correspond to the 1st and 3rd quartiles and the upper and lower whiskers roughly represent the 95% confidence interval range. The asterisks correspond to significance levels **p<0.05, ***p<0.01 between flight-trained and untrained birds.

Figure 1.2. Relative gene expression (estimated marginal means from the linear mixed models; Table 3) in the liver of European starlings that were or were not flown in the wind tunnel for 15 days, Flight-trained (N=49) or Untrained (N=38), respectively. LPL expression was greatest in flight-trained birds. Flight training did not significantly influence gene expression of CD36, Plin2, FATp1, FABPpm, PPARY, and PPARα, although there was a trend for greater expression of several of these genes (e.g., CD36, FABPpm, PPARY) in flight-trained birds. The upper and lower lines correspond to the 1st and 3rd quartiles and the upper and lower whiskers roughly represent the 95% confidence interval range. The asterisks correspond to significance level **p<0.01 between flight-trained and untrained birds.

Figure 1.3. (A) Relative LPL gene expression (estimated marginal means from the linear mixed models, ESMs; Table 2) in the pectoralis muscle was relatively greater for flight-trained European starlings fed the 32% PUFA diet but not when fed the 13% PUFA diet. The asterisks correspond to significance level $**p<0.05$ between trained and untrained birds in the 32% PUFA group (32% untrained N=20, 32% trained N=23, 13% untrained N=20, 13% trained N=26). (B) Relative CD36 expression (ESMs; Table 3) in the pectoralis muscle was relatively greater for European starlings fed the 13% PUFA diets (32% N=43, 13% N=46) whereas relative PPAR α expression (ESMs; Table 3) in the liver was greater for European starlings fed the 32% PUFA diets (32% N=41, 13% N=46). The upper and lower lines correspond to the 1st and 3rd quartiles and the upper and lower whiskers roughly represent the 95% confidence interval range. The asterisks correspond to significance levels: $**p<0.05$, $***p<0.01$ between 13% and 32% PUFA groups for each gene.

A. Flight-Trained Birds

Goodness of fit:
Fisher's C-statistic 10.39
df=8
P=0.24

B. Untrained Birds

Goodness of fit:
Fisher's C-statistic 5.17
df=8
P=0.74

Figure 1.4. The causal structure (Model 2) to explain the non-hierarchical gene expression of PPAR coactivators (PGC-1 α , PGC-1 β), and PPAR transcription factors (PPAR α , PPAR δ) on the expression of downstream metabolic genes (CD36, MCAD, CS, LPL, Plin2, and avUCP) in the pectoralis of flight-trained (A) and untrained (B) European starlings. ϵ_1 represents all unmeasured variables that could affect PPAR gene expression (e.g. ligand type and quantity), and ϵ_2 represents all unmeasured variables that could affect metabolic gene expression (e.g. other transcription factors). Regression coefficients (from linear models; Tables S4, S5) are reported to the left of each line for all causal relationships and indicate the extent of change in the number of transcripts of the metabolic genes (CD36, MCAD, CS, LPL, Plin2, avUCP) for each one-transcript change in the PPAR and coactivator genes (PPAR α , PPAR δ , PGC-1 α , PGC-1 β). The dashed lines indicate non-significant causal relationships (p-values>0.1). Solid lines indicate significant causal relationships, and the asterisks and line thickness correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01. In flight-trained birds (A) only select metabolic genes (MCAD, CS, and Plin2) are influenced by PPAR α , PPAR δ or PGC-1 α (n=48 or 49). In untrained birds (B) all metabolic genes (except Plin2) are influenced by PPAR δ and 50% of these genes are influenced by PGC-1 α (n=44 or 45).

SUPPORTING MATERIALS

Table S1.1. Sequences of primers used for reverse transcription quantitative PCR in the pectoralis muscle and liver of European Starlings.

Gene	Forward and Reverse Primers	RefSeq accession number
β -actin	F: 5'- GCTACGAACTCCCTGATGG -3' R: 5'- GACTCCATACCCAGGAAAGATG -3'	XM_014870664.1
LPL	F: 5'- AGTAAACCTCCTTGTGCAACATTCA- 3' R: 5'- CCAATAACAGCAGACTCATTTAGCCA- 3'	XM_014882395.1
CD36	F: 5'- AGTAAACCTCCTTGTGCAACATTCA - 3' R: 5'- CCAATAACAGCAGACTCATTTAGCCA - 3'	XM_014883466.1
MCAD	F: 5'- GCTTGGGAGCTTGGTCTTAT - 3' R: 5'- TGATAACTGGCATTGCCCCCT - 3'	XM_014892563.1
Plin2	F: 5'- AGCAATTGATCCAGAGCAGAACATT -3' R: 5'- ATCCTTTGTGGTGATGTAAGCTGTG -3'	XM_014871606.1
avUCP	F: 5'- AATGCCATCATCAACTGCGG -3' R: 5'- ACGTTGTCTGCCATGAGATGT -3'	XM_014888711.1
FATp1	F: 5'- CCAAGACAAGCACTTACAAGTTCCA -3' R: 5'- AAATACAACCTGTCCTTACCAGC -3'	XM_014873286.1
FABPpm	F: 5'- GTAGTGAAGAAACGAAACCTCCTCG -3' R: 5'- TTGATGCCCTGCTCGATGAAATG -3'	XM_014880748.1
PGC-1 α	F: 5'- GGCAGAAGAGCCGTCTCTA -3' R: 5'- CATGAATTCTCAGTCTTAACAACCA -3'	XM_014885152.1
PGC-1 β	F: 5'- TGGAGCAAACAGAAACACCCAG -3' R: 5'- CCACAGTCAGCATTGGCTCAAA -3'	XM_014891216.1
PPAR α	F: 5'- GTGCGTGACATCAAGGAGAA -3' R: 5'- GGTGTCATCAGGATGGTTGT -3'	XM_014880503.1
PPAR δ	F: 5'- GCATGTCACACAACGCAAT -3' R: 5'- GATCTCGCTTGCCGTCAG -3'	XM_014872751.1
PPAR γ	F: 5'- ACGACTCCTACATCAAATCCTT -3' R: 5'- ACGAAGGGTGATTTGTCTGT -3'	XM_014869773.1

Table S1.2. Mean gene expression of each metabolic gene in the pectoralis muscle relative to the reference group^R, 13L, U used for the linear models reported in text and in Table 2. Relative mean gene expression $\pm$ standard deviation are reported for each treatment group, and treatment groups are abbreviated as follows: 13 or 32% PUFA, Low (L) or High (H) antioxidant, and Untrained (U) or Flight-Trained (T) for training. The number of individuals for each treatment groups are reported in the first row or in the footnote^a

<i>Pectoralis Metabolic Genes</i>	<i>Relative Mean Gene Expression $\pm$ Standard deviation by Diet and Flight Training Treatment Groups in the Pectoralis:</i>							
	13L, U ^R	13L, T	13H, U	13H, T	32L, U	32L, T	32H, U	32H, T
<i>Number of Individuals</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>11</i>	<i>10</i>	<i>12</i>
LPL	1.00 $\pm$ 0.39	0.73 $\pm$ 0.35	0.85 $\pm$ 0.84	1.13 $\pm$ 0.59 ^a	0.76 $\pm$ 0.49	1.25 $\pm$ 0.68	0.56 $\pm$ 0.35	0.79 $\pm$ 0.35
CD36	1.00 $\pm$ 0.28	1.21 $\pm$ 0.40 ^a	0.82 $\pm$ 0.34	1.31 $\pm$ 0.64	0.79 $\pm$ 0.43	1.11 $\pm$ 0.49	0.76 $\pm$ 0.27	0.88 $\pm$ 0.39
MCAD	1.00 $\pm$ 0.35	1.19 $\pm$ 0.52	0.84 $\pm$ 0.43	1.27 $\pm$ 0.46	0.85 $\pm$ 0.50	0.98 $\pm$ 0.34	0.82 $\pm$ 0.46	1.06 $\pm$ 0.56
CS	1.00 $\pm$ 0.67	1.24 $\pm$ 0.67	0.86 $\pm$ 0.38	1.21 $\pm$ 0.69	0.61 $\pm$ 0.59	0.80 $\pm$ 0.44	1.17 $\pm$ 0.72 ^a	1.21 $\pm$ 0.90
Plin2	1.00 $\pm$ 0.44	0.77 $\pm$ 0.35	0.86 $\pm$ 0.49	0.68 $\pm$ 0.27	0.81 $\pm$ 0.32	0.72 $\pm$ 0.36	0.74 $\pm$ 0.20	0.63 $\pm$ 0.29
avUCP	1.00 $\pm$ 0.38	0.77 $\pm$ 0.29	0.77 $\pm$ 0.26	0.73 $\pm$ 0.36	0.76 $\pm$ 0.50	0.60 $\pm$ 0.38	0.98 $\pm$ 0.76	0.58 $\pm$ 0.32
PGC-1 α	1.00 $\pm$ 0.78	1.23 $\pm$ 0.85	0.83 $\pm$ 0.73	1.23 $\pm$ 0.37	0.79 $\pm$ 0.98	0.93 $\pm$ 0.35	0.76 $\pm$ 0.47	1.26 $\pm$ 1.04
PGC-1 β	1.00 $\pm$ 0.68	2.06 $\pm$ 2.05	1.14 $\pm$ 0.87	1.82 $\pm$ 0.83	1.26 $\pm$ 1.11	1.51 $\pm$ 0.80	1.06 $\pm$ 0.62	1.69 $\pm$ 1.71
PPAR δ	1.00 $\pm$ 0.63	0.68 $\pm$ 0.57	0.72 $\pm$ 0.55	0.57 $\pm$ 0.35 ^a	0.92 $\pm$ 1.01	0.50 $\pm$ 0.32	1.03 $\pm$ 0.79	0.72 $\pm$ 0.49
PPAR α	1.00 $\pm$ 0.36	1.04 $\pm$ 0.19	0.94 $\pm$ 0.18	0.97 $\pm$ 0.17	1.05 $\pm$ 0.30	0.95 $\pm$ 0.14	1.05 $\pm$ 0.27	1.00 $\pm$ 0.28

^aThere was 1 fewer sample from these treatment groups

Table S1.3. Mean gene expression of each metabolic gene in the liver relative to the reference group^R, 13L, U used for the linear models reported in text and in Table 3. Relative mean gene expression $\pm$ standard deviation are reported for each treatment group, and treatment groups are abbreviated as follows: 13 or 32% PUFA, Low (L) or High (H) antioxidant, and Untrained (U) or Flight-Trained (T) for training. The number of individuals for each treatment groups are reported in the first row or in the footnote^a.

<i>Relative Mean Gene Expression $\pm$ Standard deviation by Diet and Flight Training Treatment Groups in the Liver:</i>								
<i>Liver Metabolic Genes</i>	13L, U ^R	13L, T	13H, U	13H, T	32L, U	32L, T	32H, U	32H, T
<i>Number of Individuals</i>	10	13	10	13	10	11	8	12
LPL	1.00 $\pm$ 0.35	1.30 $\pm$ 0.63 ^a	0.90 $\pm$ 0.60	1.11 $\pm$ 0.36	1.13 $\pm$ 1.22	1.60 $\pm$ 1.35	0.74 $\pm$ 0.33	2.02 $\pm$ 2.40
CD36	1.00 $\pm$ 0.33	0.93 $\pm$ 0.40	0.83 $\pm$ 0.41	0.95 $\pm$ 0.51	0.77 $\pm$ 0.47	1.13 $\pm$ 0.57	0.86 $\pm$ 0.47	0.91 $\pm$ 0.52
Plin2	1.00 $\pm$ 0.31	0.96 $\pm$ 0.80	0.68 $\pm$ 0.35	1.04 $\pm$ 1.13	1.32 $\pm$ 1.16	0.84 $\pm$ 0.71	0.81 $\pm$ 0.95	0.81 $\pm$ 0.52
FATp1	1.00 $\pm$ 0.35	0.91 $\pm$ 0.34	0.83 $\pm$ 0.21	0.97 $\pm$ 0.41	0.95 $\pm$ 0.45	0.91 $\pm$ 0.34	0.90 $\pm$ 0.25	0.91 $\pm$ 0.32
FABPpm	1.00 $\pm$ 0.32	1.01 $\pm$ 0.40	0.85 $\pm$ 0.19	0.95 $\pm$ 0.31	0.90 $\pm$ 0.41	1.01 $\pm$ 0.34	0.85 $\pm$ 0.22	1.11 $\pm$ 0.56
PPAR γ	1.00 $\pm$ 0.44	1.44 $\pm$ 1.10	1.44 $\pm$ 0.80	1.29 $\pm$ 0.66	0.96 $\pm$ 0.43	1.19 $\pm$ 0.50	1.07 $\pm$ 0.68	1.46 $\pm$ 0.72
PPAR α	1.00 $\pm$ 0.21	1.10 $\pm$ 0.22	1.06 $\pm$ 0.08	1.02 $\pm$ 0.21	1.11 $\pm$ 0.16	1.21 $\pm$ 0.22	1.06 $\pm$ 0.06	1.13 $\pm$ 0.21

^aThere was 1 fewer sample from this treatment group

Table S1.4. Piecewise-structural equation linear model results for metabolic gene expression in the pectoralis for flight-trained birds for each alternative Causal Structure. Causal relationships are unshaded and independent claims used to calculate the C-statistic are shaded in gray. Data are reported as estimates (standard error), and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$. The sample size for each gene is 49 unless specified in the footnote.

Causal Structure 1

Goodness of fit:

C-Statistic: 61.97, df: 24 p-value < 0.001

<i>Predictor variable</i>	<i>Response variable</i>							
	LPL ^a	CD36 ^a	MCAD	CS	Plin2	avUCP	PPAR δ^a	PPAR α
PGC-1 α	-0.09 (0.23)	0.20 (0.14)	0.51*** (0.10)	0.68*** (0.14)	0.02 (0.16)	0.23* (0.13)	0.12 (0.12)	0.07 (0.05)
PGC-1 β	0.22 (0.18)	0.17 (0.135)	-0.02 (0.08)	0.0003 (0.11)	0.04 (0.12)	-0.0009 (0.10)	-0.12 0.09	0.03 (0.04)
PPAR δ	0.01 (0.30)	0.07 (0.20)	0.37** (0.18)	0.26 (0.26)	0.57*** (0.17)	0.13 (0.18)		
PPAR α	-0.74 (0.63)	1.11** (0.42)	0.81** (0.37)	1.16** (0.55)	0.86** (0.36)	0.65 (0.37)		
R ² for causal relationships	0.03	0.14	0.17	0.11	0.27	0.07	0.03	0.19

Causal Structure 2

Goodness of fit:

C-Statistic: 10.39, df: 8, p-value: 0.24

<i>Predictor variable</i>	<i>Response variable</i>							
	LPL ^a	CD36 ^a	MCAD	CS	Plin2	avUCP	PPAR δ^a	PPAR α
PGC-1 α	-0.005 (0.24)	0.15 (0.15)	0.47*** (0.10)	0.65*** (0.15)	-0.12 (0.14)	0.20 (0.14)	0.12 (0.12)	0.07 (0.05)
PGC-1 β	0.26 (0.18)	0.17 (0.11)	0.02 (0.07)	0.03 (0.11)	0.08 (0.11)	0.005 (0.11)	-0.11 (0.09)	0.03 (0.04)
PPAR δ	0.08 (0.29)	0.11 (0.19)	0.35*** (0.12)	0.24 (0.19)	0.60*** 0.18	0.12 (0.17)		
PPAR α	-1.29* (0.68)	0.49 (0.43)	-0.07 (0.28)	-0.06 (0.43)	0.91** (0.41)	0.28 (0.40)		
R ² for causal relationships	0.13	0.33	0.64	0.57	0.29	0.17		

^a The sample size for these genes are 48

Table S1.5. Piecewise-structural equation linear model results for metabolic gene expression in the pectoralis for untrained birds for each alternative Causal Structure. Causal relationships are unshaded and independent claims used to calculate the Fisher's C-statistic are shaded in gray. Data are reported as estimates (standard error), and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$. The sample size for each gene is 45 unless specified in the footnote.

Causal Structure 1

Goodness of fit:

Fisher's C-Statistic: 75.12, df: 24, p -value < 0.001

Predictor variable	Response variable							
	LPL	CD36	MCAD	CS ^a	Plin2	avUCP	PPAR δ	PPAR α
PGC-1 α	0.54*** (0.18)	0.37*** (0.07)	0.31*** (0.10)	0.28** (0.13)	0.29* (0.15)	0.03 (0.18)	0.14 (0.17)	0.07 (0.06)
PGC-1 β	-0.40 (0.13)	-0.23** (0.10)	-0.10 (0.14)	0.24 (0.18)	-0.40* (0.22)	-0.01 (0.25)	-0.25 (0.25)	-0.03 (0.09)
PPAR δ	0.40** (0.18)	0.22*** (0.08)	0.28*** (0.10)	0.30** (0.13)	0.31** (0.14)	0.70*** (0.12)		
PPAR α	0.04 (0.50)	0.16 (0.22)	-0.05 (0.26)	0.36 (0.37)	0.57 (0.39)	0.43 (0.33)		
R ² for causal relationships	0.12	0.19	0.19	0.14	0.17	0.50	0.03	0.04

Causal Structure 2

Goodness of fit:

Fisher's C-Statistic: 5.17, df: 8, p -value: 0.74

Predictor variable	Response variable							
	LPL	CD36	MCAD	CS ^a	Plin2	avUCP	PPAR δ^a	PPAR α
PGC-1 α	0.50*** (0.18)	0.34*** (0.07)	0.29*** (0.09)	0.23 (0.12)	0.22 (0.15)	-0.11 (0.13)	0.14 (0.17)	0.07 (0.06)
PGC-1 β	-0.32 (0.25)	-0.18 (0.09)	-0.04 (0.12)	0.32 (0.17)	-0.31 (0.21)	0.18 (0.18)	-0.25 (0.25)	-0.03 (0.09)
PPAR δ	0.35** (0.17)	0.19*** (0.06)	0.26*** (0.08)	0.31*** (0.11)	0.27 (0.14)	0.72*** (0.12)		
PPAR α	-0.22 (0.47)	-0.03 (0.17)	-0.23 (0.23)	0.15 (0.32)	0.49 (0.40)	0.47 (0.34)		
R ² for causal relationships	0.29	0.55	0.44	0.43	0.23	0.52		

^a The sample size for this gene is 44

CHAPTER 2

REGULAR FLYING AND DIETARY ANTIOXIDANTS, BUT NOT DIETARY FAT, INFLUENCE ANTIOXIDANT GENE EXPRESSION AND ENZYME ACTIVITY IN THE LIVER AND FLIGHT MUSCLE OF A MIGRATORY BIRD

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ABSTRACT

Ecologically-relevant factors such as exercise and diet quality can directly influence how physiological systems work including those involved in maintaining oxidative balance; however, to our knowledge, no studies to date have focused on how such factors directly affect expression of key components of the endogenous antioxidant system (i.e., transcription factors, select antioxidant genes, and corresponding antioxidant enzymes) in several metabolically active tissues of a migratory songbird. We conducted a 3-factor experiment that tested the following hypotheses: (H1) Daily flying over several weeks increases the expression of transcription factors NRF2 and PPARs as well as endogenous antioxidant genes (i.e. CAT, SOD1, SOD2, GPX1, GPX4), and upregulates endogenous antioxidant enzyme activities (i.e. CAT, SOD, GPx). (H2) Songbirds fed diets composed of more 18:2n-6 PUFA are more susceptible to oxidative damage and thus upregulate their endogenous antioxidant system compared to when fed diets with less PUFA. (H3) Songbirds fed dietary anthocyanins gain additional antioxidant protection and thus upregulate their endogenous antioxidant system less compared to songbirds not fed anthocyanins. Flight training increased the expression of 40% of the antioxidant genes and transcription factors measured in the liver, consistent with H1, but for only one gene (SOD2) in the pectoralis. Dietary fat quality had no effect on antioxidant pathways (H2) whereas dietary anthocyanins increased the expression of antioxidant enzymes and NRF2 expression in the pectoralis, but not in the liver (H3). These tissue-specific differences in response to flying and dietary antioxidants are likely explained by functional differences between tissues as well as fundamental differences in their turnover rates. The consumption of dietary antioxidants along with regular flying enables birds during

migration to stimulate the expression of genes involved in antioxidant protection likely through increasing the transcriptional activity of NRF2 and PPARs, and thereby demonstrates for the first time that these relevant ecological factors affect the regulation of key antioxidant pathways in wild birds. What remains to be demonstrated is how the extent of these ecological factors (i.e., intensity or duration of flight, amounts of dietary antioxidants) influences the regulation of these antioxidant pathways and thus oxidative balance.

Keywords

antioxidants, antioxidant enzyme expression, dietary fat quality, flight training, nuclear factor erythroid 2-related factor 2, peroxisome proliferator-activated nuclear receptors

Introduction

The challenges of oxidative balance for wild vertebrates, and specifically migratory birds

Aerobically respiring organisms must maintain a balanced oxidative status where excess reactive species (RS) are neutralized with antioxidants to minimize resulting oxidative damage or such damage must be repaired [1,2]. Maintaining oxidative balance is especially crucial when RS production is high during energetically demanding life-history stages including migration [3–6] and reproduction [7–11]. Migratory birds are particularly vulnerable to oxidative damage since they must increase their metabolism 9 times above their basal metabolic rates to complete energy-intensive long-distance flights [12–15]. However, like other vertebrates, migratory birds can avoid or ameliorate the production of RS using endogenously produced antioxidant enzymes (e.g. glutathione peroxidase), sacrificial molecules (e.g. uric acid) or dietary antioxidants (e.g. anthocyanins) to minimize oxidative damage [1,2,4]. This multifaceted antioxidant system has been typically investigated by measuring the final products of antioxidants and oxidative damage (e.g. enzyme activities, non-enzymatic antioxidant capacity, lipid peroxidation capacity, protein carbonyls), and mixed results suggest that sometimes migratory birds can maintain oxidative balance when exercising [16] but sometimes cannot [17,18]. Conflicting results may be due to indirect effects of environmental stimuli (e.g. exercise, diet) on measures of biochemical antioxidant capacity and oxidative damage levels since these measures depend on many factors including transcriptional and posttranscriptional regulation. In contrast, environmental factors such as exercise and diet can directly influence antioxidant molecular pathways; thus, studies that quantify the gene expression of antioxidant transcription factors and endogenous antioxidants in

response to environmental factors can better elucidate how this multi-faceted antioxidant system is regulated [19].

The antioxidant response of animals within the context of ecology

The antioxidant response begins with transcription factors - The major cellular pathways in all animals for regulating the antioxidant response includes peroxisome proliferator-activated nuclear receptors (PPARs) and nuclear factor erythroid 2-related factor 2 (NRF2). These transcription factors regulate endogenous antioxidant enzymes, and both transcription factors can be affected by ecologically relevant factors including exercise [20–23] and diet [24–27]. There are three PPAR isoforms, PPAR γ , PPAR δ , and PPAR α , that act as key regulators of fat metabolism [28–30] and the production of reactive oxygen species [31] and so may be important for birds which rely on primarily fat for fuel and must contend with the production of lipid peroxides [1,2]. The stimulation of PPAR pathways increase fat metabolism and oxidation, and thus may induce RS production and cause an imbalanced oxidative status [32]. However, PPARs can also protect against RS by directly transcribing superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) through PPAR response element (PPRE) sequences located in each enzyme's promotor regions [32]. To our knowledge, the PPAR regulation of antioxidant enzymes has not been examined in songbirds or other wildlife.

NRF2 binds to the antioxidant response element (ARE) to transcribe an array of 250 genes involved in antioxidant protection and redox homeostasis (Tebay et al., 2015; Yamamoto et al., 2018) including SOD [33,34] and glutathione, the precursor to GPx [35,36], whereas CAT activity is induced by NRF2 without an apparent ARE promotor region [37–39]. In most taxa NRF2 is regulated by a KEAP1 repressor that has recently

been shown to have mutated in songbirds and their relatives (i.e., Neoaves) and resulted in a constitutively active NRF2 that is able to transcribe antioxidant genes under any cellular conditions [40,41]. A constitutively active NRF2 would allow birds to quickly transcribe genes associated with antioxidant enzymes during times of high RS production, like during migratory flight. Studying molecular antioxidant pathways such as those described above in songbirds is particularly interesting due to the novel continuous activation of NRF2 recently discovered in birds, their energy-expensive lifestyle and mode of locomotion (i.e., flying is costly), and the potential importance of ecologically-relevant factors such as exercise and diet quality on the functioning of these antioxidant pathways.

Exercise stimulates specific molecular antioxidant pathways - Exercise and the associated increases in metabolism can stimulate molecular antioxidant pathways by 1. increasing the production of RS resulting in increased NRF2 transcription [20] and 2. increasing the amount of circulating fatty acid ligands (e.g. free fatty acids, eicosanoids) resulting in increased PPAR coactivator activity and PPAR signaling [21,42]. The NRF2 antioxidant pathway in muscle is stimulated by both acute exercise and exercise training in mice and humans [20,23,43]. Repeated bouts of exercise have a similar stimulatory effect on NRF2 pathways in multiple tissues including skeletal muscle, myocardium, liver, kidney, brain, testes, and prostate [20]. Wild-type mice that trained on the treadmill for five weeks compared to NRF2 deficient mice had higher NRF2 mRNA levels, improved mitochondrial biogenesis, and higher SOD and catalase expression indicating that NRF2 pathways are required for increases in endurance performance and antioxidant protection associated with exercise training [44]. The stimulatory effect of exercise on

NRF2 has not been studied in songbirds, although it is clearly relevant given their need to contend with oxidative challenges associated with regularly flying.

Exercise also stimulates PPAR pathways mainly through the generation of PPAR fat ligands and increased expression of PGC-1 cofactors that bind to PPARs to increase their transcriptional activity [21,42,45]. An eight-week cycling training regime demonstrated that exercise training increased plasma PPAR ligands, PPAR γ activity, and PPAR target gene expression (CD36, LXR α , ABCA1) within three hours post-exercise [21]. Similarly, the PGC-1 coactivators increased two-fold in within 18 hours of a single bout of swimming exercise in rats suggesting a possible increase in PPAR activity [42]. When PPARs, cofactors, and antioxidant enzymes were studied simultaneously, exercise-induced ROS production increased the mRNA expression of PGC-1 α , PGC-1 β , PPAR γ , SOD1, SOD2, GPX1, and CAT in human skeletal muscle [26]. No previous studies have investigated how exercise influences PPAR expression and antioxidant enzyme expression or activity in wild birds to determine if this pathway is important in protecting against exercise-induced RS during migration.

Dietary fat challenges the endogenous antioxidant system- Birds rely on fatty acids to fuel flight [46,47], and certain migratory songbird species optimize the relative amounts of polyunsaturated fat (PUFA) to monounsaturated fat (MUFA) in their diets, fat stores, and in circulation [48–51]. The potential benefits of consuming 18:2n-6 PUFA include faster mobilization rates, maintaining optimal membrane properties, and increases in PPAR activation [27,47]. However, all PUFA are highly susceptible to oxidative damage due to easily-oxidizable hydrogen atoms located near their double bonds [1]. Furthermore, lipid radicals are produced when RS scavenge the hydrogen

atoms from an unsaturated fat, and often this causes a self-perpetuating chain reaction damaging nearby PUFAs and other molecules [2]. Thus, there is a potential trade-off of using 18:2n-6 PUFA as substrate to enhance metabolism versus battling its associated oxidative costs that may require more endogenous and dietary antioxidant protection.

Dietary antioxidants modulate molecular antioxidant pathways-Many songbird species select fruits high in antioxidants during migration, suggesting that antioxidant consumption is important to protect against oxidative damage during this life history stage [52,53]. Water-soluble antioxidants such as anthocyanins are particularly relevant in songbirds since they are preferentially consumed by certain species during fall migration and in captivity [52–54]. Dietary anthocyanin supplements in humans stimulate NRF2 and enhance oxidative capacity in the context of the inflammatory disease atherosclerosis [55], in human diabetic aortic cells [56], cloned rat liver cells [57], human serum under mild hypoxic conditions [58], and in healthy dairy goats [24]. This ‘stimulatory’ hypothesis identifies dietary anthocyanins as enhancers of the NRF2 antioxidant pathway, yet the exact mechanisms responsible remain unclear [57,58] and have not yet been studied in birds. Interestingly, when dietary antioxidant supplements (e.g., vitamins C and E) are combined with exercise in mice and humans, RS production is reduced, there is a decrease in the transcription of NRF2 [20], and a decreased activation of PPAR pathways [25,26]. This is a potentially energetically beneficial strategy since organisms can use antioxidants gained through their diet for RS protection to avoid the energetically expensive production and maintenance of endogenous antioxidant enzymes. This ‘compensatory’ hypothesis may only be relevant when individuals are metabolically challenged, although this hypothesis has not been tested in

any non-human model system. Considered together, these studies suggest that birds consuming antioxidant-rich berries may either use dietary anthocyanins to (a) stimulate NRF2 transcription of antioxidant enzymes and/or (b) to quench RS and inhibit the energetically costly transcription of antioxidant enzymes. These scenarios may not be exclusive, but rather depend on current oxidative status and energetic demands.

How does flight training, dietary fat, and dietary anthocyanins affect the endogenous antioxidant system?

The goal of this experimental study was to investigate how flight training in a wind tunnel as well as consumption of certain dietary fats (i.e., 18:2n-6 PUFA) and dietary antioxidants (i.e., water-soluble anthocyanins) affected the expression of NRF2 and PPAR transcription factors, select antioxidant genes, and corresponding antioxidant enzymes in the liver and the pectoralis muscle of a migratory songbird. We tested the following three hypotheses: Flight training effect (H1): flying regularly over several weeks (a) increases the expression of NRF2 and PPARs, and thereby (b) increases the expression of endogenous antioxidant genes (i.e. CAT, SOD1, SOD2, GPX1, GPX4), and (c) produces a coordinated upregulation of endogenous antioxidant enzyme activities (i.e. CAT, SOD, GPx). Dietary fat effect (H2): migratory songbirds fed diets composed of more PUFA are more susceptible to oxidative damage and thus have increased expression levels of NRF2 and PPAR transcription factors, selected antioxidant genes, and corresponding antioxidant enzyme activities compared to when fed diets with less PUFA. Dietary antioxidant effect (H3): migratory songbirds fed dietary anthocyanins have less need to upregulate their endogenous antioxidant system and thus have decreased expression levels of the NRF2 and PPAR transcription factors, selected antioxidant

genes, and corresponding antioxidant enzymes compared to songbirds not fed anthocyanins. We also examined whether these three ecologically-relevant factors (flying, fat quality of diet, dietary antioxidants) significantly interacted to affect key components of the antioxidant system. For example, the compensatory function of dietary antioxidants may be most evident in birds that are flight trained due to their inhibitory effect on NRF2 and PPARs and the transcription of antioxidant enzymes. Whereas, a stimulatory function of dietary antioxidants may be evident in untrained birds due to their stimulatory effects on NRF2 activity in organisms at rest.

Materials and 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 migration [52,53]; 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, they are omnivorous and acclimate well to captivity and new diets, and they have been successfully trained and flown in wind tunnels in other studies [59,60]. 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, before fall migration, between 19-23 August 2015. Starlings from this southern Canada wild population are considered partially migratory as inferred by banding records [61]. 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; [62]) for each individual. Birds were then randomly sorted into four groups with roughly equal distributions of body size and molt score. We maintained aviaries at 21°C on a natural light cycle from capture and 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).

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 [18,63–65]. 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, we began adding a supplementary water-soluble antioxidant,

anthocyanin (elderberry powder; Artemis International, Fort Wayne, IN) to the diets of 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: 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* [54,66]. 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 35 wet g day⁻¹ (as observed in food intake trials in this study). Anthocyanins are particularly relevant to songbirds since they are prevalent in fruits consumed during migration [52–54,66], and anthocyanins are exclusively stable at acidic pH levels and are more likely to be preserved for utilization in the 2x more acidic stomachs of songbirds (pH 2) relative to mammals (pH 4.4) [67].

Experimental Timeline

On September 21st we randomly assigned 5 starlings to each of twenty cohorts. There were 5 cohorts per diet group, and the sampling order of the diet groups was randomly assigned within a cohort group ensuring that the same diet group was not consistently sampled first or last, and all diet groups were sampled within 10 days of one another. On September 23rd, and continuing every three days thereafter (Fig. S2), 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 placed in individual cages (0.6m x 0.5m x 0.5m) for two days (days -

9 and -8 relative to flight training) 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.

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 [68]. 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 (days -4 to -1) included training birds to fly between their flight cage and the wind tunnel and 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. Flight-trained starlings then participated in a fifteen-day training regimen 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. The final flight was on average 193 min +/-71 and the maximum was 360 min. At 1400hr – 1500hr on days 16 and 17 the untrained and trained birds, respectively, in each cohort 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 gene expression and enzyme activities 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.

Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed to quantify relative expression of select antioxidant genes, NRF2, and PPARs. Total RNA was extracted from liver and pectoralis muscle (25-30 mg) using RNeasy® Fibrous Mini Kit (QIAGEN®, Germantown, MD, USA) following kit instructions including the recommended DNase treatment step, but without the proteinase K digestion step for the liver. RNA concentrations and quality were verified using a NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). RNA (0.5 µg) was reverse transcribed using the SuperScript IV First-strand Synthesis System Kit (Thermofisher, Burlington, ON, CA), and cDNA was used as a template for qPCR. Each 17.5 µl PCR reaction mixture was comprised of 1:15 diluted cDNA template, 400 nM gene-specific primers and The Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (Thermofisher, Burlington, ON, CA). The temperature cycles for each PCR reaction were as follows: 2 min at 50°C, 2 min at 95°C, 45 cycles of 95°C for 15 s and a primer-specific optimal temperature (62–68°C) for 1 min. Each PCR run was completed with a melt curve analysis to confirm the presence of a single PCR product and amplification efficiency was verified for every primer pair. The gene expression values were derived from a standard curve generated for each primer set. Primer sequences were derived using NCBI's BLASTN v2.10.0 program and searching the European starling's genome

database (Sturnus_vulgaris-1.0 reference Annotation Release 100) for predicted genes. Primers were designed so that at least one primer was exon spanning. Primers in our study met the following criteria: amplification of a single product indicated by a single peak in the melting curve analysis and efficiency of amplification between 98 and 100%. In all cases, cycle threshold (Ct) values ranged from 18 to 29, except for PPAR γ which was detected between 30 and 32 range. Primer sequences and Genbank accession numbers are shown in Table S1.

Transcript expression levels were normalized to the reference gene β -actin, which codes for the beta actin gene responsible for the structure and motility of cells, and is highly conserved across tissues and avian species [69–73]. β -actin did not vary across the 8 diet and training treatments in the pectoralis ($F_{8,80}=1.68$, $P=0.12$) or liver ($F_{8,78}=0.94$, $P=0.49$) or over the course of the experiment in the pectoralis (Julian date: estimate $\pm$ standard error 0.02 ± 0.02 , $P=0.30$) or liver (Julian date: estimate $\pm$ standard error 0.01 ± 0.02 , $P=0.67$). Transcript expression normalized to β -actin was used for causal pathway analyses. Normalized transcript expression relative to the 13% 18:2n-6, low antioxidant, untrained reference group was used for all linear models.

Antioxidant Enzyme Activities

In preparation for the measurement of antioxidant enzyme activity, approximately 250 mg of tissue 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 4°C (Beckman Coulter Allegra 21R), and the supernatant was aliquoted to 3 separate tubes (~200 μ l per tube) to conduct the four separate assays (Bradford, CAT,

SOD, GPx). A chelating agent (EDTA) was added to the tubes used to measure enzymatic antioxidant activity to protect the sample from the rapid autoxidation from trace metal ions within the sample, resulting in a final buffer containing 0.05 M PBS and 0.1 mM EDTA, pH 7. Supernatant was immediately frozen at -80°C until the time of the assay (1-3 months after homogenization).

The activities of CAT, SOD, and GPx enzymes were assayed according to Cayman Chemical commercial kit protocols (Catalase Assay Kit 707002, Superoxide Dismutase Assay Kit 706002, Glutathione Peroxidase Assay Kit 703102), and all enzyme activities were normalized to soluble protein content (mg/mL) as measured by the Bradford protein assay (Biorad, 5000006) using a bovine albumin serum (BSA) standard (Fisher Scientific AAJ6477709) [18]. All assays were conducted on a microplate and read in a plate reader (BioTek Synergy HTX) in duplicate or triplicate, until the %CVs among replicates were under 13%. The final dilution factors for the assays of the pectoralis were: Bradford 1:200, CAT 1:10, SOD 1:100, GPx 1:10, and in the liver: Bradford 1:1000, CAT 1:250, SOD 1:100, GPx 1:50. CAT catalyzes the oxidation of aliphatic alcohol, which acts as an electron donor for hydroperoxides, and the assay measured the amount of oxidized aldehydes present after termination of the reaction (nmol/min/mL)[74,75]. The SOD assay measured all three types of SOD (Cu/Zn, Mn, and FeSOD) present by detecting the amount of superoxide radicals generated by xanthine oxidase and hypoxanthine using tetrazolium salt for detection (concentration unit= U/mL, one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical) [18]. GPx activity of all present GPx types (GPx 1-5) was measured indirectly, as oxidized glutathione produced upon reduction of

hydroperoxides by GPx is recycled to its reduced state by glutathione reductase and NADPH, and the resulting rate of decrease is directly proportional to GPx activity (nmol/min/mL) [16].

Statistics

Linear Models

We used R (v3.5.3; R Core Team, 2019, Vienna Austria) for all analyses. Linear models were constructed to test the hypothesis that flight training (H1), dietary fat (H2), and dietary antioxidants (H3) influenced the gene expression of NRF2 and PPAR transcription factors, their antioxidant genes, and corresponding antioxidant enzyme activities. We used a global model without interaction terms that best matched this hypothesis and included possible explanatory covariates (i.e., Julian date, sex, and wing chord). Non-significant explanatory covariates were removed from the final models. Julian date was the only covariate retained in the models for all antioxidant enzyme activities in the pectoralis and liver, but not for gene expression models. To test the hypothesis that dietary fat, dietary antioxidants, and flight training had an interactive effect on gene expression, we compared our global models to models including a 3-way interaction between dietary fat, antioxidants, and training treatment. These models also test the 2-way interactions between covariates. The models with the 3-way interactions were not among the best fit models (i.e. within 3 Δ AIC scores of the global model), or in one case when they were (SOD enzyme activity), was not the most parsimonious model (4 fewer degrees of freedom); thus we report results for only the main effects.

Piecewise Structural Equation Modelling

To test the hypothesis that NRF2 and PPAR transcription factors regulate antioxidant gene expression, and thereby regulate antioxidant enzyme activities we conducted a unidirectional path analysis that tests the causal relationships between regulatory genes (NRF2 and PPARs) and downstream antioxidant genes (CAT, SOD1, SOD2, GPX1, GPX4), and between antioxidant genes and antioxidant enzyme activities (CAT, SOD, GPx). We conducted a path analysis in the liver and pectoralis separately for flight-trained and untrained birds. We did not control for diet in our liver models since we found no effects of dietary fat and antioxidants (see Results). However, due to the positive effect of anthocyanins on select antioxidant gene expression in the pectoralis (see Results), we initially conducted path analyses for each anthocyanin diet in the pectoralis for flight-trained and untrained birds (Fig. S1). We present only the results from flight-trained and untrained birds (Fig. 5) since the causal relationships did not vary among antioxidant groups (Fig. S1). We used piecewise structural equation modeling (PSEM) using the PSEM R package [76] to calculate linear regression coefficients for each specified causal relationship in the causal model (Equations 1 and 2). Since we were not comparing causal models, we did not calculate the goodness-of-fit using tests of directed separation ('dsep'[76,77]).

Causal Equation 1.

$$Y_i = \beta_0 + \beta_1 NRF2 + \beta_2 PPAR\alpha + \beta_3 PPAR\delta \text{ or } PPAR\gamma$$

$$Y_i = \text{Genes: CAT, SOD1, SOD2, GPX1, GPX4}$$

Causal Equation 2.

$$Y_i = \beta_0 + \beta_1 CAT + \beta_2 SOD1 + \beta_3 SOD2 + \beta_4 GPX1 + \beta_5 GPX4$$

$$Y_i = \text{Enzymes: CAT, SOD, GPx}$$

Results

Flight training influences gene expression and enzyme activities in the liver and pectoralis (H1)

Flight training consistently increased the expression of 60% of the measured antioxidant genes in the liver (Fig. 1a) but affected only SOD2 in the pectoralis (Fig. 1b). In the liver, antioxidant gene expression of flight-trained starlings relative to untrained birds was greatest for CAT, SOD2, and GPX1 (Fig. 1a; CAT, $T_{87} = 2.909$, $P = 0.0047$; SOD2, $T_{87} = 2.472$, $P = 0.016$; GPX1, $T_{87} = 2.904$, $P = 0.0047$). Flight training did not significantly affect expression of SOD1 or GPX4 in the liver (Fig. 1a; SOD1, $T_{87} = 0.912$, $P = 0.364$; GPX4, $T_{87} = 0.057$, $P = 0.955$). The expression of transcription factors NRF2 (Fig. 1a) and PPAR γ (Table 2) were greatest in liver of flight-trained birds, but this trend was not significant (NRF2, $T_{86} = 1.327$, $P = 0.188$; PPAR γ , $T_{88} = 1.432$, $P = 0.156$) despite their effect sizes that were similar to the expression of CAT, SOD2, and GPX1 in response to flight training. In general, we found greater variability in the expression of transcription factors compared to antioxidant genes and this may have affected the detection of statistical significance. PPAR α was not affected by flight training (Table 2; $T_{87} = 1.517$, $P = 0.133$). In the pectoralis, flight training increased the expression of only SOD2 (Fig. 1b; SOD2, $T_{89} = 1.833$, $P = 0.070$), decreased the expression of PPAR δ (Table 3; $T_{88} = -2.268$, $P = 0.026$), and did not influence expression of the other 80% of antioxidant genes (Fig. 1b; CAT, $T_{89} = 1.101$, $P = 0.274$; SOD1, $T_{88} = 1.433$, $P = 0.156$; GPX1, $T_{89} = 1.180$, $P = 0.241$) or NRF2 (Fig. 1b; $T_{89} = -1.634$, $P = 0.106$).

Contrary to hypothesis 1, expression patterns of the antioxidant genes and antioxidant enzyme activities were not well coordinated in response to flight training in

either tissue. In fact, antioxidant enzymes displayed an opposite pattern compared to antioxidant genes in relation to flight training. For example, GPx activity in the liver and CAT activity in the liver and pectoralis were lowest in flight-trained birds relative to untrained birds (Fig. 2a; GPx activity, $T_{86} = -2.744$, $P = 0.0075$; liver CAT activity, $T_{86} = -4.093$, $P < 0.001$; pectoralis CAT activity, $T_{90} = -4.189$, $P < 0.001$) while SOD activity in the liver and pectoralis and GPX activity in the pectoralis were unchanged by flight training (Table 4; liver SOD activity, $T_{86} = -0.323$, $P = 0.747$; pectoralis SOD activity, $T_{90} = 1.779$, $P = 0.079$; pectoralis GPx activity, $T_{90} = -0.133$, $P = 0.894$).

Julian date affects antioxidant enzyme activities but not antioxidant gene expression

Overall time on the experimental diets or the progression of the fall migratory season influenced antioxidant enzyme activities, but not antioxidant gene expression or the expression of transcription factors. When including Julian date as a continuous variable in our models, we observed a negative effect of Julian date on CAT and SOD activity in the liver and pectoralis (Table 4; liver CAT activity $T_{86} = -1.790$, $P = 0.077$; liver SOD activity $T_{86} = -2.373$, $P = 0.020$; pectoralis CAT activity $T_{90} = -2.579$, $P = 0.012$; pectoralis SOD activity $T_{90} = -3.232$, $P = 0.0017$), and a positive effect of date on GPx activity in the pectoralis (Table 4; $T_{90} = 2.973$, $P = 0.004$). There was no effect of date on GPx activity in the liver (Table 4; $T_{86} = -0.022$, $P = 0.451$). Our experimental design allowed us to determine the specific time intervals that affected antioxidant enzyme activity by utilizing cohorts that were sampled across the 3-month experiment. We constructed linear models using the experimental cohort (1-5) as a covariate instead of Julian date. In accordance with the effect of Julian date, the declines in CAT and SOD activity in the liver and pectoralis were primarily due to the last cohort sampled (Table 5),

whereas the increase over the fall in GPx in the pectoralis was evident in the last two cohorts.

Differential effects of diet on gene expression: Dietary anthocyanin increases pectoralis gene expression, yet no effect of dietary fat (H2, H3)

There were no main effects of dietary 18:2n-6 on gene expression of NRF2 or antioxidant gene expression in the liver (Table 2; NRF2, $T_{86} = 0.554$, $P = 0.581$; CAT, $T_{87} = 0.768$, $P = 0.445$; SOD1, $T_{87} = -1.339$, $P = 0.184$, SOD2, $T_{87} = -0.037$, $P = 0.971$; GPX1, $T_{87} = -0.368$, $P = 0.714$; GPX4, $T_{87} = 0.526$, $P = 0.601$) or in the pectoralis (Table 3; NRF2, $T_{89} = 0.128$, $P = 0.899$; CAT, $T_{89} = -0.663$, $P = 0.509$; SOD1, $T_{89} = -1.232$, $P = 0.221$, SOD2, $T_{89} = -1.011$, $P = 0.315$; GPX1, $T_{89} = -0.334$, $P = 0.739$; GPX4, $T_{89} = -0.958$, $P = 0.341$). PPAR α was positively affected by dietary 18:2n-6 (Table 2; $T_{87} = 2.363$, $P = 0.0205$), and these results are discussed in a companion study (citation redacted for initial review). In accordance with the lack of support for hypothesis 2, we found no support for an interactive effect of dietary 18:2n-6 and flight training on NRF2 or endogenous antioxidant genes or enzymes.

Dietary anthocyanin had a more targeted effect on antioxidant genes compared to flight training. Dietary anthocyanin did not significantly influence NRF2 or antioxidant gene expression in the liver (NRF2, $T_{86} = -0.262$, $P = 0.830$; CAT, $T_{87} = -1.734$, $P = 0.087$; SOD1, $T_{87} = 1.024$, $P = 0.309$, SOD2, $T_{87} = -0.690$, $P = 0.492$; GPX1, $T_{87} = -1.351$, $P = 0.180$; GPX4, $T_{86} = -1.063$, $P = 0.291$) although there was a trend for CAT expression to be less in birds supplemented with anthocyanins (Fig. 3a). Consistent with the stimulatory hypothesis, anthocyanin supplemented birds had greater CAT and SOD1 expression in the pectoralis relative to birds not supplemented with anthocyanins (Fig.

3b; CAT, $T_{89} = 2.118$, $P = 0.0371$; SOD1, $T_{88} = 2.245$, $P = 0.0274$), although there was no other effect of dietary anthocyanin on the other antioxidant genes or NRF2 expression (Fig. 3b; SOD2, $T_{89} = 0.806$, $P = 0.423$; GPX1, $T_{89} = 0.320$, $P = 0.750$; GPX4, $T_{89} = 0.095$, $P = 0.799$; NRF2, $T_{89} = 0.507$, $P = 0.614$).

Path Analysis- how the expression of NRF2 and PPAR transcription factors, antioxidant genes, and antioxidant enzyme activities interact in flight-trained and untrained birds (H1)

In order to test hypothesis 1, that flight training increases (a) NRF2 and PPAR expression and thereby increases (b) the expression of their antioxidant genes and the (c) activities of their related antioxidant enzyme activities, we constructed a causal model for flight-trained and untrained birds for both the liver (Fig. 4) and pectoralis (Fig. 5). The regression coefficient associated with each causal relationship between the transcription factors and the antioxidant genes indicates the extent of change in the number of transcripts of the antioxidant genes (CAT, SOD1, SOD2, GPX1, GPX4) for each one-transcript change in NRF2 and PPAR transcription factors (NRF2, PPAR α , PPAR γ , PPAR δ). For example, for the liver of flight-trained birds (Fig. 4A), one of the strongest causal relationships showed that a one-transcript change in PPAR α resulted in a 2.34 transcript decrease in the SOD1 gene. Similarly, the regression coefficients between the antioxidant genes and antioxidant enzyme activities represent a 1-unit change in enzyme activity for each one-transcript change in antioxidant genes.

In general, flight training altered the relationships among transcription factors and antioxidant genes in a tissue specific manner. For example, in the liver (Fig. 4), flight training concentrated the regulation of NRF2 from all antioxidant genes in untrained

birds to 60% of genes in flight-trained birds. In addition, flight training initiated a new positive relationship between PPAR γ and SOD1, and new negative relationships between PPAR α and 80% of the antioxidant genes (Fig. 4). In contrast, in the pectoralis (Fig. 5), flight training strengthened the relationships between NRF2 and all antioxidant genes compared to only 3 significant relationships in untrained birds. In addition, flight training altered PPAR regulation of select antioxidant genes. Specifically, PPAR α and PPAR δ negatively influenced GPX1 expression and GPx activity, respectively in flight-trained birds while PPAR δ positively influenced GPX1 and GPX4 expression in untrained birds. These relationships were maintained in birds consuming different amounts of dietary antioxidants (Fig. S1) despite the significant positive effect of dietary anthocyanin on select genes in the pectoralis, thus diet groups are combined for flight-trained and untrained birds in Fig. 5.

We found little evidence that antioxidant gene expression related to antioxidant enzyme activities. For example, in the liver (Fig. 4), antioxidant enzyme activities were not significantly related to antioxidant gene expression in flight-trained birds, although CAT and GPx enzyme activities were positively related to CAT and GPX1 expression, respectively, in untrained birds. In the pectoralis (Fig. 5), GPx activity was positively influenced by GPX1 activity in flight-trained birds, although we detected no other significant relationships among the measured antioxidant genes and enzymes in flight-trained or untrained birds suggesting posttranslational modifications to these antioxidant proteins.

Discussion

Tissue-specific differences in gene expression and enzyme activity patterns in response to flight training (H1)

We found support for hypothesis 1 (flight training stimulated the expression of antioxidant genes) that was tissue specific: flight-trained birds had significantly higher expression of 3 of the 5 antioxidant genes (CAT, SOD2, GPX1) measured in liver but only one of five (SOD2) measured in pectoralis. The mitochondria produce superoxide radicals that are among the most reactive [78], and the need to convert superoxide to hydrogen peroxide is potentially why SOD2 expression was highest in both the liver and pectoralis. Furthermore, SOD2 is localized at the mitochondrial site of RS production, whereas SOD1 is localized in the cytosol and mitochondrial intermembrane space [2,78,79] which may explain the lack of upregulation of SOD1 in response to flight training. GPX and CAT are the next line of antioxidant defense after SOD2 in that they reduce the newly converted hydrogen peroxide radical. GPX1 is also localized in the mitochondria and its expression was highest in the liver of flight-trained birds, whereas there was no difference in GPX4 expression. We predicted that GPX4 would be crucial to flying birds that rely on fat as fuel since GPX4 is localized in the nuclei and mitochondria and is the only isoform that can act on peroxidized fatty acid residues [2,78,79]; thus, we are unsure why birds do not also upregulate GPX4. CAT is exclusively located in peroxisomes, a crucial site of lipid oxidation [80]. Perhaps upregulation of CAT in the liver of flight-trained birds reduced the negative effects of lipid oxidation within peroxisomes, although why this did not also occur in pectoralis muscle is puzzling. The transcription factors that we measured (NRF2, PPAR α , PPAR γ) were not differentially

expressed in either tissue in response to flight training with one exception: PPAR δ expression was lower in the pectoralis of flight-trained birds possibly reflecting its regulatory roles in lipid storage rather than lipid mobilization [81,82]. Future studies should investigate whether flight training increases the transcriptional activity of NRF2 or PPARs.

Our causal models (Fig. 4-5) provided some evidence for the regulatory pathway proposed in hypothesis 1, that flight training increases (a) NRF2 and PPAR expression and thereby increases (b) the expression of their antioxidant genes and the (c) activities of their related antioxidant enzyme activities. Our causal models indicated that NRF2 mediates the expression of a minimum of 60% of the antioxidant genes measured here. Importantly, flight training altered the relationships among NRF2, PPARs and antioxidant genes in a tissue-specific manner. In the liver, flight training concentrated the regulation of NRF2 from all antioxidant genes in the untrained state to 60% of genes (SOD1, SOD2, GPX1) in the flight-trained state. In contrast, in the pectoralis flight training broadened the relationships between NRF2 and the antioxidant genes from 60% of the antioxidant genes (CAT, SOD1, SOD2) in untrained birds to all five measured antioxidant genes when flight trained. Similarly, the NRF2 pathway was significantly upregulated in Burmese pythons (*Python molurus bivittatus*) post-feeding during which rapid organ growth and 44-fold increases in metabolism occur [83]. In contrast to our study, NRF2 and all three antioxidant enzymes (CAT, SOD, and GPX1) were consistently upregulated in the small intestine, kidney, and liver of Burmese pythons indicating that NRF2 activated the three enzymes likely to protect *all* tissues from increases in RS production associated with increases in metabolism [83]. These

contrasting results could be due to differences between studies in the focal tissues or species, the cause of metabolic increases (exercise vs. organ growth), or the scale of the measurements (i.e., our RT-qPCR analyses, transcriptome analysis used for the python study).

Some support for the proposed regulatory pathway (H1) was also provided by the PPAR transcription factors, but again the patterns were tissue specific (Fig. 4-5). In the liver, flight training initiated a new positive relationship between PPAR γ and SOD1 and new negative relationships between PPAR α and 80% of the antioxidant genes. In the pectoralis, PPAR α and PPAR δ negatively influenced GPX1 expression and GPx activity in flight-trained birds while PPAR δ positively influenced GPX expression in untrained birds. In mammals, PPARs directly transcribe CAT, GPX, and SOD [32], but it remains unknown how changes in their transcription relate to expression of downstream antioxidant target genes. We found that flight training clearly initiated PPAR regulatory involvement in songbirds; however, it is unclear how PPAR expression influences individual antioxidant enzyme expression. Transcriptome studies that characterize a complete set of differentially expressed genes will provide a more holistic picture of gene expression patterns and help to elucidate pathway responses to flight.

In contrast to our predictions, our causal models (Fig. 4-5) provided little evidence that flight training elicited a corresponding increase in both the expression of antioxidant genes and the activities of their related antioxidant enzyme activities. In general, the gene expression of antioxidant enzymes did not consistently positively correlate with enzymatic activities. This lack of correspondence between gene expression and activity of antioxidant enzymes may occur because our measures of enzyme activities

combine that for all isoforms which may mask the direct regulatory relationships among specific gene isoforms. Furthermore, enzyme activities measure the enzyme concentration at a given time and provide no information on enzymatic flux within a metabolic network [84]. The metabolic flux, or turnover, of the antioxidant enzymes may more closely reflect corresponding gene expression levels. The exceptions to this general lack of association between gene expression and activity of antioxidant enzymes included two positive relationships in the liver of untrained birds (Fig. 4b: CAT, GPX1) and two negative relationships in the pectoralis of flight-trained birds (Fig. 5a: GPX1, GPX4). We speculate that CAT and GPX expression may be more tightly linked to enzymatic activities due to their importance in reducing the more common hydrogen peroxide radical compared to SOD that neutralizes superoxide that is rapidly converted to hydrogen peroxide. The need to maintain tight regulation of CAT and GPx is also suggested by the lower antioxidant enzyme activities in flight-trained birds compared to untrained birds in the liver (CAT, GPx) and pectoralis (CAT) (Fig. 2) that may have occurred via posttranslational modifications to proteins to inactivate CAT and GPx once birds had recovered from flight training (2 days after the last flight).

Dietary fat quality does not affect molecular antioxidant pathways (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 have increased expression levels of NRF2 and PPAR transcription factors, select antioxidant genes, and corresponding antioxidant enzyme activities compared to when fed diets with less 18:2n-6. This hypothesis was informed by the biochemistry and oxidative susceptibility of PUFA [1] and the ability of 18:2n-6 to stimulate antioxidant enzymes in

fish [85,86]. Rainbow trout (*Oncorhynchus mykiss*) larvae with the highest PUFA fatty acid compositions in the first week of growth had the highest levels of CAT, SOD, and GPX expression compared to later growth periods with lower PUFA composition [85], and Darkbarbel catfish (*Pelteobagrus vachelli*) fed higher levels of linseed oil containing more PUFA had higher serum antioxidant enzyme activities (CAT, SOD, GPX) [86]. It is possible that our dietary 18:2n-6 composition did not oxidatively challenge birds even after flight training, and thereby there was no need for birds to upregulate antioxidant genes or enzymes. Consistent with our results, gene expression levels of CAT, SOD1, SOD2, and GPX were similar among rats fed different sources of n-3 and n-6 PUFA or fed no PUFA [87]. Lipid peroxidation remains a particularly relevant challenge for migratory songbirds that rely on 18:2n-6 and other fats to fuel migratory flights [48–51]. Future studies that compare the effect of different dietary 18:2n-6 levels on endogenous antioxidants and that simultaneously measure oxidative damage will be able to better elucidate the effects of dietary 18:2n-6 on the endogenous antioxidant system in migratory birds.

Dietary antioxidants stimulate antioxidant gene expression (H3) in the pectoralis but not liver

In accordance with hypothesis 3, we found evidence for a stimulatory effect of dietary anthocyanins on antioxidant pathways, but contrary to predictions this occurred only in flight-trained birds. We expected that when birds were metabolically challenged by flight training dietary anthocyanins would quench excess RS and disrupt NRF2 and PPAR signaling to have an inhibitory or compensatory effect of dietary anthocyanins on enzyme activities [20,25,26]. Instead, we observed that dietary anthocyanins maintained

their stimulatory properties, likely through NRF2 [24,55–58], to increase the gene expression of antioxidant enzymes (CAT, SOD1) in the pectoralis of flight-trained birds. Given this stimulatory effect was limited to the flight muscle of flight-trained birds, dietary antioxidants such as anthocyanins may be crucial signaling molecules especially in metabolically active tissues directly involved in responding to some challenge (e.g., flight training). Like exercise, an immune challenge produces RS at the site of injury that act as signaling molecules to recruit inflammatory mediators (e.g. cytokines, prostaglandins) and endogenous antioxidants for repair [88]. Dietary anthocyanins increased the likelihood of mounting an immune response after an inflicted immune-challenge in Blackcaps (*Sylvia atricapilla*) [66] perhaps through the amelioration of RS either directly or by stimulating endogenous antioxidants. Considered together, birds consuming dietary anthocyanins appear to gain protective benefits in response to immunological and exercise-related challenges, both of which are directly relevant to birds during migration. Furthermore, dietary anthocyanins reduced the production of corticosterone (CORT) in flying songbirds [60] indicating that antioxidant consumption protects against the metabolic costs associated with high glucocorticoid levels, like RS production. The exact mechanisms responsible for the observed antioxidant-protective effects in birds have yet to be elucidated. Future studies should investigate the protective effects of anthocyanin supplementation in migratory songbirds by characterizing the interactions between the NRF2 antioxidant pathway, NF- κ B immune pathway [88], and the HPA axis responsible for glucocorticoid production.

Possible mechanisms to explain tissue-specific differences in antioxidant gene expression

We propose that the distinct gene expression profiles for the antioxidant genes for the two tissues are driven by the metabolic state and main functions of the liver and pectoralis. A companion study demonstrated that key genes involved in fat metabolism were upregulated in the pectoralis of flight-trained songbirds, but not in the liver (citation redacted for initial review). In contrast, we demonstrated here that flight training upregulated antioxidant genes in the liver but not in the pectoralis. The pectoralis may require an upregulation of metabolic genes to match the higher demands of flight training [13,31,71,89] compared to the liver that also functions as an endocrine and exocrine gland. Additionally, the tissue-specific protein turnover rates of the liver are two times faster than the protein turnover rates of the pectoralis in migratory birds [90], and this may have allowed the liver to advance from a ‘metabolic state’ that prioritizes the expression of genes involved in fat metabolism to a ‘repair and recovery state’ that prioritizes the expression of antioxidant and pro-inflammatory genes [91,92] within two days after the longest flight. In contrast, the relatively slower turnover of the pectoralis suggests that this muscle may have remained in a metabolic state that prioritizes the expression of genes involved in fat metabolism and had not yet transitioned to the expression of antioxidant genes. In general, the time course of antioxidant gene expression changes according to tissue type, kinetics within a tissue, amount of damage generated, and with exercise (reviewed in [19]. For example in ground squirrels, CAT, SOD1, SOD2, and GPX1 were differentially expressed among different skeletal muscle types (i.e. soleus, extensor digitorum longus, gastrocnemius) potentially reflecting metabolic differences in slow-twitch, fast-twitch, and mixed muscles [93]. We propose that flight-trained birds consuming anthocyanins may have been able to upregulate

antioxidant genes CAT and SOD1 in addition to metabolic genes (companion study) because these birds incurred an energy savings, perhaps through the reduction of the glucocorticoid CORT [60]. Dietary anthocyanins did not have a stimulatory effect on antioxidant gene expression in the liver perhaps because flight training was driving increases in these enzymatic genes. Similarly, there may be less spare capacity for antioxidant genes to be upregulated by dietary antioxidants because the antioxidant pathways are already operating at a high level. In sum, evidence to date from this study and others (citation redacted for initial review) suggests that flight training and dietary antioxidants, less so dietary fat, strongly affect the gene-level regulation of the antioxidant system and fat metabolism, that these effects are tissue-specific, and likely explained by functional differences between tissues as well as fundamental differences in their turnover rates.

Relevance and significance

We provide some of the first evidence for how antioxidant pathways respond to ecological factors that are relevant to songbirds during migration. Exercise, dietary fat, and dietary antioxidants have been shown to influence antioxidant transcription factors (NRF2, PPARs) and their downstream antioxidant genes in other organisms [20,26,28], although ours is the first study that investigates all three simultaneously in a wild-caught migratory songbird. Our study confirms that repeated bouts of flight and dietary antioxidants, but not dietary fat, stimulate the downstream expression of select antioxidant genes and by inference the transcriptional activity of NRF2 and PPARs in starlings. Given that birds and their relatives have a constitutently active NRF2 because of unique mutations in the KEAP1 repressor gene [40], ecological factors such as exercise

and diet quality may be more crucial in modulating the transcriptional activity of NRF2, independent of any effect on KEAP1, and thus the expression of genes involved in antioxidant protection, as we have shown. Birds during migration are quite selective in what they eat [48–54], and these diet choices directly affect their supply of nutrients and energy but also, as we have shown, can affect the regulation of key antioxidant pathways. Likewise, birds during migration undergo regular, often daily flights interrupted by periods at stopover sites as they travel between breeding and wintering areas. Our results suggest that flying itself directly affects the regulation of key metabolic pathways involved in antioxidant protection, and a companion study has shown the same for pathways involved in fat metabolism. It remains to be demonstrated how the extent of these ecological factors (i.e., intensity or duration of flight, amounts of dietary antioxidants) influences these effects on key metabolic pathways.

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Author's contributions

SRM and BJP conceived and designed the experiment. KJD and WAC and SRM refined the methodology and KJD and WAC conducted the experiment. KJD analyzed the data. KJD and SRM interpreted the results and primarily wrote the manuscript, and WAC and BJP provided editorial advice on the manuscript.

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Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

References

1. Skrip MM, McWilliams SR. Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists. *J F Ornithol.* 2016;87: 1–20.
doi:10.1111/jofo.12135
2. Cooper-Mullin C, McWilliams SR. The role of the antioxidant system during intense endurance exercise: lessons from migrating birds. *J Exp Biol.* 2016;219: 3684–3695. doi:10.1242/jeb.123992
3. Eikenaar C, Isaksson C, Hegemann A. A hidden cost of migration? Innate immune function versus antioxidant defense. *Ecol Evol.* 2017;8: 1–8.
doi:10.1002/ece3.3756
4. Jenni-Eiermann S, Jenni L, Smith S, Costantini D. Oxidative stress in endurance flight: An unconsidered factor in bird migration. *PLoS One.* 2014;9: 1–6.
doi:10.1371/journal.pone.0097650
5. Gutiérrez JS, Sabat P, Castañeda LE, Contreras C, Navarrete L, Peña-Villalobos I, et al. Oxidative status and metabolic profile in a long-lived bird preparing for extreme endurance migration. *Sci Rep.* 2019;9: 1–11. doi:10.1038/s41598-019-54057-6
6. Skrip MM, Bauchinger U, Goymann W, Fusani L, Cardinale M, Alan RR, et al. Migrating songbirds on stopover prepare for, and recover from, oxidative challenges posed by long-distance flight. *Ecol Evol.* 2015;5: 3198–3209.
doi:10.1002/ece3.1601
7. Montesana L, Isaksson C, Goymann W, Andersson MN, Trappschuh M, Hau M.

Female variation in allocation of steroid hormones, antioxidants and fatty acids: a multilevel analysis in a wild passerine bird. *J Avian Biol.* 2018.

doi:10.1101/467258

8. Costantini D, Casasole G, AbdElgawad H, Asard H, Eens M. Experimental evidence that oxidative stress influences reproductive decisions. *Funct Ecol.* 2020;30: 1169–1174. doi:10.1111/1365-2435.12608
9. Speakman JR, Garratt M. Oxidative stress as a cost of reproduction: Beyond the simple trade-off model. *Bioessays.* 2013;36: 93–106. doi:10.1002/bies.201300108
10. Costantini D, Carello L, Fanfani A. Relationships among oxidative status, breeding conditions and life-history traits in free-living Great Tits *Parus major* and Common Starlings *Sturnus vulgaris*. *Ibis (Lond 1859).* 2010;152: 793–802. doi:10.1111/j.1474-919X.2010.01052.x
11. Wiersma P, Selman C, Speakman JR, Verhulst S. Birds sacrifice oxidative protection for reproduction. *Proc R Soc B Biol Sci.* 2004;271. doi:10.1098/rsbl.2004.0171
12. Swanson DL. Seasonal metabolic variation in birds: functional and mechanistic correlates. Thompson C. *Current Ornithology.* Thompson C. Springer, New York, NY; 2010. pp. 131–189. doi:10.1007/978-1-4419-6421-2
13. DeMoranville KJ, Corder KR, Hamilton A, Russell DE, Huss JM, Schaeffer PJ. PPAR expression, muscle size and metabolic rates across the gray catbird's annual cycle are greatest in preparation for fall migration. *J Exp Biol.* 2019;222: 1–11. doi:10.1242/jeb.209452

14. Corder KR, Schaeffer PJ. Summit metabolic rate exhibits phenotypic flexibility with migration, but not latitude in a neotropical migrant, *Parkesia noveboracensis*. J Ornithol. 2015;156: 547–550. doi:10.1007/s10336-015-1157-x
15. Butler PJ. The physiological basis of bird flight. Philos Trans R Soc B Biol Sci. 2016;371: 1–11. doi:10.1098/rstb.2015.0384
16. Cooper-Mullin C, Carter WA, McWilliams SR. Acute effects of intense exercise on the antioxidant system in birds: Does exercise training help? J Exp Biol. 2019;222: 1–10. doi:10.1242/jeb.210443
17. Skrip MM, Seeram NP, Yuan T, Ma H, McWilliams SR. Dietary antioxidants and flight exercise in female birds affect allocation of nutrients to eggs: how carry-over effects work. J Exp Biol. 2016;219: 2716–2725. doi:10.1242/jeb.137802
18. Dick MF, Guglielmo CG. Flight muscle protein damage during endurance flight is related to energy expenditure but not dietary polyunsaturated fatty acids in a migratory bird. J Exp Biol. 2019;222: 1–9. doi:10.1242/jeb.187708
19. Costantini D. Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. J Exp Biol. 2019;222: 1–9. doi:10.1242/jeb.194688
20. Done AJ, Traustadóttir T. Nrf2 mediates redox adaptations to exercise. Redox Biol. 2016;10: 191–199. doi:10.1016/j.redox.2016.10.003
21. Thomas AW, Davies NA, Moir H, Watkeys L, Ruffino JS, Isa SA, et al. Exercise-associated generation of PPAR γ ligands activates PPAR γ signaling events and

- upregulates genes related to lipid metabolism. *J Appl Physiol.* 2012;112: 806–815.
doi:10.1152/jappphysiol.00864.2011
22. Spangenburg EE, Brown DA, Johnson MS, Moore RL. Alterations in peroxisome proliferator-activated receptor mRNA expression in skeletal muscle after acute and repeated bouts of exercise. *Mol Cell Biochem.* 2009;332: 225–231.
doi:10.1007/s11010-009-0195-1
 23. Done AJ, Newell MJ, Traustadóttir T. Effect of exercise intensity on Nrf2 signalling in young men. *Free Radic Res.* 2017;51: 646–655.
doi:10.1080/10715762.2017.1353689
 24. Tian X, Xin H, Paengkoum P, Paengkoum S, Ban C, Sorasak T. Effects of anthocyanin-rich purple corn (*Zea mays L.*) stover silage on nutrient utilization, rumen fermentation, plasma antioxidant capacity, and mammary gland gene expression in dairy goats. *J Anim Sci.* 2019;97: 1384–1397.
doi:10.1093/jas/sky477
 25. Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borrás C, Pallardo F V., et al. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr.* 2008;87: 142–149. doi:10.1093/ajcn/87.1.142
 26. Ristow M, Zarse K, Oberbach A, Klötting N, Birringer M, Kiehntopf M, et al. Antioxidants prevent health-promoting effects of physical exercise in humans. *PNAS.* 2009;2: 2–7. doi:10.1073 pnas.0903485106
 27. Pierce BJ, McWilliams SR. The fat of the matter: how dietary fatty acids can affect

- exercise performance. *Integr Comp Biol*. 2014;54: 903–912.
doi:10.1093/icb/icu098
28. Wang Y-X. PPARs: diverse regulators in energy metabolism and metabolic diseases. *Cell Res*. 2010;20: 124–137. doi:10.1038/cr.2010.13
 29. Bensinger SJ, Tontonoz P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature*. 2008;454: 470–477. doi:10.1038/nature07202
 30. Ehrenborg E, Krook A. Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor δ . *Pharmacol Rev*. 2009;61: 373–393. doi:10.1124/pr.109.001560.373
 31. Zhang Y, King MO, Harmon E, Eyster K, Swanson DL. Migration-induced variation of fatty acid transporters and cellular metabolic intensity in passerine birds. *J Comp Physiol B*. 2015;185: 797–810. doi:10.1007/s00360-015-0921-9
 32. Kim T, Yang Q. Peroxisome-proliferator-activated receptors regulate redox signaling in the cardiovascular system. *World J Cardiol*. 2013;5: 164–174.
doi:10.4330/wjc.v5.i6.164
 33. Park EY, Rho HM. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. *Mol Cell Biochem*. 2002;240: 47–55.
doi:10.1023/A:1020600509965
 34. Dreger H, Westphal K, Weller A, Baumann G, Stangl V, Meiners S, et al. Nrf2-

- dependent upregulation of antioxidative enzymes: A novel pathway for proteasome inhibitor-mediated cardioprotection. *Cardiovasc Res.* 2009;83: 354–361. doi:10.1093/cvr/cvp107
35. Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT, et al. Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radic Biol Med.* 2015;88: 108–146. doi:10.1016/j.freeradbiomed.2015.06.021
 36. Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol Rev.* 2018;98: 1169–1203. doi:10.1152/physrev.00023.2017
 37. Zhu H, Jia Z, Misra BR, Zhang L, Cao Z, Yamamoto M, et al. Nuclear factor E2-related factor 2-dependent myocardial cytoprotection against oxidative and electrophilic stress. *Cardiovasc Toxicol.* 2008;8: 71–85. doi:10.1007/s12012-008-9016-0
 38. Venugopal R, Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene.* 1998;17: 3145–3156. doi:10.1038/sj.onc.1202237
 39. Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS Lett.*

2005;579: 3029–3036. doi:10.1016/j.febslet.2005.04.058

40. Castiglione GM, Xu Z, Zhou L, Duh EJ. Adaptation of the master antioxidant response connects metabolism, lifespan, and feather development pathways in birds. *Nat Commun.* 2020;11: 1–15. doi:10.1038/s41467-020-16129-4
41. Lewis KN, Mele J, Hayes JD, Buffenstein R. Nrf2, a guardian of healthspan and gatekeeper of species longevity. *Integr Comp Biol.* 2010;50: 829–843. doi:10.1093/icb/icq034
42. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J.* 2002;16: 1879–1886. doi:10.1096/fj.02-0367com
43. Wang P, Li CG, Qi Z, Cui D, Ding S. Acute exercise stress promotes Ref1/Nrf2 signalling and increases mitochondrial antioxidant activity in skeletal muscle. *Exp Physiol.* 2016;101: 410–420. doi:10.1113/EP085493
44. Merry TL, Ristow M. Nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) mediates exercise-induced mitochondrial biogenesis and the anti-oxidant response in mice. *J Physiol.* 2016;594: 5195–5207. doi:10.1113/JP271957
45. Finck BN, Kelly DP. Review series PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest.* 2006;116: 615–622. doi:10.1172/JCI27794.PGC-1
46. McWilliams SR, Guglielmo C, Pierce B, Klaassen M. Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective.

Source J Avian Biol. 2004;35: 377–393.

47. Guglielmo CG. Obese super athletes: fat-fueled migration in birds and bats. J Exp Biol. 2018;221: 1–16. doi:10.1242/jeb.165753
48. Pierce BJ, McWilliams SR. Seasonal changes in composition of lipid stores in migratory birds: causes and consequences. Condor. 2005;107: 269–279.
49. Pierce BJ, McWilliams SR, Place AR, Huguenin MA. Diet preferences for specific fatty acids and their effect on composition of fat reserves in migratory Red-eyed Vireos (*Vireo olivaceus*). Comp Biochem Physiol - A Mol Integr Physiol. 2004;138: 503–514. doi:10.1016/j.cbpb.2004.06.014
50. Price ER, Krokfors A, Guglielmo CG. Selective mobilization of fatty acids from adipose tissue in migratory birds. J Exp Biol. 2008;211: 29–34. doi:10.1242/jeb.009340
51. Smith SB, McWilliams SR. Patterns of fuel use and storage in migrating passerines in relation to fruit resources at autumn stopover sites. Auk. 2010;127: 108–118.
52. Bolser JA, Alan RR, Smith AD, Li L, Seeram NP, McWilliams SR. Birds select fruits with more anthocyanins and phenolic compounds during autumn migration. Wilson J Ornithol. 2013;125: 97–108.
53. Alan ARR, McWilliams SR, McGraw KJ. The importance of antioxidants for avian fruit selection during autumn migration. Wilson J Ornithol. 2013;125: 513–525.

54. Schaefer H, McGraw K, Catoni C. Birds use fruit colour as honest signal of dietary antioxidant rewards. *Funct Ecol.* 2008;22: 303–310. doi:10.1111/j.1365-2435.2007.01363.x
55. Aboonabi A, Singh I. Chemopreventive role of anthocyanins in atherosclerosis via activation of Nrf2-ARE as an indicator and modulator of redox. *Biomed Pharmacother.* 2015;72: 30–36. doi:10.1016/j.biopha.2015.03.008
56. Aboonabi A, Singh I, Rose' Meyer R. Cytoprotective effects of berry anthocyanins against induced oxidative stress and inflammation in primary human diabetic aortic endothelial cells. *Chem Biol Interact.* 2020;317: 108940. doi:10.1016/j.cbi.2020.108940
57. Shih PH, Yeh CT, Yen GC. Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *J Agric Food Chem.* 2007;55: 9427–9435. doi:10.1021/jf071933i
58. Cimino F, Speciale A, Anwar S, Canali R, Ricciardi E, Virgili F, et al. Anthocyanins protect human endothelial cells from mild hyperoxia damage through modulation of Nrf2 pathway. *Genes Nutr.* 2013;8: 391–399. doi:10.1007/s12263-012-0324-4
59. Hall ZJ, Bauchinger U, Gerson AR, Price ER, Langlois LA, Boyles M, et al. Site-specific regulation of adult neurogenesis by dietary fatty acid content, vitamin E and flight exercise in European starlings. *Eur J Neurosci.* 2014;39: 875–882. doi:10.1111/ejn.12456

60. Casagrande S, DeMoranville KJ, Trost L, Pierce BJ, Bryla A, Dzialo M, et al. Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird. *Proc Natl Acad Sci B*. 2020;18: 1–8.
61. Cabe P. European starling (*Sturnus vulgaris*). AF Poole & FB Gill (Eds). Ithica, NY: Cornell Lab of Ornithology; 1993.
62. Ginn H, Melville D. Moulting in birds. Thetford, UK: British Trust for Ornithology; 1983.
63. Hamilton A, Ly J, Robinson JR, Corder KR, DeMoranville KJ, Schae PJ, et al. Conserved transcriptional activity and ligand responsiveness of avian PPARs: potential role in regulating lipid metabolism in migratory birds. *Gen Comp Endocrinol*. 2018;268: 110–120. doi:10.1016/j.ygcen.2018.08.009
64. Kennedy SR, Bickerdike R, Berge RK, Porter AR, Tocher DR. Influence of dietary conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on growth, lipid composition and key enzymes of fatty acid oxidation in liver and muscle of Atlantic cod (*Gadus morhua* L.). *Aquaculture*. 2007;264: 372–382. doi:10.1016/j.aquaculture.2007.01.013
65. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proc Natl Acad Sci United States Am*. 1997;94: 4312–4317.
66. Catoni C, Martin Schaefer H, Peters A. Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird. *Funct Ecol*. 2008;22: 649–654. doi:10.1111/j.1365-2435.2007.0

67. Dangles O, Fenger JA. The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules*. 2018;23. doi:10.3390/molecules23081970
68. Engel S, Biebach H, Visser GH. Metabolic costs of avian flight in relation to flight velocity: a study in Rose coloured starlings (*Sturnus roseus*, *Linnaeus*). *J Comp Physiol B Biochem Syst Environ Physiol*. 2006;176: 415–427. doi:10.1007/s00360-006-0063-1
69. Bernard DJ, Bentley GE, Balthazart J, Turek FW, Ball GF. Androgen receptor, estrogen receptor α , and estrogen receptor β show distinct patterns of expression in forebrain song control nuclei of European starlings. *Endocrinology*. 1999;140: 4633–4643.
70. Cordes M, Stevenson S, Driessen T, Eisinger B, Riters L. Sexually-motivated song is predicted by androgen-and opioid-related gene expression in the medial preoptic nucleus of male European starlings (*Sturnus vulgaris*). *Behav Brain Res*. 2016;278: 12–20. doi:10.1016/j.bbr.2014.09.029. Sexually-motivated
71. Mcfarlan JT, Bonen A, Guglielmo CG. Seasonal upregulation of fatty acid transporters in flight muscles of migratory white-throated sparrows (*Zonotrichia albicollis*). *J Exp Biol*. 2009;212: 2934–2940. doi:10.1242/jeb.031682
72. Perfito N, Guardado D, Williams TD, Bentley GE. Social cues regulate reciprocal switching of hypothalamic Dio2/Dio3 and the transition into final follicle maturation in European starlings (*Sturnus vulgaris*). *Endocrinology*. 2015;156: 694–706. doi:10.1210/en.2014-1450

73. Zhang J, Gao Y-Y, Huang Y-Q, Fan Q, Lu X-T, Wang C-K. Selection of housekeeping genes for quantitative gene expression analysis in Yellow-feathered broilers. *Ital J Anim Sci.* 2018;17: 540–546. doi:10.1080/1828051X.2017.1365633
74. Jimenez AG, Tobin KJ, Anderson KN, McCann MKE, Briggs CW. Differences in oxidative stress across seasons and ability to cope with environmental mismatch in muscle and brain of black-capped chickadees *Poecile atricapillus* and rock pigeons *Columba livia*. *J Avian Biol.* 2020;51: 1–11. doi:10.1111/jav.02417
75. Jimenez AG, Ruhs EC, Tobin KJ, Anderson KN, Le Pogam A, Regimbald L, et al. Consequences of being phenotypically mismatched with the environment: no evidence of oxidative stress in cold- and warm-acclimated birds facing a cold spell. *J Exp Biol.* 2020;223. doi:10.1242/jeb.218826
76. Lefcheck J. Piecewise Structural Equation Modeling Package ‘piecewiseSEM .’ 2019.
77. Shipley B. Cause and correlation in biology: A user’s guide to path analysis and structural equations and causal inference with R. 2nd ed. Cambridge: Cambridge University Press; 2016. doi:doi:10.1017/CBO9781139979573
78. Halliwell B, Gutteridge JM. Free radicals in biology and medicine. 4th ed. New York, NY: Oxford University Press; 2007.
79. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev.* 2008;88: 1243–1276. doi:10.1152/physrev.00031.2007

80. Lodhi IJ, Semenkovich CF. Peroxisomes: a nexus for lipid metabolism and cellular signalling. *Cell Metab.* 2014;19: 380–392. doi:10.1038/jid.2014.371
81. Bindesbøll C, Berg O, Arntsen B, Nebb HI, Dalen KT. Fatty acids regulate perilipin5 in muscle by activating PPAR δ . *J Lipid Res.* 2013;54: 1949–1963. doi:10.1194/jlr.M038992
82. Chayama Y, Ando L, Sato Y, Shigenobu S, Anegawa D, Fujimoto T, et al. Molecular basis of white adipose tissue remodeling that precedes and coincides with hibernation in the Syrian hamster, a food-storing hibernator. *Front Physiol.* 2019;9: 1–16. doi:10.3389/fphys.2018.01973
83. Andrew AL, Perry BW, Card DC, Schield DR, Ruggiero RP, Mcgaugh SE, et al. Growth and stress response mechanisms underlying post-feeding regenerative organ growth in the Burmese python. *BMC Genomics.* 2017;18: 1–18. doi:10.1186/s12864-017-3743-1
84. Fell DA. Enzymes, metabolites and fluxes. *J Exp Bot.* 2005;56: 267–272. doi:10.1093/jxb/eri011
85. Zengin H, Yilmaz Ö. Antioxidant defence of the actively feeding *Oncorhynchus mykiss* (Walbaum 1792) larvae in relation to dietary PUFA and vitamin E contents. *Reg Stud Mar Sci.* 2016;8: 515–522. doi:10.1016/j.rsma.2016.03.003
86. Li M, Chen L, Qin JG, Li E, Yu N, Du Z. Growth performance, antioxidant status and immune response in darkbarbel catfish *Pelteobagrus vachelli* fed different PUFA/vitamin E dietary levels and exposed to high or low ammonia. *Aquaculture.* 2013;406–407: 18–27. doi:10.1016/j.aquaculture.2013.04.028

87. Tou JC, Altman SN, Gigliotti JC, Benedito VA, Cordonier EL. Different sources of omega-3 polyunsaturated fatty acids affects apparent digestibility, tissue deposition, and tissue oxidative stability in growing female rats. *Lipids Health Dis.* 2011;10: 1–14. doi:10.1186/1476-511X-10-179
88. Ahmed SMU, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: pivotal roles in inflammation. *Biochim Biophys Acta - Mol Basis Dis.* 2017;1863: 585–597. doi:10.1016/j.bbadis.2016.11.005
89. Corder KR, DeMoranville KJ, Russell DE, Huss JM, Schaeffer PJ. Annual life-stage regulation of lipid metabolism and storage and association with PPARs in a migrant species: the gray catbird (*Dumetella carolinensis*). *J Exp Biol.* 2016;219: 3391–3398. doi:10.1242/jeb.141408
90. Bauchinger U, McWilliams SR. Extent of phenotypic flexibility during long-distance flight is determined by tissue-specific turnover rates: a new hypothesis. *J Avian Biol.* 2010;41: 603–608.
91. Wilson DO, Johnson P. Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. *J Appl Physiol.* 2000;88: 1791–1796. doi:10.1152/jappl.2000.88.5.1791
92. Pillon Barcelos R, Freire Royes LF, Gonzalez-Gallego J, Bresciani G. Oxidative stress and inflammation: liver responses and adaptations to acute and regular exercise. *Free Radic Res.* 2017;51: 222–236. doi:10.1080/10715762.2017.1291942
93. Wei Y, Zhang J, Yan X, Peng X, Xu S, Chang H, et al. Remarkable protective effects of Nrf2-mediated antioxidant enzymes and tissue specificity in different

skeletal muscles of Daurian ground squirrels over the torpor-arousal cycle. *Front Physiol.* 2019;10: 1–15. doi:10.3389/fphys.2019.01449

TABLES

Table 2.1. List of key symbols and abbreviations used throughout the manuscript

List of symbols and abbreviations	
18:2n-6	linoleic acid
ARE	antioxidant regulatory element
β-actin	beta actin
CAT	catalase
GPX	glutathione peroxidase
MUFA	monounsaturated fatty acid
NRF2	nuclear factor erythroid 2-related factor 2
KEAP1	kelch like ECH associated protein 1
PGC-1	peroxisome proliferator-activated receptor gamma coactivator
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PSEM	piecewise structural equation modeling
PUFA	polyunsaturated fatty acid
qRT-PCR	reverse transcription quantitative PCR
RXR	9-cis-retinoic acid receptor
SOD	superoxide dismutase

Table 2.2. Linear model results for antioxidant gene expression in the liver in relation to flight-training, dietary antioxidant, and dietary PUFA. The intercept is the 13% 18:2n-6, Antioxidant Unsupplemented, Untrained Group (13U, UT). Data are reported as estimates (standard error) for each gene and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

<i>Covariate</i>	<i>Dependent variable:</i>							
	CAT	SOD1	SOD2	GPX1	GPX4	NRF2	PPAR γ^a	PPAR α^a
Training (Flight-trained)	0.298*** (0.103)	0.124 (0.136)	0.193** (0.078)	0.295*** (0.102)	0.006 (0.110)	0.274 (0.206)	0.219 (0.153)	0.055 (0.036)
Antioxidant (Supplemented)	-0.176* (0.102)	0.138 (0.135)	-0.053 (0.077)	-0.136 (0.101)	-0.117 (0.110)	-0.044 (0.204)	0.153 (0.152)	-0.041 (0.036)
PUFA (32% 18:2n-6)	0.078 (0.102)	-0.181 (0.135)	-0.003 (0.078)	-0.037 (0.101)	0.058 (0.110)	0.113 (0.204)	-0.108 (0.152)	0.085** (0.036)
Intercept (13U, UT)	0.998*** (0.103)	0.852*** (0.137)	0.876*** (0.099)	0.955*** (0.102)	0.939*** (0.111)	0.816*** (0.207)	1.099*** (0.154)	1.036*** (0.037)
Observations	87	87	87	87	87	86	87	87
R ²	0.124	0.044	0.072	0.109	0.017	0.025	0.043	0.098
Adjusted R ²	0.092	0.009	0.039	0.076	-0.019	-0.011	0.008	0.066
Residual Std. Error	0.474 (df = 87)	0.628 (df = 83)	0.361 (df = 83)	0.469 (df = 83)	0.511 (df = 83)	0.946 (df = 82)	0.708 (df = 83)	0.168 (df = 83)
F Statistic	3.907** (df = 3; 83)	1.263 (df = 3; 83)	2.157* (df = 3; 83)	3.373** (df = 3; 83)	0.474 (df = 3; 83)	0.689 (df = 3; 82)	1.235 (df = 3; 83)	3.022** (df = 3; 83)

^aGene expression for PPAR γ and PPAR α are presented in *citation redacted for review*

Table 2.3. Linear model results for antioxidant gene expression in the pectoralis in relation to flight-training, dietary antioxidant, and dietary PUFA. The intercept is the 13% 18:2n-6, Antioxidant Unsupplemented, Untrained Group (13U, UT). Data are reported as estimates (standard error) for each gene and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

<i>Covariate</i>	<i>Dependent variable:</i>							
	CAT	SOD1	SOD2	GPX1	GPX4	NRF2	PPAR δ^a	PPAR α^a
Training (Flight-trained)	0.112 (0.104)	0.207 (0.144)	0.180* (0.098)	0.226 (0.141)	0.069 (0.095)	-0.158 (0.097)	-0.296** (0.130)	-0.019 (0.051)
Antioxidant (Supplemented)	0.215** (0.101)	0.323** (0.144)	0.079 (0.098)	0.173 (0.140)	0.024 (0.095)	0.049 (0.096)	-0.014 (0.130)	-0.021 (0.051)
PUFA (32% 18:2n-6)	-0.067 (0.101)	-0.177 (0.144)	-0.099 (0.098)	-0.153 (0.140)	-0.091 (0.095)	0.012 (0.096)	0.047 (0.130)	0.022 (0.051)
Intercept (13U, UT)	0.926*** (0.104)	0.851*** (0.147)	0.892*** (0.100)	0.880*** (0.143)	0.906*** (0.097)	1.011*** (0.099)	0.900*** (0.132)	1.008*** (0.052)
Observations	89	88	89	89	89	89	88	89
R ²	0.068	0.095	0.057	0.019	0.018	0.067	0.060	0.006
Adjusted R ²	0.035	0.063	0.024	0.016	-0.017	0.034	0.026	-0.029
Residual Std. Error	0.478 (df = 85)	0.674 (df = 84)	0.461 (df = 85)	0.572 (df = 85)	0.447 (df = 85)	0.420 (df = 85)	0.609 (df = 84)	0.239 (df = 85)
F Statistic	2.067 (df = 3; 85)	2.94** (df = 3; 84)	1.72 (df = 3; 85)	0.546 (df = 3; 85)	0.515 (df = 3; 85)	2.030 (df = 3; 85)	1.773 (df = 3; 84)	0.168 (df = 3; 85)

^aGene expression for PPAR δ and PPAR α are presented in *citation redacted for review*

Table 2.4. Linear model results for change in antioxidant enzyme activities in the liver and pectoralis in relation to flight-training, dietary antioxidant, and dietary PUFA. The intercept is the 13% 18:2n-6, Antioxidant Unsupplemented, Untrained Group (13U, UT). Data are reported as estimates (standard error) for each enzyme and the asterisks correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01.

<i>Covariate</i>	<i>Dependent variable:</i>					
	<i>Liver</i>			<i>Pectoralis</i>		
	CAT <i>nmol/min/mL/mg</i>	SOD <i>U/mL/mg</i>	GPx <i>nmol/min/mL/mg</i>	CAT <i>nmol/min/mL/mg</i>	SOD <i>U/mL/mg</i>	GPx <i>nmol/min/mL/mg</i>
Training (Flight-trained)	-20.029*** (4.894)	-0.033 (0.102)	-2.799*** (1.020)	-0.356*** (0.092)	0.342* (0.192)	-0.038 (0.282)
Antioxidant (Supplemented)	-2.173 (4.908)	0.026 (0.102)	-0.210 (1.023)	0.077 (0.092)	0.178 (0.192)	-0.005 (0.282)
PUFA (32% 18:2n-6)	0.790 (4.869)	-0.027 (0.101)	-0.145 (1.015)	-0.064 (0.092)	0.214 (0.191)	-0.132 (0.281)
Julian Date	-0.252* (0.141)	-0.007** (0.003)	-0.022 (0.029)	-0.007** (0.003)	-0.018** (0.005)	0.024*** (0.008)
Intercept (13U, UT)	148.98*** (44.728)	3.884*** (0.932)	19.50** (9.324)	3.335*** (0.839)	12.282*** (1.744)	-0.201 (2.567)
Observations	86	86	86	90	90	90
R ²	0.199	0.067	0.091	0.231	0.1434	0.095
Adjusted R ²	0.160	0.022	0.047	0.195	0.1036	0.053
Residual Std. Error	22.62 (df = 82)	0.4711 (df = 82)	4.715 (df = 82)	0.436 (df = 86)	0.9094 (df = 86)	1.336 (df = 86)
F Statistic	5.106*** (df = 4; 82)	1.473 (df = 4; 82)	2.058* (df = 4; 82)	6.439*** (df = 4; 86)	3.599*** (df = 4; 86)	2.254** (df = 4; 86)

Table 2.5. The main effect of experimental cohort on antioxidant enzyme activities in the liver and pectoralis. The effects of dietary PUFA, dietary anthocyanin, and flight training from the linear models are not reported. The reported effects are relative to birds in Cohort 1 (in the 13% 18:2n-6, Antioxidant Unsupplemented, Untrained Group; 13U, UT). Asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$. Date ranges of final sampling for each cohort are reported in the footnote^a

<i>Δ Enzyme activity ± Standard error (p-value)</i> <i>by Cohort in the Liver and Pectoralis:</i>								
<i>Cohort Number</i>	<i>Liver</i>				<i>Pectoralis</i>			
	2	3	4	5	2	3	4	5
<i>Enzyme activity:</i>								
CAT	-16.46 ± 7.79 (0.038**)	-6.78 ± 7.29 (0.355)	-8.67 ± 7.51 (0.252)	-17.86 ± -2.38 (0.020**)	-0.075 ± 0.148 (0.615)	-0.257 ± -1.79 (0.078*)	-0.272 ± 0.062 (0.062*)	-0.307 ± 0.148 (0.041**)
SOD	-0.054 ± 0.166 (0.746)	-0.121 ± 0.155 (0.440)	-0.257 ± 0.160 (0.113)	-0.338 ± 0.160 (0.038**)	-0.377 ± 0.307 (0.223)	-0.207 ± 0.299 (0.490)	-0.544 ± 0.299 (0.072*)	-0.855 ± 0.308 (0.007***)
GPx	-2.73 ± 1.64 (0.100)	-1.32 ± 1.54 (0.394)	-1.31 ± 1.58 (0.410)	-1.93 ± 1.58 (0.227)	0.235 ± 0.450 (0.604)	0.633 ± 0.438 (0.152)	1.437 ± 0.438 (0.005***)	0.839 ± 0.451 (0.066*)

^aCohort final sampling occurred on the following dates: cohort 1, 17 October to 27 October; cohort 2, 29 October to 8 November; cohort 3, 10 November to 20 November; cohort 4, 22 November to 2 December; cohort 5, 4 December to 14 December

FIGURES

A.

B.

Figure 2.1. Relative gene expression (estimated marginal means from the linear mixed models; Tables 1, 2) in the **A.)** Liver and **B.)** pectoralis muscle of European starlings that were (N=49) or were not (N=40) flown in the wind tunnel for 15 days. Antioxidant genes CAT, SOD1, and GPX1 in the liver, and SOD2 in the pectoralis, were expressed to the greatest extent in flight-trained birds compared to untrained birds. The asterisks correspond to significance levels * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$ when comparing flight-trained and untrained birds.

A. **B.** **C.**

Figure 2.2. Antioxidant enzyme activities (estimated marginal means from the linear mixed models; Table 4) that were significantly influenced by flight-training in the liver and pectoralis. CAT activity in the liver (**A.**), GPx activity in the liver (**B.**), and CAT activity in the pectoralis (**C.**) were lowest in European starlings that were flown (N=49) in the wind tunnel for 15 days compared to unflown birds (N=40). There was no main effect of flight training on GPx or SOD activities in the pectoralis or on SOD activity in the liver (Table 3). The asterisks correspond to significance levels * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$ between flight-trained and untrained birds.

A. **B**

Figure 2.3. Relative gene expression (estimated marginal means from the linear mixed models; Table 2) in the **A.)** liver or **B.)** pectoralis muscle of European starlings that were (N=45) or were not (N=44), supplemented with the antioxidant, anthocyanin. **B.)** In the liver, antioxidant genes were not influenced by dietary anthocyanin. **B.)** In the pectoralis, antioxidant genes CAT and SOD1 were expressed to the greatest extent in anthocyanin supplemented birds compared to unsupplemented birds. SOD2, GPX1, GPX4, and were not influenced by dietary anthocyanin. The asterisks correspond to significance levels * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$ between anthocyanin supplemented and unsupplemented birds.

A.
Liver
Flight-Trained Birds

B.
Liver
Untrained Birds

Figure 2.4. The casual structures to explain the hierarchical gene expression of transcription factors NRF2, PPAR α , PPAR γ on the expression of downstream antioxidant target genes (CAT, SOD1, SOD2, GPX1 and GPX4) and antioxidant enzyme activity (CAT, SOD, GPx) in the liver of **A.** flight-trained European starlings and **B.** untrained starlings. ϵ_1 represents all unmeasured variables that could affect NRF2 and PPAR gene expression (e.g. ligand type and quantity, cofactors) while ϵ_2 represents all unmeasured variables that could affect enzyme activities (e.g. post translational modifications). Path estimates are reported to the left of each line for all causal relationships. The dashed lines indicate non-significant causal relationships (all p-values>0.1). Solid lines indicate significant causal relationships, and the asterisks and line thickness correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01. In general, flight training concentrated the regulation of NRF2 from all antioxidant genes (in untrained birds) to 60% of genes and initiates a new negative relationship between PPAR α and 80% of the antioxidant genes. Antioxidant enzyme activities were not significantly related to antioxidant gene expression in flight-trained birds, although CAT and GPx enzyme activities were significantly related to antioxidant gene expression in untrained birds.

A.
Pectoralis
All Diets
Flight-Trained Birds

B.
Pectoralis
All Diets
Untrained Birds

Figure 2.5. The casual structures to explain the hierarchical gene expression of transcription factors NRF2, PPAR α , PPAR δ on the gene expression of downstream antioxidant target genes (CAT, SOD1, SOD2, GPX1 and GPX4) and antioxidant enzyme activity (CAT, SOD, GPx) in the pectoralis of **A.)** flight-trained European starlings and **B.)** untrained starlings consuming all diets. E1 represents all unmeasured variables that could affect NRF2 and PPAR gene expression (e.g. ligand type and quantity, cofactors) while E2 represents all unmeasured variables that could affect enzyme activities (e.g. post translational modifications). Path estimates are reported to the left of each line for all causal relationships. The dashed lines indicate non-significant causal relationships (all p-values>0.1). Solid lines indicate significant causal relationships, and the asterisks and line thickness correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01. Flight training strengthens the relationships between NRF2 and antioxidant genes and broadens them to include all antioxidant genes. Flight training alters PPAR regulation on select antioxidant genes: PPAR α and PPAR δ negatively influences GPX1 expression and GPx activity, respectively in flight-trained birds while PPAR δ positively influences GPX expression in untrained birds.

SUPPORTING MATERIALS

Table S2.1. Sequences of primers used for reverse transcription quantitative PCR in the liver and pectoralis muscle of European Starlings.

Gene	Forward and Reverse Primers	RefSeq accession number
β -actin	F: 5'- GCTACGAACTCCCTGATGG -3' R: 5'- GACTCCATACCCAGGAAAGATG -3'	XM_014870664.1
CAT	F: 5'- TCATTCAGAAACGAGCTGTGA - 3' R: 5'- CCCACCCTCAGCATTGTATT - 3'	XM_014878840.1
SOD1	F: 5'- CCGGTGAAAGTCACTGGAAA - 3' R: 5'- GTGCAGCCATTAGTGTTGTC - 3'	XM_014888729.1
SOD2	F: 5'- GCAAGGAACAACAGGTCTCA - 3' R: 5'- TCACATTCCAGATGGCTTCA - 3'	XM_014874170.1
GPX1	F: 5'- CCAGTTCGGTCACCAGGAAA -3' R: 5'- CGCACTTCTCGAACAGGATG -3'	XM_014869607.1
GPX4	F: 5'- ACTTCACCAAGTTCCTCATTAACC -3' R: 5'- CTCGATCACATAGGGATCTTCCA -3'	XM_014882939.1
NRF2	F: 5'- TGGAATTAGACGAAGAGACAGGTGA -3' R: 5'- TTTGAAGGCTCTGTGGTCTGTGA -3'	XM_014890038.1
PPAR α	F: 5'- GTGCGTGACATCAAGGAGAA -3' R: 5'- GGTGTCATCAGGATGGTTGT -3'	XM_014880503.1
PPAR δ	F: 5'- GCATGTCACACAACGCAAT -3' R: 5'- GATCTCGCTTGCCGTCAG -3'	XM_014872751.1
PPAR γ	F: 5'- ACGACTCCTACATCAAATCCTT -3' R: 5'- ACGAAGGGTGATTTGTCTGT -3'	XM_014869773.1

Table S2.2. Mean gene expression of each antioxidant gene in the liver relative to the reference group^R, 13U, UT used for the linear models reported in text and in Table 2. Relative mean gene expression $\pm$ standard deviation are reported for each treatment group, and treatment groups are abbreviated as follows: 13 or 32% PUFA, Unsupplemented (U) or Supplemented (S) with antioxidants, and Untrained (UT) or Flight-Trained (T) for training. The number of individuals for each treatment groups are reported in the first row.

<i>Liver Antioxidant Genes</i>	<i>Relative Mean Gene Expression $\pm$ Standard deviation by Diet and Flight Training Treatment Groups in the Liver:</i>							
	13U, UT ^R	13U, T	13S, UT	13S, T	32U, UT	32U, T	32S, UT	32S, T
<i>Number of Individuals</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>11</i>	<i>8</i>	<i>12</i>
CAT	1.00 $\pm$ 0.29	1.26 $\pm$ 0.59	0.86 $\pm$ 0.34	1.13 $\pm$ 0.31	1.01 $\pm$ 0.57	1.47 $\pm$ 0.68	0.93 $\pm$ 0.49	1.14 $\pm$ 0.43
SOD1	1.00 $\pm$ 0.79	0.85 $\pm$ 0.72	0.73 $\pm$ 0.42	1.33 $\pm$ 0.71	0.75 $\pm$ 0.57	0.74 $\pm$ 0.50	0.85 $\pm$ 0.74	0.89 $\pm$ 0.45
SOD2	1.00 $\pm$ 0.33	1.02 $\pm$ 0.47	0.73 $\pm$ 0.22	1.04 $\pm$ 0.31	0.86 $\pm$ 0.41	1.02 $\pm$ 0.35	0.79 $\pm$ 0.31	1.08 $\pm$ 0.41
GPX1	1.00 $\pm$ 0.36	1.21 $\pm$ 0.47	0.74 $\pm$ 0.24	1.19 $\pm$ 0.46	0.92 $\pm$ 0.48	1.22 $\pm$ 0.77	0.83 $\pm$ 0.47	1.04 $\pm$ 0.39
GPX4	1.00 $\pm$ 0.42	0.91 $\pm$ 0.48	0.72 $\pm$ 0.34	0.90 $\pm$ 0.44	1.10 $\pm$ 0.73	0.89 $\pm$ 0.54	0.81 $\pm$ 0.46	0.95 $\pm$ 0.63
NRF2	1.00 $\pm$ 0.55	1.08 $\pm$ 0.62	0.71 $\pm$ 0.21	0.96 $\pm$ 0.95	0.94 $\pm$ 0.70	1.04 $\pm$ 0.60	0.72 $\pm$ 0.41	1.41 $\pm$ 2.00
PPAR γ	1.00 $\pm$ 0.44	1.44 $\pm$ 1.10	1.44 $\pm$ 0.80	1.29 $\pm$ 0.66	0.96 $\pm$ 0.43	1.19 $\pm$ 0.50	1.07 $\pm$ 0.68	1.46 $\pm$ 0.72
PPAR α	1.00 $\pm$ 0.21	1.10 $\pm$ 0.22	1.06 $\pm$ 0.08	1.02 $\pm$ 0.21	1.11 $\pm$ 0.16	1.21 $\pm$ 0.22	1.06 $\pm$ 0.06	1.13 $\pm$ 0.21

Table S2.3. Mean gene expression of each antioxidant gene in the pectoralis muscle relative to the reference group^R, 13U, UT used for the linear models reported in text and in Table 3. Relative mean gene expression $\pm$ standard deviation are reported for each treatment group, and treatment groups are abbreviated as follows: 13 or 32% PUFA, Unsupplemented (U) or Supplemented (S) with antioxidants, and Untrained (UT) or Flight-Trained (T) for training. The number of individuals for each treatment groups are reported in the first row or in the footnote^a.

<i>Pectoralis Antioxidant Genes</i>	<i>Relative Mean Gene Expression $\pm$ Standard deviation by Diet and Flight Training Treatment Groups in the Pectoralis:</i>							
	13U, UT ^R	13U, T	13S, UT	13S, T	32U, UT	32U, T	32S, UT	32S, T
<i>Number of Individuals</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>13</i>	<i>10</i>	<i>11</i>	<i>10</i>	<i>12</i>
CAT	1.00 $\pm$ 0.25	1.02 $\pm$ 0.49	1.10 $\pm$ 0.51	1.25 $\pm$ 0.53	0.79 $\pm$ 0.47	0.99 $\pm$ 0.42	1.11 $\pm$ 0.56	1.20 $\pm$ 0.56
SOD1	1.00 $\pm$ 0.57	1.03 $\pm$ 0.79 ^a	0.94 $\pm$ 0.42	1.47 $\pm$ 0.77	0.62 $\pm$ 0.59	0.83 $\pm$ 0.56	1.14 $\pm$ 0.81	1.19 $\pm$ 0.76
SOD2	1.00 $\pm$ 0.31	1.07 $\pm$ 0.35	0.89 $\pm$ 0.49	1.14 $\pm$ 0.51	0.67 $\pm$ 0.33	0.99 $\pm$ 0.38	0.97 $\pm$ 0.46	1.06 $\pm$ 0.72
GPX1	1.00 $\pm$ 0.35	0.95 $\pm$ 0.34	0.73 $\pm$ 0.21	1.17 $\pm$ 0.41	0.86 $\pm$ 0.45	0.93 $\pm$ 0.34	0.91 $\pm$ 0.25	1.02 $\pm$ 0.32
GPX4	1.00 $\pm$ 0.27	0.98 $\pm$ 0.42	0.80 $\pm$ 0.41	1.02 $\pm$ 0.58	0.76 $\pm$ 0.61	0.85 $\pm$ 0.40	0.93 $\pm$ 0.38	0.91 $\pm$ 0.44
NRF2	1.00 $\pm$ 0.24	0.94 $\pm$ 0.31	0.98 $\pm$ 0.39	0.89 $\pm$ 0.34	1.29 $\pm$ 0.65	0.82 $\pm$ 0.35	1.29 $\pm$ 0.78	0.82 $\pm$ 0.40
PPAR δ	1.00 $\pm$ 0.63	0.68 $\pm$ 0.57	0.72 $\pm$ 0.55	0.57 $\pm$ 0.35 ^a	0.92 $\pm$ 1.01	0.50 $\pm$ 0.32	1.03 $\pm$ 0.79	0.72 $\pm$ 0.49
PPAR α	1.00 $\pm$ 0.36	1.04 $\pm$ 0.19	0.94 $\pm$ 0.18	0.97 $\pm$ 0.17	1.05 $\pm$ 0.30	0.95 $\pm$ 0.14	1.05 $\pm$ 0.27	1.00 $\pm$ 0.28

^aThere was 1 fewer sample from this treatment group

Table S2.4. Mean enzyme activity relative to soluble protein content (mg/mL) of each antioxidant enzyme in the liver and pectoralis muscle. The linear models in Table 4 include Julian date as a covariate (n=2 or 3 per date), however mean enzyme activity (units for CAT and GPx, *nmol/min/mL/mg* and SOD, *U/mL/mg*) $\pm$ standard deviation are reported for each treatment group, and treatment groups are abbreviated as follows: 13 or 32% PUFA, Unsupplemented (U) or Supplemented (S) with antioxidants, and Untrained (UT) or Flight-Trained (T) for training. The number of individuals for each treatment groups are reported in the first row for the liver, pectoralis.

		<i>Mean Antioxidant Enzyme Activity $\pm$ Standard deviation by Diet and Flight Training Treatment Groups in the Liver and Pectoralis:</i>							
		13U, UT	13U, T	13S, UT	13S, T	32U, UT	32U, T	32S, UT	32S, T
<i>No. Individuals: Liver, Pect.</i>		<i>10, 10</i>	<i>13, 13</i>	<i>10, 10</i>	<i>13, 13</i>	<i>10, 10</i>	<i>11, 12</i>	<i>8, 10</i>	<i>12, 13</i>
Liver	CAT	62.16 $\pm$ 28.7	53.34 $\pm$ 19.8	71.33 $\pm$ 15.8	43.69 $\pm$ 21.1	74.93 $\pm$ 23.8	47.06 $\pm$ 20.2	61.85 $\pm$ 27.9	47.26 $\pm$ 26.4
	SOD	1.48 $\pm$ 0.76	1.74 $\pm$ 0.34	1.84 $\pm$ 0.19	1.60 $\pm$ 0.50	1.73 $\pm$ 0.35	1.63 $\pm$ 0.45	1.58 $\pm$ 0.52	1.56 $\pm$ 0.57
	GPx	12.41 $\pm$ 7.8	9.75 $\pm$ 3.6	11.57 $\pm$ 3.9	9.82 $\pm$ 4.8	12.91 $\pm$ 4.6	8.93 $\pm$ 3.7	12.00 $\pm$ 5.7	9.15 $\pm$ 3.7
Pectoralis	CAT	1.12 $\pm$ 0.35	0.85 $\pm$ 0.34	1.24 $\pm$ 0.21	0.75 $\pm$ 0.41	0.91 $\pm$ 0.45	0.78 $\pm$ 0.34	1.31 $\pm$ 0.25	0.73 $\pm$ 0.32
	SOD	6.64 $\pm$ 1.1	6.94 $\pm$ 0.96	7.18 $\pm$ 0.54	6.83 $\pm$ 1.2	6.86 $\pm$ 1.1	7.18 $\pm$ 0.84	6.50 $\pm$ 0.64	7.53 $\pm$ 0.83
	GPx	7.88 $\pm$ 2.1	6.91 $\pm$ 1.1	7.35 $\pm$ 1.5	7.61 $\pm$ 1.3	7.31 $\pm$ 1.3	7.36 $\pm$ 1.1	7.01 $\pm$ 1.4	7.62 $\pm$ 1.3

Figure S2.1. The casual structures to explain the hierarchical gene expression of transcription factors NRF2, PPAR α , PPAR δ on the gene expression of downstream antioxidant target genes (CAT, SOD1, SOD2, GPX1 and GPX4) and antioxidant enzyme activity (CAT, SOD, GPx) in the pectoralis of **A.)** anthocyanin supplemented flight-trained European starlings **B.)** anthocyanin unsupplemented flight-trained starlings **C.)** anthocyanin supplemented untrained starlings **D.)** anthocyanin unsupplemented untrained starlings. E1 represents all unmeasured variables that could affect NRF2 and PPAR gene expression (e.g. ligand type and quantity, cofactors) while E2 represents all unmeasured variables that could affect enzyme activities (e.g. post translational modifications). Path estimates are reported to the left of each line for all causal relationships. The dashed lines indicate non-significant causal relationships (all p-values>0.1). Solid lines indicate significant causal relationships, and the asterisks and line thickness correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01. Dietary anthocyanin does not strongly alter the relationships among transcription factors, antioxidant genes, and antioxidant enzymes. Whereas, flight training strengthens the relationships between NRF2 and antioxidant genes and broadens them to include all antioxidant genes. Flight training alters PPAR regulation on select antioxidant genes: PPAR α and PPAR δ differentially influence CAT expression in flight-trained birds while PPAR δ positively influences GPX expression in untrained birds.

CHAPTER 3

ACUTE FLIGHT AND FLIGHT TRAINING DEplete NON-ENZYMATIC ANTIOXIDANT CAPACITY AND PROTECT AGAINST OXIDATIVE DAMAGE IN A MIGRATORY SONGBIRD, BUT DIETARY ANTIOXIDANTS AND FAT HAVE LITTLE EFFECT ON OXIDATIVE STATUS

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ABSTRACT

1. 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 affect key components of the antioxidant system in multiple tissues of migratory songbirds.
2. We conducted a 4-factorial experiment that tested 3 hypotheses: *Stimulatory effect of flight*: plasma antioxidant capacity decreases during an acute flight to maintain low levels of circulating oxidative damage, and flying regularly over several weeks increases non-enzymatic antioxidant capacity and decreases lipid damage in the liver, flight-muscle, and plasma relative to untrained birds. *Dietary fat effect*: migratory songbirds fed diets composed of more 18:2n-6 PUFA are more susceptible to oxidative damage and thus have increased antioxidant capacity in all tissues to reduce lipid damage compared to birds fed diets with less PUFA. *Dietary antioxidant effect*: migratory songbirds fed dietary anthocyanins have increased non-enzymatic antioxidant capacity, and lower levels of lipid damage in all tissues compared to birds not fed anthocyanins.
3. 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 was similar to untrained birds in both the plasma and tissues. Thus, repeated bouts of flight separated by recovery periods may be required for maintaining oxidative balance during migration.
4. Flight-trained birds had similar levels of antioxidant capacity compared to untrained birds and oxidative damage was prevented in the flight-muscle and

reduced in the liver suggesting that the liver is preferentially protected to preserve its role in processing or protecting fatty acids during flight.

5. Flight efficiency (kJ/min) of birds during their longest flights was related to the extent to which non-enzymatic components of the antioxidant system changed during flight. Such a condition-dependent antioxidant strategy would allow birds during migration to flexibly respond to changes in the environment such as availability of dietary antioxidants or increased flight time and effort.
6. Contrary to our predictions, dietary fat did not influence oxidative status, and birds that consumed more dietary anthocyanins had similar antioxidant capacity but more plasma oxidative damage immediately after acute flight.

Keywords

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 by either neutralizing them with antioxidants to minimize resulting oxidative damage, or damage must be restored using repair mechanisms (Costantini, 2014; Halliwell & Gutteridge, 2007). Long-distance flight increases metabolism (Butler, 2016; Corder & Schaeffer, 2015; DeMoranville et al., 2019; Swanson, 2010) and poses a potential oxidative challenge for flying animals. 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, Lindecke,

Pētersons, & Voigt, 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, Jenni, Smith, & Costantini, 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 & Guglielmo, 2019). Similarly, flight-trained zebra finches *Taeniopygia guttata* had increased circulating oxidative damage compared to untrained sedentary individuals (Skrip, Seeram, Yuan, Ma, & McWilliams, 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 & 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). 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 (Jensen, Isaksson, Eikenaar, & Andersson, 2020; Pierce & McWilliams, 2005; Pierce, McWilliams, Place, & Huguenin, 2004; Price, Krokfors, & Guglielmo, 2008; Smith & McWilliams, 2010). The potential benefits of consuming certain long-chain PUFA (e.g., 18:2n-6 PUFA) (Guglielmo, 2018; Pierce & McWilliams, 2014) are also associated with potential oxidative costs because PUFA are highly susceptible to oxidative damage (McWilliams et al., 2021; Skrip & McWilliams, 2016) and the resulting lipid radicals often cause a self-perpetuating chain reaction damaging nearby PUFAs and other molecules (Cooper-Mullin, Carter, & McWilliams, 2019; Skrip & McWilliams, 2016). Birds preferentially consuming 18:2n-6 PUFA during migration to enhance their metabolism (Pierce & McWilliams, 2014) likely require an augmented antioxidant system to protect against the oxidative challenge that such PUFAs pose.

Dietary antioxidants augment the antioxidant system- Many songbird species select fruits that are high in antioxidants and fat during fall migration, suggesting that antioxidant consumption may be important to protect against oxidative damage during this life history stage (A. R. R. Alan, McWilliams, & McGraw, 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 (A. R. R. Alan et al., 2013; Bolser et al., 2013; Schaefer, McGraw, & Catoni, 2008). Anthocyanins are potent antioxidants (Halliwell & 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 2x more acidic stomachs of songbirds

(pH 2) relative to mammals (pH 4.4) (Dangles & Fenger, 2018). Anthocyanins can directly or indirectly affect the antioxidant system and also have been shown to protect birds from an immune challenge (Catoni, Martin Schaefer, & Peters, 2008) and protect against some of the metabolic costs associated with flight training (Casagrande et al., 2020) and courtship (Carbeck et al., 2018). These energy savings associated with consuming anthocyanins may allow birds to invest in increasing enzymatic or non-enzymatic antioxidants to prevent damage. Non-enzymatic antioxidant capacity increases with fat stores in songbirds on stopovers during migration and is greatest during the pre-migratory fueling stage in shorebirds (Costantini, Cardinale, & Carere, 2007; Gutiérrez et al., 2019; Skrip et al., 2015). 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 & 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.

- a. *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.
 - b. *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.
 - c. *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.
2. *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.
 3. *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. Finally, we integrated the available information from multiple

measures of the antioxidant system in three different tissues to determine the extent to which key components of the antioxidant system act in concert to protect exercising birds from oxidative damage. We combined the 5 measures of non-enzymatic antioxidant capacity measures in this study with the 18 enzymatic antioxidant measures that we characterized for each individual starling at final sampling published in a companion study (citation redacted for initial review).

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 migration (A. R. R. 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 (Casagrande et al., 2020; Hall et al., 2014; McWilliams et al., 2021; Nebel et al., 2012). 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 & Melville, 1983)) for each individual. Birds were then randomly sorted into four groups 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).

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 (Dick & Guglielmo, 2019; Forman, Chen, & Evans, 1997; Hamilton et al., 2018; Kennedy, Bickerdike, Berge, Porter, & Tocher, 2007). 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 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: 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 35 wet g day⁻¹ (as observed in food intake trials in this study).

Experimental Timeline

On September 21st we randomly assigned 5 starlings to each of twenty cohorts. There were 5 cohorts per diet group, and the sampling order of the diet groups was randomly assigned within a cohort group ensuring that the same diet group was not consistently sampled first or last, and all diet groups were sampled within 10 days of one another. On September 23rd, and continuing every three days thereafter (citation redacted for initial review), 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 in the morning at 8:00 hr in order 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, Biebach, & Visser, 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 flight duration (full methods and results reported previously, citation redacted for initial review). The final flight was on average 193 min $\pm$ 71 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 Adsorbant 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 4°C (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 -80°C 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 & Gutteridge, 2007), peroxy 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; Prior and Cao, 1999) following Jimenez et al. (2020, JEB). 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 peroxy 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 & 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 H₂O₂ 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 (Cooper-Mullin et al., 2019; Costantini, 2016; Costantini et al., 2007). 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, peroxy and hydroxyl radical absorbance capacity, d-ROMs); all coefficient of variations were under 10%, and replicates were averaged prior to statistical analyses.

Endogenous antioxidant measures included in the PCA

We report the full methods and results for the liver and pectoralis endogenous antioxidant measures included in the PCA in our companion study (citation redacted for initial review). Briefly, we homogenized tissue in phosphate buffer, collected the supernatant, and measured the antioxidant enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) using commercial kits (Cayman Chemical, Ann Arbor, MI). All enzyme activities were normalized to soluble protein content (mg/mL) as measured by the Bradford protein assay (Biorad, 5000006) using a bovine albumin serum (BSA) standard (Fisher Scientific AAJ6477709). We also conducted quantitative reverse transcription PCR (qRT-PCR) quantify relative expression of multiple isoforms of these antioxidant enzymes (SOD1, SOD2, GPX1, GPX4, CAT) and the antioxidant transcription factor NRF2. Total RNA was extracted from liver and pectoralis muscle using RNeasy® Fibrous Mini Kit (QIAGEN®, Germantown, MD, USA) following kit instructions including the recommended DNase treatment step, but without the proteinase K digestion step for the liver. RNA was reverse transcribed using the SuperScript IV First-strand Synthesis System Kit (Thermofisher, Burlington, ON, CA). The Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (Thermofisher,

Burlington, ON, CA) was used in the qPCR reaction. Each PCR run was completed with a melt curve analysis and amplification efficiency was verified for every primer pair. The gene expression values were derived from a standard curve generated for each primer set. Transcript expression levels were normalized to the reference gene β -actin, which did not vary across the 8 diet and training treatments in the pectoralis, and these normalized transcript levels were included in the PCA.

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, $\Delta AIC \geq 3$. 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 decreases oxidative damage and (H1b) that regularly flying increases antioxidant capacity and decreases oxidative damage. We conducted pairwise comparisons using least square means to determine the differences of oxidative status between specific timepoints. 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.

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). The best-fit linear models retained only experimental cohort for select measures: the change in d-ROMs during acute flight, lipid damage in the liver, and lipid damage in the pectoralis. The final models for these 3 measures were linear mixed effects models including cohort as a random effect, as cohort was more appropriately controlled for using this method. We included energy consumed (kJ/min) during the acute flight as a fixed effect in the linear models analyzing plasma oxidative status After-flight and the change in these measures during the acute flight to determine if energy expenditure influenced antioxidant capacity or oxidative damage. The best fit models for AF oxidative status included energy expenditure, whereas energy expenditure x diet was included to analyze the change in oxidative status during acute flight. 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.

Principle component analysis

We used the *stats* package in R to conduct a principle component analysis (PCA) to create multiple antioxidant status indices that incorporated the 5 antioxidant capacity measures in this study with the 18 antioxidant measures that we characterized for each individual starling at final sampling published previously (citation redacted for initial review). The 23 total antioxidant measures consisted of 1 measure of non-enzymatic antioxidant capacity in the plasma (OXY), 2 measures of non-enzymatic antioxidant capacity in both the liver and pectoralis (ORAC: peroxy and hydroxyl scavenging capacity), 3 antioxidant enzyme activities (SOD, GPx, CAT) in the liver and pectoralis, and gene expression for multiple isoforms of these antioxidant enzymes (SOD1, SOD2, GPX1, GPX4, CAT) and the antioxidant transcription factor NRF2 in both tissues. We used the top three PCs that together explained 45% of the variation in our data to represent an individual's antioxidant index. We determined the effect of each antioxidant index on each of the 3 measures of oxidative damage (d-ROMs in the plasma, LPO in the liver, LPO in the pectoralis) during Recovery sampling using linear models.

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. Plasma d-ROMs was highest after two weeks of daily flight training (PF blood sample, Fig. 1) 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 min $\pm$ 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 was substantially lower than BG after two weeks of daily flight as well as two days later without further flights (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: $T_{49} = -8.544$, $P < 0.0001$). These results support H1a (acute effects of flight) since plasma antioxidant capacity decreased during a given long-duration flight and levels of circulating oxidative damage remained lower than or at baseline levels.

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 but was related to the decrease in non-enzymatic antioxidant capacity during the acute flight (Fig. 3A,B,C, Fig. S1). Only a quarter of birds 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; 3C, 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. 3C and S1), well below the mean decrease ($-22.7 \pm 10.4 \text{ mmol l}^{-1}$ of HClO neutralized). These birds expended energy in the upper 50% range ($>0.51 \text{ kJ/min}$ was consumed by the upper 50% of individuals) (Fig. 3C and S1), 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 ($0.5 \pm 0.06 \text{ mmol}$); 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, although this trend was not apparent among individuals sampled before (BG) and after (RC) the two-week period.

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.

Effect of antioxidant indices on lipid damage in the plasma, liver, pectoralis during Recovery

The top 3 PCs explained a total of 45% of the variation in the 23 antioxidant measures ($N=81$; PC1: 24.8%, PC2: 11.4%, PC3: 9%). The PC loadings (Table S5) described 3 independent antioxidant indices that we generalized as: birds with high PC1 scores had relatively high tissue (liver, pectoralis) antioxidant gene expression; birds with high PC2 scores had relatively high liver antioxidant enzyme activities and gene expression (highest loadings were on GPx and CAT enzyme activities, and GPX1, GPX4, SOD2, and CAT gene expression); birds with high PC3 scores had relatively low liver antioxidant enzyme activities. PC1 and PC2 antioxidant indices did not explain plasma d-ROMs (Table 1; PC1, $T_{81} = -0.332$, $P = 0.741$, PC2, $T_{81} = 1.065$, $P = 0.290$), liver (PC1, $T_{81} = 0.777$, $P = 0.439$, PC2, $T_{81} = -0.701$, $P = 0.486$), or pectoralis lipid hydroperoxide concentration (PC1, $T_{46} = 0.492$, $P = 0.625$, PC2, $T_{46} = -0.214$, $P = 0.832$). The PC3 antioxidant index did not explain plasma or pectoralis damage (Table 1; d-ROMs: $T_{81} = 0.818$, $P = 0.416$, Pectoralis LPO: $T_{81} = -0.201$, $P = 0.842$); however, PC3 significantly explained liver damage levels, but contrary to our predictions. For a 1 standard deviation increase in PC3 score, liver damage decreased by 1.449 ($T_{81} = -2.965$, $P = 0.004$). In general, the lack of overall predictive power of the 3 PCs on oxidative damage was not

surprising given the low correlations among the antioxidant and damage measures analyzed (Correlation matrix, Table S6).

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, Vasilaki, & Jackson, 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, H2b). 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 (Cooper-Mullin et al., 2019; Skrip et al., 2016). Most studies report increases in markers of circulating oxidative damage shortly after a migratory flight in free-living birds (Costantini et al., 2018; Jenni-Eiermann et al., 2014), after a 200 km experimental flight

in homing pigeons (Costantini, Dell'Araccia, & Lipp, 2008), or in the pectoralis after an experimental flight in a wind tunnel (Dick & Guglielmo, 2019). Given that previous flight training history for birds in these studies was unknown, it is not possible to determine whether these increases in oxidative damage were caused by acute or longer-term effects of flying. 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 during their longest flight (Fig. 3). We propose that birds with higher flight efficiencies 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 & 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 seems to deplete non-enzymatic antioxidant capacity with an associated increase in oxidative damage immediately after a flight yet after 48 hrs of rest (without wind tunnel flying) both non-enzymatic antioxidant capacity and oxidative damage returned to baseline levels. 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. 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, 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 peroxy 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 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 (Skrip & McWilliams, 2016) and the demonstrated responsiveness of the antioxidant system in migratory birds (Dick & Guglielmo, 2019; Jenni-Eiermann et al., 2014; Skrip et al., 2015). Additionally, 18:2n-6 has been shown to stimulate antioxidant enzymes in fish (Li et al., 2013; Zengin & Yilmaz, 2016) but not in rats (Tou, Altman, Gigliotti, Benedito, & Cordonier, 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 & 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 (R. R. Alan & 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, Källstig, Andersson, Herrera-Dueñas, & Isaksson, 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 relying on their antioxidant system for protection. 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 & McWilliams, 2005; Pierce et al., 2004; Price et al., 2008; Smith & 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), and increased testosterone levels and breeding behaviors in male European starlings (Carbeck et al., 2018). 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 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. 1. Dietary anthocyanins prevented the accumulation of reactive species used to stimulate the 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 (Merry & Ristow, 2016; Ristow et al., 2009). 2. 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 (Cooper-Mullin et al., 2019; Jenni-Eiermann et al., 2014), 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 & 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.

Antioxidant index 3 explained liver lipid damage during Recovery

The principle components analyses that integrated 23 measures of the antioxidant system measured in the same individuals explained 45% of the variation in oxidative damage in the plasma, liver, and pectoralis. The three measures of antioxidant capacity reported in this study (OXY in plasma, ORAC in liver and pectoralis) were not significant predictors of oxidative damage compared to gene expression and enzyme activities in the liver and pectoralis (Table S5), reinforcing the notion that using multiple measures of antioxidant capacity per individual is needed for interpreting how the antioxidant system to ecologically-relevant oxidative challenges (Costantini, 2008). Only

one of the three antioxidant indices (PC3) explained a significant portion of the variation in oxidative damage in the three tissues - specifically, lower liver antioxidant enzyme activities were associated with less lipid damage. Enzyme activities measure the enzyme concentration at a given time, and it is possible the relationship we detected between low enzyme activities and low damage levels in the liver is an artifact of those enzyme activities being greater up to a day prior to sampling. Such a strategy would be beneficial to flight-trained birds, as high enzyme activities in the tissues immediately after flight would protect against damage, as demonstrated by (Dick & Guglielmo, 2019), but might be unnecessary or too costly to maintain during recovery.

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 (Costantini, 2014; Halliwell & Gutteridge, 2007). 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 & McWilliams, 2016). Individual variation in the ability to utilize antioxidants must exist for phenotypic flexibility of the antioxidant system to persist, based on this general concept proposed by (Piersma & Van Gils, 2011). The varying degrees to which individuals modulate OXY and uric acid during acute flight provides evidence for condition-dependent antioxidant strategies in birds when faced with an energetic challenge (Fig. 3). Studies such as those done with mosquitofish (Loughland & 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. We also found that birds seem able to rapidly adjust their antioxidant system to maintain overall low levels of circulating damage although the extent was tissue-dependent. We suggest that researchers remain mindful of the tissue-dependency of antioxidant capacity and oxidative damage demonstrated here and that future studies that assess oxidative status select biologically relevant tissues to measure that directly relate to the hypotheses of interest.

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Author's contributions

SRM and BJP conceived and designed the experiment. KJD and WAC and SRM refined the methodology and KJD and WAC conducted the experiment. LC conducted critical lab analyses. KJD primarily analyzed the data and CCM assisted with analyses and coding. CCM designed and coded the figures. KJD and SRM interpreted the results and primarily wrote the manuscript, and WAC, CCM, LC, and BJP provided editorial advice on the manuscript.

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Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

References

- Alan, A. R. R., McWilliams, S. R., & McGraw, K. J. (2013). The importance of antioxidants for avian fruit selection during autumn migration. *Wilson Journal of Ornithology*, 125(3), 513–525.
- Alan, R. R., & McWilliams, S. R. (2013). Oxidative stress, circulating antioxidants, and dietary preferences in songbirds. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 164(3), 185–193. doi: 10.1016/j.cbpb.2012.12.005
- Bolser, J. A., Alan, R. R., Smith, A. D., Li, L., Seeram, N. P., & McWilliams, S. R. (2013). Birds select fruits with more anthocyanins and phenolic compounds during autumn migration. *Wilson Journal of Ornithology*, 125(1), 97–108.
- Butler, P. J. (2016). The physiological basis of bird flight. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371, 1–11. doi: 10.1098/rstb.2015.0384
- Cabe, P. (1993). European starling (*Sturnus vulgaris*). In A.F. Poole & F.B. Gill (Eds.): *Vol. Version 2*. Ithica, NY: Cornell Lab of Ornithology.
- Carbeck, K. M., DeMoranville, K. J., D’Amelio, P. B., Goymann, W., Trost, L., Pierce, B., ... McWilliams, S. R. (2018). Environmental cues and dietary antioxidants affect breeding behavior and testosterone of male European starlings (*Sturnus vulgaris*). *Hormones and Behavior*, 103. doi: 10.1016/j.yhbeh.2018.05.020
- Casagrande, S., DeMoranville, K. J., Trost, L., Pierce, B. J., Bryla, A., Dzialo, M., ... McWilliams, S. R. (2020). Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird. *Proceedings of the*

National Academy of Sciences B, 18(29), 1–8.

- Catoni, C., Martin Schaefer, H., & Peters, A. (2008). Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird. *Functional Ecology*, 22, 649–654. doi: 10.1111/j.1365-2435.2007.0
- Cooper-Mullin, C., Carter, W. A., & McWilliams, S. R. (2019). Acute effects of intense exercise on the antioxidant system in birds: Does exercise training help? *Journal of Experimental Biology*, 222(19), 1–10. doi: 10.1242/jeb.210443
- Corder, K. R., & Schaeffer, P. J. (2015). Summit metabolic rate exhibits phenotypic flexibility with migration, but not latitude in a neotropical migrant, *Parkesia noveboracensis*. *Journal of Ornithology*, 156(2), 547–550. doi: 10.1007/s10336-015-1157-x
- Costantini, D. (2008). Oxidative stress in ecology and evolution: Lessons from avian studies. *Ecology Letters*, 11(11), 1238–1251. doi: 10.1111/j.1461-0248.2008.01246.x
- Costantini, D. (2014). *Oxidative stress and hormesis in evolutionary ecology and physiology: A marriage between mechanistic and evolutionary approaches*. doi: 10.1007/978-3-642-54663-1
- Costantini, D. (2016). Oxidative stress ecology and the d-ROMs test: facts, misfacts and an appraisal of a decade's work. *Behavioral Ecology and Sociobiology*, 70(5), 809–820. doi: 10.1007/s00265-016-2091-5
- Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress:

- the opportunities and challenges ahead. *Journal of Experimental Biology*, 222, 1–9.
doi: 10.1242/jeb.194688
- Costantini, D., Cardinale, M., & Carere, C. (2007). Oxidative damage and anti-oxidant capacity in two migratory bird species at a stop-over site. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, 144(4), 363–371. doi: 10.1016/j.cbpc.2006.11.005
- Costantini, D., Dell’Ariccia, G., & Lipp, H.-P. (2008). Long flights and age affect oxidative status of homing pigeons (*Columba livia*). *Journal of Experimental Biology*, 211(3), 377–381. doi: 10.1242/jeb.012856
- Costantini, D., Lindecke, O., Pētersons, G., & Voigt, C. C. (2018). Migratory flight imposes oxidative stress in bats. *Current Zoology*.
- Dangles, O., & Fenger, J. A. (2018). The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules*, 23(8). doi: 10.3390/molecules23081970
- Davies, M. J. (2016). Protein oxidation and peroxidation. *Biochemical Journal*, 473(7), 805–825. doi: 10.1042/BJ20151227
- DeMoranville, K. J., Corder, K. R., Hamilton, A., Russell, D. E., Huss, J. M., & Schaeffer, P. J. (2019). PPAR expression, muscle size and metabolic rates across the gray catbird’s annual cycle are greatest in preparation for fall migration. *Journal of Experimental Biology*, 222, 1–11. doi: 10.1242/jeb.209452
- Dick, M. F., & Guglielmo, C. G. (2019). Flight muscle protein damage during endurance

- flight is related to energy expenditure but not dietary polyunsaturated fatty acids in a migratory bird. *Journal of Experimental Biology*, 222, 1–9. doi: 10.1242/jeb.187708
- Dogan Comert, E., & Gokman, V. (2017). Antioxidants bound to an insoluble food matrix: their analysis, regeneration behavior, and physiological importance. *Comprehensive Reviews in Food Science and Safety*, 16, 382–339. doi: 10.1111/1541-4337.12263
- Eikenaar, C., Källstig, E., Andersson, M. N., Herrera-Dueñas, A., & Isaksson, C. (2017). Oxidative challenges of avian migration: A comparative field study on a partial migrant. *Physiological and Biochemical Zoology*, 90(2), 223–229. doi: 10.1086/689191
- Engel, S., Biebach, H., & Visser, G. H. (2006). Metabolic costs of avian flight in relation to flight velocity: a study in Rose coloured starlings (*Sturnus roseus*, Linnaeus). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 176(5), 415–427. doi: 10.1007/s00360-006-0063-1
- Forman, B. M., Chen, J., & Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proceedings of the National Academy of Sciences of the United States of America*, 94(9), 4312–4317.
- Ginn, H., & Melville, D. (1983). *Moult in birds*. Thetford, UK: British Trust for Ornithology.
- Guglielmo, C. G. (2018). Obese super athletes: fat-fueled migration in birds and bats. *Journal of Experimental Biology*, 221, 1–16. doi: 10.1242/jeb.165753

- Gutiérrez, J. S., Sabat, P., Castañeda, L. E., Contreras, C., Navarrete, L., Peña-Villalobos, I., & Navedo, J. G. (2019). Oxidative status and metabolic profile in a long-lived bird preparing for extreme endurance migration. *Scientific Reports*, 9(1), 1–11. doi: 10.1038/s41598-019-54057-6
- Hall, Z. J., Bauchinger, U., Gerson, A. R., Price, E. R., Langlois, L. A., Boyles, M., ... Macdougall-Shackleton, S. A. (2014). Site-specific regulation of adult neurogenesis by dietary fatty acid content, vitamin E and flight exercise in European starlings. *European Journal of Neuroscience*, 39, 875–882. doi: 10.1111/ejn.12456
- Halliwell, B., & Gutteridge, J. M. (2007). *Free radicals in biology and medicine* (4th ed.). New York, NY: Oxford University Press.
- Hamilton, A., Ly, J., Robinson, J. R., Corder, K. R., DeMoranville, K. J., Schaeffer, P. J., & Huss, J. M. (2018). Conserved transcriptional activity and ligand responsiveness of avian PPARs: potential role in regulating lipid metabolism in migratory birds. *General and Comparative Endocrinology*, 268, 110–120. doi: 10.1016/j.ygcen.2018.08.009
- Ito, F., Ito, T., Suzuki, C., Yahata, T., Ikeda, K., & Hamaoka, K. (2017). The application of a modified d-ROMs test for measurement of oxidative stress and oxidized high-density lipoprotein. *International Journal of Molecular Sciences*, 18(2), 454. doi: 10.3390/ijms18020454
- Jenni-Eiermann, S., Jenni, L., Smith, S., & Costantini, D. (2014). Oxidative stress in endurance flight: An unconsidered factor in bird migration. *PLoS ONE*, 9(5), 1–6. doi: 10.1371/journal.pone.0097650

- Jensen, J. K., Isaksson, C., Eikenaar, C., & Andersson, M. N. (2020). Migrant blackbirds, *Turdus merula*, have higher plasma levels of polyunsaturated fatty acids compared to residents, but not enhanced fatty acid unsaturation index. *Ecology and Evolution*, (July), 1–11. doi: 10.1002/ece3.6681
- Kennedy, S. R., Bickerdike, R., Berge, R. K., Porter, A. R., & Tocher, D. R. (2007). Influence of dietary conjugated linoleic acid (CLA) and tetradecylthioacetic acid (TTA) on growth, lipid composition and key enzymes of fatty acid oxidation in liver and muscle of Atlantic cod (*Gadus morhua* L.). *Aquaculture*, 264(1–4), 372–382. doi: 10.1016/j.aquaculture.2007.01.013
- Li, M., Chen, L., Qin, J. G., Li, E., Yu, N., & Du, Z. (2013). Growth performance, antioxidant status and immune response in darkbarbel catfish *Pelteobagrus vachelli* fed different PUFA/vitamin E dietary levels and exposed to high or low ammonia. *Aquaculture*, 406–407, 18–27. doi: 10.1016/j.aquaculture.2013.04.028
- Loughland, I., & Seebacher, F. (2020). Differences in oxidative status explain variation in thermal acclimation capacity between individual mosquitofish (*Gambusia holbrooki*). *Functional Ecology*, 34(7), 1380–1390. doi: 10.1111/1365-2435.13563
- McArdle, A., Vasilaki, A., & Jackson, M. (2002). Exercise and skeletal muscle ageing: cellular and molecular mechanisms. *Aging Research Reviews*, 1, 79–93. doi: 10.1016/b978-012597951-1/50016-0
- McWilliams, S. R., Guglielmo, C., Pierce, B., & Klaassen, M. (2004). Flying, fasting, and feeding in birds during migration: A nutritional and physiological ecology perspective. *Journal of Avian Biology*, 35(5), 377–393. doi: 10.1111/j.0908-

- McWilliams, S. R., Pierce, B. J., Wittenzellner, A., Langlois, L., Engel, S., Speakman, J., ... Bauchinger, U. (2021). The energy savings-oxidative cost trade-off for birds during migration. *ELife*.
- Merry, T. L., & Ristow, M. (2016). Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training? *Journal of Physiology*, 594(18), 5135–5147. doi: 10.1113/JP270654
- Nebel, S., Bauchinger, U., Buehler, D. M., Langlois, L. A., Boyles, M., Gerson, A. R., ... Guglielmo, C. G. (2012). Constitutive immune function in European starlings, *Sturnus vulgaris*, is decreased immediately after an endurance flight in a wind tunnel. *Journal of Experimental Biology*, 215(2), 272–278. doi: 10.1242/jeb.057885
- Pierce, B. J., & McWilliams, S. R. (2005). Seasonal changes in composition of lipid stores in migratory birds: causes and consequences. *The Condor*, 107(2), 269–279.
- Pierce, B. J., & McWilliams, S. R. (2014). The fat of the matter: how dietary fatty acids can affect exercise performance. *Integrative and Comparative Biology*, 54(5), 903–912. doi: 10.1093/icb/icu098
- Pierce, B. J., McWilliams, S. R., Place, A. R., & Huguenin, M. A. (2004). Diet preferences for specific fatty acids and their effect on composition of fat reserves in migratory Red-eyed Vireos (*Vireo olivaceus*). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 138(4), 503–514. doi: 10.1016/j.cbpb.2004.06.014

- Piersma, T., & Van Gils, J. A. (2011). *The flexible phenotype: a body-centred integration of ecology, physiology, and behaviour*. New York, NY: Oxford University Press.
- Price, E. R., Krokfors, A., & Guglielmo, C. G. (2008). Selective mobilization of fatty acids from adipose tissue in migratory birds. *Journal of Experimental Biology*, 211(1), 29–34. doi: 10.1242/jeb.009340
- Rattan, S. I. S. (2008). Hormesis in aging. *Ageing Research Reviews*, 7(1), 63–78. doi: 10.1016/j.arr.2007.03.002
- Ristow, M., Zarse, K., Oberbach, A., Klötting, N., Birringer, M., Kiehntopf, M., ... Blüher, M. (2009). Antioxidants prevent health-promoting effects of physical exercise in humans. *PNAS*, 106(12), 2–7. doi: 10.1073/pnas.0903485106
- Schaefer, H., McGraw, K., & Catoni, C. (2008). Birds use fruit colour as honest signal of dietary antioxidant rewards. *Functional Ecology*, 22, 303–310. doi: 10.1111/j.1365-2435.2007.01363.x
- Skip, M. M., Bauchinger, U., Goymann, W., Fusani, L., Cardinale, M., Alan, R. R., & McWilliams, S. R. (2015). Migrating songbirds on stopover prepare for, and recover from, oxidative challenges posed by long-distance flight. *Ecology and Evolution*, 5(15), 3198–3209. doi: 10.1002/ece3.1601
- Skip, M. M., & McWilliams, S. R. (2016). Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists. *Journal of Field Ornithology*, 87(1), 1–20. doi: 10.1111/jfo.12135
- Skip, M. M., Seeram, N. P., Yuan, T., Ma, H., & McWilliams, S. R. (2016). Dietary

antioxidants and flight exercise in female birds affect allocation of nutrients to eggs: how carry-over effects work. *Journal of Experimental Biology*, 219, 2716–2725.
doi: 10.1242/jeb.137802

Smith, S. B., & McWilliams, S. R. (2010). Patterns of fuel use and storage in migrating passerines in relation to fruit resources at autumn stopover sites. *The Auk*, 127(1), 108–118.

Swanson, D. L. (2010). Seasonal metabolic variation in birds: functional and mechanistic correlates. In *Current Ornithology* (Thompson C, Vol. 17, pp. 131–189). doi: 10.1007/978-1-4419-6421-2

Tou, J. C., Altman, S. N., Gigliotti, J. C., Benedito, V. A., & Cordonier, E. L. (2011). Different sources of omega-3 polyunsaturated fatty acids affects apparent digestibility, tissue deposition, and tissue oxidative stability in growing female rats. *Lipids in Health and Disease*, 10, 1–14. doi: 10.1186/1476-511X-10-179

Zengin, H., & Yilmaz, Ö. (2016). Antioxidant defence of the actively feeding *Oncorhynchus mykiss* (Walbaum 1792) larvae in relation to dietary PUFA and vitamin E contents. *Regional Studies in Marine Science*, 8, 515–522. doi: 10.1016/j.rsma.2016.03.003

TABLES

Table 3.1. Linear model results for d-ROMs in the plasma and Lipid Hydroperoxide concentration (LPO) in the liver and pectoralis at the Recovery timepoint in relation to the three top antioxidant indices (PC1, PC2, PC3). See PC variables and loadings in Table S5. Data are reported as estimates (standard error) for each measure and the asterisks correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01.

<i>Covariate</i>	<i>Dependent variable:</i>		
	<i>Tissue:</i> <i>Plasma</i> d-ROMs <i>mmol⁻¹ H₂O₂ equivalents</i>	<i>Liver</i> Lipid [Hydroperoxide] <i>μM</i>	<i>Pectoralis</i> Lipid [Hydroperoxide] <i>μM</i>
PC1: High liver and pectoralis antioxidant gene expression	-0.159 (0.480)	0.230 (0.295)	0.053 (0.248)
PC2: High liver enzymatic antioxidant capacity	0.753 (0.707)	0.305 (0.436)	0.011 (0.366)
PC3: Low liver antioxidant enzyme activities	0.649 (0.793)	-1.449*** (0.489)	-0.082 (0.410)
Intercept	38.374*** (1.137)	12.074*** (0.700)	13.599*** (0.588)
Observations	81	81	81
R ²	0.024	0.113	0.001
Adjusted R ²	-0.014	0.078	-0.037
Residual Std. Error	10.3 (df = 78)	6.431 (df = 78)	5.323 (df = 78)
F Statistic	0.638 (df = 3; 78)	3.296** (df = 3; 78)	5.323*** (df = 3; 78)

FIGURES

Figure 3.1. Experimental timeline displaying the diet acclimation phase for all birds, blood sampling timepoints for flight-trained birds (Background, BG; Pre-flight, PF; After flight, AF; Recovery, RC) and the paired-in-time untrained birds (BG; RC), as well as when all three tissues were sampled (RC). For the flight-trained birds, the 4 days of pre-training are indicated by PT followed by the 15-day flight-training, FT, regime. The long-term effects of flight-training (gray shaded) 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. The acute effects of flight (red shaded) were assessed by comparing the change in plasma OXY and d-ROM at the AF and PF time points. Overall, the 20 cohorts were tested in groups of 4-5 birds every 3-5 days beginning on 9/23/2015 until the experiment ended on 12/14/2015, as shown in the bottom panel.

A) Plasma Oxidative Damage

B) Plasma Non-enzymatic Antioxidant Capacity

Figure 3.2. Changes in **A).** oxidative damage (d-ROMs) and **B).** non-enzymatic antioxidant capacity (OXY) in the plasma (means $\pm$ standard error) in relation to flight-training. Background (BG) levels of d-ROMs and OXY (solid horizontal line $\pm$ 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).

Figure 3.3. The change in **A).** oxidative damage (d-ROMs) and **B).** non-enzymatic antioxidant capacity (OXY) in the plasma (means $\pm$ standard error) (AF-PF timepoints, see Fig. 1) in relation to the energy consumed during the flight (kJ/min). The four diets are depicted with unique colors. Birds generally decreased (inverted triangle) d-ROMs and OXY during flight, although some birds slightly increased (triangle) d-ROMs and OXY during flight. 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. **C).** Change in OXY and Uric acid during an acute flight ($\pm$ standard deviation, dotted lines; AF-PF timepoints) filled by energy expenditure (purple-fill indicates energy expended was in the upper 50% range (>0.51 kJ/min) and open circles indicate energy expended was in the lower 50% range). Each individual is labeled with their change in d-ROMs during the flight.

Figure 3.4. A). Lipid hydroperoxide concentration and **B).** Oxygen radical absorbance capacity (ORAC) (means $\pm$ 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 peroxy scavenging capacities between flight-trained and untrained birds in the liver or pectoralis. Asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

SUPPORTING MATERIALS

Table S3.1. Linear mixed effect model results for longitudinal d-ROMs and OXY in the plasma in relation to flight-training and diet (dietary antioxidants and 18:2n-6 PUFA). Bird identification number and cohort number (1-5) were included as random effects. The intercept is the 13% 18:2n-6 PUFA, Antioxidant Supplemented, Untrained Group for the Background timepoint. The pairwise comparisons for the additional timepoints are reported using least square means. Data are reported as estimates (standard error) for each measure. The asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

<i>Covariate</i>	<i>Dependent variable:</i>	
	<i>d-ROMs $\text{mmol}^{-1} \text{H}_2\text{O}_2 \text{ equivalents}$</i>	<i>OXY $\text{mmol}^{-1} \text{HClO neutralized}$</i>
Training (Flight-trained)	-0.549 (1.412)	4.787 (7.725)
13% PUFA, Unsupplemented	1.361 (1.636)	13.445 (9.089)
32% PUFA, Unsupplemented	-1.91 (1.684)	3.738 (9.351)
32% PUFA, Supplemented	1.556 (1.635)	6.325 (9.076)
Timepoint, Pre-flight	5.072*** (1.667)	-54.139*** (8.901)
Timepoint, After-flight	-1.774 (1.677)	-76.397*** (8.957)
Timepoint, Recovery	-0.079 (1.321)	0.489 (7.037)
Intercept	38.377*** (1.645)	252.522*** (10.546)
Observations, df	278, 11	279, 11
Conditional R^2	0.135	0.381
<i>Least Square Means for Timepoint pairwise comparisons:</i>		
Pre-flight vs After-flight	6.866*** (1.774)	22.210** (9.532)
Pre-flight vs Recovery	5.156*** (1.664)	-54.628*** (8.937)
After-flight vs Recovery	-1.710 (1.675)	-76.838*** (8.993)

Table S3.2. Linear model results for Background and Recovery d-ROMs and OXY and the change in d-ROMs and OXY during Training (RC-BG) in the plasma in relation to flight-training and diet (dietary antioxidants and 18:2n-6 PUFA). Δ d-ROMs results are from a linear mixed effects model including Cohort as a random effect^a. The intercept is the 13% 18:2n-6 PUFA, Antioxidant Supplemented, Untrained Group. Data are reported as estimates (standard error) for each measure and the asterisks correspond to significance levels: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

<i>Dependent variable:</i>						
<i>Covariate</i>	<i>Timepoint: Background (BG)</i>		<i>Recovery (RC)</i>		<i>Δ During Training (RC-BG)</i>	
	d-ROMs <i>mmol⁻¹ H₂O₂</i> <i>equivalents</i>	OXY <i>mmol⁻¹ HClO</i> <i>neutralized</i>	d-ROMs <i>mmol⁻¹ H₂O₂</i> <i>equivalents</i>	OXY <i>mmol⁻¹ HClO</i> <i>neutralized</i>	d-ROMs ^a <i>mmol⁻¹ H₂O₂</i> <i>equivalents</i>	OXY <i>mmol⁻¹ HClO</i> <i>neutralized</i>
Training (Flight-trained)	2.314 (2.065)	-0.581 (9.825)	-3.457 (2.120)	10.906 (9.899)	-5.426** (2.680)	11.487 (11.699)
13% PUFA, Unsupplemented	4.262 (2.868)	13.107 (13.649)	2.611 (2.956)	22.336 (13.753)	-0.930 (3.743)	9.228 (16.253)
32% PUFA, Unsupplemented	1.772 (2.937)	4.331 (13.976)	-2.193 (3.027)	13.410 (14.082)	-3.297 (3.833)	9.079 (16.643)
32% PUFA, Supplemented	4.181 (2.868)	15.017 (13.649)	-1.321 (2.956)	20.003 (13.753)	-4.818 (3.739)	4.986 (16.253)
Intercept	34.510*** (2.340)	253.440*** (11.135)	40.430*** (2.457)	241.797*** (11.219)	5.129 (3.348)	-11.643 (13.259)
Observations	89	89	88	89	89	89
R ² ^b	0.049	0.019	0.062	0.0493	0.061	0.015
Adjusted R ² ^c	0.004	-0.027	0.017	0.005	0.105	-0.031
Residual Std. Error	9.727 (df = 85)	46.29 (df = 85)	9.912 (df = 84)	46.64 (df = 85)	- (df = 85)	55.12 (df = 85)
F Statistic	1.091 (df = 4; 85)	0.407 (df = 4; 85)	1.384 (df = 4; 84)	1.103 (df = 4; 85)	- (df = 4; 85)	0.342 (df = 4; 85)

^aResults for Δ d-ROMs are from a linear mixed effects model including Cohort as a random effect

^bMarginal R² is reported for Δ d-ROMs

^cConditional R² is reported for Δ d-ROMs

Table S3.3. Linear model results for Pre-flight and After-flight d-ROMs and OXY and the change in d-ROMs and OXY during an acute flight (AF-PF) in the plasma of flight-trained birds in relation to diet (dietary antioxidants and 18:2n-6 PUFA). Energy consumed (KJ/min) during flight is included only for the models for change in oxidative status during flight. The intercept is the 13% PUFA, Antioxidant Supplemented, Untrained Group. Data are reported as estimates (standard error) for each measure and the asterisks correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01.

Covariate	Dependent variable:					
	Timepoint: Pre-flight (PF)		After-flight (AF)		Δ During flight (AF-PF)	
	d-ROMs $\text{mmol}^{-1} \text{H}_2\text{O}_2$ equivalents	OXY $\text{mmol}^{-1} \text{HClO}$ neutralized	d-ROMs $\text{mmol}^{-1} \text{H}_2\text{O}_2$ equivalents	OXY $\text{mmol}^{-1} \text{HClO}$ neutralized	d-ROMs $\text{mmol}^{-1} \text{H}_2\text{O}_2$ equivalents	OXY $\text{mmol}^{-1} \text{HClO}$ neutralized
13% PUFA, Unsupplemented	0.487 (3.509)	-16.77 (23.06)	-5.687** (2.632)	11.584 (23.552)	-78.55* (40.36)	498.5* (284.4)
32% PUFA, Unsupplemented	-4.164 (3.665)	-34.62 (24.08)	-7.154** (2.758)	-5.285 (24.684)	-27.80 (41.51)	-380.5 (255.4)
32% PUFA, Supplemented	3.356 (3.509)	-27.69 (23.06)	-1.440 (2.622)	-19.080 (23.464)	-35.47 (26.28)	-235.8 (161.7)
Energy consumed (KJ/min)			-30.220* (14.977)	-419.989*** (134.036)	-52.40 (35.30)	-612.9*** (217.2)
Energy consumed x 13% PUFA, Unsupplemented					138.43* (75.19)	-880.1* (462.7)
Energy consumed x 32% PUFA, Unsupplemented	<i>Energy consumed not included in best fit model</i>		<i>Energy consumed x diet not included in best fit model</i>		46.10 (76.84)	756.1 (472.8)
Energy consumed x 32% PUFA, Supplemented					61.10 (76.84)	477.5 (305.0)
Intercept	43.146*** (2.482)	228.68*** (16.31)	56.053*** (8.299)	183.985*** (18.527)	23.80 (19.26)	281.9*** (118.5)
Observations	49	49	46	48	46	46
R ²	0.085	0.050	0.2554	0.014	0.120	0.383
Adjusted R ²	0.025	-0.012	0.1845	-0.052	-0.038	0.272
Residual Std. Error	8.947 (df = 46)	58.79 (df = 46)	6.441 (df = 42)	64.18 (df = 45)	9.452 (df = 39)	58.17 (df = 39)
F Statistic	1.415 (df = 3; 46)	0.8123 (df = 3; 46)	3.602** (df = 4; 42)	0.215 (df = 3; 45)	0.759 (df = 7; 39)	3.46*** (df = 7; 39)

Table S3.4. Linear model results for Lipid Hydroperoxide Concentration (LPO), and Peroxyl and Hydroxyl scavenging capacities in the liver and pectoralis in relation to flight-training and diet (dietary antioxidants and 18:2n-6 PUFA). Lipid damage in the liver and pectoralis results are from a linear mixed effects model including Cohort as a random effect^a. The intercept is the 13% 18:2n-6 PUFA, Antioxidant Supplemented, Untrained Group. Data are reported as estimates (standard error) for each measure and the asterisks correspond to significance levels: *p<0.1, **p<0.05, ***p<0.01.

<i>Covariate</i>	<i>Dependent variable:</i>					
	<i>Tissue:</i>					
	<i>Liver</i>			<i>Pectoralis</i>		
	Lipid [Hydroperoxide] μM ^a	Peroxyl <i>a.u.g⁻¹x10⁻⁶</i>	Hydroxyl <i>a.u.g⁻¹x10⁻⁴</i>	Lipid [Hydroperoxide] μM ^a	Peroxyl <i>a.u.g⁻¹x10⁻⁵</i>	Hydroxyl <i>a.u.g⁻¹x10⁻⁴</i>
Training (Flight-trained)	-4.342*** (1.253)	-1.131 (0.754)	-2.498 (3.356)	-1.625 (1.000)	2.046 (2.994)	-6.499* (3.352)
13% PUFA, Unsupplemented	-0.115 (1.752)	-8.067 (1.055)	-8.762* (4.700)	-2.4610* (1.417)	-6.509 (4.289)	-1.206 (4.802)
32% PUFA, Unsupplemented	0.401 (1.736)	-1.491 (1.046)	-2.279 (4.657)	-0.703 (1.404)	-4.742 (4.204)	-5.999 (4.707)
32% PUFA, Supplemented	1.620 (1.772)	-1.068 (1.067)	-6.002 (4.753)	-2.446* (1.402)	-1.124 (4.200)	0.223 (4.703)
Intercept	14.111*** (1.417)	9.577*** (0.843)	36.676** (3.756)	16.097*** (1.129)	25.374*** (3.410)	50.063*** (3.818)
Observations	93	92	92	95	96	96
R ^{2 b}	0.126	0.046	0.052	0.074	0.037	0.060
Adjusted R ^{2 c}	0.133	0.002	0.008	0.074	-0.005	0.019
Residual Std. Error	- (df = 89)	3.616 (df = 88)	16.1 (df = 88)	- (df = 91)	14.7 (df = 92)	16.45 (df = 92)
F Statistic	- (df = 4; 89)	1.052 (df = 4; 88)	1.194 (df = 4; 88)	- (df = 4; 91)	0.883 (df = 4; 92)	1.455 (df = 4; 92)

^aResults for liver and pectoralis lipid hydroperoxide concentration are from a linear mixed effects model including Cohort as a random effect

^bMarginal R² is reported for lipid hydroperoxide concentration in the liver and pectoralis

^cConditional R² is reported for lipid hydroperoxide concentration in the liver and pectoralis

Table S3.5. The top 3 PCs and their loadings from the principle component analysis (PCA) that was conducted to create antioxidant status indices by incorporating the 5 antioxidant capacity measures in this study with the 18 antioxidant measures that we characterized for each individual starling at final sampling published previously^a. *Note:* The loadings for the antioxidant measures with the largest magnitude in each PC are bolded.

		<i>PC Loadings for the Top 3 Antioxidant Indices</i>		
<i>Antioxidant measures included:</i>		<i>PC1: High tissue antioxidant gene expression</i>	<i>PC2: High liver enzymatic antioxidant capacity</i>	<i>PC3: Low liver antioxidant enzyme activities</i>
<i>Liver Enzyme activities^a</i>	Glutathione Peroxidase (GPx)	-0.06201	0.266350228	-0.45151
	Superoxide Dismutase (SOD)	-0.0835	0.134996581	-0.49184
	Catalase (CAT)	-0.09053	0.278047963	-0.40979
<i>Pectoralis Enzyme Activities^a</i>	Glutathione Peroxidase (GPx)	-0.07653	0.028327208	-0.18831
	Superoxide Dismutase (SOD)	-0.04943	0.138192735	0.133663
	Catalase (CAT)	-0.10824	-0.045406058	-0.06308
<i>Liver Enzyme Gene Expression^a</i>	Transcription Factor NRF2	0.221526	0.136032141	-0.11722
	GPX1	0.269857	0.359297803	0.091007
	GPX4	0.244158	0.362354903	0.065794
	SOD1	0.24392	0.104708484	0.1504
	SOD2	0.292647	0.340861613	0.121187
	CAT	0.223865	0.332322403	0.155727
	Transcription Factor NRF2	0.24476	-0.158187772	-0.22411
<i>Pectoralis Enzyme Gene Expression^a</i>	GPX1	0.303344	-0.180966928	-0.12414
	GPX4	0.328466	-0.276833129	-0.14548
	SOD1	0.295248	-0.228921804	-0.01562
	SOD2	0.331171	-0.205415657	-0.14675
	CAT	0.320647	-0.171068728	-0.1401
<i>Liver ORAC</i>	Peroxyl Scavenging Capacity	-0.11379	-0.089168516	0.068996
	Hydroxyl Scavenging Capacity	0.012707	0.031178232	-0.29396
<i>Pectoralis ORAC</i>	Peroxyl Scavenging Capacity	-0.02252	0.10268473	0.015437
	Hydroxyl Scavenging Capacity	-0.09313	-0.120364066	-0.00709
<i>Plasma OXY</i>	Non-enzymatic Antioxidant Capacity (OXY)	0.04941636	-0.053319565	0.146337

^aThese measures are published previously (citation redacted for initial review)

Table S3.6. Pearson's correlation matrix of the antioxidant measures and oxidative damage measures reported in this study in relation to energy consumption during flight and flight duration (minutes).

	OXY during training	d-ROMs during training	Flight duration	Energy per min	Liver hydroxyl	Liver peroxyl	Liver LPO	Pectoralis hydroxyl	Pectoralis peroxyl	Pectoralis LPO	OXY at Recovery	d-ROMs at Recovery
OXY during training	1.0	0.39	-0.03	0.07	-0.16	-0.19	0.04	-0.01	0.01	0.002	0.63	0.17
d-ROMs during training	0.39	1.0	0.12	-0.07	-0.06	-0.12	0.04	-0.20	-0.05	0.05	0.10	0.59
Flight duration	-0.28	0.12	1.0	-0.34	-0.07	0.05	-0.16	0.09	0.12	0.18	-0.13	0.13
Energy per min	0.07	-0.07	-0.34	1.0	-0.13	-0.12	0.14	-0.02	-0.25	0.08	0.04	-0.13
Liver hydroxyl	-0.16	-0.06	0.07	-0.13	1.0	0.11	0.14	0.05	-0.09	0.18	-0.29	-0.05
Liver peroxyl	-0.18	-0.12	0.05	-0.12	0.11	1.0	0.04	0.10	0.04	-0.23	-0.15	0.08
Liver LPO	0.04	0.04	-0.16	0.14	0.14	0.04	1.0	0.05	0.05	-0.01	-0.04	-0.006
Pectoralis hydroxyl	-0.009	-0.20	0.09	-0.02	0.05	0.10	0.05	1.0	0.14	-0.29	-0.08	-0.04
Pectoralis peroxyl	0.01	-0.05	0.12	-0.25	-0.09	0.04	0.05	0.14	1.0	0.07	-0.002	0.10
Pectoralis LPO	0.002	0.05	0.18	0.08	0.18	-0.23	-0.01	-0.29	0.07	1.0	-0.02	-0.01
OXY at Recovery	0.63	0.10	-0.14	0.04	-0.29	-0.15	-0.04	-0.08	-0.002	-0.02	1.0	-0.14
d-ROMs at Recovery	0.17	0.59	0.13	-0.13	-0.05	0.08	-0.006	-0.04	0.10	-0.01	-0.14	1.0

Figure S3.1. An alternative display of Figure 3. The change in **A).** oxidative damage (d-ROMs) and **B).** non-enzymatic antioxidant capacity (OXY) in the plasma (means $\pm$ standard error) (AF-PF timepoints, see Fig. 1) related to the energy consumed during the flight (KJ/min). The four diets are depicted with unique colors. 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.