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HOW BIRDS RESPOND TO ANTIOXIDANT CAPACITY, OXIDATIVE DAMAGE, AND FUEL STORES DURING MIGRATION

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HOW BIRDS RESPOND TO ANTIOXIDANT CAPACITY,
OXIDATIVE DAMAGE, AND FUEL STORES DURING
MIGRATION

BY
CLARA COOPER-MULLIN

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DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

During migration, birds have an elevated metabolic rate and rely heavily on fat for fuel, creating a state where oxidative stress may be high if not counterbalanced by antioxidants. However, no previous studies have examined how birds utilize different aspects of the antioxidant system to combat oxidative damage during flight training, whether dietary antioxidants are absorbed and transported to the site of reactive species generation, the mitochondria, or how oxidative status interacts with fuel stores to shape decisions birds make during migratory stopovers. In this dissertation, I first introduce the physiological challenges of increased production of reactive species confronted by birds during migration, and the interacting roles of antioxidants in protecting birds from oxidative damage (Chapter 1). Next, I examine how a bird's multifaceted antioxidant system (enzymatic, nonenzymatic, and dietary) responds to increased oxidative demands associated with flight across time (Chapters 2 and 3). Finally, I examine how fat stores and oxidative status influence stopover behaviors in wild songbirds during fall migration.

To investigate the acute and chronic effects of an energy-intensive activity (flight) on redox homeostasis (Chapter 2), I drew blood samples from Zebra Finches (*Taeniopygia guttata*) exposed to an energy-intensive activity (60 min of perch-to-perch flights twice a day) on the first day of flight, after 13 days of training and after 44 days of training. Although well documented in mammals, this is the first study to examine how energy-intensive training affects the antioxidant system and damage by reactive species in flight-trained birds over many weeks. I measured multiple components of the antioxidant system: an enzymatic antioxidant (glutathione

peroxidase, GPx) and non-enzymatic antioxidants (measured by the OXY-adsorbent test) as well as a measure of oxidative damage (d-ROMs). At no point during the experiment did oxidative damage change. I discovered that exposure to energy-intensive exercise training did not alter baseline levels of GPx (flight day, $F_{2, 15} = 2.42$, $P = 0.122$; training group, $F_{1, 15} = 0.02$, $P = 0.97$; Interaction, $F_{2, 15} = 1.63$, $P = 0.227$), but induced exercise-trained birds to maintain a higher non-enzymatic antioxidant status as compared with untrained birds (untrained, $F_{2, 25} = 5.51$, $P = 0.010$; training group, $F_{1, 18} = 0.01$, $P = 0.893$; Interaction, $F_{2, 25} = 4.26$, $P = 0.026$). Novel intense exercise (Day 1) increased the enzymatic but not the non-enzymatic antioxidant system relative to baseline. GPx activity was elevated above baseline in trained birds immediately after completion of the second one-hour flight on each of the three sampling days (flight day, $F_{2, 13} = 0.54$, $P = 0.593$; training group, $F_{1, 15} = 23.17$, $P = 0.002$; Interaction, $F_{2, 13} = 3.05$, $P = 0.082$), and non-enzymatic antioxidants were acutely depleted during flight after 13 and 44 days of training (flight day, $F_{2, 13} = 1.27$, $P = 0.300$; training group, $F_{1, 15} = 4.36$, $P = 0.028$; Interaction, $F_{2, 13} = 4.15$, $P = 0.041$). The primary effect of exercise training on the acute response of the antioxidant system to 2-hr flights was a more coordinated response between the enzymatic (GPx) and non-enzymatic components of the antioxidant system of birds and this reduced the oxidative damage associated with exercise.

To better understand the bioavailability of dietary antioxidants for birds exposed to the energetic and oxidative demands of flight, I gavaged a subset of Zebra Finches with deuterated α -tocopherol (Chapter 3). I show for the first time that an ingested lipophilic antioxidant, α -tocopherol, reached the mitochondria in the flight

muscles of a songbird and that its bioavailability depended on exercise. I also examined the time course over which deuterated α -tocopherol appeared in the blood and mitochondria isolated from pectoral muscle of Zebra Finches exposed to 60 min of perch-to-perch flights two times in a day, or that were relatively sedentary in cages. Deuterated α -tocopherol was found in the blood of Zebra Finches within 6.5 hrs ($R^2 = 0.83$, $F_{5,15} = 19.74$, $P < 0.001$) and in isolated mitochondria within 22.5 hrs ($R^2 = 0.77$, $F_{7,10} = 4.77$, $P = 0.01$), but only if the birds were exercise-trained. These results indicate that exercise affected the timecourse and facilitated the absorption and deposition of vitamin E to tissues and organelles.

To examine how antioxidants interact with fat stores to influence stopover decisions (how long to stay on stopover and which direction to leave) during fall migration, I conducted a field experiment and manipulated the condition (fat stores and antioxidant capacity) of wild Hermit Thrush, Yellow-rumped Warblers, Red-eyed Vireos, and Blackpoll Warblers on Block Island, Rhode Island (41°130N, 71°330W; Chapter 4). I tested the hypothesis that birds with greater fuel stores and antioxidant capacity have shorter stopovers and depart in a seasonally appropriate direction compared to lean birds with low antioxidant capacity. I used a 2 X 2 factorial experiment (high, *ad libitum*, or low, maintenance, food availability; dietary anthocyanins or no anthocyanins) in four species of birds that differed in migration strategy: Yellow-rumped Warblers (Myrtle subspecies, *Setophaga coronata coronata*), Hermit Thrushes (*Catharus guttatus*), Red-eyed Vireos (*Vireo olivaceus*), and Blackpoll Warblers (*Setophaga striata*). Prior to release, I attached digitally coded VHF transmitters to assess stopover duration and direction of departure from the

stopover site using the Motus network. Non-enzymatic antioxidant capacity increased during the captive refueling period for Hermit Thrushes, Red-eyed Vireos, and Blackpoll Warblers fed *ad lib* diets ($F_{1,2} = 6.79$, $P = 0.01$; $F_{1,2} = 10.63$, $P = 0.03$; $F_{1,2} = 13.60$, $P = 0.004$, respectively). Oxidative damage decreased in plasma from Red-eyed Vireos and Hermit Thrushes across all treatment groups during captivity ($F_{1,2} = 1.15$; $P = 0.31$; $F_{1,3} = 1.12$, $P = 0.30$), but increased in the plasma of Blackpoll Warblers fed an *ad lib* diet ($F_{1,2} = 104.90$; $P = 0.009$). No measure of oxidative capacity changed during captivity in Yellow-rumped Warblers ($F_{1,3} = 3.21$, $P = 0.89$). Stopover duration was shorter for Hermit Thrushes, Red-eyed Vireos and Blackpoll Warblers fed *ad lib* as compared to maintenance food ($F_{1,3} = 7.52$, $P = 0.01$; $F_{1,2} = 2.96$, $P = 0.09$; $F_{1,2} = 18.01$, $P = 0.004$ respectively), but not for Yellow-rumped Warblers ($F = 0.69$, $P = 0.41$). Departure direction was not related to fuel stores or antioxidant condition, and birds from all species primarily reoriented north when departing Block Island. This is the first study to show the importance of antioxidant capacity as a physiological cue for migratory behavior, and the results highlight the influence of fat stores and antioxidant capacity on the timing of migratory stopovers.

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DEDICATION

This thesis is dedicated to the memory of:

John Fulham Cooper-Mullin

The best dad and ultimate inspiration for always wanting to be outside

PREFACE

The following dissertation appears in Manuscript Format, with chapters prepared in accordance with the formatting guidelines of each respective target publication. Two of the four chapters have been published in peer-reviewed journals, one has been submitted for review, and the last will be submitted. Chapter 1 is an invited review paper published by the *Journal of Experimental Biology* in November, 2016 (volume 219, issue 23, pages 3684-3695; doi: 10.1242/jeb.123992). This paper reviews what was currently known about the role of the antioxidant system during exercise and, in particular, for migrating birds. Chapter 2 was published online by the *Journal of Experimental Biology* in October, 2019 (volume 222, pages jeb.210443; doi: 10.1242/jeb.210443). This paper summarizes the results of a captive experiment on the effect of exercise training (flight) on enzymatic and non-enzymatic aspects of the antioxidant system in Zebra Finches. Chapter 3 is submitted for peer-review at *Biology Letters* and reports the results of an experiment examining how exercise influences the availability (absorption and deposition into muscle mitochondria) of a labeled dietary antioxidant. Chapter 4 is formatted to be submitted to *The Condor: Ornithological Applications* and describes the how manipulating fat and antioxidant capacity affects stopover duration and departure decisions in Blackpoll Warblers, Red-eyed Vireos, Hermit Thrush, and Yellow-rumped Warblers.

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CHAPTER 1

The role of the antioxidant system during intense endurance exercise: lessons from migrating birds

by

Clara Cooper-Mullin and Scott R. McWilliams

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Key Words: Fat oxidation, Bird migration, Reactive species

Summary Statement: We describe the physiological challenge of increased production of reactive species confronted by birds during migration, and the interacting roles of antioxidants in protecting birds from oxidative damage.

Abstract

During migration, birds substantially increase their metabolic rate and burn fats as fuel and yet somehow avoid succumbing to overwhelming oxidative damage. The physiological means by which vertebrates such as migrating birds can counteract an increased production of reactive species (RS) are rather limited: they can upregulate their endogenous antioxidant system and/or consume dietary antioxidants (prophylactically or therapeutically). Thus, birds can alter different components of their antioxidant system to respond to the demands of long-duration flights, but much remains to be discovered about the complexities of RS production and antioxidant protection throughout migration. Here, we use bird migration as an example to discuss how RS are produced during endurance exercise and how the complex antioxidant system can protect against cellular damage caused by RS. Understanding how a bird's antioxidant system responds during migration can lend insights into how antioxidants protect birds during other life-history stages when metabolic rate may be high, and how antioxidants protect other vertebrates from oxidative damage during endurance exercise.

Introduction

Migratory birds fly hundreds to thousands of kilometers to escape areas of decreasing resources and poor conditions, and return to reproduce when resources are plentiful (Rappole, 2013). Although some extraordinary bird species can make their migratory journey in one flight, most migrations involve relatively short periods of endurance flight interspersed with longer periods of hyperphagia and fat deposition at stopover sites (Lupi et al., 2016; Moore, 2000; Rappole and Warner, 1976; Wikelski et al., 2003). During migratory flights, birds are fasting, have an elevated metabolic rate and must store and burn fat for fuel, creating a state where the level of reactive species (RS) production and oxidative challenges may be high (Costantini, 2014; Jenni-Eiermann et al., 2014; McWilliams et al., 2004; Skrip et al., 2016; Weber, 2009). RS are pro-oxidant molecules that can cause considerable cellular damage (Halliwell and Gutteridge, 2007), which could limit a bird's ability to successfully fly long distances.

Indeed, regular aerobic metabolism in all organisms generates an impressive variety of RS and free radicals (defined as molecular species with one unpaired electron). RS are produced primarily in the mitochondria during respiration in metabolic reactions with oxygen (producing reactive oxygen species, ROS) and, less commonly, with nitrogen (producing reactive nitrogen species, RNS) (Fig. 1; Brand et al., 2004; Halliwell and Gutteridge, 2007). In cells, the primary RS generated by the electron transport chain, immune responses and/or cytochrome P450s are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\bullet}$) (Brand et al., 2004; Halliwell and Gutteridge, 2007; Imlay, 2003; Murphy, 2009). These molecules can readily react with lipids, proteins, DNA and each other, leading to the production of

additional RS and damage to cells. For example, although H₂O₂ is relatively unreactive, in the presence of ferrous ions it forms the highly reactive hydroxyl radical (Fig. 1) that plays a large role in lipid peroxidation cascades (Brand et al., 2004; Hulbert et al., 2007). The production of RS can lead to the accumulation of cellular damage, unless it can be counterbalanced by antioxidants that act to quench RS and prevent the oxidation of other important biological molecules (Fig. 2).

Animals have a multifaceted antioxidant system made up of endogenous antioxidants, micromolecular sacrificial molecules and dietary antioxidants (see Glossary; Fig. 2) that work synergistically to protect against oxidative damage (Alan et al., 2013; Costantini, 2008; Costantini et al., 2007, 2008, 2011; Jenni-Eiermann et al., 2014; Skrip and McWilliams, 2016). For birds in migration, the relationship between RS production, antioxidant protection and oxidative damage is not straightforward, and various aspects of the antioxidant system may respond differently depending on the type of damage, the duration of flight or the physiological state of the bird (Cohen and McGraw, 2009; Costantini et al., 2007; Jenni-Eiermann et al., 2014; Skrip et al., 2015).

Regulating oxidative balance is important for all aerobically respiring organisms as it may affect a variety of demanding life-history stages, such as reproduction, migration, thermal regulation or lactation (e.g. Beaulieu et al., 2011, 2014; Jenni-Eiermann et al., 2014; Speakman, 2008; Tsahar et al., 2006). The regulation of oxidative damage may act as an underlying driver of aging or longevity (Montgomery et al., 2012; Selman et al., 2012). Other reviews have focused on the regulation of RS production from an evolutionary perspective (Costantini, 2008, 2014;

Costantini et al., 2010b; Monaghan et al., 2009; Speakman, 2008; Speakman et al., 2015; Williams et al., 2010), or on the role of RS in conservation physiology (Beaulieu and Costantini, 2014; Isaksson, 2010), in signaling (Garratt and Brooks, 2012) and as an important indicator of bird health for field ornithologists (Hutton and McGraw, 2016; Skrip and McWilliams, 2016), or on the importance of dietary antioxidants for wild animals (e.g. Beaulieu and Schaefer, 2013; Catoni et al., 2008b). Here, we aim to summarize for physiological ecologists how organisms maintain oxidative balance during exercise, using bird migration as an ecologically relevant form of endurance exercise. Thus, we will focus on how RS are produced during migratory flights, and how birds use key aspects of their multifaceted antioxidant system to protect themselves against damage during flights and at stopover sites. We also consider whether there is evidence for apparent trade-offs in the use of the endogenous antioxidant system or dietary antioxidants. Given that altered physiology and behavior during spring and autumn migration can have consequences that spill over into the non-migratory season (Finch et al., 2014; Legagneux et al., 2012; Skrip et al., 2016), we also discuss how RS production and the antioxidant system response during migration may have overarching fitness consequences for migratory birds, and the implications for other organisms that undergo periods of intense endurance exercise. For physiological ecologists, this Review can serve as an easily digestible summary of RS production and antioxidant defense in birds and other organisms, especially in the context of very demanding periods of the annual cycle.

RS production, oxidative damage and antioxidant capacity: achieving balance

Conventionally, the term ‘oxidative stress’ is used to describe the situation where the amount of RS produced overwhelms the antioxidant capacity of the organism (Halliwell and Gutteridge, 2007); the oxidative status of an organism is determined by the balance of RS produced, the quenching of RS by the antioxidant system and the damage caused by unquenched RS (Costantini, 2014; Halliwell and Gutteridge, 2007). Unfortunately, directly measuring RS production in whole organisms is not yet feasible (Cohen and McGraw, 2009; Cohen et al., 2007; Costantini and Verhulst, 2009; Skrip and McWilliams, 2016), although there are a variety of indirect measures of RS production that can be useful (Abuja and Albertini, 2001; Meitern et al., 2013; Miller et al., 1993; Monaghan et al., 2009). Instead, most studies examine the damage caused by RS (e.g. products of lipid peroxidation or protein oxidation) and probe various aspects of the antioxidant system (Abuja and Albertini, 2001; Alan and McWilliams, 2013; Costantini, 2014; Costantini et al., 2007; Dalle-Donne et al., 2003; Jenni-Eiermann et al., 2014; Prior and Cao, 1999; Skrip et al., 2015). Interpreting oxidative damage and antioxidant capacity without direct measures of RS production can be complicated, because if antioxidant levels are adequately high, they may limit damage even when RS production is high (Fig. 2; Cohen and McGraw, 2009; Costantini and Verhulst, 2009).

Metabolic Rate and RS Production

Because RS are a primary product of aerobic metabolism, it is often assumed that species with higher metabolic rates have a higher rate of RS production. It is thus

relevant to ask whether RS production changes with aerobic metabolism. The majority of studies testing this relationship in wild animals have focused on whether species with high basal metabolic rates (BMRs; see Glossary) have a correspondingly higher RS production relative to species with lower BMRs, and we evaluate this evidence below. Far fewer studies in ecology have examined how short-term increases in metabolic rate (such as those observed during exercise) change RS production within an individual. The metabolic rate of birds is high during migratory flights (Bishop and Butler, 2015; Hedenström et al., 2009), which could lead to an increase in RS production and an increase in damage to cells and tissues (Costantini et al., 2008; Jenni-Eiermann et al., 2014; Skrip et al., 2015).

Among Species Differences in BMR

This Review focuses mainly on how the antioxidant and oxidative status of birds responds to short-term changes in metabolic rate during exercise (such as that associated with migratory flights), but most studies that examine the link between RS production and metabolic rate assess differences among species, and often focus on differences in lifespan and/or BMR (Abele et al., 2008; Barja, 1998; Hulbert et al., 2007; Jimenez et al., 2013; McGraw, 2011; Wiersma et al., 2007). Such studies have revealed differences in RS physiology among birds and non-flying mammals. Surprisingly, birds have higher BMRs, yet lower RS production in isolated mitochondria and lower free radical leak (see Glossary) than non-flying mammals (Barja, 2007; Costantini, 2008; Hulbert et al., 2007; Ku and Sohal, 1993; Perez-Campo et al., 1998). In addition, the cell membranes of birds are composed of fatty

acids that are more resistant to lipid peroxidation than those of mammals, and birds show lower overall lipid peroxidation rates in tissues (Hulbert and Else, 1999; Hulbert et al., 2007). Most mammals primarily use carbohydrates as fuel during exercise, whereas birds primarily burn fats to fuel flight, and this reliance on fatty acid oxidation results in relatively lower RS production in exercising birds than in mammals (Kuzmiak et al., 2012; Montgomery et al., 2012; Weber, 2009). Although burning fat may result in lower RS production in muscle mitochondria, stored fat is highly susceptible to lipid peroxidation, and, during migration, birds must build and store large amounts of fat (Costantini, 2014; Costantini et al., 2007; Pierce and McWilliams, 2014; Skrip et al., 2015). Thus, the amount of RS produced during exercise may be different if measured in a bird as opposed to a non-flying mammal, and birds may be more susceptible to RS damage via lipid peroxidation of stored fats. Further information on the interspecific relationship between BMR, lifespan and RS production can be found in other thorough reviews (Barja, 2007; Brand et al., 2004; Cohen et al., 2008; Costantini et al., 2010b; Hulbert and Else, 2000; Hulbert et al., 2007; Jimenez et al., 2014; Munshi-South and Wilkinson, 2010; Perez-Campo et al., 1998; Selman et al., 2012).

Short-term effects of exercise

We now focus on how short-term changes in metabolic rate during endurance exercise, such as that associated with migratory flight, directly affect RS production. Metabolic rate during flapping flight is as much as 30× BMR (Hedenström et al., 2009), and this short-term increase in metabolism associated with flight affects RS

production and the response of the antioxidant system (Costantini et al., 2008; Jenni-Eiermann et al., 2014). Interestingly, RS production does not vary proportionally to the rate of oxygen consumption during mitochondrial respiration, most likely due to the action of uncoupling proteins (UCPs). UCPs are part of a superfamily of anion carriers that are located in the inner mitochondrial membrane where oxidative phosphorylation and ATP production occur (Criscuolo et al., 2005). These proteins allow protons to cross the inner mitochondrial membrane without producing ATP, and are thought to be important for non-shivering thermogenesis and/or the modulation of RS production in the mitochondria. Because UCPs act to uncouple the electron transport chain, and a 10 mV decrease in mitochondrial membrane potential is associated with a 70% decrease in superoxide production, it follows that UCPs may regulate RS production in the mitochondria (Brand et al., 2004; Hulbert et al., 2007; Miwa et al., 2003). While UCPs have been discovered and studied widely in mammals, the avian homolog of mammalian UCPs has only been examined in turkeys, poultry, ducks, quails, zebra finches and king penguins, and, to our knowledge, never in the context of endurance flight (Criscuolo et al., 2005).

Although UCPs may reduce RS production (Barja, 2007), birds are working at or near maximal metabolic rates during migration, which is potentially associated with a higher production of RS (Bishop and Butler, 2015; Engel et al., 2006; Jenni-Eiermann et al., 2002; Schmidt-Wellenburg et al., 2007). Several studies have demonstrated an increase in damage to lipids and proteins during the migratory season that is consistent with the effects of unquenched RS (Table 1). Protein oxidative damage was high in red blood cells from European robins (*Erithacus rubecula*) during a long-distance

migratory flight, as compared with resting individuals (Jenni-Eiermann et al., 2014). Levels of hydroperoxides – indicators of oxidative damage caused by peroxidation of lipids, proteins and DNA – were 54% higher in the plasma of homing pigeons (*Columba livia*) after flights of 60–200/km (Costantini et al., 2008) and were higher in plasma from garden warblers (*Sylvia borin*) newly arrived at stopover sites than in those that had rested for up to 8/h (Skrip et al., 2015). Overall, these studies indicate that migratory flights cause oxidative damage to the cells and tissues of birds (Table 1). However, the non-enzymatic antioxidant capacity and lipid oxidative damage in serum from Northern bald ibis (*Geronticus eremita*) trained to follow an ultra-light aircraft during migration did not change before or after flights (Bairlein et al., 2015).

One reason to explain why migration is not always associated with increased levels of oxidative damage is that the amount of damage caused by RS during migration is closely tied to the condition of an individual bird. For instance, prior to migratory flight in the autumn, non-enzymatic antioxidant capacity in plasma (discussed below) was positively correlated with fat stores in blackpoll warblers (*Setophaga striata*) and red-eyed vireos (*Vireo olivaceus*) (Skrip et al., 2015). Therefore, these birds may concomitantly build antioxidant capacity along with fat stores, perhaps to protect against damage to fats, or against an increase in RS production during the next migratory flight (Skrip et al., 2015). Directly after an endurance flight, fat stores in the garden warbler did not correlate with antioxidant capacity in plasma, but did positively correlate with lipid oxidative damage in plasma (Costantini et al., 2007). These results suggest that lipid oxidation may be an ‘inescapable hazard’ of using fat to fuel migratory flights (Skrip et al., 2015). Because

accumulation of RS damage can lead to cell senescence, a decline in organ viability and even death, an efficient antioxidant system to protect against damage is crucial for birds in migration, and for other animals undergoing endurance exercise.

The role of the endogenous antioxidant system

Because RS are produced in the mitochondria, the ‘endogenous’ antioxidant system (which includes antioxidants directly produced or available within cells) is a particularly important component of the antioxidant defense system for migrating birds (Fig. 2; Brigelius-Flohé, 1999; Halliwell and Gutteridge, 2007). Endogenous antioxidants can be in the form of antioxidant enzymes or other non-enzymatic sacrificial molecules that interact with and quench RS (Fig. 2). These molecules and enzymes work by preferentially oxidizing RS that would otherwise damage essential molecules in a cell. During migration, the circulating concentrations of endogenous antioxidants may be upregulated when RS production is high (Cohen et al., 2014; Costantini et al., 2008; Jenni-Eiermann et al., 2014; Tsahar et al., 2006). We provide more information on components of the endogenous antioxidant system below.

Sacrificial molecules

By definition, RS are molecules with one unpaired electron, and sacrificial antioxidant molecules, such as glutathione (GSH), bilirubin, albumin or uric acid, generally serve to donate an electron to RS to prevent oxidation of other important biological molecules (Fig. 2). Birds and mammals are able to synthesize these antioxidants, which act as nucleophiles for RS. GSH is present at relatively high

concentrations in cells, and directly acts to neutralize H_2O_2 and hydroperoxides, which are abundant RS in migrating birds (Costantini, 2014; Deponce, 2013; Halliwell and Gutteridge, 2007; Kamiński et al., 2009; Margis et al., 2008). Unlike other reactions between sacrificial molecules and RS, the GSH reaction is catalyzed by the antioxidant enzyme glutathione peroxidase (GPx, see below), which means that GSH and GPx must be present at high concentrations for GSH to be effective (Fig. 2). Further, the oxidized form of GSH, glutathione disulfide (GSSG), can be recycled back to GSH by the enzyme glutathione reductase (Halliwell and Gutteridge, 2007; Upton et al., 2009). Other sacrificial molecules that can be enzymatically recycled include bilirubin in mammals or biliverdin in birds. Both have strong antioxidant properties (McDonagh, 2001). Bilirubin is preferentially oxidized to biliverdin by RS (Stocker et al., 1987); once oxidized, biliverdin is rapidly recycled to bilirubin by biliverdin reductase (Sedlak et al., 2009). The bilirubin- and GSH-based antioxidant systems play complementary roles: bilirubin acts preferentially to protect against lipid peroxidation, and GSH acts preferentially to protect against the oxidation of water-soluble proteins (Sedlak et al., 2009).

Although fat is the primary source of fuel during migration, birds burn some protein during flight for energy and to provide water to prevent dehydration (Gerson and Guglielmo, 2013). Uric acid is the main form of nitrogenous waste in birds, and its production increases during protein catabolism (Alan and McWilliams, 2013; Cohen et al., 2014; Gerson and Guglielmo, 2013; Rokitzki et al., 1994; Simoyi et al., 2003). Uric acid is a powerful antioxidant that is able to scavenge RS and to chelate RS-producing metal ions (Halliwell and Gutteridge, 2007). When uric acid interacts

with RS, it is oxidized to allantoin (Fig. 2), which can be measured in the circulation (Tsahar et al., 2006). As such, uric acid largely determines the total antioxidant capacity of serum (Cohen and McGraw, 2009), although non-enzymatic antioxidant capacity measured by the OXY adsorbent test does not include uric acid (Beaulieu et al., 2011). Measuring uric acid in addition to performing the OXY adsorbent test therefore provides a means to determine the importance of uric acid relative to other non-enzymatic antioxidant defenses. Birds lack the enzyme urate oxidase that oxidizes uric acid to allantoin in other vertebrates, so any allantoin in the avian circulatory system is generally considered to be a product of neutralizing RS (Tsahar et al., 2006). It has been found that non-enzymatic antioxidant capacity, but not lipid peroxidation, is higher after re-feeding following fasting in captive northern wheatears (*Oenanthe oenanthe*), and this was mostly explained by an increase in circulating uric acid produced during protein metabolism (Eikenaar et al., 2016). This experiment highlights the impact of uric acid on antioxidant capacity in birds undergoing hyperphagia before migration and at stopover sites. Thus, birds produce an antioxidant as a byproduct of protein catabolism during migration, and metabolic markers such as allantoin are available that can indicate the extent to which this antioxidant is used as a part of the antioxidant system.

Endogenous Enzymes

Three main groups of endogenous antioxidant enzymes – superoxide dismutases (SODs), GPxs and catalase (CAT) – have been investigated in birds. These enzymes operate in different cells and tissues to quench RS, and to stop the propagation of

damage by H₂O₂. It is important to examine the subcellular and tissue-specific locations of these enzymes in order to more fully understand how birds alter enzyme concentrations when RS production is elevated during migration.

SODs are a group of enzymatic antioxidants that accelerate the dismutation of superoxide to H₂O₂ (Halliwell and Gutteridge, 2007). H₂O₂ can then be converted to oxygen and water by GPx or CAT. There are three types of SODs (Fig. 1); in birds, MnSOD is localized in the mitochondria, CuZnSOD is found in the cytosol, blood, lysosomes, nucleus and between the inner and outer mitochondrial membrane, and SOD3 is plasma specific (Halliwell and Gutteridge, 2007; Oropesa et al., 2013; Smith et al., 2011).

GPxs are a large family of enzymes that catalyze the reduction of H₂O₂ to water using GSH as the electron donor (Fig. 1; Halliwell and Gutteridge, 2007). Several important GPxs that act as antioxidant enzymes are selenoproteins, and have a selenocystine (Sec) residue at their active site (Johansson et al., 2005). Four major selenium-dependent GPxs have been identified in mammals and birds in different tissues, and in distinct subcellular locations (Gibson et al., 2014; Johansson et al., 2005; Kong et al., 2003; Margis et al., 2008). GPx1 is found in red blood cells, the liver, lung and kidney and is restricted to the cytosol, nucleus and mitochondria. GPx2 is found in the gastrointestinal tract, confined to the cytosol and nucleus of cells. GPx3 is found in plasma, kidneys, lungs, epididymis, vas deferens, placenta, seminal vesicles, heart and muscle, and is found in the cytosol or is secreted into the plasma. GPx4, otherwise known as phospholipid GPx, is broadly distributed across tissues, and is found in the nucleus, cytosol and mitochondria, as well as existing in a

membrane-bound form (Kong et al., 2003; Margis et al., 2008). Although these enzymes are located in different tissues or subcellular locations, they all catalyze the same reaction, using the sacrificial molecule GSH as a substrate (Brigelius-Flohé, 1999; Halliwell and Gutteridge, 2007; Johansson et al., 2005; Margis et al., 2008; Martensson et al., 1990). GPxs are the most ubiquitous antioxidant enzymes across the body (Halliwell and Gutteridge, 2007; Margis et al., 2008), indicating that they may be the most important antioxidant enzymes when RS production is high.

The third group of antioxidant enzymes, CAT, is completely located in the peroxisome (Fransen et al., 2012; Halliwell and Gutteridge, 2007; Martensson et al., 1990; Scott et al., 1969). CAT cannot remove any H₂O₂ produced in the mitochondria unless the RS diffuses from the mitochondria to peroxisomes (Halliwell and Gutteridge, 2007). However, once H₂O₂ enters the peroxisomes it can be directly removed by CAT – no cofactor is required to drive the reaction (Hulbert et al., 2007).

How is the endogenous antioxidant system modified during migration?

During migration, many of these antioxidants can be upregulated to protect against damage resulting from increased RS production, although too few studies to date have assessed how antioxidant enzyme activity changes during flight (Table 1). European robins caught during a long-distance migratory flight had elevated GPx in red blood cells, as well as increased RS damage to proteins in red blood cells, compared with resting individuals (Jenni-Eiermann et al., 2014). This suggests that GPx was upregulated in response to RS-mediated damage during flight, although not to the extent that damage was entirely avoided. Accordingly, plasma non-enzymatic

antioxidants were higher in great tits (*Parus major*) with clipped feathers (which increases flight effort) than in individuals with unclipped feathers (Vaugoyeau et al., 2015). Interestingly, zebra finches (*Taeniopygia guttata*) flown at rapid speeds had increased levels of plasma uric acid but not antioxidant enzymes compared with those of control birds (Costantini et al., 2013). Many studies have shown that uric acid levels increase during flapping flight and migration. For example, Tsahar et al. (2006) demonstrated that the rate of uric acid oxidation to allantoin was highest in white-crowned sparrows (*Zonotrichia leucophrys*) immediately after exercise, in accordance with the role of uric acid as an antioxidant. Uric acid was also elevated in the plasma of garden warblers, European robins and pied flycatchers (*Ficedula hypoleuca*) that were in active migration as compared with birds feeding on stopover or birds fasted in captivity for 2 days (Jenni-Eiermann and Jenni, 1991). In contrast, homing pigeons (*Columba livia*) flown for 200 km depleted their plasma non-enzymatic antioxidant capacity, presumably because these antioxidants were ‘used up’ by the RS produced during exercise (Costantini et al., 2008). It should be noted that these studies examined different aspects of the antioxidant system, making comparisons among studies complicated. Clearly, studies are needed on many different bird species and these studies should simultaneously examine multiple components of the antioxidant system – such data would allow us to better understand how birds manage their oxidative status during migration.

Exogenous antioxidants: do birds use dietary antioxidants?

Dietary antioxidants are a group of hundreds of molecules that operate as secondary compounds in plants, and may serve multiple functions including protection against damage from sunlight; once consumed (e.g. by birds during migration), these molecules may potentially act prophylactically to provide protection against damage by RS, or therapeutically to repair existing oxidative damage. Dietary antioxidants can be sorted into two broad groups based on their chemical solubility, which also relates to whether they can be stored by consumers for later use: lipophilic antioxidants (vitamin E or carotenoids) can be stored, whereas hydrophilic antioxidants (vitamin C or polyphenols) cannot be stored in the long term (Halliwell and Gutteridge, 2007; Johnson and Hill, 2013). Birds acquire exogenous antioxidants by consuming foods including seeds (Beaulieu et al., 2014; Cohen et al., 2008), insects (Catoni et al., 2008b; Eeva et al., 2010), leaves (Catoni et al., 2008b) and fruits (Alan et al., 2013; Bolser et al., 2013; Cohen and McGraw, 2009). As many songbirds switch to a diet consisting almost exclusively of fruit during migration (e.g. Herrera, 1984; McWilliams et al., 2004; Parrish, 1997; Thompson and Willson, 1979), we will focus our discussion of dietary antioxidants on those found in fruits. However, the action of these antioxidants in potentially preventing or repairing oxidative damage would be similar for all diet items as long as they are properly absorbed. The fruits that birds eat are seasonally abundant and provide a cheap and easy source of fat, carbohydrates and, potentially, dietary antioxidants when refueling at stopover sites or after migration has ended (Alan et al., 2013; Bolser et al., 2013; Eggers, 2000; Hernández, 2009; Orłowski et al., 2011; Parrish, 1997; Piersma and Jukema, 2002). Below, we

discuss how hydrophobicity can affect the uptake and/or tissue and cellular targets for dietary antioxidants, and the main types of lipophilic or hydrophilic antioxidants.

Can dietary antioxidants prevent or repair damage by RS?

Generally, it is thought that dietary hydrophilic antioxidants act in the bloodstream or cytoplasm, and lipophilic antioxidants are stored in cell membranes and fats, and can counteract RS within cells (Catoni et al., 2008b; Ge et al., 2015; Halliwell and Gutteridge, 2007). This, however, varies among tissues, and understanding the direct link between consumption, deposition and use is complicated by the interactive nature of these antioxidants (Bohn, 2014; Catoni et al., 2008b). For example, simultaneous intake of polyphenols with vitamin C or vitamin E reduces the absorption and bioavailability of these vitamins (Bohn, 2014; Halliwell and Gutteridge, 2007). Further, under certain physiological conditions, dietary antioxidants can have pro-oxidant properties; therefore, dietary antioxidants are not universally beneficial in all contexts (Berger et al., 2012; Halliwell and Gutteridge, 2007). Below, we provide further details on lipophilic and hydrophilic dietary antioxidants.

Lipophilic dietary antioxidants

The main dietary lipophilic antioxidants available to birds comprise eight forms of vitamin E (tocopherols) and >700 carotenoid molecules (Brigelius-Flohe and Traber, 1999; Halliwell and Gutteridge, 2007). Carotenoids are responsible for most pigmentation in bird plumage, and the role of these molecules as advertisements for an individual's antioxidant or immune defense capacity has been widely studied (Chui et

al., 2011; Giraudeau et al., 2013; Hill et al., 2006; Marri and Richner, 2014; McGraw, 2011; Negro et al., 2014). In plants, carotenoids serve as powerful scavengers of singlet oxygen radicals (Halliwell and Gutteridge, 2007), but in animals, their role as antioxidants is less clear. For example, carotenoids seem to inhibit lipid peroxidation at low oxygen concentrations, but not at high oxygen concentrations (Hatta and Frei, 1995); although carotenoids are able to neutralize RS, the contribution of carotenoids to plasma antioxidant capacity is small (Costantini and Møller, 2008). Great tits supplemented with carotenoids and experimentally manipulated to increase physical activity showed increased circulating carotenoid concentrations, but also displayed increased oxidative damage and reduced antioxidant capacity (Vaugoyeau et al., 2015). Therefore, the role of carotenoids as potent antioxidants is debated (Costantini and Møller, 2008; Halliwell and Gutteridge, 2007; Hill and Johnson, 2012; Marri and Richner, 2014; Pamplona and Costantini, 2011), and recent research has emphasized that carotenoids may act as a signal for oxidative health rather than as antioxidants themselves (Costantini and Møller, 2008; Hill and Johnson, 2012; Johnson and Hill, 2013). Other evidence suggests that carotenoids stored in subcutaneous fat depots may protect fats from damage by RS or act as a reservoir of antioxidants for activities that result in oxidative challenges, such as flight (Metzger and Bairlein, 2011; Pamplona and Costantini, 2011; Tomášek et al., 2016).

Vitamin E functions to stop the propagation of lipid peroxidation by interacting with lipid peroxyl radicals to prevent them from oxidizing fatty acids (Brigelius-Flohe and Traber, 1999; Halliwell and Gutteridge, 2007). In mammals, vitamin E is important for preventing oxidative damage during exhaustive exercise (Ham and

Liebler, 1997; Rokitzki et al., 1994). The role of vitamin E as a dietary antioxidant in birds has been widely studied in domestic poultry, whereas vitamin E has primarily been studied in wild birds as a protectant in egg yolk, and for its role in affecting plumage coloration and nestling growth rates (Giraudeau et al., 2013; Matrková and Remeš, 2014; McLean et al., 2005; Williamson et al., 2006). Dietary vitamin E along with carotenoids was studied in captive-reared budgerigars and was found to reduce oxidative damage but not plasma antioxidant concentration compared with those of control birds not given these dietary antioxidants (Larcombe et al., 2008), indicating that lipophilic dietary antioxidants were either used up in the plasma or were stored to protect cells. Although no studies have directly examined how wild birds utilize vitamin E during migration, investigating whether vitamin E gleaned from fruits could be stored alongside fat to prevent lipid peroxidation seems worthwhile.

Hydrophilic dietary antioxidants

Although less studied than lipophilic antioxidants in birds, hydrophilic antioxidants may be just as important for scavenging RS in the circulation or in the aqueous portion of cells (Catoni et al., 2008b; Halliwell and Gutteridge, 2007). For example, vitamin C acts as a powerful reducing agent of the more reactive free radicals and can be recycled or can help to recycle oxidized vitamin E (Halliwell and Gutteridge, 2007), although its role during exercise and bird migration still remains ambiguous.

Polyphenols are a large group of hydrophilic molecules that range from simple molecules, such as phenolic acids, to polymerized compounds, such as tannins, with

antioxidant properties. Depending on their structure, polyphenols may give fruits their pigment and odor, and contribute to a bitter taste (Bravo, 2009; El Gharras, 2009).

Flavonoids, polyphenols with a benzo- γ -pyrone structure, are a family of molecules that are abundant antioxidants in the fruits that birds consume during migration, and have higher antioxidant potency than other dietary antioxidants in vitro (Alan et al., 2013; Bolser et al., 2013; Bravo, 2009; El Gharras, 2009; Rice-Evans et al., 1996).

Although not all polyphenols are absorbed easily, there is evidence that birds are able to absorb and circulate certain polyphenols. For example, absorbed polyphenols have been detected in the plasma of European blackcaps (*Sylvia atricapilla*) (Catoni et al., 2008a). More study is needed to determine how polyphenols are utilized to protect against or repair damage to the aqueous portion of cells during flights or while birds are recovering on stopovers.

Evidence that birds benefit from choosing foods high in dietary antioxidants

During demanding life-history stages, many animals may use antioxidants from their diet to protect themselves or their young against overwhelming oxidative damage. For example, in mammals, dietary vitamin E is important for lactating sows in order to have healthy piglets (Lauridsen et al., 2002). In birds, Gouldian finches (*Erythrura gouldiae*) that were cold stressed and fed polyphenol-rich seeds had 25% lower oxidative damage with no change to their antioxidant capacity compared with those fed seeds with a lower polyphenol content (Beaulieu et al., 2014). Accordingly, finches significantly increased their intake of seeds with high polyphenol content under cold conditions, but not their consumption of seeds with low polyphenol content

(Beaulieu et al., 2014). In a choice experiment, wild blackcaps during the breeding season chose food that contained flavonoids over a diet without flavonoids (Catoni et al., 2008a). After migratory flights in the spring, Eurasian golden plovers (*Pluvialis apricaria*) consume large amounts of crowberries (*Empetrum* spp.) that have high concentrations of anthocyanins (Ogawa et al., 2008; Piersma and Jukema, 2002), indicating that the antioxidants in the berries could be used therapeutically to help birds cope with the oxidative damage incurred during migratory flights. During autumn migration, many species of songbirds rely on seasonally abundant fruit to refuel during stopovers, and the presence of fruiting species may be more important than habitat structure in determining habitat use by birds during migration (Eggers, 2000; Parrish, 1997; Sapir et al., 2004; Smith and McWilliams, 2014; Smith et al., 2007; Suthers et al., 2000). Birds consume fruit during migration as an important source of fat (Smith and McWilliams, 2010; Smith et al., 2007; Thompson and Willson, 1979), but there is also evidence that birds select fruits based on dietary antioxidant content (Fig. 3; Alan et al., 2013; Bolser et al., 2013; Schaefer et al., 2008; Skrip et al., 2015). Blackcaps and garden warblers on stopovers chose fruits that had high lipid content and were darker in color, indicating that they contained more polyphenols (Schaefer et al., 2014). Birds at a stopover site during autumn migration consumed arrowwood fruits (*Viburnum* spp.) more readily than other fruits, and arrowwood had higher total antioxidants, total anthocyanins and total phenolics than the other abundant fruits at the stopover site (Bolser et al., 2013). Arrowwood fruits also had high fat content ($41.3 \pm 5.8\%$), total lipophilic antioxidants and total tocopherols (Alan et al., 2013; Bolser et al., 2013; Smith et al., 2007). However, birds

do not always choose fruits with high antioxidant content. For instance, Virginia creeper berries are fat rich but relatively poor in antioxidants, and are preferentially consumed by birds (Fig. 3). Taken together, these studies indicate that birds in migration may be able to visually assess and choose fruits with high antioxidant content, perhaps to prophylactically build antioxidant stores before subsequent migratory flights (Skrip et al., 2015) or to therapeutically repair damage after migratory flights (Piersma and Jukema, 2002). However, the relative roles of fat and antioxidant content in food choice by birds during migration clearly need further investigation.

Utilizing endogenous and dietary antioxidants

The antioxidant system consists of many molecules, some derived from the diet and some synthesized endogenously. When birds are exposed to an increase in RS, it is the shared action of these molecules that protects birds against damage, but there may be different costs associated with foraging for antioxidants versus synthesizing enzymes or sacrificial molecules. Birds in migration alternate between fasting while flying and then feeding at stopover sites to rebuild fat and muscle. However, stopover is by no means energy inexpensive for birds. In fact, estimates of energy expenditure of songbirds through migration suggest that birds expend twice as much energy during stopovers as during flights (Hedenström and Ålerstam, 1997; Wikelski et al., 2003). If foraging for fruits (dietary antioxidants and fat) is costly at sites that are lower in quality (e.g. sites with more non-native than native fruiting shrubs), then birds at stopover sites may upregulate their endogenous antioxidant system in preparation for

migration (Hutton and McGraw, 2016; Smith et al., 2013). However, endogenous production of antioxidants requires resources such as amino acids or dietary metal cofactors, thus preventing their use for other physiological processes, such as rebuilding muscle on stopovers. For instance, synthesis of GPxs requires selenium (Cockell et al., 1996; Johansson et al., 2005; Surai, 2002, 2006), which consequently cannot be used in other selenoproteins, such as those important for immune function (Arthur et al., 2003). There is some evidence that animals may reduce the synthesis of endogenous antioxidants if they are able to consume dietary antioxidants. Costantini et al. (2011) measured six molecular biomarkers for the redox system in zebra finches, and found a negative association between non-enzymatic and enzymatic antioxidant capacity, indicating that these antioxidant types are differentially regulated. Further, mice supplemented with vitamin C had lower levels of SOD, CAT and GPx, but no change to levels of oxidative damage, suggesting that antioxidants in the diet may offset synthesis and fulfill the role of antioxidant enzymes (Selman et al., 2006). However, vitamin E was found to be beneficial for rats during acute exercise, but adversely affected GPx capacity during exercise training (Venditti et al., 2014). Further investigation into the interplay between consumption of dietary antioxidants and the synthesis of endogenous antioxidants is needed for birds in migration, and the knowledge acquired may help to inform us about the interactions between RS production and the antioxidant system during periods of high energy demand in other organisms.

Future directions

Much remains to be discovered about how the antioxidant system of birds copes with the oxidative challenges associated with migratory flights. In this section, we outline some potential avenues of research that seem worthwhile given the limited studies that have been done to date, and discuss some ways in which these questions might be addressed.

Coping with an increase in RS during spring versus autumn migrations

Fruits can provide migrating birds with an inexpensive source of antioxidant protection (Alan et al., 2013; Bolser et al., 2013; Schaefer et al., 2008), but their availability varies seasonally. In general, fruits from fruiting shrubs are most abundant during autumn migration (Parrish, 1997; Smith et al., 2013), although there are some interesting exceptions where berries are abundant directly after spring migration (e.g. Piersma and Jukema, 2002). Are there other sources of dietary antioxidants besides fruits that are available to birds during spring migration, or must spring-migrating birds upregulate their endogenous antioxidant system to a greater extent because of the reduced availability of dietary antioxidants? Alternatively, do birds consume antioxidants on their wintering grounds prior to migration and store them for use during spring migration, thus creating carryover effects? Because the breeding season is another life stage that may cause an increase in RS (Romero-Haro et al., 2016), it is also possible that birds in spring migration utilize their antioxidant system differently to reduce the costs of migration while preparing for breeding. These questions could be addressed via field experiments that longitudinally measure antioxidant capacity

(both enzymatic and non-enzymatic) and oxidative damage in migratory birds across the year [i.e. winter, spring migration, summer (reproduction), and autumn migration].

Melatonin as an antioxidant during night-time flight

Many songbirds migrate under the cover of darkness, switching from primarily diurnal activity during other parts of the annual cycle to nocturnal activity during migration. Melatonin is an important messenger for establishing circadian rhythms, and its levels are generally highest at night (Fusani and Gwinner, 2005). Melatonin is also a potent antioxidant (reviewed in Tan et al., 2015). Is it possible that elevated night-time melatonin serves to also protect birds from oxidative damage while they fly at night? Particularly revealing would be controlled experiments with captive birds that examine antioxidant capacity and oxidative damage while carefully regulating melatonin levels either directly or by altering the duration of daylight as well as physical activity.

How are dietary antioxidants used?

Although it is generally thought that hydrophilic antioxidants cannot be stored, and lipophilic antioxidants are stored, few studies have directly examined how birds metabolize dietary antioxidants and their bioavailability. Especially helpful would be studies that track the absorption and mode of action of certain antioxidants acquired in the diet. For example, are ingested hydrophilic antioxidants used primarily to quench RS in the plasma or can they cross cell membranes?

Can lipophilic antioxidants reach the main source of RS production, the mitochondria, or are they exclusively used for protecting stored fats? If so, are these antioxidants used differently by birds? Can lipophilic antioxidants reach the main source of RS production, the mitochondria, or are they exclusively used for protecting stored fats? If so, are these antioxidants used differently by birds while at stopover sites? For instance, do they store lipophilic antioxidants to protect their fat stores, but use hydrophilic antioxidants therapeutically to recover from previous migratory flights? Experiments to address these issues could use labeled antioxidant molecules to determine specific cellular targets of dietary antioxidants.

The role of early, short endurance flights

Birds may upregulate their endogenous antioxidant system in preparation for their first migratory flight; alternatively, short endurance flights early in migration may prime a bird's antioxidant system for subsequent flight bouts. Endurance flights may activate a number of molecular pathways that regulate anti-stress, damage repair and inflammatory responses (Bayly et al., 2012; Lucas et al., 2014). Exposure to a relatively low level of RS may upregulate antioxidant defenses, and RS may trigger a hormetic response whereby exposure to an endurance flight may activate the antioxidant system so that it is better able to respond when exposed to stressors in the future (Costantini, 2008, 2014; Costantini et al., 2010a; Hollander et al., 2011). There remains much to learn about the dynamics of this relationship between RS production and endogenous antioxidant defenses for wild birds during migration.

How UCPs modulate RS production during endurance flights

UCPs may act as a crucial safety valve for organisms during times of stress, allowing protons to cross the inner mitochondrial membrane without generating ATP (Brand et al., 2004; Criscuolo et al., 2005). Upregulating the actions of UCPs may serve to decrease the amount of RS produced during respiration (Brand et al., 2004). However, no study to date has examined whether UCPs are activated by RS production during endurance flights.

The use of dietary antioxidants for other migratory organisms

Much of the work on how antioxidants (endogenous or dietary) affect exercising animals in the wild has focused on songbirds. However, many insects, large mammals (e.g. zebras or whales), seabirds, bats and raptors also migrate by running, flying or swimming. Understanding how animals protect their cells, tissues and DNA from RS while covering long distances and whether there are associated fitness consequences could lend insights into their habitat use, food requirements and ultimately their population dynamics. Studies are needed that compare the response of the antioxidant system to increasing oxidative demands across the wide diversity of organisms that undergo various extents of migration.

Conclusions

RS production associated with endurance exercise causes damage to lipids, proteins and DNA in organisms, unless counterbalanced by an antioxidant system. Because migratory birds fly hundreds to thousands of kilometers during migration, they are especially at risk of oxidative damage. Like other vertebrates, birds have a multifaceted antioxidant system that includes endogenous enzymes, sacrificial

molecules and dietary antioxidants that can respond flexibly to oxidative demands and so provide protection from RS. Further research needs to be done – not only in migrating birds but also during periods of high energy expenditure in other animal species – in order to more fully understand how the various facets of the antioxidant system respond and interact to avoid oxidative damage during endurance exercise.

List of Symbols and Abbreviations

Reactive Oxygen Species (ROS)

Reactive Nitrogen Species (RNS)

Hydrogen Peroxide (H_2O_2)

Nitric Oxide ($NO\bullet$)

Superoxide ($O_2\bullet^-$)

Hydroxyl Radical ($HO\bullet$)

Peroxy Nitrate ($ONOO^-$)

Nitrous Acid (HNO_2)

Basal Metabolic Rate (BMR)

Glutathione (GSH)

Glutathione Disulfide (GSSG)

Glutathione Peroxidase (GPx)

Superoxide Dismutase (SOD)

Catalase (CAT)

Glossary

Basal Metabolic Rate: The metabolic rate of organisms in their thermoneutral zone and in a post-absorptive, resting, non-growing, non-reproductive state

Dietary antioxidants: Antioxidants produced by plants and consumed by animals in their diets. The two broad classes of dietary antioxidants include lipophilic antioxidants (vitamin E or carotenoids) and hydrophilic antioxidants (vitamin C or polyphenols).

Endogenous antioxidants: Antioxidants directly produced or available within cells.

Free radical leak: The percentage of the total electron flow in the mitochondria that produces reactive species.

Micromolecular sacrificial molecules: Antioxidants that donate an electron to RS to prevent oxidization of other important biological molecules.

Oxidative stress: The situation when the amount of RS produced in an organism overwhelms the capacity of the antioxidant system and causes damage.

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Competing interests

The authors declare no competing or financial interests.

Author Contributions

Conceptualization: S.M., C.C.M.; Writing - Original draft preparation: C.C.M;

Writing - review and editing: S.M., C.C.M.; Visualization: S.M., C.C.M.; Funding

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Table 1.1. Summary of studies that examined antioxidant capacity and oxidative damage during flight

Bird Species	Comparison	Antioxidants		Damage		Citation
		Measurement	Result	Measurement	Result	
Non-Migratory						
Homing pigeon (<i>Columbia liva</i>)	≥ 200 km flight vs ≤ 60 km flight	Non-enzymatic serum antioxidant capacity _a	↓	Serum hydroperoxides _b	↑	Costantini et al. (2008)
Great tit (<i>Parus major</i>)	Clipped feathers vs unclipped feathers	Non-enzymatic serum antioxidant capacity _a	↑	Serum hydroperoxides _b	↑	Vaugoyeau et al. (2015)
Zebra finch (<i>Taeniopygia guttata</i>)	Fast flight (≥ 911 m h ⁻¹) vs. control flight (≤ 55.2 m h ⁻¹)	Total thiols _c Carotenoids, catalase, GPx _d , SOD _e Uric acid	↓ — ↑	Serum hydroperoxides _b	↑	Costantini et al. (2013)
Migratory						
European robin (<i>Erithacus rubecula</i>)	Migratory flight vs resting/ refueling on stopover	GPx _d	↑	Protein carbonyls	↑	Jenni-Eiermann et al. (2014)
Garden warbler (<i>Sylvia borin</i>)	New to stopover after migratory flight	Non-enzymatic serum antioxidant capacity _a	—	Serum hydroperoxides _b	↑	Skrip et al. (2015)

vs on stopover for up to 8h					
Northern bald ibis (<i>Geronticus eremita</i>)	Before flight vs after flight	Non-enzymatic serum antioxidant capacity ^a	—	Serum hydroperoxides ^b	—
					Bairlein et al. (2015)

The few studies that have directly measured antioxidant status and oxidative damage during flight include data from three species of non-migratory birds and two species in migration. The direction of the arrow indicates whether antioxidant capacity or oxidative damage increased (↑) or decreased (↓), or did not change (—) for the experimental group (first listed of the comparison groups). Flight caused oxidative damage in all species examined, but changes in antioxidant capacity were less consistent. This is likely to be because of differences in the extent to which the antioxidant system quenched reactive species produced during flight, or due to the different groups of antioxidants measured.

^aMeasured using the OXY-adsorbent test.

^bMeasured using the test for reactive oxygen metabolites (d-ROMs)

^cTotal thiols (e.g. glutathione, thioredoxin) measured using the –SHp test

^dGlutathione peroxidase (GPx), an antioxidant enzyme

^eSuperoxide dismutase (SOD), an antioxidant enzyme

Figure 1.1. Examples of primary ways in which reactive species are generated and endogenous antioxidant protection occurs in the mitochondria, cytosol and plasma. Processes that generate reactive species (RS) are shown in red, and antioxidant protection is shown in blue. Non-reactive end products of the interactions between RS and antioxidants are in black. The primary RS generated are superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^\bullet$). $O_2^{\bullet-}$ can react with nitric oxide ($NO^\bullet$) to create peroxynitrate ($ONOO^-$), a lipid-soluble radical, or $NO^\bullet$ can react with $OH^\bullet$ to form non-reactive nitrous acid (HNO_2). In the mitochondria, $O_2^{\bullet-}$ is primarily broken down by manganese superoxide dismutase (MnSOD) to H_2O_2 , which either forms $OH^\bullet$ via the Fenton reaction or can be broken down to water (H_2O) in the glutathione (GSH) to glutathione disulfide (GSSG) cycle catalyzed by glutathione peroxidase (GPx). The thiol group on GSH is able to donate an electron to H_2O_2 , briefly causing the GSH itself to be reactive. However, because of the high concentration of GSH in the cell, this briefly reactive GSH quickly interacts with a second molecule of GSH to form GSSG, water and alcohol. Lipid-soluble RS, such as H_2O_2 or $ONOO^-$, can diffuse to the cytosol (dotted arrows), and cause lipid peroxidation in the mitochondrial membranes. In the cytosol, RS are formed in the cytochrome P450 pathways, or during lipid peroxidation or protein oxidation. The main enzymes that prevent RS-associated damage here are copper-zinc superoxide dismutase (CuZnSOD), catalase

and the enzymes involved in the GSH cycle. In the plasma, interactions between H_2O_2 and transition metals, such as ferrous ions, can create RS, but these metals can be sequestered by metal-binding proteins. Additionally, $\text{O}_2^{\bullet-}$ is produced by activated white blood cells in the plasma. The main antioxidants in the plasma for birds and other uricotelic organisms are uric acid, SOD3 and GPx. Not shown here are the modes of action for dietary antioxidants (see text for discussion). The phospholipid bilayers are representative of areas where RS and antioxidants must cross membranes.

Figure 1.2. The multifaceted antioxidant filter available to birds. Although endurance flight produces reactive species (RS), birds and other animals have a multifaceted antioxidant filter that can mitigate damage. The antioxidant filter consists of micromolecular sacrificial molecules, endogenous enzymes and dietary antioxidants. Uric acid is an example of a sacrificial molecule. Uric acid donates an electron to RS and is oxidized to allantoin, and the ratio of uric acid to allantoin can be measured and used as an indicator of oxidative balance. Glutathione peroxidase (GPx) is an example of an endogenously produced enzyme that catalyzes the glutathione (GSH) to glutathione disulfide (GSSG) cycle. During the reaction catalyzed by GPx, GSH donates an electron to RS, and is oxidized to GSSG, which can be recycled back to GSH by glutathione reductase. Vitamin E (α -tocopherol) is an example of a lipophilic antioxidant from the diet, and vitamin C (ascorbate) is an example of a hydrophilic antioxidant from the diet. As RS production increases during endurance flight, the antioxidant system must also be upregulated to prevent an increase in damage. Here, we represent a scenario in which a large number of RS are being produced (thick arrows and lines, left) but RS are quenched by a strong antioxidant system, preventing damage to cells, tissues and DNA (thin arrows and lines, right). There are other scenarios in the wild where the antioxidant system may not be able to overwhelm the RS being produced, and the amount of oxidative damage would be higher.

Figure 1.3. Birds consume fruits for fat and/or antioxidant content while on stopover. (A) A hermit thrush (*Catharus guttatus*) during autumn migration eating fruit. Photo by Ryan Brady; printed with permission. (B) Relative consumption index for seven fruiting shrub species in relation to the total lipophilic antioxidant content of the fruit. Fruits with a higher consumption index were preferentially consumed by songbirds during autumn migration stopover on Block Island, Rhode Island, USA (adapted from Alan et al., 2013). (C) Relative consumption index for the same fruiting shrubs in relation to their relative fat content. Northern arrowwood (*Viburnum dentatum*) had the highest total lipophilic antioxidant content, ranked high on the scale for fat content

and was the preferred fruit eaten by birds. Northern bayberry (*Myrica pensylvanica*) ranked highest for fat content, and was high for total lipophilic antioxidant content, but was not highly preferred by birds as only a few species can digest its waxy coating. Other preferred fruits such as winterberry (*Ilex verticillata*) and Virginia creeper (*Parthenocissus quinquefolia*) had a lower antioxidant content but more fat than less-preferred fruits such as chokeberry (*Aronia* sp.), Asiatic bittersweet (*Celastrus orbiculatus*) or multiflora rose (*Rosa multiflora*) (adapted from Alan et al., 2013). Thus, antioxidant content alone does not determine fruit preferences of migrating songbirds although fruits clearly provide good sources of antioxidants for consumers.

CHAPTER 2

Acute effects of intense exercise on the antioxidant system in birds: does exercise training help?

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Acute effects of intense exercise on the antioxidant system in birds: does exercise training help?

Summary Statement: Daily exercise training of Zebra Finches over many weeks increased the coordination between the enzymatic and non-enzymatic components of their antioxidant system and this reduced the oxidative damage associated with exercise.

Abstract:

The acute effects of an energy-intensive activity such as exercise may alter an animal's redox homeostasis, although these short-term effects may be ameliorated to some extent by chronic exposure to that activity, or training, over time. Although well documented in mammals, how energy-intensive training affects the antioxidant system and damage by reactive species has not been investigated fully in flight-trained birds. We examined changes to redox homeostasis in Zebra Finches exposed to energy-intensive activity (60 min of perch-to-perch flights twice a day), and how exercise training over many weeks affected this response. We measured multiple components of the antioxidant system: an enzymatic antioxidant (glutathione peroxidase, GPx) and non-enzymatic antioxidants (measured by the OXY-adsorbent test) as well as a measure of oxidative damage (d-ROMs). At no point during the experiment did oxidative damage change. We discovered that exposure to energy-intensive exercise training did not alter baseline levels of GPx, but induced exercise-trained birds to maintain a higher non-enzymatic antioxidant status as compared with untrained birds. Novel intense exercise (Day 1) increased the enzymatic but not the non-enzymatic

antioxidant system relative to baseline. GPx activity was elevated above baseline in trained birds immediately after completion of the second one-hour flight on each of the three sampling days, and non-enzymatic antioxidants were acutely depleted during flight after 13 and 44 days of training. The primary effect of exercise training on the acute response of the antioxidant system to 2-hr flights was a more coordinated response between the enzymatic (GPx) and non-enzymatic components of the antioxidant system of birds and this reduced the oxidative damage associated with exercise.

Introduction:

Animals must engage in energy-intensive activities (e.g. migration, escape from predators, reproduction) to enhance their survival and fitness. These activities increase metabolic rates, which can potentially lead to an increase in reactive species (RS) generation, and a need for antioxidant defenses to protect against damage to cells, tissues, and organs (Beaulieu et al., 2011; Cooper-Mullin and McWilliams, 2016; Halliwell and Gutteridge, 2007; Morosinotto et al., 2018; Pingitore et al., 2015). Antioxidant defenses are multifaceted, and include enzymatic antioxidants, micromolecular sacrificial molecules and dietary antioxidants, and are ubiquitous across most taxa (Cohen and McGraw, 2009; Cooper-Mullin and McWilliams, 2016; Halliwell and Gutteridge, 2007). However, studies that assess how the antioxidant system reacts to energy-intensive activities have demonstrated a wide variety of responses, even when examining the same type of energy-intensive activity (Hõrak and Cohen, 2010). For example, activation of the immune system via an injection of

phytohemagglutinin increased lipid peroxidation and total antioxidant defenses in greenfinches (*Chloris chloris*) (Hörak et al., 2007), whereas challenging the immune system by injecting a bacterial lipopolysaccharide reduced the antioxidant barrier in the blood of Zebra Finches (*Taeniopygia guttata*) (Bertrand et al., 2006). These antioxidant defenses may respond differently depending on which aspect of the antioxidant system is measured, the type, duration or fuel used for an activity, the type of damage, and an animal's physiological state (Cohen and McGraw, 2009; Costantini et al., 2011a; Halliwell and Gutteridge, 2007; Jenni-Eiermann et al., 2014; Skrip and McWilliams, 2016). Particularly lacking are studies that identify which key components of the antioxidant system (i.e., sacrificial molecules such as uric acid, endogenous antioxidant enzymes such as glutathione peroxidase, GPx, non-enzymatic antioxidants, OXY) predictably respond, interact, or are unresponsive to certain challenges and activities (Cooper-Mullin and McWilliams, 2016).

The acute effects of an energy-intensive activity may alter an animal's redox homeostasis, although these short-term effects may be ameliorated to some extent by chronic exposure to that activity, or training, over time. Numerous studies in mammals have demonstrated that intense exercise can induce acute oxidative stress, but regular exercise training can reduce exercise-induced damage by increasing endogenous antioxidant capacity (Gomes et al., 2012; Gomez-Cabrera et al., 2008; Jackson, 2005; Nikolaidis et al., 2012; Pingitore et al., 2015; Smarsh and Williams, 2016; Sun et al., 2010; Vaanholt et al., 2010). In contrast, only a few studies have examined the effects of exercise training in other taxa. Southern Corroboree frogs (*Pseudophryne*

corroboree) given dietary carotenoids had improved exercise endurance during initial aquatic and terrestrial escape-response trials, but not after five consecutive weeks of repeated escape-response trials (McInerney et al., 2017; Silla et al., 2016). A comparison of several species of elasmobranch and teleost fishes that differed in their swimming ability and activity revealed that those capable of reaching faster swimming speeds had higher antioxidant enzyme activities than species with lower swimming capacities (Vélez-Alavez et al., 2015). These studies indicate that exercise training can beneficially stimulate multiple components of the antioxidant system of at least a few species of running or swimming vertebrates, although no such studies on antioxidant responses to flight training have been conducted to date on volant vertebrates.

Flying birds offer a particularly interesting suite of species for investigating the immediate effects of exercise on the antioxidant system and the potential benefits of exercise training. Flapping flight often increases metabolic rates up to 30x basal metabolic rates (Nudds and Bryant, 2000; Tatner and Bryant, 1986; Wikelski et al., 2003), with associated increases in acute RS production (Costantini, 2014; Costantini et al., 2010; Jenni-Eiermann et al., 2014). In addition, passerines primarily use fat as fuel during flights (Bairlein, 1990; Hambly et al., 2002; Jenni and Jenni-Eiermann, 1998), particularly during migration when the capacity for fat metabolism is highest (DeMoranville et al., 2019; Gerson and Guglielmo, 2013; Jenni-Eiermann et al., 2002; McWilliams et al., 2004; Price et al., 2011). Relying on fat as fuel may also increase RS production as fats are catabolized (Costantini et al., 2007; Skrip et al., 2015). Studies of free-living migratory birds have demonstrated that the antioxidant system of

birds is quite dynamic during the alternating bouts of flying and fasting, resting and feeding that is typical for most migrating birds (Cooper-Mullin and McWilliams, 2016; Costantini, 2008). For example, protein oxidative damage was high in red blood cells from European Robins (*Erithacus rubecula*) captured during a nocturnal migratory flight as compared with resting or foraging individuals, but actively migrating robins also had higher levels of glutathione peroxidase, an antioxidant enzyme (Jenni-Eiermann et al., 2014). In contrast, serum non-enzymatic antioxidant capacity did not differ between freshly arrived and rested Garden Warblers (*Sylvia borin*) at a spring stopover site (Skríp et al., 2015) and did not change before or after flights in Northern Bald Ibis (*Geronticus eremita*) trained to follow an ultra-light aircraft during migration (Bairlein et al., 2015). In the only study to directly test the relationship between training and oxidative damage, Larcombe et al (2010) found that takeoff flight training reduced circulating lipid damage in captive Budgerigars (*Melopsittacus undulates*) compared to a single bout of exercise. Thus, the immediate effect of flight on the antioxidant system remains equivocal, the effect of flight training on the antioxidant system is largely untested, and how the collective action and integration of key components of the antioxidant system responds to the production of RS during acute and trained flight remains an open question.

In this study, we exercised Zebra Finches (*Taeniopygia guttata* Vieillot, 1817) for two hours each day for 6+ weeks to determine how key components of the circulating antioxidant system responded to the acute challenge of such daily flight, as well as how such exercise training over many weeks influenced this response. We evaluated

the following hypotheses and predictions: Hypothesis 1: (Training Effect) Exercise training alters baseline levels of enzymatic and non-enzymatic antioxidants.

Hypothesis 2: (Acute Exercise Effect) Short-term intense exercise alters the antioxidant system, and the magnitude or direction of that acute change associated with a given exercise bout will be affected by training. Hypothesis 3: (Inevitable Damage) Short-term intense exercise causes oxidative damage in part because the slow response of the antioxidant system.. Hypothesis 4: (Coordinated Antioxidant Responses) Within individuals, different aspects of the antioxidant system should work in concert and acute changes in enzymatic antioxidants should be correlated with changes in non-enzymatic antioxidants.

Methods:

Exercise Training:

The Zebra Finches used in this experiment were a part of a larger experiment on lipid turnover rates in exercised and non-exercised birds (Carter et al., 2018; Carter et al., 2019), and were acquired from a known-age captive population of birds at Sacred Heart University. All care and experimental procedures were reviewed and approved by the University of Rhode Island's Institutional Animal Care and Use Committee under protocol AN11-12-009. Zebra Finches are nomadic in the wild, may travel long distances across the interior of Australia to find suitable breeding conditions (Griffith and Buchanan, 2010; Mariette and Griffith, 2012), and they are relatively easily trained to fly in small groups within aviaries (Carter et al., 2018; Carter et al., 2019; Griffith and Buchanan, 2010; Skrip and McWilliams, 2016). Prior to exercise training, 65 Zebra Finches were kept in same-sex aviaries (2.1 x 0.9 x 1.8 m = L x W x H) for 8

weeks on a 14 h:10 h light:dark cycle under full-spectrum light, with lights on at 06:00. Birds were banded with an aluminum numbered band on their right leg for individual recognition with one color band on the left leg to indicate treatment (trained = red; untrained = green). During these 8 weeks, birds acclimated to the aviaries, and to a standard mixed-seed diet (Hagen #B2405, Mansfield, MA USA) primarily composed of millet (*Pennisetum glaucum*) supplemented with fresh kale, a source of ascorbic acid and α -tocopherol, once weekly (Podsdek, 2007).

Birds were randomly assigned to an exercise-trained treatment group ($N = 33$: 16 males, 17 females) or an untrained, sedentary group ($N = 32$: 15 males, 17 females). The exercise-trained Zebra Finches were subjected to two one-hour periods of stop-and-go perch-to-perch flights (11:00 – 12:00 and 13:30 – 14:30) in a 6 (L) x 3 (W) x 2 m tall flight arena (see Bauchinger *et al.* (2010), Skrip *et al.* (2016), and Carter *et al.* (2018) for further details). The birds flew to and from two perches 4 m apart in opposite corners of the arena, with a cloth wall partially dividing the arena in the middle. A handler walked clockwise around the arena during flight training sessions for 300 laps per hour while tracking the number of circuits with a hand counter. This resulted in about 1200 flights of at least 4 m per bird each day, or approximately 4.8 km day⁻¹ (or 2.4 km hr⁻¹). Short-burst flights of this type incur energetic costs that are approximately three times higher than sustained flight in small songbirds (Nudds and Bryant, 2001), and respiratory quotients for Zebra Finches exposed to perch-to-perch flights were indicative of primarily burning fats (0.75 ± 0.01 , Nudds and Bryant, 2001), a fuel type that increases the potential for oxidative damage (Skrip and

McWilliams, 2016). However, short-burst exercise training was previously demonstrated to decrease oxidative damage in the plasma of captive budgerigars (Larcombe et al., 2008). During the two one-hour exercise periods, we removed food and water from the cages housing untrained birds to ensure both treatment (exercise-trained) and control (untrained sedentary) birds were similarly fasted. Although not subjected to perch-to-perch flight training, untrained birds were allowed to voluntarily move and fly unrestricted in their same-sex aviaries (2.1 x 0.9 x 1.8 m = L x W x H). All birds were measured at the beginning of the experiment (tarsus, wing chord, weight, and fat), and condition indices (fat and weight) were measured once a week to monitor their health. Trained birds in this study were exercised every day for 44 days.

Blood Sampling:

To examine changes in the oxidative status of finches before, during, and after the 44 days of exercise training, we randomly selected 10 male Zebra Finches from the untrained group and 10 male Zebra Finches from the trained group for blood sampling. Using blood allowed us to take repeated measures from the same individual over time, and previous work in mammals has indicated that although the magnitude of the effect of exercise on the antioxidant system likely varies depending on the tissue measured, acute exercise still alters redox homeostasis in almost every fluid, blood cell, tissue and organ regardless of large differences in basal rates of RS generation among tissues (Nikolaidis et al., 2012). We sampled only males to control for any sex differences (Isaksson, 2013; López-Arrabé et al., 2018), and to avoid confounding problems with reproduction, as females can deposit antioxidants into their eggs (Skríp

et al., 2016). We obtained blood samples (200 μ L) at Days 0-1, 12-13, and 43-44 of exercise training from the same individuals prior to training in the morning (08:00 h) to obtain baseline measurements of antioxidant status, and again within 10 min after training in the afternoon (14:30 h) to examine acute changes in antioxidants and damage. The untrained and trained birds were sampled on two subsequent days so that all birds could be bled within 10 minutes of entering the room to control for possible short-term increases in blood metabolites associated with our activities. On days 0-1 and 12-13 of the exercise regime, we took blood samples from the same 10 untrained and trained Zebra Finches before and immediately after exercise. On days 43-44, we took blood samples from 5 of the original 10 trained and untrained birds, as the other 5 birds from each group had been randomly selected to be sacrificed as a part of the larger experiment (Carter et al., 2018). All blood samples were centrifuged within 10 min of collection to separate the red blood cells from the plasma and stored at -80°C until further analysis.

Analysis of Oxidative Status:

We measured several indicators of the antioxidant status and oxidative damage in blood of these birds including non-enzymatic antioxidant capacity, glutathione peroxidase (GPx), an important enzymatic antioxidant (Cooper-Mullin and McWilliams, 2016; Costantini et al., 2011b; Masaki et al., 1998), and reactive oxygen metabolites (ROMs). Non-enzymatic antioxidant capacity was measured with the OXY-adsorbent test (OXY) 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 and provides an index of non-enzymatic antioxidant capacity, without being complicated by inclusion of uric acid (Alan and McWilliams, 2013; Costantini, 2011; Skrip and McWilliams, 2016). GPx activity in red blood cells was measured indirectly via a coupled reaction with glutathione reductase following the manufacturer's protocol (concentration unit = nmol/min/mL; Cayman Chemical Glutathione Peroxidase Assay Kit). Oxidized glutathione produced upon reduction of hydroperoxides by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A₃₄₀ is directly proportional to the GPx activity (Arazi et al., 2017; Celi et al., 2013).

Oxidative damage was measured using the d-ROMs test (concentration unit = mmol l⁻¹ H₂O₂ equivalents; Diacron International, Grosseto, Italy). This test works by first decreasing the pH of the plasma to release metal ions from proteins to cleave circulating reactive oxygen metabolites (ROMs) through incubation with a solution of 0.01 M 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 ROMs in the plasma and was measured at 505 nm (Costantini, 2016; Costantini et al., 2007). ROMs measured in this test are primarily hydroperoxides, which are produced when RS interact with many different biological macromolecules (Costantini, 2016), but in plasma, are primarily produced during lipid oxidation events (Davies, 2016; Ito et al., 2017), and are correlated with levels of

circulating isoprostanes, an end product of lipid peroxidation events (Lubrano et al., 2002). Since hydroperoxides are precursors of lipid peroxidation products such as malondialdehyde or isoprostanes (Halliwell and Gutteridge, 2007), they are more likely to reflect a whole animal response to changes in RS as they occur earlier in the oxidative cascade than those end products.

OXY and dROMs were run in duplicate and GPx was run in triplicate. Sample sizes reported in the figure legends indicate individuals with sufficient repeatability among samples to be included in the analysis ($CV < 9\%$).

Statistics:

We used R version 3.6.0 (R Core Team, Vienna, Austria 2019) with the nlme package (Pinheiro et al., 2019) to perform linear mixed effects analyses for longitudinal data to determine the influence of the treatment group (trained vs. untrained), blood sampling stage and their interaction for each of the three oxidative parameters: plasma non-enzymatic antioxidant capacity (OXY), GPx activity in red blood cells, and plasma oxidative damage (dROMs). The best model for each oxidative parameter was chosen based on Akaike's information criterion, and then fit with restricted maximum likelihood (Bolker et al., 2009). We first inspected whether measures of condition (weight or fat score) changed within individuals and among flight groups over the experiment. We then examined the change in baseline (pre-flight levels) across the experiment to determine the effect of training on all the baseline oxidative parameters. We also examined the change from baseline within a training day (post-flight minus

pre-flight levels). As random effects, we included intercepts for the individual bird. Since our sampling time points were not evenly spaced, we used the autocorrelation structure of order 1, with a continuous time covariate (corCAR1). We visually inspected residual plots, and this did not reveal any noticeable deviations from homoscedasticity or normality. We used Pearson correlations to examine how changes in GPx during a given flight were associated with changes in non-enzymatic antioxidants, and how that relationship was affected by exercise training.

Results:

Changes in weight and fat score over time were not different among untrained and trained Zebra Finches throughout the experiment (Weight: flight day, $F_{8,59} = 5.22$, $P = 0.01$; training group, $F_{1,15} = 0.46$, $P = 0.51$; Interaction, $F_{8,59} = 0.87$, $P = 0.55$; Fat Score: flight day, $F_{8,59} = 2.00$, $P = 0.06$; training group, $F_{1,15} = 0.35$, $P = 0.56$; Interaction, $F_{8,59} = 1.46$, $P = 0.19$). Additionally, including these factors in our models of antioxidant capacity or oxidative damage did not change our results. Therefore, we excluded these condition measures as covariates in our final analyses of oxidative status.

Training Effect: Does baseline oxidative status change with exercise?

Baseline GPx activity did not change during training:

Baseline GPx activity did not change over time in untrained or trained Zebra Finches throughout the experiment (Fig. 1A: flight day, $F_{2,15} = 2.42$, $P = 0.122$; training group, $F_{1,15} = 0.02$, $P = 0.97$; Interaction, $F_{2,15} = 1.63$, $P = 0.227$). Baseline GPx prior to

exercise training varied among individuals (Range: 935.83 - 2572.39) but was not different among the trained or untrained Zebra Finches (lsmeans $\pm$ s.e.m.: trained = 1597.30 ± 205.14 , untrained = 1586.17 ± 191.96 , $t = |0.040|$, $P = 0.98$).

Baseline OXY was maintained in trained birds:

Baseline OXY decreased over time in untrained birds, while it remained unchanged in trained Zebra Finches throughout the experiment (Fig. 1B: untrained, $F_{2, 25} = 5.51$, $P = 0.010$; training group, $F_{1, 18} = 0.01$, $P = 0.893$; Interaction, $F_{2, 25} = 4.26$, $P = 0.026$).

Baseline OXY prior to exercise training varied among individuals (Range: 198.48 – 361.76) but was not different among trained and untrained birds (lsmeans $\pm$ s.e.m.: trained = 301.81 ± 15.51 , untrained = 298.86 ± 15.30 , $t = |0.14|$, $P = 0.99$).

Baseline ROMs did not change during training:

Baseline reactive oxygen metabolites (dROMs) did not change over time in untrained and trained Zebra Finches throughout the experiment (Fig. 1C: flight day, $F_{2, 17} = 0.03$, $P = 0.969$; training group, $F_{1, 15} = 0.017$, $P = 0.898$; Interaction, $F_{2, 15} = 0.057$, $P = 0.945$).

Acute Exercise Effect: Does short-term intense exercise acutely alter the antioxidant system in birds, and does exercise training affect this alteration?

Elevated GPx activity immediately after flight:

GPx activity was similarly elevated above baseline in trained Zebra Finches immediately after completion of the second one-hour flight on each of the three

sampling days (Fig. 2A: flight day, $F_{2, 13} = 0.54$, $P = 0.593$; training group, $F_{1, 15} = 23.17$, $P = 0.002$; Interaction, $F_{2, 13} = 3.05$, $P = 0.082$). GPx activity did not change during this same time in untrained birds (Fig. 2A).

OXY decreased with acute exercise after training:

By 12-13 days of the exercise training, non-enzymatic antioxidant capacity was on average lower than baseline in trained Zebra Finches immediately after completion of the second one-hour flight while non-enzymatic antioxidant capacity was consistently positive in untrained birds (Fig. 2B: flight day, $F_{2, 13} = 1.27$, $P = 0.300$; training group, $F_{1, 15} = 4.36$, $P = 0.028$; Interaction, $F_{2, 13} = 4.15$, $P = 0.041$).

Inevitable Damage: Does short-term intense exercise causes oxidative damage?

ROMs did not change during acute exercise:

Reactive oxygen metabolites did not change between baseline and immediately after flights, or between exercised-trained and untrained sedentary Zebra Finches (flight day, $F_{2, 13} = 0.33$, $P = 0.722$; training group, $F_{1, 15} = 0.48$, $P = 0.72$; Interaction, $F_{2, 13} = 0.23$, $P = 0.797$).

Coordinated Antioxidant Response: Do non-enzymatic and enzymatic components of the antioxidant system respond in concert?

GPx and OXY were negatively correlated after exercise training:

Prior to the start of exercise training (Day 1), change in GPx activity between baseline and immediately after their second one-hour flight was not significantly correlated

with change in non-enzymatic antioxidant capacity (Fig. 3, Training Day 1, Pearson $R^2 = 0.002$). However, after 13 days and 44 days of training the changes in GPx activity associated with acute flight were negatively correlated with non-enzymatic antioxidant capacity (Fig 3, Training Day 13: Pearson $R^2 = 0.763$, Training Day 44: Pearson $R^2 = 0.645$).

Discussion:

This experiment is one of very few studies of birds to examine changes in an individual's antioxidant system over time, and the first to examine the effects of long-term flight training on multiple aspects of the antioxidant system. We found that Zebra Finches protected against potential damage associated with acute flight by rapidly and reversibly increasing the activity of GPx and utilizing their non-enzymatic antioxidant capacity during exposure to two one-hour flight bouts per day. Exercise training (6+ weeks of such daily flights) did not alter baseline levels of these enzymatic (GPx) and non-enzymatic (OXY) components of the antioxidant system. The primary effect of exercise training was an increased coordination of these two key components of the antioxidant system; specifically, the acute increase in GPx associated with acute flight became negatively correlated with that of OXY but only after a couple weeks of training.

Hypothesis 1 (Training Effect): Does energy-intensive training alter baseline levels of enzymatic and non-enzymatic antioxidants?

Our results for enzymatic antioxidants were not consistent with Hypothesis 1 (Training Effect) whereas those for non-enzymatic antioxidants provided some support for this hypothesis. Daily exercise training did not alter the baseline levels of enzymatic antioxidants (GPx) in individual Zebra Finches throughout our 44-day experiment. However, trained birds had higher levels of OXY compared to untrained birds on Day 13 and Day 44 of training, indicating that training caused birds to maintain levels of circulating non-enzymatic antioxidants. The drop in baseline OXY in untrained birds was most likely due to natural temporal changes in antioxidant activities, as has been demonstrated in wild birds (Cohen et al., 2008a; Norte et al., 2009; Raja-aho et al., 2012). Diet-independent changes in circulating non-enzymatic antioxidants across 10 species of birds in the wild from June to July in Michigan likely reflect changes in physiology (e.g. circulating hormones, oxidative costs, or changes in how antioxidants are stored) among breeding and non-breeding stages (Cohen et al., 2008a). It is possible that the untrained Zebra Finches in this experiment experienced similar physiological changes even when not exposed to changes in photoperiod or temperature, and that subjecting birds to daily training suppressed those changes. Birds that were trained for more than one day maintained their non-enzymatic antioxidant capacity by consistently replenishing it after exercise. These results suggest that birds preserve non-enzymatic antioxidant capacity, but the timescale of this response is relatively slow as birds did not invest in or use OXY until Day 13.

Hypothesis 2 (Acute Exercise Effect): Does the antioxidant system in birds respond to short-term intense activity? Does training change how the antioxidant system responds relative to baseline?

Consistent with Hypothesis 2, Zebra Finches increased the activity of their enzymatic antioxidant system (GPx) during exposure to two one-hour flight bouts each day, and since baseline GPx did not change over time, seemingly returned to baseline by the next morning. Furthermore, training did not alter the magnitude of this acute GPx response. European Robins caught during flight also had higher levels of GPx activity compared to birds resting on stopover (Jenni-Eiermann et al., 2014). The rapid up- and down-regulation of GPx activity during flight and after rest suggests that maintaining high levels of GPx may be costly, perhaps due to the selenium co-factors needed for GPx synthesis and function (Cockell et al., 1996; Franson et al., 2011; Halliwell and Gutteridge, 2007; Zigo, 2017). Broiler chicks reared under constant cold conditions (12-14°C), and therefore unable to rest, increased oxygen requirements by 185%, had higher GPx activity initially (< 3 weeks) followed by a decrease in GPx activity (> 3 weeks) (Pan et al., 2005), further supporting the idea that maintaining upregulated GPx activity was too costly after a certain amount of time.

The consistent upregulation of enzymatic antioxidants (GPx) in Zebra Finches in response to acute exercise throughout training was different than that documented in exercising mammals (Gomez-Cabrera et al., 2008). Exercise training promotes mitochondrial biogenesis in skeletal muscle of mammals which results in an enhanced upregulation of superoxide dismutase and GPx activity with training (Powers and

Criswell, 1996; Vilela et al., 2018). In contrast, many wild birds are able to physiologically adjust to changes in exercise levels without the need for training (Dietz et al., 1999; Vezina et al., 2006; Zúñiga et al., 2016). Our results extend this to adjustments associated with a bird's antioxidant system, which can be modulated without the need for exercise training, and are in agreement with previous studies (Jenni-Eiermann et al., 2014). Racing pigeons exposed to a simulated flight by inducing muscle contractions (standardized electric muscle simulation) acutely increased GPx activity (Schoonheere et al., 2009), although whether the pigeons were able to return to pre-exercise levels quickly during rest was not measured. Additionally, reactive species production is generally higher in songbird mitochondria than rat mitochondria (Barja Gustavo, 1998; Herrero and Barja, 1997; Perez-Campo et al., 1998), which may in turn have led to different levels and timescales of response of the endogenous antioxidant system to intense exercise.

The timescale and intensity of exercise training are important to consider when examining the functional links among training, metabolic changes, and oxidative parameters (Costantini, 2019). Zebra Finches that were experimentally manipulated to fly at a pace of $\geq 911 \text{ m h}^{-1}$ had increased damage to lipids and proteins, higher uric acid, depleted thiols, and experienced no change to enzymatic antioxidants as compared to birds flying at a pace of $\leq 55.2 \text{ m h}^{-1}$ (Costantini et al., 2013). Our birds experienced a faster pace of exercise during daily flight (2.4 km h^{-1}), and experienced training for a much longer period of time. We found that the timescale on which the antioxidant system responded to exercise training was different for OXY as compared

to GPx. Non-enzymatic antioxidant capacity decreased relative to baseline (pre-flight levels) after birds were exposed to two one-hour flight sessions within 13 days of the start of exercise training, while untrained birds increased non-enzymatic capacity over the same time period. Although there may be circadian rhythms associated with antioxidant capacity (Cohen et al., 2008b; Norte et al., 2009), increased non-enzymatic antioxidant capacity from morning to afternoon in untrained Zebra Finches was likely a byproduct of the two one-hour bouts of fasting; although more work to determine how time of day, food intake, and fasting affect circulating non-enzymatic antioxidants in passerines is needed. Our test for non-enzymatic antioxidant capacity (OXY) quantifies the contribution of any antioxidants that react with hypochlorous acid, which includes proteins, thiols, ascorbate, tocopherols, and carotenoids (Costantini, 2011), and a decrease in OXY or thiols during flight perhaps indicates that those antioxidants were used and not yet recycled or replaced (Costantini et al., 2013; Skrip and McWilliams, 2016).

Hypothesis 3 (Inevitable Damage): Do energy-intensive activities cause oxidative damage to lipids in birds?

Exposure to two one-hour flight sessions each day did not cause oxidative damage to lipids in Zebra Finches at any point during training, counter to Hypothesis 3. There are several plausible explanations for why we failed to see changes in oxidative damage: (1) the antioxidant capacity of these birds sufficiently responded to protect against oxidative damage, (2) exposure to two one-hour flights per day was not sufficiently strenuous enough to cause an increase in RS to cause damage, (3) mitochondrial RS

production did not increase proportionally with an increase in oxygen consumption traditionally associated with flight, (4) an increase in oxygen consumption (state 3 to state 4 energy transition) instead caused a decrease in RS production (Barja Gustavo, 1998; Barja Gustavo, 2007; Herrero and Barja, 1997; Perez-Campo et al., 1998). However, given that Zebra Finches increased enzymatic antioxidant capacity during flight training, the first explanation seems most likely although the other three possibilities cannot be fully tested until direct measures of RS production are available and validated for birds (Logan et al., 2014; Salin et al., 2015). Tissues of animals with high rates of RS production should have a high capacity for enzymatic antioxidants to protect against damage from RS (Barja Gustavo, 2007; Cooper-Mullin and McWilliams, 2016; Costantini, 2014; Skrip and McWilliams, 2016), and any change in antioxidant enzymes reflects a disruption of oxidative status (Costantini, 2019). Our Zebra Finches increased GPx over a day of flight training, indicating they were likely exposed to a concomitant increase in RS. Although trained and untrained Zebra Finches had similar body mass and fat reserves at each stage of the experiment, we did not measure changes in body condition over a single day of flight training. Therefore, it is possible that trained birds increased protein catabolism during flight, leading to a concomitant increase in circulating uric acid, an important non-enzymatic antioxidant (Tsahar et al., 2006). An increase in uric acid along with the measured increase in GPx could further explain why we observed no differences in oxidative damage associated with flight.

In contrast to our findings, the few studies to examine oxidative challenges in wild migratory passerines have found increased oxidative damage associated with flight (Jenni-Eiermann et al., 2014; Skrip et al., 2015). However, migratory birds undergo seasonal, photoperiod induced, physiological changes to enhance fat catabolism and ultimately, their metabolic output (DeMoranville et al., 2019; Dick, 2017; Guglielmo et al., 2002; Jenni and Jenni-Eiermann, 1998; Price et al., 2011) that may result in an especially high oxidative challenge not experienced by our non-migratory Zebra Finches. Additionally, how quickly a bird shifts to using fat as fuel may inherently affect the risk of lipid peroxidation associated with flight. In general, birds use glycogen and proteins during the early stages (i.e., in the first ca. 15-20 min) of a longer-duration flight as lipid metabolism is upregulated (McWilliams et al., 2004). Birds rapidly (< 20 mins) increase lipid metabolism during these long-duration flights and lipids account for 85-95% of fuel use (Jenni-Eiermann, 2017; McWilliams et al., 2004). Although pigeons exhibit a more gradual shift (1-2 hrs) to fat oxidation during flight (Schwilch et al., 1996), American Robins (*Turdus migratorius*) in migratory condition were primarily burning fat as fuel within 20 minutes of continuous flight in a wind tunnel (Gerson and Guglielmo, 2013). Importantly, lipid fuels even short-duration, hovering flights measured as Peak Metabolic Rate (Pierce, 2005), and non-migratory, captive, Zebra Finches were relying on primarily fat with some protein catabolism for short-burst flights (Hambly et al., 2002; Nudds and Bryant, 2000; Nudds and Bryant, 2001). Therefore, the risk of damage by RS during flight may vary based on the species measured or migratory state, although more studies are needed to explore these impacts.

Hypothesis 4 (Coordinated Antioxidant System Response): Is training beneficial for birds as it is in mammals?

Novel intense exercise (Day 1) increased the enzymatic (GPx) but not the non-enzymatic antioxidant system relative to baseline in Zebra Finches. However, after 13 and 44 days of training, the acute changes in GPx activity and non-enzymatic antioxidant concentration associated with flights became strongly and negatively correlated. Our interpretation of this coordinated response requires making some assumptions about (1) the timescale over which each antioxidant parameter responds, and (2) the potential prioritization of each response. We show that GPx activity increased immediately after exercise on all days during training and, since baseline GPx did not change over time, seemingly returned to baseline by the next morning. In contrast, trained birds maintained baseline non-enzymatic antioxidants on all days during training, but non-enzymatic antioxidant capacity did not respond to acute exercise until Day 13. These results suggest that exercise induces a rapid and prioritized upregulation of GPx relative to OXY.

Careful scrutiny of Figure 3 reveals that after a couple of weeks of exercise-training the coordinated response of GPx and OXY to 2-hrs of flying occurs because individuals that more strongly upregulate GPx also “use up” more of their non-enzymatic antioxidant capacity, while individuals that less strongly upregulate GPx in response to acute exercise use less of their non-enzymatic antioxidant system. Since GPx can be localized inside cells near the mitochondria where most RS are produced

(Brigelius-Flohé, 1999; Cooper-Mullin and McWilliams, 2016), it may be beneficial for a bird to invest briefly in a costly enzymatic antioxidant as an immediate response to an energy-intensive activity. After many days of exercise-training, birds exposed to the 2-hr daily flights apparently invested in this consistent, short-term GPx upregulation as well as used more non-enzymatic antioxidant capacity perhaps in response to RS diffusing out of the cells, interacting with other molecules to form lipid peroxidation cascades, and accumulating damage.

This experiment is one of very few studies in birds to examine changes in the antioxidant system over multiple timepoints, and the first to examine the effect of long-term flight training on multiple components of the antioxidant system. Therefore, our experiment provides several insights into how the antioxidant system in songbirds responds to a given energy-intensive activity (120 min of perch-to-perch flights), and how exercise training over many weeks affects this response. The primary antioxidant response to a given energy-intensive activity was to rapidly and reversibly increase antioxidant enzyme (GPx) activity and utilize non-enzymatic antioxidants (OXY). Exercise training for 44 days did not alter pre-flight, baseline levels of GPx, while baseline levels of OXY were maintained in exercise-trained birds compared to untrained birds. The principal effect of exercise-training was an increased coordination of these two key components of the antioxidant system; specifically, the acute increase in GPx associated with acute flight became negatively correlated with that of OXY but only after a couple weeks of training.

List of Symbols/Abbreviations Used:

RS: Reactive species

GPx: Glutathione peroxidase activity

OXY: Non-enzymatic antioxidants measured by the OXY-adsorbent test

dROMs: Reactive oxygen metabolites

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Competing interests

No competing interests declared.

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Figure 2.1. Baseline changes to the antioxidant system in response to daily exercise training. A) Baseline enzymatic glutathione peroxidase activity (GPx: nmol/min/mL) in red blood cells of Zebra Finches prior to training on Day 0-1 (n = 10 Untrained, 8 Trained), prior to exercise after 12-13 days (n = 10 Untrained, 9 Trained) and prior to exercise after 43-44 days of training (n = 5 Untrained, 5 Trained; flight day $P = 0.122$; training group $P = 0.97$; Interaction $P = 0.227$). B) Baseline non-enzymatic antioxidant capacity (OXY: nmol⁻¹HClO neutralized) from plasma of Zebra Finches prior to training on Day 0-1 (n = 10 Untrained, 10 Trained), prior to exercise after 12-13 days (n = 10 Untrained, 10 Trained) and prior to exercise after 43-44 days of training (n = 5 Untrained, 5 Trained; flight day $P = 0.010$; training group $P = 0.893$;

Interaction $P = 0.026$). C) Baseline reactive oxygen metabolites (dROMs) were not significantly different for trained or untrained Zebra Finches over time ($n = 10$ Untrained, 10 Trained; flight day $P = 0.969$; training group $P = 0.898$; Interaction $P = 0.945$). Trained birds are triangles with solid lines; untrained birds are circles with dotted lines.

Figure 2.2. Acute antioxidant responses to daily exercise training over time. A)

Change in enzymatic glutathione peroxidase activity (GPx: nmol/min/mL) in red blood cells between baseline and immediately after the two-hour flight training on Day 0-1 (n = 10 Untrained, 8 Trained), Day 12-13 (n = 10 Untrained, 9 Trained), and Day 43-44 of exercise training (n = 5 Untrained, 5 Trained; flight day $P = 0.593$; training group $P = 0.002$; Interaction $P = 0.082$). B) The change in non-enzymatic antioxidant capacity (OXY: nmol⁻¹HClO neutralized) in plasma between baseline and immediately after the two-hour flight training on Day 0-1 (n = 10 Untrained, 10 Trained), Day 12-13 (n = 10 Untrained, 10 Trained), and Day 43-44 (n = 5 Untrained, 5 Trained; flight day $P = 0.300$; training group $P = 0.028$; Interaction $P = 0.041$).

Trained birds are in dark gray, untrained birds are in light gray. In contrast, reactive oxygen metabolites (dROMs) did not change between baseline and immediately after flights or between exercised-trained and untrained sedentary birds (see text for details).

Figure 2.3. The correlation between the acute change in glutathione peroxidase (GPx: nmol/min/mL) activity and acute change in non-enzymatic antioxidant capacity (OXY: nmol⁻¹HClO neutralized) on Day 1 (n = 8, stars), Day 13 (n = 9, solid squares), and Day 44 (n = 5, solid triangles) for Zebra Finches that were actively trained in a flight arena. Each point represents an individual Zebra Finch. R² values indicate Pearson correlation coefficients. Acute change represents baseline values subtracted from those measured after the 2-hr flight. For antioxidant capacity (OXY), positive values indicate an increase in non-enzymatic antioxidant capacity during the 2-hr flight, whereas negative values indicate a decrease in antioxidant capacity during the 2-hr flight.

CHAPTER 3

Exercise affects whether dietary antioxidants reach the mitochondria in the flight muscle of birds

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Exercise affects whether dietary antioxidants reach the mitochondria in the flight muscle of birds

Abstract

Whether dietary antioxidants are effective for alleviating oxidative costs associated with energy-demanding life events first requires they are successfully absorbed in the digestive tract and transported to sites associated with reactive species production (e.g. the mitochondria). Flying birds are under high energy and oxidative demands, and although birds commonly ingest dietary antioxidants in the wild, the bioavailability of these consumed antioxidants is poorly understood. We show for the first time that an ingested lipophilic antioxidant, α -tocopherol, reached the mitochondria in the flight muscles of a songbird and that its bioavailability depended on exercise. We examined the time course over which deuterated α -tocopherol appeared in the blood and mitochondria isolated from pectoral muscle of zebra finches (*Taeniopygia guttata*) exposed to an energy-intensive activity (60 min of perch-to-perch flights two times in a day) or that were relatively sedentary in cages. Deuterated α -tocopherol was found in the blood of zebra finches within 6.5 hrs and in isolated mitochondria within 22.5 hrs, but only if the birds were exercise-trained. These results indicate that exercise affected the timecourse and facilitated the absorption and deposition of vitamin E to tissues and organelles.

Introduction

Although animals have a complex endogenous antioxidant system that can and does respond to oxidative challenges, upregulating this endogenous system costs energy [1–

3]. Availability of dietary antioxidants (e.g. tocopherols, carotenoids, polyphenols) may mitigate this cost [1] and play an essential role in preventing oxidative damage during demanding life events such as growth, exercise (e.g. migration), breeding, or when faced with an immune challenge [2,4–6]. Generally, these life events increase metabolic rates, which in turn may lead to an increase in reactive species (RS) production in the mitochondria, and without antioxidant intervention, increased oxidative costs for an individual [7–9], but see [10,11]. To illustrate, blue tit (*Cyanistes caeruleus*) chicks born to mothers given dietary α -tocopherol had faster growth rates than controls in a cross-fostering experiment, and avoided an increase in oxidative damage that is often associated with faster growth [12]. Magnificent frigatebird (*Fregata magnificens*) chicks supplemented with resveratrol had lower oxidative damage to lipids caused by a viral disease and induced an immune response earlier in the progression of the disease [13]. Supplementing the diets of athletes (humans, birds, or mammals) with tocopherols prior to high-intensity exercise training protects against oxidative damage to lipids and muscles [14–16]. All these important physiological functions and benefits of dietary antioxidant supplementation require effective absorption of consumed antioxidants in the gut, and transport to the primary site of reactive species production, the mitochondria, although such essential requirements have rarely been demonstrated in wild animals [17–20].

α -Tocopherol, one of eight stereoisomers of vitamin E, is an essential dietary antioxidant (i.e., cannot be produced de novo by any vertebrate and so must be consumed) that acts as a chain-breaker in the propagation of lipid peroxidation [21–

23], and once oxidized can be recycled by vitamin C *in vivo* [2]. The livestock and poultry industries have long acknowledged vitamin E to be crucial for preventing oxidation of meat and have focused on understanding how to use dietary supplementation to increase subcellular concentrations of vitamin E [24,25]. Holstein and beef steers that consumed vitamin E-supplemented diets had higher tocopherol concentrations in muscle mitochondrial fractions as compared to steers not given dietary vitamin E [26]. Additionally, dietary supplementation increased α -tocopherol content of the subcellular membranes of various porcine and chicken muscles [20,27]. Although mitochondria are known to have high α -tocopherol concentrations, especially in the inner mitochondrial membrane [28,29], the timing and deposition of α -tocopherol from the diet to plasma to mitochondria in different tissues remains largely unknown. Further, as α -tocopherol can be recycled [2], it is unclear whether an observed increase in α -tocopherol concentrations in subcellular fractions are a direct result of deposition from the diet or an indirect effect on the recycling of α -tocopherol by ascorbic acid.

To our knowledge, no previous studies, have directly demonstrated (a) that α -tocopherol from the diet is metabolically routed to the muscle mitochondria in a volant species with relatively higher metabolic rates and potential reactive species production, and (b) how the metabolic routing is directly affected by very high-energy demanding exercise, in this case in actively-flying birds [30–32]. This is the first study in passerines to trace vitamin E from the diet into circulation and then mitochondria of the primary flight muscle, and the first in birds to examine whether exercise changes

the absorption and deposition of vitamin E into tissues and organelles. Given the high energy and oxidative costs of flying in birds, elucidating the pace and extent of metabolic routing of a commonly consumed dietary antioxidant would reveal the efficacy of such dietary sources for paying these oxidative costs. Specifically, we tested the following hypotheses: (1) Lipid-soluble dietary antioxidants are routed to the mitochondria within 2-30 hours of ingestion in agreement with previous studies on the appearance and half-life of vitamin E in plasma of livestock and humans [25,33,34] (2) The pace of this routing of dietary antioxidants depends on exercise with faster routing in flown vs. sedentary birds. We also provide the first estimates of the time course of metabolism of lipid-soluble dietary antioxidants in sedentary and flying birds.

Methods:

Exercise Training:

The zebra finches used in this experiment were part of a larger experiment focused on lipid turnover rates and changes in the antioxidant system in exercised and non-exercised birds [35,36]. The finches were obtained from a known-age captive population of birds at Sacred Heart University [35,36]. Prior to exercise training, 65 zebra finches were kept in same-sex aviaries (2.1 x 0.9 x 1.8 m = L x W x H) for 8 weeks on a 14 h:10 h light:dark cycle under full-spectrum light, with lights on at 06:00. Birds were banded with an aluminum numbered band on their right leg for individual recognition with one color band on the left leg to indicate treatment (trained males = red; untrained males = green). During those 8 weeks, birds acclimated to the

aviaries, and to a standard mixed-seed diet (Abbaseed #3700, Hillside, NJ USA) composed mainly of canarygrass (*Phalaris canariensis*). All Zebra Finches were given fresh kale, a source of ascorbic acid and α -tocopherol, once weekly [35–37].

Zebra finches (n = 65) were randomly assigned to an exercise-trained treatment group (N = 33, 16 males, 17 females) or an untrained, sedentary group (N = 32, 15 males, 17 females). The exercise-trained zebra finches were subjected daily to two one-hour periods of stop-and-go perch-to-perch flights (11:00 – 12:00 and 13:30 – 14:30) in a 6 (L) x 3 (W) x 2 m tall flight arena (see: [36, 38-40]). Each day during the 48 or 70 days of exercise training, Zebra Finches flew between perches located 4 m apart in opposite corners of the arena. A cloth wall partially divided the arena in the middle such that birds departing from a given perch flew to one side of the arena and returned on the opposite side. During the two one-hr sessions each day, a handler walked clockwise around the arena for 300 laps per hour, which resulted over the two hrs in about 1200 8-m flights or approximately 8.5 km per day. This type of short-burst flight incurs energetic costs that are approximately 3x higher than sustained flight [41]. During the two one-hour exercise periods, we removed food and water from the cages housing untrained birds to ensure both treatment (exercise-trained) and control (untrained sedentary) birds were similarly fasted.

Blood Sampling and Mitochondrial Isolation:

After 48 days of exercise training, we randomly selected 8 male zebra finches from the untrained group and 4 male zebra finches from the trained group to be gavaged with

150 μ L of deuterated α – tocopherol ((d₆)-RRR- α – tocopherol acetate, a gift from Dr. John Lodge, St. Thomas’ Hospital, London) dissolved in olive oil. The gavaged amount of deuterated α – tocopherol (30 IU/dose) was chosen based on recommended supplements for poultry and to avoid any negative effects of over supplementation of dietary antioxidants [42,43]. After 70 days of exercised training, we randomly selected a second group of 3 male zebra finches from the trained group to be gavaged with 150 μ L of deuterated α – tocopherol (δ D α -tocopherol), and 3 male zebra finches from the untrained group to be gavaged with 150 μ L of olive oil. This latter group was used to determine the natural background level of deuterated hydrogen in the blood and isolated mitochondria of zebra finches. To determine a time course for incorporation of δ D α -tocopherol into blood and mitochondria, we randomly selected whether birds were gavaged the night before (22.5 hours prior to sampling) or in the morning (6.5 hours prior to sampling). Directly after the second one-hr flight training, we sacrificed the 8 trained and paired untrained birds, took a blood sample from the severed carotid artery, and removed the right pectoral muscle. Blood samples were flash frozen in liquid nitrogen and stored at -80°C until further analysis. Pectoral muscle was placed in an ice-cold high sucrose isolation buffer solution modified from Scott et al. [44]. Mitochondria from each bird was isolated using differential centrifugation and a Percoll gradient [44–47].

Mass Spectrometry:

Sample Preparation:

Both mitochondria and blood samples were freeze dried and run on a mass spectrometer to detect amount of deuterated α – tocopherol. Blood samples, isolated mitochondria and two muscle samples were freeze dried at the University of Rhode Island and mailed to Los Alamos National Laboratories for further analysis. One muscle sample was obtained from a bird gavaged with δD - α -tocopherol in olive oil and one from a bird gavaged with only olive oil. Aliquots of each muscle sample in 15-ml glass vials (uncapped) equilibrated with the local air for approximately 4 days to allow hydrogen isotopic exchange prior to analysis. We examined these samples first to establish appropriate sample aliquot sizes for analysis of blood and isolated mitochondria and determined that 150- μg tissue yielded sufficient signal for analysis.

Statistics:

We used separate linear models for whole blood and mitochondria to determine if the amount of δD - α -tocopherol in a tissue varied with treatment group (Trained, Untrained) as referenced by background deuterium levels for each tissue, the time that a bird was gavaged (22.5 hrs or 6.5 hrs prior to sampling), and the amount of administered δD α -tocopherol. We used the information-theoretic approach based on Akaike's Information Criterion for small sample sizes (AICc) and Akaike weights (w_i) to select the best model for each tissue [48]. We report the results of the best model for each tissue (Table 3.1). We visually inspected residual plots and did not reveal any noticeable deviations from homoscedasticity or normality. Finally, we used Tukey post hoc testing with estimated marginal means to determine significance at $\alpha=0.05$ among groups.

We estimated mean retention time of $\delta\text{D-}\alpha\text{-tocopherol}$ in whole blood (τ) using an exponential decay function: $y_t = y_\infty + ae^{(-t/\tau)}$, where y_t is the measured δD value of blood at time t , y_∞ is the estimated asymptotic, or background, δD value of the blood, a is the estimated difference between the δD values of blood sampled 6.5 hrs after gavage and at equilibrium with background δD samples, t is the measured time since gavage in hours, and τ is the estimated mean retention time of $\delta\text{D-}\alpha\text{-tocopherol}$. Since the time at which δD in blood would return to background was unknown, we fit the model using a range of specified time values for the asymptote (30 to 250 hours) until the model converged, and then estimated τ from the model with the best fit. All statistics were performed with R Core Team version 3.6.0 (2019).

Results:

Whole blood $\delta\text{D-}\alpha\text{-tocopherol}$ was higher than background for trained zebra finches at both 6.5 hrs and 22.5 hrs but no time points were significantly different from background for untrained zebra finches (Figure 1, $R^2 = 0.83$, $F_{5,15} = 19.74$, $P < 0.001$). Further, trained birds sampled 22.5 hrs after gavage had 45.38 ± 14.10 lower $\delta\text{D } \alpha\text{-tocopherol}$ values as compared with those sampled 6.5 hrs after gavage (Training group: $F_{1,15} = 56.77$, $P < 0.001$; gavage time: $F_{2,15} = 7.62$, $P < 0.001$; Interaction: $F_{2,15} = 13.33$, $P < 0.001$). Deuterated $\alpha\text{-tocopherol}$, controlled for the amount given (mL $\delta\text{D-}\alpha\text{-tocopherol}$ in olive oil), appeared in mitochondria isolated from pectoral muscle of trained birds within 22.5 hrs, but $\delta\text{D-}\alpha\text{-tocopherol}$ was never higher than baseline in mitochondria isolated from untrained birds (Figure 2, $R^2 = 0.77$, $F_{7,10} = 4.77$, $P =$

0.01). The retention time (τ) estimate in whole blood converged on a value of 24.72 ± 12.76 by 180 + hrs after gavage and the median residence time, or half-life ($\ln(2) * \tau$), of gavaged Vitamin E in whole blood of trained zebra finches was estimated at 17.1 hrs. In other words, 90% of gavaged δD - α -tocopherol that appeared in the blood would be replaced or deposited into tissues within 2.4 days (Figure 3.3).

Discussion:

We show for the first time that an ingested lipophilic antioxidant reached the mitochondria in the flight muscles of a songbird and, importantly, that uptake and metabolism of this dietary antioxidant depended on exercise. Specifically, deuterated α -tocopherol was found in the blood and mitochondria of zebra finches within 6.5 and 22.5 hrs, respectively, but only if the birds were exercise-trained. These results indicate that exercise facilitated the absorption and deposition of vitamin E likely due to a need for external antioxidants to combat increased reactive species production associated with intense exercise [5,49], and/or by inducing muscle cells to actively produce more α -tocopherol rich mitochondria [50–52]. As the most important physiological function of vitamin E is as an antioxidant against free radical-mediated lipid peroxidation [53,54], the first explanation seems more likely. The absence of δD - α -tocopherol levels above baseline in untrained zebra finches suggests that any baseline reactive species generation or that associated with short flights within the cages or perching could be neutralized via endogenous antioxidants (enzymes, sacrificial molecules, or other stores of dietary antioxidants), or were in low enough of a dose to act solely as cellular messengers rather than cause overwhelming damage

[7,55]. It also indicates that birds, like mammals, have mechanisms to maintain plasma α -tocopherol concentration [34,56], and prevent the buildup of excess α -tocopherol by preventing absorption or through fast and efficient metabolism and excretion by the liver.

Although δD - α -tocopherol was detected in whole blood from trained zebra finches within 6.5 hours after gavage, deposition of α -tocopherol into pectoral muscle mitochondria took more than 6.5 hrs and less than 22.5 hrs. We estimated that 50% of ingested vitamin E in whole blood would be replaced within ~17 hrs, and 90% replaced within 2.4 days in exercised finches. This residence time of vitamin E in the blood of trained zebra finches is more rapid than in plasma of humans given deuterium-labeled foods (half-life = ~ 30 hrs), and more rapid than uptake into human erythrocytes [56–58]. Many more such studies of residence time of Vitamin E across a broader suite of species and body sizes are required before any conclusions can be made about the allometry of such metabolism (references). The implication for migratory passerines is that vitamin E available in fruits such as Arrowwood Viburnum (*Viburnum dentatum*) at fall migration stopover sites [59] would be available in their muscle mitochondria within 22.5 hours after ingestion. In other words, vitamin E in fruits eaten by birds today would be available the next night during (nocturnal) migration to defend against reactive species production in their mitochondria.

What remains to be determined is the exact mechanism by which exercise training in small songbirds enables α -tocopherol to be absorbed into the blood and then transported to the mitochondria, although our exercise training regime seems a reasonable approach. Two non-mutually exclusive hypotheses are worth testing: (1) exercise upregulates features of the gut (e.g., digestive enzymes, nutrient transporters, microbial communities) that enhance absorption of α -tocopherol to blood; (2) exercise increases fat metabolism which then facilitates metabolism and transport of dietary antioxidant into muscle mitochondria. Birds rely primarily on fatty acid oxidation to fuel the demands of intense exercise and, in the wild, mechanisms associated with fatty acid oxidation (e.g. activation of PPARs, enzymes such as β -hydroxyacyl-Coenzyme-A dehydrogenase or carnitine palmitoyl transferase) are upregulated prior to seasonal migration [60,61]. Further, migratory flights are fueled by high levels of circulating triglycerides transported by very low density lipoproteins (VLDLs) [62]. Since vitamin E is lipid soluble and is transported in circulation and delivered into cells and organelles via VLDLs [63], increased transport of fatty acids and fatty-acyl CoA into muscle mitochondria for β -oxidation likely facilitates the absorption and transport of vitamin E into the mitochondria. Therefore, the ability to benefit from ingested antioxidants where the risk of damage by peroxy radicals is highest (the mitochondria), could be an inevitable benefit of burning fats as fuel.

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Table 3.1. Model Selection to explain δD - α -tocopherol values in whole blood and mitochondria

Number of model parameters (K), maximized log-likelihood [$\log(L)$], change in second-order Akaike Information Criterion for small sample sizes (AIC_c), and AIC_c weights (w_i) for each of the candidate models used to determine if the amount of δD - α -tocopherol in a tissue varied with treatment group (Trained, Untrained) as referenced by background deuterium levels, the time that a bird was gavaged (22.5 hrs or 6.5 hrs prior to sampling), and the amount of administered δD α -tocopherol (mL olive oil mixture). Separate models were run for each tissue measured.

Blood Samples				
Model	K	$\log L$	ΔAIC_c	w_i
Treatment * Time Since Gavage	4	-87.50	0.00	0.92
Treatment * Time Since Gavage + Amount Gavaged	5	-87.26	4.95	0.08
Treatment	2	-101.50	12.80	0.002
Null	1	-108.77	24.60	0.000
Mitochondria Samples				
Model	K	$\log L$	ΔAIC_c	w_i
Treatment * Time Since Gavage + Treatment * Amount Gavaged	6	-57.51	0.00	0.88
Treatment* time since gavage	4	-60.79	4.57	0.09
Time Since Gavage	2	-68.44	7.29	0.018
Null	1	-47.68	12.4	0.002

Figure 3.1. Whole blood δD - α -tocopherol values from background (dark blue circle), trained (red) and untrained (light blue) Zebra Finches. Only trained birds had deuterium values that were higher than background, and trained birds gavaged 6.5 hours prior to sampling (red star) had higher deuterium values than birds gavaged 22.5 hours prior to sampling (red triangle). Deuterium values in the blood of untrained birds were not different from background at either sampling timepoint (gavaged 6.5 hours prior to sampling = light blue star; gavaged 22.5 hours prior to sampling = light blue triangle).

Figure 3.2. δD - α -tocopherol values of mitochondria isolated from the pectoral muscles of background (dark blue circle), trained (red) and untrained (light blue) Zebra Finches. Only trained birds had deuterium values that were higher than background, and trained birds gavaged 6.5 hours prior to sampling (red star) had lower deuterium values than birds gavaged 22.5 hours prior to sampling (red triangle). Deuterium values in the blood of untrained birds were not different from background at either sampling timepoint (gavaged 6.5 hours prior to sampling = light blue star; gavaged 22.5 hours prior to sampling = light blue triangle).

Figure 3.3 Turnover of Vitamin E (D₆-RRR- α – tocopherol acetate) calculated from whole blood collected from trained Zebra Finches. The median residence time, or half-life, of gavaged Vitamin E in whole blood of trained zebra finches was estimated at 17.1 hrs (star = δD-α-tocopherol values 6.5 hours after gavage, triangle = δD-α-tocopherol values taken 22.5 hours after gavage, open circle = background values).

CHAPTER 4

Fat stores and antioxidant capacity affect stopover duration decisions in migratory passerines: an experimental approach

Clara Cooper-Mullin and Scott R. McWilliams

Is to be submitted to *The Condor: Ornithological Applications*

Fat stores and antioxidant capacity affect stopover duration decisions in migratory passerines: an experimental approach

ABSTRACT

During migratory stopovers, birds must make decisions about when to travel and these decisions are likely contingent on their fuel stores, food availability, and antioxidant capacity. We conducted a field experiment on an offshore stopover site (Rhode Island: 41°130N, 71°330W) during fall migration to test the hypothesis that birds with greater fuel stores and antioxidant capacity have shorter stopovers and depart in a seasonally appropriate direction compared to lean birds with low antioxidant capacity. We used a 2 X 2 factorial experiment (high, *ad libitum*, or low, maintenance, food availability; dietary anthocyanins or no anthocyanins) in four species of birds that differed in migration strategy: Yellow-rumped Warblers (Myrtle subspecies, *Setophaga coronata coronata*), Hermit Thrushes (*Catharus guttatus*), Red-eyed Vireos (*Vireo olivaceus*), and Blackpoll Warblers (*Setophaga striata*). Prior to release, we attached digital VHF transmitters to assess stopover duration and direction of departure from the stopover site using the Motus network. Non-enzymatic antioxidant capacity increased during the captive refueling period for Hermit Thrushes, Red-eyed Vireos, and Blackpoll Warblers fed *ad lib* diets. Oxidative damage decreased in plasma from Red-eyed Vireos and Hermit Thrushes across all treatment groups during captivity but increased in the plasma of Blackpoll Warblers fed an *ad lib* diet. No measure of oxidative capacity changed during captivity in Yellow-rumped Warblers. Stopover duration was shorter for Hermit Thrushes, Red-eyed Vireos and Blackpoll Warblers fed *ad lib* as

compared to maintenance food but not for Yellow-rumped Warblers. Departure direction was not related to fuel stores or antioxidant condition, and birds from all species primarily reoriented north when departing Block Island. These results highlight the influence of fat stores and antioxidant capacity on the timing of migratory stopovers.

INTRODUCTION

Almost 19% of all extant bird species (~1,855 species) undergo seasonal migrations, maximizing their fitness by taking advantage of habitats that vary in resources, environmental conditions, predation, parasites, or competition (Somveille et al. 2013, 2015; Steadman 2005). Although some avian species are capable of travelling thousands of kilometers during a migratory flight (DeLuca et al. 2019, 2015; Gill et al. 2009), most migratory journeys, especially among passerines, are characterized by shorter flights punctuated by longer rest and refueling periods on stopover (Covino and Holberton 2011, Gómez et al. 2014, Pomeroy et al. 2006, Seewagen and Guglielmo 2010). These stop and go strategies vary in duration (short-distance migrants vs. Neotropical migrants) and distance of a given flight (flying over land or over the ocean) (Cohen et al. 2017a, DeLuca et al. 2019, Toews et al. 2014, Woodworth et al. 2015) but all such migratory birds must contend with the alternating gaining and expending of energy and nutrients at stopover and during flight, respectively.

A given bird will spend ~70% of its time and energy during migration at stopover sites and decisions made at those sites can ultimately affect the timing and success of a migration (Åkesson et al. 1996, Gómez et al. 2017, Warnock 2010, Weber

et al. 1998, Wikelski et al. 2003). In general, birds stop during migration to rest and refuel prior to their next migratory flight, but to do so at a high rate exposes an individual to risk of predation (Sapir et al. 2004, Woodworth et al. 2014, Ydenberg et al. 2004) and depends on the quality of the habitat (Clark and Seewagen 2019, Oguchi et al. 2017, Smith et al. 2013, Smith and McWilliams 2010). Therefore, intrinsic (current nutritional reserves, oxidative capacity, genetically determined migratory routes) and extrinsic (weather, habitat quality) conditions continuously shape behavioral decisions about when and in what direction to leave a stopover site (Lupi et al. 2019, Newton 2007a, Skrip et al. 2015, Watts et al. 2018, Winkler et al. 2014). Additionally, a species' migratory strategy or remaining distance to breeding or wintering grounds may affect the degree to which a given physiological or environmental cue influences its behavior.

Much is known about how migratory songbirds and shorebirds integrate extrinsic factors (e.g., precipitation, cloud cover wind speed and direction, day length (Able 1973, Matthews and Rodewald 2010a, b; Newton 2007b, c; Stokke et al. 2005) with intrinsic cues (e.g. fat stores;(Carlisle et al. 2012, Cohen et al. 2017b, Gudmundsson et al. 1991, Newton 2006, Seewagen and Guglielmo 2010, Smith and McWilliams 2014a) to decide when to depart stopover sites during migration. Prior research has highlighted how energetic condition can be a driving force behind departure decisions from stopover sites (Deppe et al. 2015, Eikenaar et al. 2014, Seewagen and Guglielmo 2010, Smith and McWilliams 2014a, Wikelski et al. 2003). The evidence for a relationship among fuel stores, time on a stopover site, and/or direction of subsequent flights, however, is generally derived from observations of a

migrant's natural body condition at capture and its "minimum stopover duration" (Schmaljohann 2018, Schmaljohann and Eikenaar 2017, Seewagen and Guglielmo 2010). Such evidence is at best correlational and suggestive of causation (Seewagen 2008).

We know of only two studies to date that have directly manipulated fuel stores in migratory birds to examine how body condition affects stopover duration (Dossman et al. 2016, Smith and McWilliams 2014a). Experimentally-manipulated Hermit Thrush (*Catharus guttatus*) with large fat reserves were twice as likely to depart a stopover site than lean birds, and more likely to depart the stopover site in a seasonally appropriate direction (Smith and McWilliams 2014a). Experimentally food-restricted American Redstarts (*Setophaga ruticilla*) were four times less likely to depart a spring stopover site on a given night than paired controls (Dossman et al. 2018). Given the diversity of passerine species that pass-through a specific stopover site during migration (Baird et al. 1959, Parrish 1997, Smith and Paton 2011), additional experiments are needed that examine the impact of fuel stores on stopover behavior across multiple species that differ in migration and feeding strategies.

Stopover sites may also be important for replenishing dietary antioxidant stores and recovering from oxidative damage caused during previous flights (Jenni-Eiermann et al. 2014, Skrip et al. 2015). During migratory flights, birds have elevated metabolic rates and rely on stored fats as an efficient source of fuel (McWilliams et al. 2004), which may increase the production of reactive species (RS) and propagation of oxidative damage (Costantini 2014, Jenni-Eiermann et al. 2014, Skrip et al. 2015, Weber 2009). Birds have evolved a multifaceted antioxidant system of endogenous

molecules (non-enzymatic and enzymatic) and dietary-derived vitamins to combat or repair the damage created by RS (Cooper-Mullin and McWilliams 2016). During fall migration, many passerines primarily find dietary antioxidants in the seasonally-abundant fruit they rely on for refueling (Sapir et al. 2004, Schaefer et al. 2008, Smith and McWilliams 2014b). For example, the fruit with the highest consumption rate in New England, arrowwood (*Viburnum* spp.) was highest in fat, total lipophilic antioxidant content, anthocyanins and other phenolics as compared with other local fruits (Alan et al. 2013, Bolser et al. 2012). Therefore, the ability of a bird to forage effectively for dietary antioxidants and to rebuild fat stores on a stopover site may influence behavioral decisions (e.g. length of stay on a stopover, direction of subsequent migratory flights) that affect overall timing and success of migration, but this has not been thoroughly studied.

We conducted an experimental manipulation of fat stores and availability of dietary antioxidants in four species of migratory passerines (Table 1; Blackpoll Warbler, *Setophaga striata*; Red-eyed Vireo, *Vireo olivaceus*; Hermit Thrush, *Catharus guttatus*; Yellow-rumped Warbler, Myrtle subspecies, *Setophaga coronata coronata*) on an offshore stopover site in southern New England. We then tracked these birds with miniature digital VHF transmitters using automated radio-telemetry stations (Taylor et al. 2017) to determine how condition affected the length of stopover duration and departure direction. We tested the following hypotheses: (1) non-enzymatic antioxidants increase with increased rest and refueling on stopover, (2) oxidative damage is an “inescapable hazard” of consuming fats, (3) individuals with greater antioxidant capacity and/or fuel stores are able to depart the stopover more

quickly and are less likely to exhibit reverse migration, (4) species that migrate farther (Blackpolls and Vireos), or those that are more likely to undertake a long-distance flight (Blackpolls) exhibit a greater degree of condition-dependent behavior while on stopover, and those behaviors are driven both by fat stores and antioxidant capacity.

METHODS

Study Site

Block Island (41°130N, 71°330W) is an offshore stopover site located 15.5 km off the coast of Rhode Island, USA and 22.5 km northeast of Long Island, New York, USA (Figure 1) that serves as a major stopover site for migratory birds in the fall (Reinert et al. 1981). Once on Block Island, migrating songbirds rest and refuel, consume large quantities of fruit, and then continue migration to the south, or reorient back towards the mainland of Rhode Island (Bolser et al. 2012, Parrish 1997, Smith and McWilliams 2014a).

Target Species, Capture, and Blood Sampling

We manipulated body condition of four passerine species during fall migratory stopover in 2015 and 2016 (Table 1). These species represented a range of migration strategies based on the expected distance of migration prior to and after stopover on Block Island, as well as alternative migratory strategies and food resources that may affect behavioral decisions on stopover. Yellow-rumped Warblers likely migrate shorter distances than many of the other species passing through New England in the fall, and winter farther north than any other wood warbler species (Holberton 1999, Place and Stiles 1992). Red-eyed Vireos are long-distance neotropical migrants that regularly stopover on Block Island during fall migrations. After departing from New

England, Red-eyed Vireos follow mountain ranges south until the Gulf of Mexico (Callo et al. 2013, Reinert et al. 1981), and are presumably able to stopover over many more times after they depart Block Island (Callo et al. 2013, Parrish 1997, Skrip et al. 2015). In contrast, Blackpoll Warblers stage in New England during fall migration prior to a 3-5 day non-stop migratory flight over the open ocean before reaching a wintering destination in the Caribbean and South America (Brown and Taylor 2015, 2017; DeLuca et al. 2019, 2015; Morris et al. 2016, Nisbet 1970, Skrip et al. 2015, Smetzer et al. 2017). Hatch year (HY) Red-eyed Vireos and Blackpoll Warblers exhibit prolonged stopovers (> 7 days) in the Gulf of Maine suggesting that New England stopover sites (including Block Island) could represent a crucial ecological bottleneck for these declining species (Smetzer et al. 2017). Finally, Hermit Thrushes are medium-distance migrants, spending winter in the southern United States and Central America (Smith and McWilliams 2014a). Although primarily insectivorous during the breeding season, these species shift to a fruit-rich diet in late summer (Marshall et al. 2016, Mudrzynski and Norment 2013, Parrish 1997, Woodworth et al. 2014). Although all four species consume fruits, only Yellow-rumped Warblers are able to consume and digest fruits from the waxy bayberry (*Myrica spp.*) (Afik et al. 1995, McWilliams and Karasov 2014, Parrish and Martin 1977, Place and Stiles 1992).

Mist nets were operated daily on Block Island from 60 min before dawn until sunset on all fair-weather days between September 1 and November 11, 2015 and 2016. When a target species was captured, we drew a ca. 150 μ L blood sample to measure antioxidant status and oxidative damage at capture (see below for details).

Blood was collected within 1.5 hrs of a bird entering the mist net, and within 25 mins of extraction, although the majority of individuals were sampled within 10 mins of extraction. All blood samples were centrifuged within 20 min of capture for 6 min at 5000 rpm, and the separated plasma and red blood cells were flash frozen under liquid nitrogen and later stored at -80°C prior to analysis. After blood sampling, we measured subcutaneous fat score on a 0–8 scale (Kaiser 1993), muscle score on a 0-3 scale (Bairlein et al. 1995), body mass (± 0.1 g), wing chord (± 0.5 mm) and tarsus (± 0.5 mm) to determine if an individual was suitable for inclusion in a cohort for the manipulative field experiment.

Manipulation of Fuel Stores:

Across the ca. two-month fall season, we captured 8 cohorts of 2-4 HY birds per species. Individuals for a given cohort were captured within the same capture morning (1 hr prior to sunrise to 6 hrs after sunrise) to minimize the effect of season and to maximize the chance they arrived on the same day on Block Island. We also controlled for body size by minimizing variation in wing chord ($< \pm 1.5$ mm), tarsus ($< \pm 2.5$ mm), and body weight ($< \pm 1.3$ g) at capture. All individuals had a fat score = 0 and muscle score = 0-1 at capture.

We randomly assigned individuals within a cohort to a fuel manipulation group. We manipulated food availability by providing individuals with *ad libitum* food (*ad lib* treatment; Blackpoll Warblers: n = 8, Red-eyed Vireos: n = 8, Hermit Thrush: n = 8; Yellow-rumped warblers: n = 8) or restricting food availability (maintenance treatment; Blackpoll Warbler: n = 8, Red-eyed Vireo: n = 8), Hermit Thrush: n = 8, Yellow-rumped warblers: n = 8). *Ad lib* treatment groups were provided with more

live wax moth larvae (hereafter, waxworms, *Galleria mellonella*) than could be consumed per day. Maintenance groups were given enough waxworms to maintain an individual's weight at capture (Hermit Thrush: 10 g per day; Blackpoll Warblers, Red-eyed Vireos, Yellow-rumped Warblers: 5 g per day). We manipulated antioxidant capacity by providing an additional group of *ad lib* birds (Hermit Thrush: n = 8, Yellow-rumped Warblers: n = 8) and maintenance birds (Hermit Thrush: n = 8, Yellow-rumped Warblers: n = 8) water-soluble dietary antioxidants (dietary AO). Birds fed dietary AO were gavaged once a day with anthocyanins (25 IU/day: Standard Elderberry Powder 35% per ml; Artemis International, Inc., Fort Wayne, IN, USA).

We housed birds for 3-6 days in standard stainless-steel cages (36 cm x 43 cm x 60 cm) in an outdoor aviary with protection from the elements (e.g. wind, sun, precipitation), but exposure to natural photoperiod and temperature fluctuations. We monitored food intake, body weight, fat scores and muscle scores daily. On the day of release, we obtained a second ca. 150 μ l blood sample to examine changes in antioxidant capacity and damage during captivity.

Radio-Tracking

On the day of release, we fit all birds with a digital VHF transmitter (Blackpoll Warblers, Red-eyed Vireos and Yellow-rumped Warblers: Avian NanoTag model NTQB-1; Hermit Thrush: Avian NanoTag model NTQB-2) attached with a leg-loop harness adjusted to body size (Naef-Daenzer 2007). Tags transmitted a coded burst at 166.380 MHz every 6.2 s. Tags and harness weighed 0.28 to 0.33 g ($\leq$ 3.0% body weight).

We released all individuals in a cohort on the same night. To ensure that the release location was standardized across cohorts and species, we constructed a “soft-release aviary” (following Smith and McWilliams 2014a) around a bayberry (*Myrica pensylvanica*) shrub that was 200 m from their experimental aviary and not visible from any dwelling. The soft-release aviary had a wooden frame (2.5 m tall x 1.5 m wide x 1.5 m long) with a heavy canvas covering with the bottom 1 m of the aviary left open to provide birds an exit when they voluntarily chose to do so (Figure 4.1). We placed birds in the soft-release aviary 1.5-2.5 hours after sunset by removing them from their experimental aviary, placing them in a cloth bag for transport to the soft-release aviary (e.g. 10 min walk), and then quietly removing from the cloth bag and gently placing them on a perch to rest on the branches in the aviary where they remained as we left the area. Birds exited the soft-release aviary on their own the following morning.

We used automated telemetry receiving stations in the Motus Wildlife Tracking System (Taylor et al. 2017) to determine how long individuals spent on stopover after release and their departure orientation. Importantly, two automated telemetry receivers were active on Block Island during Fall 2015 and Fall 2016 (Figure 4.1): our station was located near the north end of the island (NBI) and a station operated by the U.S. Fish and Wildlife Service was located near the south end of the island (BISE). Both receiver stations consisted of six 9-element Yagi antennas mounted 12 m above the ground. All antennas monitored a frequency of 166.38 MHz continuously, allowing birds to be detected when within range (Crewe et al. 2019b).

Analysis of Antioxidant Status:

We measured the non-enzymatic antioxidant capacity with the OXY-adsorbent test (OXY) 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 and provides an index of non-enzymatic antioxidant capacity, without being complicated by inclusion of uric acid (Alan and McWilliams 2013, Costantini 2011, Skrip et al. 2016). Oxidative damage was measured using the d-ROMs test (concentration unit = mmol l⁻¹ H₂O₂ equivalents; Diacron International, Grosseto, Italy). This test works by first decreasing the pH of the plasma to release metal ions from proteins to cleave circulating reactive oxygen metabolites (ROMs) through incubation with a solution of 0.01 M 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 ROMs in the plasma and was measured at 505 nm (Costantini 2016, 2019; Costantini et al. 2007).

Statistics:

We used R version 3.6.0 (<http://www.R-project.org/>; 2019) with the nlme package (<https://CRAN.R-project.org/package=nlme>) to perform linear mixed effects analyses to determine the influence of treatment group, change in body condition (weight, fat score, muscle score) and their interaction for each of the three oxidative parameters: plasma non-enzymatic antioxidant capacity (OXY), GPx activity in red blood cells and plasma oxidative damage (d-ROMs). We included a random effect of cohort to control for or change over the season or environmental conditions during captivity.

The best model for each oxidative parameter was chosen based on Akaike's information criterion, and then fitted with restricted maximum likelihood (Bolker 2017, Bolker et al. 2009)

To examine stopover duration differences across all species, we first pooled together all *ad lib* and maintenance groups and used a linear mixed model with fixed effects for species and treatment group (*ad lib* or maintenance) and a random effect of cohort. We then ran separate linear mixed models for Hermit Thrush and Yellow-rumped Warblers with fixed effects for species and all treatment groups (*ad lib*, *ad lib* + antioxidants, maintenance, maintenance + antioxidants) and a random effect of cohort. We used AICc model selection for each species and antioxidant measurement (Bates et al. 2015, Bolker 2017). We report associated results as mean $\pm$ SE.

Determining Orientation:

We visually inspected plots of signal strength over time to determine when and from which tower each individual departed Block Island. We calculated vanishing bearings for all individuals and obtained mean bearings for species and treatment group (Crewe et al. 2019a). We used AICc model selection to compare models for random orientation (uniform), a single preferred departure direction (unimodal), or two preferred departure directions (bimodal) using the package CircMLE (Fitak and Johnsen 2017, Schnute and Groot 1992). To maintain appropriate sample sizes, we pooled across antioxidant treatments to compare departure orientation for birds given an *ad lib* or maintenance diet using a pairwise Mardia-Watson-Wheeler test.

RESULTS

Change in Condition Measures During Captivity:

All cohorts spent an average of 2.93 ± 0.99 days in captivity prior to release (range = 2 – 6 days). Individuals on a maintenance diet did not significantly change weight, fat scores, or muscle scores during captivity as prescribed, whereas birds on an *ad lib* diet significantly increased on all indicators of fuel stores during captivity (Figure 4.2, $P < 0.001$).

Non-enzymatic antioxidant capacity (OXY) increased during captivity for Blackpolls fed an *ad lib* diet as compared with Blackpolls fed a maintenance diet (Figure 4.3, Food Manipulation: $F = 13.60$, $df = 1$ and 2 , $P = 0.004$). OXY also increased during captivity for Red-eyed Vireos fed an *ad lib* as compared with Vireos fed a maintenance diet (Figure 4.3, $F = 10.63$, $df = 1$ and 4 , $P = 0.03$). OXY increased during captivity for Hermit Thrushes fed an *ad lib* diet and increased more for the treatment groups given dietary AO (Figure 4.3, Food Manipulation: $F = 6.79$, $df = 1$ and 2 , $P = 0.01$; Antioxidants: $F = 13.72$, $df = 1$ and 2 , $P = 0.001$). Non-enzymatic antioxidant capacity was not affected by the dietary treatments in Yellow-rumped Warblers (Figure 4.3, Food Manipulation: $F_{1,2} = 3.79$, $df = 1$ and 2 , $P = 0.21$; Antioxidants: $F = 3.27$, $df = 1$ and 2 , $P = 0.10$).

Oxidative damage (dROMs) increased in the plasma from Blackpolls fed an *ad lib* diet, and did not change in plasma from Blackpolls fed a maintenance diet (Figure 4.3, Food Manipulation: $F = 104.90$, $df = 1$ and 2 , $P = 0.009$, $F = 2.40$, $df = 1$ and 2 , $P = 0.026$; Interaction, $F = 8.31$, $df = 1$ and 2 , $P = 0.011$). Oxidative damage decreased during captivity in Red-eyed Vireo plasma, but was not different among treatments (Figure 3, Food Manipulation: $F = 1.15$, $df = 1$ and 3 , $P = 0.31$). Oxidative damage decreased during captivity in Hermit Thrush plasma, but was not different among

treatments (Figure 3, Food Manipulation: $F = 1.12$, $df = 1$ and 3 , $P = 0.30$; Dietary AO Treatment: $F = 5.62$, $df = 1$ and 3 , $P = 0.02$). Oxidative damage was not affected by dietary treatment (fuel stores or dietary AO) and did not change consistently during captivity in the plasma from Yellow-rumped Warblers ($F = 3.21$, $df = 1$ and 3 , $P = 0.89$).

Stopover Length and Condition:

Blackpoll Warblers that were lean departed Block Island later than their *ad lib* fed counterparts (Figure 4.4, *ad lib*: 2.75 ± 4.71 days, maintenance: 16.51 ± 4.71 days, GLMM, $F = 7.52$, $P = 0.001$). Similarly, Red-eyed Vireos that were lean departed Block Island later than their *ad lib* fed counterparts (Figure 4.4, *ad lib*: 3.35 ± 2.29 days, maintenance: 9.15 ± 1.51 days, $F = 2.96$, $P = 0.09$; respectively). Hermit Thrushes fed a maintenance diet but not given dietary antioxidants had the longest stopovers (maintenance: 16.01 ± 3.02 days, GLMM, $F = 6.57$, $df = 3$ and 20 , $P = 0.003$). Hermit Thrushes fed a maintenance diet with dietary AO had stopover lengths that were similar to those given an *ad lib* diet (maintenance + dietary AO: 9.62 ± 2.27 , *ad lib*: 6.47 ± 0.59 , *ad lib* + dietary AO: 5.81 ± 0.52). There was no effect of diet treatment on the number of days Yellow-rumped Warblers spent on stopover (maintenance: 18.41 ± 7.37 , maintenance + dietary AO: 13.82 ± 5.42 , *ad lib*: 11.96 ± 9.00 , *ad lib* + dietary AO: 11.68 ± 9.29 , GLMM, $F = 0.69$, $df = 3$ and 20 , $P = 0.41$). Across species, Red-eyed Vireos fed maintenance diets had shorter stopover durations than Blackpoll Warblers fed maintenance diets ($P = 0.02$), and Yellow-rumped Warblers had the longest stopover durations ($P = 0.04$).

Across the *ad lib* and maintenance groups, Red-eyed Vireos that increased non-enzymatic antioxidant capacity the most during the captive period tended to have the shortest stopover lengths (Figure 4.5, Food Manipulation: $F = 23.22$, $P = 0.009$; Change in OXY: $F = 9.28$, $P = 0.04$; Interaction: $F = 5.81$, $P = 0.074$). For Blackpolls, however, change in antioxidant capacity influenced stopover length only for birds on the maintenance diet (Figure 4.5, Treatment: $F = 6.09$, $P = 0.04$, Change in OXY: $F = 23.22$, $P = 0.017$, Interaction: $F = 0.65$, $P = 0.46$). Individual change in OXY during captivity was not associated with stopover length in Hermit Thrush and Yellow-rumped Warblers (Hermit Thrush, Treatment: $F = 0.09$, $P = 0.68$, Change in OXY: $F = 0.03$, $P = 0.82$, Interaction: $F = 0.62$, $P = 0.56$; Yellow-rumped Warblers, Treatment: $F = 0.81$, $P = 0.24$, Change in OXY: $F = 0.72$, $P = 0.15$, Interaction: $F = 0.65$, $P = 0.46$).

Departure Directions:

About 50% of Blackpoll Warblers oriented northeast after departure from Block Island, whereas the rest exhibited no clear orientation pattern and orientation did not appear to be related to fat stores at release (Figure 4.6, $\phi_1 = 77.81^\circ$, $k_1 = 7.01$, $\lambda = 0.50$). Similarly, about 50% of Red-eyed Vireos oriented northeast, whereas the rest exhibited no clear orientation pattern and orientation did not appear to be related to fat stores at release (Figure 4.6, $\phi_1 = 81.88^\circ$, $k_1 = 58.44$, $\lambda = 0.50$). Hermit Thrushes had a bimodal pattern of orientation when departing Block Island. Thrushes oriented north towards the mainland of Rhode Island or to the southeast (Figure 4.6, $\phi_1 = 140.15^\circ$, $k_1 = 1.45$, $\phi_2 = 355.35^\circ$, $k_2 = 1.45$, $\lambda = 0.41$). There was no evidence that Hermit Thrushes oriented differently based on fat stores at the time of release ($W = 0.25$, $df =$

2, $P = 0.88$). Half of the Yellow-rumped Warblers released oriented north towards mainland Rhode Island, but the other half exhibited no clear orientation pattern (Figure 4.6, $\phi_1 = 341.25^\circ$, $k_1 = 58.44$, $\lambda = 0.50$). There was no evidence that Yellow-rumped Warblers oriented differently based on fat stores at the time of release ($W = 1.09$, $df = 2$, $P = 0.58$).

DISCUSSION

Hatch-year Hermit Thrush, Red-eyed Vireos, and Blackpoll Warblers fed *ad libitum* for several days in captivity had higher fuel stores (fat and antioxidant capacity) and left this offshore stopover site at least 2 times faster than lean birds, while there was not a similar response for Yellow-rumped Warblers. These results indicate that an individual's fuel stores can act as a cue to continue migration, but that these cues may not be consistent among species. Ultimately, we found that migrants adjust their stopover strategies based on physiological cues for habitat quality as well as distance to their wintering destination.

Hypothesis 1: Non-enzymatic antioxidants increase with increased rest and refueling on stopover

During captivity, Blackpoll Warblers, Red-eyed Vireos, and Hermit Thrushes fed an *ad lib* diet increased their non-enzymatic antioxidant capacity higher than individuals of the same species fed a maintenance diet, indicating that food availability on stopover allows individuals to build antioxidant capacity. These results confirm previous work from Block Island that found fat stores positively correlated with non-enzymatic antioxidant capacity in Red-eyed Vireos and Blackpoll Warblers (Skríp et

al. 2015). Fasting-refeeding trials also boosted non-enzymatic antioxidant capacity in Northern Wheatears (*Oenanthe oenanthe*), although those results were primarily attributed to increased circulating uric acid (Eikenaar et al. 2016). Since OXY-adsorbent test does not measure uric acid, a highly potent but hard to interpret antioxidant, these results indicate that birds were also able to rebuild other circulating non-enzymatic antioxidants (e.g. other sacrificial molecules, precursors for enzymatic antioxidants) while building fat stores (Carro et al. 2012, Costantini 2011, Skrip and McWilliams 2016, Tsahar et al. 2006).

Hermit Thrushes fed an *ad lib* diet with daily doses of anthocyanins (a hydrophilic antioxidant) were able to increase their non-enzymatic antioxidant capacity higher than those on an *ad lib* diet alone, and Blackpolls and Vireos were able to increase their non-enzymatic antioxidant capacity to similar levels without additional dietary antioxidants. Since waxworms can have up to 69.2 mg/kg of vitamin E (Finke 2015), it seems likely this lipophilic antioxidant was responsible for increased non-enzymatic antioxidant capacity of the *ad lib* fed birds. These results suggest that Blackpolls and Vireos may be able to absorb lipophilic antioxidants (vitamin E from waxworms) better than Hermit Thrushes, and that Hermit Thrushes may rely more heavily on hydrophilic antioxidants. Additionally, Hermit Thrushes on a maintenance diet with dietary antioxidants had comparable levels of non-enzymatic antioxidants to those on an *ad lib* diet without additional antioxidants. This confirms that (1) Hermit Thrushes are likely able absorb these hydrophilic antioxidants and (2) dietary sources of antioxidants can influence circulating levels of non-enzymatic antioxidant capacity directly. On stopover in the fall, birds primarily forage on locally

abundant fruits, but not all fruits are high in both fat and antioxidant content (Alan et al. 2013, Benvenuti et al. 2004, Bolser et al. 2012, Schaefer et al. 2008). These results also suggest that consuming fruits with low fat content but sufficient antioxidants, such as Winterberry (*Ilex verticillata*; (Alan et al. 2013), while on stopover may still be beneficial for birds to rebuild antioxidant capacity. However, further research on the absorption (Beaulieu and Schaefer 2013, Skrip et al. 2016), possible microbial contribution (Laparra and Sanz 2010, Saag et al. 2011), or inhibition of dietary antioxidants in migratory birds is necessary.

In contrast, Yellow-rumped Warblers did not build antioxidant capacity when fueling with fats or dietary antioxidants. There are three possible, non-mutually exclusive, explanations for why Yellow-rumped Warblers responded differently to our dietary treatments than Hermit Thrushes, Blackpoll Warblers, and Red-eyed Vireos. First, Yellow-rumped Warblers have specialized digestive enzymes that facilitate their ability to digest bayberry fruits (Afik et al. 1995, Place and Stiles 1992). It is possible that this adaptation has changed how Yellow-rumped Warblers absorb these dietary antioxidants. Second, Yellow-rumped Warblers may rely more heavily on other aspects of their antioxidant system (e.g. enzymatic antioxidants) to combat damage during migration (Cooper-Mullin and McWilliams 2016). Third, these warblers are shorter-distance migrants that winter relatively farther north than the other species in this study, and may not need to build reserve antioxidant capacity (Metzger and Bairlein 2011). Therefore, the degree to which birds build antioxidant capacity while refueling may be linked to their migratory strategy, distance to migratory destination, and species-specific physiology.

Hypothesis 2: Oxidative damage is an “inescapable hazard” of consuming fats

Although all birds given an *ad lib* diet in captivity increased fat stores, only Blackpoll Warblers had higher circulating lipid damage. Red-eyed Vireos and Hermit Thrushes had decreased circulating lipid damage across treatment groups, and there was no change in circulating lipid hydroperoxides during captivity in Yellow-rumped Warblers. Although Skrip et al. (2015) found that fat stores were positively correlated with circulating lipid hydroperoxides in Blackpoll Warblers and Red-eyed Vireos on Block Island during fall migration, oxidative measures in Red-eyed Vireos not strongly correlated with fat mass, and oxidative damage was not correlated with fat score. Therefore, within an individual, circulating oxidative damage to lipids may decline as that individual builds fat, but variation among individuals may still result in population level correlations (Costantini 2019, Skrip et al. 2016). Further, Northern Wheatears exposed to a fasting – refeeding experiment had similar circulating malondialdehyde levels (a secondary byproduct of damage to polyunsaturated fatty acids) to controls, and malondialdehyde levels were not correlated with food intake (Eikenaar et al. 2016). Our results indicate that there is variation among how species respond to the oxidative challenge associated with building fat stores.

Hypothesis 3: individuals with greater antioxidant capacity and/or fuel stores are able to depart a stopover site more quickly and are less likely to exhibit reverse migration

For the first time across multiple species, we demonstrated that Blackpoll Warblers, Red-eyed Vireos and Hermit Thrushes fed an *ad lib* diet departed an offshore stopover site prior to their maintenance-fed counterparts. However, we saw no impact of treatment on stopover duration of Yellow-rumped Warblers. These results generally agree with the other two studies that have experimentally manipulated fuel stores of migrating songbirds on stopover sites (Dossman et al. 2018, Smith and McWilliams 2014a), and suggest that individuals on stopover with sufficient fuel stores are likely to leave as soon as weather allows (Schmaljohann et al. 2012, Smith and McWilliams 2014a). Past studies have also shown that if birds experience a loss of energy stores or a low fuel deposition rate, the probability of departure from stopover was high, whereas high rates of energy accumulation were observed in birds staying at a stopover site (Aamidor et al. 2011, Morganti et al. 2011, Paxton and Moore 2017, Schaub et al. 2008, Schmaljohann et al. 2013). Maintenance birds spent more time on Block Island than their *ad lib* counterparts, indicating that Block Island is a higher quality stopover site as compared with other coastal stopovers.

For the first time, we demonstrated that antioxidant stores can influence stopover duration in long and short distance migratory birds. Hermit Thrushes fed a maintenance diet alone had longer stopovers than those fed a maintenance diet supplemented with anthocyanins, indicating that availability of dietary antioxidants without fat deposition can influence stopover duration. Further, Red-eyed Vireos that were able to increase their non-enzymatic antioxidant capacity during captivity had shorter stopovers than those that did not, as did Blackpoll Warblers fed a maintenance

diet. Therefore, large antioxidant stores seem to provide migratory birds with an additional cue indicating “good condition”, although more research on the mechanisms and molecular underpinnings of these signals is needed.

Further, if individuals that build high antioxidant capacity can leave stopover faster, stopovers with fruits that are high in both fats and antioxidants, such as Northern Arrowwood, *Viburnum recognitum*, are extremely valuable resources for Neotropical and other frugivorous migrants (Alan et al. 2013, Bolser et al. 2012). Since passerines may not be able to distinguish lower caloric content of non-native fruits (Drummond 2005, Mudrzynski and Norment 2013, Smith and Hatch 2017), stopovers with non-native fruits that are low in antioxidant capacity or fat resources could act as stopover sinks, causing delays if birds are not able to refuel quickly.

There are several possibilities for why we did not observe differences in stopover duration for Yellow-rumped Warblers fed different diets. First, Yellow-rumped Warblers have a diet source (bayberry) that is unavailable to all other species in this study and, therefore, do not have to compete for food resources to the same degree as other migrants (Bolser et al. 2012, Place and Stiles 1992, Podlesak et al. 2005). Birds fed a maintenance diet may have been able to fuel relatively quickly after leaving captivity, essentially erasing any differences in stopover duration when compared with *ad lib* fed Yellow-rumped Warblers. Second, Yellow-rumped Warblers were likely near the end of their migratory journey once they reach New England (Murray Jr. and Murray 1979), although clear understanding of the migratory patterns among different populations of this species remains murky. Therefore, minimizing

time on stopover may not be as important for this species as conserving energy (Hedenström and Ålerstam 1997).

The majority of the individuals in this study departed stopover heading north, or reorienting, after stopover. Only Hermit Thrushes exhibited a true bimodal departure, half the birds departed in a direction suggesting reorientation, and half departed to the southeast (forward migration towards New York). Orientation of departure flight was not associated with condition for any species studied. This is in contrast to a previous experiment on Block Island that found Hermit Thrushes given an *ad lib* diet had a higher probability of continuing migration in a seasonally appropriate direction (Smith and McWilliams 2014a). As fruit resources on Block Island can vary from year to year, as well as within a season, it is possible that the distribution of food on the stopover landscape influenced Hermit Thrush departure orientation (Bolser et al. 2012, Smith and McWilliams 2014b, Smith et al. 2007).

In the Gulf of Maine, Blackpoll Warblers and Red-eyed Vireos often leave coastal stopovers by orienting inland (Brown and Taylor 2017, Smetzer and King 2018). However, those movements are often explained as birds fleeing poor refueling habitat at coastal sites (Mehlman et al. 2005, Woodworth et al. 2015). Since we experimentally manipulated birds to have large fat stores, and Block Island offers good habitat for refueling (Reinert et al. 1981, Skrip et al. 2015, Smith and McWilliams 2010), there are likely other drivers of reorientation behavior for these species. Most birds that stopover on Block Island are HY individuals that presumably were displaced offshore on nights with strong northwest winds, so birds that reorient may be retracing their steps back to familiar habitat before continuing migration

(Åkesson et al. 1996, Baird et al. 1959, Taylor et al. 2011). Although further research is necessary to determine whether individuals departing Block Island on a given night were exhibiting true migratory movement or were exhibiting extended stopover or landscape scale movement (Brown and Taylor 2017, Mills et al. 2011, Taylor et al. 2011), initial departure direction was not influenced by condition.

Hypothesis 4: Species that migrate farther, or those that are more likely to undertake a long-distance flight exhibit a greater degree of condition-dependent behavior while on stopover, and those behaviors are driven both by fat stores and antioxidant capacity.

Blackpoll Warblers fed a maintenance diet stayed at this offshore stopover site up to 6 times longer than those fed an *ad lib* diet and Red-eyed Vireos fed a maintenance diet stayed up to 3 times longer than those fed an *ad lib* diet. These results indicate that departure decisions are strongly tied to fuel stores for these Neotropical migrants, and that differences in migratory strategies among these two species may fine-tune these decisions, especially when a bird is lean. Red-eyed Vireos fed a maintenance diet left this stopover site faster than Blackpoll Warblers of the same treatment, possibly because Blackpoll Warblers were facing a greater geographical barrier (DeLuca et al. 2019, 2015). Many Blackpoll Warblers initiate a trans-Atlantic crossing from New England and fly nonstop over the North Atlantic Ocean to the Caribbean or South America (DeLuca et al. 2019, 2015; Nisbet et al. 1995), a journey that requires at least 10-12g of fat stores (Boal 2014, Klaassen 1996). Among Blackpolls measured at 3 coastal stopover sites and 9 inland stopover sites

during migration, individuals on Block Island were the heaviest and had the largest fat stores (Morris et al. 2016). Thus, building fat stores may be particularly important for this species in this region. However, there were no differences in stopover duration for birds that are facing an ecological barrier (Blackpoll Warblers), shorter-distance migrants (Hermit Thrush) and migrants that are more likely to have multiple stopovers after Block Island (Red-eyed Vireos) fed an *ad lib* diet.

Yellow-rumped Warblers had the longest stopovers of all the species.

Although Yellow-rumped Warblers and Hermit Thrush have similar migration travel distances, the wintering ecology of these species may shape time constraints on stopover (Alvarado et al. 2014, Hunt and Flaspohler 1998, Kwit et al. 2004). Hermit Thrush often set up territories on their wintering grounds, and individuals that arrive first choose the highest quality areas (Kwit et al. 2004). In contrast, Yellow-rumped Warblers usually flock up and forage together on the wintering grounds, a much less competitive lifestyle (Kwit et al. 2004). Therefore, Hermit Thrushes may be more time constrained on migration than Yellow-rumped Warblers.

Since delays during migration caused by poor condition due to poor weather or food availability or quality at stopover sites are likely to carry over to influence population dynamics (Marra et al. 2005), understanding how differences in condition (fat and antioxidants) affects different species of birds during migration can help shape conservation and management decisions about the types of plants birds rely on during migration (Deppe and Rotenberry 2008, Smith and Hatch 2017) and where best to put those resources. Further, the importance of fat stores and antioxidant capacity in shaping the length of stopovers across three of four migratory species underscores the

value of high-quality habitat in coastal areas, especially prior to geographical barriers. Since these locations and habitats are under threat from human encroachment and sea level rise (Both et al. 2010, Rosenberg et al. 2019), foraging opportunities for migratory songbirds may be reduced, limiting birds' ability to gain sufficient fat for nonstop flights over water.

Migratory bird numbers have experienced shocking declines since the 1970s, likely due to the difficulty of preserving habitat across large spatial scales of migration (Rosenberg et al. 2019). For example, Blackpoll Warbler populations have lost an estimated 0.75 billion breeding individuals since 1970. The median capture date of Blackpoll Warblers on stopover is shifting later (Morris et al. 2016), but native fruiting plants are not necessarily responding in a similar fashion. Therefore, there is the potential for a developing mismatch in peak fruit and migratory bird abundance. Additionally, as invasive plants have later fruiting phenology than native ones (Gallinat et al. 2018), shifts in the timing of migration may also increase the chance that migratory birds will consume and propagate the spread of invasive plants.

The strength of this study lies in an experimental approach based on temporary short-term captivity of birds undergoing actual migration across multiple species, and the ability to automatically track birds after release. The paired design allows us to confidently state that fat stores and antioxidant capacity lead birds to leave stopover sooner and that condition-dependent behavior is linked to a species migratory ecology.

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TABLE 4.1 Total migration distances and experimental treatment groups for Blackpoll Warblers, Red-eyed Vireos, Hermit Thrushes, and Yellow-rumped Warblers

Species	Breeding Area	Wintering Area	Total Fall Migration Distance	References	Manipulation Treatment
NEOTROPICAL MIGRANTS					
Blackpoll Warbler (<i>Setophaga striata</i>)	Boreal Forest (western Alaska to Newfoundland, Nova Scotia and Northern New England)	Northern South America east of the Andes Mountains	3820 – 21600 km (up to 2770 km nonstop: New England to South America) ₁	DeLuca et al. 2019, 2015	- Ad Lib - Maintenance
Red-eyed Vireo (<i>Vireo olivaceus</i>)	Eastern North America	Northern South America (Columbia, Venezuela, Guyana, Suriname, Ecuador, Peru, Western Brazil)	5264 km (nonstop over Gulf of Mexico) ₁	Callo et al. 2013	- Ad Lib - Maintenance
NORTH AND CENTRAL AMERICAN MIGRANTS					

Hermit Thrush (<i>Catharus guttatus</i>)	North America (above ~37° Latitude)	North and Central America	0 – 2887 km ^{1,2}	Alvarado et al. 2014, Smith and McWilliams 2010	<ul style="list-style-type: none"> - Ad Lib - Ad Lib + Dietary Antioxidants - Maintenance - Maintenance + Dietary Antioxidants
Yellow-rumped Warbler (Myrtle subspecies, <i>Setophaga coronata coronata</i>)	Coniferous forests of Canada and north east United States	Primarily southern United States, Baja, Mexico, and Caribbean	0 – ~5000 km ^{2,3}	Hunt and Flaspohler 1998	<ul style="list-style-type: none"> - Ad Lib - Ad Lib + Dietary Antioxidants - Maintenance - Maintenance + Dietary Antioxidants

1 Estimated based on geolocator studies (cited)

2 Occasionally winters on Block Island, Rhode Island

3 Coarse estimate based on species range

Figure 4.1 A) Location of Block Island ($41^{\circ}13'N$, $71^{\circ}33'W$), Rhode Island, USA with the two automated radio telemetry towers marked (NBI: northern Block Island and BISE: south east Block Island). B) Picture from the ground of a Hermit Thrush in the soft-release aviary. Note that this was a re-enactment of what it would look like for a thrush to leave in the morning. Radio-tagged birds were placed in this soft-release aviary early in the night and were allowed to leave when they wanted, usually at dawn the next day. Photo by ©KarineAigner.

Figure 4.2. Boxplot for change in body mass (g) for individuals of each species (Blackpoll Warbler, Hermit Thrush, Yellow-rumped Warbler, Red-eyed Vireo) after the *ad lib* diet treatment (red), *ad lib* diet with dietary antioxidants (yellow), the maintenance diet with dietary antioxidants (light blue) or after the maintenance diet treatment (navy). Boxes represent 95% confidence intervals; whiskers represent 1.5 times the interquartile range and outliers are represented by black dots.

A) Blackpoll Warblers

B) Red-eyed Vireo

C) Hermit Thrush

D) Yellow-rumped Warbler

Figure 4.3. Change in non-enzymatic antioxidant capacity (OXY) and change in oxidative damage (dROMs) for each individual by cohort between when sampled at capture and at release. Each dot represents an individual and the line indicates the magnitude of change during captivity (sample at release – sample at capture). For Blackpoll Warblers (A) and Red-eyed Vireos (B) fed an *ad lib* diet (red) or a maintenance diet (navy). For Hermit Thrush (C) and Yellow-rumped Warblers (D) fed an *ad lib* diet (red), an *ad lib* diet with dietary anthocyanins (yellow), a maintenance diet (navy), or a maintenance diet with dietary anthocyanins (light blue).

Figure 4.4. Stopover duration (days on Block Island after release from captivity) for (A) Blackpoll Warblers fed an *ad lib* diet (red) or a maintenance diet (navy). (B) Red-eyed Vireos fed an *ad lib* diet (red) or a maintenance diet (navy). (C) Hermit Thrush fed an *ad lib* diet (red), an *ad lib* diet with dietary anthocyanins (yellow), a maintenance diet (navy), or a maintenance diet with dietary anthocyanins (light blue). (D) Yellow-rumped Warblers fed an *ad lib* diet (red), an *ad lib* diet with dietary anthocyanins (yellow), a maintenance diet (navy), or a maintenance diet with dietary anthocyanins (light blue). Stars indicate significant differences among groups (* < 0.05; ** < 0.005).

Figure 4.5. Relationship between non-enzymatic antioxidant capacity during captivity (blood sample prior to release – blood sample at capture) and days spent on stopover after release for (A) Blackpoll Warblers fed an *ad lib* diet (red) or a maintenance diet (navy). (B) Red-eyed Vireos fed an *ad lib* diet (red) or a maintenance diet (navy). There were no significant relationships for Hermit Thrush or Yellow-rumped Warblers (see text for details).

Figure 4.6. Vanishing bearings for birds departing from Block Island, RI for (A) Blackpoll Warblers fed an *ad lib* diet (red) or a maintenance diet (navy). (B) Red-eyed Vireos fed an *ad lib* diet (red) or a maintenance diet (navy). (C) Hermit Thrush fed an *ad lib* diet (red), an *ad lib* diet with dietary anthocyanins (yellow), a maintenance diet (navy), or a maintenance diet with dietary anthocyanins (light blue).