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Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists

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ABSTRACT. All air-breathing organisms must face the challenge of oxidative damage, and understanding how animals cope can lend insight into their ecology. Unlike other vertebrates, birds rely primarily on fats to fuel endurance exercise such as migration, and therefore face a greater potential for damage from the reactive by-products of their own metabolism. We review the physiological ecology of migrating birds through the lens of oxidation–reduction chemistry, underscoring how oxidative balance in wild birds may affect their dietary choices and use of critical stopover habitats during migration. Recent studies reveal that migratory birds prepare for oxidative challenges either by up-regulating endogenous antioxidants or by consuming them in their diet, and they repair oxidative damage after long flights, although much remains to be discovered about how birds maintain oxidative balance over the course of migration. We conclude by describing some of the most used and useful measures of antioxidant status and oxidative damage that field ornithologists can include in their tool kit of techniques to probe the oxidative balance of wild birds.

RESUMEN. **Balance oxidativo en aves: introducción para ornitólogos desde los átomos hocia los organismos y a la ecología**

Todos los organismos que respiran aire deben enfrentarse al reto del daño por oxidación. Entender como los animales pueden manejar este daño puede dar indicios sobre su ecología. Contrario a otros vertebrados, las aves se basan primordialmente en grasas como combustible para ejercicios que requieren una resistencia alta, como la migración. Por lo tanto, las aves se enfrentan a un mayor potencial daño oxidativo por parte de los subproductos reactivos de su propio metabolismo. Aquí revisamos la ecología fisiológica de aves migratorias a través de la lupa de la química de oxido-reducción, resaltando como el balance oxidativo en aves silvestres puede afectar sus preferencias en la dieta y uso de hábitats críticos de escala durante la migración. Estudios recientes revelan que las aves migratorias se preparan para los retos oxidativos mediante el incremento de antioxidantes endógenos, o mediante el consumo en su dieta y reparan daños oxidativos después de vuelos largos. Sin embargo, aun queda mucho por descubrir sobre como las aves mantienen su balance oxidativo a lo largo del transcurso de la migración. Concluimos describiendo algunos de las medidas útiles y mas utilizadas del estatus antioxidante y daño oxidativo que los ornitólogos de campo puede incluir dentro de sus técnicas para la exploración del balance oxidativo en aves silvestres.

Key words: antioxidants, lipid oxidation, measurement, migration, stopover

Compared to other vertebrates, birds are unique endurance athletes capable of sustaining high metabolic rates while relying on fat as their primary fuel. All vertebrates generate reactive by-products during oxygen-based metabolism, and exercise requires an increase in energy production and consequently can increase oxidative by-products. Endurance flights of migrating birds are particularly remarkable because the high metabolic rates required to stay aloft for long periods are fueled by fats that produce more oxidative by-products per capita than other fuels such as proteins and carbohydrates. In this review, we present evidence from recent studies that reveal how migratory birds overcome or accommodate the risks of oxidative stress during long-duration flights, and discuss how this understanding can provide deep insight into the physiological and behavioral ecology of birds during migration.

Oxidative stress and its association with aging, disease, and life-history trade-offs have received considerable recent attention (e.g., Cohen et al. 2008, 2010, Costantini 2008, 2014, Monaghan et al. 2009, Buttemer et al. 2010, Costantini et al. 2010, Lushchak 2011, McGraw 2011, Pamplona and Costantini 2011, Beaulieu and Costantini 2014). In this review, we focus on the nutritional and physiological ecology of birds, with special reference to the oxidative challenges of migration, to highlight the relevance of oxidative balance research to field investigations. We first summarize current understanding of

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the atomic origin of oxidative damage and the particular vulnerability of fats used by birds, followed by an overview of the various components of the antioxidant system that help maintain oxidative balance at the organismal level. We then place these aspects of biochemistry within an ecological context, describing the oxidative costs of long-distance flight and how the dietary choices of migrating birds on stopover may help prevent oxidative stress. We conclude with a primer of measurements practical for use by field researchers to study the oxidative status of migratory birds, and end with a brief discussion of gaps in our knowledge that may be addressed by future field research into oxidative balance.

ATOMIC LEVEL: THE ESSENTIALS OF OXIDATIVE BALANCE

Any investigation into oxidative balance requires at least a basic understanding of oxidation–reduction chemistry. Amateur and professional ornithologists alike will recall high school chemistry lectures in which the handy mnemonic device OIL RIG was presented: oxidation is loss, and reduction is gain, of electrons. Oxidation–reduction chemistry explains both the source of oxidative damage in bird tissues and how birds can counter damage-causing molecules. This understanding allows field ornithologists to more wisely choose among the possible measurements available to probe the oxidative status of migratory birds.

How does oxidative damage happen? Oxidative balance in birds begins at the molecular level (Fig. 1) and with the chemical reactions taking place in mitochondria, the organelles responsible for generating adenosine triphosphate (ATP), the energetic currency of cells. Inside each mitochondrion—on the inner mitochondrial membrane—enzyme complexes collectively known as the electron transport chain (ETC) transfer electrons generated from the metabolism of food to a final electron acceptor, molecular oxygen (O_2), reducing it to water (H_2O) in the process. The energy released during this transfer of electrons is used to pump hydrogen ions (H^+) into the intermembrane space of the mitochondrion, and the diffusion of these ions back into the mitochondrial matrix drives the production of ATP by ATP-synthase, an enzyme also located on the inner mitochondrial membrane. Simply put, then, food is burned to convert oxygen into water, all to generate energy that cells can use. However, an estimated 1–6% of the molecular oxygen is not reduced to water by the enzyme responsible for doing so (cytochrome oxidase; Dreosti 1991, Ji 2008). Rather, intermediates of the reduction process escape this enzyme and enter the mitochondrial matrix (Fig. 2A). Some of these intermediates are radicals (denoted by a raised dot [•] next to an atom); radicals are atoms or molecules with an unpaired electron, and so they readily scavenge electrons from other compounds, damaging them and making them radicals in turn. Highly reactive non-radical compounds that escape the ETC may

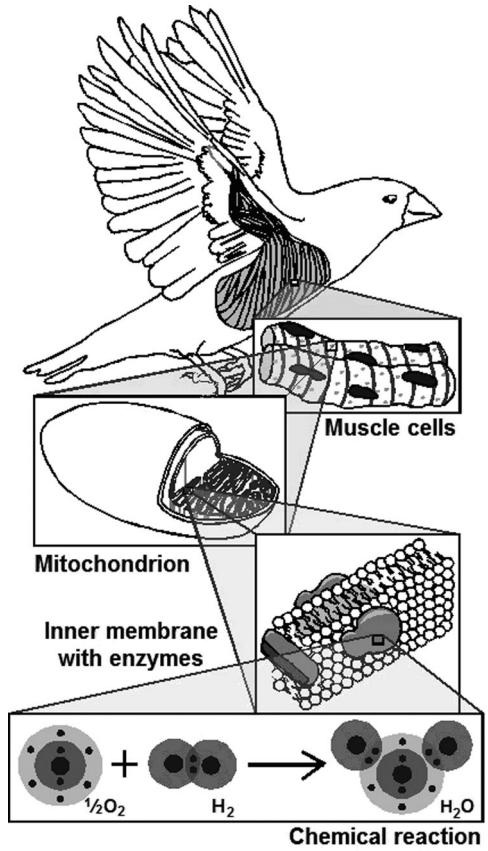

Fig. 1. Although the oxidative status of birds has important implications at the organismal and ecological levels, understanding how oxidative damage occurs begins at the atomic level. Chemical reactions taking place in the mitochondria generate pro-oxidants during aerobic respiration.

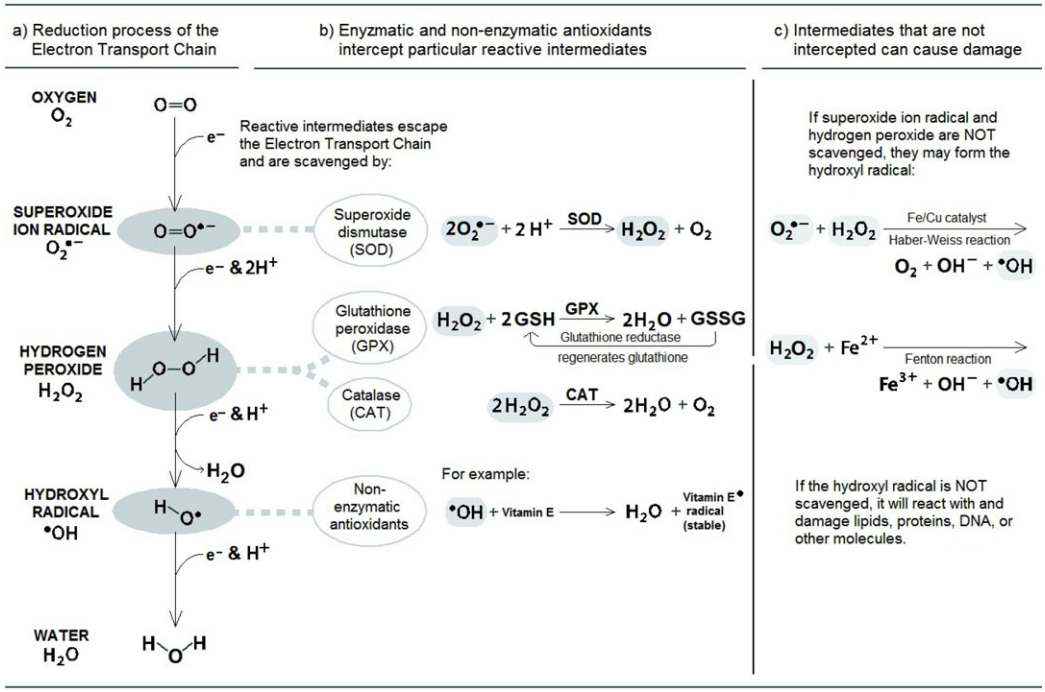

Fig. 2. The reduction of oxygen to water in mitochondria results in production of reactive intermediates, and layers of protection from by-products prevent oxidative damage.

also undergo reactions that generate radicals. Ignoring their radical or non-radical nature, we call this group of dangerous compounds reactive oxygen species (ROS) or pro-oxidants because they cause oxidation.

If antioxidant enzymes (or other antioxidant compounds) are not available in suitable quantity to convert or quench these reactive intermediates (Fig. 2B), they are free to react with (read: "damage") other molecules in the mitochondrion, the cell, and body (depending on how far they, or their products, get). Superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$) are particularly reactive free radicals and will quickly take electrons from other molecules (e.g., lipids, proteins, and DNA) to satisfy their unbalanced chemical structure. Meanwhile, hydrogen peroxide is less reactive (not being a radical, but rather an oxidizing acid) and can cross membranes, and hence exert its effects further from the site of production; contact with metals (chiefly iron ions) can convert it to the highly reactive hydroxyl radical (Fig. 2C).

Damage to proteins can destroy their structural or enzymatic function, damage to DNA can generate mutations or diminish a mitochondrion's ability to replicate, and damage to lipids can affect membrane structure and function (e.g., decreasing membrane fluidity, affecting permeability, and deactivating membrane-bound proteins; Dreosti 1991). Protection against the damaging effects of these reactive intermediates is, therefore, clearly important.

All of this thus described applies to any vertebrate that uses oxygen-based metabolism to generate energy. Animals performing exercise such as birds during a migratory flight, and those that rely on large amounts of fat as fuel such as migratory birds, face extra complications because (1) an increase in activity may increase production of ROS in excess of the immediate capacity of antioxidants to quench them, and (2) stored and structural fats, particularly polyunsaturated fatty acids (PUFAs), are especially vulnerable to oxidative damage because of their chemical structure, and may generate their own variety of pro-oxidants.

Why are PUFAs so important to understanding oxidative damage in birds? PUFAs are particularly relevant molecules in the

context of bird physiology. We know that birds and mammals differ in the saturation level of fatty acids in their cellular membranes, migratory birds select particular unsaturated fatty acids in their diets, chain length of unsaturated dietary fatty acids can affect exercise performance in birds, and birds appear to be capable of controlling their fatty acid composition in relation to their diet (McWilliams et al. 2004, Costantini 2008, Weber 2009, Pamplona and Costantini 2011, Pierce and McWilliams 2014).

Fatty acids are essential for birds during migration, given that fat is the primary fuel used for long-distance flights, but they are also the foundational components of cellular membranes and therefore crucial to maintaining the integrity and function of cells and cellular compartments. These membranes are particularly vulnerable to oxidative damage for two reasons: (1) their proximity to the site of pro-oxidant production (the membrane-bound ETC), and (2) the ease with which their electrons are stolen by free radicals, especially when double bonds are present. Membrane damage may then cause further damage in stored fats to be used as fuel.

PUFAs are vulnerable to oxidative damage simply because hydrogen atoms located near double bonds are easily oxidized, i.e., they have low oxidative potential, which simply means it takes very little chemical energy to steal them away; hydrogen atoms on carbons between double bonds are particularly vulnerable (Wagner et al. 1994, Milbury and Richer 2008; Fig. 3). When an unsaturated fatty acid encounters a free radical like the hydroxyl radical, the fatty acid loses an electron and becomes a lipid radical (Fig. 3A); when that lipid radical encounters oxygen, the resultant lipid peroxyl radical (Fig. 3B) becomes free to react with another intact PUFA and generate a new radical (Fig. 3C). Overall, the entire process can be visualized as a chain reaction (Fig. 3D). As shown in Figure 3, new lipid radicals can interact with oxygen to generate another lipid peroxyl radical. Thus, one hydroxyl radical can initiate a self-perpetuating chain reaction that needs only the continual input of oxygen and other PUFAs to persist. This is why PUFAs are so vulnerable to ROS, i.e., the reaction will continue either until all PUFAs are oxidized or an antioxidant compound breaks the chain. Vitamin E is an important antioxidant here because it can quench both hydroxyl radical and radicals produced by PUFAs, thereby "breaking the chain" of lipid peroxidation (Dreosti 1991, Milbury and Richer 2008).

The more double bonds present in a fatty acid, the more vulnerable it is to oxidation and the more likely it is to participate in this runaway lipid peroxidation cascade (Wagner et al. 1994). As noted above, damage to these fatty acids changes their properties and function in cells. Equally problematic, however, is their potential to further damage other tissues. Just as by-products of the ETC are called ROS, radicals produced by lipid oxidation are called reactive carbonyl species (RCS), but these RCS pro-oxidant compounds persist much longer in cells than ROS (half-lives of minutes to hours, rather than fractions of a second) and can diffuse through and out of cells, thereby causing damage far from their site of origin (Buttemer et al. 2010). Migrating birds store, mobilize, and burn considerable amounts of fat during their journeys, and ROS and RCS compounds generated in cells can place that fat at risk. As such, for exercising birds with fat stores vulnerable to pro-oxidant attack, protecting PUFAs from damage is important for preserving the integrity of those molecules and their function, as well as preventing the risk of exacerbated damage produced by RCS.

ORGANISM LEVEL: OXIDATIVE STRESS AND HOW TO AVOID IT

What is oxidative stress, really? Although the term "oxidative stress" is often used in the literature, truly quantifying it remains a challenge, and one that is unlikely to be resolved soon. As Costantini (2014) pointed out, "oxidative stress" is really a latent variable, conceptually constructed rather than actually observable because there is no one metric that defines it. Researchers have provided various definitions of what oxidative "stress" represents, whether it manifests in fitness consequences (Cohen et al. 2010), and how it can be visualized (e.g., seesaw balance of ROS and antioxidant defense, Monaghan et al. 2009; gradient diagrams between extremes of pro-oxidants and antioxidants, Costantini and Verhulst 2009).

For practical design of studies, however, perhaps it is more helpful to think of a bird in

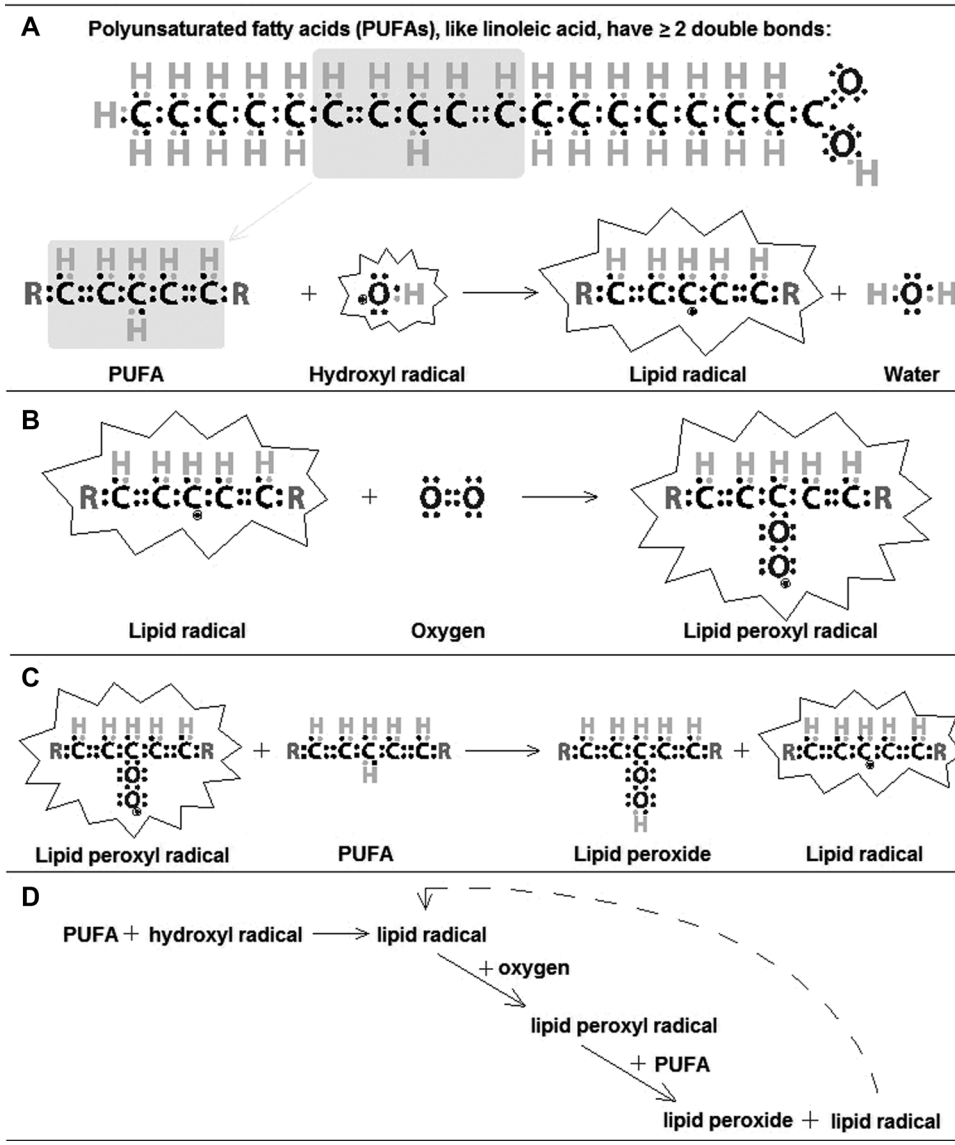

Fig. 3. Polyunsaturated fatty acids (PUFAs) such as linoleic acid are particularly vulnerable to oxidative damage. (A) A hydrogen atom near the double bonds is easily stolen by the hydroxyl radical (˙OH), which has an unpaired electron, forming a lipid radical and water. (B) When the lipid radical encounters oxygen, it forms a lipid peroxyl radical. (C) The lipid peroxyl radical is then free to react with another intact PUFA, forming another lipid radical. (D) Overall, the entire process can be visualized as a chain reaction.

terms of the components of its "oxidative status." Take, for example, two scenarios for an exercising (read: migrating) bird (Fig. 4A and B). If a bird exercises, producing ROS, and has limited antioxidant capacity (which we can measure in several ways), then a substantial amount of oxidative damage can occur (as indexed by damage to lipids, proteins, and so on; Fig. 4A). However, if the antioxidant capacity of the exercising bird is matched to the increased pro-oxidant levels, there may be minimal oxidative damage (Fig. 4B). What should be clear in such a system

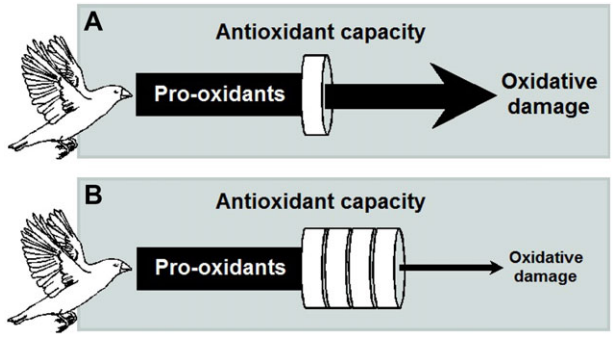

Fig. 4. The antioxidant capacity of an organism such as a flying bird can be visualized as a filter, intercepting pro-oxidants to prevent oxidative damage. Facing the same oxidative challenge, a bird (A) with little antioxidant capacity can be expected to suffer more oxidative damage than one (B) with greater antioxidant capacity (whether endogenous, dietary, or a combination of both). As described in the text and detailed in Table 1, birds can increase their antioxidant capacity in two ways: (1) up-regulate their endogenous antioxidant capacity (e.g., antioxidant enzymes) and (2) consume more dietary antioxidants in preparation for migration and during stopovers.

is that we must always at least measure both sides—antioxidants and pro-oxidants, or antioxidants and oxidative damage—to understand the oxidative status of a bird. Unfortunately, there are no direct, field-ready measures of pro-oxidant production. However, there are several useful indicators of oxidative damage and antioxidant capacity (the most popular of which we will discuss below).

Oxidative "stress" then logically manifests itself in some physical or functional consequence of damage. For the purposes of this review, we will define oxidative stress as the accumulation of oxidative damage that affects the performance of a migrating bird. How do migrating birds, therefore, prevent or alleviate the accumulation of such damage?

How do birds avoid oxidative stress? Migrating birds have two main ways of dealing with the heightened threat of oxidative damage during their flights: (1) up-regulate their endogenous antioxidant capacity (e.g., antioxidant enzymes) and (2) consume more dietary antioxidants in preparation for migration and during stopovers (Table 1, Fig. 4). These are not mutually exclusive. Phenotypic flexibility is a hallmark of both strategies, and discovering which strategy (or combination of strategies) free-living birds adopt in various ecological contexts will enrich our understanding of bird–habitat associations during migration and the parameters that constrain their migratory abilities.

Enzymatic antioxidants, their function, and up-regulation. As shown in Figure 2B, three main varieties of enzymes act as the first line of defense against oxidative damage, given that they intercept the first-escaped by-products of the ETC. Superoxide dismutase (SOD) functions to neutralize the superoxide anion radical ($O_2^{\bullet -}$), whereas both glutathione peroxidase (GPX) and catalase (CAT) convert hydrogen peroxide (H_2O_2) to water. GPX is a particularly important scavenger of this compound in animal tissues, and even converts hydroperoxides, such as lipid peroxides produced by the lipid oxidation cascade, into alcohols. GPX relies on the endogenous antioxidant glutathione (GSH) to perform its reactions, and the oxidized form of GSH is consequently recycled for repeated use by the enzyme glutathione reductase.

The utility of SOD, GPX, and CAT extends beyond the inner mitochondrial membrane, however, and various forms of these enzymes may be found in different cellular compartments. Their activity also tends to vary across tissues, with highest activities in those tissues with the greatest oxygen consumption (Ji 2008). Indeed, a distinct advantage of these enzymes is that they can be up- or down-regulated in response to need, and the effect of physical exertion on their activity levels has been examined in multiple studies (reviewed by Ji 2008). Importantly, however, SOD, GPX, and CAT are not effective in quenching all pro-oxidants

Table 1. Summary of antioxidants mentioned in text.

Endogenous antioxidants (i.e., those produced by birds)		Exogenous antioxidants (i.e., those eaten by birds)	
Enzymatic		Fat-soluble (lipophilic)	
Superoxide dismutase (SOD)	First line of defense, neutralizes the superoxide anion radical ($O_2^{\bullet-}$), generating hydrogen peroxide (H_2O_2)	Carotenoids	Large group of chiefly orange, red, and yellow pigments with antioxidant properties
Glutathione peroxidase (GPX)	First line of defense, converts hydrogen peroxide to water and lipid peroxides to alcohols	Vitamin E	Large group of antioxidants chiefly responsible for preventing lipid oxidation; alpha-tocopherol is the most common/studied form
Catalase (CAT)	First line of defense, converts hydrogen peroxide to water		
Non-enzymatic		Water-soluble (hydrophilic)	
Glutathione	Neutralizes radicals independently, recycles other antioxidants, assists GPX, is recycled to reduced form by glutathione reductase	Polyphenols	Large group of compounds with antioxidant (and other) properties, includes the anthocyanins (red, blue, purple pigments)
Uric acid	Catabolically derived as final waste product of protein metabolism, with antioxidant properties	Vitamin C	Compound that supports immune function and has antioxidant properties, can recycle oxidized vitamin E back to its reduced form
Albumin	A circulating protein that scavenges free radicals		
Estrogen and melatonin	Circulating hormones with antioxidant properties		
Ferritin and ceruloplasmin	Proteins that sequester metal ions that might otherwise react with molecules to produce pro-oxidants		

or preventing all manner of oxidative damage. In short, these enzymes do not work alone.

Non-enzymatic endogenous antioxidants and their function. We have already mentioned one non-enzymatic endogenous compound important to antioxidant protection, GSH. GSH not only assists GPX, but can also scavenge pro-oxidants; this compound is synthesized from amino acid precursors by glutathione synthetase and, as mentioned above, is converted back to its active form from its oxidized form (glutathione disulfide) by glutathione reductase (McGraw 2011, Costantini 2014). Given that GSH is such an active and important component of the antioxidant system, measurement of the ratio between its reduced and oxidized forms has been used as one indicator of oxidative balance (see "Measuring oxidative balance in a field context" section).

Perhaps one of the best known endogenous antioxidants circulating in birds, however, is uric acid, the final product of nitrogen metabolism in these taxa. It is commonly known that a high-protein diet in birds results in high circulating levels of uric acid (e.g., Alan et al. 2013). Uric acid is a potent scavenger of pro-oxidants, and its level in blood has been used as an indicator of antioxidant capacity. The oxidized form of uric acid, allantoin, is produced when uric acid reacts with a pro-oxidant, and measurement of the uric acid:allantoin ratio in birds has been proposed as a useful indicator of oxidative balance, as with GSH (Tsahar et al. 2006, McGraw 2011). Unlike GSH, however, uric acid cannot be recycled

to its active form once oxidized (Milbury and Richer 2008).

Another compound particularly relevant to endogenous antioxidant protection is the circulating plasma protein albumin. Like GSH and uric acid, albumin scavenges free radicals by being easily oxidized, sparing other molecules by reacting with pro-oxidants itself (Casagrande et al. 2015). Researchers speculate that albumin may protect PUFAs to which it binds, but any oxidized albumin loses its antioxidant capability and must be replaced (Roche et al. 2008).

Circulating hormones may also have antioxidant functions, either as scavengers of pro-oxidants (e.g., estrogen) or as regulators of other endogenous compounds (e.g., glucocorticoids). Melatonin is a hormone with both properties (Pandi-Perumal et al. 2006, McGraw 2011, Beaulieu and Schaefer 2014, Costantini 2014). Given that melatonin is secreted chiefly at night, the potential importance of this hormone for nocturnally migrating birds is high and deserves further study.

Lastly, it is important to acknowledge the endogenous compounds that help prevent conversion of hydrogen peroxide to the hydroxyl radical by sequestering the metals that catalyze that reaction. Ferritin and ceruloplasmin are two examples of molecules that chelate free metal ions so that they cannot react with peroxides and therefore facilitate oxidative damage (Costantini 2014).

Dietary antioxidants, their origin, function, and interactions. Although enzymes and other endogenous antioxidant compounds must be synthesized by cells in response to demand, dietary antioxidants can be acquired through feeding and may therefore be a "cheaper" alternative (in terms of both time and energy) if they can supplement or replace the action of endogenous components of the antioxidant system (Pamplona and Costantini 2011). Endogenous and exogenous antioxidants are not broadly interchangeable, but evidence suggests that supplementation of some dietary antioxidants may result in lower activity of antioxidant enzymes with no concomitant change in total antioxidant protection (Costantini 2014). Broadly speaking, the benefits of dietary antioxidants span a range of protective mechanisms, from scavenging pro-oxidants such as the hydroxyl radical, to chelating metals, strengthening the immune system, and stimulating the up-regulation of endogenous antioxidants (Lushchak 2011, Costantini 2014). Dietary antioxidants are typically classified as either water-soluble (hydrophilic) or fat-soluble (lipophilic), and therefore may be distributed, stored, or mobilized in various tissues depending on their solubility.

Dietary antioxidants used by animals originate in photosynthetic plants where they act as pigments and protect plant tissues from sunlight, the radicals generated during photosynthesis, oxidation from the air, and reactive by-products of oxygen metabolism. Thus, plants produce and use antioxidant compounds to protect their own cells and invest them in tissues, e.g., leaves, nectar, seeds, and fruits, and animals gain protection by ingesting them. Antioxidants in edible plant parts may have originated to protect those tissues, but they also appear to offer a nutritional reward to birds that eat fruits and consequently scatter the seeds. That is, plants use birds just as the birds use plants (Izhaki and Safriel 1985, Smith and McWilliams 2014a). Seeds and fruits may vary widely in their total antioxidant content, and particular constitutive compounds, and birds may choose these foods based on their antioxidant content (e.g., Schaefer et al. 2008, Alan et al. 2013, Bolser et al. 2013). Several main classes of dietary antioxidant are particularly relevant to bird studies, including carotenoids, vitamin E, and polyphenols.

Carotenoids represent a large group of chiefly orange, red, and yellow pigments with antioxidant properties. Specifically, they are capable of scavenging hydroxyl and other radicals, becoming relatively stable radicals themselves in the process. Although their contribution to circulating antioxidant capacity has been estimated to be quite low (Costantini and Møller 2008, Simons et al. 2012), their value out of circulation should not be ignored (Cohen and McGraw 2009); carotenoids are fat-soluble, rapidly absorbed, and therefore readily integrated into cellular membranes and lipid-dense tissues, offering them antioxidant protection. As pigments, they are responsible for the color of furcular fat deposits and egg yolks, as well as the sexual ornaments of many birds. Carotenoids may be mobilized from fat deposits at time of need (Metzger and Bairlein 2011), they contribute to the survivability of growing embryos and hatchlings (McGraw et al. 2005), and carotenoids may even help protect muscles

during flight exercise or otherwise enhance flight performance (Blount and Matheson 2006, Mateos-Gonzalez et al. 2014). Lutein and its isomer, zeaxanthin, are two of the most common carotenoids in plants (Shahidi and Ho 2007), and therefore of primary relevance to bird studies (Koutsos et al. 2003, McGraw et al. 2005, Casagrande et al. 2015).

Vitamin E also represents a group of molecules with common structures and functions. Like carotenoids, vitamin E is fat-soluble and therefore serves its primary protective function in fatty tissues, e.g., cellular membranes, fat deposits, and egg yolk. More than any other dietary antioxidant, vitamin E is considered key to protecting lipids from oxidative damage, given its ability to break the chain reaction of the lipid oxidation cascade (Fig. 3; Cohen and McGraw 2009, Teixeira et al. 2009, Pamplona and Costantini 2011, Costantini 2014). Specifically, vitamin E can react with lipid peroxyl radicals, preventing further radicalization of additional fatty acids and becoming a relatively stable radical itself in the process. Alpha-tocopherol is the major form of vitamin E typically examined in bird studies, and has been widely studied for its benefits to domestic poultry (e.g., Surai 2002).

So far we have mentioned two major groups of fat-soluble antioxidants, both of which have been well studied in birds. An emerging area of research now focuses on an enormous group of water-soluble, plant-derived compounds, the polyphenols. The subset of polyphenols most relevant to our discussion is the flavonoids, which include the anthocyanins—itself a class of >200 sugar-bound pigmented compounds (Shahidi and Ho 2007). Flavonoids are the most ubiquitous and potent (at least *in vitro*) antioxidants in nature, appearing in high concentrations in vegetables and fruits, including those that birds choose to consume during migration (Schaefer et al. 2008, Alan et al. 2013, Bolser et al. 2013). In plant tissues, flavonoids serve various functions, including protecting cells against UV radiation and scavenging pro-oxidants generated during photosynthesis (Pietta 2000). Pigmented under acidic conditions, anthocyanins specifically are responsible for the dark blue, red, and purple colors present in many fruits, and birds appear to use this coloration to distinguish the antioxidant content of those fruits (Schaefer et al. 2008). In the bodies of animals, polyphenols display a variety of beneficial functions, scavenging pro-oxidants, including in the digestive system where they may protect ingested food from oxidation (Pietta 2000, He et al. 2006), facilitating the mounting of an immune response (Catoni et al. 2008), chelating metal ions and preventing them from participating in reactions that generate pro-oxidants (Pietta 2000), and up-regulating the expression of antioxidant enzymes and production of GSH (Moskaug et al. 2005, Spanier et al. 2009, Yeh et al. 2009).

Debate has arisen concerning the extent to which polyphenols can be absorbed and stored in the tissues of birds and other animals, but recent studies provide convincing evidence that they are indeed metabolized and available for an appreciable period of time after ingestion. Over a decade ago, Cao and Prior (1999) showed for the first time that anthocyanins can be absorbed in their sugar-bound form in humans, and they are measurable in the circulation for at least 1 h. Catoni et al. (2008) demonstrated that European Blackcaps (*Sylvia atricapilla*) absorb dietary flavonoids and circulate them in blood, and Beaulieu and Schaefer (2014) showed that Gouldian Finches (*Erythrura gouldiae*) circulate dietary polyphenol metabolites for at least several hours.

Furthermore, polyphenol compounds may undergo several chemical changes during digestion (removal of associated sugar, conversion to a phenolic acid) that nevertheless do not diminish their antioxidant potency, and their chemical properties (type of associated sugar, methylation) may affect how they are integrated into tissue (Pietta 2000, He et al. 2006). Clearly, there is ample opportunity to examine how birds choose dietary polyphenols in foods (as first demonstrated by Catoni et al. 2008 and Schaefer et al. 2008) and how they may alter their structure to utilize them *in vivo*.

A complex interacting system. All the antioxidants we have discussed so far have unique chemical structures, solubilities, scavenging properties, and capabilities, and none of them act exclusively alone. Rather, research is continuously revealing how multiple antioxidants act in concert, and deficiencies in one can lower the efficacy of others. Although not often a sole focus of wild bird research, vitamin C (ascorbic acid) is another potent dietary antioxidant of note, mainly because of its interactions with other antioxidants, particularly vitamin E.

Before we discuss how these antioxidants interact, understanding why they do so is important. Whether or not a molecule can be (or will be) oxidized by another molecule comes down to comparing the "oxidation potential"—the amount of energy required to remove yet another electron from the first molecule. These oxidation potentials explain why vitamin E (alpha-tocopherol) protects PUFAs from oxidative damage because an electron on the vitamin E molecule is stolen much more readily than, i.e., has a lower oxidation potential than, an electron on a PUFA. All things being equal, a hydroxyl radical will steal an electron from vitamin E before it steals one from a PUFA, leaving the PUFA intact.

Vitamin E molecules become oxidized when they serve their protective function and therefore lose their antioxidant properties unless they recover an electron. This is where other antioxidants come in; vitamin C has an even lower oxidation potential than vitamin E and can "restore" vitamin E to its protective form. Vitamin C then itself becomes a radical, but can be "restored" by reduced GSH, which has an even lower oxidation potential and is easily recycled (when oxidized to oxidized GSH, GSSG) by the enzyme glutathione reductase (Milbury and Richer 2008).

Because of its very low oxidation potential, reduced GSH can be easily oxidized by pro-oxidants, sparing other molecules from oxidation or restoring other recyclable antioxidants, and is, therefore, a very important endogenous antioxidant (Milbury and Richer 2008). Additionally, polyphenols may help maintain high levels of vitamin E in membranes by protecting that important antioxidant from oxidation and indirectly protecting lipids despite their water-solubility (Pietta and Simonetti 1998, Pietta 2000); furthermore, some oxidized polyphenols may rely on regeneration from vitamin C and GSH (Pietta 2000). Clearly, circulating non-enzymatic antioxidants interact to prevent oxidative damage in animals.

ECOLOGICAL LEVEL: OXIDATIVE BALANCE AS A MIGRATORY CONSTRAINT

Oxidative damage occurs at the atomic level and birds do have ways to prevent damage on an organismal level, but how does oxidative balance relate to avian life history and patterns/processes at the ecological level? We would like to make the case that considering oxidative balance is crucially important to understanding several aspects of the behavioral choices of birds and their distribution during migration. In particular, we posit that oxidative balance is relevant to thinking about (1) flight distance and migration strategy and (2) nutritional choices at stopover sites.

How does flight exercise affect oxidative balance in birds? Given the demands of intense flight exercise, migrating birds should seek to minimize the potential for oxidative stress during each flight bout, whether by reducing the time spent aloft, consuming prophylactic antioxidants, up-regulating endogenous antioxidants, repairing damage after landing, or a combination of all of these strategies. Because of several pioneering studies, we know that oxidative damage is a real hazard of flight by volant birds, both domesticated and free-living (Costantini et al. 2007, 2008, Jenni-Eiermann et al. 2014). However, we also know that birds can prepare for and repair such damage (Jenni-Eiermann et al. 2014, Skrip et al. 2015).

Costantini et al. (2008) provided some of the first direct evidence that flying causes oxidative damage in birds. They flew trained pigeons for short (60 km) and long durations (200 km) and took blood samples within 15 min after flights to acutely measure oxidative damage and antioxidant capacity. Pigeons that flew for 200 km exhibited a 54% increase in oxidative damage (as measured by serum reactive oxygen metabolites [ROMs]), and a 19% decrease in total serum non-enzymatic antioxidant capacity (measured by the OXY-adsorbent test), presumably because some of the antioxidant capacity was used to quench pro-oxidants, although not enough to avoid oxidative damage.

Subsequently, Jenni-Eiermann et al. (2014) examined European Robins (*Erithacus rubecula*) captured in flight at night as they migrated through a Swiss mountain pass. Both a marker of oxidative damage (protein carbonyls) and marker of enzymatic antioxidant capacity (GPX) were significantly higher in birds captured during the night than in conspecifics captured while on stopover during the day, presumably because flight caused muscle damage and the antioxidant system was up-regulated during migration. These researchers further showed that antioxidant enzyme levels were higher in

after-hatch-year birds than hatch-year birds, suggesting that experienced animals were better able to prepare for the oxidative challenge.

The results of laboratory studies also suggest that animals can up-regulate their own endogenous defenses in response to exercise training, decreasing levels of oxidative damage, and even compensating for an antioxidant-deficient diet (Teixeira et al. 2009, Janiak et al. 2010, Larcombe et al. 2010). Oxidative damage in the plasma of adult Budgerigars (*Melopsittacus undulatus*) can be reduced by exercise training regardless of antioxidant levels in the diet, indicating that endogenous antioxidants are up-regulated by repeated flight bouts (Larcombe et al. 2010). In horses, long-duration training programs for distance racing can increase levels of antioxidant enzymes (particularly GPX) in red blood cells (Janiak et al. 2010). Furthermore, oxidative damage is highest in the tissues (e.g., liver, heart, and skeletal muscle) of rats fed on antioxidant-deficient diet and not exercised, but low and equivalent in exercised rats on either a deficient or standard diet (Teixeira et al. 2009).

Regardless of whether a bird increases its antioxidant capacity for migration, some damage will inevitably occur. Birds appear, however, to be able to readily remove or repair damaged molecules. Jenni-Eiermann (2014) showed that European Robins resting during the day exhibited lower protein damage levels than those actively migrating, and Skrip et al. (2015) showed that lipid peroxidation levels in Garden Warblers (*Sylvia borin*) decreased with time on stopover after spring migration over the Sahara Desert and Mediterranean Sea. The ability of birds to recover from damage, and prepare for the demands of further flights, likely depends heavily on their use of stopover sites. Although not yet explicitly tested at a large scale, the arrangement of stopover sites on the landscape may dictate not only how birds can refuel, but how they can cope with oxidative damage.

How do stopover sites help birds manage oxidative balance? During migration, stopover sites provide opportunities for birds to rest, refuel on fats and protein, acquire water, and potentially to recover from accumulated oxidative damage, but they also offer, especially during fall migration, seasonally abundant fruits rich in antioxidants. An opportune area of future research concerns the use of stopover sites for building non-enzymatic antioxidant capacity in concert with energy stores, particularly through dietary choices (Skrip et al. 2015).

Many insect-eating and seed-eating birds shift to eating fruits during fall migration (Parrish 1997), and this "diet-switching" has been widely understood as a common strategy by many species to use nutritious, and seasonally abundant, fruits to quickly and efficiently build fat stores. Building fat stores of good quality in a short time is necessary for animals that must burn large amounts of high-energy fuel to complete their migrations (McWilliams et al. 2004). Studies show that fruit-rich stopover habitats produce birds with higher fattening signatures (circulating triglycerides) in their blood than similar, but fruit-poor, habitats (Smith and McWilliams 2010). However, birds may also consume fruits for additional nutritional benefits.

Although migrating birds can be expected to select foods with high energy density—and they do—antioxidant content may also influence their decisions. Research has shown that birds are able to discriminate among fruits based on their color, and hence anthocyanin content, and prefer anthocyanin-enriched foods when presented a choice (Schaefer et al. 2008). On Block Island, a stopover site for millions of songbirds each autumn off the coast of Rhode Island, USA, arrowwood viburnum (*Viburnum dentatum*) fruits are highly prized by birds, presumably due to their unique combination of abundant, high-quality fats, and both fat- and water-soluble antioxidants (Smith et al. 2007, 2015, Alan et al. 2013, Bolser et al. 2013). Influxes of migratory birds to Block Island during peaks in fall migration are regularly followed by increases in rates of arrowwood removal (Smith and McWilliams 2014a). Even in years of low arrowwood fruit yield, birds seek and choose to consume this species at a greater rate than fruits of other species that are more abundant, suggesting the importance of this shrub to the diet of migrating birds in this region (Bolser et al. 2013).

Several authors have speculated that birds may face trade-offs while feeding, depending on whether their needs are greater for energy or for particular compounds such as antioxidants (Monaghan et al. 2009, Beaulieu and Schaefer 2013, 2014). However, these conceptual trade-offs may not exist when birds consume fruits that are high in both energy density and antioxidants. Birds on Block Island show strong correlations

between fat stores and circulating non-enzymatic antioxidants, suggesting that they may acquire both in their diet as they prepare for migration (Skrip et al. 2015). In Europe, researchers have also found anthocyanin and caloric content to be positively correlated in autumn fruits (Schaefer et al. 2008).

The type of antioxidants in high-fat fruits may also be particularly advantageous for consumers, given that high-fat fruits should contain antioxidants that specifically protect fats from spoiling before consumption by animal dispersers. For example, arrowwood fruits on Block Island contain abundant water-soluble polyphenols (the many benefits of which were discussed above), and most of their fat-soluble antioxidants are represented by vitamin E (Alan et al. 2013), the benefits of which may be twofold: protecting lipids in the fruits against oxidation, and conferring valuable protection to the fat depots and membranes of migrating birds that rely heavily on fat metabolism.

Especially when abundant in foods that offer other nutritional benefits, dietary antioxidants may be considered relatively "inexpensive" to acquire and use (Beaulieu and Schaefer 2013). Oxidative damage may occur because endogenous defenses take time, energy, and molecular resources to up-regulate, and so organisms can take advantage of dietary antioxidants to compensate. Dietary or non-enzymatic antioxidants may be most essential to animals not accustomed to long-distance exercise, e.g., birds making a migratory flight for the first time or early in the migration season (Costantini 2008, Larcombe et al. 2010, Beaulieu and Schaefer 2013). How birds may strategically use these antioxidants, however, still remains an open question. Beaulieu and Schaefer (2013), in particular, speculated that migrating birds may use specific antioxidants prophylactically, in anticipation of impending need, and may take advantage of a series of stopover sites, or a combination of fat- and water-soluble antioxidants that may or may not be stored in tissue, during the course of their migrations. Their logical arguments now require empirical evidence (e.g., Skrip et al. 2015).

MEASURING OXIDATIVE BALANCE IN A FIELD CONTEXT

Now that we have covered the basics of oxidative challenges in migrating birds, we can provide some recommended field measurements for oxidative damage and antioxidant capacity that ornithologists can include in their tool kit of techniques (Table 2). As hopefully is clear from Figure 4, measuring multiple aspects of an animal's oxidative status, i.e., both antioxidant capacity and damage, is important for understanding how a bird is coping with an oxidative challenge, and so we highlight ways of evaluating both.

As described above, different antioxidant compounds work in different ways, and oxidative damage may be represented by multiple indicators (e.g., protein or fat). Antioxidants may also be concentrated or most active in different sites in the body, e.g., fatty tissue, muscle, egg, plasma, or red blood cells. Choosing the most appropriate metric (and tissue) for a research question is also important. We acknowledge that many researchers will prefer to sample blood as a non-invasive, non-lethal way to answer different questions, especially field ornithologists sampling protected species, and so we emphasize here what can be measured in blood. For researchers new to sampling avian blood, we highly recommend reading Owen (2011).

We should also make clear that our intention is not to provide an exhaustive treatment of all of the measures that can be used to gauge oxidative status, or their various uses. For those interested in additional information, we recommend Urso and Clarkson (2003), Hõrak and Cohen (2010), and Halliwell and Gutteridge (2015). In addition, Cohen et al. (2007), Cohen and McGraw (2009), and Cohen et al. (2009) provide additional applications. Here, we highlight some commonly used measures that are most practical for field studies (Table 2).

Measuring antioxidant capacity in blood. Emerging research suggests that multiple components (enzymatic or non-enzymatic) of the antioxidant system of birds may co-vary or operate independently—depending on the components—and so there may be animal-wide integration or compartmentalization of antioxidant responses (Costantini et al. 2011, 2013). Therefore, in general, we recommend pairing a measure of non-enzymatic antioxidant capacity in plasma and/or red blood cells with a measure of antioxidant enzyme activity (chiefly GPX) in red blood cells for questions that require a broad, whole-animal gauge of antioxidant levels. We

Table 2. Recommended methods for measuring the oxidative status of birds using blood samples.

Measure	Method	Helpful methodological references
Antioxidant capacity		
Total non-enzymatic antioxidant capacity		
TEAC	Colorimetric assay to measure ability of sample to reduce radicals formed by reaction with hydrogen peroxide	Cohen et al. (2007)
OXY-adsorbent test	Commercial kit; colorimetric assay to measure ability of sample to neutralize hypochlorous acid	Costantini (2011), Costantini et al. (2011)
Enzymatic protection of red blood cells		
GPX	Commercial kit; kinetic colorimetric assay to measure activity of GPX	Costantini et al. (2011, 2013), Jenni-Eiermann et al. (2014)
Specific circulating antioxidants		
Uric acid	Many colorimetric commercial kits are available to measure uric acid in circulation	Tsahar et al. (2006), Cohen et al. (2007), Costantini (2011)
Uric acid:allantoin ratio	Uric acid and allantoin colorimentric assays run separately to compare values of reduced and oxidized forms	Tsahar et al. (2006)
Glutathione and other thiols	Commercial kit, colorimetric assay to measure compounds with sulfhydryl (–SH) groups that can reduce pro-oxidants	Costantini et al. (2011, 2013)
GSH:GSSG ratio	Colorimetric determination of reduced and oxidized glutathione, with or without a commercial kit	Mahmoud and Edens (2003), Isaksson et al. (2005)
Vitamin E	Detection of compound-specific peak via HPLC	Supporting Information (Appendix 4) in Cohen and McGraw (2009), Online Supplementary Material in Cohen et al. (2009)
Carotenoids	Detection of compound-specific peak via HPLC	Supporting Information (Appendix 4) in Cohen and McGraw (2009), Online Supplementary Material in Cohen et al. (2009)
Polyphenols	Detection of compound-specific peak via HPLC	Cao and Prior (1999), He et al. (2006), Catoni et al. (2008)
Oxidative damage		
Markers of lipid oxidation		
d-ROMs test	Commercial kit; colorimetric assay to measure hydroperoxides	Costantini et al. (2007, 2011, 2013)
Markers of protein oxidation		
Protein carbonyls	Commercial kit is available although not always used; colorimetric assay to detect carbonyl groups per unit protein	Costantini et al. (2013), Jenni-Eiermann et al. (2014)

recommend further measurement of individual antioxidant compounds for questions that address dietary effects on circulating antioxidant levels or questions targeted to the effects of oxidative challenges on particular aspects of the antioxidant system.

Total non-enzymatic antioxidant capacity. There are several general assays that can be

used to assess the capacity of the non-enzymatic antioxidant pool of a plasma sample to quench pro-oxidants, e.g., OXY-adsorbent test (OXY), Trolox equivalent antioxidant capacity (TEAC; also known as total antioxidant capacity [TAC]), ferric reducing ability of plasma (FRAP), and oxygen radical absorbance capacity (ORAC; Monaghan et al. 2009, Costantini 2011). These assays, however, do not measure the same components, and their usefulness under different circumstances has been discussed elsewhere (Costantini 2011, Alan and McWilliams 2013).

Two spectrophotometric assays that have been used widely and are relevant for field studies are TEAC and OXY (Table 2). As modified by Cohen et al. (2007), the TEAC test can be run on small volumes of blood and accounts for a variety of circulating micromolecular antioxidants, excluding albumin and other proteins. TEAC works by activation of a chromogenic free radical by hydrogen peroxide. Antioxidants in the sample being tested quench this radical to convert it back to its clear form. Recently, however, avian studies—including our own—favored use of the OXY test, a commercial kit (Diacron International, Grosseto, Italy) that measures the ability of a plasma sample to neutralize an oxidizing assault of hypochlorous acid (HOCl), an endogenous pro-oxidant. Like TEAC, this test accounts for a variety of non-enzymatic antioxidants, including vitamins C and E, carotenoids, and thiols (Costantini 2011), and can be run on small volumes of plasma.

A main difference between the two tests is whether they include the action of uric acid, and hence must be corrected for it. Although non-enzymatic endogenous antioxidants such as GSH are up- or down-regulated in response to need, and exogenous antioxidants such as vitamin E and carotenoids are acquired through the diet, uric acid is a catabolically derived antioxidant; it is the final product of protein catabolism in birds and, therefore, its increased presence in the circulation is the result of increased breakdown of dietary or body protein. Whether an increase or decrease in circulating uric acid can reliably indicate a bird's preparation for or response to an oxidative challenge is therefore unclear. Researchers often prefer to evaluate uric acid separately, given that its origin and function differ from those of other antioxidants.

The different chemical processes of assays dictate what they measure. The TEAC test measures the contribution of uric acid to circulating antioxidant capacity and so uric acid is typically measured along with TEAC in a separate assay; a simple linear regression analysis can then yield "residual TEAC" values used to evaluate the non-uric acid portion of total antioxidant capacity (e.g., Cohen et al. 2007, Cohen and McGraw 2009, Alan and McWilliams 2013, Cram et al. 2015). OXY does not include the antioxidant properties of uric acid, so is useful for studies where investigators do not want to integrate this potentially confounding contribution into their measure of antioxidant capacity (Costantini 2011, Alan et al. 2013).

Enzymatic protection of red blood cells. Pairing measurement of non-enzymatic antioxidant capacity of plasma with measurement of GPX in red blood cells (Table 2) is emerging as an important research strategy because the non-enzymatic and enzymatic portions of the antioxidant system appear to work in concert through different mechanisms (Costantini et al. 2011). Costantini et al. (2011:1151–1152) suggested that the two systems may be regulated differently, and specifically suggested that "measuring both antioxidant capacity and GPX activity may produce a better estimate of blood antioxidant status than measurements of a single component." Remember that GPX is specifically useful in neutralizing hydrogen peroxide and other hydroperoxides, including lipid peroxides from the lipid peroxidation cascade; this enzyme therefore appears to be particularly important in protecting red blood cells from oxidative damage from these pro-oxidants (Jenni-Eiermann et al. 2014). We suggest that this aspect of antioxidant capacity should be favored in future studies as an indicator of the acute endogenous responses of birds to oxidative challenges, along with erythrocyte activities of SOD and CAT if sample volumes permit (e.g., Oropesa et al. 2013). Measuring GPX in red blood cells typically involves the spectrometric Ransel assay, where the activity of this enzyme and its ability to oxidize GSH is assessed by a decrease in absorbance (Costantini et al. 2011, 2013, Jenni-Eiermann et al. 2014).

Specific circulating antioxidants. The antioxidants most commonly measured in the circulation of birds include uric acid, GSH and other thiols (compounds with a sulfhydryl [–SH]

group), vitamin E, carotenoids, and polyphenols (Table 2). Although uric acid, GSH, and other thiols are endogenously produced and therefore their levels can be internally modified, vitamin E, carotenoids, and polyphenols are acquired by birds through their diets. The choice to track individual antioxidants, therefore, depends on the research question. For example, uric acid may be measured specifically in studies focused on dietary or muscular protein catabolism, whereas vitamin E may be measured and tracked during supplementation experiments, and carotenoids or polyphenols may be assayed during feeding studies, studies that track how these compounds are assimilated and/or mobilized, and/or studies that link antioxidant capacity with dietary habits on stopover.

As discussed above, uric acid has antioxidant properties in addition to serving as the final, excreted, product of protein metabolism. Researchers may find measuring this antioxidant most useful in contexts where they expect protein (whether dietary or from muscle) to be metabolized in large amounts during an oxidative challenge (e.g., Alan and McWilliams 2013). Although uric acid is an antioxidant in its reduced form, the ratio of uric acid and its oxidized form, allantoin, has also been suggested to be an indicator of oxidative status (see Tsahar et al. 2006 for some methodological considerations for measurement). Birds lack the enzyme urate oxidase, and therefore the presence of allantoin in these species indicates non-enzymatic oxidation of uric acid—in other words, an oxidative challenge (Tsahar et al. 2006).

The benefits of GSH were noted previously, particularly as a substrate for the enzyme GPX; circulating levels of GSH and GPX seem to be integrated (Costantini et al. 2011). Measuring the ratio between the reduced (helpful) form of GSH and its oxidized form (GSSG) in particular may provide a useful index of oxidative status, a concept similar to the ratio of reduced-to-oxidized uric acid. This ratio between GSH and GSSG has been examined in relation to exercise in humans (see summary and citations in Urso and Clarkson 2003) and in the context of toxicant challenges in birds (Henny et al. 2002, Hoffman 2002, Isaksson et al. 2005). Its applicability to avian reproductive and migratory contexts deserves further study.

Vitamin E, carotenoids, and polyphenols (including anthocyanins and related compounds) can be measured in plasma via high performance liquid chromatography (HPLC) (vitamin E and carotenoids: Cohen and McGraw 2009, Cohen et al. 2009; polyphenols: Cao and Prior 1999, He et al. 2006, Catoni et al. 2008). Given the increasing recognition of anthocyanins in the diet of birds during migration (Alan et al. 2013, Bolser et al. 2013), we believe future researchers would benefit from tracking these compounds in wild birds on stopover, particularly when fruit consumption is high. Remember, however, that anthocyanins, and other flavonoids, may be degraded to other metabolites during digestion, and so researchers may benefit by looking for not only the original compounds in blood, but also derivative metabolites. Furthermore, there is evidence that carotenoids are stored in, and mobilized from, fatty tissues and may not contribute highly to circulating antioxidant capacity in a chronic way (Costantini and Møller 2008, Cohen and McGraw 2009, Metzger and Bairlein 2011, Simons et al. 2012); therefore, these measures may be most helpful for tracking the acute accumulation or mobilization of dietary antioxidants at stopover sites or during oxidative challenges.

Measuring oxidative damage in blood. Measures of oxidative damage are as varied as measures of antioxidants, and can provide different glimpses into a bird's oxidative status. For birds during migration, the most relevant markers are those for lipid and protein oxidation. Wherever possible, we recommend that these two types should be used in concert.

Markers of lipid oxidation. Given the importance of fats to migrating birds, measuring lipid damage will be important for gauging how exercise and other stressors affect their oxidative status. Lipid oxidation can be assessed in various forms, e.g., isoprostane assays, detection of malondialdehyde (MDA) via HPLC, thiobarbituric acid reactive substances (TBARS), or d-ROMs test (Monaghan et al. 2009, Cohen et al. 2010). A prospective problem with all these assays is the potential for detection of confounding molecules, inflating estimates of natural damage (Monaghan et al. 2009).

The d-ROMs test kit (Diacron International, Grosseto, Italy; Table 2) has emerged as a favored assay in avian studies (e.g., Costantini et al. 2007, 2008, 2011, 2013, 2014, 2015, Alan

and McWilliams 2013, Skrip et al. 2015). The test measures oxidative damage as the presence of circulating hydroperoxides, which include products of the lipid oxidation cascade, as well as protein and nucleic acid oxidation. Kilk et al. (2014) suggested that the copper-containing protein ceruloplasmin may contribute to test signal, but Costantini et al. (2014, 2015) found otherwise. The d-ROMs test has revealed many useful results regarding the oxidative status of exercising birds, including, e.g., higher d-ROMs levels in exercised pigeons (Costantini et al. 2008), a decrease in levels of d-ROMs the longer birds were on stopover after flights (Skrip et al. 2015), and d-ROMs values predicted return of seabirds to their nesting sites in multiple breeding seasons (Costantini and Dell'Omo 2015). In addition, circulating levels of d-ROMs covaried with circulating triglyceride levels in birds (Pérez-Rodríguez et al. 2015) and were affected by the fat quality in the diets of birds (Alan and McWilliams 2013). Furthermore, we have found that d-ROMs are correlated with fat stores in migratory birds on stopover both before and after long flights (Skrip et al. 2015).

Markers of protein oxidation. In addition to fats, protein is also essential for migration performance, not only to support fat-based metabolism, but also as a source of metabolic water (Gerson and Guglielmo 2011) and to restore flight and other muscles. Reactions with pro-oxidants can introduce carbonyl groups (an oxygen double-bonded to carbon [C=O]) into proteins, and these stable changes are easily detected in plasma (e.g., Costantini et al. 2013) and red blood cells (e.g., Jenni-Eiermann et al. 2014). Protein carbonyls (Table 2) are commonly accepted as a useful indicator of protein damage from oxidation, and can be measured via colorimetric assay while accounting for total protein in samples, including via use of commercial kits (e.g., Heiss and Schoech 2012). Recently, high levels of this protein damage have been associated with low muscle score in actively migrating songbirds, suggesting that individuals in poor condition may suffer more oxidative damage (Jenni-Eiermann et al. 2014). The effect of diet and flight exercise on protein damage deserves further study, particularly in the context of preparation for and recovery from long-duration flights.

FUTURE DIRECTIONS

Condition-dependent aspects of oxidative state in birds during migration. Recent work during autumn migration has shown the circulating non-enzymatic antioxidant capacity of birds preparing for long flights to be condition-dependent, with fatter birds showing higher antioxidant capacity (Skrip et al. 2015). The mechanisms underlying this phenomenon have yet to be investigated. Particularly revealing would be the studies that manipulate fat condition of migrants (e.g., Smith and McWilliams 2014b) and/or track fattening rates in birds using plasma metabolites such as triglycerides (e.g., see Smith and McWilliams 2010), and then also measure antioxidant capacity, damage, or particular antioxidants (e.g., Pérez-Rodríguez et al. 2015). Partitioning blood sampling to focus on dawn, midday, and evening feeding, when hungers for different requirements are expected to differ (e.g., Beaulieu and Schaefer 2014), would also be most illuminating. In addition, future researchers may focus on how migration distance, protein content of diet, and stopover duration (and hence extent of damage repair) affect damage levels in wild birds.

Availability of dietary antioxidants during migration. Thus far, research concerning dietary antioxidants on stopover has focused on autumn migration in temperate regions. Whether and how birds acquire dietary antioxidants before their spring journeys from tropical areas remains a considerable gap in our understanding of migratory birds. Furthermore, for both seasons, we have yet to fully understand how use of stopover sites helps birds overcome oxidative costs. How does the specific mixture of antioxidants in bird blood and fat depots change within and between stopovers? What is the effect of flight on levels of particular antioxidant compounds, and does diet dictate the mixture? The distribution and seasonal availability of antioxidant- or calorie-rich plant communities could shape the migration strategies of songbirds, and so learning how birds choose and utilize foods on the landscape may aid conservation efforts.

Processing and storage of dietary antioxidants. It is well established that birds are capable of assimilating dietary antioxidants,

and that fat-soluble antioxidants such as carotenoids and vitamin E can be stored in tissues, but a major gap in our understanding concerns the storage and use of water-soluble antioxidants such as polyphenols, and how the processing and routing of dietary antioxidants prevent damage in the most vulnerable tissues. If birds eat antioxidants, do they make it to the mitochondria where pro-oxidants are generated? If there is greater demand in some tissues for antioxidants (e.g., flight muscle in exercising birds) does the routing of antioxidants change? Given that antioxidants interact and recycle each other (see above), do volant birds manage combinations of antioxidants differently than non-flying vertebrates?

Carryover effects and competing demands. Studies that evaluate the oxidative condition of migratory birds on breeding grounds will be instrumental in teasing apart the costs of oxidative damage, e.g., whether effects of flight (and inadequate preparation or recovery) are immediate and brief, and whether they have fitness costs (e.g., Costantini and Dell'Omo 2015). Given that birds begin breeding in spring shortly after performing challenging long-distance flights, how do the competing demands of reproduction versus migration influence allocation of antioxidants? Are birds with higher antioxidant levels before and after migration better able to provision their eggs with antioxidants, or suffer less oxidative damage during chick-rearing?

Measurement of acute change and marker sensitivity to external change. Further research on the utility of different oxidative markers for various study questions, and their sensitivity to confounding factors such as handling, time since capture, and time of day will help improve study design. For example, in several studies, investigators have failed to detect directional changes in OXY and d-ROMs between first capture and restraint in a cloth handling bag for between 20 and 162 min (Costantini et al. 2007, Skrip et al. 2015). However, researchers have yet to determine if acute changes occur within a 20-min period or confirm whether time of day is an important confounding factor for evaluation of oxidative status. Further methodological research will be of utmost importance in evaluating the oxidative state of birds in field contexts.

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