What do we really know about oxidative stress?
Facing the problems with current oxidative stress studies in passerine birds.

by

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Abstract

Oxidative stress occurs in organisms when there are not enough antioxidants to satiate reactive oxygen species formed during oxidative phosphorylation within the mitochondria. The excess in reactive oxygen species can cause oxidative damage to lipids, proteins, and DNA. This damage has proposed involvement in numerous biological processes, including certain diseases, immune response, and energetically demanding life-history traits, such as reproduction and longevity. The proposed importance necessitates that investigators determine how to best measure oxidative damage and what causes damage to differ between species and individuals. Recently, methods for quantifying oxidative stress as well as the relationships proposed between certain biological processes and oxidative damage have been questioned. Herein, I investigate the value of erythrocytes and plasma as indicators of oxidative damage present within pectoralis, heart, and liver tissues of Northern Cardinals, as well as measure the effect of energy expenditure on oxidative damage in pectoralis and liver tissues of House Finches.

The preferred method for evaluating the products of oxidative damage in evolutionary and ecological studies is through plasma or erythrocyte samples. This is assumed to be indicative of oxidative damage occurring throughout the body, yet variations between organ generation of oxidative damage and rate of oxidative damage by-product transfer into circulation make this questionable. Some mammalian studies have garnered support for this assumption, however it has never been investigated in birds. This relationship is further confounded in birds due to the

presence of functioning mitochondria within avian erythrocytes. To determine if erythrocytes and plasma provide valuable insight into oxidative damage throughout the body of birds, in chapter 1 I measured two different markers of oxidative damage, 4-hydroxynonenal and protein carbonyls, as well as antioxidant potential in erythrocytes, plasma, pectoralis, liver, and heart of 18 wild Northern Cardinals. I found no evidence to support the use of either erythrocytes or plasma as valuable indicators of oxidative damage to other tissues.

When investigating the effects of oxidative damage on life-history traits, it is often assumed that reactive oxygen species production is positively correlated with an animal's metabolic rate as more electrons must be moved through the electron transport system to accommodate increased energy demands. Yet the effect of energy expenditure on oxidative damage has never been directly evaluated in birds. In chapter 2, I measured levels of oxidative damage and antioxidants in pectoralis and liver tissues of 24 wild caught House Finches that spent at least two weeks in one of three cage sizes to promote low-, medium-, and high-energy expenditure. I found no significant evidence supporting an increase in oxidative damage relative to an increase in energy expenditure. The results of these two chapters indicate that the relationships surrounding oxidative damage are more complicated than originally proposed, and that further investigations need to be made into establishing more reliable methods of oxidative damage measurement.

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List of Abbreviations

4HNE	4-hydroxynonenal
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ETS	Electron transport system
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
OXPHOS	Oxidative phosphorylation
pmf	Proton motive force
RBC	Red blood cell
ROS	Reactive oxygen species
TBARS	Thiobarbituric reactive substances

Introduction

Oxidative stress, its importance in science, and major problems investigators face when testing oxidative stress hypotheses

As aerobic respiration allows for a much greater rate of energy production than its anaerobic counterpart, the initial appearance of aerobic respiration was a substantial evolutionary development. This transition ultimately permitted the evolution of complex eukaryotic life (Dismukes et al. 2001; Jiang et al. 2010; Lane and Martin 2010; Stamati et al. 2011). Aerobic respiration within the mitochondria relies on the transfer of electrons through a series of energy transferring enzymatic complexes known as the electron transport system (ETS), at the end of which they are paired with molecular oxygen and hydrogen to produce water at the same time as adenosine diphosphate (ADP) is bound to inorganic phosphate to from the energy storing molecule, adenosine triphosphate (ATP) (Ludwig et al. 2001; Fernie et al. 2004; Arnold 2012; Srinivasan and Avadhani 2012; Friedrich 2014; Mailloux 2015). While this mechanism supports the efficient production of ATP, it often allows unpaired electrons to escape and rapidly bind to molecular oxygen to form reactive oxygen species (ROS), which are highly unstable molecules capable of causing a cascade of damage to cellular components (Chance et al. 1979; Brand 2000; Liu et al. 2002; Brand et al. 2004; Murphy 2009). Organisms rely on enzymatic and non-enzymatic antioxidants to transform these ROS into less harmful conformations before any damage is done (Burton and Ingold 1984; Cadenas 1997; Carr and Frei 1999; Mates et al. 1999;

Sies 1991; Halliwell and Gutteridge 2007; Valko et al. 2007). When this balance in antioxidants and ROS is disrupted, the cells are said to experience oxidative stress (Chance et al. 1979; Felton 1995; Sies 1991; Zelko et al. 2002; Halliwell and Gutteridge 2007). In a state of oxidative stress, there are not enough antioxidants to combat the levels of ROS, and these unquenched ROS go on to cause damage to lipids, protein, and DNA, known as oxidative damage (Chance et al. 1979; Liu et al. 2002; Murphy 2009; Kowaltowski et al. 2009).

Oxidative damage has proposed involvement in many biological processes. In the medical field, oxidative damage has been linked to a myriad of diseases, including cancer, diabetes, cardiac disease, and arthritis (Trush and Kensler 1991; Wiseman and Halliwell 1996; Valko et al. 2006; Valko et al. 2007). Similarly, immune response cells may release reactive oxygen species to combat pathogens, which in turn, increases oxidative damage in organisms (Klasing 2004; Halliwell and Gutteridge 2007; Costantini and Möller 2009). Oxidative damage has been suggested to play a role in the development of sexually selected traits (Alonso-Alvarez et al. 2008; but see Koch et al. 2016) and has also been implicated in energetically demanding life-history traits, such as reproduction and longevity (Beckman and Ames 1998; Wiersma et al. 2004; Speakman and Garratt 2013).

Much of the damage that animals experience from ROS is derived from unpaired electrons that have escaped the ETS (Chance et al. 1979; Brand 2000; Liu et al. 2002; Brand et al. 2004; Murphy 2009; Brand 2016). Damage includes, but is not limited to, key enzymatic proteins in the ETS, phospholipid bilayers, and mitochondrial DNA (mtDNA) (Barja 1999; Monaghan et al. 2009; Circu and Aw 2010). Damage to any of the enzymatic proteins in the ETS can change their conformation, causing dysfunction in the chain and less efficient ATP synthesis (Dröge 2002; Monaghan et al. 2009), less available energy for the organisms, and, consequently,

the inability to meet energy demands. Damage to the phospholipid bilayer that composes the inner and outer mitochondrial membranes compromises the hydrogen gradient required to fuel the ETS (Gutteridge 1995, Hulbert et al. 2007, Monaghan et al. 2009). Damage to the mtDNA can cause deleterious mutations, which could also cause dysfunction in oxidative phosphorylation (OXPHOS) (Richter et al. 1988, Monaghan et al. 2009). Furthermore, mitochondria with ROS damaged DNA may replicate before the damage is repaired, leading to the proliferation of damaged mitochondria throughout the cell (Richter et al. 1988; Zhang and Hood 2016) that can reduce an animal's performance and hasten aging.

Many published studies that have evaluated oxidative damage in ecological or evolutionary context have quantified the products of oxidative damage in the blood, which frees investigators from destructive sampling and allows for longitudinal data collection (Speakman et al. 2015). However, this approach assumes that damage detected in blood is indicative of processes that are occurring throughout the body (Arguelles et al. 2004; Veskoukis et al. 2009; Speakman and Garratt 2013). For this assumption to be true, organs would need to equally contribute to a cumulative pool of products of oxidative damage found in blood, which would need to enter and leave the bloodstream at similar and steady rates. In addition, many studies have been based on the assumption that ROS production is positively correlated with an animal's metabolic rate (Wiersma et al. 2004; Speakman and Garratt 2013). Because a higher metabolic rate requires more electrons to be shuttled through the ETS, it seems feasible that an individual with a heightened metabolic rate would release more electrons, leading to greater levels of ROS production. Yet as our understanding of the mechanisms behind OXPHOS and ROS production increase, this assumption becomes less probable. Both approaches have recently been challenged

and, thus, it is necessary to evaluate the validity of each approach to provide prospective on published work and to determine if alternative methods should be considered for future works.

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Chapter One

Is blood a valuable indicator of oxidative stress in birds?

Abstract

The oxidative stress status of an individual is often determined by measuring markers of oxidative damage and antioxidant concentrations in blood. Though collecting blood is preferable to organs due to its noninvasive nature, it is unclear if these results provide adequate information about the oxidative stress status of specific organs and if specific organs effect the oxidative stress status of the whole organism. Though some investigations have been made on these relationships in mammals, no such steps have been taken with birds. Avian erythrocytes have functioning mitochondria, which could potentially cause localized oxidative damage and a local upregulation of antioxidants in blood and confound possible relationships between the oxidative damage and antioxidant biomarkers found in specific organs and blood. The aim of this study was to determine if blood provides an accurate indication of relative oxidative damage and antioxidant production by different organ systems of birds. Two biomarkers of oxidative damage and total antioxidant potential were measured in erythrocytes, plasma, pectoralis, liver, and heart of Northern Cardinals. Pearson correlation analyses were used to determine the relationship of damage biomarkers and antioxidant potential between blood components and organs. Our results indicate that neither erythrocytes nor plasma are reliable indicators of organ specific

oxidative stress. No significant correlations were found between any of the organs for either oxidative damage biomarker. Antioxidant potentials were moderately correlated only between heart and erythrocyte.

Introduction

Oxidative stress occurs when the number of reactive oxygen species (ROS) formed as a product of electrons escaping the electron transport system (ETS) outnumber available antioxidants that lessen or negate their negative effects (Chance et al. 1979; Felton 1995; Sies 1991; Zelko et al. 2002; Halliwell and Gutteridge 2007; Weydert and Cullen 2010). If not neutralized, ROS damage lipids, proteins, and DNA (Chance et al. 1979; Richter et al. 1988; Felton 1995; Gutteridge 1995; Herrero and Barja 1999; Brand 2000; Dröge 2002; Liu et al. 2002; Brand et al. 2004; Hulbert et al. 2007; Kowaltowski et al. 2009; Murphy 2009; Monaghan et al. 2009; Circu and Aw 2010; Zhang and Hood 2016). This oxidative damage is thought to play a role in many biological processes, including select diseases, immune response, and aging (Trush and Kensler 1991; Wiseman and Halliwell 1996; Beckman and Ames 1998; Klasing 2004; Valko et al. 2006, 2007; Halliwell and Gutteridge 2007; Costantini and Möller 2009). Because of its proposed importance, investigators have developed several methods for measuring oxidative damage through the detection and quantification of products and by-products of oxidative damage, as well as the presence of enzymatic and non-enzymatic antioxidants. Both oxidative damage and antioxidant concentration are often measured within the same organism to better understand how the status of an individual's oxidative stress, not just their oxidative damage or use of antioxidants, effects biological processes.

In studies of birds, all published work completed to date has quantified the products of oxidative damage in blood, rather than other organs (Speakman and Garratt 2013; Blount et al. 2016). This approach has been preferred because blood collection is minimally invasive and longitudinal sampling is possible. Oxidative damage detected in blood is then interpreted to be a cumulative measure of damage occurring throughout the body (Speakman and Garratt 2013). While most oxidative damage is localized within the cells, quantifying oxidative damage in blood could prove valuable if all organs display relatively similar patterns of damage and if the processes that allow the products of damage to move into the circulation are similar between organ systems (Barja 1999). If these characteristics were true, a linear relationship between oxidative damage in the blood and oxidative damage in the largest and/or many of the small organs within the body would be expected. Likewise, data from the blood could also be valuable if it reliably represents a single organ that plays an important role in animal performance.

Our understanding of how oxidative damage in organs is related to the markers of damage in blood is largely based on work in laboratory animals. We know that by-products of lipid damage in the organs may be delayed relative to their appearance in the blood and they can be short lived. Levels of lipid oxidative damage to the liver increased more rapidly than that of plasma, indicating that the release of damage by-products into circulation is slow (Morrow et al. 1992). Other by-products of lipid damage, such as alkane and pentane, are quickly released by exhalation (Hageman et al. 1992). Thymine glycol and thymidine glycol, by-products of DNA oxidative damage, are only in circulation in the blood for 24 hours before being excreted in urine (De Zwart et al. 1999). In addition, time of day appears to influence the levels of malondialdehyde (MDA) circulating in the blood independent of patterns of organ damage; MDA is an indicator of lipid peroxidation (Akbulut et al. 2003). The circadian changes in MDA

may be associated with circadian changes in DNA synthesis (Berger and Daniel 1982; Rodgers and Borst 1983), which could alter relative rates of damage repair. Sohal et al. (1994) found that DNA oxidative damage in rats was greatest in long-lived skeletal muscle, heart, and brain cells relative to short-lived liver and kidney cells and proposed that the relative contribution of each organ to circulating levels of markers of DNA damage may vary. If the propensity to release products of damage into the circulation varies between cells types and with relative stage of the cell in its life cycle, blood could be an ambiguous measure of even recent oxidative damage.

While data on the relationship between oxidative damage in blood and organs in birds are lacking (Speakman and Garratt 2013; Blount et al. 2016), several studies have found little or no relationship between the products of damage in blood and other organs in mammals (but see Arguelles et al. 2004 and Veskoukis et al. 2009). For example, the liver constitutes one of the largest and most metabolically demanding organs in the body that doubles in size during lactation in rodents (Hamosh et al. 1970). Yet, several investigators have found that protein carbonyls are reduced in liver but elevated in the blood of lactating female rodents (Yang et al. 2013; Xu et al. 2014). Thus, quantifying damage in blood clearly misses the pattern of damage displayed by this organ.

Arguelles et al. (2004) induced change in ROS production and tested for correlations between markers of oxidative damage in blood and other individual organs. Rats were fed altered diets to induce different levels of oxidative damage and then thiobarbituric reactive substances (TBARS), lipid peroxides, and protein carbonyl groups in blood serum, liver, spleen, heart, and kidney of the rats were measured. They found that, while levels of lipid peroxides (a measure of lipid oxidative damage) in the blood and other organs were correlated, TBARS (an alternative measure of lipid oxidative damage) and protein carbonyls were not correlated between blood and

other organs. Protein carbonyls were not correlated to TBARS or lipid peroxides in any other the tissues, save the kidney, which had a negative correlation between protein carbonyls and lipid peroxides. This suggests that not only do different forms of damage differ in their relationship between blood and organs, but the relationship between different by-products of the same form of damage may also vary. Veskoukis et al. (2009) measured TBARS and protein carbonyls in plasma, red blood cells (RBCs), skeletal muscle, heart, and liver in rats. Rats were assigned to control, exercise, or allopurinol administered groups, as the latter two are known to affect redox status. Their results indicated that TBARS in the plasma and RBCs satisfactorily correlated with damage to the heart and liver, under all three conditions, and protein carbonyls in the plasma and RBCs satisfactorily correlated with damage to skeletal muscle and heart. Their results also showed that all by-products of oxidative damage were nearly identical between RBCs and plasma, indicating that it is not necessary to measure both.

In a compilation of results from numerous prior studies in mammals, Blount et al. (2016) found several inconsistencies in the patterns damage displayed between the blood and organs when reproductive and non-reproductive females, and females with low and high reproductive effort, were compared. Thus, the work of Blount et al. (2016) and prior empirical studies suggest that, at least in mammals, blood should not be considered a strong indicator of relative oxidative stress incurred by individuals.

While interested in oxidative stress in birds has been high, how oxidative damage in blood is related to that of the organ systems is unknown and may differ from that of mammals. Unlike mammals, avian erythrocytes contain functioning mitochondria (Stier et al. 2013). In theory, mitochondria in erythrocytes should produce ROS and cause localized damage to the erythrocytes or other cells in blood. Thus, it is feasible that oxidative damage in avian blood may

more strongly reflect local damage than damage incurred by other organ systems. As all studies completed to date on oxidative damage in birds have solely been based on damage detected in the blood (Speakman and Garratt 2013; Blount et al. 2016), it is important to determine if blood gives an accurate representation of what is happening in other organ systems and, therefore, it is necessary to compare oxidative damage in multiple organs to that of blood.

Like oxidative damage, antioxidants in blood have also been commonly measured based on similar assumptions. Antioxidants play a vital role in quenching ROS before they cause damage, and thus it can valuable in understanding individual variation in the expression of antioxidants. While the function and transport of many antioxidants have been well studied, comparisons between blood and organs are less common than oxidative damage (but see Veskoukis et al. 2009).

Thus, the aim of this investigation was to determine if blood provides an accurate indication of the relative patterns of oxidative damage and antioxidants in different organ systems of birds. I hypothesize that markers of oxidative damage and antioxidants in blood will not consistently reflect that of other organ systems. Specifically, I expect to find no correlations between oxidative damage and antioxidant potentials in the blood and oxidative damage and antioxidant potentials in liver, pectoralis, or heart of a passerine bird. If blood does reflect oxidative damage in organs, then I would predict the following: 1) Larger organs are more likely to be correlated with damage found in the plasma than smaller organs because they have relatively more mitochondria that contribute products of oxidative damage into the circulation. Further, skeletal muscle and liver, two of the largest and most active organs in the body, are more likely to be correlated with circulating markers of damage in the blood than small vital organs such as the heart that are vital to immediate survival and theoretically should experience a

higher level of protection (Zera and Harshman 2001). 2) If erythrocytes contribute to the oxidative damage in plasma, then plasma and erythrocytes are likely correlated due to their proximity.

Methods

All methods were approved by Auburn University's (AU) Institutional Animal Care and Use Committee (PRN #2015-2698).

Animal and tissue collection

Eighteen Northern Cardinals (*Cardinalis cardinalis*) were collected in Tuskegee National Forest, Auburn University Campus, and Auburn University's E.V. Smith Research Center in Alabama between June 16th and September 6th, 2016. This species of passerine bird is abundant in the United States and easily trapped in Alabama. They are larger than many other common passerine species, which allowed for a large blood sample to be collected. Cardinals were collected using mist nets placed in the flight paths of the birds and finch traps place around established feeders (Hill 2002). Birds were removed from nets immediately upon capture. Cardinals trapped in finch traps continue to display social feeding behaviors while in the traps, which attracts additional birds and increases trapping success and thus, cardinals were allowed to stay in the finch traps up to 20 minutes before removal. Birds were then placed in individual brown paper bags and brought back to the Hood Laboratory on Auburn University Campus and weighed. Birds were then euthanized with a small dose of isoflurane vapors and their core blood

collected and centrifuged at 13,000 rpm for 15 minutes and subsampled into erythrocytes and plasma. The pectoralis muscle, heart, and liver were excised and all tissues frozen at -80 °C until further use.

Oxidative damage measurements

Pectoralis, liver, heart, and packed erythrocytes were homogenized 1:10 wt (mg):vol (uL) in phosphate-buffered saline (PBS) with 10uL of Protease Inhibitor Cocktail (PIC, P8340, Sigma-Aldrich, St. Louis, MO, USA) for every 100uL of PBS. An OMNI Tip homogenizer (OMNI International, Kennesaw, GA, USA) was used and samples were kept on ice during homogenization. Resulting homogenates were centrifuged at 13,000 rpm for 10 minutes in a refrigerated centrifuge and supernatants saved in aliquots to avoid multiple freeze-thaw cycles. Total protein content in the supernatants and plasma were quantified using the Bradford method (Bradford 1976).

Oxidative damage to proteins in the form of protein carbonyls were quantified using OxiSelect Protein Carbonyl ELISA Kit (STA-310, Cell Biolabs, Inc., San Diego, CA, USA). Prepared pectoralis, liver, heart, plasma, and erythrocytes were diluted to 10ug protein/mL PBS and ELISAs performed following manufacturer's specifications. Protein carbonyl derivatives are the most common product of oxidative damage, by both direct reactions of proteins with ROS and indirect reactions of proteins with other by-products of oxidative damage. These derivatives are results of covalent modifications to proline, arginine, lysine, and threonine, and are chemically stable (Reznick and Packer 1994). Therefore, protein carbonyls are thought to be reliable and inclusive indicators of oxidative damage to proteins.

Oxidative damage to lipids in the form of 4-hydroxynonenal (4HNE) protein adducts were quantified using OxiSelect HNE Adduct Competitive ELISA Kit (STA-838, Cell Biolabs, Inc., San Diego, CA, USA) following manufacturer's specifications. Lipid peroxides from oxidative damage to lipids are highly unstable and quickly break down to form unstable by-products, making direct measurement of lipid peroxidation difficult. These by-products are also highly reactive and continue to cause secondary damage. However, 4HNE, a common by-product of lipid peroxidation, is known to form stable adducts with lysine, histidine, and cysteine protein residues that can be measured as a reliable indicator of oxidative damage to lipids (Hoff and O'Neil 1993). Due to sample limitations, 4HNE adducts were not measured for the heart.

Absorbance of samples and standards for both ELISAs were determined using PowerWave XS plate reader (BioTek, Winooski, VT, USA).

Antioxidant measurements

The OxiSelect Ferric Reducing Antioxidant Power (FRAP) Assay Kit (STA-859, Cell Biolabs, Inc., San Diego, CA, USA) was used to determine the antioxidant potential in pectoralis, liver, heart, plasma, and erythrocytes samples. To satiate reactive oxygen species, antioxidants donate electrons to free radicals. This assay simulates that environment by supplying the antioxidants in the samples with ferric iron (Fe^{3+}) that the antioxidants reduce to ferrous iron (Fe^{2+}). This reduction causes a blue coloration that is measured by absorbance to determine the quantity of reduced iron. Samples were prepared per manufacturer's specifications. Pectoralis, liver, heart, and erythrocytes were homogenized (10mg each) in 1.5mL of the provided Assay Buffer, then centrifuged at 12,000 rpm for 15 minutes in a refrigerated

centrifuge and the supernatant saved. Total protein content in the supernatants and plasma were quantified using the Bradford method (Bradford 1976). Supernatants and plasma were diluted to 140 ug of protein per mL in Assay Buffer to standardize protein content between samples. The assay was then performed following manufacturer's specifications. Antioxidants can degrade over time, but antioxidant comparisons were only made between samples from the same individuals and a single individual's tissues were all collected and frozen at the same time. Therefore, any discrepancies due to degradations can be assumed to have occurred equally between each statistically compared group. Absorbance of samples and standards were determined using PowerWave XS plate reader (BioTek, Winooski, VT, USA).

Kit validations

All kits were validated prior to use. For each kit, a full standard curve was made and samples were randomly chosen from each tissue type and diluted stepwise to four different known concentrations in PBS or Assay Reagent, according to kit protocol. Each tissue showed a strong correlation between the concentration of tissue in each sample and detected level of protein carbonyls (R^2=0.98985 (pectoralis), 0.97723 (liver), 0.99755 (heart), 0.99919 (plasma); no validations for erythrocytes due to space limitations), 4HNE adducts (R^2=0.99422 (pectoralis), 0.92458 (liver), 0.95852 (heart), 0.97262 (plasma); no validations for erythrocytes due to space limitations), and antioxidant potential (R^2=0/98768 (pectoralis), 0.99809 (liver), 0.99984 (heart), 0.99596 (plasma), 0.99986 (erythrocytes)).

Statistics

The relationship between variables was determined using Pearson's correlation. P-values were obtained from a one-tailed test hypothesis, that true correlation is greater or less than zero, depending on the direction of the correlation. Though this approach increases the probability of type 1errors and finding correlations that do not exist, it reduces the probability of incorrectly rejecting an established method. Linear models using categorical data were performed to assess differences between groups for all biological variables measured. Significance was established at $p \leq 0.05$ for all analyses. All data were analyzed using R, version 3.2.4 (R Core Team 2016).

Results

Protein carbonyls

There were no detectable levels of protein carbonyls present in plasma, and only nine of nineteen erythrocyte samples had detectable levels. Eleven heart samples also had no detectable levels of protein carbonyls (Table 1; Table 2). Correlation analyses between protein carbonyls in erythrocytes and pectoralis, liver, and heart were run twice, once with all erythrocyte data and again with erythrocyte zeroes and their corresponding values from all other tissues removed (Fig. 1; Fig. 2; Fig. 3).

Significant moderate correlations were found in protein carbonyls between erythrocytes and pectoralis (positive), liver (negative), and heart (positive), as well as between protein carbonyls in pectoralis and heart (positive); however, in the pairwise deletion analyses, the correlations were weak and insignificant (Fig. 1; Fig. 2). No significant correlation was found

between protein carbonyls in the pectoralis or liver, indicating that these organs do not display similar patterns of oxidative stress (Fig. 3).

4HNE

Unlike the protein carbonyls, 4HNE adducts were detected in all tissues measured (Table 1; Table 2). No significant correlations were found between any of the examined tissues (Fig. 3; Fig. 4).

Antioxidant potential

Antioxidant potentials were detected in all tissues measured (Table 2). A significant, moderate negative correlation was found between antioxidant potentials in heart and erythrocytes, but no other significant correlations were found between erythrocytes or plasma and other tissues (Fig. 5; Fig. 6).

Antioxidants and oxidative damage

Full and pairwise deletion data sets were used to analyze correlations between antioxidant potentials and protein carbonyls in heart and erythrocytes to determine correlations with and without the undetectable levels of protein carbonyls. Results of correlation analyses for antioxidants and oxidative damage markers are presented in Table 3. A significant, strong positive correlation was found in the pairwise deletion analysis between antioxidant potential and

protein carbonyls in erythrocytes, though the correlation was weak and borderline significant in the full analysis (Table 3). A significant, moderate negative correlation was found between antioxidant potential and protein carbonyls in pectoralis (Table 3). No other correlations between antioxidants and oxidative damage markers within tissues were significant (Table 3).

Discussion

The aim of this study was to determine if markers of oxidative damage present in the blood provide an accurate indication of the relative patterns of oxidative damage in different organ systems of birds. To my knowledge, this is the first study of its kind to focus on an avian instead of mammalian species. Relationships between oxidative damage in erythrocytes, plasma, pectoralis, liver, and heart tissues were determined with two different biomarkers of oxidative damage: protein carbonyls and 4HNE adducts (Table 2). Antioxidant potentials were also measured to determine if differences of antioxidant abilities may affect the correlations of oxidative damage biomarkers between blood and organs (Table 2; Fig. 5; Fig. 6).

Even though the ELISA kit used to measure protein carbonyls is reportedly very sensitive according to the manufacturer, many erythrocytes, heart, and all plasma samples had undetectable levels of carbonyls, and those samples with detectable levels were still low (Table 1; Table 2; Fig. 1; Fig. 2; Fig. 3). It is well established that birds incur less oxidative damage than similarly sized mammals (Ku and Sohal 1993; Barja et al. 1994; Barja 1998; Perez-Campo et al. 1998; Herrero and Barja 1999; Holmes et al. 2001; Pamplona et al. 2005), which may explain oxidative damage markers at levels below detection in many samples. Though it is necessary to be cautious about drawing any conclusions from the correlational analyses obtained

from these data, the lack of protein carbonyl presence in blood indicates oxidative stress in the blood is limited and the blood failed to detect low levels of damage that were found in the liver and pectoralis muscle (Fig. 1; Table 1; Table 2). Should these measurements have been made in a study solely based on the results detectable in blood, many individuals would have been reported to not have detectable oxidative damage.

Further, the significant correlation coefficients from the full analyses suggest that protein damage to the heart is more correlated to damage found in blood than either liver or pectoralis, with pectoralis having the smallest correlation coefficient of any of the organs (Fig. 1). Considering the masses of these different organs, this is the opposite of what I had predicted. Unfortunately, correlations between erythrocytes and plasma were not possible as all plasma samples contained undetectable levels of protein carbonyls (Table 1). Without understanding the exact mechanisms of the transfer of damage from tissues into circulation, it is impossible to say whether the damage found in the erythrocytes was generated from the mitochondria within or passively gained through damage to other tissues.

No correlations were found in markers of lipid damage between blood and any other organs, including between the plasma and the erythrocytes (Fig. 3; Fig. 4). Plasma 4HNE adducts were significantly higher than those found in the erythrocytes, liver, and pectoralis tissues (Table 1). Considering the lack of any correlations or any nearly significant patterns, it is likely that 4HNE adducts from different tissues move into plasma at different rates, and that the relative sizes of the tissues does not increase or decrease their likelihood of correlation with lipid damage in blood. There are many factors that could affect the presence of 4HNE adducts in plasma. Avian erythrocytes contain functioning mitochondria that theoretically produce localized lipid damage (Stier et al. 2013), as well as traverse the whole of the circulatory system,

encountering 4HNE by-products produced by other cells in other tissues (Kiefer and Snyder 2000; Stier et al. 2015). Because of the partial lipid composition of the erythrocytes cell wall, lipid by-products of oxidative damage are thought to travel between tissues and erythrocytes with relative ease, as well as from erythrocytes into the plasma (Kiefer and Snyder 2000; Stier et al. 2015). If investigators are trying to determine the effect of a certain activity, such as reproduction or migration, on an organism's oxidative stress, then plasma results could be misleading as it impossible to deduce whether the culmination of damage within plasma is current or residual or both. Further, the lack of correlation and the inability to know precisely from where the 4HNE adducts in plasma originate makes plasma an inadequate tool for investigating tissue specific questions.

Lack of a correlation between plasma and erythrocyte 4HNE adducts (Fig. 4) does not align with results found in mammalian erythrocytes and plasma, which showed nearly perfect correlation between the two parts (Veskoukis et al. 2009). This suggests that there are differences between how avian and mammalian lipid oxidation products form and/or move between different cells and that their proximity does not increase their likelihood for correlation.

Only one significant correlation was found between antioxidant potentials in the blood and different organs (Fig. 5; Fig. 6). The negative correlation between antioxidants in the heart and erythrocytes suggests that, as antioxidants decrease in the heart, they increase in erythrocytes. Interestingly, protein carbonyls in both tissues were either undetectable or extremely low. However, the moderate correlation coefficient indicates that this relationship is not very strong. Further, the antioxidant potential in erythrocytes was significantly higher than those measured in all other tissues, while 4HNE adducts in erythrocytes were significantly lower than all other tissues and protein carbonyls significantly lower than those in pectoralis and liver

tissues (Table 1). It is possible that ROS are generated within the heart at a much slower rate, decreasing the necessity for a large antioxidant presence while simultaneously causing less damage within the heart. The significantly larger potentials of antioxidants within the erythrocytes may be due to high rates of ROS generation within their mitochondria.

Antioxidants are a major player in mitigating oxidative stress, and the oxidative damage observed from these measurements indicates what ROS remain after antioxidant intervention. The fact that no correlations were observed between antioxidants in plasma or erythrocytes and other organs suggest that differing amounts of ROS are being mitigated in each tissue before damage occurs and, thus, likely effects the relationship of oxidative damage between erythrocytes and plasma and the pectoralis, liver, and heart.

Conclusion

Based on the result of this investigation, there seems to be no relationship between the size of each organ and its likelihood to contribute to products of damage found in the blood. Similarly, the proximity of plasma to erythrocytes does not appear to cause a correlation between their oxidative damage products. Both factors suggest that the diffusion of damage between organs and the circulation is unpredictable. Therefore, oxidative damage markers in blood do not appear to be valuable indicators of oxidative stress in many of a bird's organ systems, at least not under the low stress conditions of this study. Under similar conditions, conclusions drawn about the relative oxidative stress of a bird from blood could be erroneous. Markers of oxidative damage in blood relative to other organ systems should be reevaluated under natural conditions known to elevate oxidative stress, such as migration, and experimentally induced oxidative

conditions. Under conditions that promote oxidative stress, organ systems may be more likely to have similar responses to ROS exposure (Zhang et al. in review). The function of mitochondria in erythrocytes is currently unknown. If mitochondria in erythrocytes are related to some measure of individual performance, it is possible that data from these cells could prove useful in the future.

Table 1

Fraction of samples with non-detectable levels of damage markers in each tissue.

	Erythrocytes	Plasma	Pectoralis	Liver	Heart
Protein carbonyls	10/19	19/19	0/19	1/18	11/19
4HNE	2/19	0/19	0/19	0/19	NA

Table 2

Oxidative damage and antioxidant potential measurements from erythrocytes, plasma, pectoralis, liver, and heart tissues.

	Erythrocytes	Plasma	Pectoralis	Liver	Heart
Protein carbonyls (nmol/mg)	0.070±0.023^{ML}	NA	0.393±0.038^{EH}	0.343±0.068^{EH}	0.098±0.038^{ML}
4HNE (ug/mL)	0.868±0.160*	4.79±0.17*	3.00±0.13*	2.38±0.14*	NA
Antioxidant potential (uM)	106±8*	34.4±2.3*	11.4±1.2^{EPL}	46.8±4.4*	16.4±1.5^{EPL}

Means are presented ± SE. Sample size: liver n=18, all other tissues n=19. Significance established at $P < 0.05$. E = significant difference from erythrocytes; P = significant difference from plasma; M = significant difference from pectoralis; L = significant difference from liver; H = significant difference from heart. * = significant difference between all.

Table 3

Pearson correlation coefficients between oxidative damage and antioxidant potentials in each tissue measured

Tissue		Antioxidants-Protein Carbonyls		Antioxidants-4HNE	
		Correlation Coefficient	*P*-value	Correlation Coefficient	*P*-value
Full					
Erythrocyte		0.347	0.073	0.271	0.131
Plasma		NA	NA	0.046	0.426
Pectoralis		-0.494	0.016*	-0.289	0.115
Liver		0.150	0.276	0.375	0.063
Heart	0.172	0.241	NA	NA	
Pairwise deletion					
Erythrocyte	0.674	0.023*	NA	NA	
Heart	0.528	0.089	NA	NA	

Results from correlational analyses between oxidative damage and antioxidant potentials in each tissue measured, both including zeros from undetectable levels of protein carbonyls in erythrocytes and heart (full) and excluding zeroes (pairwise deletion) *****Significance established at $P < 0.05$

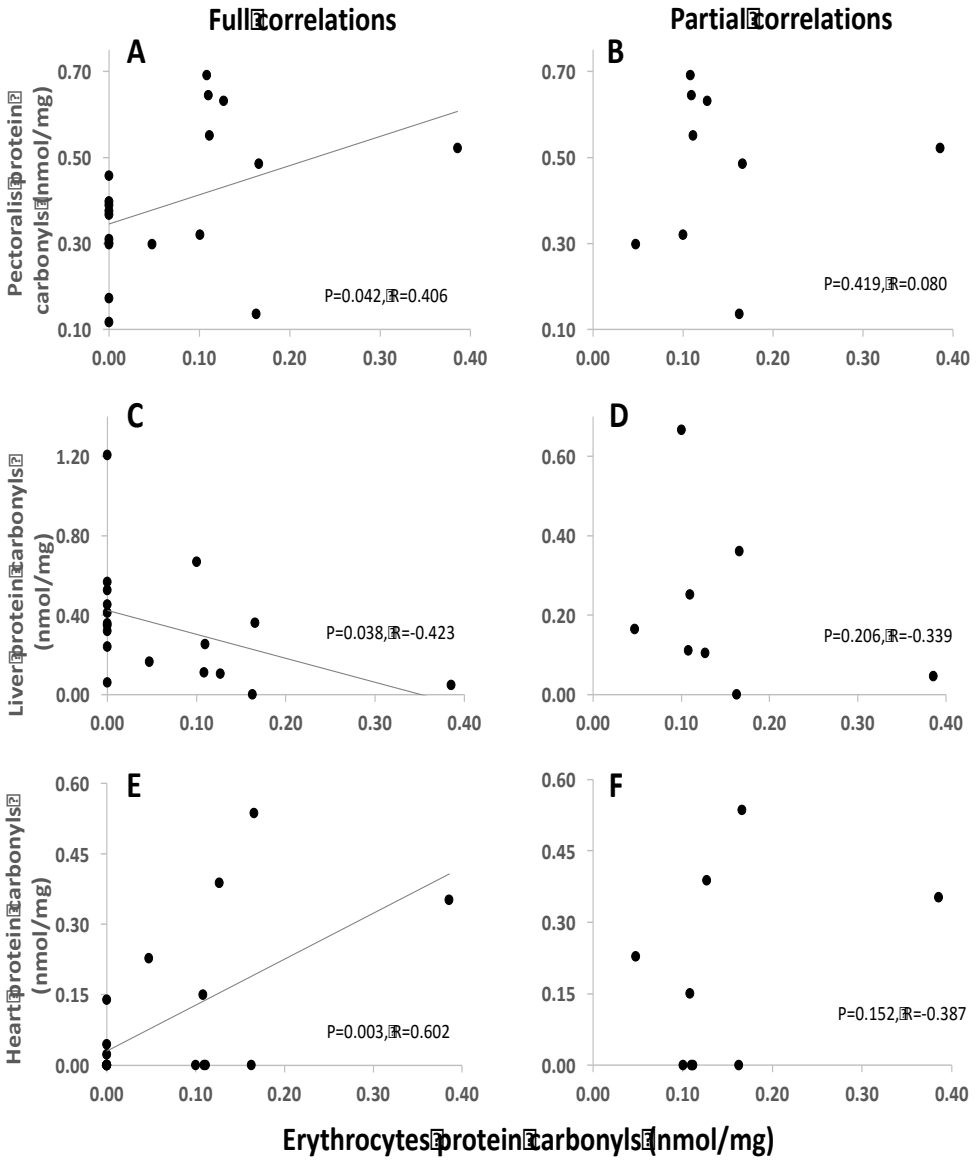

Fig. 1 Correlations between protein carbonyl levels in erythrocytes and other organs. Data includes pectoralis (A, B), liver (C,D), and heart (E, F) in full (A, C, E) and pairwise (B, D, F) comparisons. Full and partial Pearson correlation coefficients are given. Partial correlations are based on a pairwise deletion of samples with without detectable carbonyls. Regression line is shown for significant correlations.

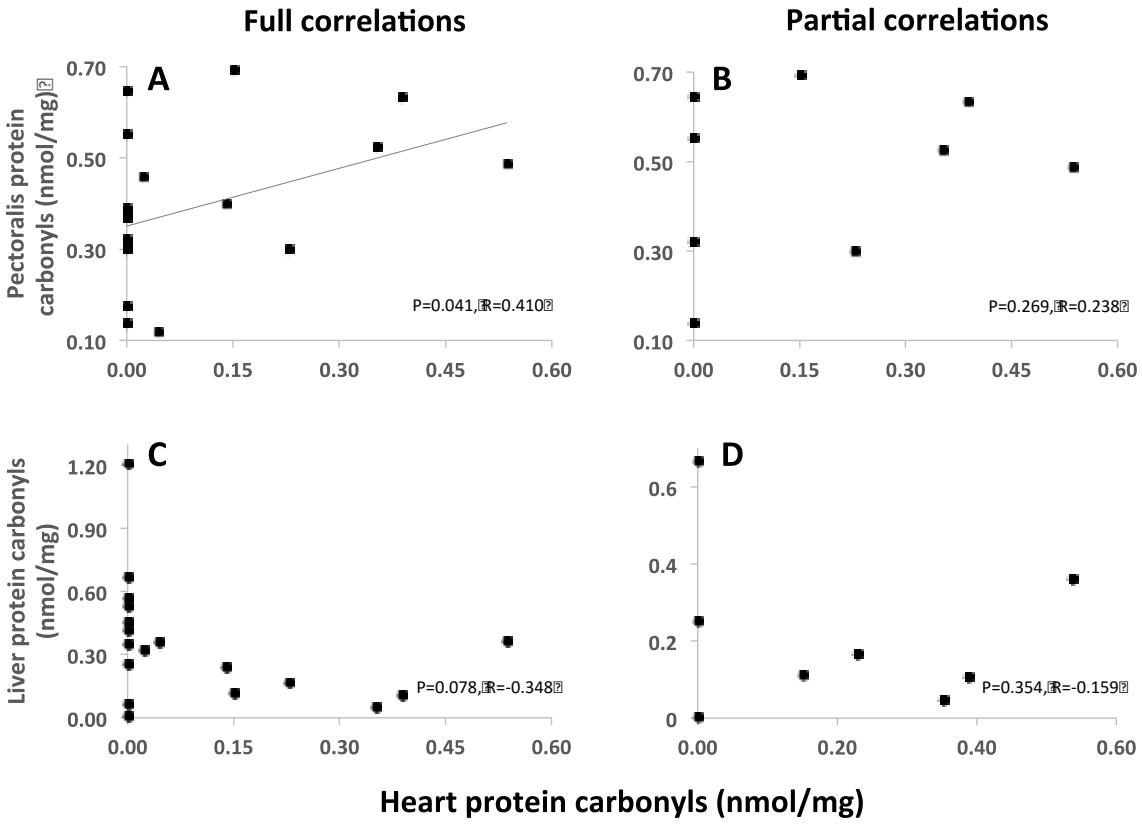

Fig. 2 Correlations between protein carbonyl levels in the heart and other organs. Data includes pectoralis (A, B), liver (C, D) in full (A, C) and pairwise (B, D) comparisons. Full and partial Pearson correlation coefficients are given. Partial correlations are based on a pairwise deletion of samples with without detectable carbonyls. Regression line shown for significant correlations

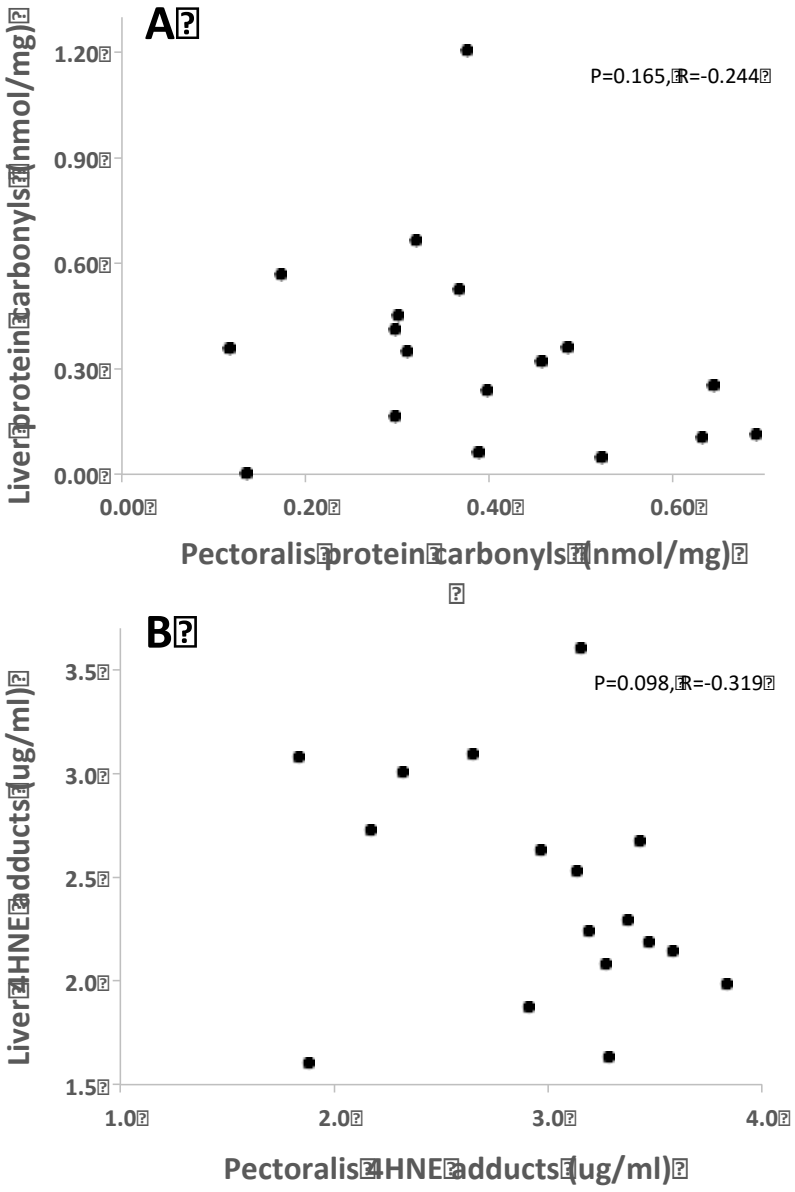

Fig. 3 Correlations between liver and pectoralis protein carbonyl levels (A) and 4HNE adduct levels (B). Full Pearson correlation coefficients are given. Correlations were not significant.

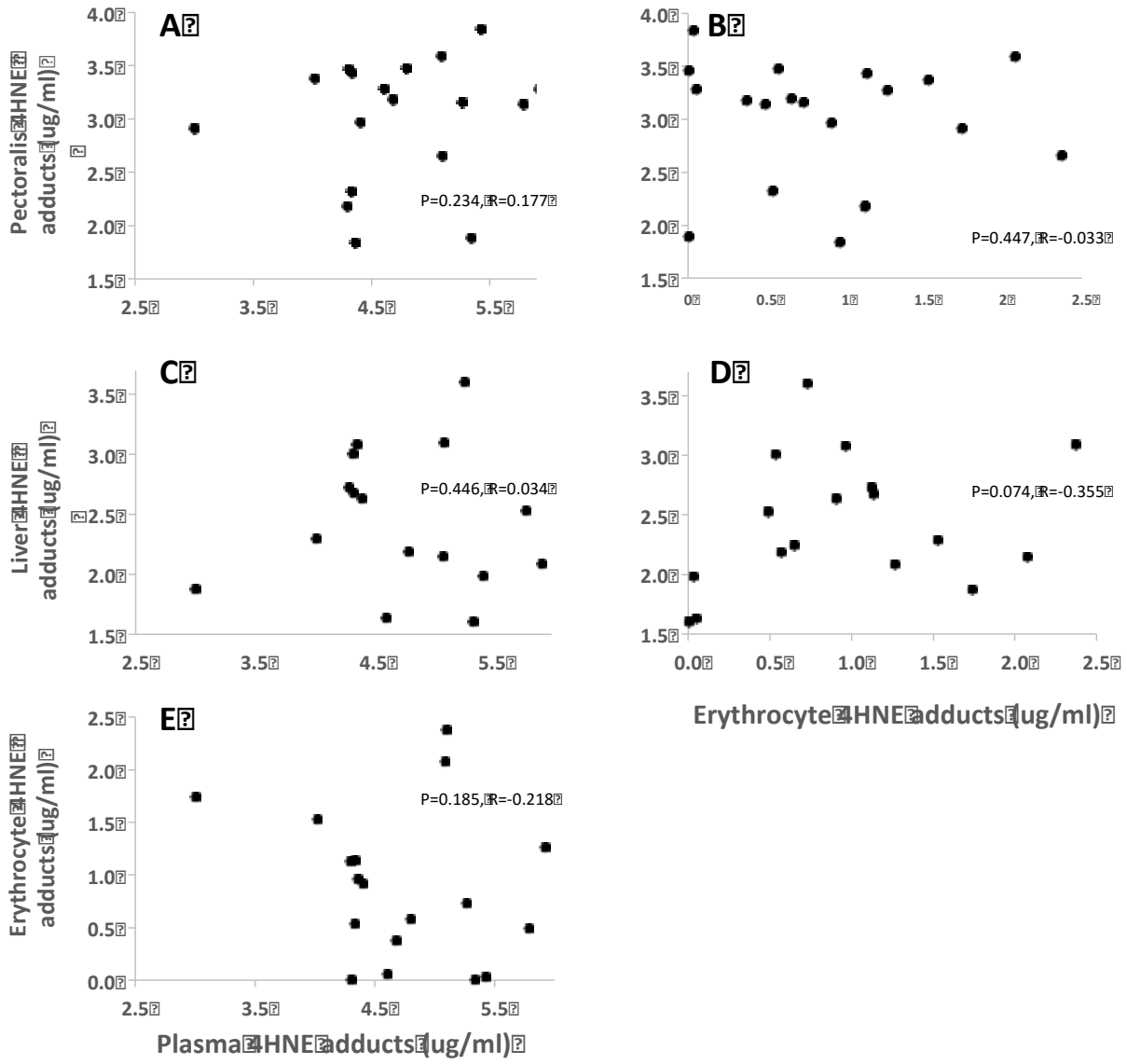

Fig. 4 Correlations between 4HNE adduct levels in the plasma (A, C, E) and erythrocytes (B, D) and other organs. Data includes pectoralis (A, B), liver (C, D) and the relationship between plasma and erythrocytes (E). Full Pearson correlation coefficients are given. Partial correlations are based on a pairwise deletion of samples with without detectable carbonyls. Correlations were not significant.

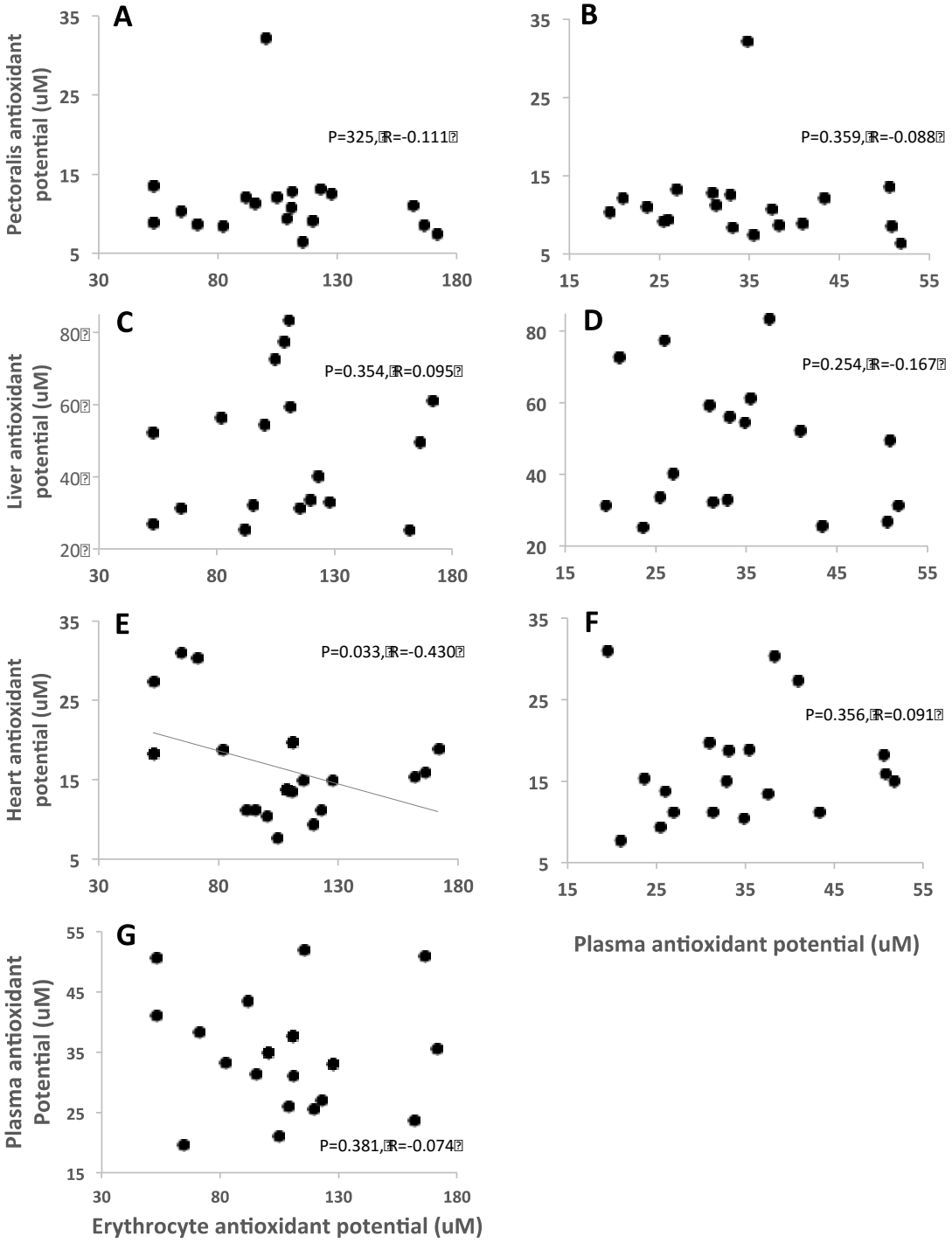

Fig. 5 Correlations between antioxidant potential of erythrocytes (A, C, E, G) and plasma (B, D, F) and other organs. Data includes pectoralis (A, B), liver (C, D), heart (E, F) and the relationship between plasma and erythrocytes (G). Full Pearson correlation coefficients are given. Regression line is shown for significant correlations.

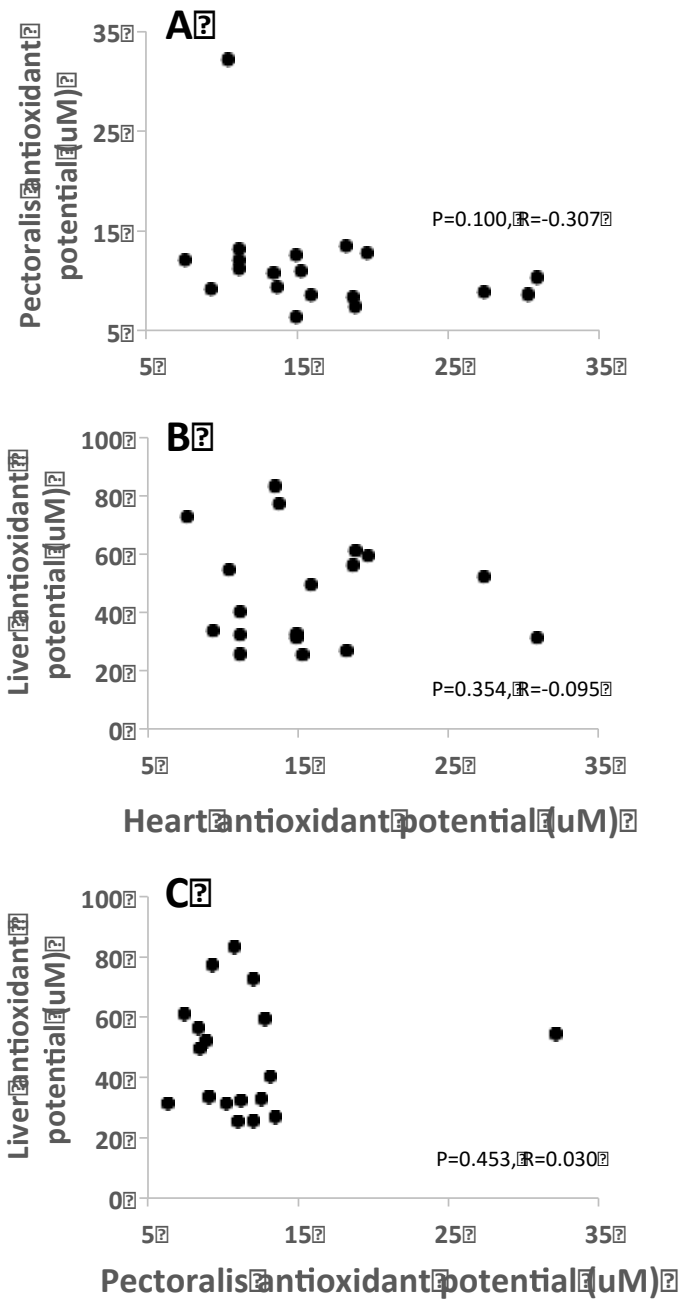

Fig. 6 Correlations between antioxidant potential of the heart and pectoralis (A) heart and liver (B), and pectoralis and liver (C). Full Pearson correlation coefficients are given. Correlations were not significant.

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Chapter Two

A test of the relationship between oxidative damage and energy expenditure in a passerine bird

Abstract

Oxidative damage caused by reactive oxygen species has been implicated as the mechanism responsible for tradeoffs between energetically demanding life-history traits, such as reproduction and longevity. When investigating the effects of oxidative damage on life-history traits, it is often assumed that reactive oxygen species production is positively correlated with an animal's metabolic rate as more electrons must be moved through the electron transport system to accommodate increased energy demands. However, limited empirical data and increasing knowledge of the mechanism behind oxidative damage suggest this assumption is likely incorrect. To more accurately evaluate relationships between cellular metabolism and oxidative production, directly evaluating the relationship between an animal's energy expenditure and reactive oxygen species production *in vivo* is critical. Yet the effect of energy expenditure on oxidative damage has never been directly evaluated in birds. Oxidative damage and antioxidants were measured in pectoralis and liver tissues of 24 wild caught House Finches that spent at least two weeks in one of three cage sizes to promote low-, medium-, and high-energy expenditure.

No significant evidence was found supporting an increase in oxidative damage relative to an increase in energy expenditure.

Introduction

Aerobic respiration, which occurs within mitochondria, is a necessity for complex life (Dismukes et al. 2001; Lane and Martin 2010; Stamati et al. 2011). The final stages of aerobic respiration are carried out by the electron transport system (ETS), which moves electrons through enzymatic complexes to create a proton gradient necessary to produce adenosine triphosphate (ATP) by way of oxidative phosphorylation (OXPHOS) (Skulachev 1999; Ludwig et al. 2001; Fernie et al. 2004; Arnold 2012; Papa et al. 2012; Srinivasan and Avadhani 2012; Friedrich 2014; Mailloux 2015). As electrons move through the ETS, some unbound electrons are released into the surrounding area, which can rapidly bind with molecular oxygen to form reactive oxygen species (ROS) (Brand 2000; Liu et al. 2002; Brand et al. 2004; Murphy 2009; Brand 2016). Antioxidants are the cell's defense against ROS, catalyzing them to less harmful or neutral structures (Burton and Ingold 1984; Cadenas 1997; Carr and Frei 1999; Mates et al. 1999; Sies 1991; Halliwell and Gutteridge 2007; Valko et al. 2007) When the quantity of antioxidants is outnumbered by ROS, oxidative stress occurs (Chance et al. 1979; Felton 1995; Brand 2000; Sies 1991; Zelko et al. 2002; Halliwell and Gutteridge 2007). A cell in the state of oxidative stress is prone to oxidative damage, the damage caused by ROS to lipids, protein, and DNA (Chance et al. 1979; Richter et al. 1988; Felton 1995; Gutteridge 1995; Barja 1999; Brand 2000; Dröge 2002; Brand et al. 2004; Hulbert et al. 2007; Kowaltowski et al. 2009; Murphy 2009; Monaghan et al. 2009; Circu and Aw 2010; Zhang and Hood 2016). Through the ETS and

OXPHOS, mitochondria are responsible for creating 95% of the fuel needed for cellular functions in the form of ATP (Skulachev 1999; Papa et al. 2012; Srinivasan and Avadhani 2012). Thus, the capacity for OXPHOS underlies essentially all energetically demanding processes within the body. Reproduction is among the most energetically demanding events that an animal experiences (Speakman 2008). Animals with higher reproductive output are thought to have reduced longevity (Promislow and Harvey 1990; Zera and Harshman 2001; Mukhopadhyay and Tissenbaum 2007). While several mechanisms have been proposed to underlie this phenomenon, the idea that ROS production could be responsible for reduced longevity in breeders gained traction in the last decade (reviewed by Sena and Chandel 2012; reviewed by Speakman and Garratt 2013). These studies are based on the assumption that an increase in energy demand, ATP production, and oxygen consumption contributes to an increase in the frequency of electron loss from the ETS and, therefore, higher probability of oxidative stress and higher rates of ROS formation resulting in greater levels of oxidative damage (Kirkwood and Holliday 1979; Wiersma et al. 2004; but see Barja 2002 and Speakman and Garratt 2013). Yet support for this assumption is equivocal and counters our understanding of how ROS is generated by the ETS. Theoretical data suggests that ROS production may be reduced as ATP production increases (Loschen and Flohé 1971; Barja 2002; Kavazis et al. 2009; Speakman and Garratt 2013; Willis et al. 2016; but see Brand 2016). Further, empirical data are needed to confirm this relationship *in vivo*.

In tissues with reduced energetic demand, ATP production drops and protons are less frequently being pumped back into the matrix by Complex V to create ATP. This change increases the proton motive force (pmf) within the inner membrane space, and proton pumping into the intermembrane space slows, which consequently slows the flow of electrons through the

chain (reviewed by Schönfeld and Wojtczak 2008). The increase in pmf causes ROS producers, largely complexes I and III, in the chain to become more reduced, as electrons are more likely to remain in one place longer and the buildup causes a release of unbound electrons (Barja 1999; Brand et al. 2004; Murphy 2009; Schönfeld and Wojtczak 2008). Further, the increase in oxygen partial pressure as less oxygen is being reduced to water by complex IV raises the likelihood of ROS formation (Speakman and Garratt 2013). As a result, mitochondria performing closer to the basal rate present a less coupled ETS, allowing greater electron release from the chain and into the surrounding matrix where they can potentially form damaging ROS (Barja 2002; Kavazis et al. 2009; Murphy 2009; Speakman and Garratt 2013).

In tissues with high-energy demand, the production of ATP by the ATP synthase is enhanced, the ETS complexes move electrons at a higher rate, and consume more oxygen. Protons are quickly being pumped through Complex V, which decreases the pmf and allows electrons to be shuttled through the chain quickly, as the speed of proton pumping into the intermembrane space needs to increase to replace those protons moved by Complex V (reviewed by Schönfeld and Wojtczak 2008). Consequently, ROS producers are in a less reduced state and, thus, are less likely to need to release unbound electrons into the mitochondria (Barja 1999; Brand et al. 2004; Murphy 2009; Schönfeld and Wojtczak 2008). ROS formation is also less likely due to the decreased partial pressure of oxygen (Speakman and Garratt 2013). Therefore, mitochondria performing closer to a maximal state present a more coupled ETS, decreasing electron release and production of ROS (Barja 2002; Kavazis et al. 2009; Murphy 2009). With this knowledge, it is possible that the relationship between energy expenditure and ROS production is opposite that which is widely accepted in the ecophysiology literature, and that increased energy expenditure (not including increases for energetically demanding, taxa or

species specific activities such as migration) reduces the production of ROS (Kavazis et al. 2009; Speakman and Garratt 2013). In fact, many empirical studies have provided evidence for such a relationship. Garratt et al. (2011) found that reproductive house mice had reduced protein and lipid oxidative damage to liver and skeletal muscle compared to non-reproductive mice, and reduced lipid oxidative damage was found in livers and kidneys of lactating voles than that of non-lactating voles by Oldakowski et al. (2012). Similar results from Xu et al. (2014) found that protein carbonyls in the livers of lactating voles were significantly less than in the livers of non-reproductive voles.

This is not to say, however, that there are not exceptions to this correlation. In the same study by Garratt et al. (2011), female mice at the peak of lactation had increased liver protein oxidative damage as her litter size increased. Though, they were not significantly higher than non-reproductive control females and the lipid damage was, in fact, lower in peak lactating females than in non-reproductive controls. Yang et al. (2013) found that serum protein oxidative damage was increased in lactating gerbils compared to non-reproductive gerbils, but also found that liver protein and lipid oxidative damage was lowered in lactating compared to non-reproductive gerbils. It is likely that oxidative damage is affected by multiple variables and is not solely determined by energy expenditure. Animals experience many changes associated with a reproductive event, including diverse change in physiology, morphology, and social environment. These changes may affect processes that are responsible for ROS production independent of relative energy expenditure. Thus, to empirically test the impact of relative energy expenditure on ROS production, it is important to uncouple this question from a reproductive event.

The effect of antioxidants on oxidative damage must also be considered, as an individual's oxidative stress status is determined by the disparagement between available antioxidants and ROS production. Endogenous antioxidants are enzymes found within cells and mitochondria that catalyze the transformation of highly reactive and damaging ROS to less reactive states (Chance et al. 1979, Felton 1995, Zelko et al. 2002, Weydert and Cullen 2010). When ROS production is greater than the available antioxidants, oxidative damage arises (Sies 1991, Halliwell and Gutteridge 2007). Superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT), and glutathione peroxidase (GPx) are the four most common and primary endogenous antioxidants (Weydert and Cullen 2010). SOD1 and SOD2 are the most important enzymes for defense against ROS damage (Zelko et al. 2002). They catalyze the change of superoxides, which are the most common ROS that originate from the ETS, to hydrogen peroxide or molecular oxygen (Chance et al. 1979, Zelko et al. 2002, Weydert and Cullen 2010). SOD1 is almost exclusively present in cellular cytosol, while SOD2 exists in mitochondria (Zelko et al. 2002). CAT, which is mostly active in subcellular peroxisomes, converts hydrogen peroxide to water and oxygen (Chance et al. 1979, Weydert and Cullen 2010). GPx, located in cellular cytosol and mitochondrial matrix, transforms hydrogen peroxide into water (Weydert and Cullen 2010).

Early investigations on endogenous antioxidants in relation to longevity hypothesized that organisms with longer maximum life-span potentials (MLSP) would have greater levels of endogenous antioxidants (Barja 2002; reviewed in Buttemer et al. 2010). Yet many results found that endogenous antioxidants were negatively correlated with MLSP (Barja 2002; reviewed in Buttemer et al. 2010). Similarly, it has often been shown that ROS production by mitochondria is negatively correlated with MLSP (Barja 2002; reviewed in Buttemer et al. 2010). It is likely that

the negative correlation between antioxidants and MLSP is simply a result of reduced ROS production (Perez-Campo et al. 1998; Barja 2002; Buttemer et al. 2010; Costantini et al. 2010). On an intraspecific basis, if increased energy expenditure within normal realms of energy utilization reduces ROS production relative to oxygen consumption, then it is likely that those with increased energy expenditure would also have lower levels of endogenous enzymatic antioxidants. However, it is possible that an organism with lower levels of oxidative damage is offsetting high rates of ROS production by way of antioxidant defense (Sies 1991).

To more accurately evaluate the possibility of cellular metabolism and ROS production as physiological mechanisms of life-history traits, it is critical to empirically test the relationship between an animal's energy expenditure and oxidative damage as a proxy for ROS production. It is also necessary to account for antioxidant activity, as it is possible that individuals with reduced oxidative damage may owe this reduction to an increase in antioxidants, and vice versa.

This study aimed to determine if oxidative damage (as a proxy for ROS) and energy expenditure are correlated. I hypothesized that there is a negative correlation between energy expenditure and oxidative damage in passerine birds. I predicted that birds with increased energy expenditure would have reduced oxidative damage to lipids and proteins relative to birds with lower energy expenditure, and birds with higher energy expenditure would have lower quantities of endogenous enzymatic antioxidants relative to those birds with lower energy expenditure.

Methods

All methods were approved by AU's International Animal Care and Use Committee (PRN #2015-2661).

Animal Collection

Twenty-four male and female juvenile House Finches (*Haemorhous mexicanus*) were collected from Auburn University Campus in Lee County, Alabama from June 8-15, 2015. This species was chosen due to high rates of trapping success in previous years, their abundant population in the area, and their distinct juvenile plumage. Collection of juveniles eliminated possible confounding effects of previous life history events (i.e.: breeding and molt) on oxidative damage to tissues. Finches were collected using mist nets placed in the flight paths of the birds and finch traps place around established feeders (Hill 2002). Birds were removed from nets immediately upon capture. Finches trapped in finch traps continue to display social feeding behaviors while in the traps, which attracts additional birds and increases trapping success. Thus, finches were allowed to stay in the finch traps up to 20 minutes before removal. Upon collection, birds were brought to the Auburn University Aviary where the birds were weighed and measurements were taken (tarsus length, wing chord, culmin length, and tail length) as indicators of individual condition. Based on these data, no significant differences were found in the condition of the birds between each treatment group at the onset of the experiment (ANOVA; mass P=0.151; culmin P=0.793; tail P=0.61; wing P=0.695; tarsus P=0.10). Finches were then banded with a unique combination of color bands and an aluminum numerical band for individual identification.

Energy Expenditure Treatments

Finches were randomly assigned to one of three treatment groups to allow for low-, medium-, and high-energy expenditure, with eight finches per group. Cage size is expected to impact relative ability to fly and, thus, daily energy expenditure. Low-energy expenditure birds were placed in indoor cages measuring 0.19 m^3, with two birds per cage (four cages total). Medium-energy expenditure birds were placed in indoor free flight rooms measuring 4.98 m^3, with four birds per room (two rooms total). High-energy expenditure birds were placed in an outdoor flight cage measuring 73.4 m^3, with eight birds in a single flight cage. All treatments received *ad lib* food (black oil sunflower seed, meal worms, and broccoli) and water, perches, and cuttlebones. High-energy group birds maintained in the flight cage were subject to ambient light, temperature, and weather. However, the roof of the flight cage was covered with a tarp to provide protection from sun and rain, and the flight cage is in a relatively shaded area. Birds maintained inside (low and medium groups) were all in rooms with a 2-foot by 2-foot window, allowing for natural light cycles. The inside of the aviary was kept between 65 and 75 °F. No personnel accessed the areas housing birds except during daylight hours as to sustain the birds' natural sleep and wake cycles. Beginning the day after the last birds were collected, finches were given a two-week acclimation period (June 16-30).

Oxidative Damage and Antioxidants

Following the acclimation period, birds were euthanized using an overdose of isoflurane vapors. The pectoralis was immediately excised and flash frozen in liquid nitrogen. Frozen tissues were then stored at -80 °C for later analysis.

To prepare for this ELISA, pectoralis tissues were homogenized in phosphate-buffered saline (PBS) (liver 1:4 wt:vol; pectoralis 1:6 wt:vol) using a dounce homogenizer, first with a course pestle and then a fine pestle. Homogenized samples were then sonicated on 50% power for three to seven seconds and then centrifuged at 13,000 rpm for 10 minutes in a refrigerated centrifuge and the supernatant saved. The Bradford method (Bradford 1976) was used to quantify protein content, and samples were diluted to 10 ug/mL protein/PBS. Samples were then tested with OxiSelect Protein Carbonyl ELISA Kit (STA-310, Cell Biolabs, Inc., San Diego, CA, USA) following manufacturer's specifications.

Oxidative damage to lipids in the form of 4-hydroxynonenal (4HNE; ab46545, Abcam, Cambridge, MA;) bound proteins and antioxidants superoxide dismutase 1 (SOD1; GTX100554, GeneTex, Irvine, CA), superoxide dismutase 2 (SOD2; GTX116093, GeneTex), glutathione peroxidase 1 (GPx; GTX116040, GeneTex), and catalase (CAT; GTX110704, GeneTex) were quantified relative to total protein content in liver and pectoralis tissue using western blotting. Tissues were homogenized 1:10 wt:vol on ice in 5mM Tris-HCl, 5mM EDTA with 10uL of protease inhibitor cocktail (PIC) for every 100uL of PBS, using a glass-on-glass homogenizer. Homogenized tissues were then centrifuged at 1500 g for 10 minutes at 4°C and the supernatant saved. The Bradford method (Bradford 1976) was then used to quantify protein content in the supernatants. Samples were then prepared with Loading Buffer mixture 1:9 ß-Mercaptoethanol:4X Laemmli and deionized water. Protein content in the final samples used for Western Blots was standardized by adjusting added amounts of loading buffer, distilled water, and homogenate supernatant as determined by individual sample protein content. Samples were then heated in a heating block for 10 minutes at 100°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins, with a total SDS content of 1%.

Twelve percent polyacrylamide separating gels were prepared with distilled water, 1.5M Tris base (pH=8.8), 10% sodium dodecyl sulfate (SDS), 40% acrylamide, 10% ammonium persulfate (APS), and tetramethylethylenediamine (TEMED). Four percent polyacrylamide stacking gels were prepared with distilled water, 1.0M Tris Base (pH=6.7), 10% SDS, 40% acrylamide, 10% APS, and TEMED. Proteins were separated in gels with 120 V (constant volts) for two to three hours. Proteins were then transferred to a PVDF membrane with 220 mA (constant amps) for two hours. Protein transfer to membranes was confirmed using a 2% Ponceau S stain. Membranes were then washed with PBS containing 0.1% tween (PBS-T). A 5% milk protein PBS-T blocking buffer was used to block non-specific binding sites. Membranes were then submerged for at least one hour while on a rocker in primary antibodies (one membrane for each target protein, one primary antibody per membrane) (GeneTex, Irvine, CA). Membranes were then washed in PBS-T three times for five minutes each, incubated in secondary antibody (GeneTex, Irvine, CA) for at least one hour while on a rocker, and then washed again. Labeled proteins on the membrane were detected using an enhanced chemiluminescent (ECL) reagent mixture (GE Healthcare) and then imaged with ChemiDoc-It2 Imaging System (UVP, LLC, Upland, CA). Membranes were then submerged in 2% Ponceau Stain for two to three minutes and then allowed to dry. Once dry, membranes were once again imaged with the same system and the images used to determine loading control of the proteins. The total raw density total raw density of each sample for the labeled proteins as well as the loading control were determined. The total raw density of each sample was then normalized by dividing it by the total raw density of its corresponding loading control.

Statistics

Data are reported as mean ± standard error (SE) with number of birds (n) per assay, and significance established at $p \leq 0.05$. 4HNE, SOD1, SOD2, CAT, and GPx results from western blots are reported in arbitrary units. For western blot analyses, low-energy group was used as the control (averaged to 1), with medium- and high-energy groups each averaged and compared to control to show % change from control. Linear models using categorical data were performed to assess differences between low-, medium-, and high-energy groups for all biological variables measured. All data were analyzed with R, version 3.2.4 (R Core Team 2016).

Results

Oxidative damage

To determine the amount of oxidative damage to pectoralis and liver, I measured protein carbonyls as an indicator of protein damage and 4HNE bound proteins as an indicator of lipid peroxidation. Lipid peroxidation results were limited to pectoralis samples as the western 4HNE data for liver were inconclusive. There were no significant differences in oxidative damage to proteins between the low-, medium-, or high-energy expenditure groups in either pectoralis or liver (Fig. 7; Table 4); and no significant differences in lipid peroxidation levels between any groups in pectoralis (Fig. 8; Table 4).

Antioxidants

To determine the levels of enzymatic antioxidants in pectoralis and liver, we measured SOD1, SOD2, CAT, and GPx. CAT and GPx results were limited to pectoralis samples (Fig. 9; Table 4). Liver CAT and GPx antioxidant data were thrown out due to inconclusive western blots. There were no significant differences in any of the enzymatic antioxidant levels in pectoralis between low-, medium-, or high-energy expenditure groups. Levels of liver SOD1 in the low energy expenditure birds were significantly higher than in the high-energy expenditure birds, with 1.00 ± 0.08 and 0.76 ± 0.06, respectively. However, no significant difference between low and medium or medium and high were found, as well as no significant differences in liver SOD2 levels between any of the energy expenditure groups (Table 4).

Discussion

As we continue to learn about oxidative stress and all its components, it becomes clearer that the relationship between oxidative stress and life-history traits is much more complicated than originally proposed. This study aimed to examine the direct relationship between energy expenditure and oxidative stress, and the results suggest that there is no significant, direct relationship. No significant differences were found for either tissue between any of the energy expenditure groups and levels of protein or lipid oxidative damage (Fig. 7; Fig. 8; Table 4). The significantly greater quantities of SOD1 present in the liver of the low- compared to medium- or high-energy expenditure groups (Fig. 9; Table 4) could suggest that more antioxidants are required to combat higher levels of ROS within the low-energy group, which is consistent with the decreased levels of protein carbonyls found in the livers of the high- versus low-energy expenditure groups, but the protein carbonyls were not decreased in the medium- versus low-

energy expenditure groups. The same pattern is observed in SOD2 quantities within the liver; however, as the differences in damage and SOD2 between these two groups were not statistically significant, this conclusion cannot be made with certainty.

Other patterns are present throughout the data, though they also lack in statistical significance. In the pectoralis, protein carbonyls increased as the energy expenditure treatment increased, which was opposite of the prediction. In conjunction, quantities of SOD1 present within the pectoralis also increased as energy expenditure increased. 4HNE quantities in pectoralis displayed the same pattern as SOD2 quantities, which were highest in the medium- and lowest in the high-energy expenditure group. These patterns between these two different products of oxidative damage and antioxidants suggests the possibility that certain types of damage are more likely to arise and be mitigated in different parts of the cell, as SOD1 is found exclusively in the cellular cytosol while SOD2 are present within the mitochondria (Zelko et al. 2002).

To my knowledge, this study was the first of its kind where birds were used instead of mammals. As such, several complications arose in the implementation which I feel lessen the impact and reliability of the results presented herein. The results of such a study are necessary to furthering our understanding and refining our investigations of the effects of oxidation stress on life-history traits and, therefore, I believe additional studies need to be undertaken which take into consideration the following issues:

Energy expenditure must be directly measured instead of basing it on cage size. Though increasing cage size allows birds greater capability of flight and additional energy expenditure, this assumption alone is not enough to draw significant conclusions. Birds in large cages are not certain to fly more, just as birds in small cages may spend all day fluttering back and forth.

Similarly, no measure was made to control for chronic or acute stress, which may contribute to greater ROS production and oxidative damage (Bagchi et al. 1999; Costantini et al. 2008). Birds experience a stress response when moved into captivity (Dickens et al. 2009) and it has been well established that male house finches kept in cages of any size during molt do not produce the red carotenoid coloration that has been so well categorized in non-captive males (Hill 2002). To disentangle stress-induced oxidative damage from metabolically-induced oxidative damage, it is necessary to have some measure of stress. Without this, one is unable to conclude if increases or decreases in oxidation damage are truly results from differing energy expenditures or if stress responses are skewing results.

In addition to better controls, adding a variety of tissues for measurements, as well as increasing the sample size of each tissue, will provide more conclusive results. This study was limited to damage measurements in pectoralis and liver and, though these are two oft-measured tissues when investigating oxidative stress, other tissues may be responding in entirely different ways. Understanding how a large variety of tissues respond to, promote, and mitigate oxidative stress provides a more complete understanding of not only the needs and functions of individual organ systems, but how they all work together and regulate as a whole.

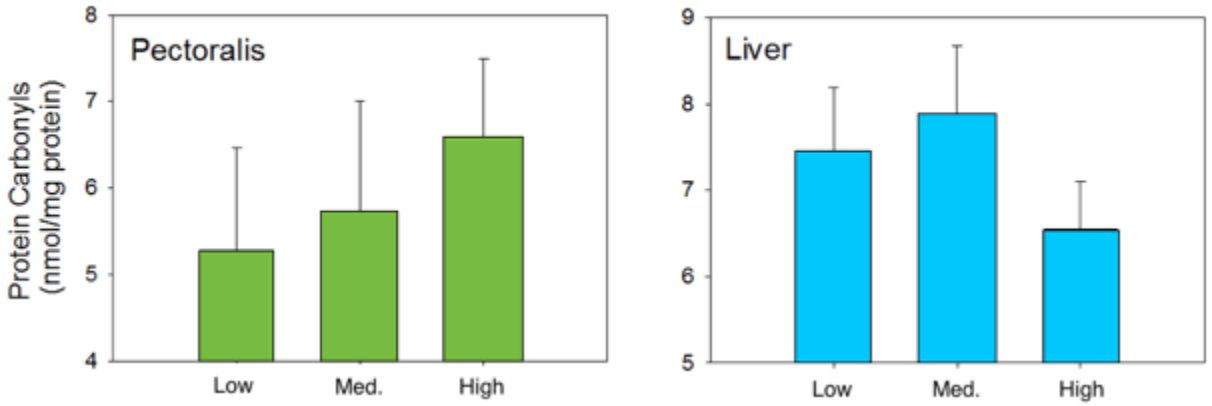

Fig. 7 Average protein carbonyl concentrations from pectoralis and liver tissues of low-, medium-, and high-energy expenditure birds. No significant differences in protein carbonyl concentrations between any of the groups in either pectoralis or liver were found. Significance established at $P < 0.05$. Standard error bars are given.

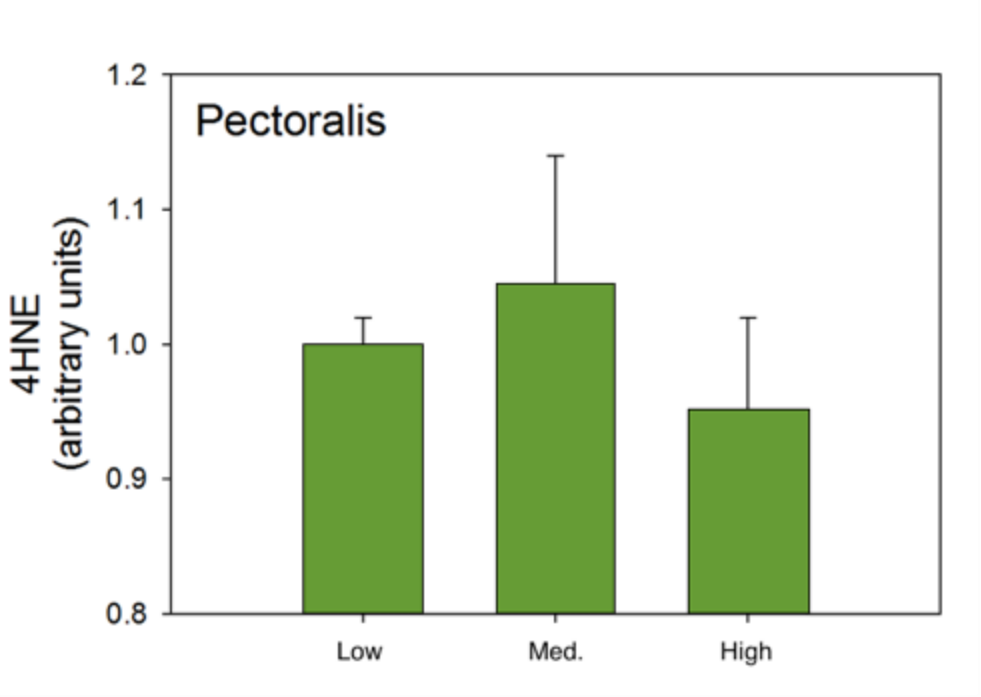

Fig. 8 Average 4HNE levels from pectoralis tissues of low-, medium-, and high-energy expenditure birds. No significant differences in 4HNE levels were found between any of the groups. Significance established at $P < 0.05$. Standard error bars are given.

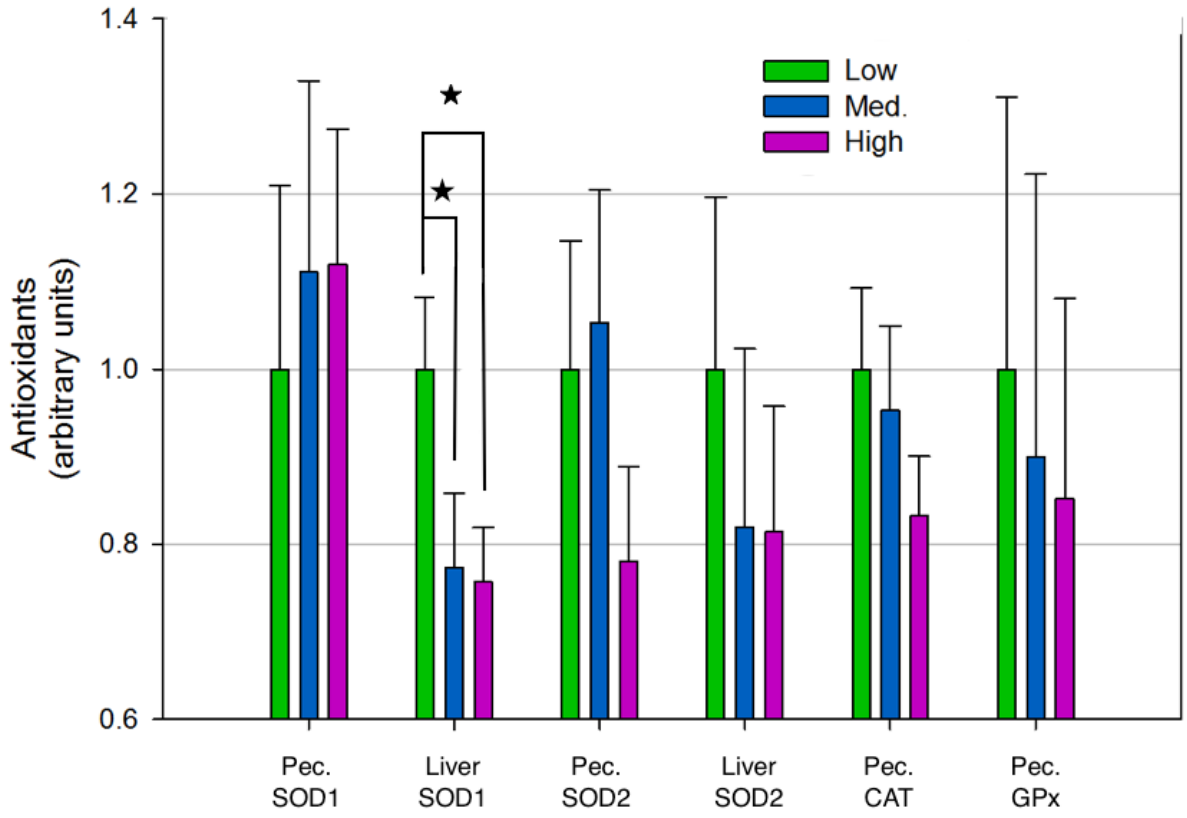

Fig. 9 Average SOD, SOD2, CAT, and GPx levels from pectoralis and SOD1 and SOD2 from liver tissues of low-, medium-, and high-energy expenditure birds. Significance established at $P < 0.05$. Standard error bars are given.

Table 4

Oxidative damage and antioxidant measurements from liver and pectoralis tissues of birds in low-, medium-, and high-energy expenditure groups

	Energy expenditure			***P* value**		
	Low	Medium	High	L:M	L:H	M:H
Pectoralis						
PC	5.28 ± 1.19	5.73 ± 1.27	6.59 ± 0.90	0.711	0.289	0.511
4HNE	1 ± 0.092	1.044 ± 0.095	0.952 ± 0.067	0.636	0.608	0.347
SOD1	1 ± 0.210	1.111 ± 0.218	1.119 ± 0.154	0.604	0.578	0.970
SOD2	1 ± 0.146	1.052 ± 0.152	0.781 ± 0.107	0.725	0.154	0.0927
CAT	1 ± 0.093	0.953 ± 0.096	0.832 ± 0.068	0.618	0.0895	0.229
GPx	1 ± 0.311	0.900 ± 0.323	0.852 ± 0.228	0.752	0.641	0.884
Liver						
PC	7.45 ± 0.74	7.89 ± 0.79	6.55 ± 0.56	0.562	0.236	0.107
SOD1	1 ± 0.082	0.773 ± 0.085	0.757 ± 0.060	**0.014***	**0.009***	0.848
SOD2	1 ± 0.196	0.820 ± 0.204	0.814 ± 0.144	0.371	0.357	0.978

Overall mean values ± SE; *Significant differences established at $P < 0.05$

For protein carbonyls (PC), low n=8; medium and high n=6 and means are reported as nmol/mg

For all others, low n=7; medium and high n=6 and means are reported with no units.

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