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Comparing Oxidative Balance of Summer and Winter Acclimatized Birds

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COMPARING OXIDATIVE BALANCE OF SUMMER AND WINTER ACCLIMATIZED BIRDS

by

Olivia R. Gulseth

A Thesis Submitted in Partial Fulfillment
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The members of the Honors Thesis Committee appointed

to examine the thesis of Olivia Gulseth

find it satisfactory and recommend that it be accepted.

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ABSTRACT

Comparing Oxidative Balance of Summer and Winter Acclimatized Birds

Olivia R. Gulseth

Director: David Swanson, Ph.D.

The oxidative stress system allows air-breathing animals to deal with the potentially harmful byproducts of metabolism. How this system reacts to the increasing thermoregulatory demands in winter for birds from cold winter climates is poorly understood. The purpose of this project was to compare the oxidative balance of summer (warm acclimatized) and winter (cold acclimatized) phenotypes of overwintering songbirds in South Dakota to determine if there is a significant difference between the seasonal phenotypes and among species. Seasonal differences in oxidative balance were expected due to the increased metabolic costs of elevating metabolism to maintain body temperature during winter. Blood samples were taken from four resident songbird species near Vermillion, South Dakota, during the summer and winter of 2023-2024. The overwintering species studied were the American goldfinch (*Spinus tristis)*, black-capped chickadee (*Poecile atricapillus*), house finch (*Haemorhous mexicanus*), and house sparrow (*Passer domesticus*). The red blood cells and plasma from the samples were analyzed for reactive oxygen species production, levels of antioxidant activity, total antioxidant capacity, and oxidative damage. There were two predicted outcomes. The first was that a higher level of oxidative damage would be present in the winter acclimatized birds due to the elevated metabolism. The second was that all antioxidants would be present in higher amounts in the winter to offset the elevated metabolism. The results supported neither of these predictions, with levels of lipid damage being higher in the summer and varying levels of enzymes across seasons and species. These data suggest that the higher thermoregulatory costs in winter do not result in consistently elevated oxidative damage or antioxidant capacities relative to summer in small resident birds in cold climates.

Keywords: Oxidative balance system, antioxidants, seasonal flexibility, songbirds, South Dakota

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INTRODUCTION

Birds that overwinter in temperate regions must face the consequences of living in an environment that experiences both hot and cold temperatures on a seasonal basis. In South Dakota, extreme winters and scorching summers regularly push inhabitants of the region to find ways to survive. While many Northern Hemisphere breeding birds migrate to warmer climates to winter in the subtropics or tropics, with some migrating as far as the Southern Hemisphere, some birds remain in the northern winter conditions and manage to persevere. The mechanisms allowing birds to survive in seasonally variable climates is of particular interest due to the increasing fluctuations in temperature resulting from climate change, as these seasonal and shorter-term shifts have been linked to reduced avian fitness (Taff & Shipley, 2023).

Examining the mechanisms underlying the ability of birds to survive seasonally variable climates has been the subject of numerous recent studies (e.g., Jimenez et al., 2020a; Jimenez et al., 2020b; Nord et al., 2021; Sonmez et al., 2023). Researchers in the field of ornithology have extensively hypothesized about the mechanisms that explain the ability of birds to survive the demands of changing environmental conditions, including the increase of basal and maximal metabolic rates in winter (Swanson, 2010). As metabolic rate is increased, however, the body must account for a potentially increased production of reactive oxygen species (ROS) that occur as a byproduct of metabolism (Brown et al., 2009; Skrip & McWilliams, 2016). These ROS contribute to the formation of free radicals, which can cause cell and tissue damage and may contribute to neurodegenerative diseases such as Lou Gehrig's disease in humans (Silverthorn et al., 2018). The regulation of potential tissue damage resulting from the production of free radicals is accomplished by antioxidant defense and damage repair systems which are interchangeably referred to as either free-radical balance or oxidative balance (Skrip & McWilliams, 2016; Cohen et al., 2008).

The group of compounds labeled as ROS are dangerous to organisms because these highly reactive compounds can produce radical elements in the body. Free radicals have an unpaired electron that allows them to scavenge other elements for electrons, which in turn causes the scavenged element to become a radical (Skrip & McWilliams, 2016). This creates a cascading reaction that can damage proteins, lipids, and nucleic acids, which affects the ability of these molecules to function properly within the body. This can particularly affect birds, due to the reliance of birds on fats to support long distance flights, especially during migration (Skrip & McWilliams, 2016), and to fuel prolonged shivering in cold winter climates (Vaillancourt et al., 2005; Swanson, 2010).

The presence of polyunsaturated fatty acids (PUFAs) as a part of the diet of birds has been studied recently in conjunction with antioxidant pathways (DeMoranville et al., 2022). PUFAs are at a particularly high risk of oxidative damage due to the presence of hydrogen close to double bonds present in the chemical backbone of PUFAs, which are easily oxidized when radicals are encountered (Skrip & McWilliams, 2016). This process begins when an unsaturated fatty acid encounters a ROS (such as the highly reactive hydroxyl radical byproduct) that removes an electron from the fatty acid, creating a lipid radical (Skrip & McWilliams, 2016). This lipid radical becomes free to interact with other PUFAs after encountering oxygen, creating a chain reaction that continues unbroken until an antioxidant interrupts the cycle (Skrip & McWilliams, 2016). In a recent study, DeMoranville et al. (2022) predicted that migratory birds fed a diet rich in 18:2n-6 PUFAs would be more susceptible to oxidative damage from flight as a result of ROS interacting with the PUFAs, resulting in increased levels of antioxidant enzyme activity when compared to migratory birds fed diets lower in PUFAs. The outcome of the study found that the hypothesis was not supported, as the migratory birds did not have elevated levels of antioxidant enzyme activities. The “migratory birds” in this study were European starlings (*Sturnus vulgaris*) that were subjected to flight-training designed to mimic biannual migration. It is possible that the flight training did not produce a response akin to migration, but this was unlikely as the study followed protocols that were deemed effective in eliciting the desired migratory response. The authors declared the reason for the unsupported hypothesis was due to the dietary PUFA composition not being oxidatively challenging enough even with flight training. There was also no measure of oxidative damage done in the study, rather DeMoranville et al. (2022) focused on levels of antioxidant gene expression and the corresponding antioxidant enzyme activities, failing to fully account for the oxidative stress system. The present study measures the levels of total and enzymatic antioxidant activities and the amount of oxidative damage in blood, which will address the question of whether seasonal variation in the oxidative balance system can counteract potential oxidative damage resulting from winter increases in thermoregulatory demands that may result in potentially elevated levels of oxidative damage.

The Oxidative Stress System

The defense employed by animals against oxidative damage is a multifaceted system. Monaghan et al. (2009) outline five aspects to the total oxidative stress system that describe the layers of protection. The first part of the system is the ability to minimize the release of uncontrolled ROS in the cell. Due to different factors such as the makeup of the mitochondrial membrane, age of mitochondria, and energetic demands, the amount of ROS generated can be regulated to some degree. In cases where heat production is required via the uncoupling of the electron transport chain, ROS production is minimized. The concept of birds using uncoupling as a source of non-shivering thermogenesis is a controversial topic, as birds lack the brown fat and uncoupling protein (UCP) that mammals use to undergo this process (Swanson, 2010). There is an uncoupling gene present in birds (avUCP) that has been studied, but a definitive conclusion of the function of the gene has not yet been determined (Swanson, 2010). A recent paper from Nord et al. (2021) explored the mitochondrial function in red blood cells of coal tits (*Perparus ater*), blue tits (*Cyanistes caeruleus*), and great tits (*Parus major*) during the winter. The findings indicated that increased uncoupling of the electron transport chain occurred in the winter phenotype of the coal and great tits, with the winter phenotype of the blue tits demonstrating increased routine respiration but no significant increase in uncoupled respiratory capacity. This uncoupling produces heat (lowering metabolic efficiency) but also reduces the production of ROS, which could mitigate potential oxidative damage from ROS in winter acclimatized birds, although such seasonal differences in uncoupling appear not to occur uniformly across all bird species.

The second aspect of the oxidative stress system involves the multiple types of antioxidant enzymes inside of the cell that counteract superoxide anions (a destructive and short-lived type of ROS) (Monaghan et al., 2009). These enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which work together to break down the superoxide anion into hydrogen peroxide, and then to water. The third facet includes the chain-breaking antioxidant compounds that are able to neutralize the harmful effects of ROS without continuing the chain of reactivity. These antioxidants are either produced by the body or obtained through diet. Some examples of these are glutathione, vitamin E, and vitamin C. Together, these antioxidants work to facilitate chain-breaking and recycling of antioxidants that have been used in the defense against ROS.

The next defense against ROS damage is the structural configurations of biological molecules. Generally, more important structures are structurally configured to resist reacting with ROS (Monaghan et al. 2009). However, because the molecular makeup of structures varies across species and with diet, the resistance of these structures to ROS also varies. For example, fatty acids found in phospholipids are the primary components of the cellular membranes that play the important role of maintaining cellular compartmentalization. As membrane elements, lipids are highly susceptible to damage, especially in the inner mitochondrial membrane where the electron transport chain is located. Although this threat of damage is present in any animal, birds selectively incorporate particular types of unsaturated fatty acids into their membranes to maintain fitness (Skrip & McWilliams, 2016). Because unsaturated fatty acids are more susceptible to oxidative damage, this increases the risk for ROS damage to the membranes of birds. Finally, the last defense of the oxidative stress system is to repair damage to molecules that occur in response to ROS (Monaghan et al., 2009). This may include replacement of damaged DNA, proteins, and lipids via molecular repair mechanisms. The multiple layers of defense against ROS damage highlights the complexities of attempting to study the oxidative stress system.

Study Design

This project explores whether oxidative balance differs between cold-acclimatized (winter) and warm-acclimatized (summer) birds of the same species. By researching these specific physiological responses, more can be learned about how birds phenotypically adjust to survive cold winter climates. I addressed this question by measuring blood cell levels of CAT, SOD, GPx, plasma levels of total antioxidant capacity (TAC), and lipid oxidative damage for four species of overwintering songbirds captured in both summer and winter. There is limited research on seasonal variation in oxidative balance in overwintering songbirds residing in cold winter climates, such as those in the Midwest, where extreme winters and summers are present (Jimenez et al., 2020a). The samples for this study were collected at sites near Vermillion, Clay County, South Dakota (SD) in both winter (December-February) and summer (June-August). Average temperatures (1980-2016) in Vermillion, SD, in January are -1°C (average daily high) and -10°C (average daily low; Weather Spark, n.d.). Corresponding average daily high and low temperatures in July for Vermillion, SD, are 30°C and 18°C, respectively (Weather Spark, n.d.). The four species in this study were the American goldfinch (*Spinus tristis*; AMGO), black-capped chickadee (*Poecile atricapillus*; BCCH), house finch (*Haemorhous mexicanus*; HOFI), and house sparrow (*Passer* domesticus; HOSP), all of which are year-round residents in South Dakota (Tallman et al., 2022) and show winter increases in basal and maximal thermogenic (i.e., summit) metabolic rates (Cooper & Swanson, 1994; Liknes et al., 2002; Swanson & Liknes, 2006; Oboikovitz & Swanson, 2022).

There were two predicted outcomes of this study. First, a higher level of oxidative damage would occur in the winter due to the increased metabolic rates associated with thermogenesis and cold temperatures, which is necessary to survive the cold weather. Alternatively, antioxidants would be elevated in the winter to mitigate the consequences of elevated metabolism, resulting in the same amount or less oxidative damage than in the summer months.

MATERIALS AND METHODS

Data Collection

To obtain blood samples, AMGO, BCCH, HOFI, and HOSP were captured using mist nets at multiple locations near and within Vermillion, South Dakota (SD) in the summer of 2023 (June-August) and the winter of 2023-2024 (December-February). After capture, a blood sample (<150 µl) was collected by pricking the brachial vein with a 26-gauge needle. Blood was collected in a heparinized micro-capillary tube, after which blood samples were transferred to a microcentrifuge tube and immediately placed on ice in a cooler until transport back to the lab. In the lab, the blood samples were placed in a refrigerated centrifuge to separate the plasma from the blood cells. The plasma was drawn off using a micropipette. Both the blood and plasma samples were then placed into a -80°C freezer until being shipped on dry ice to a collaborator at Colgate University after each season for analysis of oxidative stress system metrics (see below). Sample sizes from summer and winter for the four study species were: AMGO (summer n = 10, winter n = 13), BCCH (summer n = 8, winter n = 10), HOFI (summer n = 10, winter n = 11), and HOSP (summer n = 10, winter n = 16; Table 1). All procedures were approved by the University of South Dakota Institutional Animal Care and Use Committee under protocol number 08-05-21-24C.

Sample Processing

Commercially available assay kits (Cayman Chemicals, Ann Harbor, MI) were used to measure the levels of CAT (cat. No. 707002), SOD (cat. No. 706002), and GPx (cat. No. 703102) activities, which have been used in other studies to estimate antioxidant capacity in blood cells (e.g., Jimenez, 2020a.). From the gathered samples, 4 µl of red blood cells were diluted with 396 µl of 20 mM HEPES, 1 mM EGTA, and 90 mM mannitol buffer solution. After dilution, samples were vortexed prior to each assay. We then followed the manufacturer's protocol to determine each of these enzyme activities. All enzyme assays were run on the same day as sample dilution. Furthermore, we quantified the concentration of proteins in each diluted RBC sample using a protein determination kit (Cayman chemicals cat. No704002) to standardize enzyme activities to a per protein basis for subsequent comparisons (Jimenez et al., 2019).

In addition, the ability of plasma to neutralize hypochlorous acid was also measured using the commercial kit for the OXY-Adsorbent test, which revealed the total antioxidant capacity (TAC) of the plasma (Diacron International, Grosseto, Italy). Oxidative damage was measured as the presence of circulating hydroperoxides, including products of lipid oxidation, by performing the d-ROMs test on plasma (Diacron International). These tests concur with methods used in previous studies designed to measure the oxidative balance in birds (Skrip & McWilliams, 2016).

Data Analysis

A two-way ANOVA was used for each oxidative stress metric, with season, species, and their interaction as predictor variables. If significant differences were detected in the two-way ANOVA, the Holm-Sidak method was used for pairwise multiple comparisons to identify which values differed significantly. The Holm-Sidak method was used to identify significant differences of values between species within seasons. The same method was also used to identify seasonal differences within species. We considered $P < 0.05$ as a statistically significant difference. These analyses were performed using SigmaPlot (Version 13.0, Systat Software, Palo Alto, CA).

RESULTS

The season x species interaction term was significant for all oxidative stress metrics except for SOD activity, signifying that seasonal variation in the oxidative stress metrics differed among species (Table 1). Species within season comparisons for lipid peroxidation in summer revealed that the HOSP had higher lipid damage than the other three study species and BCCH had higher levels of lipid damage than AMGO (Table 3). No differences in lipid damage among species were detected in winter. For seasonal within species comparisons, BCCHs and HOSPs had greater levels of lipid peroxidation in summer than winter, but the AMGO and HOFI showed no significant seasonal differences of lipid peroxidation (Table 2). For TAC, species within season comparisons in summer revealed higher levels in HOSPs than in the AMGO and HOFI, but no other significant differences (Table 3). TAC did not vary significantly among species in winter. For season within species comparisons, all species showed higher values of TAC in summer than in winter (Table 2).

Species within season comparisons of CAT activity in summer revealed significantly higher levels in BCCHs than the other three species in both summer and winter (Table 3). The only other significant within season difference was HOSP had higher CAT activity than AMGO in summer. For season within species comparisons, all species showed higher CAT activities in summer than in winter (Table 2). GPx activity showed higher values in HOSP than the other three species in both summer and winter for species within season comparisons (Table 3). Likewise, GPx activity was higher in AMGOs than in BCCH and HOFI in both summer and winter. Finally, GPx activity was higher in BCCH than HOFI in the summer (Table 3. The only significant season within species comparison was higher GPx activity in summer than in winter for BCCH (Table 2). For SOD activity, winter values exceeded summer values for all species (t85 = 4.880, P < 0.001) and BCCHs showed higher activities than the other three species (t41 = 5.284, P < 0.001 for HOSP; t36 = 4.791, P < 0.001 for HOFI; t38 = 3.221, P = 0.007 for AMGO).

OS Metric	**Variable**	**df**	***F***	***P***
Lipid Peroxidation	Season	1	28.554	< 0.001
	Species	3	8.715	< 0.001
	Season x Species	3	8.168	< 0.001
TAC	Season	1	277.912	< 0.001
	Species	3	5.042	0.003
	Season x Species	3	4.601	0.005
Catalase	Season	1	486.243	< 0.001
	Species	3	27.338	< 0.001
	Season x Species	3	3.365	0.023
GPx	Season	1	0.015	0.902
	Species	3	73.574	< 0.001
	Season x Species	3	3.564	0.018
SOD	Season	1	23.817	< 0.001
	Species	3	11.024	< 0.001
	Season x Species	3	0.679	0.565

Table 1: Statistics from two-way ANOVA for season, species, and season x species interactions for the different oxidative stress metrics measured in this study.

OS Metric	**Species**	***t***	***P***
Lipid Peroxidation	AMGO	0.127	0.899
	BCCH	2.483	0.015
	HOFI	1.798	0.076
	HOSP	6.814	< 0.001
TAC	AMGO	6.359	< 0.001
	BCCH	7.792	< 0.001
	HOFI	7.670	< 0.001
	HOSP	11.864	< 0.001
Catalase	AMGO	9.055	< 0.001
	BCCH	12.005	< 0.001
	HOFI	13.377	< 0.001
	HOSP	12.720	< 0.001
GPx	AMGO	0.890	0.376
	BCCH	2.660	0.009
	HOFI	0.879	0.382
	HOSP	1.514	0.134

Table 2: Results of season within species comparisons by Holm-Sidak post hoc tests after two-way ANOVA for the different oxidative stress metrics with significant species x season interaction terms.

OS Metric	**Season**	**Species Comparison**	***t***	***P***
Lipid Peroxidation	Summer	HOSP v AMGO	6.347	< 0.001
		HOSP v HOFI	4.502	< 0.001
		HOSP v BCCH	3.142	0.009
		BCCH v AMGO	2.843	0.017
TAC	Summer	HOSP v AMGO	4.848	< 0.001
		HOSP v HOFI	3.182	0.010
Catalase	Winter	BCCH v HOSP	4.818	< 0.001
		BCCH v AMGO	4.084	< 0.001
		BCCH v HOFI	3.761	< 0.001
	Summer	BCCH v AMGO	7.597	< 0.001
		BCCH v HOFI	5.911	< 0.001
		BCCH v HOSP	5.289	< 0.001
		HOSP v AMGO	2.448	0.049
GPx	Winter	HOSP v BCCH	10.757	< 0.001
		HOSP v HOFI	10.418	< 0.001
		AMGO v BCCH	6.555	< 0.001
		AMGO v HOFI	6.106	< 0.001
		HOSP v AMGO	4.229	< 0.001
	Summer	HOSP v HOFI	8.619	< 0.001
		AMGO v HOFI	5.616	< 0.001
		HOSP v BCCH	5.194	< 0.001
		HOSP v AMGO	3.002	0.011
		BCCH v HOFI	2.931	0.009
		AMGO v BCCH	2.364	0.021

Table 3: Results of species within season comparisons by Holm-Sidak post hoc tests after two-way ANOVA for the different oxidative stress metrics with significant species x season interaction terms. Only significant results are listed.

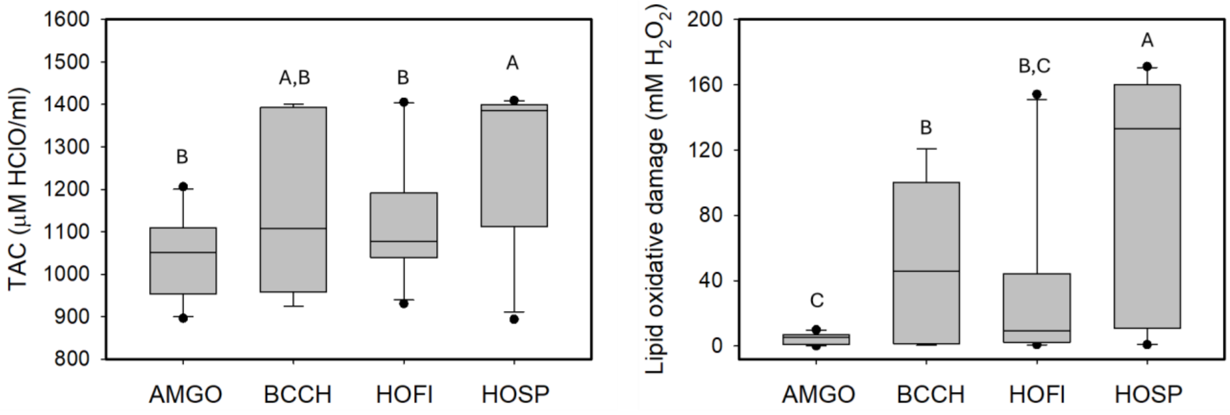

Figure 1: Box plots of the differences in TAC and lipid oxidative damage from blood samples of AMGO, BCCH, HOFI, and HOSP collected during the summer. Species with the same capital letters indicate that the data sets do not differ significantly. Species with different letters are significantly different.

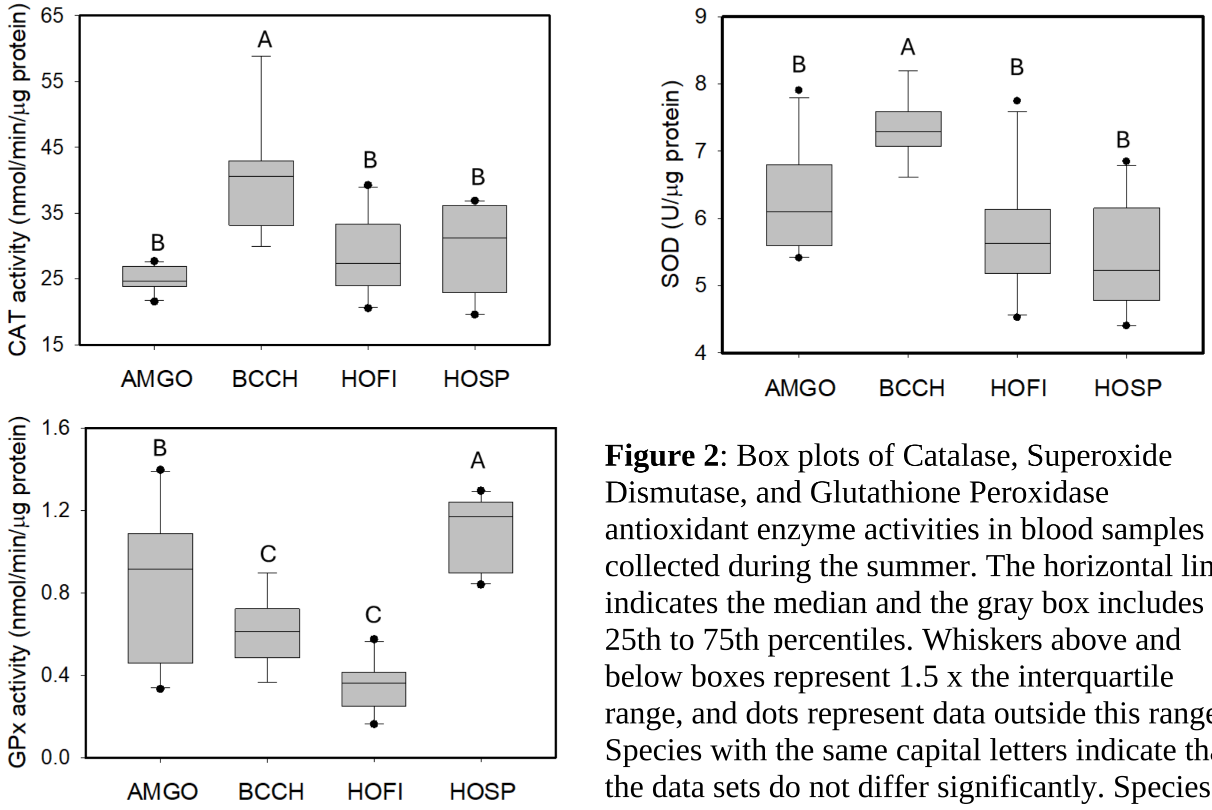

Figure 2: Box plots of Catalase, Superoxide Dismutase, and Glutathione Peroxidase antioxidant enzyme activities in blood samples collected during the summer. The horizontal line indicates the median and the gray box includes the 25th to 75th percentiles. Whiskers above and below boxes represent 1.5 x the interquartile range, and dots represent data outside this range. Species with the same capital letters indicate that the data sets do not differ significantly. Species with different letters are significantly different.

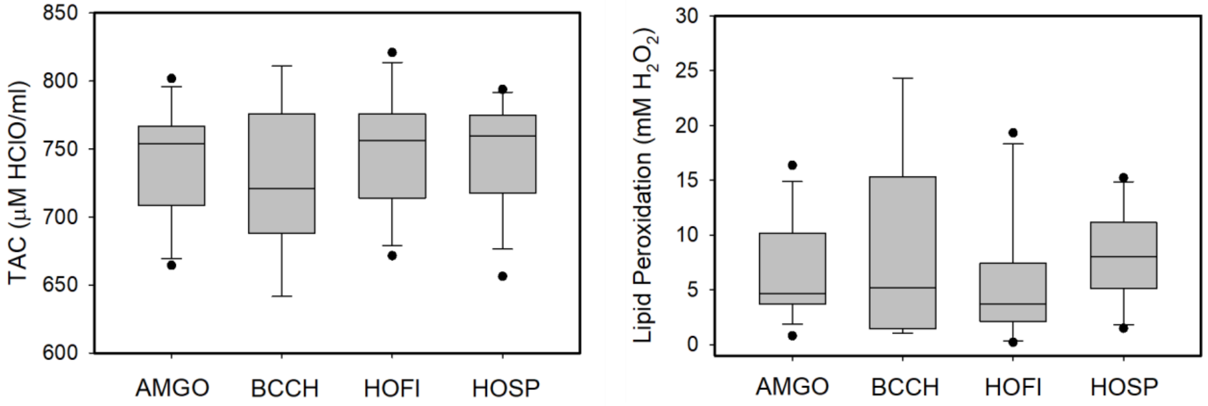

Figure 3: Box plots of the differences in TAC and lipid oxidative damage from blood samples of AMGO, BCCH, HOFI, and HOSP collected during the winter.

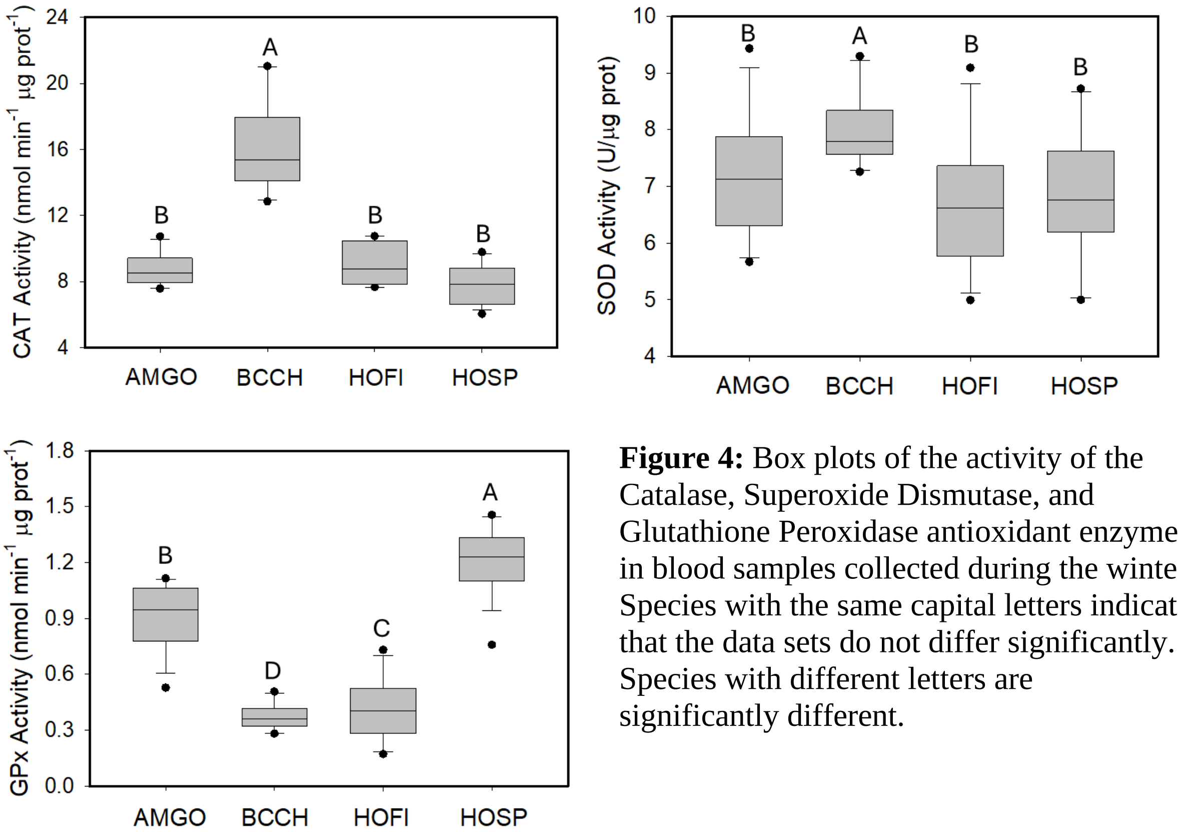

Figure 4: Box plots of the activity of the Catalase, Superoxide Dismutase, and Glutathione Peroxidase antioxidant enzymes in blood samples collected during the winter. Species with the same capital letters indicate that the data sets do not differ significantly. Species with different letters are significantly different.

DISCUSSION

This study sought to explore the differences in the oxidative stress system between summer and winter phenotypes of overwintering songbirds in South Dakota by examining the seasonal differences in blood cell antioxidant enzyme activities, plasma oxidative damage and total antioxidant capacity. The results provide interesting insight into the complex ways that potential oxidative stress associated with increased metabolic rates in winter for thermoregulation is dealt with by the oxidative stress system. Instead of finding a clear relationship between increased lipid damage (as measured by lipid peroxidation) in winter relative to summer, there was significantly increased lipid damage present in two out of the four studied species in the summer. The higher lipid peroxidation in summer birds may be due to the demands of reproduction (e.g., female birds making individual flexible adjustments in metabolism in response to reproduction demands, as seen with female zebra finches *Taeniopygia guttata*; Williams et al., 2009). Van de Crommenacker et al. (2011) also found that ROS production varied inversely with territory quality (as measured by food availability). Although we did not measure territory quality in the present study, differences in available resources among breeding sites cannot be ruled out as a causative factor for the higher and more variable lipid peroxidation detected for summer relative to winter birds. In the future, including measures of territory quality along with measures of the oxidative stress system, similar to Crommenacker et al. (2011), may allow testing of whether habitat quality influences the oxidative stress system in our study system. These data indicate that the prediction of higher lipid damage in the winter resulting from increased energy expenditures for thermoregulation must be rejected.

I also predicted that overall antioxidant capacity may be increased in winter to offset potentially higher ROS production associated with the increased metabolic demands. Such increases in antioxidant capacity could subsequently result in lowered oxidative damage. The TAC of the birds was significantly decreased in the winter, which is the opposite of what was hypothesized (Table 2). This result may be partially explained by the dietary changes birds face in the winter. As the birds do not have fruit or other dietary sources of antioxidants during the winter, the circulating exogenous antioxidants may be decreased causing overall TAC to be decreased. However, decreased TAC in the winter is not consistently supported in other studies. Ramírez-Otarola et al. (2023) found that two species of birds residing in a seasonal Mediterranean climate in Chile had increased TAC during the colder months, albeit the weather does not get below freezing in that region. Sonmez et al. (2023) found no significant difference in TAC between seasons for blackbirds (*Turdus merula*) from Turkey. Further research into how TAC and other OS system elements vary (or fail to vary) between seasons in birds from regions with cold winter climates will help define the relationships among ROS production, metabolic rates, oxidative stress, and the defenses birds mount against ROS.

In addition to the measurement of TAC, the seasonal measurements of blood cell GPx, CAT, and SOD activities test whether the second predicted outcome of the experiment was supported. If the second prediction was valid, elevated levels of antioxidant enzymes during the winter would be expected to offset the energetic costs of increased metabolic rates associated with thermogenesis in the winter. There is not a consistent relationship between enzymes when comparing the winter and summer phenotypes of each species. Instead, some enzyme activities increased significantly during the winter, but other enzymes were significantly elevated in the summer. For example, CAT activity was significantly elevated during the summer for all species, but SOD activity was significantly elevated in the winter for all species (Table 2). Notably, the season x species interaction was not significant for SOD activity, illustrating that similar upregulation in winter may be important to oxidative balance for all four study species (Table 1). GPx showed conflicting seasonal results among species, with most species showing seasonal stability, but BCCH showing a statistically significant increase in summer. Seasonal variation in these enzymes may respond to species-specific adjustments of the oxidative stress system resulting from differences in diet, lifestyle, and/or habitat types or seasonal differences in other antioxidants not measured in this study (e.g., glutathione, uric acid, dietary antioxidants, or peroxiredoxin enzymes). For example, the BCCH consumes the most animal matter of the four study species, with a winter diet that is half plants and half animals (insects, spiders, and frozen meat) and a summer diet of approximately 80-90% animal matter (Foote et al., 2020). In contrast, house sparrows consume diets of > 90% plant matter at both seasons (Lowther and Cink, 2020) and the two finch species both have diets composed almost exclusively of seeds at all seasons (Badyaev et al., 2020; McGraw and Middleton, 2020). The influence of these dietary differences may result in differential intake of dietary antioxidants and, subsequently, the differential regulation of plasma or cellular antioxidant capacities, potentially explaining some of the species-specific variation within and between seasons. For example, the higher intake of protein from animal matter in BCCH relative to the other study species may increase uric acid (as a nitrogenous waste product of protein breakdown) thereby enhancing the role of uric acid as an antioxidant relative to other species in this study. This possibility, however, is not necessarily supported by plasma TAC and LPO data, as TAC did not vary significantly among BCCH and the other study species at either season, and lipid peroxidation was higher in BCCH than in AMGO in summer. In addition, HOSP had significantly higher plasma TAC than the two finch species but also significantly higher levels of plasma lipid peroxidation relative to the other study species in summer (Fig. 1, Table 3). The HOSP generally lives in areas inhabited by humans, which might expose this species to more environmental pollutants (Lowther and Cink, 2020), which could, in turn, affect oxidative status (Isaksson, 2010).

Cohen et al. (2008) found that gray catbirds (*Dumetella carolinensis*) and house sparrows increased antioxidants when exposed to cold temperatures. It would be expected therefore that enzymes specifically needed in winter would be increased in response to the stresses of cold weather. Following this hypothesis, SOD may be an important enzyme in multiple species of birds to deal with seasonal changes in temperature. SOD catalyzes the conversion of oxygen free radicals to hydrogen peroxide, followed by conversion of hydrogen peroxide to water and oxygen by both CAT and GPx (Skrip & McWilliams, 2016). The finding that SOD increases during winter while CAT is down-regulated and GPx shows lesser seasonal changes suggests that the initial step of converting ROS to hydrogen peroxide is critical for winter birds whereas the conversion of hydrogen peroxide to water and oxygen is important for summer birds. In addition, perhaps the redox signaling role for hydrogen peroxide (Veal & Day, 2011; Marinho et al., 2014) is more important for winter birds, resulting in a lower demand to convert hydrogen peroxide to water and oxygen.

One aspect of the metabolic process that may cause these unexpected results is the uncoupling of the electron transport chain for thermogenesis. When oxygen consumption and ATP generation are uncoupled, ROS production is reduced (Monaghan et al., 2008). If ATP uncoupling to reduce ROS in winter occurs, this could aid in explaining the decreased levels of lipid oxidative damage seen for the winter-acclimatized birds. Expression of uncoupling proteins avUCP and adenine nucleotide translocase (ANT) in pectoralis muscle with cold acclimation does occur in some birds (Pani & Bal, 2022), although seasonal changes in gene expression for avUCP and ANT does not occur in AMGO and BCCH (Cheviron & Swanson, 2017). Non-shivering thermogenesis via ATP uncoupling was associated with an increase in the mitochondrial volume of red blood cells during winter for three bird species (Nord et al., 2021). If this experiment was repeated with our study species, it could reveal whether these birds use non-shivering thermogenesis in red blood cells and to what extent this process may be lessening the levels of ROS production and therefore levels of oxidative damage in the form of lipid peroxidation in winter.

Future Research

I focused on seasonal differences in the oxidative stress system in this study, but other factors could also influence this system and complicate seasonal interpretations of these results.

Including factors such as the age and sex of each bird could also be useful in future studies of this nature. The demands of breeding, nesting, and foraging may produce differing energetic and metabolic demands of breeding between males and females, which could impact oxidative balance differently in the two sexes and influence seasonal comparisons. For example, female birds may have higher energetic or metabolic demands during breeding than males due to the energetic demands of supporting daily survival as well as producing and incubating eggs and caring for the young, resulting in elevated levels of antioxidant defenses and/or elevated levels of lipid damage relative to males. Our sex ratios were similar between seasons for the three species that showed plumage dimorphism (not including BCCH which is not sexually dimorphic in plumage), with percent males in the samples being 80.0 and 76.9% for AMGO, 20.0 and 36.4% for HOFI, and 50.0 and 43.8% for HOSP in summer and winter, respectively. This suggests that seasonally differing sex ratios are not a major factor influencing the seasonal results in this study. Age may also have an effect as the demands of each phase of life and bodily development could theoretically affect the functioning and prioritization of the oxidative stress system. However, in this study, we used all adult birds except for BCCH, where six fully grown hatch-year birds (out of 8 total) were included in the summer sample. These fully grown hatch-year BCCH, however, showed oxidative system metrics that overlapped with adult birds, so we expect that age-related differences in this study are minimal.

In future studies examining the differences between summer and winter acclimatized birds, it may also be useful to examine the oxidative stress system in other metabolically active organs (such as pectoralis muscle, heart, liver and intestine) for these bird species to determine if tissue function is related to oxidative stress. Tissue samples from other metabolically active organs for measures of SOD, CAT, and GPx activities (Monaghan et al., 2008) would allow multiple data points from the same bird to elucidate the variations in the oxidative balance system across the body. Analyses of these data could further reveal how some structures in the body may upregulate antioxidant levels while others may not, as noted by DeMoranville et al. (2021). Birds may preferentially protect the DNA structure from oxidative damage because of the importance of high-fidelity DNA to survival and reproduction. Whether this results in a tradeoff with damage to other structures, such as lipids in cell membranes, could provide another avenue to explore the oxidative balance system. If there are higher levels of oxidative damage in certain organs over others, this would reveal where specifically the oxidative stress system is promoted for various ecological or energetic demands.

Studies that perform experiments on the seasonal flexibility of the oxidative stress system may also become more important as climate change continues to affect the planet. Results of these studies may change as seasons become more extreme and less predictable. Eventually, there is a potential for extreme temperatures (e.g., cold snaps) to overwhelm the oxidative stress system as the frequency of such cold snaps are predicted to increase with climate change. If birds are acclimatized to warmer winter temperatures under climate change, this could lead to lower capacities to modulate the oxidative stress system, as well as other metabolic pathways, in response to cold, resulting in mismatches between phenotypes and prevailing environmental conditions (Jimenez et al. 2020a). Such mismatches could, in turn, lead to reduced abilities to tolerate extreme cold periods or to carry-over effects reducing reproductive success in the subsequent breeding season.

Implications

The outcomes of this research demonstrate the complexity of the oxidative balance system and the challenges in capturing an accurate image of the mechanisms used by the body to maintain homeostasis. Further research examining the various metrics of this defense system, including more direct measures of ROS production (e.g., electron spin resonance (ESR) with spin trapping and flow cytometry; Monaghan et al., 2008) will provide a clearer understanding of how birds are able to maintain oxidative balance and metabolic homeostasis across seasons. The results of this study suggest that SOD is particularly important in the response of birds to cold temperatures. More data on the levels of SOD across seasons for other songbirds residing in seasonal regions may confirm the importance of this enzyme in response to the cold. Uncoupling of ATP production in winter may also play an important role in maintaining homeostasis while also lowering the production of ROS, thereby lowering levels of lipid peroxidation. Further research into the relationship between seasonal changes in metabolic rates, mitochondrial uncoupling, and their impact on ROS production and oxidative balance will further elaborate on this potentially important relationship.

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