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BEYOND THE FAT: PROTEIN METABOLISM AND MUSCLE FUNCTION AS PART OF THE MIGRATORY SYNDROME OF SONGBIRDS

Cory R. Elowe
University of Massachusetts Amherst

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BEYOND THE FAT: PROTEIN METABOLISM AND MUSCLE FUNCTION AS PART OF THE MIGRATORY SYNDROME OF SONGBIRDS

A Dissertation Presented

by

CORY ELOWE

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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February 2023

Organismic and Evolutionary Biology
BEYOND THE FAT: PROTEIN METABOLISM AND MUSCLE FUNCTION AS
PART OF THE MIGRATORY SYNDROME OF SONGBIRDS

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CORY ELOWE

Approved as to style and content by:

_____________________________________________
Alexander Gerson, Chair

_____________________________________________
Courtney Babbitt, Member

_____________________________________________
Stephen McCormick, Member

_____________________________________________
Matthew Fuxjager, Member

_____________________________________________
Craig Albertson, Graduate Program Director
Organismic & Evolutionary Biology
DEDICATION

To Emily, Teddy, and the birds
ACKNOWLEDGEMENTS

This work was supported by innumerable people who lent their time and energy, expertise, advice, and encouragement. For that reason, there are so many to thank that I’m sure to omit entire groups of folks without whom this would not have been possible. Please know that I’m eternally grateful, and that I will likely remember to praise you after this is all done and over with!

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Migration allows animals to seasonally exploit favorable habitats that are geographically disparate, and migratory animals have a suite of adaptations to complete their extraordinary journeys. This “migratory syndrome” of traits is comprised of seasonally-responsive adaptations that together result in changes to metabolism, body composition, and reproductive status, all of which act to increase the probability of a successful migration. The focus of my dissertation is to investigate the suite of changes in metabolism and muscle function that enable birds to undertake long-duration flights. In chapter 1, I compare fuel use in flight between two related migratory warbler species that migrate vastly different distances, showing that both species catabolized fat and lean mass similarly in flight, with fat burned at a steady rate while protein loss starts off high
early in flight and declines exponentially over time. In chapter 2, I investigated seasonal changes in protein metabolism between White-throated Sparrows in the non-migratory and migratory condition. I found that birds in the migratory condition increased activity of fat catabolism enzymes as expected, but also increased activity of protein catabolism enzymes and should greater water loss and lean mass catabolism in the migratory condition. In chapter 3, I performed RNA-seq analysis on muscle and liver tissues from a subset of birds in the migratory and non-migratory conditions in Chapter 2 to show metabolic changes to the two tissues in preparation for migration and potential changes to the capacity for rapid muscle remodeling in the migratory condition. Finally, in chapter 4 I explored the potential for altered Ca$^{2+}$-pumping efficiency in the muscle of migratory birds, finding increased expression of the sarcolipin uncoupling gene and proposing that seasonal changes to gene expression in the flight muscle may precede the fattening that is associated with migratory body composition. These studies move beyond our current understanding of fuel use during migration to suggest that dynamic protein metabolism and unique alterations to muscle physiology are important, and underexplored, aspects of the migratory syndrome in songbirds.
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CHAPTER 1

INTRODUCTION

Migration allows animals to seasonally exploit favorable habitats that are geographically disparate, and migratory animals have a suite of adaptations that allow them to cope with seasonal changes in habitat quality and resource availability by completing extraordinary journeys between habitats (Liedvogel and Lundberg, 2014). The physiological demands of migration are enabled by a “migratory syndrome” of traits that are highly coordinated, seasonally-responsive adaptations that together result in changes to metabolism, body composition, and reproductive status, all of which act to increase the probability of a successful migration (Piersma et al., 2005). The focus of my thesis is to investigate the suite of changes in metabolism and muscle function that enable birds to fly for long durations. One well-documented physiological example of the migratory syndrome is the seasonal upregulation of fatty acid transport and metabolism to fuel migratory flight (Guglielmo, 2018; Guglielmo et al., 2002; McFarlan et al., 2009). Flapping flight is extremely demanding, with birds operating at metabolic rates 8 - 18 times basal levels for extended periods of time (Butler, 2016; Jenni-Eiermann, 2017). Fatty acids are by far the best fuel for migratory flight, since storage without water results in high energy density providing 8 to 10 times more ATP per gram than protein or glycogen (Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann, 2017). The adaptive use of fat to fuel migratory flight is clearly visible in the seasonal changes of fat stores and increased metabolic capacity for fat catabolism. Photostimulation of captive migrants with a long-day spring photoperiod results in higher activity of the β-oxidation enzyme 3-Hydroxyacyl-CoA Dehydrogenase (HOAD) and the rate-limiting enzyme carnitine
palmitoyl transferase (CPT) in the pectoralis flight muscle, even in untrained conditions (McFarlan et al., 2009; Zajac et al., 2011). During the migratory season, Western Sandpipers increase enzyme activity of fatty acid synthase and Δ^9-desaturase (Egeler et al., 2000), as well as CPT and HOAD, and show a 70% relative increase in heart-type fatty acid binding proteins (H-FABP) in pectoralis muscle (Guglielmo et al., 2002), indicating the importance of fatty acid synthesis, transport, and catabolism to fuel migratory flight.

Although fatty acids are the primary fuel source for long-distance flight and receive the vast majority of research attention, catabolism of protein from flight muscles and organs during fasting and flight has also been well documented (Battley et al., 2000; Bauchinger and Biebach, 1998; Gerson and Guglielmo, 2011b; Jenni-Eiermann, 2017; Swain, 1992). Protein is estimated to contribute around 4-7% of the total flight energy (Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002a). Several hypotheses exist for the loss of protein in migratory flight. The protein turnover hypothesis suggests that suppression of protein synthesis during fasting or flight fails to offset baseline levels of protein catabolism, leading to the documented tissue-specific rates of organ breakdown and a net reduction in total lean mass (Bauchinger and McWilliams, 2012; Bauchinger et al., 2010). Protein breakdown may also supply amino acids for gluconeogenesis or anaplerotic replenishment of TCA cycle intermediates as they are lost through fatty acid catabolism and other reactions (Bauchinger and Biebach, 1998; Gibala et al., 2000; Jenni-Eiermann, 2017; Sahlin et al., 1990). The mass-power hypothesis stipulates that protein breakdown in flight is primarily a mechanism to reduce the power needed in flight as fuel is consumed and weight is reduced (Bauchinger and Biebach, 1998; Lindstrom et al.,
Recent studies indicate that more lean mass is catabolized in response to high water loss conditions during flight or fasting (Gerson and Guglielmo, 2011a; Gerson and Guglielmo, 2011b; Groom et al., 2019; Swain, 1992), supporting an alternative hypothesis that protein is catabolized as a means to offset water losses, since lean mass breakdown yields both metabolic and stored water from lean tissues to generate nearly 6 times more water than fat catabolism (Bauchinger and Biebach, 1998; Jenni-Eiermann, 2017). Therefore, lean mass breakdown may enable birds to overcome water-limitation during extended flights with no opportunity to land or access water (Gerson and Guglielmo, 2011a; Klaassen, 2004; Klaassen and Biebach, 2000; Schmaljohann et al., 2008; Skrip et al., 2015). These hypotheses about the purpose of lean mass catabolism are not mutually exclusive, Recent evidence also unifies these two hypotheses, as lean mass loss during flight and fasting shows consistent protein turnover with an additional degree of loss in response to drier conditions (Groom et al., 2019). However, it remains untested if these aspects of protein metabolism are part of the “migratory syndrome”.

While the enhanced capacity for fuel mobilization and catabolism is an essential component of the migratory syndrome, there is a suite of other adaptations that allow the completion of migratory journeys, and one of the most relevant would include mechanisms for enhanced fatigue resistance in the flight muscles. Changes to the size and isoforms of myosin heavy chain (Velten et al., 2016), fiber hypertrophy (DeMoranville et al., 2019; Marsh, 1984), capillary density (Lundgren and Kiessling, 1988), and corticosterone signaling (Pradhan et al., 2019) may all contribute to tighter coupling between energy consumption and power output during the migratory life history stage.
One particularly energy-consuming process in skeletal muscle that has not been previously investigated in migratory animals is calcium cycling. During muscle contractions, sarcolemmal and t-tubular membranes are depolarized, enabling Ca\(^{2+}\) release from the lumen of the sarcoplasmic reticulum (SR) (Dulhunty, 2006). As Na\(^+/K^+\)-ATPases restore the ionic equilibrium in the cytosol for subsequent depolarization, the Ca\(^{2+}\) release stimulates ATP hydrolysis and cross-bridge cycling by myosin ATPases, leading to muscle contraction (Clausen, 2003). Muscle relaxation requires the sequestration of Ca\(^{2+}\) in the lumen of the SR against its concentration gradient to lower the cytosolic free Ca\(^{2+}\) concentration. This is accomplished by the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), which under ideal conditions pumps Ca\(^{2+}\) into the SR at a coupling ratio of 2 Ca\(^{2+}\) ions for every 1 ATP hydrolyzed (Toyoshima, 2008). The maintenance of this Ca\(^{2+}\) gradient by SERCA requires a substantial amount of energy in skeletal muscle that can account for as much as half of the resting metabolic rate of mouse skeletal muscle (Smith et al., 2013).

The high energetic cost of maintaining the Ca\(^{2+}\) gradient may be exacerbated by the addition of sarcolipin (SLN), which binds to SERCA and uncouples Ca\(^{2+}\) transport from ATP hydrolysis, increasing the duration of Ca\(^{2+}\) transients in the cytoplasm and futile cycling of SERCA. In rodent models, this leads to heat production (Bal et al., 2018; Nowack et al., 2017; Periasamy et al., 2017), slower muscle relaxation (Tupling et al., 2011), higher resting energy expenditure (Bombardier et al., 2013), and reduced cases of diet-induced obesity (Maurya et al., 2015). However, recent studies also implicate SLN-mediated increases in cytosolic Ca\(^{2+}\) in signaling for greater PGC1\(\alpha\) expression, mediating a physiological switch to increased mitochondrial biogenesis and fatty acid
catabolism, and greater fatigue resistance in skeletal muscle (Fajardo et al., 2017; Maurya et al., 2018; Sopariwala et al., 2015; Summermatter et al., 2011). However, the role of Ca$^{2+}$ cycling efficiency in the development of fatigue-resistant muscle in migratory birds has gone unexplored.

The regulatory basis for seasonal flexibility of metabolism and tissue growth and breakdown are also poorly understood. Numerous studies have shown changes in the gene transcription of tissues in birds in response to photoperiod or temperature changes (Sharma and Kumar, 2019; Singh et al., 2015; Stager et al., 2015). Frequently, these studies focus on brain tissues to determine the control of migratory behavior in songbirds (Boss et al., 2016; Frias-Soler et al., 2020; Sharma et al., 2018). However, detailed studies of tissues involved in the metabolism of migratory songbirds, such as the liver and flight muscle, have the potential to reveal novel pathways that enable birds to accomplish remarkable feats of endurance. The liver plays an important role in metabolic activity, regulating glycogenesis, gluconeogenesis, lipoprotein synthesis, and controls the availability of fatty acids for use by other tissues (Guglielmo, 2010). As migratory birds consume and rebuild a substantial portion of their muscle and organs with each flight and subsequent stopover recovery (Battley et al., 2000; Lindstrom et al., 2000; Piersma et al., 1999), the liver also serves an important role in processing amino acids and nitrogen waste (Hayamizu, 2017; Paulusma et al., 2022; Salway, 2018; Wu, 2009). During migratory flight, the pectoralis flight muscles demand enormous amounts of fuel and may undergo substantial damage, raising the demand for nitrogenous waste processing and amino acid recycling (Wagenmakers, 1998). In addition to the potential for higher rates of protein consumption, migratory birds have remarkable potential to restore muscle mass
after severe depletion (Bayly et al., 2021; Lindstrom et al., 2000; Piersma et al., 1999), and Young et al. (2021) recently shown extraordinary rates of cultured muscle satellite cell proliferation from migratory bird flight muscles. However, the regulatory basis of rapid lean mass remodeling in migratory birds still largely unknown. Muscle flexibility may be determined primarily by satellite cell activation (Hoppeler, 2016; Swanson et al., 2022), and these stem cells are quiescent unless stimulated by hormonal signals or injury, after which they proliferate and fuse with muscle fibers. One of the established pathways of muscle growth—via the mTOR signaling pathway and upstream regulators myostatin (MSTN) and IGF1—has shown conflicting results in photo-stimulated White-throated Sparrows and other migratory birds (King et al., 2015; Price et al., 2011) while supporting the larger muscle sizes found in overwintering passerines in cold environments (Swanson et al., 2009; Swanson et al., 2014; Zhang et al.). When comparing migratory and sedentary subspecies of Dark-eyed Juncos (Junco hyemalis) in the spring, lipid transport and fatty acid catabolism genes were expressed more in the flight muscle of migratory birds, but the authors also noted that small muscle protein was also highly expressed in the migratory juncos, potentially contributing to rapid muscle growth in the migratory season (Fudickar et al., 2016). In a recent study investigating changes in gene expression in the heart and liver of migratory White-throated Sparrows, Horton et al. (2019) found increased transcripts associated with immune function, including wound healing pathways, in the migratory condition, which may sustain the rapid tissue remodeling seen during migratory stopovers. However, the mechanisms underlying flexible tissue remodeling through either growth or degradation pathways have not been thoroughly explored (Swanson et al., 2022), and the connection between
flight muscle changes and liver metabolism in birds in migratory condition have been understudied.

The central hypothesis of this thesis is that preparatory changes to protein metabolism and muscle function enable birds to undertake long-duration flights. In chapter 2, I compared two related migratory species—long-distance Blackpoll Warblers (*Setophaga striata*) and short-distance Yellow-rumped Warblers (*Setophaga coronata*)—during fall migration used wind tunnel flights to investigate differences in energy expenditure, overall fuel use, and fuel mixture. Current flight modelling programs, such as Flight v. 1.24 (Pennycuick, 2008), assume static contributions of protein that typically make up 5% of the total energy, allowing the program to run until the fat stores of the simulated bird are exhausted. Based on these simulations, a 19 g migratory bird should be able to fly up to 119 hours until its fat is depleted, leaving ~8 g of fat-free mass at the destination. I tested the underlying assumptions that these flights are primarily limited by fat stores and that protein provides 5% of flight energy for long- and short-distance migratory birds. I measured fat and fat-free body mass before and after flight using quantitative magnetic resonance (QMR) and calculated energy expenditure from body composition changes and doubly-labeled water for flights lasting up to 28 hours. I found that both species used fat and lean mass similarly, with fat burned at a steady rate while the rate of protein loss was high early in flight and declined exponentially over time, suggesting that protein may be a dynamic, and often overlooked, fuel for long distance migratory birds.

Chapter 3 explores the potential for adaptive seasonal modulation of lean mass storage and catabolism during migration and to investigate the mechanisms of fatigue
resistance through reduced SERCA uncoupling. To accomplish this, I conducted a photoperiod manipulation using captive migratory White-throated Sparrows (*Zonotrichia albicollis*). I monitored changes in body composition using QMR and measured water-restricted metabolic rate and water loss at multiple time points using flow-through respirometry. I found that birds in the migratory condition increased activity of various fat catabolism enzymes as expected. However, I also found evidence of increased activity of protein catabolism enzymes greater water loss and lean mass catabolism in the migratory condition, indicating that protein breakdown may be adaptive during migration in songbirds, potentially in association with higher rates of water loss.

In chapter 4, to investigate regulatory pathways involved in seasonal changes to metabolism and tissue plasticity, I performed RNA-seq analysis on muscle and liver tissues from a subset of birds in the migratory and non-migratory conditions in Chapter 3. I isolated RNA and prepared libraries for sequencing, followed by differential expression analysis of the reads aligned to the White-throated Sparrow genome. This showed expression patterns consistent with reduced energy use but higher fat export from the liver for consumption by active pectoralis flight muscle in the migratory condition. Expression patterns in the muscle also suggest that the capacity for rapid muscle remodeling in migratory birds may be mediated through inflammation pathways.

In chapter 5, I further explored my findings from chapter 3 showing that migratory White-throated Sparrows showed a substantial increase in muscle *sarcolipin* mRNA, which uncouples Ca^{2+} transport from ATP hydrolysis upon sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) binding. This surprising result suggests that a substantial energetic process in skeletal muscle is less efficient in the migratory
condition. Using a photoperiod manipulation with Gray Catbirds (*Dumetella carolinensis*), I confirmed higher sarcolipin gene transcription in the migratory condition for this species alongside unchanged SERCA2 transcription. However, while both male and female catbirds showed these changes in gene transcription and demonstrated migratory restlessness overnight, only the males gained fat and showed higher muscle Ca^{2+}-dependent ATPase activity, suggesting that the development of the migratory phenotype in flight muscle may occur independently from the fattening that is associated with migratory body composition.

Migratory songbirds are an ideal system for the study of physiological flexibility. Substantial physiological changes enable their remarkable feats of endurance exercise, but along with endurance capacity comes the ability to respond to staggering changes in body composition over a period of days, or even hours. Furthermore, this capacity is seasonally adjusted each year for many years, even in relatively small songbirds. Within this fascinating system, my thesis addresses the role of lean mass breakdown in these migratory birds—a seemingly negative consequence of their demanding journeys—as another contribution to the migratory syndrome of traits that enable successful migration. Furthermore, I generate new hypotheses around altered regulatory mechanisms that may play a role in rapid tissue remodeling and the signaling pathways that trigger physiological changes in preparation for migration. This shifts the focus of migration research to an understudied aspect of migratory physiology, which may provide avenues for the discovery of novel mechanisms for withstanding radical changes in body composition and metabolic intensity.
CHAPTER 2

LONG-DURATION WIND TUNNEL FLIGHTS REVEAL EXPONENTIAL DECLINES IN PROTEIN CATABOLISM OVER TIME IN SHORT- AND LONG-DISTANCE MIGRATORY WARBLERS

2.1 ABSTRACT

During migration, long-distance migratory songbirds may fly non-stop for days, whereas shorter-distance migrants complete flights of 6-10 hours. Fat is the primary fuel source, but protein is also assumed to provide a low, consistent amount of energy for flight. However, little is known about how the use of these fuel sources differ among bird species and in response to flight duration. Current models predict that birds can fly until fat stores are exhausted, with little consideration of protein’s limits on flight range or duration. We captured two related migratory species—ultra long-distance Blackpoll Warblers (*Setophaga striata*) and short-distance Yellow-rumped Warblers (*Setophaga coronata*)—during fall migration and flew them in a wind tunnel to examine differences in energy expenditure, overall fuel use, and fuel mixture. We measured fat and fat-free body mass before and after flight using quantitative magnetic resonance and calculated energy expenditure from body composition changes and doubly-labeled water. Three blackpolls flew voluntarily for up to 28 hours—the longest wind tunnel flight to date—and ended flights with substantial fat reserves but concave flight muscle, indicating that protein loss, rather than fat, may actually limit flight duration. Interestingly, while blackpolls had significantly lower mass-specific metabolic power in flight than Yellow-rumped Warblers, fuel use was remarkably similar in both species, with consistent fat use but exceptionally high rates of protein loss at the start of flight that declined
exponentially over time. This suggests that protein may be a critical, dynamic, and often overlooked fuel for long distance migratory birds.

2.2 INTRODUCTION

Migratory birds cover extraordinary distances to seasonally exploit favorable habitats and conditions in distant locations. In songbirds (Passeriformes), these journeys are accomplished in a series of non-stop flapping flights operating at roughly 8-10 times their basal metabolic rate alongside increased rates of water loss (Engel et al., 2006; Jenni-Eiermann, 2017; Wikelski et al., 2003). These journeys can range from short- to long-distances even within closely related species, with long-distance migrants crossing vast ecological barriers over thousands of kilometers in non-stop flights lasting for days while short-distance migrants complete overland routes in a series of overnight flights with ample landing opportunities (DeLuca et al., 2019; McKinnon et al., 2017; Rappole, 2013; Wikelski et al., 2003). Migratory birds seasonally alter their capacity to transport and catabolize fat to fuel their demanding flights, which provides the vast majority the fuel needed to make the journey (Guglielmo, 2018; Jenni-Eiermann, 2017; Jenni-Eiermann et al., 2002a). Indeed, fat is the most energy-dense fuel available to the bird and it is stored efficiently in lightweight adipose tissue with minimal bound water (Guglielmo, 2010).

Though fat is the primary fuel source for migratory flight, protein is estimated to contribute 4-7% of the total flight energy (Battley et al., 2001; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002a). While the role of protein catabolism in migratory flight is not fully understood, it was long assumed that protein was spared as a fuel of last
resort because it comes from functional organs and tissues (Biebach, 1992; Mcnair et al., 2002; Nisbet et al., 1963), and reduced vital organs and muscles following non-stop flight has been well-documented (Battley et al., 2000; Bauchinger and Biebach, 2001). Therefore, the estimated ~5% of flight energy coming from protein may lead to functional losses that can impose important limits on flight duration and animal performance after flight (Gerson et al., 2020).

Our current understanding of fat and protein use and aerodynamics is frequently used to estimate chemical and mechanical power as well as migration distance and duration (Biebach, 1992; Bolus et al., 2017; Pennycuick et al., 2013). *Flight v. 1.24* (Pennycuick, 2008) is one steady-flight modelling program that incorporates physical principles of flight alongside assumptions of fuel contributions and muscle efficiency, and it has been applied to estimates of flight cost and migration distance for multiple species of birds (Bayly et al., 2012; Bayly et al., 2020; Pennycuick and Battley, 2003) and bats (McGuire et al., 2012). The bird model applies the assumption that protein catabolism consistently contributes 5% of flight energy, with the remaining 95% coming from fat (Battley et al., 2001; Pennycuick and Battley, 2003; Piersma et al., 2022). The ‘Migrate’ function of the program therefore performs a time-marching calculation of changes to body mass—and accompanying alterations to drag, power coefficients, wingbeat frequency, and other covariates—until the fat stores of the simulated bird are exhausted (Pennycuick, 1998; Pennycuick, 2008). These models often result in extremely low final fat-free mass, but the role of protein in determining flight range has not been carefully examined. However, records of birds arriving at their destinations with
emaciated muscles but remaining fat stores (Bayly et al., 2021) suggest that real-world flight range may be constrained by protein loss.

Protein may also be a more responsive and beneficial fuel than previously acknowledged. For example, migratory White-throated Sparrows (Zonotrichia albicollis) show a seasonal increase in the capacity to catabolize protein (Elowe and Gerson, 2022) and migratory songbirds flown in a wind tunnel show greater lean mass loss in response to drier conditions, indicating that lean mass may serve as a source for endogenous water during flight (Gerson and Guglielmo, 2011a; Gerson et al., 2020; Groom et al., 2019). Protein catabolism in migratory flight may also restore Kreb’s cycle intermediates that enable continued fatty acid catabolism (Jenni-Eiermann and Jenni, 1991), maintain optimal power to weight ratios as fuel loads change in flight (McWilliams and Karasov, 2005), or reflect an imbalance between consistent, tissue-specific rates of protein catabolism and suppressed rates of protein synthesis (Bauchinger and McWilliams, 2010). If protein catabolism serves an important function in migratory flight independent of fat catabolism, it may show variability that exceeds our current view of fuel use in migratory flight.

One way to uncover the importance of protein catabolism in flight is to compare flight fuel use in related migratory species that differ greatly in their migratory strategies. Many studies generalize migration physiology to all migratory species, but the extent to which flight physiology differs between short- and long-distance migrants is unclear. These migratory strategies lead to selective pressures of different types and severity, leading to differences in enzyme activity (Lundgren and Kiessling, 1985) endurance capacity (Hahn et al., 2022), and muscle physiology (Kelsey et al., 2021; Lundgren and
Kiessling, 1988), but they may also differ in flight performance, efficiency, water balance, and fuel use. If protein catabolism serves a vital function for migratory flight, such as alleviating water stress, we might expect to see canalization of this trait for a more prominent humidity response in long-distance migrants during flight relative to short-distance migrants. Alternatively, if protein catabolism is simply a byproduct of flight metabolism, such as a source of Kreb’s Cycle intermediates for fat catabolism, we may expect to see protein breakdown correlate closely with the degree of fat catabolism, and the degree of this relationship may be determined by migratory strategy.

We tested the underlying assumption that endurance flights are fueled by consistent rates of fat and protein catabolism in migratory birds. To accomplish this, we compared the fuel use strategies of related ultra-long and short-distance migratory Setophaga warblers in captive wind tunnel flights under dry or humid conditions. While Blackpoll Warblers have been documented flying non-stop for over 100 hours across open water during their migratory flights (DeLuca et al., 2015), Yellow-rumped Warblers complete shorter 6-10 hour hops overnight and primarily over land. This provides an exceptional comparison for drastically different migratory strategies. Before and after flights, we weighed the birds and measured body composition using quantitative magnetic resonance (QMR) (Guglielmo et al., 2011) and compared calculations of flight metabolic power using QMR as well as doubly-labeled water (DLW). We predicted that energy expenditure, overall fuel use, and fuel mixture in response to the humidity conditions would differ between the short- and long-distance migrants. If protein is adaptive for migratory flight and a humidity response, we might expect to see a more prominent protein loss response to dry conditions in long-distance migrants.
Alternatively, if protein loss is detrimental, we may expect to see a lower overall reliance on protein as a fuel source in long-distance than short-distance migrants (Jenni and Jenni-Eiermann, 1998; Kelsey et al., 2021).

2.3 METHODS

2.3.1 Capture and housing

In fall 2018, 20 Blackpoll Warblers *Setophaga striata* (Forster 1772) and 44 Yellow-rumped Warblers *Setophaga coronata* (Linnaeus 1766) were captured using mist-nets at Long Point Bird Observatory in Long Point, ON, Canada. Blackpoll Warblers are ultra-distance neotropical migratory songbirds that depart their boreal forest breeding range in North America in the fall and gather on the Atlantic coast before flying non-stop for 100 hours over open water to their winter range in northern South America (DeLuca et al., 2015; DeLuca et al., 2019). Meanwhile, Yellow-rumped Warblers migrate shorter distances primarily over land to the southern USA, Caribbean islands, and Central America with nocturnal flights that likely last 6-10 hours (Wikelski et al., 2003).

Birds were color banded for identification and transported to the Advanced Facility for Avian Research at Western University in London, ON, Canada, where they were housed in free-flight rooms (2.4 m × 3.6m) or paired in cages (121 × 68 × 185 cm) under a 12 h light/12 h dark photoperiod and approximately 20°C and 60% relative humidity. Birds were maintained on an ad libitum synthetic high-carbohydrate diet modified from Guglielmo et al. (2017) with supplemental *Tenebrio* mealworms provided daily (Dick and Guglielmo, 2019). Procedures were approved by the University of
2.3.2 Experimental design

All birds were given at least two weeks to acclimate to captivity prior to flight experiments in the wind tunnel. Infrared camera recordings overnight show that the Yellow-rumped Warblers showed migratory restlessness in their cages, confirming their continued migratory condition in captivity (unpublished obs.). For a description of the wind tunnel, see Gerson and Guglielmo (2011a). To determine whether the environmental conditions influence fuel use, we flew birds under both high evaporative water loss (HEWL; 2 g H2O m\(^{-3}\); 13% relative humidity (RH)) and low evaporative water loss (LEWL; 12 g H2O m\(^{-3}\); 80% RH) conditions at 18°C at 8 m/s wind speed. Flights began approximately 30 minutes after lights were turned off as both species are nocturnal migrants, and access to food was restricted for 1 hour prior to flight. Additional flight protocol details can be found in Gerson et al. (2020). Eighteen Blackpoll Warblers were selected for experiments, with nine flying in the wind tunnel at the facility and others serving as rest birds that were concurrently held in a covered cage in the plenum of the wind tunnel room to experience the same conditions alongside the flight. For Yellow-rumped Warblers, 29 birds were included in the experiment and 16 birds were flown. Before and after flights, we weighed the birds and measured fat mass using quantitative magnetic resonance (QMR) body composition analysis (Guglielmo et al., 2011). We regularly test the accuracy of fat mass measurements in the QMR by scanning known oil
standards, but we calculated fat-free mass (total mass – fat mass) as a proxy for lean mass since we have no suitable standard for regularly testing lean mass measurement accuracy. Although fat-free mass constitutes all non-fat body components including the beak, feathers, bones, and carbohydrates, the bulk of the mass that is changing during flight comes from lean mass changes due to protein catabolism. We eliminated 5 rest and 3 flight Blackpoll Warblers from the analysis to account for instances where the QMR showed abnormal variability in the oil standard readings or bird scans, such as those leading to an estimated gain in fat or fat-free mass after the flight or rest.

Initially, flights were capped at 6 hours and birds were flown under HEWL and LEWL for repeat measures. Birds flew up to four times each, and after these capped flights we allowed three Blackpoll Warblers to fly until they voluntarily stopped (landing or failing three times within 5 minutes). We measured total body mass changes with a balance (0.001 g) while fat was measured immediately before and after flight using quantitative magnetic resonance (QMR) body composition analysis (Guglielmo et al., 2011). Fat-free mass was used as our proxy for wet lean mass by subtracting dry fat mass from total body mass.

2.3.3 Energy Expenditure

Flight costs are typically measured using mask respirometry, $^{13}$C-labeled sodium bicarbonate, doubly-labeled water (DLW), or QMR (Hedh et al., 2020; Ward et al., 2004). QMR has been used repeatedly for wind tunnel flights, and QMR-measured changes in fat and fat-free mass between scans are used to estimate energy expenditure
by multiplying these changes by the energy density of the tissue (37.6 kJ/g for fat mass or 5.3 kJ/g for wet lean mass). However, this method has not been directly compared to measurements from traditional methods such as DLW.

We calculated energy expenditure from DLW using breath isotope enrichment measurements (Mitchell et al., 2015) using a LGR Water Vapor Isotope Analyzer (Los Gatos Research, San Jose, CA) and standard calculations from (Speakman, 1997). Initially, we collected background breath isotope enrichment on each bird to apply a correction to enriched breath. This was only measured once for each bird after they were in captivity for over two weeks due to the consistent background enrichment measurements over time. After lights out, we injected birds intraperitoneally with approximately 19 mg (range: 10.9 – 26.9 mg) of prepared 2:1 $^{18}$O:$^{2}$H doubly-labeled water (Cambridge Isotope Laboratories, Tewksbury, MA) The syringe was weighed to the nearest 0.001 g before and after injection to determine the dose. One hour after injection, we measured the breath enrichment of the birds using a face mask setup until the isotope measurements plateaued (equilibration measurement). After the bird flew or rested, we collected breath enrichment again. The logarithm of the turnover in these enrichment values for $^{18}$O and $^2$H over the time between measurements was used to calculate the elimination constants for each isotope during flight per hour ($k_o$ and $k_d$ respectively). Because QMR total body water measurements were inconsistent, we compared pre- and post-flight body water pools using 66% of the fat-free mass and took the average of these for the estimated flight body water pool (N). We calculated rCO$_2$ (mol/h) using the one-pool model equation 7.17 in Speakman (1997), simplified to $r$CO$_2$ = $N((.48123 \times k_o) - (.48743 \times k_d))$, which we then converted to Watts.
2.3.4 Flight simulations

We used the ‘Migrate’ function in *Flight 1.24* (Pennycuick, 2008) to simulate flights for Blackpoll Warblers. Aside from the defaults, we incorporated parameters for Blackpoll Warblers used by Bayly et al. (2020), including wing span (0.2108 m), wing area (0.0080 m\(^2\)), and flight muscle fraction from closely-related Bay-breasted Warblers (*Setophaga castanea*) and Blackburnian Warblers (*Setophaga fusca*) reported in Graber and Graber (1962), and the flight muscle fraction was set to 0.156. We also used a Basal Metabolic Rate (BMR) Factor of 1.2 as this was the closest approximation of the pre-flight resting metabolic rate of Yellow-rumped Warblers from a previous wind tunnel study (Gerson et al., 2020) and the fat-free mass-corrected estimate of the metabolic rate measured in our rest birds using QMR and DLW. The altitude of the simulations was set to 0 m above sea level to be comparable to the wind tunnel. The speed control was set to approximately using the V/V\(_{mp}\) ratio (flight speed vs. minimum power speed), and when possible we adjusted this ratio to match 8 m/s constant flight speed. For nine of the birds with higher pre-flight masses it was not possible to set this ratio below 1 so the simulated speed was higher than 8 m/s (maximum speed was 9.07 m/s). Therefore, for these birds we acknowledge that the range estimate would be higher than expected if the bird was flown at 8 m/s due to the higher flight cost speeds lower than V\(_{mp}\). We ran the ‘Migrate’ program with these parameters and adjusting the total mass and fat fraction for all of the Blackpoll Warblers that flew in the wind tunnel. For each time step in the simulated migratory flights, we calculated fat-free mass by subtracting the fat mass from total mass, then calculated the cumulative fat and fat-free mass lost since the starting point and the
rate of fat and fat-free mass loss over the elapsed time. We also estimated the power
curve for Blackpoll Warblers across flight speeds using the `afpt` (1.1.0.2) package in R
using the same measurement parameters as described above.

2.3.5 Statistical Analysis

All statistics were performed in R software (v 4.1.1, R Foundation for Statistical
Computing, Vienna, Austria). We used the function ‘lmer’ (lme4 package, version 1.1-
17) and performed backward fixed-effects stepwise model selection on linear mixed-
effects models with a \( \alpha = 0.05 \) cutoff for fixed effects. Bird ID was included as a random
effect to account for repeated measures for each individual bird. For starting models, we
were interested in how the rate of mass loss (fat or fat-free) relates to the starting body
mass, flight or humidity treatment, species, duration in the wind tunnel, and the
interaction of duration x species or duration x flight treatment.

All energy expenditure statistics were conducted using the mass-specific
metabolic power (Watts/gram) due to the significant difference in starting masses for
Yellow-rumped Warblers and Blackpoll Warblers (\( t = 3.34, df = 16.6, p = 0.004 \)). Our
starting model examined the difference in mass-specific metabolic power with fixed
effects for the two measurement techniques and their interaction with the flight status, the
humidity treatment, species, and duration in the wind tunnel, with bird ID as a random
effect due to repeated measures.
2.4 RESULTS

2.4.1 Body Composition

Fat was burned consistently as a fuel for both species, with a higher rate of fat loss in flight (0.15±0.01 g/h) than at rest (0.06±0.01 g/h; F$_{1,88.99}$ = 52.79, p < 0.001) (Fig. 1). A similar pattern appeared with the rate of fat-free mass loss (F$_{1,72.23}$ = 14.49, p < 0.001), but the rate of fat-free mass loss also declined exponentially with flight duration (F$_{1,66.60}$ = 47.64, p < 0.001) (Fig. 1). Three Blackpoll Warblers flew until they voluntarily stopped, leading to flights of approximately 10, 20, and 28 hours. After these long-duration flights, substantial fat reserves remained (> 3 g), but concave flight muscle and fat-free mass of ~8.5 g indicate that flight was limited by lean body mass rather than fat loss. As in the shorter flights, fat was burned consistently higher in flight (0.14±0.01 g/h) than rest (0.04±0.01 g/h; F$_{1,42}$ = 118.41, p < 0.001), and the rate of fat-free mass loss was slightly lower in rest birds than flight birds (F$_{1,40.70}$ = 6.60, p = 0.014) and declined exponentially with flight duration (F$_{1,24.14}$ = 76.23, p < 0.001) to the point where fat-free mass contributions dropped from approximately 10% to less than 3% of total energy (Fig. 2).
**Figure 1.** Fat mass is lost consistently over flight or rest while the rate of fat-free mass loss is high initially and declines exponentially over time in both Yellow-rumped Warblers and Blackpoll Warblers. The two species are merged in this figure since there was no significant difference in fuel use for these comparable flights lasting up to ~6 hours. Rate of fat mass loss is shown in yellow and fat-free mass loss is shown in red, with flight birds shown with solid lines/triangles and birds at rest depicted in dashed lines/circles.
Figure 2. The rate of fat-free mass loss in wind tunnel flights for Blackpoll Warblers is exceptionally high initially and declines exponentially over time in flight, in contrast to the predictions of consistent protein loss using Flight 1.24. Simulations (blue/triangles/dotted line) incorporated the starting total and fat masses of all Blackpoll Warblers flown in the wind tunnel in the ‘Migrate’ program, with 5% of flight energy coming from protein and flight speed set to 8 m/s. Corresponding measurements for rates of fat-free mass loss in Blackpoll Warbler flights up to 28 hours are shown in green/circles/solid line.

2.4.2 Energy Expenditure

Estimated metabolic power for Blackpoll Warblers was 1.46±0.2 W using QMR and 1.62±0.2 W using DLW, and for Yellow-rumped Warblers it was 1.43±0.2 W with QMR and 1.50±0.1 W with DLW (Fig. 3). When using mass-specific metabolic power, we found a significant interaction between the measurement technique (DLW or QMR) and the flight status (flight or rest) (F1,51.1=9.64, p = 0.003). Therefore, we split the model by flight status and continued with backwards model selection. For flight birds, we found a significantly greater mass-specific metabolic power in Yellow-rumped Warblers (F1,11.8=11.76, p = 0.005) with a trend toward 0.005±0.003 W/g lower estimates using QMR than DLW (F1,28.3=4.12, p = 0.052). For rest birds, the final model showed that
QMR estimates were approximately $0.005 \pm 0.002$ W/g higher than DLW ($F_{1,20.0}=7.21$, $p = 0.014$) and mass-specific metabolic power was higher for the HEWL treatment ($F_{1,32.8}=4.28$, $p = 0.047$).

**Figure 3.** Mass-specific metabolic power is significantly lower in Blackpoll Warblers than Yellow-rumped Warblers in flight, but not in rest. Data are subset for flights in which quantitative magnetic resonance (QMR) and doubly-labeled water (DLW) energy expenditure measurements were both completed for flights up to ~6 hours. In general, QMR measurements (left panel) of metabolic rate were higher in rest birds and lower in flight birds than DLW measurements (right panel). Blackpoll Warblers are shown in green/circles and Yellow-rumped Warblers in orange/triangles.

### 2.4.3 Flight Simulations

We ran *Flight 1.24* ‘Migrate’ simulations using the pre-flight total masses and fat fractions for all Blackpoll Warblers flown in our experiment (Table 1). These simulations
predicted that the birds in our study would fly between 27 and 164 hours (average of 82 hours) at approximately 8 m/s for a final distance of approximately 2431 km (range 769 – 5260 km), ending with no fat remaining and body masses ranging from 6.6 to 13.7 g (average of 9.6 g). This was a more severe ending body composition than we saw in the Blackpoll Warblers that were allowed to fly to a voluntary end point, with shorter flight durations overall in the wind tunnel and the lowest fat-free mass at the end being

Table 1. Flight 1.24 ‘Migrate’ results from Blackpoll Warbler wind tunnel flights and actual results from long-duration flights.

<table>
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<tr>
<th>Bird ID</th>
<th>Starting mass (g)</th>
<th>Starting fat (g)</th>
<th>Starting fat-free (g)</th>
<th>Actual end mass (g)</th>
<th>Actual end fat (g)</th>
<th>Actual end fat-free (g)</th>
<th>Actual Duration (h)</th>
<th>Predicted Duration (h)</th>
<th>Predicted Distance (km)</th>
<th>Predicted Final mass (g)</th>
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2.5 DISCUSSION

Despite vast differences in the distances and barriers traversed by short- and long-distance migrants, we found remarkably similar fuel use for Yellow-rumped Warblers and Blackpoll Warblers flown in a wind tunnel. Though Yellow-rumped Warblers showed higher mass-specific metabolic power, fat was used consistently as a fuel. Importantly, both warblers initially burned protein at a higher rate than expected—making up over 10% of flight energy—followed by a dramatic reduction over the first several hours of flight. This pattern was most apparent with the addition of three voluntary extreme-duration Blackpoll Warbler wind tunnel flights lasting up to 28 hours—the longest wind tunnel flight to our knowledge. These flights ended with ample fat stores but depleted flight muscles. These wind tunnel flights in closely related *Setophaga* warblers shed new light on the dynamic use of protein in migratory flight.

Despite their difference in their migratory strategy, there were surprisingly few differences between Yellow-rumped Warbler and Blackpoll Warbler fuel use in wind tunnel flights up to 6 hours. Fat was a consistent fuel, with more burned in flight than in rest, but the rate of fat-free mass loss was highest early in flight. This rate declined exponentially with flight duration, eventually settling around 2.5% of flight energy in the longest flights for Blackpoll Warblers. A similar pattern appeared in birds at rest, but to a lower degree than the corresponding flight birds. High fat-free mass loss early in flight may point to protein as an important fuel initially during migration. In American Robins (*Turdus migratorius*), a gradual reduction in lean mass catabolism within the first hour of flight in a wind tunnel was shown previously by Gerson & Guglielmo (2013), but this is the first evidence that this reduction continues over several hours of flight. These
temporarily high rates of protein catabolism contradict the assumption that protein contributes consistently to flight fuel over each iterative step of a simulation and could help explain why models predict shorter flight ranges for extreme migratory shorebirds over the Pacific than are observed (Pennycuick and Battley, 2003). For example, if this is a vital weight adjustment early in flight, models may be missing the lighter loads that ultimately save fuel for the remainder of the journey.

Rapid protein loss in the first hours of flight provides insight into the use of protein to fuel migratory flight. This may indicate digestive organ catabolism early in flight to reduce the weight and energy expenditure of an active tissue that does not contribute to flight (Piersma and Gill, 1998), though this is typically seen over longer time scales. Furthermore, Bayly et al. (2021) found that Blackpoll Warblers caught after arriving on the coast of Colombia after their long-duration flight did not appear to have reduced digestive capacity upon arrival. These Blackpoll Warblers may not show the same degree of digestive organ catabolism shown previously in shorebirds, although gut enzymes may compensate for reduced gut mass to maintain digestive function (Griego et al., 2021). Furthermore, if this were simply a way to save weight in flight, it is unlikely that we would see a similar pattern in the resting (and fasting) birds unless it is a predictive adjustment when birds are in migratory condition, which could be tested by measuring rates of protein catabolism in the same species in non-migratory condition. This would be particularly interesting if rapid protein breakdown at the start of flight or fasting provides an influx of uric acid as an antioxidant in the migratory condition (Gutiérrez et al., 2019; McWilliams et al., 2020). By showing a reduction in the protein contribution to flight fuel over time, we also show that the protein loss, at least initially in
flight, is unlikely to be an iterative adjustment to fuel loads throughout flight (Bauchinger and Biebach, 2001; Pennycuick, 1998). As fat was burned consistently, we would have expected correlated, gradual fat-free mass loss to adjust to these changing fuel loads. The difference in the degree of fat-free mass loss between flight and rest birds does provide some support for the hypothesis that protein serves to replenish Kreb’s Cycle intermediates during fatty acid metabolism. However, if this were the case, we might also expect correlated rates of both fat and fat-free mass loss over the duration of flight or rest to replenish depleted intermediates, or perhaps even an increase in protein catabolism over time as the pool is depleted below a threshold (Jenni et al., 2000), although previous wind tunnel studies suggest that the switch to fat catabolism occurs within the first hour of flight (Jenni-Eiermann et al., 2002a) and the initial protein catabolism may have a different purpose than what is seen later in flight or may be a result of higher corticosterone levels early in flight (Jenni-Eiermann et al., 2009). Finally, the protein turnover hypothesis suggests that protein catabolism occurs at consistent, organ-specific rates regardless of the exercise status and must be balanced by protein anabolism, which is typically suppressed during flight or fasting. In this hypothesis, the different degree of fat-free mass loss seen between flight and rest in this study may be explained by different degrees of suppression of protein synthesis, while the exponential reduction in fat-free mass loss rates over the duration of flight or rest may reflect degradation of organs with high rates of protein turnover followed by those with lower rates while synthesis is suppressed (Bauchinger and McWilliams, 2012).

Interestingly, we did not see a significant effect of the humidity treatment on the fat or fat-free mass loss in either species. While there was a trend toward higher fat-free
mass loss in the HEWL flight birds, this was not significant. Although this response has been consistent and robust in many species of migratory birds, including Yellow-rumped Warblers in previous studies (Gerson and Guglielmo, 2011a; Gerson et al., 2020; Groom et al., 2019), long distance migratory birds like Blackpoll Warblers may have an attenuated response to spare protein during extreme flight durations (Kelsey et al., 2021). Additionally, protein breakdown early in flight may be related to a corticosterone (CORT) stress response (Sapolsky et al., 2000), which may be greatest prior to flight or early in flight (Jenni-Eiermann et al., 2009). The use of DLW in this study required an additional degree of handling over the hour prior to starting the flight compared to previous studies flying Yellow-rumped Warblers, which could have increased the pre-flight CORT response more uniformly for all individuals. The period of substantial fat-free mass loss we found early in flight may also be the time period where a humidity effect leads to the greatest differences if it is a CORT-mediated response to maintain water balance (Landys et al., 2006), and by handling the birds more prior to flight we may have dampened the degree of this difference. However, it is unlikely that the pattern of fat-free mass loss seen was fully the result of handling-induced CORT as we found no significant difference in the amount of fat-free mass loss between birds that did or did not receive the DLW treatment (F_{1,39.5}=0.187, p = 0.67) and studies have shown that CORT responses to acute stress return to baseline levels within 30 – 60 min (Rich and Romero, 2005).

Despite finding no significant differences in fuel use for the two species, we did find lower mass-corrected metabolic power for Blackpoll Warblers than Yellow-rumped Warblers. Metabolic power for Blackpoll Warblers has not been measured directly in a
wind tunnel flight before, but the \( afpt \) power curve estimate of approximately 1.67±0.3W at 8 m/s was close to our measurement using DLW (1.62±0.2W) and about 14% higher than our QMR estimate (1.46±0.2 W). For Yellow-rumped Warblers, our measurement was comparable to published flight costs using the sodium bicarbonate (NaBi) method (1.5±0.4W), though our QMR estimate was 9-40% lower than previous measurements for the species using QMR (Hedh et al., 2020). The higher mass-specific flight metabolic power in Yellow-rumped Warblers than Blackpoll Warblers, measured by both QMR and DLW, may reflect selective pressures for efficient flight in ultra-long distance migratory species crossing major ecological barriers (Conklin et al., 2017; Kelsey et al., 2021; Piersma et al., 2022) where poor condition or inefficient flight can lead to high mortality (Boal, 2014; Morris et al., 2016). In contrast, Yellow-rumped Warblers typically remain over land and even those with poor flight performance would likely survive the migratory journey through a series of shorter-duration hops. Hahn et al. (2022) speculated that physiological differences such as muscle fiber sizes and vasculature (Lundgren and Kiessling, 1988) or mitochondrial efficiency (Toews et al., 2014) could lead to differences in exercise endurance seen between short- and long-distance migrants. Since flight energy expenditure differences appeared using both QMR and DLW measurements, it is surprising that no differences in fuel use appeared. However, slight, non-significant differences in fuel use, such as the minor trend we see toward a higher rate of fat loss in Yellow-rumped Warblers, could compound over long flight durations and lead to this variation in flight energy expenditure. Furthermore, as these differences appeared in the mass-specific metabolic power in flight only, it may reflect more efficient
flight behavior or aerodynamics in the Blackpoll Warblers that allows them to transport a greater fuel load for roughly the same total fuel cost as the Yellow-rumped Warblers.

While calculations of energy expenditure using QMR and DLW supported the higher mass-specific metabolic power in Yellow-rumped Warblers and the correlation between the two measurements was strong, QMR measurements were slightly higher than DLW for the rest groups and slightly lower for flight. Nagy (1983) notes that 1-2 half-lives of \(^{18}\)O elimination from peak enrichment is an ideal washout to estimate metabolic rate, with errors being higher with less washout. The elimination of \(^{18}\)O for our flight birds was close to, but significantly different, from one half-life (\(\delta^{18}\)O elimination: 440\(\pm\)110 vs. 547\(\pm\)104 for one half-life; \(t = -3.14, df = 38, p = 0.003\)), but rest birds were showed significantly less washout (\(\delta^{18}\)O elimination: 147\(\pm\)35.6 vs. 577\(\pm\)61.6 for one half-life; \(t = -23.45, df = 28, p < 0.001\)) so there may be greater error in the DLW estimate for rest birds. Despite \(^{18}\)O turnover less than one half-life, this method has been successful for other bird flights of approximately 6 h and is likely to be relatively accurate (Ward et al., 2004).

Using the geolocator data from 20 Blackpoll Warblers in Deluca et al. (2019), and integrating the minimum and maximum flight time and distance from the uncertainty in the data, we estimate that the birds in that study flew for 62.4 h on average (range: 42 to 102 h). Flight simulations predicted that Blackpoll Warblers from our study would fly for 98\(\pm\)7 hours and end their flights at 8.7\(\pm\)0.3 g, with a minimum estimate of 6.6 g. Blackpoll Warblers arriving on the coast of South America during fall migration often arrive emaciated (Wetmore, 1939), with Nisbet et al. (1963) estimating that 7.9 g appeared to be the lowest limit for fat-free mass in Blackpoll Warblers arriving in
Venezuela and Bayly et al (2021) observing the lowest mass upon arrival in Colombia at 8.3 g. In our study, we allowed three captive Blackpoll Warblers to fly until they voluntarily ended the flights by landing at least 3 times within 5 minutes. In the resulting flights lasting up to 28 h, each of these birds still had substantial (at least 3 g) fat remaining, but we observed slightly concave flight muscles and a final fat-free body mass of at least 9.6 g. Flight in a wind tunnel may be more erratic and consume more energy than wild flights with steady, consistent wingbeats (Pennycuick and Battley, 2003), but by voluntarily ending flights earlier than predicted with ample fat reserves but concave flight muscle we may find that factors other than fat mass, such as protein or water, may be important for determining flight duration. This is consistent with recent evidence from Bayly et al (2021) that out of over 1000 Blackpoll Warblers captured after arriving in Colombia, only 14% and 21% of birds were fat depleted in 2017 and 2018, respectively, while 87% of the birds had depleted flight muscles (muscle scores of 1 or lower).

We found remarkably similar use of fuels in these short- and long-distance migratory warblers. Despite the difference in mass-specific metabolic power, both species used fat consistently and lost substantially more fat-free mass early in flight. When allowed to fly to voluntary completion, the Blackpoll Warblers ended their flights with ample fat stores, but evidence of fat-free mass depletion, suggesting that protein may be a critical, dynamic, and overlooked fuel for determining the capabilities of migratory birds.
CHAPTER 3

MIGRATORY DISPOSITION ALTERS LEAN MASS DYNAMICS AND PROTEIN METABOLISM IN MIGRATORY WHITE-THROATED SPARROWS (ZONOTRICHIA ALBICOLLIS)


3.1 ABSTRACT

Migratory birds seasonally increase fat stores and the capacity to use fat to fuel long-distance migratory flights. However, lean mass loss also occurs during migratory flights and, if adaptive, should exhibit seasonal changes in the capacity for protein metabolism. We conducted a photoperiod manipulation using captive White-throated Sparrows (Zonotrichia albicollis) to investigate seasonal changes in protein metabolism between the non-migratory “winter” condition and after exposure to a long-day “spring” photoperiod to stimulate the migratory condition. After photostimulation, birds in the migratory condition rapidly increased fat mass and activity of fat catabolism enzymes. Meanwhile, total lean mass did not change, but birds increased activity of protein catabolism enzymes and lost more water and lean mass during water-restricted metabolic testing. These data suggest that more protein may be catabolized during migratory seasons, corresponding with more water loss. Counter to predictions, birds in the migratory condition also showed an approximately 30-fold increase in muscle expression of sarcolipin, which binds to sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and uncouples Ca^{2+} transport from ATP hydrolysis. Our documented changes to protein
catabolism enzymes and whole-animal lean mass dynamics may indicate protein breakdown or increased protein turnover is adaptive during migration in songbirds.

3.2 INTRODUCTION

Migration allows animals to seasonally exploit habitats that are geographically disparate, and a “migratory syndrome” of coordinated, seasonally-responsive adaptations alter metabolism, body composition, and reproductive status together increase the probability of successful migration and reproduction (Dingle, 2006). This study aims to broaden our understanding of the changes to metabolism and muscle function that enable long duration flight in migratory birds. One well-documented physiological example of the migratory syndrome is the seasonal upregulation of fatty acid transport and metabolism to fuel migratory flight, which is metabolically expensive (Jenni-Eiermann, 2017; McFarlan et al., 2009). Fatty acids are the best fuel for flight due to the high energy density compared to protein or glycogen (Jenni-Eiermann, 2017). Photostimulation of captive migrants with a long-day spring photoperiod results in higher activity of the β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (HOAD) and the rate-limiting enzyme carnitine palmitoyl transferase (CPT) in the pectoralis flight muscle, even in untrained conditions (McFarlan et al., 2009; Zajac et al., 2011), and migratory Western Sandpipers increase enzyme activity of fatty acid synthase and Δ⁹-desaturase (Egeler et al., 2000) alongside CPT and HOAD in pectoralis muscle (Guglielmo et al., 2002).

Although fatty acids are the primary fuel source for long-distance flight, protein breakdown from flight muscles and organs during fasting and flight has been well documented (Battley et al., 2000; Swain, 1992), but it remains unclear if the capacity for protein catabolism is part of the seasonally-responsive migratory syndrome. The purpose
of protein catabolism during migratory flight is unknown, but several hypotheses seek to
address this question (Bauchinger and Biebach, 1998; Bauchinger and McWilliams,
2012). The protein turnover hypothesis suggests that suppression of protein synthesis
during fasting or flight fails to offset baseline levels of protein catabolism, leading to
tissue-specific rates of organ breakdown and a net reduction in total lean mass
(Bauchinger and McWilliams, 2012). If migratory birds seasonally shift their baseline
capacity for protein catabolism, then this could lead to greater rates of lean mass loss in
the migratory condition during flight or fasting. However, recent studies also show
greater loss of lean mass in drier conditions (Gerson and Guglielmo, 2011a; Gerson et al.,
2020; Groom et al., 2019). As lean mass breakdown yields both metabolic and stored
water from tissues to generate ~6 times more water than fat catabolism (Bauchinger and
Biebach, 1998; Jenni-Eiermann, 2017), this supports the hypothesis that protein is
catabolized as a means to offset the high rate of respiratory water losses during flight
(Engel et al., 2006). Therefore, lean mass breakdown may enable birds to overcome
water-limitation during extended flights with no opportunity to land or access freshwater
(Gerson and Guglielmo, 2011a; Klaassen, 2004; Schmaljohann et al., 2008). Recent
evidence also unifies these two hypotheses, comparable lean mass loss in migratory birds
during flight or fasting, but with an additional degree of loss in response to drier
conditions (Groom et al., 2019). However, it remains untested whether the capacity for
increased protein metabolism shifts seasonally as part of the migratory syndrome.

The enhanced capacity for fuel mobilization and catabolism is an essential
component of the migratory syndrome, but a suite of other adaptations also allow the
completion of migratory journeys, including mechanisms for enhanced fatigue resistance
in flight muscles. Changes to the size and isoforms of myosin heavy chain (Velten et al., 2016), fiber hypertrophy (DeMoranville et al., 2019; Marsh, 1984), capillary density (Lundgren and Kiessling, 1988), and corticosterone signaling (Pradhan et al., 2019) may all contribute to changes in energy consumption and power output during the migratory life history stage. One particularly energy-consuming process in skeletal muscle that has not been previously investigated in migratory birds is calcium cycling. Active pumping of calcium into the lumen of the sarcoplasmic reticulum (SR) to allow repetitive muscle contraction is accomplished through sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), accounting for nearly half of the resting metabolic rate of mouse skeletal muscle (Smith et al., 2013). This energetic cost may be exacerbated by sarcolipin (SLN), which binds to SERCA and uncouples Ca$^{2+}$ transport from ATP hydrolysis, increasing Ca$^{2+}$ transients in the cytoplasm and possibly augmenting muscle heat production (Bal et al., 2018; Nowack et al., 2017; Periasamy et al., 2017). Although beneficial for thermogenesis in small mammals, SLN reduces efficient use of ATP during muscle contraction, leading to lower force, lengthened contraction and relaxation, and fatigue in rats (Tupling et al., 2002). Therefore, we predicted that migratory birds would seasonally reduce SERCA uncoupling to trade off thermoregulatory capacity for greater mechanochemical efficiency supporting economical flight.

To explore the potential for adaptive seasonal modulation of lean mass storage and catabolism during migration, and to investigate the mechanisms of energetic efficiency through reduced SERCA uncoupling, we conducted a photoperiod manipulation using captive migratory White-throated Sparrows (Zonotrichia albicollis). We tracked body composition through the transition from a short-day “winter” (8 h
Light: 16 h Dark) to long-day “spring” photoperiod (16L: 8D) and conducted water-restricted metabolic testing before and after the photoperiod change to measure seasonal changes in the catabolism of endogenous fuel stores that may contribute to differences in overall metabolic rate and water loss. At two time points following the photoperiod switch we measured organ masses and collected muscle and liver tissue samples to test fatty acid (HOAD, CPT) and oxidative metabolism (citrate synthase (CS)) enzyme activities (Fig. 4A). To assess protein metabolism, we measured aminopeptidase capacity to cleave amino acids from peptides within the cell; alanine aminotransferase (ALT), which fixes skeletal muscle ammonia from protein breakdown to pyruvate, forming alanine to be processed in the liver; glutamate dehydrogenase (GDH), which deaminates amino acids in the liver and feeds ammonia into the uric acid cycle; and xanthine oxidase (XOD) as the final step in uric acid formation (Fig. 4B). We also measured mRNA expression of genes in the flight muscle that may be associated with seasonal shifts in muscle efficiency and energetics (SLN, SERCA2, and PGC1α) to explore a potential mechanism for seasonal changes in fatigue resistance. By tracking body composition through seasonal changes, relative loss of lean mass and water during metabolic testing may be connected to shifts in the molecular capacity for protein catabolism. We hypothesized that birds increase their capacity for protein catabolism during spring migration, which would manifest in migratory animals as greater baseline lean mass catabolism during water and food restriction along with tissue-level increases in activity of protein catabolism enzymes.
Figure 4. (a) Enzyme activities were measured for the fatty acid transport enzyme carnitine palmitoyl transferase (CPT), beta-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (HOAD), and oxidative metabolism enzyme citrate synthase (CS). (b) Protein metabolism enzyme assays measured amino acid cleavage by aminopeptidases (AP), alanine formation by alanine aminotransferase (ALT), glutamate dehydrogenase (GDH) amino acid deamination to feed ammonia into the uric acid cycle, and xanthine oxidase (XOD) as the final step in uric acid formation.

3.3 MATERIALS AND METHODS

3.3.1 Animal collection

The White-throated Sparrow is a 25–30 g passerine bird that breeds from the boreal forests of northern Ontario and Quebec to the northeastern USA, and migrates south to the mid-Atlantic latitudes and the coast of the Gulf of Mexico. We captured 26 sparrows (13 male, 13 female) by mist-netting during fall migration between 29 September and 11 October 2017 on the University of Massachusetts Amherst campus (42°23'45.9"N
Birds were color-banded and transported to captive facilities at the University of Massachusetts Amherst and housed in cages with 2 – 3 individuals (77.5 x 30.5 x 39 cm) at 21°C. Birds were exposed to a decreasing fall photoperiod to match the duration of civil dawn to civil twilight, with full-spectrum lights adjusted every 2 – 3 days until a short-day “winter” photoperiod (8L:16D) was achieved on 22 December, 2017. A dim light at night provided ~1 lux in the room (Ramenofsky et al., 2003). Birds had ad libitum access to water, mixed wild bird seed with canola oil and ground small bird diet (Mazuri Small Bird Breeder, PMI Nutrition International, Brentwood, MO), millet sprays (Nutriphase, Petsmart Inc., Phoenix, AZ, USA), egg food supplement (Quiko, Bowling Green, OH), and Tenebrio mealworms. Sparrows were kept on the short-day winter photoperiod (8L:16D) for ~70 days to break photorefractoriness (Barceló et al., 2016). Our study followed the Institutional Animal Care and Use Committee guidelines approved by the University of Massachusetts Amherst (protocol #2015-0019). A collection permit was granted by the US Fish and Wildlife Service (permit #MB65968B-0) and the State of Massachusetts (#097.18SCB) to ARG.

3.3.2 Photoperiod switch

Sex was determined by gel electrophoresis using blood before the photoperiod manipulation (Griffiths et al., 1998). We randomly divided half of the sparrows—equal numbers male and female—into two adjacent rooms where they were singly housed (38.5 x 30.5 x 39 cm) and allowed to acclimate for 4 days. On 5 March, One room was then switched to a long day “spring” photoperiod (16L:8D; N = 13; LD photoperiod treatment, hereafter) to induce a migratory disposition (Gwinner, 1990; Ramenofsky et al., 2003)
while the other room remained on the short-day “winter” light regime (N = 13; SD photoperiod treatment, hereafter). Infrared cameras (Ailipu Technology Co., Ltd., Guangdong, China) were used to record nighttime activity, and two LD sparrows began to show signs of migratory restlessness starting on day 12 following the light change, with all LD birds displaying restlessness by day 23, while SD sparrows exhibited little to no movement overnight.

3.3.3 Body composition

We monitored body composition using a quantitative magnetic resonance body composition analyzer (QMR) (Echo-MRI Echo-Medical Systems, Houston, TX) with fat and wet lean mass accuracies of ±6-11%, and ±1-2%, respectively (Guglielmo et al., 2011). Food was removed from cages ~2 h prior to scanning to ensure birds were postabsorptive. We monitored bird condition through the short-day photoperiod, and after the photoperiod switch birds were weighed and scanned approximately every 3 days to accurately track changes. Birds were sampled 16 days after the photoperiod switch (N = 6 for SD and LD; hereafter “Early” time point) and 25 days after the photoperiod switch (N = 7 for SD and LD birds, hereafter “Late” time point). Samples of pectoralis, liver, and heart were frozen on dry ice within 5 minutes of sacrifice for enzyme activity assays and qPCR.

3.3.4 Metabolic testing

Prior to the photoperiod switch and at the Early and Late time points we measured overnight resting metabolic rate (RMR) using standard push flow-through respirometry in
dry air at 30°C for 12 h from ~ 20:00 – 08:00. For details, see Griego et al. (2021). Our conditions approximate basal metabolic rate (BMR), but after the photoperiod switch the lights turned on earlier for the LD photoperiod treatment group (04:00) than the SD group (08:00), and therefore we refer to this as overnight RMR. Each overnight RMR was followed by an additional 4 h daytime RMR (~ 08:30 – 12:30) with lights on, resulting in 18+ hours of total water restriction during metabolic testing. Overnight metabolic testing took place in complete darkness, thus reducing the likelihood that LD birds were showing active Zugunruhe while in the chambers (Ramenofsky et al., 2008). We scanned birds using QMR both before and after the overnight and daytime RMR. We calculated total water loss and total volume of O₂ consumed for the duration of metabolic testing by calculating the area under the curve for the \( \dot{V}\text{H}_2\text{O} \) and \( \dot{V}\text{O}_2 \) traces (MESS package version 0.5.2 in R version 3.4.2).

3.3.5 Organ masses

After the switch to spring photoperiod, birds were weighed and scanned approximately every 3 days to accurately track mass changes. Birds were sacrificed by isoflurane overdose and cervical dislocation in compliance with all animal care guidelines 16 days after the photoperiod switch (N = 6 for SD and LD; hereafter “Early” time point) and 25 days after the photoperiod switch (N = 7 for SD and LD birds, hereafter “Late” time point). The Early time point was chosen based on the timing of Zugunruhe noted in other photoperiod manipulations using related sparrow species while the Late time point was chosen to ensure that all birds had been showing overnight restlessness for several days (Agatsuma and Ramenofsky, 2006; Zajac et al., 2011). Samples of pectoralis, liver, and
heart were frozen on dry ice within 5 minutes of sacrifice for enzyme activity assays and qPCR. We also weighed the left pectoralis, liver, heart, gizzard, and gut to the nearest 0.0001 g and measured the total gut length to the nearest millimeter. We dried small samples of the pectoralis, liver, and heart in an oven at 60°C for ~48 h to estimate water content and organ dry mass.

3.3.6 Enzyme Activity

Homogenization

Fifty to 100 mg of pectoralis and liver were homogenized using beadmill homogenization (NextAdvance, Troy, NY; speed 8, time 3 for liver 4 for pectoralis, 4°C) in 9 volumes of 20 mM Na$_2$PO$_4$, 0.5 mM EDTA, 0.2% BSA, 0.1% Triton x-100, and 50% Glycerol, pH = 7.4. Total protein concentrations were quantified in duplicate for each sample using a Bradford assay (BioRad, Bradford Quick Start, Hercules, California).

Enzyme assays

Enzyme assays were performed in duplicate or triplicate wells in a Biotek Synergy H1 microplate reader (BioTek, Winooski, VT) at 39°C, a typical avian body temperature. Activities of CS and HOAD were measured using the difference between background absorbance at 340 nm and maximal rates following addition of acetoacetyl-CoA, for a final well concentration of 0.1 mM Acetoacetyl-CoA, 0.5 mM NADH, 0.2 mM DTT, 50 mM Tris, pH = 8.56. For carnitine palmitoyl transferase (CPT), absorbance at 412 nm was measured to detect the appearance of CoA-TNB after the addition of carnitine with a final well concentration of 5 mM carnitine, 0.035 mM palmitoyl-CoA, 0.15 mM DTNB,
50 mM Tris, pH = 8.56. We measured ALT activity in a tandem reaction with LDH, monitoring change in absorbance at 340 nm in 10 mM NaHCO₃, 0.18 mM NADH, 0.10 mM Pyridoxal-5-phosphate, 500 mM L-Alanine, 20 μkat/L Lactate Dehydrogenase, 15 mM α-ketoglutarate, and 50 mM Tris, pH = 8. For GDH, we measured absorbance at 340 nm relative to blank wells in 10 mM Glutamate, 3 mM NAD+, 1 mM EDTA, and 50 mM K-phosphate buffer, pH = 7.4. Relative XOD activity was measured using an Amplex Red fluorescence kit (Invitrogen, Molecular Probes, Eugene, OR). Aminopeptidase activity was measured at 380 nm in 2 mM L-alanine-p-nitroanilide (pNA), 1 M Na₂PO₄, pH = 7.4 as in Griego et al. (2021). We determined the extinction coefficient of nitroanilide to be 3.86 mmol⁻¹ cm⁻¹ in the conditions of this assay.

3.3.7 RNA extraction and qPCR

*Homogenization*

Approximately 50 mg of pectoralis muscle were homogenized (as above) in 1 mL of TriZol (Invitrogen, Carlsbad, CA) and RNA was separated into an aqueous phase using a chloroform ethanol procedure. Total RNA was purified using a PureLink RNA kit (Invitrogen) and DNase treated using TURBO I DNase (Invitrogen). Samples were quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) and RNA quality (RIN > 8.2) was confirmed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA was reverse transcribed to cDNA using NEB Luna RT (New England Biolabs, Ipswich, MA). Each cDNA sample was diluted 1:5 with nuclease-free water before analysis.
We selected glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene for qPCR using a previously published primer sequence used in White-throated Sparrows (McFarlan et al., 2009). We used NCBI Primer-Blast (Ye et al., 2012) to design primers for each gene of interest in White-throated Sparrows: SLN, SERCA2, and PGC1α (see Table 2 for primer sequences). While the SERCA1 isoform has been implicated in the control of non-shivering thermogenesis, it was not annotated in the White-throated Sparrow genome and therefore we selected SERCA2 for this study.

Table 2. Primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>F primer</th>
<th>R primer</th>
<th>Product Length</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CAGCAATGCTTCCTGCACTA</td>
<td>CCTCTGCCCATCTCTCCAAAG</td>
<td>162</td>
<td>(McFarlan et al., 2009)</td>
</tr>
<tr>
<td>SERCA2</td>
<td>AGTGGCAGTTGGAGACAAGG</td>
<td>GATCTCCGTGTTCAACCC</td>
<td>241</td>
<td></td>
</tr>
<tr>
<td>SLN</td>
<td>CTGCTGACCTCTGCTCTCTC</td>
<td>GCACACAGTGAAATCACCTCC</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td>PGC1α</td>
<td>CTATTGTGAGTCCAGCCACT</td>
<td>GCGGTATTCTCCCCTTCTCTCA</td>
<td>151</td>
<td></td>
</tr>
</tbody>
</table>

We confirmed primer specificity on a 2% agarose gel in TAE with SYBR-safe stain and tested for amplification efficiency on a serial dilution series of pooled cDNA (1:2 – 1:40; efficiencies ranged from 98.4 – 99.5%). All reactions were prepared on ice in 10 μL volumes with 0.2 μM primers and 1 μL cDNA in Luna Universal qPCR master mix (New England Biolabs, Ipswich, MA) and run on StepOnePlus qPCR (Applied Biosystems, Foster City, CA) for 60s at 95°C, then 40 cycles of 95°C for 15s followed by 60°C for 30s, with melt curve analysis from 60-95°C. We performed comparative cycle threshold (C_T) analysis according to Schmittgen and Livak (2008). Briefly, each sample was run in duplicate wells and the average C_T for the corresponding housekeeping gene reading (GAPDH) was subtracted from the C_T for the target genes (SLN, SERCA2, and...
PGC1α) to obtain the ΔCT for each individual and gene. Fold change calculations incorporated the average and standard error of the mean (SEM) for ΔCT by photoperiod and time point, subtracting the Early SD mean ΔCT from other group means for ΔΔCT. Fold change was calculated from the mean, upper, and lower SEM limits of ΔΔCT using $2^{\Delta \Delta CT}$.

3.3.8 Statistical analyses

All statistics were performed in R (v 3.4.4, R Foundation for Statistical Computing, Vienna, Austria). To account for structural body size and sex differences, we used PC1 from a principal components analysis of morphometric measurements that showed sex differences (wing, tarsus, and bill width) using ‘prcomp’ (stats package, version 3.5.2) (McCabe and Guglielmo, 2019). This “Structure” term was significantly related to lean mass ($F_{1,64}= 38.2$, $R^2 = 0.36$, $P < 0.001$) but not fat mass ($F_{1,64}= 2.22$, $R^2 = 0.02$, $P = 0.142$), making it indicative of body size. We evaluated linear mixed models with the function ‘lmer’ (lme4 package, version 1.1-17) and performed backward fixed-effects stepwise model selection with a $\alpha = 0.05$ cutoff for fixed effects to determine seasonal differences in body composition after the photoperiod switch, metabolic rate, fat and lean mass changes, and water loss. Bird ID was included as a random effect for repeated measures. For body composition (total, fat, and lean mass), fixed effects included a time point and photoperiod interaction and structural body size. For metabolic rate, we included initial lean and fat mass, photoperiod, and structural body size. For total water loss, our starting model included lean and fat mass lost during testing, photoperiod, total O$_2$ consumed, initial body mass and structural body size. Finally, models for fat and lean
mass loss rates during metabolic testing began with fixed effects for the photoperiod, the duration of time in metabolic testing (including both overnight and daytime RMR testing), mean $\dot{V}O_2$ and $\dot{V}H_2O$, initial mass, and structural body size.

For $\Delta C_T$ gene expression data, we started with an ANCOVA with the interaction between photoperiod and time point, along with body mass as a covariate. For all organ masses, we started with an ANCOVA with the interaction between treatment and time point, along with the structural body size as a covariate. For enzyme activity data, we performed a similar ANCOVA including pectoralis or liver mass. In each of these tests, non-significant ($p > 0.05$) interactions and covariates were sequentially removed from the models. All data are available on the Open Science Framework (osf.io/uqehx).

3.4 RESULTS

3.4.1 Body composition

Prior to photostimulation, birds weighed 29.50 ± 0.73 g, with 5.67 ± 0.64 g of fat and 20.48 ± 0.24 g of lean mass. There were no significant differences between groups at the start of the study. For total body mass, we split our final model by photoperiod to account for a significant time point and photoperiod interaction ($F_{2,44.08}= 4.94$, $p = 0.012$), showing that LD birds significantly increased mass after the photoperiod switch ($F_{2,22.00}= 7.20$, $p = 0.004$) while SD birds remained the same ($F_{2,21.90}= 1.69$, $p = 0.210$; Fig. 5). This mass gain was primarily due to fat accumulation in LD birds ($F_{2,22.07}= 9.42$, $p = 0.001$).

For lean mass our final model included only time point ($F_{2,46.25}=10.10$, $p = 0.0002$), with
both LD and SD birds gaining on average $0.46 \pm 0.12$ g of lean mass by the Early time point and decreasing to $0.13 \pm 0.15$ g below starting mass by the Late time point.

Figure 5. Body composition of White-throated Sparrows through the experimental time course. Birds were weighed and scanned at the end of the winter as a baseline (5 days pre-stimulation), then again at the Early (15 days post-stimulation; $N = 13$ each LD and SD) and Late (25 days post-stimulation; $N = 7$ each LD and SD) time points. Photoperiod switch for LD birds is indicated by the gray line. Mass change is relative to the winter time point (~15 days before photostimulation). LD birds are shown in orange and SD birds in blue. Significant differences between groups are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).
3.4.2 Organ masses

Pectoralis and heart masses were greater for LD birds, but gizzard and gut masses were reduced relative to SD birds (Fig. 6). We found that pectoralis muscle showed a higher, though non-significant, mass for both the LD treatment (F1,22, p = 0.069) and Late time point (F1,22, p = 0.064) while controlling for a correlation with the structural size of the bird (F1,22, p = 0.057). Dry pectoralis muscle mass was significantly greater in the LD treatment (F1,22=9.86, p = 0.005) with a trend towards greater mass for the Late time point (F1,22=3.57, p = 0.072) when accounting for the structural size of the bird (F1,22=4.16, p = 0.054). Wet (F1,23=8.56, p = 0.025) and dry liver mass (F1,23=5.50, p = 0.028) were significantly greater for both LD and SD birds at the Late time point. Dry heart mass was significantly greater in the LD treatment (F1,22=6.96, p = 0.015) with a significant correlation with the structural size of the bird (F1,22=10.37, p = 0.004). For measures of gut mass and length as well as gizzard mass, body size was not a significant covariate. Gut mass was significantly lower for LD birds overall (F1,23=4.40, p = 0.047) and at the Late time point for both groups (F1,23=6.93, p = 0.015), although the gut showed a trend toward being longer at the Late time point for both groups (F1,23=4.197, p = 0.052). Gizzard mass was significantly lower in spring birds than winter (F1,23=9.94, p = 0.004).
3.4.3 Enzyme activity

*Fat metabolism*

Fatty acid and oxidative metabolism enzymes increased in activity in the pectoralis muscle but decreased activity in the liver for LD birds (Fig. 7). In the pectoralis, citrate
synthase activity trended higher in the LD birds (F_{1,22} = 4.179, p = 0.053). Pectoralis HOAD activity was significantly correlated with the muscle mass (F_{1,22} = 6.43, p = 0.029) and increased in LD birds (F_{1,23} = 12.01, p = 0.002). CPT showed no significant differences in activity. Liver citrate synthase activity was significantly lower in the LD birds (F_{1,23} = 4.56, p = 0.044) and liver HOAD showed no differences in activity, but CPT activity was slightly lower, though non-significant, in the LD photoperiod (F_{1,23} = 3.54, p = 0.073), and was significantly lower at the Late time point (F_{1,23} = 5.57, p = 0.027).

**Figure 7.** Fat metabolism and oxidative enzyme activity at the Early (15 days post-stimulation; N = 6 each LD and SD) and Late (25 days post-stimulation; N = 7 each LD and SD) time points. LD birds are shown in orange and SD birds in blue. CS = citrate synthase, HOAD = 3-hydroxyacyl-CoA dehydrogenase, CPT = carnitine palmitoyl transferase. Significant differences between groups are indicated by asterisks (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).
**Protein metabolism**

Activity of aminopeptidase increased in both the liver and pectoralis of LD birds alongside increased GDH and decreased XOD (Fig. 8). Since the interaction between photoperiod and time point was nearly significant for pectoralis aminopeptidase ($F_{1,21} = 3.99$, $p = 0.059$) when accounting for a significant negative relationship with pectoralis muscle mass ($F_{1,21} = 5.46$, $p = 0.030$), we split the model by time point. LD birds show significantly higher aminopeptidase activity than SD birds at the Late time point ($F_{1,11} = 5.70$, $p = 0.027$). Pectoralis ALT showed no significant differences in activity.

Liver aminopeptidase activity showed a non-significant tendency to be higher in LD vs SD birds ($F_{1,22} = 3.84$, $p = 0.063$) when accounting for a significant negative relationship with liver mass ($F_{1,22} = 15.99$, $p < 0.001$). Liver ALT showed no significant differences in activity. GDH activity was significantly higher in LD birds ($F_{1,23} = 10.61$, $p = 0.003$).

There was a significant interaction between photoperiod and time point for liver XOD activity ($F_{1,21} = 13.85$, $p = 0.001$) when accounting for a significant negative relationship with liver mass ($F_{1,21} = 10.73$, $p = 0.004$). After splitting by time point, LD birds showed significantly lower XOD activity than SD birds at the Late time point ($F_{1,11} = 35.28$, $p < 0.001$).
Figure 8. Protein metabolism enzyme activity at the Early (15 days post-stimulation; N = 6 each LD and SD) and Late (25 days post-stimulation; N = 7 each LD and SD) time points. LD birds are shown in orange and SD birds in blue. aminopeptidase = Aminopeptidase, ALT = Alanine Aminotransferase, GDH = Glutamate Dehydrogenase, XOD = Xanthine Oxidase. Significant differences between groups are indicated by asterisks (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).

3.4.4 Metabolic testing

Overnight resting metabolic rate was significantly higher with larger structural body size (F1,62 =10.84, p = 0.002), with a significant difference between the Winter SD birds and Late LD birds only (F4,62 =2.93, p = 0.028; Fig. 9). Water loss increased with lean mass loss (F1,102.6 =115.15, p < 0.001), fat mass loss (F1,119.2 =16.23, p < 0.001), and total O2 consumed (F1,124.9 =15.93, p < 0.001). Total water loss was significantly higher for LD than SD groups (F4,112.58 =4.41, p = 0.002). Across all metabolic tests, the strongest
predictor of water loss was lean mass loss ($F_{1,123} = 527.4$, Adj. $R^2 = 0.809$, $p < 0.001$).

However, the effect of the photoperiod treatment on water loss required further testing since the duration of darkness in the overnight metabolic testing differed between LD and SD birds, with LD birds experiencing ~ 4 h of light at the end of overnight testing. We selected metabolic data for the same time period between hours 3 and 6 of overnight testing, corresponding to approximately 23:00 and 02:00, to make comparisons between these two groups. As respiratory water loss typically increases with breathing frequency, we explored additional increases in this water loss using metabolic rate-corrected water loss ($\dot{V}H_2O/\dot{V}O_2$). With MR-corrected water loss as the independent variable, we found significantly greater MR-corrected water loss in LD birds ($F_{4,44.79} = 5.95$, $p < 0.001$). After repeating this for the daytime RMR testing (corresponding to approximately 08:30-11:30) we found significantly lower MR-corrected water loss in LD birds ($F_{4,44.15} = 3.12$, $p = 0.024$; Fig. 10).
Figure 9. Minimum metabolic rate at the winter (~15 days pre-stimulation; N = 13 each LD and SD), Early (~12 days post-stimulation; N = 13 each LD and SD), and Late (~20 days post-stimulation; N = 7 each LD and SD) time points. Photoperiod switch for LD birds is indicated by the gray line. LD birds are shown in orange and SD birds in blue. Groups with shared letters are not significantly different at p ≤ 0.05.

Figure 10. Metabolic rate-corrected water loss at the winter (~15 days pre-stimulation; N = 13 each LD and SD), Early (~12 days post-stimulation; N = 13 each LD and SD), and Late (~20 days post-stimulation; N = 7 each LD and SD) time points during the quiescent period (23:00-02:00) of overnight RMR followed by morning RMR (08:30-11:30). Photoperiod switch for LD birds is indicated by the gray line. LD birds are shown in orange and SD birds in blue. Groups with shared letters are not significantly different at p ≤ 0.05.
Fat and lean mass loss

For the rate of lean mass loss, the only significant fixed effect in the final model was the rate of water loss ($F_{1,125} = 121.47, p < 0.001$). Meanwhile, the rate of fat loss was correlated only with the duration of time in the test ($F_{1,125} = 8.91, p < 0.003$). These differences are primarily driven by the duration of the test (12-hour overnight RMR versus 4-hour daytime RMR), so we ran the same models separately for overnight and daytime RMR and found no significant effects in the daytime. However, the rate of fat loss overnight was positively related to the initial mass of the bird entering metabolic testing ($F_{1,30.52} = 5.83, p = 0.022$) and was higher in the Late time point for LD birds ($F_{4,51.49} = 6.80, p < 0.001$).

3.4.5 Gene transcription

In evaluating GAPDH expression as a housekeeping gene we found that it did show minor, but significant, photoperiod differences. GAPDH C<sub>T</sub> was significantly correlated with total body mass ($F_{1,22} = 8.73, p = 0.008$) and was higher in LD birds than SD ($F_{1,22} = 7.51, p = 0.012$; LD C<sub>T</sub> = 12.82 ± 0.69, SD C<sub>T</sub> = 12.21 ± 0.60). Continuing with GAPDH as the housekeeping gene, SERCA2 expression showed no significant differences across photoperiod, time point, or with body size and mass (Fig. 11). However, SLN expression increased ~30-fold in LD birds ($F_{1,21} = 20.1, p < 0.001$; Fig. 11), accounting for a significant positive relationship with total body mass ($F_{1,21} = 9.06, p = 0.007$). PGC1α expression was significantly higher in LD birds ($F_{1,21} = 9.66, p = 0.005$) and at the Late time point for both photoperiod groups ($F_{1,21} = 6.75, p = 0.017$; Fig. 11).
when accounting for a significant positive relationship with total body mass ($F_{1,21}=12.29$, $p = 0.002$).

**Figure 11.** Pectoralis gene expression at the Early (15 days post-stimulation; $N = 6$ each LD and SD) and Late (25 days post-stimulation; $N = 7$ each LD and SD) time points. Fold change is relative to the Early SD group. LD birds are shown in orange and SD birds in blue. Significant differences between groups are indicated by asterisks (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$).

### 3.5 DISCUSSION

To explore the adaptive potential of lean mass loss for birds in the migratory condition, we tested whether components of protein metabolism are under photoperiodic control similar to those seen in fatty acid metabolism. After demonstrating the expected photoperiodic changes in fat storage and metabolism, we found changes to key pathways of protein catabolism in muscle and liver tissue alongside higher rates of lean mass...
breakdown and water loss in birds in the migratory condition. Together, these data suggest that protein catabolism is altered during spring migration in response to photoperiod, complementing recent data showing that molecular pathways involved in tissue breakdown and remodeling increase in preparation for migratory challenges (Horton et al., 2019).

After the photoperiod switch, birds gained fat stores and showed higher pectoralis HOAD activity with moderate increases in CS activity, confirming that our photoperiod manipulation resulted in the migratory phenotype. McFarlan et al. (2009) showed that wild-caught White-throated Sparrows have higher muscle HOAD, CS, and CPT activities during migration and Zajac et al. (2011) found similar results in captive birds after three weeks on a long-day photoperiod. However, a similar study with White-crowned Sparrows found no significant differences in enzyme activities, despite exhibiting overnight Zugunruhe (Price et al., 2010). In captivity, the shift to a migratory condition is likely more gradual than that of wild birds (Zúñiga et al., 2016), so our muted CS response and non-significant change in CPT activity may be a result of sampling too soon after Zugunruhe began.

Interestingly, CS and CPT activities in the liver were both lower in LD birds. Studies have shown increased fatty acid synthase activity during pre-migratory hyperphagia and migration to generate the substantial fat stores required to sustain migratory flight (Egeler et al., 2000), but this lipogenesis must be balanced by reduced basal lipolysis, which was recently shown to be highest during tropical overwintering in Gray Catbirds (Corder et al., 2016). We speculate that reduced activity of CPT and CS in
the liver of migratory birds indicates fat sparing while fat stores are generated in
preparation for migration.

While LD birds rapidly gained fat mass after switching to a long day photoperiod, both LD and SD birds maintained similar total lean mass. However, when accounting for body size, LD birds had greater dry pectoralis and heart mass than SD birds, likely increasing power and aerobic output during the migratory season (DeMoranville et al., 2019; Trivedi et al., 2014). Interestingly, the LD birds also had reduced gut and gizzard mass. While increases in heart and flight muscles are beneficial for migratory flights, the gut is often catabolized before or early in long-distance flight (Battley et al., 2000; Piersma and Gill, 1998). As the gut has been implicated as a key driver of BMR (Barceló et al., 2016; Scott and Evans, 1992; Zheng et al., 2014), the reduced gut size in LD sparrows may help to save energy, particularly when balanced against energy requirements of active skeletal and heart muscles.

While total lean mass did not change with the photoperiod, we found that LD birds lost more lean mass and water over the ~18 hours of water-restricted metabolic testing. There are multiple hypotheses for lean mass loss during migratory flight and fasting (Bauchinger and Biebach, 1998), but with higher rates of protein loss correlating with water loss our results appear to support the protein-for-water hypothesis. However, without testing a 24-h cycle we cannot rule out an inversion of circadian activity levels and metabolism during migration. For example, in addition to altered molecular timekeeping in the liver (Trivedi et al., 2014), Black-headed Buntings appear to shift to fat rather than carbohydrate metabolism overnight during migration (Trivedi et al., 2015).
Horton et al. (2019) also found numerous genes that were responsive to the interaction between sampling time (night or day) and migratory condition in the heart and liver of White-throated Sparrows. Likewise, Landys et al. (2004) showed that early evening migratory restlessness was associated with a rise in corticosterone in White-crowned Sparrows, which may trigger some of the metabolic shifts we see in the migratory birds, including increased protein catabolism and water loss overnight. This suggests that we may be seeing shifts in the circadian organization of metabolic activity and fuel use during migration. Unlike in SD birds, metabolic rate-corrected water loss in LD birds was roughly the same between the dark and light hours. Therefore, the migratory disposition may dampen metabolic rhythmicity, leading to consistently higher baseline protein catabolism throughout the day and night resulting in higher rates of water loss.

Alternatively, greater lean mass loss overnight may be a result of selective protein catabolism in anticipation of migratory flight. During Zugunruhe, baseline levels of lean mass loss may occur in preparation for flight by reducing organ masses and selectively catabolizing sarcoplasmic or myofibrillar proteins of muscle to achieve the optimal balance of power to mechanical output prior to an expected migratory flight (Bauchinger and Biebach, 2001; Bordel and Haase, 2000; Piersma and Gill, 1998). Lean mass loss is particularly evident early in long-duration flights for American Robins (Gerson and Guglielmo, 2013) and Yellow-rumped Warblers in wind tunnel studies (Groom et al., 2019). Overnight lean mass loss may also reflect selective protein catabolism to generate a reservoir of free amino acids to repair anticipated damage during migratory flight. In LD birds, higher liver GDH activity alongside reduced XOD activity—catabolizing the
final step in uric acid synthesis—could favor GDH activity toward alanine rather than excreting amino acid waste products as ammonia (Gibala, 2001).

Unfortunately, we also cannot rule out that the nightly activity as birds increased their Zugunruhe, including repeated hopping and wing fluttering, could have influenced protein catabolism due to an exercise effect rather than a migratory preparation effect alone. While this activity allowed us to monitor the migratory state of the birds, keeping the birds in complete darkness at night may have offered the opportunity to prevent this confounding effect (Ramenofsky et al., 2008). However, this overnight activity is likely not representative of the endurance exercise associated with migration, and during metabolic testing the birds were maintained in complete darkness and showed no evidence of activity in the chambers through $\dot{V}CO_2$ and $\dot{V}O_2$ traces. Furthermore, by monitoring activity in cages overnight we know that Zugunruhe was only prevalent in the LD birds by the Late time point, with only five birds showing any signs of restlessness at the Early time point, mostly within the previous three nights, and only one of these birds was sampled for tissues and enzyme activity at the Early time point. For this reason, the Early time point captures changes in response to the photoperiod without a substantial influence of overnight activity.

One of our most surprising findings was the substantial increase in SLN transcripts in the pectoralis after photostimulation. SLN is a known uncoupler of SERCA Ca$^{2+}$-pumping efficiency in skeletal muscle, which produces heat in non-shivering thermogenesis (NST) and has been proposed as a key determinant of basal metabolic rate in mice (Maurya et al., 2015; Periasamy et al., 2017). We expected that SLN would be
reduced in the migratory condition to enhance SERCA efficiency and reduce wasteful ATP consumption. If SLN expression leads to SERCA uncoupling in spring migratory birds, this may explain variations in summit metabolic rate that are not directly related to differences in changes in organ and tissue masses (Barceló et al., 2016). Additionally, recent work in cold-acclimated Dark-eyed Juncos suggests that SLN may instead be a hindrance to avian thermoregulation, counter to expectations of SLN-induced NST demonstrated in mammalian studies (Stager and Cheviron, 2020). If this is the case, lower SLN may instead lead to improved thermogenic capacity for White-throated Sparrows overwintering in relatively cold climates.

SLN uncoupling may also act as a signaling mechanism for a suite of changes in muscle physiology during spring migration. SLN-mediated increases in cytosolic Ca\(^{2+}\) may lead to PGC1\(\alpha\) expression, mediating a physiological switch to increased mitochondrial biogenesis and fatty acid catabolism, and greater fatigue resistance in skeletal muscle (Fajardo et al., 2017; Maurya et al., 2018; Sopariwala et al., 2015; Summerrmatter et al., 2011). We found \(~2\)-fold higher PGC1\(\alpha\) mRNA in the pectoralis of LD birds, providing some support for this hypothesis. However, we acknowledge that our GAPDH housekeeping gene showed a minor, but significant, photoperiod difference—\(\Delta C_T\) was approximately 0.6 cycles higher in LD birds—that could account for the higher PGC1\(\alpha\) expression, but the effect of this difference on the \(\Delta C_T\) for SLN is negligible given the large increases in SLN expression. Despite these caveats, we hypothesize that SLN-mediated calcium signaling in the muscle may facilitate the shift towards fat metabolism and mitochondrial biogenesis in the flight muscle of migratory birds.
CHAPTER 4

WHITE-THROATED SPARROW (ZONOTRICHIA ALBICOLLIS) LIVER AND PECTORALIS FLIGHT MUSCLE TRANSCRIPTOMIC CHANGES IN PREPARATION FOR MIGRATION

4.1 ABSTRACT

Migratory songbirds undertake challenging journeys to reach their breeding grounds each spring. They accomplish these non-stop flapping feats of endurance through a suite of physiological changes, including the development of substantial fat stores and flight muscle hypertrophy and an increased capacity for fat catabolism. Additionally, migratory birds may show large reductions in organ masses during flight, including the flight muscle and liver, which they must rapidly rebuild during their migratory stopover prior to replenishing their fat stores. However, the molecular basis of this capacity for rapid tissue remodeling and energetic output has not been thoroughly investigated. We performed RNA-seq analysis of the liver and pectoralis flight muscle of captive White-throated Sparrows in the migratory and non-migratory condition to explore the mechanisms of seasonal change to metabolism and tissue mass regulation that may facilitate these migratory journeys. We identified transcriptional alterations to glucose and fatty acid metabolism as well as the production and metabolism of ketone bodies. Based on transcriptional changes, we propose that tissue-specific adjustments in preparation for migration may alleviate the damaging effects of long-duration activity, including a potential increase to the inflammatory response in the muscle. Furthermore, based on transcriptional changes, we hypothesize that seasonal hypertrophy balances satellite cell recruitment and apoptosis, while little evidence appeared in the transcriptome to support MSTN, IGF1, and mTOR-mediated pathways for muscle growth. These findings can encourage more targeted molecular studies on
the unique integration of pathways that we find in the development of the migratory endurance phenotype in songbirds.
4.2 INTRODUCTION

Migration allows animals to seasonally exploit favorable habitats that are geographically disparate, and migratory animals have a suite of adaptations that allow them to cope with seasonal changes in habitat quality and resource availability by completing extraordinary journeys between habitats. The physiological demands of migration are enabled by a “migratory syndrome” of traits that are highly coordinated, seasonally-responsive adaptations that together result in changes to metabolism, body composition, and reproductive status, all of which act to increase the probability of a successful migration (Piersma et al., 2005). One well-documented physiological example of the migratory syndrome is the seasonal upregulation of fatty acid transport and metabolism to fuel migratory flight, which is metabolically expensive (Jenni-Eiermann, 2017; McFarlan et al., 2009). Fatty acids are the best fuel for flight due to the high energy density compared to protein or glycogen (Guglielmo, 2018; Jenni-Eiermann, 2017). Photostimulation of captive migrants with a long-day spring photoperiod leads migratory songbirds to develop migratory restlessness, or Zugunruhe, characterized by hopping activity and beak-up flapping overnight (Agatsuma and Ramenofsky, 2006; Ramenofsky et al., 2003). Additionally, these birds show higher activity of the β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase (HOAD) and the rate-limiting enzyme carnitine palmitoyl transferase (CPT) in the pectoralis flight muscle, even in untrained conditions (McFarlan et al., 2009; Zajac et al., 2011). Although fatty acids are the primary fuel source for long-distance flight, protein breakdown from flight muscles and organs during fasting and flight has been well documented (Battley et al., 2000; Gerson et al., 2020; Groom et al., 2019; Swain, 1992), and recent evidence from White-throated Sparrows (Zonotrichia albicollis) indicates that the capacity for protein catabolism is part of the seasonally-responsive migratory syndrome (Elowe and Gerson, 2022).

Numerous studies have shown changes in the gene expression of tissues in birds in response to photoperiod or temperature changes (Sharma and Kumar, 2019; Singh et al., 2015;
Stager et al., 2015). Frequently, these studies focus on brain tissues to determine the control of migratory behavior in songbirds (Boss et al., 2016; Frias-Soler et al., 2020; Sharma et al., 2018). However, detailed studies of tissues involved in the metabolism of migratory songbirds, such as the liver and flight muscle, have the potential to reveal novel pathways that enable birds to accomplish remarkable feats of endurance. The liver plays an important role in metabolic activity, regulating glycogenesis, gluconeogenesis, lipoprotein synthesis, and controls the availability of fatty acids for use by other tissues (Guglielmo, 2010). However, we have recently shown that White-throated Sparrows in migratory condition have higher activity of aminopeptidase and glutamate dehydrogenase, with lower xanthine oxidase activity, indicating a seasonal change in the handling of protein catabolism in the liver in addition to fat metabolism (Elowe and Gerson, 2022).

Migratory birds consume and rebuild a substantial portion of their muscle and organs with each flight (Battley et al., 2000), and we found greater aminopeptidase activity in migratory sparrow flight muscle (Elowe and Gerson, 2022). During migratory flight, the pectoralis flight muscles demand enormous amounts of fuel and may undergo substantial damage, and these muscles show remarkable plasticity. In addition to the potential for higher rates of protein consumption, migratory birds have recently shown extraordinary rates of cultured muscle satellite cell proliferation (Young et al., 2021). However, the regulatory basis of rapid lean mass remodeling in migratory birds is still largely unknown. Mature muscle cells are large and multinucleated, and during hypertrophy and atrophy there is typically a fluctuation in these nuclei as the cytoplasmic area is reduced (Jimenez, 2020; Swanson et al., 2022). Muscle flexibility may be determined primarily by satellite cell activation (Hoppeler, 2016; Swanson et al., 2022). These stem cells are quiescent unless stimulated by hormonal signals or injury, after which they proliferate and fuse with muscle fibers. One of the established pathways of muscle growth—via the mTOR signaling pathway and upstream regulators myostatin (MSTN) and IGF1—has shown
conflicting results in photo-stimulated White-throated Sparrows and other migratory birds (King et al., 2015; Price et al., 2011) while supporting the larger muscle sizes found in overwintering passerines in cold environments (Swanson et al., 2009; Swanson et al., 2014; Zhang et al.). When comparing migratory and sedentary subspecies of Dark-eyed Juncos (Junco hyemalis) in the spring, lipid transport and fatty acid catabolism genes were expressed more in the flight muscle of migratory birds, but the authors also noted that small muscle protein was also highly expressed in the migratory juncos, potentially contributing to rapid muscle growth in the migratory season (Fudickar et al., 2016). In a recent study investigating changes in gene transcription in the heart and liver of migratory White-throated Sparrows, Horton et al. (2019) found increased transcripts associated with immune function, including wound healing pathways, in the migratory condition, which may sustain the rapid tissue remodeling seen during migratory stopovers. However, the mechanisms underlying flexible tissue remodeling through either growth or degradation pathways have not been thoroughly explored (Swanson et al., 2022).

While most studies examine a broad array of genes and classify differential expression by larger functional groups including all genes measured, we sought to explore physiologically-relevant changes to gene transcription in two tissues with key roles in successfully completing migratory journeys. Specifically, our goal was to explore seasonal changes in protein metabolism, tissue remodeling and flexibility, and regulatory genes within the muscle and liver that support adaptive lean mass flexibility. We sequenced the transcriptome from flight muscle and liver tissue of captive White-throated Sparrows in migratory disposition (exposed to a long-day photoperiod 16h light: 8h dark) and non-migratory disposition (exposed a short-day 8 h L: 16 h D). We hypothesized that seasonal shifts in protein catabolism and lean mass dynamics may in fact be part of the migratory syndrome, and that photoperiod stimulates unexplored regulatory pathways for flexible remodeling of flight muscle and capacity for protein catabolism.
4.3 METHODS

4.3.1 Animal collection

Details of the animal collection and sampling were previously outlined in Elowe & Gerson (2022). Briefly, we captured White-throated Sparrows (13 male, 13 female) by mist-netting during fall migration between 29 September and 11 October 2017 on the University of Massachusetts Amherst campus (42°23'45.9"N 72°31'04.5"W) and color-banded prior to transport to captive facilities at the University of Massachusetts Amherst. Birds were housed at 21°C and the photoperiod was gradually reduced a short-day “winter” photoperiod (8L:16D), which we maintained for ~70 days to break photorefractoriness (Barceló et al., 2016). A dim light at night provided ~1 lux in the room (Ramenofsky et al., 2003). Our study followed the Institutional Animal Care and Use Committee guidelines approved by the University of Massachusetts Amherst (protocol #2015-0019). A collection permit was granted by the US Fish and Wildlife Service (permit #MB65968B-0) and the State of Massachusetts (#097.18SCB) to ARG.

4.3.2 Photoperiod switch and tissue collection

We randomly divided sparrows into two adjacent rooms where they were singly housed (38.5 x 30.5 x 39 cm) and allowed to acclimate for 4 days. On 5 March, one room was switched to a long day “spring” photoperiod (16L:8D; N = 13; LD photoperiod treatment, hereafter) to induce a migratory disposition (Gwinner, 1990; Ramenofsky et al., 2003) while the other room remained on the short-day “winter” light regime (N = 13; SD photoperiod treatment, hereafter). Infrared cameras (Ailipu Technology Co., Ltd., Guangdong, China) were used to record nighttime activity, and all LD birds displayed
migratory restlessness by day 23, while SD sparrows exhibited little to no movement overnight. For RNA-seq, we sampled only a subset of the birds in the experiment (N = 5 for SD and N = 5 LD birds) collected 25 days after the photoperiod switch. Birds were sacrificed by isoflurane overdose and cervical dislocation in the morning to early afternoon in compliance with all animal care guidelines and samples of pectoralis and liver tissue were frozen on dry ice within 5 minutes of sacrifice.

4.3.3 RNA extraction and library preparation

Approximately 50 mg of pectoralis muscle and liver tissue were homogenized using beadmill homogenization (NextAdvance, Troy, NY; speed 8, time 3 for liver 4 for pectoralis, 4°C) in 1 mL of TriZol (Invitrogen, Carlsbad, CA) and RNA was separated into an aqueous phase using a chloroform ethanol procedure. Total RNA was purified using a PureLink RNA kit (Invitrogen) and DNase treated using TURBO I DNase (Invitrogen). Samples were quantified using Qubit (Invitrogen) and RNA quality (RIN > 8.2) was confirmed using an Agilent 2100 Bioanalyzer. Libraries were prepared using an NEB Ultra II Directional kit (New England Biolabs, Ipswich, MA) with poly(A) mRNA magnetic isolation and dual-index primers according to the manufacturer’s protocol. We confirmed library quality and fragment sizes using the Bioanalyzer 7500 kit for dsDNA (Agilent, Santa Clara, CA) prior to pooling 96 samples (including additional samples from a separate study) to a final library concentration of 50 µM. We sequenced the pooled libraries using the NovaSeq 6000 platform (Illumina, San Diego, CA) multiplexed on a single S4 flow cell for paired-end 150 bp sequencing (GENEWIZ, South Plainfield, NJ).
4.3.4 Transcript processing and mapping

Raw reads were trimmed to remove low quality bases and adapters using paired-end Trimmomatic v.0.32 (Bolger et al., 2014). We used a 4 bp sliding window to remove 5’ and 3’ bases when average phred quality dropped below 20 and maintaining a minimum read length of 80 bp. We confirmed trimmed sequence quality by comparing FastQC v. 0.11.5 (Babraham Bioinformatics, Babraham, England) reports of pre- and post-processed reads. We used the Bowtie 2 read alignment program (Langmead and Salzberg, 2012) to align sequences to the *Zonotrichia albicollis* genome (GCF_000385455.1_Zonotrichia_albicollis-1.0.1). Due to a high number of PCR duplicates in one sample from the sequencing run, we ran the `markdup` program in samtools v. 1.9 (Li et al., 2009) to mark and remove duplicate reads from each sample.

4.3.5 Differential expression and enrichment analysis

We used HTseq v. 0.10.0 (Putri et al., 2022) to generate read counts for each sample. These read counts were subsequently used for differential expression analysis between the SD and LD groups in both the liver and pectoralis muscle using DESeq2 (Love et al., 2014) or the non-parametric NOISeq program (Tarazona et al., 2015). Outlier samples were detected and removed from differential expression analysis if they were abnormally separated in a PCA due to excessively low or high read counts relative to the other samples even after pre-analysis filtering. Using DESeq2 we applied independent filtering and used a Benjamini-Hochberg multiple testing cutoff of q < 0.1 to detect significantly up- or down-regulated genes. NOISeq was used for liver samples, beginning with low count filtering to eliminate genes with average CPM < 9 and a CV cutoff of 40 to
eliminate genes with excessive variability within conditions. Remaining counts were normalized by TMM. Differential expression was detected using NOISeqBIO on the normalized filtered data with the number of $k$ clusters set to 15, within-cluster random sampling set to 50, kernel smoothing for distributions set to 1.5, the default sampling error cutoff of 0.9, and $k=.5$ added to zero counts to avoid undefined logarithms. With NOISeq, we considered features significantly differentially expressed with a probability cutoff (prob) $> 0.9$, signifying a false-discovery rate cutoff comparable to Benjamini-Hochberg $q < 0.1$ (Tarazona et al., 2015). We used KOBAS-i (Bu et al., 2021) with a Benjamini-Hochberg multiple testing cutoff of 0.1 to detect enrichment within the Gallus gallus database for KEGG (Kanehisa and Goto, 2000), PANTHER (Thomas et al., 2003), or gene ontology (GO) term (Ashburner et al., 2000) membership in the list of significantly up- or down-regulated genes in the liver and pectoralis.

4.4 RESULTS

4.4.1 Pectoralis differential expression analysis

For the pectoralis, we used DESeq2 (Love et al., 2014) to perform differential expression analysis on all but two samples (bird 2 from SD and bird 6 from the LD group) that clustered separately on a PCA and showed lower read counts than the other eight samples. Excluding these two samples improved the cluster between the two treatment groups (Fig. 12A). Out of 12,279 genes with a nonzero total read count, we found 239 (1.9%) that were upregulated in the LD condition and 69 (0.56%) that were downregulated, with 9,548 (78%) features flagged as low counts (Fig. 13A and B). With relatively few differentially expressed genes, our interpretation also included genes and
pathways for which we had an *a priori* hypothesis with an unadjusted cutoff of $p < 0.05$. Using KOBAS-i to detect significantly enriched pathways or terms within the differentially expressed genes, we found that upregulated genes were enriched for the actin cytoskeleton (GO:0015629, $q = 0.057$), protein-containing complex binding (GO:0044877, $q = 0.057$). The top three enriched categories for down-regulated genes included glycolysis/gluconeogenesis (KEGG gga00010, $q < 0.001$), calcium signaling (KEGG gga04020, $q = 0.003$), and metabolic pathways (KEGG gga01100, $q = 0.005$) (Table 3).

![Figure 12](image)

**Figure 12.** Principal components analysis of all normalized non-zero read counts for (A) pectoralis genes used in DESeq2 differential expression analysis, (B) liver genes used for DESeq2 analysis, and (C) liver genes used for NOISeq analysis after outlier samples were excluded. Short day (SD) photoperiod treatment birds are shown in blue and long day (LD) birds are shown in orange.

Within the muscle, the top 100 most abundant transcripts made up approximately 50% of the average total mRNA in the samples. Among these genes, 25 were significantly differentially expressed between the SD and LD photoperiod treatments, with all of them upregulated in the LD group. These genes include cytoskeletal and structural proteins (EML6, NEXN, with a trend in MYH7), muscle growth (MYORG), signaling (NMRAL1, PXK, NIM1K, and a trend in IL18), golgi and vesicle transport (KDELR3, ANKRD27), and metabolism (COQ10B, and a trend in ACSBG1).
Interestingly, the most highly expressed gene in the dataset was a member of the endogenous retrovirus group K proteins (ERVK-6).

**Figure 13.** (A) Volcano plot showing significantly up- and down-regulated genes in the pectoralis flight muscle of photostimulated White-throated Sparrows as identified by DESeq2 and (B) heatmap showing significantly up- and down-regulated genes identified by NOISeq. Yellow indicates higher expression in the LD treatment group and blue indicates lower expression in the LD birds. Genes were identified as significantly differentially expressed when the FDR $\leq 0.1$.

4.4.2 Liver differential expression analysis

We removed one liver sample (bird 7) from the LD group that showed unusually high read counts and clustered separately along PC1 of a principal components analysis. DESeq2 was not the most effective at picking out differentially expressed genes for this tissue. The liver data showed a large amount of variation among non-zero read counts, with overlap in clustering between the treatment groups across PC1 and PC2 (Fig. 12B). Out of 10,858 genes with a nonzero read count we found 8 (0.074%) that were
upregulated in the LD group and 10 (0.92%) that were downregulated, with 7,954 (73%) flagged as low counts (Fig. 14A).

Figure 14. (A) Volcano plot showing significantly up- and down-regulated genes in the liver of photostimulated White-throated Sparrows as identified by DESeq2. (B) Volcano plot of significant genes identified by NOISeq (FDR = 1 – probability of differential expression) and (C) heatmap showing significantly up- and down-regulated genes identified by NOISeq. Yellow indicates higher expression in the LD treatment group and blue indicates lower expression in the LD birds. Genes were identified as significantly differentially expressed when the FDR ≤ 0.1.

In light of the variability in expression in the liver tissue, we decided to apply the non-parametric NOISeq method (Tarazona et al., 2015) to pick differentially expressed genes, beginning with low count filtering to eliminate genes with average CPM < 9 and a CV cutoff of 40 to eliminate genes with excessive variability within conditions. This retained 1632 features that were normalized using TMM for DE analysis, and these features clustered along PC2 of a principal components analysis (Fig. 12C). NOISeqBIO
detected 179 (11%) downregulated genes in the LD condition and 108 (6.6%) upregulated genes (Fig. 14B and C). As with the pectoralis, we allowed a more flexible significance cutoff for genes of interest with an *a priori* hypothesis from other publications. Within the list of differentially expressed genes, upregulated features were significantly enriched for the centriole (GO:0005814, q = 0.095) and the hormone biosynthetic process (q = 0.095). The top three categories for downregulated genes included metabolic pathways (KEGG gga001100, q = 0.009), endoplasmic reticulum (GO:0005783, q = 0.009), and iron binding (GO:0005506, q = 0.012) (Table 3).

In the liver, approximately half of the mRNA in the dataset was made up of 25 genes. Within these 25 genes, 21 were differentially expressed between the photoperiod treatments. However, unlike in the pectoralis, only 6 of these genes were upregulated in the LD group. These upregulated genes included free fatty acid receptor 4, NIM1 serine/threonine protein kinase, interleukin 16, and an AMPK regulatory subunit. Additionally, as in the pectoralis, one of the most highly expressed genes in the liver, making up over 4% of the total mRNA, was a member ERVK-6, and this gene was upregulated in the LD birds. The most highly expressed gene in the liver was SEC31 homolog B, COPII coat complex component (SEC31B), a component of golgi vesicle transport, which made up nearly 10% of the transcript abundance on average.

**Table 3.** Top ten KOBAS-i significantly enriched gene categories within the up- and down-regulated genes in the liver and pectoralis.
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</table>
4.5 DISCUSSION

We examined the liver and pectoralis flight muscle transcriptomes of captive White-throated Sparrows maintained under a short day winter photoperiod or stimulated with a long day photoperiod to develop and express the migratory condition. This revealed roughly 300 differentially expressed genes (DEGs) in the liver and pectoralis, and based on these changes we explored the tissue-specific changes to metabolism and inflammation pathways, along with patterns associated with the control of muscle mass. While this is a powerful method for hypothesis development regarding metabolic shifts, signaling pathways, and the mechanisms of tissue plasticity as birds prepare to migrate, there are multiple regulatory steps between the measurement of mRNA and the phenotypic expression of a functional protein, including post-transcriptional regulation (Mata et al., 2005) and post-translational protein modification (Hurley et al., 2018; Ryšlavá et al., 2013) and degradation (Boisvert et al., 2012; Lück et al., 2014). Therefore, while differences in transcript abundance can reveal patterns of regulation and inform new avenues of investigation, we caution against drawing definitive conclusions from RNAseq data (Evans, 2015; Feder and Walser, 2005).

4.5.1 Variation in transcription over time

We were surprised to find relatively few DEGs in the liver between the SD and LD conditions using DESeq2, and recognized that this was due in large part to substantial variation in expression within treatment groups. This may relate to the progression of migratory preparation. Frias-Soler (2022) recently showed that liver gene expression varies greatly between the lean, fattening, fat, and defatting stages of migration in
Northern Wheatears (*Oenthae oenthae*). Signs of *Zugunruhe* appeared initially in some of our White-throated Sparrows beginning on day 12 and only appeared in all birds by day 23, indicating that there is a high degree of variability in the onset of migratory preparation that could lead also to substantial variation in the gene transcription.

Previous studies have noted a change to circadian signaling during the migratory period, including gene transcription of core circadian oscillators (Sharma et al., 2021; Trivedi et al., 2014). This has been demonstrated in both the hypothalamus (Sharma et al., 2018; Sharma et al., 2021) and the liver (Frias-Soler et al., 2022; Horton et al., 2019; Sharma and Kumar, 2019), with many predominant metabolic processes biased toward the evening, in some cases showing a reversal from the non-migratory condition. Given that we sampled tissues between morning and midday in our sparrows, this may have contributed to the overall variability and lack of differences between our SD and LD groups for many metabolic genes. However, while we found no differences in circadian-related genes in the liver, we did find lower expression of cryptochromes (CRY1/2), period genes (PER2/3), and the melatonin synthesis gene ASMTL in the flight muscle (Fig. 15). This suggests dampened or, more likely, inverted circadian rhythms in migratory birds that may alter expression of numerous metabolic and regulatory genes and ensure that the correct fuel is catabolized at the correct time. Therefore, we may expect metabolic genes to show higher expression in the evening in the migratory period when nocturnal migrants undertake their migratory flights. Furthermore, given that skeletal muscle myogenesis has shown circadian rhythmicity, particularly through the important muscle growth transcription activator myogenic differentiation 1 (MYOD1)
(Harfmann et al., 2015), by sampling only during the daylight hours we may be missing seasonal or diurnal rhythms in regulatory processes.

Circadian shifts may also explain the tissue-level differences in DEGs. Migratory and non-migratory phenotypes are superficially similar during the day, particularly in terms of activity levels and foraging (Ramenofsky et al., 2017). Therefore, the liver, as primarily a metabolic organ, may not show such dramatic differences between the two life history stages during time points with similar activities, leading to relatively fewer DEGs in our data. Meanwhile, the muscle may shift baseline fuel use in preparation for higher activity levels and a higher constitutive response to potential damage during high-intensity exercise, even during the day. While we cannot rule out that the upregulation of certain genes involved in the control of muscle mass—such as MSTN or Atrogin/MuRF and MYOD—may be more highly expressed during the overnight period of increased exercise, they do not appear to be higher at all times in the migratory condition.

**Figure 15.** Normalized counts of transcripts involved in circadian rhythms in the pectoralis between short day (SD) and long day (LD) photoperiod treatments. Significant differentially expressed genes are shown by Benjamini-Hochberg q-value ≤ 0.1 and trends are indicated by unadjusted p-values.
4.5.2 Carbohydrate metabolism

Despite the variability in expression within treatment groups, we found evidence that the liver underwent a shift from the expected anabolic condition seen in many pre-migratory birds to a lower-energy state with downregulation across many processes. This may be related to body condition, as the later stage of migratory preparation is characterized by high fat stores and migratory restlessness (Eikenaar et al., 2016; Frias-Soler et al., 2022). Additionally, as gene expression is highly variable over relatively short time spans (Hodgins-Davis and Townsend, 2009), early and late stages of preparation can have drastically different transcriptomic profiles (Franchini et al., 2017). Therefore, short-distance migrants in a late stage of migratory condition may shift from an overall anabolic to catabolic state, with fuel use primarily directed toward the flight muscle. Reduced energy consumption in the liver was also evident through lower enzyme activity for citrate synthase and carnitine palmitoyl transferase in these sparrows in the LD condition (Elowe and Gerson, 2022). We found higher X-box binding protein 1 transcript abundance, involved in reducing gluconeogenesis, alongside lower expression of Wnt signaling genes that would activate forkhead box O1 (FOXO1) and stimulate expression of gluconeogenic genes (Fig. 16) (Rui, 2014). We identified higher transcription of cytosolic phosphoenolpyruvate carboxykinase 1 (PCK1)—a regulator of gluconeogenesis/lipogenesis, glyceroneogenesis, and TCA cycle anaplerosis—that also matches the transcriptomic changes from the ‘defatting’ time point in the liver of migratory Northern Wheatears (Friass-Soler et al., 2022). However, downregulated cytosolic malate dehydrogenase 1 would reduce shuttling of glycolytic electrons to the mitochondria and would generate less oxaloacetate for PCK1 to generate glucose,
glycerol and triglycerides, or acetyl-CoA for the TCA cycle (Yang et al., 2009). Given that PCK1 activity is strongly related to its transcription and oxaloacetate concentrations, with limited allosteric regulation (Yang et al., 2009), the reduced MDH1 transcription that we found in the liver indicates that there is both a shift away from using glucose as a fuel and an overall lower flux through PCK1 for anabolic purposes (Fig. 16).

In the pectoralis, many of the prominent changes in metabolic pathways aligned with our expectations of the metabolic state of flight muscle in the migratory condition. We saw lower lactate dehydrogenase (LDHA; Fig. 16), which is consistent with lower LDH activity in the migratory condition (Banerjee and Chaturvedi, 2016; McFarlan et al., 2009). Similarly, a reduction in two genes associated with glycogenolysis (phosphorylase kinase and the glycogen debranching enzyme AGL) are consistent with a shift away from glycogen catabolism in the muscle of migratory birds (Blem, 1976). We found an overall reduction in glycolytic gene expression, including the pyruvate dehydrogenase (PDH) complex, glycerol-3-phosphate dehydrogenase, and bisphosphoglycerate 3-phosphate, bisphosphoglycerate mutase. Interestingly, we found lower expression of pyruvate dehydrogenase kinase 1 (PDK1), which reduces PDH activity and allows a higher entry of pyruvate into the tricarboxylic acid cycle. While lower PDK1 could result in higher PDH activity, it also appears to be linked to a hypoxic response (Peng et al., 2018), and lower PDK1 expression through a dampened hypoxia response is linked to increased endurance in the skeletal muscle of athletes (Lindholm et al., 2014).

We also found increased expression of the platelet form of phosphofructokinase (Fig. 16), consistent with higher activity in the muscle of migratory birds seen previously (Banerjee and Chaturvedi, 2016). This may be linked to fatty acid metabolism by a
reduction in glycerol-3 phosphate dehydrogenase (GPD1), which shuttles glycolytic NADH to the mitochondrial electron transport system. While phosphofructokinase would increase the production of dihydroxyacetone phosphate, reduced GPD1 may convert less of this to G3P. Despite a trend toward higher glycerol kinase expression, which would produce G3P and favor triglyceride formation, we found lower expression of phospholipid phosphatase and a non-significant trend toward lower diacylglycerol O-acyltransferase 2, suggesting that the pathway shifts toward G3P rather than triglyceride formation, while higher expression of phosphofructokinase as a key regulator of glycolysis may prevent this accumulation leading to gluconeogenesis. Given that triglycerides are stored and transported to provide fatty acids to fuel flight muscle, this may indicate that G3P is a prominent endpoint for its catabolism in the muscle, and as it accumulates it exits the muscle through upregulated aquaporin 9 (Jelen et al., 2012) and is transported to the liver or adipocytes for triglyceride synthesis. In long-distance migratory birds, increased circulating glycerol has been documented following migratory flight (Gerson and Guglielmo, 2013; Landys et al., 2005).
Figure 16. Fold change of metabolic genes in the liver (dark red) or pectoralis (bright red) associated with carbohydrate, fat, and ketone metabolism relative to the short day (SD) photoperiod treatment. Significant differentially expressed genes identified through DESeq2 are shown by Benjamini-Hochberg q-value ≤ 0.1 and trends are indicated by unadjusted p-values while genes from the liver using NOISeq are significant with Prob ≥ 0.9.

4.5.3 Fatty acid metabolism

Fatty acid metabolism in migratory birds is intricately connected to liver function. During fattening, liver lipid droplets may be tightly regulated, with fatty acids being used...
to synthesize membrane lipids, β-oxidation, or exported through the ER for secretion in VLDLs (Natarajan et al., 2017). We found few changes in fatty acid synthesis gene expression, and even some reduced lipogenic transcripts, including fatty acid desaturase (Fig. 16). This agrees with the findings in Zajac et al. (2011), in which captive White-throated Sparrows photostimulated into the migratory condition also showed lower fatty acid synthase activity in the liver than the birds in non-migratory condition. Similarly, Sharma et al. (2018) showed lower lipogenic gene expression in the liver of migratory Black-headed Buntings. While they speculated that this may be a result of *ad libitum* food availability in captivity and increased fat production for winter birds under a shorter photoperiod with relatively opportunity to feed in the light, it is possible that it is also a result of the migratory fattening stage, as in Frias-Soler et al. (2022). Rather than *de novo* synthesis of fatty acids, the liver may demonstrate a high capacity for fatty acid uptake—indicated by higher expression of long-chain fatty acid transport, free fatty acid receptor, and a trend toward higher lipoprotein lipase—after which fatty acids are repackaged for export from the liver or converted to ketone bodies for extrahepatic metabolism (Jenni-Eiermann and Jenni, 1992). Furthermore, we found two acyltransferases (LPGAT1 and AGPAT) with a trend toward lower expression in LD birds. These enzymes are involved in the formation of triacylglycerides (TAGs), and it could support the reduction in *de novo* fatty acid and TAG synthesis in the liver during this pre-migratory period. Alternatively, these genes may be expressed at higher levels overnight to coincide with nocturnal activity in the initial hours of migratory flight.

Despite a trend toward higher expression of peroxisome proliferator-activated receptor α (PPARα; Fig. 16), a master regulator of fatty acid catabolism in migratory
birds (Corder et al., 2016; DeMoranville et al., 2019), we found relatively few differences in expression of fatty acid β-oxidation genes in the liver. Instead, we found evidence that ER and golgi transcripts were differentially expressed in the migratory condition, along with apolipoprotein H, indicating that fat export from the liver is altered in their current state. While high lipid storage in the liver of migratory Black-headed Buntings correlated with reduced gene expression related to golgi and vesicle transport (Sharma et al., 2018), the birds in our study may have developed to a later pre-migratory stage where liver lipogenesis and energy consumption are reduced in favor of processing extrahepatic fatty acids, a reduction which was also supported through enzyme activity assays (Elowe and Gerson, 2022).

The liver of LD birds also showed upregulation of ERVK-6, an endogenous retrovirus (ERV) gene that made up an average of roughly 4% of all mRNA in the White-throated Sparrow liver samples (Fig. S1). Certain ERVs have been shown to be responsive to feeding in the liver of birds, with increased expression in the liver of overfed geese developing fatty livers while chickens increased expression during fasting, alongside immune genes (Liu et al., 2021). This suggests that ERVs may be highly responsive to nutritional status, though their role in migratory birds is unclear; in our study, the upregulation of ERVK-6 could be indicative of the hyperphagic stage of migratory fattening or, as proposed, a ‘defatting’ stage of reduced energy use in the liver (Frias-Soler et al., 2022).

In the flight muscle, genes involved in fatty acid catabolism were surprisingly limited among the DEGs. Along with the reductions in the triglyceride synthesis pathway, fatty acid elongation was reduced, as evident in lower fatty acid elongase 5
expression and a trend toward lower acyl-CoA synthetase 3 (Fig. 16). Meanwhile, we found a trend toward higher expression of the fat metabolism regulator PPARα and its cofactor NFE2L2 alongside increased expression of fat transport proteins, including long-chain fatty acid transport protein 6, a trend toward higher expression of acylCoA synthetase bubblegum family member 1, and altered peroxisomal fat metabolism with reduced expression of ABCD2 for fatty acid import and higher expression of exit transport gene carnitine O-octanoyltransferase (Fig. 16). We also found that glycine-N-acyltransferase was significantly higher in the muscle of LD birds. This has been proposed as pool for bound coenzyme A (CoASH) to buffer its concentrations for release as needed for metabolic processes (Badenhorst, 2013). Furthermore, we found higher expression of acetyl-CoA acetyltransferase 2 and acyl-CoA thioesterase 12, which may regulate the levels of acyl-CoA in cells following rapid influx of fatty acids (Fujita et al., 2011) (Fig. 16). A high capacity for balancing levels of coenzyme A (CoA) and fatty acids may be important for rapidly shifting energetic needs between rest and endurance flight in migratory birds.

4.5.4 Ketone bodies

Another source of fuel for extrahepatic tissues in migratory birds is ketone bodies (Frias-Soler et al., 2021; Fudickar et al., 2016; Jenni-Eiermann and Jenni, 2001). In the liver of our birds, we found higher expression of SLC16A7 that transports ketone bodies, higher 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), with a trend toward lower acetoacetyl-CoA synthetase and HMGCS2 (Fig. 16). While postexercise ketosis has been recognized in birds after flight (Jenni-Eiermann and Jenni, 2001) and circulating
ketones may correlate with fasting in migratory birds (Jenni-Eiermann et al., 2002b), Frias-Soler et al. (2021) only recently indicated that ketones may play a dynamic role in migratory fattening and the shift to a reliance on lipids as a fuel. The expression patterns we found in the production of ketone bodies support our hypothesis that our birds were in a ‘defatting’ stage as in Frias-Soler et al. (2021). Furthermore, in the flight muscle, we also found higher expression of 3-hydroxybutyrate dehydrogenase 1, along with a trend toward lower acyl-CoA acyltransferase 2 and higher acetyl-CoA acetyltransferase 2, consistent with the pattern of ketone body metabolism in Frias-Soler et al. (2021) (Fig. 16). This suggests that even without the influence of exercise as indicated in Jenni-Eiermann and Jenni (2001), ketone bodies produced in the liver may provide important fuel for muscles at rest in the defatting stage (Beis et al., 1980).

4.5.5 Control of muscle mass

A primary goal of this study was to investigate potential pathways for muscle hypertrophy and flexibility in the migratory condition, particularly beyond the typical IGF1 and MSTN pathways that have been evaluated in the past to mixed results (reviewed in Swanson et al., 2022). Both of these genes, along with many of the other expected atrogenes (Atrogin/MuRF, MYOD, etc.) showed no significant difference in expression in the muscle of our White-throated Sparrows between seasons. Instead, we found substantial evidence that the mTORC signaling pathway for muscle growth is reduced at the stage in which these sparrows were sampled (Fig. 17A), alongside higher ubiquitin-mediated proteolysis (Fig. 17B). For example, the beta-transducin repeat containing E3 ubiquitin protein ligase which targets inhibitors of protein translation and
the inhibitor of mTORC1, DEP domain-containing mTOR-interacting protein (which was significantly upregulated in LD birds), as well as MAPK activated protein kinase 5, another potential mTOR suppressor, showed reduced expression (Fig. 17A). However, insulin-like growth factor 1 receptor (IGF1R) showed higher expression in the LD birds, potentially indicating that the muscle does show higher sensitivity to growth factor signaling in the migratory condition.

Satellite cell differentiation can occur through multiple different pathways, and we found mixed expression patterns in those genes. The Wnt signaling pathway may increase the size of fibers, potentially through multiplying satellite cells and a link to mTOR signaling (Hoppeler, 2016). We found a reduction in the autocrine motility factor receptor involved in negative Wnt signaling alongside an increase in ring finger protein 220, a ubiquitin protein ligase involved in positive Wnt signaling (Fig. 17A), suggesting that this pathway may be involved in muscle growth through satellite cells in the LD birds (Hoppeler, 2016; Snijders et al., 2015). Additionally, multiple genes related to Notch signaling showed higher expression in the LD birds (Fig. 17A). Notch signaling is important for satellite cell maintenance and proliferation when activated (Bjornson et al., 2012; Snijders et al., 2015), and association between transmembrane Notch on satellite cells and the Delta ligand following injury may signal for local satellite cell proliferation to repair the damage (Yin et al., 2013). Additionally, small muscle protein X (SMPX) expression was significantly higher in LD birds and has been shown to be expressed more in migrant relative to resident birds (Fudickar et al., 2016). This protein may play an important role in coordinating muscle growth and repair, and may serve a mechanosensory function in the muscle under exercise conditions (Eftestøl et al., 2014;
Ghilardi et al., 2020). Differentiation of these satellite cells may also be facilitated by the upregulation of myogenesis-regulating glycosidase (Datta et al., 2009). Therefore, the capacity for muscle repair through satellite cell recruitment may be increased in the migratory condition.

Given the importance of local associations and responses to muscle activity and stress, it is not surprising that we found altered expression of genes associated with the extracellular matrix, vesicular trafficking, and ER/golgi processing in the LD group (Fig. S2). Expression of extracellular proteins may be required for rapid response to local conditions between mature myocytes and satellite cells (Yin et al., 2013). For example, a trend toward upregulation of extracellular matrilin 2 (MATN2) in the LD condition may be important for secondary fiber formation in satellite cell differentiation (Yuan et al., 2021). The increased expression of the growth factor signaling receptor integrin subunit beta 4 in LD birds suggests that extracellular receptors increase the capacity for injury detection and growth factor response in the migratory condition (Fig. 18). Increased exocytosis of peptidases and may serve to rapidly alter the activity of growth factors within a local reservoir upon injury, stimulating satellite cell responses (Yin et al., 2013).

Additionally, adjustment to an endurance phenotype may be facilitated by inflammatory signaling pathways. We found multiple inflammation-related genes within the nuclear factor-κB (NF-κB) response pathway, including a trend toward higher toll-like receptor 2 (TLR2) and NF-κB expression itself in the muscle, though the pro-inflammatory activity of this pathway may be modulated by several inhibitors of NF-κB, including NMRAL1 and NKIRAS1 (Fig. 17A). Interestingly, the most highly expressed gene in the muscle dataset was ERVK-6, making up approximately 7% of mRNA
transcripts and showing a trend toward upregulation in the LD birds. In muscle, ERVKs have recently been shown to have high expression in broiler chicken myoblasts during growth, potentially regulating the transcription of multiple neighboring genes (Takaya et al., 2021), and ERVKs may be involved in proinflammatory transcription, including via NF-κB (Manghera and Douville, 2013). The pectoralis also showed higher expression of TNF receptor associated factor 6 (TRAF6), which may be responsive to inflammatory tissue repair in the flight muscle via NF-κB signaling. The function of NF-κB in the muscle may be diverse, ranging from immune response and cell proliferation to anti-apoptotic regulation and angiogenesis (Liu et al., 2017), making this regulator at the nexus of the inflammatory response a candidate for master regulation of muscle plasticity in the migratory condition. Indeed, several interleukin genes (IL17D, IL16, and IL18) and genes associated with angiogenesis were also increased in the LD birds (Fig. 17A), suggesting that NF-κB was exerting a diverse signaling function. Meanwhile, in the liver we found evidence of a reduction in the NF-κB response pathway in LD birds (Fig. 17A) alongside an overall reduction in extracellular matrix and signaling gene expression (Fig. 18). This pattern appeared in other studies as well (Horton et al., 2019; Johnston et al., 2016; Sharma et al., 2018), suggesting that extracellular signaling and cell-cell interactions are reduced in the liver in the migratory condition, perhaps to reduce the typical scarring response during migration-induced tissue damage. Therefore, while the NF-κB pathway appeared to be suppressed in the liver, the flight muscle may be more responsive to this regulatory network during the migratory season.
Figure 17. Fold change of genes involved in (A) inflammation and muscle mass changes and (B) degradation pathways in the liver (dark red) or pectoralis (bright red) associated with carbohydrate, fat, and ketone metabolism relative to the short day (SD) photoperiod treatment. Significant differentially expressed genes identified through DESeq2 are shown by Benjamini-Hochberg q-value ≤ 0.1 and trends are indicated by unadjusted p-values while genes from the liver using NOISeq are significant with Prob ≥ 0.9.

Higher TRAF6 expression also serves as a link between multiple proteolytic pathways and inflammation through NF-κB (Paul et al., 2010; Vainshtein and Sandri, 2020). In migratory birds, muscle mass may be maintained through degradation pathways. We found that the pectoralis of LD birds had a generally increased expression of many genes involved in protein degradation, primarily through ubiquitin-mediated
proteolysis, with the exception of proteasome 20S subunit beta 4 (Fig. 17B). This is consistent with the evidence of increased protein breakdown through enzyme activity in the muscle of these same sparrows (Elowe and Gerson, 2022). However, we previously found that the liver of the sparrows showed higher activity of glutamate dehydrogenase and lower xanthine oxidase activity, neither of those genes showed differential expression. Instead, we found general reductions in the expression of genes involved in protein breakdown in the liver. Therefore, the ubiquitin-proteasome system may be increased in the pectoralis in the migratory condition, perhaps indicating that selective protein degradation is increased in this active tissue while the liver is primarily involved in processing the products of this protein breakdown. Meanwhile, the autophagy gene microtubule associated protein 1 light chain 3 beta was lower in the LD group and increased expression of the FoxO autophagy repressor FOXK2 suggests that autophagy pathways may be reduced in the muscle (Sandri, 2013; Vainshtein and Sandri, 2020). The fate of catabolized protein is unclear in our dataset, though we do find a reduction in three genes involved in the purine nucleotide cycle, which is involved in anaplerosis of the TCA cycle through fumarate (Gibala et al., 2000; Sahlin et al., 1990) (Fig. 17B), suggesting a limited role of this proposed function of protein catabolism in these birds (Bauchinger and Biebach, 1998; Jenni-Eiermann, 2017). However, given that the purine nucleotide cycle is increased primarily in response to exercise, these genes may be reduced to prevent the loss of TCA cycle intermediates during rest and instead increase during activity (Sahlin et al., 1990).

We also found mixed expression of apoptosis regulators in the flight muscle, including both pro-apoptotic genes, reduced expression of negative regulators of
apoptosis, and anti-apoptotic genes (Fig. 17B). This may indicate satellite cell apoptosis following proliferation (Schwartz, 2008), and given the heterogenous nature of muscle tissue, which includes not only muscle fibers but also satellite cells of different stages and endothelial cells, it is not possible to distinguish between these cell types in our samples (Schwartz, 2019). As muscle hypertrophy and rapid satellite cell proliferation in the migratory condition has been documented (Young et al., 2021), including greater pectoralis mass in these sparrows (Elowe and Gerson, 2022), we may be seeing a later stage of migratory preparation in which proliferation has been reduced and instead the mature myocytes are maintained while additional satellite cells are reduced. Indeed, satellite cell proliferation may be high while differentiation may be suppressed overall in these LD birds, as indicated by a reduction in the protein kinase A and cAMP signaling pathway for muscle hypertrophy including adrenoceptor 2, adenylate cyclase, and CREB1, as well as increased expression of genes involved in cAMP hydrolysis (Fig. 17A). We also found higher expression of the myocardin-related transcription factor A (MRTFA; Fig. 17A), which has been shown to enhance proliferation in skeletal muscle, though primarily in conjunction with serum response factor (SRF) (Cenik et al., 2016), which was downregulated in the LD birds and potentially moderating the growth effects of MRTFA (Fig. 17A). Therefore, we hypothesize that birds at the late pre-migratory stage may show a balance of satellite cell proliferation and apoptosis, with reduced differentiation, as flight muscle hypertrophy slows.
4.5.6 Ca2+ signaling in muscle

We found a uniform reduction in the calcium signaling pathways in the LD condition, from calmodulins and other regulatory genes to downstream targets such as phosphorylase kinase and mitochondrial calcium transporters (Fig. 18). This was interesting considering the significantly higher sarcolipin (SLN) expression in the muscle seen here and using qPCR in a larger sample size (Elowe and Gerson, 2022), as well as in migratory Gray Catbird muscle (Elowe and Gerson, unpublished). We had hypothesized that increased SLN in the migratory condition would lead to uncoupling of sarco/endoplasmic reticulum ATPase (SERCA) activity and more calcium transients in the cytoplasm, signaling for mitochondrial biogenesis and a switch to fatty acid metabolism (Maurya et al., 2018), but this reduction in calcium signaling pathways does not appear to support that hypothesis. However, SLN uncoupling may instead lead to protection against oxidative stress, atrophy, or a protective hypermetabolic state in preparation for the stress of high-endurance migratory flight as reviewed in Chambers et al. (2022). Alternatively, a reduction in Ca2+-signaling sensitivity in the muscle may suggest that SLN serves as an uncoupler for SERCA with the purpose of heat production (Bal and Periasamy, 2020; Pani and Bal, 2022). Indeed, migratory birds appear to show higher thermogenic capacity during spring migration (Swanson and Dean, 1999). An increase in the capacity for non-shivering thermogenesis through SLN-induced futile Ca2+ cycling, while reducing the resulting signaling capacity, may indicate an adaptive role for greater heat production in coping with unpredictable weather conditions in the spring (Boelman et al., 2017; Swanson and Dean, 1999).
Figure 18. Fold change of genes involved in signaling mechanisms in the liver (dark red) or pectoralis (bright red) associated with carbohydrate, fat, and ketone metabolism relative to the short day (SD) photoperiod treatment. Significant differentially expressed genes identified through DESeq2 are shown by Benjamini-Hochberg q-value ≤ 0.1 and trends are indicated by unadjusted p-values while genes from the liver using NOISeq are significant with Prob ≥ 0.9.

4.5.7 CORT signaling

Given that protein breakdown in muscle is partly induced by glucocorticoid signaling, it is interesting that we found three enzymes involved in this process to be
increased in the migratory condition (Fig. 18). We found higher expression for 11β-hydroxysteroid dehydrogenase 1 (HSD11β1) and HSD11β2, which activate and inactive corticosterone (CORT), respectively. HSD11β2 has previously been shown to increase expression in the muscle of White-crowned Sparrows (Zonotrichia leucophrys) during the spring migratory season (Pradhan et al., 2019), potentially to reduce the catabolic effects of CORT on flight muscle. However, by finding higher expression of both dehydrogenases, with no changes in the glucocorticoid or mineralocorticoid receptors (NR3C1/2, respectively), the effect of CORT on the muscle of our sparrows is less clear. The increase for HSD11β1 was more substantial than HSD11β2, potentially indicating a different state of migratory preparation than was seen in the White-crowned Sparrows. While CORT may promote migratory readiness, the influence of CORT on protein breakdown may also be increased as birds get closer to migratory flight and prepare to dynamically alter muscle mass. For example, rapid lean mass loss in the early hours of flight may be indicative of CORT responses (Elowe et al., 2022, in review).

4.5.8 Thyroid hormones

Thyroid hormones may be important mediators of migratory readiness. The typically less active product of the thyroid gland is the hormone thyroxine (T4), which is converted to a more active form (3,5,3’-triiodothyronine, T3) by iodothyronine deiodinase 2 (DIO2) or inactive form by DIO3, with less selective deiodinase activity by DIO1. Through deiodinase activity, the effects of broad changes in hormone levels can be modulated at a tissue level (Pérez et al., 2016; Sharma et al., 2018; Watanabe et al., 2007). In the liver, we found significantly lower expression of the non-selective DIO1
with higher expression of DIO2 and no change in DIO3 (Fig. 18), which agrees with findings from Frias-Soler et al. (2022). Interestingly, exogenous T₄ administration after thyroid inhibition has been shown to rescue the development of spring migratory traits in White-crowned Sparrows, but T₃ administration specifically rescued mass gain, fattening, and muscle hypertrophy without any migratory restlessness (Pérez et al., 2016). Therefore, increased DIO2 levels in the liver may show that T₃ has specific tissue-level function in migratory fattening, particularly given the lack of expression changes for deiodinase enzymes in the pectoralis despite generally higher expression of thyroid hormone receptors (MED26, NCOR1, NR2C2, and THRAP3).

4.5.9 Conclusions

In response to long-day photoperiod stimulation of the migratory condition in captive White-throated Sparrows, we show numerous changes to gene expression in two vital tissues—the flight muscle and liver—that may facilitate migratory flight. These changes include alterations to the production and metabolism of ketones and fatty acids, a shift away from glucose as a fuel, and changes to circadian genes that may mediate the timing of metabolism. We also propose numerous tissue-specific changes that may alleviate the damaging effects of long-duration activity, including a suppression of the inflammatory response in the liver and an increase in the response in the muscle. In the muscle, we propose that seasonal hypertrophy balances satellite cell recruitment into existing muscle fibers and apoptosis, with a lower influence from mTOR and cAMP-mediated pathways, alongside proteasomal control of muscle mass. We also found little support for Ca²⁺-mediated signaling in the development of the migratory phenotype in the
pectoralis. Therefore, these data can encourage more targeted molecular studies on the unique integration of pathways that we find in the development of the migratory endurance phenotype in songbirds.

Figure S1. Normalized counts of highly expressed DEGs in the liver (dark red) and pectoralis (bright red) between short day (SD) and long day (LD) photoperiod treatments. Significant differences between treatments identified through DESeq2 are shown by Benjamini-Hochberg q-value ≤ 0.1 and trends are indicated by unadjusted p-values while genes from the liver using NOISeq are significant with Prob ≥ 0.9.
**Figure S2.** Fold change of genes involved in vesicle trafficking in the liver (dark red) or pectoralis (bright red) associated with carbohydrate, fat, and ketone metabolism relative to the short day (SD) photoperiod treatment. Significant differentially expressed genes identified through DESeq2 are shown by Benjamini-Hochberg q-value \( \leq 0.1 \) and trends are indicated by unadjusted p-values while genes from the liver using NOISeq are significant with Prob \( \geq 0.9 \).
CHAPTER 5
SARCO/ENDOPLASMIC RETICULUM CA\textsuperscript{2+}-ATPASE (SERCA) UNCOUPLING MAY PLAY A ROLE IN THE DEVELOPMENT OF THE MIGRATORY PHENOTYPE INDEPENDENT OF FATTENING IN CAPTIVE GRAY CATBIRDS

5.1 ABSTRACT

In order to complete their energetically demanding journeys, migratory birds undergo a suite of physiological changes that prepare them for long-duration endurance flight. This “migratory syndrome” of changes includes a shift to hyperphagia, fat deposition, a reliance on fat as a fuel source, and flight muscle hypertrophy. We previously documented significant increases to muscle sarcolipin (SLN) mRNA abundance, which binds to sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) and uncouples Ca\textsuperscript{2+} transport from ATP hydrolysis. This novel finding has unknown functional consequences for migratory birds, but may exacerbate energetic costs and increase heat production or signal for mitochondrial biogenesis, fatigue resistance, and a shift to fatty acid oxidation. We used a photoperiod manipulation of captive Gray Catbirds (Dumetella carolinensis) to investigate the role of SLN in the development of the migratory phenotype. We found that both male and female catbirds demonstrated migratory restlessness following the shift to a long day photoperiod, but males deposited more body fat while females did not. Similarly, only male catbirds showed a trend toward higher SERCA activity in the flight muscle. Despite these sex-specific changes, both males and females increased expression of h-FABP, PGC1\textalpha, and SLN in the migratory condition. This suggests that involvement of these three genes in the development of the
migratory phenotype in flight muscle may precede the fattening that is associated with migratory body composition.

5.2 INTRODUCTION

Migratory songbirds undergo physically demanding journeys each year between breeding and non-breeding locations. They accomplish this through a series of long-duration, non-stop flapping flights operating at metabolic rates up to 18 times their basal metabolic rates (Jenni-Eiermann, 2017). This physiological demand is met by a “migratory syndrome” of traits that enable successful migration through a suite of physiological changes (Dingle, 2006), including adaptive changes to metabolism and body composition that are particularly evident in the increased fat stores and capacity to transport and catabolize fat as a fuel (Guglielmo, 2010; McFarlan et al., 2009; Ramenofsky et al., 2017; Zajac et al., 2011).

To accommodate extended periods of flapping flight, bird muscle undergoes hypertrophy and changes to efficiency in preparation for migration. Alterations to the myosin heavy chain (Velten et al., 2016), fiber hypertrophy (DeMoranville et al., 2019; Marsh, 1984), capillary density (Lundgren and Kiessling, 1988), and corticosterone signaling (Pradhan et al., 2019) have all been documented in migratory birds. During muscle contractions, sarcolemmal and t-tubular membranes are depolarized, enabling Ca^{2+} release from the lumen of the sarcoplasmic reticulum (SR) (Dulhunty, 2006). As Na^{+}/K^{+}-ATPases restore the ionic equilibrium in the cytosol for subsequent depolarization, the Ca^{2+} release stimulates ATP hydrolysis and cross-bridge cycling by myosin ATPases, leading to muscle contraction (Clausen, 2003). Muscle relaxation
requires the sequestration of $\text{Ca}^{2+}$ in the lumen of the SR against its concentration gradient to lower the cytosolic free $\text{Ca}^{2+}$ concentration. This is accomplished by the sarco/endoplasmic reticulum $\text{Ca}^{2+}$ ATPase (SERCA), which under ideal conditions pumps $\text{Ca}^{2+}$ into the SR at a coupling ratio of 2 $\text{Ca}^{2+}$ ions for every 1 ATP hydrolyzed (Toyoshima, 2008). The maintenance of this $\text{Ca}^{2+}$ gradient by SERCA requires a substantial amount of energy in skeletal muscle that can account for as much as half of the resting metabolic rate of mouse skeletal muscle (Smith et al., 2013).

The high energetic cost of maintaining the $\text{Ca}^{2+}$ gradient may be exacerbated by the addition of sarcolipin (SLN), which binds to SERCA and uncouples $\text{Ca}^{2+}$ transport from ATP hydrolysis, increasing the duration of $\text{Ca}^{2+}$ transients in the cytoplasm and futile cycling of SERCA. In rodent models, this leads to heat production (Bal et al., 2018; Nowack et al., 2017; Periasamy et al., 2017), slower muscle relaxation (Tupling et al., 2011), higher resting energy expenditure (Bombardier et al., 2013), and reduced cases of diet-induced obesity (Maurya et al., 2015). However, recent studies also implicate SLN-mediated increases in cytosolic $\text{Ca}^{2+}$ in signaling for greater PGC1$\alpha$ expression, mediating a physiological switch to increased mitochondrial biogenesis and fatty acid catabolism, and greater fatigue resistance in skeletal muscle (Fajardo et al., 2017; Maurya et al., 2018; Sopariwala et al., 2015; Summermatter et al., 2011). We recently found evidence of ~ 2-fold higher PGC1$\alpha$ mRNA in the pectoralis flight muscle of White-throated Sparrows (Zonotrichia albicollis) in the migratory condition alongside approximately 25-fold increased SLN expression and unchanged SERCA2 transcript levels (Elowe and Gerson, 2022), providing some support for the hypothesis that SLN uncoupling may act as a signaling mechanism for a suite of changes in muscle physiology.
during spring migration. While the uncoupling action of SLN could logically impede efficient use of ATP and slow rates of muscle contraction during long-distance migratory flight, calcium signaling in the muscle may facilitate the shift towards fat metabolism and mitochondrial biogenesis in the flight muscle of migratory birds.

To investigate the role of SLN in the development of the migratory phenotype, I conducted a photoperiod manipulation using Gray Catbirds (*Dumetella carolinensis*) and monitored body composition in their transition to the migratory condition. We tracked body composition changes from a short day “winter” photoperiod (8-h light: 16-h dark) to a long-day “spring” photoperiod (16L:8D) and monitored the development of migratory restlessness overnight, or *Zugunruhe*. After three weeks and consistent overnight *Zugunruhe*, we measured organ masses and collected pectoralis flight muscle tissue to measure gene transcription of multiple indicators of the migratory phenotype, including fatty acid binding (h-FABP), fat transport (CD36), and β-oxidation (HOAD), and genes involved in the regulation of Ca$^{2+}$ cycling and signaling (SERCA2, SLN, and PGC1α). We also measured the activity of Ca$^{2+}$-dependent ATPases in the muscle to explore changes in SERCA function as birds prepare for migration. We hypothesized that Gray Catbirds show alterations to SERCA activity and coupling ratios in the migratory condition, potentially signaling seasonal changes to PGC1α, fat metabolism, and body composition.
5.3 MATERIALS AND METHODS

5.3.1 Animal collection

Gray Catbirds (Dumetella carolinensis, ‘catbirds’ hereafter) are 30 - 50 g passerines that breed from Nova Scotia to eastern Washington and into the southern reaches of Alberta and British Columbia. They migrate south in the winter to the Gulf of Mexico, with some birds remaining in the southern USA and others to the Yucatán Peninsula down to Panama, with a small number of birds lingering along the eastern seaboard of the USA up to New York. Band recaptures suggest that birds from the northeastern USA migrate to Florida and the Caribbean islands (Ryder et al., 2011). We captured 20 catbirds (10 male, 10 female) by mist-netting during fall migration between 29 September and 10 October 2019 on the University of Massachusetts Amherst campus (42°23'45.9"N 72°31'04.5"W). We immediately transported them to captive facilities at the University of Massachusetts Amherst where they were initially housed in cages with 2 individuals (77.5 x 30.5 x 39 cm) at 21°C and exposed to a decreasing fall photoperiod to match the duration of civil dawn to civil twilight, with full-spectrum lights adjusted every 2 – 3 days until a short-day winter photoperiod (8L:16D) was achieved. A dim light at night provided ~1 lux in the room (Ramenofsky et al., 2003). Birds had ad libitum access to water and a synthetic high-carbohydrate diet modified from Guglielmo et al. (2017) with supplemental Tenebrio mealworms provided daily (Dick and Guglielmo, 2019). They were kept on the short-day winter photoperiod for 70 days to break photorefractoriness (Barceló et al., 2016). Our study followed the Institutional Animal Care and Use Committee guidelines approved by the University of Massachusetts Amherst (protocol #2018-0038). A
collection permit was granted by the US Fish and Wildlife Service (permit #MB65968B-1) and the State of Massachusetts (#192.19SCB) to ARG.

5.3.2 Photoperiod switch

Sex was determined by molecular sexing using whole blood prior to the photoperiod manipulation (Griffiths et al., 1998). We randomly divided half of the catbirds, with equal numbers of male and female, into two adjacent rooms where they were singly housed (38.5 x 30.5 x 39 cm) and allowed to acclimate for 19 days to the new space. On March 24, 2020, one room was switched to a long day spring photoperiod (16L:8D) to induce a migratory disposition (Gwinner, 1990; Owen et al., 2006; Ramenofsky et al., 2003) while the other room remained on the same short-day winter light regime. Infrared cameras (Ailipu Technology Co., Ltd., Guangdong, China) were used to monitor nighttime activity in both rooms following the change in light cycle. Catbirds held on the long-day light regime (N = 5 Male, N =5 Female; “LD” treatment birds, hereafter) showed substantial variation in their timing for displaying migratory restlessness, with one outlier starting right away and the next beginning on day 7 following the light change, with all birds showing migratory restlessness by day 14 aside from one bird that did not develop Zugunruhe. Males and females did not appear to show a difference in the development of Zugunruhe. Catbirds kept on the short-day photoperiod (N = 5 Male, N =5 Female; “SD” treatment birds, hereafter) displayed little to no nocturnal activity.
5.3.3 Body composition

During the photorefractory period, we weighed birds on 3 separate occasions and inspected for mass changes, health, and muscle and fat scores. Starting on the day of the photoperiod switch, we tracked changes in body composition using a quantitative magnetic resonance body composition analyzer (QMR) customized for small birds (Echo-MRI Echo-Medical Systems, Houston, TX). The QMR measures fat and wet lean masses with accuracies of ±6-11%, and ±1-2%, respectively (Guglielmo et al., 2011). To ensure scans were always of post-absorptive birds, food was removed from cages approximately 2 h prior to scanning. Birds were scanned on day 1, 7, 14, and immediately prior to sacrifice. All catbirds were sacrificed by isoflurane overdose and cervical dislocation in compliance with all animal care guidelines 21-22 d after the photoperiod switch between 10:30 and 16:30. Pectoralis samples were frozen on dry ice within 5 min of sacrifice for gene expression. We weighed the left pectoralis, liver, heart, gizzard, and gut to the nearest 0.0001 g and measured the total gut length to the nearest millimeter. We dried small samples of the pectoralis and liver in an oven at 60°C for ~48 h to estimate water content and organ dry mass.

5.3.4 RNA extraction and qPCR

We homogenized approximately 50 mg of pectoralis muscle using beadmill homogenization (NextAdvance, Troy, NY; speed 8, time 4, 4°C) in 1 mL of TriZol (Invitrogen, Carlsbad, CA) and RNA was separated into an aqueous phase using a chloroform ethanol procedure. Total RNA was purified and DNase-treated using NEB Monarch RNA Cleanup Kit (New England Biolabs, Ipswich, MA). Samples were
quantified and checked for RNA quality using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) prior to reverse transcription to cDNA using NEB Luna RT (New England Biolabs). Each cDNA sample was diluted 1:5 with nuclease-free water before analysis.

We designed primers for Gray Catbirds using conserved sequence alignments. We began by using NCBI Blast (Madden, 2003) for each gene transcript against *Gallus gallus* and the Passeriformes taxonomy to obtain transcript sequence alignments across diverse songbirds. Using PrimerIdent (Pessoa et al., 2010), we selected candidate primers for target genes based on conserved locations in the transcript sequences. Our target genes were sarcolipin (SLN), sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase 2 (SERCA2), fatty acid translocase (CD36), heart-type fatty acid binding protein (h-FABP), 3-hydroxyacyl-CoA dehydrogenase (HOAD), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1\(\alpha\)). For housekeeping genes, we selected hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a stably expressed gene in multiple tissues in rats (Kim et al., 2014) and ribosomal protein lateral stalk subunit P0 (RPLP0), which did not show seasonal changes in Gray Catbirds previously (DeMoranville et al., 2019) and has been evaluated as a reference gene in several birds species (Olias et al., 2014) (see Table 4 for primer information). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is frequently used as a housekeeping gene, but we previously found minor differences in expression under photoperiod manipulations (Elowe and Gerson, 2022) that make this gene a less reliable option.
Table 4. Primers used for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F Primer</th>
<th>R Primer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC1α</td>
<td>GTGGTCTCAGCACACAAAAAC</td>
<td>CAGGGACGTCCTTTAGGGCCTT</td>
<td>Demoranville et al. 2019</td>
</tr>
<tr>
<td>CD36</td>
<td>TGAATGAGTCTCGTTGATGTTGG</td>
<td>GCATATGAAGTGGAGCCCATG</td>
<td>Demoranville et al. 2019</td>
</tr>
<tr>
<td>RPLP0</td>
<td>GCAGACAACGTTGGATCCAGCAGAT</td>
<td>GATCTCCCTTAGTGAAGACAAAGCC</td>
<td>Demoranville et al. 2019</td>
</tr>
<tr>
<td>HPRT1</td>
<td>TTGGGTTGCTGTTTTGG</td>
<td>ACCAAAGGTGCAACTATCC</td>
<td></td>
</tr>
<tr>
<td>h-FABP</td>
<td>CACCTGGAAGCTGTTGGACA</td>
<td>TTAGGTTGCTCCTGGGTTCCT</td>
<td></td>
</tr>
<tr>
<td>SERCA2</td>
<td>AGCAAGTGAAGAAGCTGAGG</td>
<td>TTCTTTCCCTGCCACACTCC</td>
<td></td>
</tr>
<tr>
<td>SLN</td>
<td>CAAAATCCAGAGCGACACCC</td>
<td>CTTCACAAGAGGCCACATGA</td>
<td></td>
</tr>
<tr>
<td>HOAD</td>
<td>AGGCTGTTGCCAGTTTCATT</td>
<td>GCAAGGATGAAGTGTGGCTT</td>
<td></td>
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</tbody>
</table>

Prior to running samples, we confirmed primer specificity on a 2% agarose gel in TAE with SYBR-safe stain and tested efficiency on a serial dilution series of pooled cDNA (1:2 – 1:40). All amplification efficiencies fell between 97.9% and 99.7%. We prepared reactions on ice in 10 μL volumes with 0.2 μL primers and 1 μL cDNA in Luna Universal qPCR master mix (New England Biolabs, Ipswich, MA) to run on a fast protocol in a StepOnePlus qPCR machine (Applied Biosystems, Foster City, CA) for 60s at 95°C, then 40 cycles of 95°C for 15s followed by 60°C for 30s, with melt curve analysis from 60-95°C. We performed comparative cycle threshold (C_T) analysis according to Schmittgen and Livak (2008). Each sample was run in duplicate wells and we subtracted average C_T values for each target gene (SLN, SERCA2, CD36, h-FABP, HOAD, and PGC1α) from the average C_T for the two corresponding housekeeping gene readings (HPRT1 and RPLP0) to obtain the ΔC_T for each individual and gene. Both HPRT1 and RPLP0 were stably expressed between the photoperiod treatments (HPRT1:
\[ t_{14.8} = 0.55, \ p = 0.589; \ \text{RPLP0: } t_{12.8} = -1.05, \ p = 0.314; \ \text{Average: } t_{12.6} = -0.25, \ p = 0.808 \]

and sex (HPRT1: \[ t_{14.8} = 0.55, \ p = 0.589; \ \text{RPLP0: } t_{12.7} = -1.47, \ p = 0.165; \ \text{Average: } t_{13.7} = -1.30, \ p = 0.214 \]). Fold change calculations incorporated the average and standard error of the mean (SEM) for \( \Delta C_T \) by subtracting the SD mean \( \Delta C_T \) from LD mean to provide \( \Delta \Delta C_T \), from which we calculated average fold change and upper and lower limits of the SEM using \( 2^{\Delta \Delta CT} \).

**5.3.5 SERCA activity assay**

We measured total SERCA activity using an enzyme-linked spectrophotometric method as outlined in (Fajardo et al., 2015; Gamu et al., 2019; Jannas-Vela et al., 2020; Tupling and Green, 2002). At the time of sacrifice, pectoralis muscle tissue (~160 mg) was homogenized in 1:10 ice-cold buffer (in mM: 250 Sucrose, 5 HEPES, 0.2 PMSF, 15 NaN3) with two 30-s bursts of a Tissue Tearor (BioSpec Products, Bartlesville, OK, USA) at ~15,000 RPM with 30 s of rest in between bursts. Aliquots of ~115 µL of the homogenate were immediately stored at -80°C until use. The SERCA ATPase reaction buffer contained (in mM) 200 KCl, 20 HEPES, 15 MgCl2, 10 phosphoenolpyruvate, and 1 EGTA (pH 7.0 at 39°C). Prior to starting the reaction, we added 18 U/ml lactate dehydrogenase (LDH), 18 U/ml pyruvate kinase (PK), 5 mM ATP, and 0.3 mM NADH. We added 2 µl homogenate to 648 µl of the SERCA ATPase reaction buffer and added samples in triplicate to a clear 96-well plate with 200 µl reaction volumes to measure the conversion of NADH to NAD\(^+\) using 340 nm absorbance on a Biotek Synergy H1 microplate reader (BioTek, Winooski, VT) at 39°C, a typical avian body temperature. We first measured total ATPase activity in the absence of Ca\(^{2+}\) with the disappearance of NADH for 5 min, followed by the addition of 10 µl of 2 mM CaCl\(_2\) and absorbance
readings for 25 min. The difference in these rates were attributed to Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+}-ATPase activity, which we hereafter attribute primarily to SERCA activity.

**5.3.6 Statistical analysis**

All statistics were performed in R (v 4.2.1, R Foundation for Statistical Computing, Vienna, Austria). To account for body size, we included sternum measurements in each model. Sternum size was significantly related to lean mass measures (F\textsubscript{1,18}= 5.64, R\textsuperscript{2} = 0.20, P = 0.03), but not fat mass (F\textsubscript{1,18}= 1.69, R\textsuperscript{2} = 0.04, P = 0.210), making it a suitable proxy for the structural body size of an individual. For body composition changes over time, we evaluated linear mixed models with the function ‘lmer’ (lme4 package, version 1.1-17) and performed backward fixed-effects stepwise model selection with a \( \alpha = 0.05 \) cutoff for fixed effects. We evaluated changes in body composition (total, fat, or lean mass) with interaction terms for time point × photoperiod, sex × photoperiod, and sex × time, with sternum size as fixed effects while controlling for repeated measures in individual birds with a bird ID random effect. For organ masses, we started with an ANCOVA with the interaction between treatment × sex, along with sternum size as a covariate to account for differences in body size. For SERCA activity data and \( \Delta C_T \) gene expression data we started with an ANCOVA with the interaction between treatment × sex, with sternum size to account for structural body size. For all models, we evaluated for normal distribution and equal variance of residuals. If non-significant (p > 0.05), covariates and interactions were sequentially removed from the models.
5.4 RESULTS

5.4.1 Body composition

Total mass

Relative to the start of the photoperiod switch, we found a significant time × sex interaction (F_{3,60}=7.40, p < 0.001; Fig. 19), so we split the model by sex going forward. We found that within the male catbirds there was a significant time × treatment interaction (F_{3,30}=32.12, p < 0.001), and after further splitting the model by treatment we found that SD males lost significant body mass by day 7 and remained below starting mass for the full three weeks (F_{3,15}=13.92, p < 0.001). In contrast, LD males gained mass by day 14 and stayed above their starting mass (F_{3,15}=23.11, p < 0.001). Females in both treatment groups lost body mass by day 14 and remained below their starting mass (F_{3,30}=4.33, p = 0.012), but also showed a significantly lower body mass with larger sternum size (F_{1,10}=6.71, p = 0.027).

Fat mass

As with total body mass, we found a significant time × sex interaction (F_{3,60}=7.92, p <0.001; Fig. 19), and after separating the model by sex we found a significant time × treatment interaction in the male catbirds (F_{3,30}=61.22, p < 0.001). While the SD males lost fat over the three weeks (F_{3,15}=51.43, p<0.001), LD males gained fat by day 7 and peaked at day 14 (F_{3,15}=20.52, p<0.001). Female catbirds showed no change in fat mass for SD or LD birds, but within both treatment groups there was a negative relationship between fat mass and sternum size in the females (F_{1,10}=5.635, p =0.039).

Lean mass
We found a significant time × treatment interaction (F\(_{3,60}=4.79, \ p=0.005\); Fig. 19), so we separated data by treatment. Within the SD birds there was also a significant time × sex interaction (F\(_{3,30}=5.19, \ p=0.005\)). After splitting the model by sex, we found that males and females both lost around 1 gram of lean mass over the first 2 weeks, but males rebounded exceeding their starting lean mass (F\(_{3,15}=21.80, \ p<0.001\)) while females were still below their starting mass (F\(_{3,15}=11.29, \ p<0.001\)). The LD birds lost significant lean mass by day 14 and did not recover the lost lean mass after that time (F\(_{3,30}=8.65, \ p<0.001\)).
Figure 19. Relative body composition changes in female and male Gray Catbirds following long-day photostimulation or continued short-day conditions. Females (N = 5 SD, N = 5 LD; left) and males (N = 5 SD, N = 5 LD; right), were weighed and scanned on the day of the photoperiod change for the LD group and each week for three weeks. The photoperiod change after the baseline time point is denoted with the vertical gray line. SD birds are shown in blue and LD birds in orange. Significant differences from the baseline measurement are indicated with asterisks (*p ≤ 0.1, **p ≤ 0.05, ***p ≤ 0.01).

5.4.2 Organ masses

We found no significant difference in wet pectoralis muscle mass between the photoperiod treatment groups, but females had a non-significant trend toward lower
pectoralis mass ($F_{1,18}=3.63, p = 0.073$). Dry pectoralis showed a non-significant trend toward lower mass in LD birds ($F_{1,17} = 3.97, p = 0.063$) but greater mass in males ($F_{1,17} = 4.50, p = 0.049$; Fig. 20). Both wet ($F_{1,18} = 6.22, p = 0.023$) and dry ($F_{1,18} = 10.67, p = 0.004$) liver mass were lower in LD birds than SD birds. Heart mass was significantly greater for LD birds ($F_{1,18} = 5.17, p = 0.035$). However, the final model of heart mass in relation to photoperiod treatment showed unequal variance between groups using a stringent Levene’s Test ($F_{1,18}=8.93, p = 0.008$), but a t-test with unequal variances also showed significantly higher heart mass in the LD birds ($t = -2.27, df = 10.56, p = 0.045$). Gut measurements did not show any photoperiod treatment differences, but intestine length showed a non-significant trend toward greater length with increasing sternum size ($F_{1,18}=3.89, p=0.064$) and gizzard mass showed a non-significant trend toward lower mass in males than females ($F_{1,18}=3.30, p=0.096$).
Figure 20. Catbirds organ measurements at day 21 following the photostimulation of LD birds (orange) or birds on a continued SD photoperiod (blue). N=5 each for males and females in the SD and LD photoperiod treatment groups. Significant differences between groups are denoted by asterisks (*p ≤ 0.1, **p ≤ 0.05, ***p ≤ 0.01).

5.4.3 Gene transcription

We found no significant photoperiod treatment effect for SERCA2 or CD36, but found a nearly significant treatment effect for PGC1α ($F_{1,18}=4.36$, $p = 0.051$; Fig. 21). For HOAD, there was a non-significant trend toward lower HOAD expression in the LD males ($F_{1,17}=3.03$, $p=0.10$). We found 3.8-fold higher h-FABP transcription in LD birds...
SLN transcription was approximately 6-fold higher in LD than in the SD treatment birds (F_{1,18}=11.20, p=0.004; Fig. 21).

**Figure 21.** Pectoralis gene expression at day 21 following the photostimulation of LD birds (orange) or continued SD photoperiod (blue). Fold change shown is relative to the SD birds. N=10 each for the SD and LD photoperiod treatment groups. Significant differences are denoted by asterisks (*p \leq 0.1, **p \leq 0.01).

### 5.4.4 SERCA activity

SERCA activity was nearly significant for the photoperiod × sex interaction (F_{1,15}=3.10, p = 0.099). After splitting the model by sex, we found no difference in the activity for females between the treatment groups, but LD males showed a non-significant trend
toward higher SERCA activity than the SD males ($F_{1,8}=3.84, p=0.086$; Fig. 22). Given that only LD males showed higher fat accumulation as well, we examined the correlation between SERCA activity and fat mass to find a non-significant positive trend ($F_{1,18}=3.15, p = 0.093$, adjusted $R^2 = 0.102$).

**Figure 22.** Maximal activity of Ca$^{2+}$-dependent ATPases in the pectoralis of female (left) or male (right) catbirds at day 21 following the photostimulation of LD birds (orange) or continued SD photoperiod (blue). Ca$^{2+}$-dependent ATPase activity is attributed to SERCA. N=5 each for males and females in the SD and LD photoperiod treatment groups. Significant differences are denoted (*$p \leq 0.1$).

**5.5 DISCUSSION**

We used a photoperiod manipulation of captive Gray Catbirds to investigate the role of SLN in the development of the migratory phenotype. While male and female catbirds both demonstrated migratory restlessness after exposure to a long day photoperiod, males deposited significantly more body fat over the subsequent three
weeks. Similarly, only male catbirds showed a trend toward higher SERCA activity in the flight muscle and there was a non-significant correlation between SERCA activity and fat mass. Independently of these sex-specific changes, both males and females increased transcription of h-FABP, PGC1α, and SLN in the migratory condition. This suggests that expression of these three genes are key to the development of the migratory phenotype in flight muscle and precede the fattening that is associated with pre-migratory body composition.

Our study revealed drastically different patterns of fat accumulation between male and female catbirds in the migratory condition. Due to the similar development of Zugunruhe and similar expression of SLN and h-FABP we were able to conclude that the females were developing the migratory condition but had not yet gained the fat stores seen in the males. This may indicate differences in migratory timing or earlier arrival on the breeding grounds by male catbirds (protandry). However, protandry is not fully explored in species that do not display sexual dimorphism, in part due to the absence of sex differentiation in typical banding records (Hatch and Smith, 2009). While past records of protandry in Gray Catbirds were suggested by Darley et al. (1971), protandry was not seen in Hatch and Smith (2009). However, in a captive photoperiod manipulation with catbirds, Owen et al. (2014) found that males exhibited migratory behavior 11.3 days earlier than females on average. This suggests that a lack of documented arrival time differences may not encompass differences in migratory departure timing and/or speed between males and females. We did not quantify overnight activity, but qualitatively we did not note any differences in the development of Zugunruhe between the sexes in our study. However, Owen et al. (2014) also found that while all of their catbirds
demonstrated overnight Zugunruhe, none of them gained significant mass following photostimulation. While this differed from our study, without measuring fat mass directly it is difficult to tell whether their body composition changed. DeMoranville et al. (2019) found significantly more fat in wild-caught catbirds during the fall and spring migratory seasons, but only fall migratory birds showed a significant increase in total body mass. Given that even our male catbirds showed significantly more fat but also a reduction in lean mass after photostimulation, it’s plausible that measurements of overall body mass in spring migratory catbirds could miss sex-specific differences in body composition changes, and therefore females may not develop greater fat stores given more time after photostimulation.

We were also surprised to find that pectoralis mass did not increase in the LD catbirds and that liver mass declined in LD birds. In White-throated Sparrows, flight muscle tends to increase in photo-stimulated captive migrants (Elowe and Gerson, 2022; Price et al., 2011) and wild catbirds tend to show pectoralis hypertrophy during migration (DeMoranville et al., 2019; Marsh, 1984). However, pectoralis mass is known to respond to exercise conditions (Price et al., 2011; Zhang et al., 2015a), and DeMoranville et al. (2019) showed that the increases to pectoralis mass in wild catbirds was greater in the fall than the spring, and unadjusted wet pectoralis mass in the spring was not significantly different from the winter mass.

SLN and h-FABP were higher in LD birds than SD birds in both sexes, despite differences in fat stores between males and females in migratory condition. This suggests that increased transcription of SLN and h-FABP, and PGC1α to an extent, precede the deposition of fat stores. Meanwhile, the levels of CD36 and HOAD mRNA did not show
the expected increase in our catbirds. Previous studies have shown that CD36 mRNA levels did not change in captive White-throated Sparrows in the migratory condition, even with upregulated SLN (Elowe and Gerson, 2022; Price et al., 2010; Elowe & Gerson, unpublished), but in wild-caught migratory sparrows CD36 does show higher transcription (McFarlan et al., 2009). This suggests that these genes are more responsive to exercise (McFarlan et al., 2012), increasing with migratory flight rather than the development of migratory behavior in captive conditions. Furthermore, Zhang et al. (2015b) demonstrated in migratory Yellow-rumped Warblers (Setophaga coronata) and Warbling Vireos (Vireo gilvus) that not only did the patterns of CD36 transcription in the flight muscle differ between the two species, but those patterns did not necessarily translate to protein levels. This may be the case for lower HOAD transcript abundance seen in our catbirds as well, given that many studies have shown an increase in HOAD enzyme activity in the migratory condition (Marsh, 1981; McFarlan et al., 2009; Zhang et al., 2015b). However, DeMoranville et al. (2019) did find that HOAD activity, while trending higher in the muscle of wild-caught migratory catbirds, was not significantly different between the seasons, and Price et al. (2010) also showed that captive White-throated Sparrows in the migratory condition did not show significantly increased HOAD activity. Therefore, it is unclear whether lower HOAD transcript levels in the pectoralis of our catbirds would lead to lower protein levels and, ultimately, HOAD enzyme activity.

The increased transcription of SLN in the muscle of captive Gray Catbirds in the migratory condition, matching the pattern seen in White-throated Sparrows (Elowe and Gerson, 2022), poses interesting questions. While the effects of SLN on SERCA activity
and efficiency has been thoroughly examined in rodents, few studies have addressed this in birds (Elowe and Gerson, 2022; Pani and Bal, 2022; Price et al., 2019; Stager and Cheviron, 2020). If SLN acts as an uncoupler of SERCA Ca\(^{2+}\)-pumping efficiency in skeletal muscle for non-shivering thermogenesis (NST), we would expect to see higher SLN expression as a benefit of cold acclimation and lower SLN expression to favor efficient muscle function and reduce excessive heat production during migration. However, in addition to our findings of higher SLN transcription in the migratory condition, Stager and Cheviron (2020) found that resident Dark-eyed Juncos (Junco hyemalis) were more susceptible to hypothermia when they had higher pectoralis muscle SLN mRNA. In this case, perhaps lower SLN expression is associated with improved thermogenic capacity in cold-acclimated birds, such as White-throated Sparrows that overwinter in relatively cold climates. However, catbirds tend to overwinter in milder climates (Ryder et al., 2011), leading us to believe that this pattern of SLN expression is not functioning to increase thermogenic capacity in these migratory species.

Instead, SLN-mediated SERCA uncoupling may lead to increased cytosolic Ca\(^{2+}\) transients in the muscle. This cytosolic Ca\(^{2+}\) could act as a signaling mechanism for PGC1\(\alpha\) (Maurya et al., 2018; Sopariwala et al., 2015), including increased fatty acid catabolism, mitochondrial biogenesis, fiber type transitions, and fatigue resistance (Fajardo et al., 2017; Summermatter et al., 2011), each of which align with expected seasonal changes to flight muscle in migratory songbirds. In our study, we found higher SLN transcription in both male and female catbirds alongside unchanged SERCA2 mRNA levels, suggesting that uncoupling would increase in the pectoralis in the migratory condition, and therefore the higher PGC1\(\alpha\) transcription we found could be the
result of increased Ca\(^{2+}\) signaling, though we cannot comment on the causality from our data. However, the phenotypic effects of this potential signaling mechanism—namely increased muscle hypertrophy, mitochondrial biogenesis, and a switch to fatty acid metabolism—are unclear in our study. PGC1\(\alpha\) isoforms may have different targets, including oxidative phosphorylation genes or the induction of muscle hypertrophy (Ruas et al., 2012), though the latter appears unlikely in our study due to the reduced pectoralis mass seen in conjunction with PGC1\(\alpha\) expression. In Sln-knockout mouse studies, the loss of SLN expression leads to impaired expression of multiple genes associated with the migratory phenotype, including carnitine palmitoyltransferase 1 (CPT1), long-chain acyl-CoA dehydrogenase (LCAD), and HOAD, as well as reduced fatty acid oxidation. While we did not measure these transcripts directly, their increased transcription and enzyme activities have been noted in other studies of migratory birds (Marsh, 1981; McFarlan et al., 2009; Price et al., 2010; Sharma and Kumar, 2019; Zhang et al., 2015b). Therefore, increasing the SLN/SERCA2 ratio in the muscle of birds in the migratory condition may facilitate a shift to the endurance migratory phenotype (Maurya et al., 2018; Sopariwala et al., 2015). More puzzling is the negative correlation between SLN transcription and thermogenic capacity in Dark-eyed Juncos (Stager and Cheviron, 2020). Given that the cold-acclimated phenotype should maximize heat production, SLN-induced NST may generate only ~2% of the heat that shivering would produce (Campbell and Dicke, 2018) and represent a functional detriment relative to cold-induced phenotypic changes for enhanced shivering thermogenesis in songbird flight muscles (Pani and Bal, 2022; Swanson, 2010). Therefore, the heat generation and disrupted SERCA efficiency through SLN upregulation during migration may be negligible relative
to the adaptive benefits of altered Ca\textsuperscript{2+} signaling in preparation for migration. Due to the different demands for shivering thermogenesis and flight, in which one requires rapid isometric contraction and the other consists of contraction-relaxation cycles, perhaps SLN-induced reductions to Ca\textsuperscript{2+} cycling efficiency are more detrimental to the shivering than the exercise phenotype. Of interest is whether this pattern of upregulated SLN also exists during the fall migratory season, given that Swanson and Dean (1999) showed increases in the thermogenic capacity of migratory birds in the spring but not the fall.

While an increased SLN/SERCA ratio is expected to alter the efficiency of SERCA, we were unable to fully test the functional consequences of increased SLN. While measurements of maximal SERCA activity may be compared to the rate of Ca\textsuperscript{2+} uptake to estimate an apparent coupling ratio and thus the influence of SLN on SERCA efficiency (Fajardo et al., 2015; Gamu et al., 2019; Tupling and Green, 2002), we only present SERCA activity in the pectoralis of our catbirds. However, the pattern of SERCA activity in our catbirds was similar to that of migratory fattening, where only males in the LD condition showed a trend toward higher activity. Although SLN upregulation, and subsequent changes to a slow fiber phenotype, may affect maximal SERCA activity alone in mice (Fajardo et al., 2017), the uniform increase in SLN transcription between male and female catbirds suggests that SLN did not directly influence SERCA activity itself. More likely, the effects of SLN on SERCA uncoupling would be reflected in a lower rate of Ca\textsuperscript{2+} uptake relative to Ca\textsuperscript{2+}-dependent ATPase activity, potentially leading to altered fiber types that would accompany pre-migratory fattening and migratory restlessness.
The extent to which SLN influences SERCA activity leading to NST, as well as the potential for post-translational modification to regulate this interaction, is not fully understood. Within mammals, the SLN sequence is remarkably conserved (Campbell and Dicke, 2018). A SLN protein sequence alignment using Clustal Omega (Sievers et al., 2011) between 9 passerines, 9 non-passerines, and 39 mammalian species (57 species total) reveals several amino acid substitutions in birds (Table 5). While proposed sequence locations (£² and £⁷) for association to disrupt SERCA Ca²⁺ binding are well conserved between mammals and birds, the degree of variability in the sequence among other mammal lineages has called that function into question (Campbell and Dicke, 2018). However, based on sequence conservation in mammals, the C-terminal sequence from amino acids 27-31 has been proposed as a candidate for the uncoupling function due to its role in proper localization of the SLN protein in the SR (Gorski et al., 2013). Deletion of the three amino acids in this C-terminal sequence produced partial inhibition without altering the SLN/SERCA complex (Wang et al., 2021). Not only do all of the birds appear to show a R²⁷→K²⁷ substitution, but the Y³¹→D³¹ substitution we find in each of the passerine birds at the C-terminus of SLN could provide an alteration to the inhibitory function on SERCA. Additional post-translational modifications to SLN could also alter its effects on SERCA. For example, in rabbits and pigs Montigny et al. (2014) found that depalmitoylation of the C⁹ residue led to a 30% increase in Ca²⁺-ATPase activity. The F⁹→C⁹ substitution is only seen in some mammals, but it is surprisingly consistent in passerine bird SLN sequences. In our alignment, we found that 8 of 9 passerines and only 1 of 9 non-passerines showed the cysteine amino acid at position 9, and in the mammals 15 of 39 showed the sequence, including the Trichechidae family.
(manatees), some members of Artiofabula (blue whale, sheep, pig, goat), and prominently in bats (Chiroptera). Furthermore, a neighboring $L^8 \rightarrow I^8$ substitution appears in all passerine birds and 3 of 9 non-passerines, but in mammals only appears in the goat and sheep, which both contain the $C^9$ substitution. Due to nucleotide sequence differences giving rise to this $F^9 \rightarrow C^9$ substitution, Montigny et al. (2014) speculated that this appears due to convergent evolution. While the function of this potential regulatory site is not fully understood, particularly if it evolved convergently in passerine birds and bats it may present an avenue for altered SLN/SERCA interaction, and the dependence on acylation as a post-translational modification may relate the increased activity of SERCA to a dependence on fatty acids. Based on the uncertainty around the SLN/SERCA interaction due to sequence differences, it’s possible that SLN plays a limited, or flexible, role in NST in bird muscle. Therefore, it is likely that the trend toward increased SERCA activity in the pectoralis of LD male catbirds is simply a byproduct of the physiological changes in preparation for migration alongside migratory fattening rather than a direct result of higher SLN expression. However, determining the relative efficiency of SERCA $Ca^{2+}$ pumping in each condition would be key to understanding the effects SLN transcription in migratory birds.
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Table 5. SLN protein sequence alignment between mammals (white), passerine birds (light gray), and non-passerine birds (dark gray), with notable amino acid substitutions highlighted in red.
This study confirmed that photostimulated migratory birds show higher transcription of SLN in the absence of a correlated change in SERCA2. These changes appear to match expected changes in the migratory condition, including higher transcription of h-FABP, and may relate to the coordination of physiological changes by PGC1α. However, these changes in transcription are not necessarily matched by corresponding changes to body composition, as only the males in migratory condition showed greater fat stores and SERCA activity. Therefore, while SLN does appear to play a role in the development of the migratory condition, further studies are needed to determine its effect on SERCA Ca\(^{2+}\)-pumping efficiency and the role it plays in altered Ca\(^{2+}\) signaling pathways in the shift to a migratory phenotype. Depending on the effect of SLN on SERCA efficiency, this may reveal important trade-offs in the development of an endurance flight phenotype as well as the role of SLN in cold-acclimation in birds.
REFERENCES


hydrogen ($\delta^2$H) and oxygen ($\delta^{18}$O) isotope concentrations in water vapor from breath. *Physiological and Biochemical Zoology* **88**, 599–606.


