

REVIEW

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

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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.

KEY WORDS: Fat oxidation, Bird migration, Reactive species

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 and McWilliams, 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 ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO^{\cdot}) (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 H_2O_2 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

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List of symbols and abbreviations

BMR	basal metabolic rate
CAT	catalase
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
H ₂ O ₂	hydrogen peroxide
NO [•]	nitric oxide
O ₂ ^{•-}	superoxide
ONOO ⁻	peroxy nitrate
ROS	reactive oxygen species
RNS	reactive nitrogen species
RS	reactive species
SOD	superoxide dismutase
UCPs	uncoupling proteins

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

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 oxidation 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.

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.

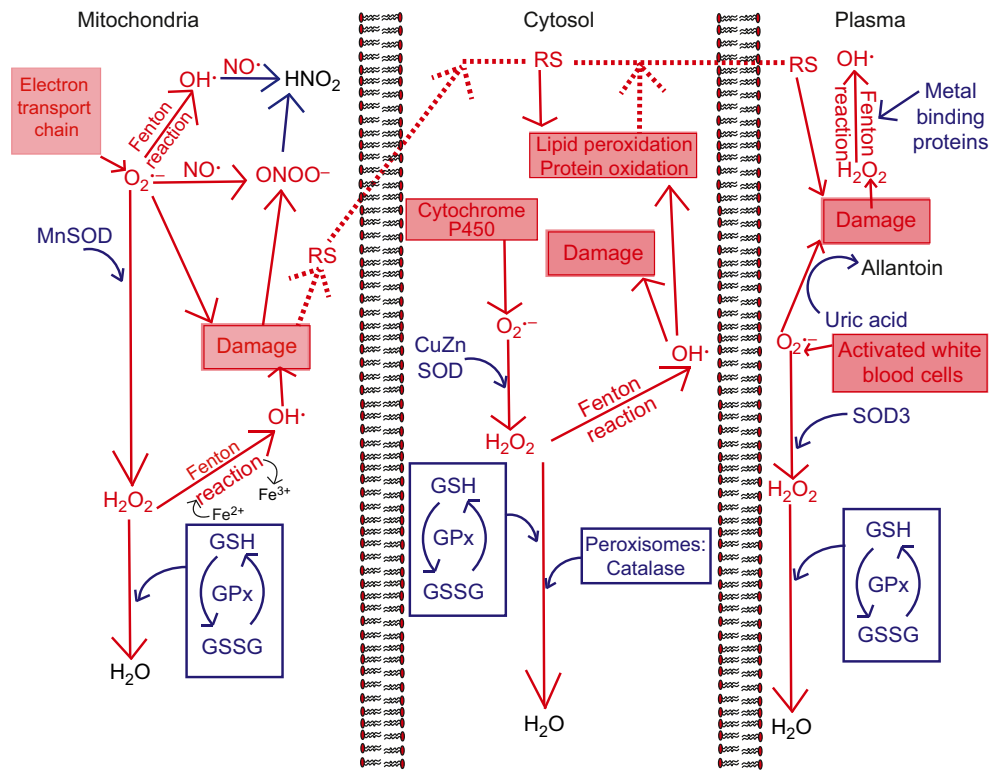


Fig. 1. Examples of primary ways in which reactive species are generated and endogenous antioxidant protection occurs in the mitochondria, cytosol and plasma. Processes that generate reactive species (RS) are shown in red, and antioxidant protection is shown in blue. Non-reactive end products of the interactions between RS and antioxidants are in black. The primary RS generated are superoxide ($O_2^{\cdot-}$) and the hydroxyl radical (HO^{\cdot}). $O_2^{\cdot-}$ can react with nitric oxide (NO^{\cdot}) to create peroxynitrate ($ONOO^{\cdot}$), a lipid-soluble radical, or NO^{\cdot} can react with OH^{\cdot} to form non-reactive nitrous acid (HNO_2). In the mitochondria, $O_2^{\cdot-}$ is primarily broken down by manganese superoxide dismutase (MnSOD) to H_2O_2 , which either forms HO^{\cdot} 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 $ONOO^{\cdot}$, 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, $O_2^{\cdot-}$ 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.

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 $30\times$ 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 (Crisuolo 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 (Crisuolo 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-

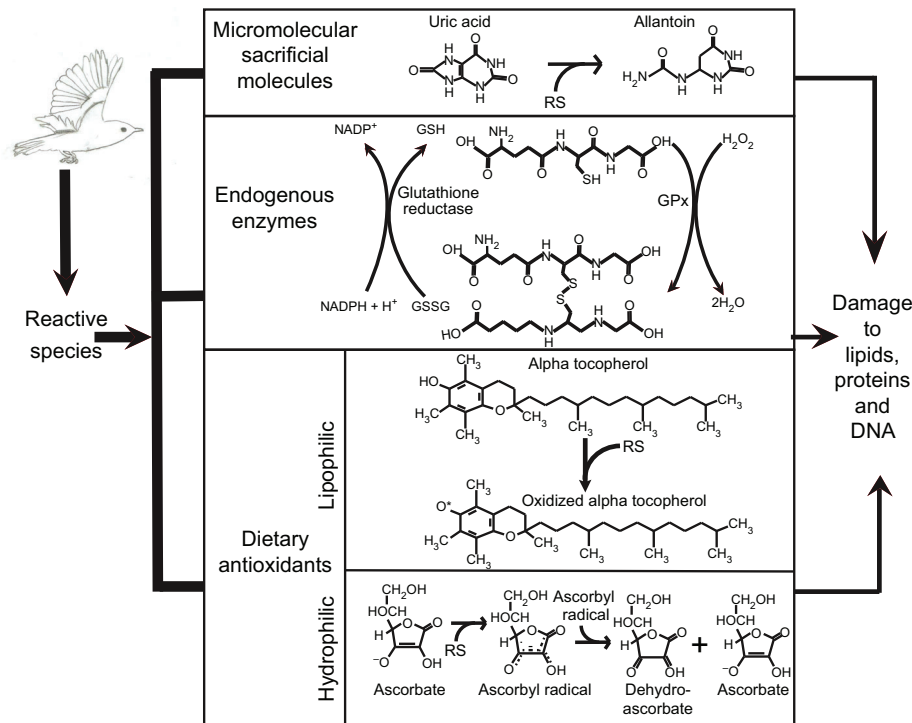


Fig. 2. The multifaceted antioxidant filter available to birds. Although endurance flight produces reactive species (RS), birds and other animals have a multifaceted antioxidant filter that can mitigate damage. The antioxidant filter consists of micromolecular sacrificial molecules, endogenous enzymes 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.

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 (Skríp 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*) (Skríp 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 (Skríp 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 (Skríp 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

Table 1. Summary of studies that examined antioxidant capacity and oxidative damage during flight

Bird species	Comparison groups	Antioxidants		Damage		Citation
		Measurement	Result	Measurement	Result	
Non-migratory						
Homing pigeon (<i>Columba livia</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 versus 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	↓	Serum hydroperoxides ^b	↑	Costantini et al. (2013)
		Carotenoids, catalase, GPx ^d , SOD ^e	–	Protein carbonyls	↑	
		Uric acid	↑			
Migratory						
European robin (<i>Erithacus rubecula</i>)	Migratory flight versus 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 versus on stopover for up to 8 h	Non-enzymatic serum antioxidant capacity ^a	–	Serum hydroperoxides ^b	↑	Skrip et al. (2015)
Northern bald ibis (<i>Geronticus eremita</i>)	Before flight versus 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 results show whether antioxidant capacity or oxidative damage increased (↑), 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.

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 H₂O₂ and hydroperoxides, which are abundant RS in migrating birds (Costantini, 2014; Deponte, 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 H_2O_2 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 selenocysteine (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 H_2O_2 produced in the mitochondria unless the RS diffuses from the mitochondria to peroxisomes (Halliwell and Gutteridge, 2007). However, once H_2O_2 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 peroxy 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±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 off-set 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

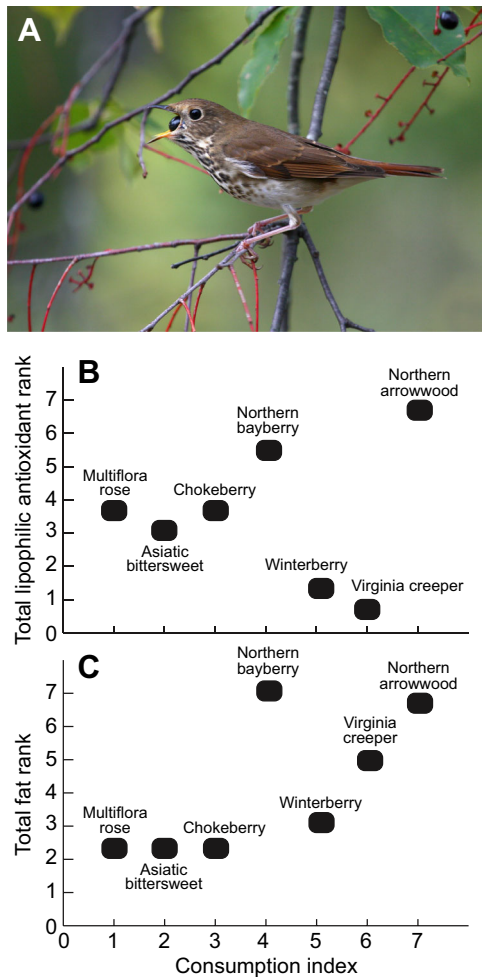


Fig. 3. Birds consume fruits for fat and/or antioxidant content while on stopover. (A) A hermit thrush (*Catharus guttatus*) during autumn migration eating fruit. Photo by Ryan Brady; printed with permission. (B) Relative consumption index for seven fruiting shrub species in relation to the total lipophilic 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.

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 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.

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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 acquisition: S.M.; Resources: S.M.; Supervision: S.M.

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