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Effects of evolved migration strategy, seasonal flexibility, and endurance flight on songbird mitochondrial bioenergetics

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

Abstract

Migratory birds make large-scale movements using flight twice per year, which are physiologically challenging due to high energetic costs and oxidative damage from reactive oxygen species (ROS). Muscle mitochondria play central roles in energy supply and ROS homeostasis during exercise, but the role of mitochondrial function in overcoming the demands of migratory flight is presently unclear. In this dissertation, I explore how mitochondrial function is modulated in three contexts relevant to avian migration: seasonal flexibility, endurance flight and evolved variation in migration strategy. For my first aim, I compared flight muscle mitochondrial respiration and ROS emission between a migratory and short-day photoperiod-induced non-migratory phenotype in yellow-rumped warblers (*Setophaga coronata*). I found that fatty acid-fuelled respiration was higher and ROS emission was lower in the migratory compared to the non-migratory phenotype, indicating that mitochondria are seasonally remodeled to support greater fatty acid oxidation and lower risk of oxidative stress during migration. For my second aim, I assessed mitochondrial respiration and ROS emission and lean tissue catabolism before and after up to eight hour endurance flight using a wind tunnel in blackpoll warblers (*Setophaga striata*). I found that endurance flight had little effect on flight muscle size, ultrastructure, mitochondrial respiration or ROS emission, but a modest reduction in the mass of liver, gizzard and proventriculus. These findings suggest that mitochondria are resilient against the damaging aspects of endurance flight and that the flight muscle may be preferentially protected to support endurance flight performance. For my third aim, I assessed pectoralis size, mitochondrial physiology, and mitochondrial abundance across 19 passerine species of varying migratory strategy. I found that long-distance migration was associated with lower pectoralis size and fatty acid oxidation capacity, while mitochondrial abundance and ROS emission were unaffected. Surprisingly, these data suggest that longer migration distance is associated with a lower oxidative capacity, which may reflect evolved reductions in energy expenditure during migration. Together, my findings indicate that mitochondrial metabolism is modulated by migratory songbirds to better meet the demands of migratory flight.

Keywords

Exercise physiology; skeletal muscle; metabolism; locomotion; enzymes; adaptation; plasticity; comparative

Summary for Lay Audience

Migratory birds make some of the largest scale movements in nature, but how they accomplish these feats of endurance exercise is poorly understood. One way that migratory birds can facilitate these movements is by changing the function of structures within their muscles called mitochondria, which supply the energy used during exercise, but can produce potentially harmful chemicals called reactive oxygen species (ROS). In my thesis, I investigated the role of mitochondria in bird migration by comparing mitochondrial function across three different aspects of migration: 1) during and after migratory season, 2) before and immediately after extended flights using a wind tunnel and 3) comparing between species that migrate different distances. For all studies, I measured how quickly flight muscle mitochondria consumed oxygen and produced ROS when burning fat as a fuel. I found that in the migratory season, mitochondria consume more oxygen and produce less ROS compared to after migration, suggesting that migratory birds modify their mitochondria to increase energy supply and lower the risk of damage during flight. Next, I found that eight hour wind tunnel flight had no effect on mitochondrial oxygen consumption or ROS production, indicating that mitochondria are not damaged during flight. Finally, I found that long-distance migratory species had lower oxygen consumption, similar ROS production and smaller flight muscles compared to short-distance migrants. This surprising finding suggests that long-distance migrants (which are assumed to have the most difficult migrations) have flight muscles that have a lower capacity for exercise, which indicates that long-distance migrants use energy-efficient flight. The results of my studies show that mitochondrial function is dynamic with respect to migration in birds. These findings improve our understanding of how muscle metabolism can be changed to better meet the demands of endurance exercise.

Co-Authorship Statement

This dissertation contains a modified version of a peer-reviewed publication (Chapter 2) and two manuscripts in preparation for submission for publication (Chapters 3 and 4). In all cases, I am the first author. The main body of the manuscript for Chapter 2 has been incorporated into the data chapter, with supplementary materials in the appendices. The formatting of the manuscript for Chapter 2 has been modified for consistency with the dissertation.

A version of Chapter 2 was published in the *Journal of Experimental Biology* [citation: Coulson, S. Z., Guglielmo, C. G., & Staples, J. F. (2024). Migration increases mitochondrial oxidative capacity without increasing reactive oxygen species emission in a songbird. *Journal of Experimental Biology*, 227(9)]. I am the first author and Christopher Guglielmo (CGG) and James Staples (JFS) are co-authors. I designed the experiment, collected and analyzed data, drafted the manuscript and CGG and JFS contributed to experimental design, writing of this manuscript and provided funding.

A version of Chapter 3 is in review following submission to the *Journal of Avian Biology*. I am the first author, and Catherine Ivy (CMI), JFS and CGG are co-authors. CMI contributed to data collection (pectoralis ultrastructure) and analysis, and JFS and CGG contributed to experimental design and writing of this manuscript and provided funding.

Chapter 4 is in preparation for submission to *Proceedings of the Royal Society B*. I am the first author, and JFS and CGG are co-authors. CGG and JFS contributed to experimental design and writing of this manuscript and provided funding.

Acknowledgments

"Start a new chapter, find what I'm after
It's changing every day
The change of a season's enough of a reason
To want to get away" -Neil Peart

A Ph.D. is often regarded as the pinnacle of modern education. While intelligence, cleverness, etc. are certainly important drivers of success during doctoral studies, I think that persistence and resilience are equally (if not more) important. To this end, I am certain that the successes and progress that I have enjoyed during my doctoral studies could not have happened without the extensive support that I have received throughout my time at Western.

I would first like to thank my supervisors, Drs. Jim Staples and Chris Guglielmo. It has been a pleasure working on this dissertation under your supervision and I thank you for the endless guidance that you have given me over the past five years. Jim's focus on the present and fine attention to detail with Chris's focus on the future and ambition make for an effective supervisory team. First-hand experience in how your labs are organized and how your grad students are supported has given me much to reflect on how I might supervise students in the future. I would also like to thank Drs. Natasha Mhatre and Tim Regnault for their efforts on my advisory committee. I also thank the examiners of my dissertation, Drs. Mark Bernards, Rob Cumming, Carrie Branch and Wendy Hood for their insightful comments and great discussion during my thesis defence.

Next, I would like to thank my friends and colleagues in the Guglielmo lab, including Scott Walters, Kevin Young, Corrine Genier and Drs. Catie Ivy, Sara Lupi, Andrew Beauchamp, Jessica Deakin and Morag Dick. I would especially like to thank Catie for all of the work that she has done at AFAR that allowed me to do the work presented in this dissertation, including (but not limited to) watching blackpoll warblers flying in a wind tunnel for more than 40 hours cumulatively. Thank you for all the stimulating discussions over the past several years and fun at social events. Thank you to Sara Lupi and Kevin Young for initiating me into the world of ornithology with training on bird handling and wind tunnel operation. I would also like to thank all of the students in the Staples lab, including Brynne Duffy, Amalie Hutchinson and Lauren Rego for all of the fun that we have had and for making my Ph.D. thesis an enjoyable one. As

the senior student in the lab, it has been gratifying to see you all develop throughout grad school and I am certain that you will go on to do great things.

I am also grateful for the friends that I have made in and outside the biology department, including Hershy Japraikash, Kaitlyn Ludba, Brad Bork, Lauren Witterick, Garth Casbourne and Drs. Jackie Lebenzon and Ryley Yost. Thank you for the many fun evenings at the grad club and thank you to Jackie for serving as a model grad student, both in the department and at CSZ. I am also grateful to the friends that I have made before my time at Western that supported me during my studies, including Tom Chen, Lionel Alforque, Sherry Du, Ryan Lau, Taylor Brooks, Liz Toop, Nicole Pranckevicius, Audrey Wilson, Grant Holman and Drs. Manreet Dhaliwal, Katie Pelletier, David Green, Zach Dickson, Andrea Murillo. I value the time that we spent together, particularly during the social isolation associated with the Covid-19 lockdowns. I would also like to thank the Natsuoka karate club in Toronto for their support.

I also thank Francis Boon and Michela Rebuli for their supporting efforts in maintaining birds in captivity at the AFAR. I would also like to thank Long Point Bird Observatory and Birds Canada for their support of this research, as well as NSERC and the Government of Ontario for financially supporting my research.

Lastly, I would like to thank my family. Mom, Dad, Lauren, Megan, Zack, Spencer, thank you for your continuing, unwavering support over these last eleven years of post-secondary education. There were many trials and tribulations where I wanted to quit along the way, but thanks to your support, I pulled through. I am grateful to my partner, Stefane Saruhashi for her direct support in improving the writing of this dissertation, formulating hypotheses and patiently listening to me talk about birds *ad nauseam*. Stefane, I am grateful for your trust, confidence, demeanour, excellent cooking and most of all, I am grateful that you showed me the value of the ‘life’ aspect of work-life balance, including a newfound hope for the future, because you are in it.

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List of Abbreviations

ANT	Adenine nucleotide translocase
AUP	Animal use protocol
BSA	Bovine serum albumin
CPT	Carnitine palmitoyltransferase
CS	Citrate synthase
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
DTPA	Diethylenetriaminepentaacetic dianhydride
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ETS	Electron transport system
FRL	Free radical leak
HB	Homogenizing buffer
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HOAD	B-hydroxyacyl-CoA dehydrogenase
IMM	Inner mitochondrial membrane
LDH	Lactate dehydrogenase
NADH	β-Nicotinamide adenine dinucleotide
O_2^-	Superoxide
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TMPD	N,N,N′,N′-Tetramethyl-p-phenylenediamine
$\dot{V}O_2max$	Maximum rate of O2 uptake

Chapter 1

1 Literature review

1.1 Animal migration

Access to environmental resources such as food and shelter is critical for animals to survive and thrive. However, resource availability varies across space and time. In seasonal habitats, environmental resource availability has fluctuated fairly predictably over evolutionary time scales, so animals have evolved a suite of seasonally flexible physiological and behavioural traits to survive. Animals may remain active year-round within an environment and tolerate seasonal environmental challenges, or animals can avoid seasonal environmental challenges via dormancy (e.g. hibernation) or by moving to a different environment with better resource availability. These large-scale movements are typically performed via migration. Migration can be defined as a syndrome of behavioural and physiological traits that results in movements that are persistent, more direct and larger scale than any other movement throughout the annual cycle (Dingle, 2014). The migratory syndrome is expressed following exposure to signals that herald environmental degradation (e.g. changing daylength). This mechanism gives migratory animals sufficient time to re-allocate physiological resources and prepare for endurance locomotion. Animals use long-distance movements as part of a 'round trip' between two spatially disparate environments (Shaw, 2016). By timing migration to seasonal changes in resource availability, migratory animals can track areas of high resource abundance throughout the year, as shown in Palearctic-African migratory birds (Thorup et al., 2017). Migration allows animals to exploit spatiotemporal variation in resource abundance to invade seasonal environments.

Migration is expressed among diverse animal taxa, including mammals (Avgar et al., 2014), fish (Brönmark et al., 2014), flying insects (Satterfield et al., 2020), reptiles (Southwood and Avens, 2010), crustaceans (Adamczewska and Morris, 2001) and birds (Berthold et al., 2013), but see (Shaw, 2016) for a more comprehensive list of migratory taxa. As a result, there is considerable interspecific diversity in the migration syndrome. This diversity includes the hypothesized proximate benefits of migration, such as refuge

from seasonally inhospitable conditions, access to specialized breeding grounds or to track food resources (Shaw, 2016). Inter-specific diversity is also apparent in functional metrics of migration performance, such as speed of migration and total distance travelled. Migration speed (i.e. average speed across the migration season) is partly determined by mode of locomotion and body size in animals: at a body size of 1 kg, migration speed in flying animals is approximately 5-fold greater than in animals that use running or swimming (Hedenström, 2003). Migration speed in volant animals is high due to exceptionally high speed during movement, but at a high metabolic cost, which results in an intermediate cost of transport, relative to other modes of locomotion (i.e. cost per unit distance) (Schmidt-Nielsen, 1972). As a result, long-distance migration (10,000 km) has been hypothesized to be feasible (i.e. can be completed between winter seasons) in flying animals of all sizes, but not in small runners or swimmers (Alexander, 1998). The high migration speed of flight gives volant animals the potential for exceptionally long-distance movements. Indeed, the highest reported migration distance per mode of locomotion is ~38,000 km for fliers (Arctic tern (c. 0.1 kg); Egevang et al., 2010), ~13,000 km for swimmers (whale shark (c. 15,000 kg; Eckert and Stewart, 2001) and ~1,500 km for runners (blue wildebeest (c. 250 kg); Musiega and Kazadi, 2004). The disparity in maximal migration distance among modes of locomotion becomes more apparent after accounting for variation in body size: small flyers migrate two to three orders of magnitude more body lengths than swimmers or runners, respectively (Hein et al., 2012). As a result, volant animals regularly conduct the largest-scale movements on Earth. The underlying causes and consequences associated with these migration patterns are worthy of future study.

1.2 Avian migration

Among volant animals, birds are an excellent model to study migration. Migration occurs widely in birds; an estimated one in five of the c. 10,000 global bird species migrate as part of their annual life-history cycle (Kirby et al., 2008). Seasonal migration is the most common type of migration in birds, where birds travel biennially between breeding and non-breeding grounds. Migration is expressed in avian species from a wide variety of taxa that vary in body size, morphology, ecological niche, habitat, etc. The use of migration is partly determined by seasonality experienced on the breeding site. For example, the

proportion of migratory species that breed in the tropics is low (~10%) compared to birds that breed in more seasonal environments (temperate and arctic latitudes), reaching >80% above 65°N (Newton and Dale, 1996a; Newton and Dale, 1996b). Functional aspects of migration also vary among migratory species. For example, migration distance is highly diverse: Hermit thrush (*Catharus guttatus*) migrate ~2500 km, while Swainson's thrush (*C. ustulatus*) migrate ~7100 km (Pegan et al., 2024). Similarly, red-eyed vireos (*Vireo olivaceus*) migrate roughly double the distance compared to warbling vireos (*Vireo gilvus*), despite similar body size, morphology, life-history, etc. (Figure 1.1). Within species, short-distance migrant populations fly <250 km, while long-distance migrants fly >4500 km in lesser black-backed gulls (*Larus fuscus*) (Brown et al., 2023). Increasing migration distance is beneficial due to lower thermoregulatory costs and competition for resources at low latitudes, as shown in Northern Gannets (*Morus bassanus*) (Pelletier et al., 2020).

While migration may provide several benefits, it is not without consequences. Migratory flight is physiologically demanding and carries a high energetic cost, making migratory flight the most energy-expensive activity performed throughout the year (Brown et al., 2023). Migration also incurs a temporal cost: migratory flight may require birds to stop on route to replenish fuel. These stopovers may account for 7-fold more time than flight over a migration season (Hedenström and Alerstam, 1997). Lastly, migrants pass through novel, often inhospitable environments that ultimately increase the risk of mortality due to hazards like exposure to novel pathogens (Altizer et al., 2011) or predators, such as Eleonora's falcon (*Falco eleonorae*) (Samraoui et al., 2022; Xirouchakis et al., 2019). These challenges are hypothesized to increase with migration distance and may ultimately reduce survival and/or breeding opportunities. Indeed, among 46 species of Nearctic boreal forest-breeding birds (mostly passerine), long-distance migrants have fewer total offspring per breeding season yet higher annual adult survival rates relative to short-distance migrants (Winger and Pegan, 2021). This higher apparent survival despite the challenges associated with long-distance migration may be the result of behavioural and physiological adaptations that permit and support endurance locomotion. These adaptations are only partially understood and are an active area of investigation.

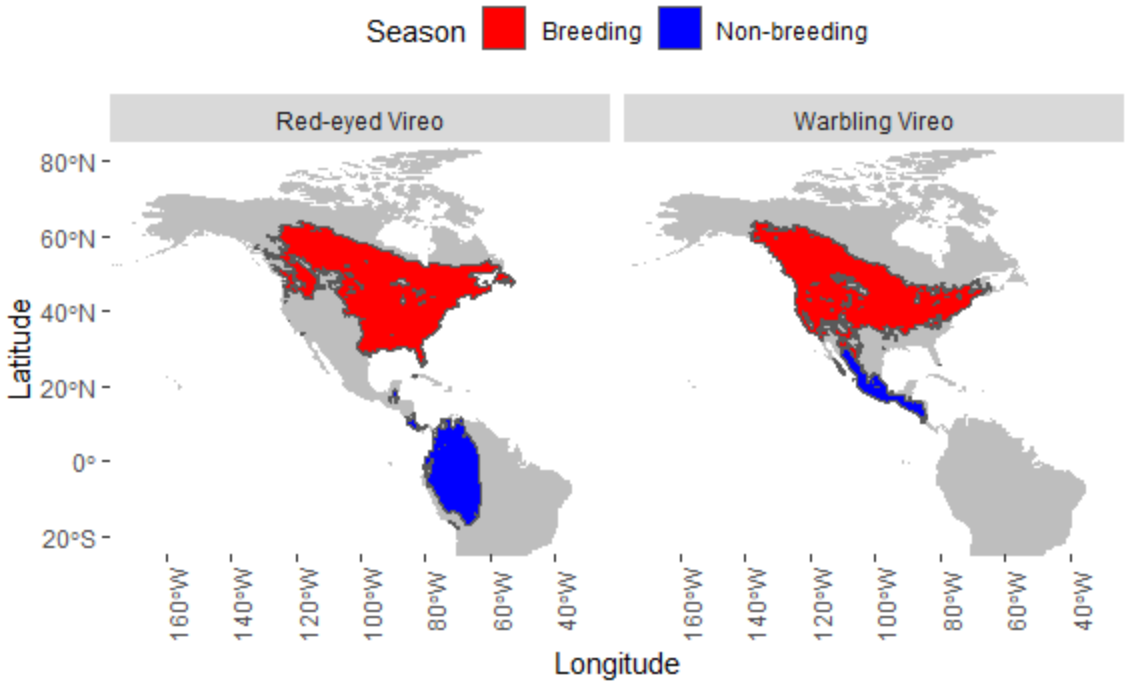

Figure 1.1: Evolved variation in migration strategy in songbirds. Range maps plotted for red-eyed vireo (*Vireo olivaceus*) and warbling vireo (*Vireo gilvus*) for breeding and non-breeding seasons. Red-eyed vireos migrate a further distance between breeding and non-breeding ranges than warbling vireos. Range maps generated using data from eBird Status and Trends (ebird.org).

1.3 Migration cycle in birds

Migration is variable among avian taxa, but the migration season for a typical migrant can be divided into a general schematic (Figure 1.2). Many birds use photoperiod as an exogenous cue for timing phases of the annual cycle (Helm and Liedvogel, 2024; MacDougall-Shackleton and Hahn, 2007). At a point midway through the non-migratory season, birds become photosensitive (i.e. responsive to photoperiod) and upon exposure to long-day photoperiods, birds become 'photo-triggered' and begin transitioning into a migratory phenotype. Alternatively, some species respond to different environmental cues, such as food availability (DeSimone et al., 2021). This stimulation initiates an endocrine signalling cascade, which is incompletely understood, but includes signalling from melatonin (Fusani et al., 2013) and thyroid hormones (Pathak and Chāndolā, 1982; Pérez et al., 2016) to induce the physiological and behavioural traits associated with the migratory syndrome. For example, migratory birds greatly increase fat mass by increasing energy intake (Coiffait et al., 2011; Cornelius and Hahn, 2012; Fry et al., 1972; Kobylkov et al., 2014; Pradhan et al., 2019; Tobolka et al., 2024; Vézina et al., 2021). Nocturnal activity also increases in passerines, as many species conduct migratory flights at night (Schmaljohann et al., 2015). Lastly, the flight muscle is remodelled at several levels of physiological organization to better support endurance flight performance (see section 1.5.1).

Once birds have fully transitioned into a migratory phenotype, they enter the next phase of migration: endurance flight. In this phase, birds make long-distance movements across diverse landscapes using a combination of flapping flight (especially in small or medium-sized birds) or soaring flight (large birds). Birds typically fly continuously for several hours, often covering distances of hundreds of kilometres. Some species migrate across ecological barriers (e.g. ocean, desert, mountain) that do not contain stopover habitats, so these flights may last several days non-stop, such as in blackpoll warblers (*Setophaga striata*) (DeLuca et al., 2015) or bar-tailed godwits (*Limosa lapponica*) (Gill et al., 2005). Once birds find a suitable environment to stop flight, they will stopover if that area is not their ultimate destination. Stopover ecology is multifaceted and is an active area of research (Schmaljohann et al., 2022). While there are many processes active during stopover, it is

accepted that one of the major aspects of stopover is recovery from flight and fuel replenishment. The decision-making process of when birds leave stopover is also complex but may be linked to fat load, wind conditions (Dossman et al., 2016) and appetite-regulating hormones, such as ghrelin (Goymann et al., 2017). Once birds depart their stopover site, they re-enter the flight phase and cycle between stopover and flight until they reach their ultimate destination. At this time, birds become photorefractory, where they are no longer responsive to changes in photoperiod (MacDougall-Shackleton and Hahn, 2007) and then transition out of the migratory phenotype, which includes losses in fat mass and pectoralis oxidative capacity.

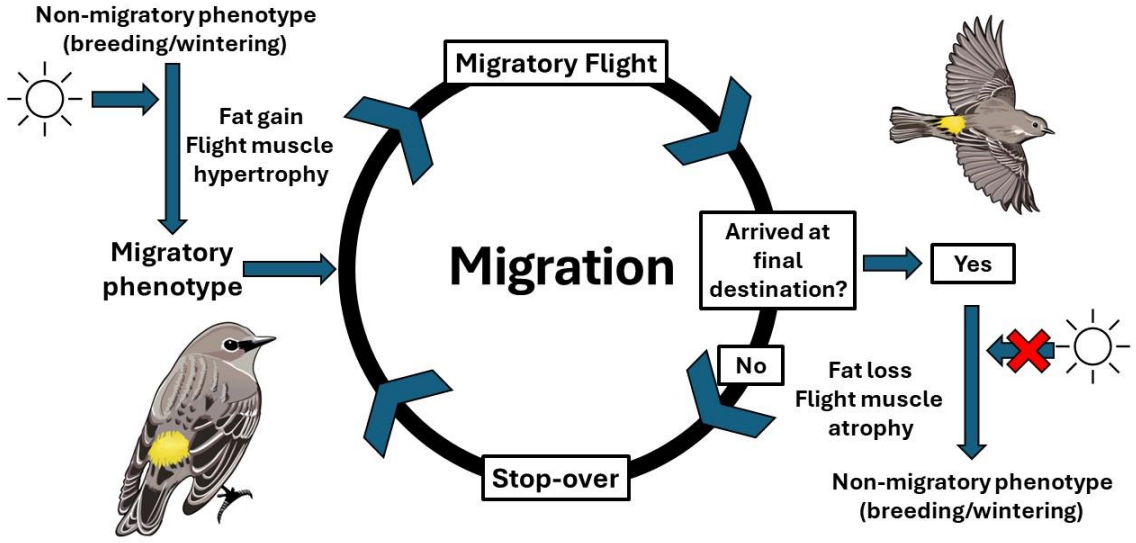

Figure 1.2: Schematic of typical migration cycle in birds. Birds transition into a migratory phenotype in response to photoperiod, where birds gain fat mass and hypertrophy their flight muscles. Once the migratory phenotype is fully expressed, birds repeatedly cycle between migratory flight and stop-over until reaching their destination. Birds are no longer sensitive to photoperiod and will transition to a non-migratory phenotype, which includes loss of fat mass, and flight muscle atrophy.

1.4 Physiological challenges of avian migratory flight

Migratory birds face a gauntlet of challenges during flight. Flapping flight is the most energetically demanding mode of locomotion (Schmidt-Nielsen, 1972): metabolic rates during flight are ~10-fold greater than at rest, more than double the maximal aerobic capacity ($\dot{V}O_2max$) of similarly sized running mammals (Butler, 1991) and may be sustained for hours to days (Guglielmo, 2018). This energetic burden is met primarily (i.e. >90%) with the oxidation of fatty acids (Dick and Guglielmo, 2019a; Guglielmo et al., 2017; Jenni and Jenni-Eiermann, 1998; Klaassen et al., 2000), although carbohydrate and protein oxidation are prominent early in flight (Dick and Guglielmo, 2019b; Elowe et al., 2023a; Gerson and Guglielmo, 2013; Rothe et al., 1987). Protein oxidation occurs throughout flight via catabolism of lean tissues (Battley et al., 2000; Bauchinger and Biebach, 2001; Bauchinger et al., 2005; Biebach, 1998; Schwilch et al., 2002), which is hypothesized to be important to maintain water balance and generate intermediate metabolites for the mitochondrial tricarboxylic acid (TCA) cycle (Jenni and Jenni-Eiermann, 1998). Fatty acids are the best available fuel source for endurance flight, because they are more energy-dense and have a significantly greater storage capacity than carbohydrates or proteins (Jenni and Jenni-Eiermann, 1998). The high reliance on fatty acids during flight in birds is in stark contrast with running mammals which must rely primarily on carbohydrate oxidation to fuel exercise at medium to high intensities (Weber, 2011). The high use of fatty acids during flight is associated with elevated capacities for their uptake by flight muscles and delivery to mitochondria (Guglielmo, 2010).

A second challenge faced by birds during migratory flight is damage incurred to muscles that are active during flight. For example, muscle plasma membranes exhibit higher permeability (as inferred by increased circulating creatine kinase activity) following migratory flight in shorebirds (Guglielmo et al., 2001; Thomas and Swanson, 2013). This higher membrane permeability may be the result of oxidative damage accumulation during migratory flight, which has been extensively reviewed previously (Cooper-Mullin and McWilliams, 2016; Costantini, 2008; Jenni-Eiermann et al., 2014; McWilliams et al., 2021). During exercise, the production of oxidants, such as reactive oxygen species (ROS), increases with exercise intensity in contracting muscle (Lovlin et al., 1987). Increased ROS

production can disturb cellular redox balance (Sies et al., 2024), with significant consequences for muscle function, including lower force production and fatigue development (Powers and Jackson, 2008). At high exercise intensities, ROS production overcomes the quenching capacity of antioxidants, resulting in oxidative stress in active myocytes, as shown in mammals (Powers et al., 2020). Currently, most assessments of oxidative stress in migrating birds rely on plasma samples and find that migratory flight is associated with elevated oxidative damage markers and diminished total antioxidant capacity, as shown in several passerine species (Costantini et al., 2007; Eikenaar et al., 2020; Eikenaar et al., 2023b; Jenni-Eiermann et al., 2014; Skrip et al., 2015). Similar trends have been observed following 5-hour flights in homing pigeons (*Columbia livia domestica*) (Costantini et al., 2008) and 3 hours of perch-to-perch flights in zebra finches (*Taeniopygia guttata*) (Costantini et al., 2013). However, the physiological origin of these circulating markers is unknown, so inferences to oxidative stress in the flight muscle are limited. To my knowledge, flight muscle oxidative stress associated with migratory flight has been assessed in only one study to date, which found that 6-hour wind tunnel endurance flight increased flight muscle protein carbonyl accumulation in yellow-rumped warblers (*Setophaga coronata*) (Dick and Guglielmo, 2019a). Migratory birds must therefore manage the various physiological challenges associated with endurance flight, or risk potentially lethal losses in flight performance.

Migratory birds may mitigate oxidative stress incurred during flight using diverse physiological strategies that act in concert. Migratory birds may lower the risk of oxidative stress by two non-mutually exclusive mechanisms: lower ROS production and increased antioxidant capacity. Whether migratory birds suppress ROS production is unknown, but potential underlying mechanisms include lower flight muscle activities of xanthine oxidase and NADPH oxidase, which produce ROS in the cytosol (Powers and Jackson, 2008). An additional mechanism to lower ROS production is to decrease rates of electron leak from the mitochondrial electron transport system (ETS). In turn, lower electron leak may occur by decreasing ATP synthesized per O_2 consumed, thereby ‘uncoupling’ respiration from ATP synthesis (Brand, 2000). Alternatively, electron leak may be lowered by increasing ‘spare capacity’ of the ETS, as seen in house sparrows (*Passer domesticus*) compared to mice (Brown et al., 2009). Migratory birds may increase antioxidant capacity via intake of

dietary antioxidants and/or by increasing expression of antioxidant proteins (Cooper-Mullin and McWilliams, 2016). Indeed, non-enzymatic antioxidant capacity (concentrations of molecules including uric acid, α-tocopherol, ascorbate (Cooper-Mullin and McWilliams, 2016)) is higher in migratory than non-migratory populations, as shown in common blackbirds (*Turdus merula*) (Eikenaar et al., 2017). Furthermore, non-enzymatic antioxidant capacity increases in anticipation of long flights in songbirds (Skrip et al., 2015) and shorebirds (Gutiérrez et al., 2019). Dietary antioxidant supplementation via anthocyanin intake has complex effects on oxidative balance in migratory birds. Generally, high anthocyanin intake decreases oxidative damage and increases antioxidant capacity, though this effect varies among (Frawley et al., 2021b). In contrast, anthocyanin supplementation has little effect on total antioxidant capacity in blood, potentially due to lower circulating uric acid (Frawley et al., 2021a). Notably, some dietary interventions may increase the risk of oxidative stress during migratory flight, such as increased intake of n-6 polyunsaturated fatty acids (McWilliams et al., 2020). A second strategy available to migratory birds is prompt repair of oxidative damage and replenishment of antioxidant capacity. Indeed, circulating oxidative damage markers decrease and antioxidant capacity increases with time spent at stopover (Eikenaar et al., 2020; Eikenaar et al., 2023b; Skrip et al., 2015). It is presently unknown if migratory birds modify oxidative damage repair mechanisms (reviewed by Davies, 2000) in preparation for, or in response to endurance flight. However, the importance of oxidative stress management in avian migration has recently been called into question, as stopover departure decision-making is unrelated to circulating concentrations of malondialdehyde (lipid oxidation damage marker) or total antioxidant capacity in song thrush or northern wheatears (Eikenaar et al., 2023a).

1.5 Determinants of flight muscle metabolism

Migratory birds have evolved a high capacity for endurance exercise performance to meet the physiological demands of long-distance flight. Endurance exercise performance is multifaceted and difficult to quantify but has previously been expressed in human exercise physiology literature as the average velocity maintained by an individual during continuous long-duration (i.e. several minutes to hours) exercise (Joyner and Coyle, 2008). Several aspects of skeletal muscle phenotype have emerged as strong predictors of overall

endurance performance and its underlying determinants as per the mammalian model (van der Zwaard et al., 2021), so study of skeletal muscle physiology is likely to provide insights on endurance flight performance in migratory birds. Briefly, the determinants of endurance performance are $\dot{V}O_2max$, critical power (i.e. the highest exercise intensity that can be maintained), locomotory efficiency (i.e. efficiency of converting metabolic power to mechanical power) (Joyner and Coyle, 2008) and resilience (i.e. the degree of change of the previous determinants over an exercise bout) (Jones, 2023). While the mammalian model of endurance exercise performance has yet to be validated in migratory birds, the highly conserved nature of vertebrate skeletal muscle form and function in relation to locomotion is likely to make the mammalian model applicable to exercising birds. The flight muscle (pectoralis major) is a large skeletal muscle in the thoracic cavity that is remodeled in response to variation in energy demands (Swanson et al., 2022). The pectoralis is responsible for generating wing downstroke and is therefore highly active during migratory flight (Biewener, 2022), so changes in pectoralis phenotype are likely to translate to changes in migratory flight performance. The form and function of skeletal muscle is determined by phenotypic plasticity and selective pressure, so flight muscle physiology in migratory birds is hypothesized to be seasonally flexible and vary with evolved variation in locomotory demand.

1.5.1 Seasonal flexibility in flight muscle metabolism

Migratory birds use seasonal phenotypic flexibility to remodel whole-animal metabolism to better support endurance locomotion in migratory seasons. In preparation for migration, some bird species increase overall flight muscle size (DeMoranville et al., 2019; Elowe and Gerson, 2022; Fry et al., 1972) via hypertrophy of muscle fibers (Evans et al., 1992; Gaunt et al., 1990; Marsh, 1984; Vézina et al., 2021), which may occur in the absence of exercise training (Dietz et al., 1999; Price et al., 2011). However, some other migratory species show no seasonal changes in pectoralis size (King et al., 2015; Pradhan et al., 2019), or no changes in fiber size or lower fiber size in the migratory phenotype (Ivy and Guglielmo, 2023; Lundgren and Kiessling, 1988). Explanations for these disparities remain to be drawn. The mechanisms underlying seasonal hypertrophy are poorly understood but may include increased expression of insulin-like growth factor 1 (Pradhan et al., 2019;

Price et al., 2011), but see (Elowe et al., 2023b). Satellite cells are hypothesized to mediate changes in muscle fiber diameter in birds (Jimenez, 2020), but little is known about how satellite cells function in migratory birds other than that *in vitro* proliferation rates are among the highest reported for vertebrates (Young et al., 2021). Pectoralis fiber type proportions are also seasonally modulated to contain more fast oxidative glycolytic fibers and fewer fast glycolytic fibers in the migratory phenotype, but this seasonal flexibility varies with phylogeny and migration distance (Chang et al., 2024). Lastly, seasonal flexibility in pectoralis capillary supply remains unclear: capillary density is higher in the migratory phenotype than wintering in some Nearctic-Neotropical migratory passerine species (yellow-rumped warbler, hermit thrush, Swainson's thrush), but capillary density is similar between seasons in warbling vireo (*Vireo gilvus*), red-eyed vireo (*Vireo olivaceus*) and blackpoll warbler (Ivy and Guglielmo, 2023). In contrast, capillary density is lower in the migratory phenotype than breeding in some Palearctic-Afrotropical migratory passerine species - European robin (*Erithacus rubecula*), common blackbird, common reed warbler (*Acrocephalus scirpaceus*), willow warbler (*Phylloscopus trochilus*) and reed bunting (*Emberiza schoeniclus*) – while capillary density is unaffected by season in 7 other species (Lundgren and Kiessling, 1988). Together, these seasonal changes contribute to a greater capacity for force production, which is hypothesized to better meet the increased flight demands associated with greater body mass during migration.

Like muscle ultrastructure, muscle metabolism is seasonally flexible in migratory birds. The activities of several mitochondrial enzymes (citrate synthase, carnitine palmitoyl-transferase, β-hydroxyacyl-CoA dehydrogenase) are higher in the migratory than non-migratory phenotype when expressed relative to tissue mass (Banerjee and Chaturvedi, 2016; Dick, 2017; Lundgren and Kiessling, 1985; Lundgren and Kiessling, 1986; Marsh, 1981; Rhodes et al., 2024; Sharma et al., 2021; Zajac et al., 2011), but see (DeMoranville et al., 2019; Lundgren, 1987; Price et al., 2010). Similarly, the abundance of proteins associated with fat transport from the circulation to the mitochondria are also increased during migration (Zajac et al., 2011; Zhang et al., 2015). These seasonal metabolic changes contribute to an overall greater oxidative capacity of the flight muscle during migration, which increases the capacity for fat use during flight. In turn, a greater muscle oxidative

capacity may increase endurance flight performance, as predicted by mammalian endurance exercise models (van der Zwaard et al., 2021).

1.5.2 Evolved variation in flight muscle metabolism

Variation in evolved migration strategy partially determines flight muscle phenotype. In contrast to seasonal flexibility, little is known about how evolved variation in migratory strategy has affected flight muscle ultrastructure and oxidative metabolism. For example, one study reported similar pectoralis mass across 84 Palearctic-breeding passerine species that migrate between ~5 to ~7900 km (Vágási et al., 2016). However, these measurements were made in the breeding season, presumably when flight muscles have atrophied post-migration, so it remains unclear how flight muscle size varies with migration distance. Other studies have shown that long-distance migration is associated with smaller diameter fibers and greater capillary density (Lundgren and Kiessling, 1988). Fiber type proportions are unaffected by migration distance when compared between short- and long-distance congeners in wood-warblers (Parulidae) and vireos (Vireonidae), but fast oxidative glycolytic fiber density is greater in long-distance migratory thrush (Turdidae) (Chang et al., 2024). Intraspecifically, mitochondrial enzyme activities are higher in migratory compared to non-migratory populations in three Palearctic-breeding passerine species (Lundgren, 1988). Similarly, flight muscle mitochondrial abundance is greater in migratory compared to non-migratory subspecies in white-crowned sparrows (Rhodes et al., 2024), but not in yellow-rumped warblers, though these birds were sampled in the breeding season (Toews et al., 2014).

1.6 Mitochondrial determinants of exercise performance

Mitochondria fuel movement. During endurance exercise, muscle mitochondria synthesize most of the ATP required to fuel contractions at rates that may be 100-fold greater than at rest (Hochachka and Matheson, 1992). Mitochondrial physiology is also a direct determinant of muscle oxidative capacity, which is a significant predictor of endurance exercise performance in mammalian models (van der Zwaard, 2021). High oxidative capacity (and therefore high endurance exercise performance) may be achieved by two non-mutually exclusive mechanisms: increases in total mitochondrial abundance or

increases in individual mitochondrial capacity. Both traits may increase in response to higher endurance locomotory demands on long timescales (i.e. across generations), or short timescales (e.g. training) to improve endurance exercise performance.

1.6.1 Mitochondrial physiology

Mitochondria synthesize ATP via oxidative phosphorylation (OXPHOS), which involves the use of free energy liberated by substrate oxidation to power the phosphorylation of adenosine diphosphate (ADP) into ATP. Substrate oxidation in the mitochondrial matrix yields high-energy electrons that transit through protein complexes of the ETS, ultimately reducing O_2 to H_2O (i.e. respiration). Electron movement through the ETS is used to actively pump H^+ from the matrix to the inter-membrane space. This H^+ flux establishes and maintains a H^+ concentration gradient and electric potential (i.e. membrane potential) across the inner mitochondrial membrane, together known as the proton-motive force. This proton-motive force is 'dissipated' by the passive movement of H^+ back into the matrix. H^+ may re-enter the matrix through F_1/F_o-ATPase (complex V) to phosphorylate ADP into ATP, or H^+ may bypass complex V and re-enter the matrix without contributing to ATP synthesis in a process termed 'proton leak'.

Mitochondria also contribute to cellular ROS dynamics. However, these dynamics are complex because ROS are both formed and quenched in mitochondria and each process is regulated by a diverse array of factors (Kowaltowski et al., 2009). The primary ROS generated by mitochondria is the highly reactive, yet membrane-impermeable superoxide ($O_2^{\bullet -}$). Superoxide is formed when an electron prematurely leaks from the ETS and reduces O_2 (Hernansanz-Agustín and Enríquez, 2021). While there are multiple sites in the ETS from which electrons can leak to form superoxide, complexes I and III are the predominant sites (Quinlan et al., 2012). Superoxide formation rates are sensitive to several factors, including O_2 concentration, substrate availability and membrane potential (Treberg et al., 2018). Most superoxide is quenched by intramitochondrial antioxidants. First, superoxide is dismuted by the enzymatic antioxidant superoxide dismutase into the less reactive, yet membrane-permeable H_2O_2. In turn, H_2O_2 is either quenched by another antioxidant (e.g. glutathione peroxidase) or emitted into the cytosol (Treberg et al., 2015). The contribution of mitochondrial ROS to cellular oxidative stress is controversial, with some authors

arguing for (Ježek and Hlavatá, 2005) and against (Palma et al., 2023; Zhang and Wong, 2021) mitochondria as a major source of ROS. Other authors have suggested that the primary effect of mitochondrial ROS is to induce intracellular signalling (Palma et al., 2023). Additional work in this area is required to resolve the physiological significance of mitochondrial ROS dynamics.

1.6.2 Acute effects of exercise on mitochondrial function

Exercise causes significant changes in the intracellular milieu in muscle. For example, oxidative stress is a common result of muscle contractions (Powers et al., 2020). This oxidative stress has been linked to mitochondrial dysfunction, wherein mitochondria suffer decreases in capacity for ATP synthesis (Ostojic, 2016). Intense endurance exercise results in lower mitochondrial respiratory capacity, as shown in horses (Chen and Gollnick, 1994; Gollnick et al., 1990), rats (Ding et al., 2010) and humans (Larsen et al., 2016; Layec et al., 2018; Lewis et al., 2021). Furthermore, extreme endurance exercise (24 hours of kayaking/running/cycling at 55% $\dot{V}O_2$peak) in humans results in increased mitochondrial ROS emission (Sahlin et al., 2010). The mechanisms underlying exercise-induced mitochondrial dysfunction have yet to be revealed. Potential mechanisms include compromised ETS function, which may occur with carbonylation of proteins in the ETS, or peroxidation of inner mitochondrial membrane phospholipids, such as cardiolipin (Chicco and Sparagna, 2007). It also remains unclear whether wild animals experience mitochondrial dysfunction in response to exercise.

1.6.3 Mitochondrial metabolism in migratory birds

As sources of ATP and ROS in muscle, mitochondrial function may play a large role in supporting endurance flight in migratory birds. Indeed, flight muscle mitochondrial abundance is seasonally flexible in migratory birds and increases during migration (e.g. Banerjee and Chaturvedi, 2016; Rhodes et al., 2024; Zajac et al., 2011). Furthermore, the capacity for mitochondrial phosphorylating respiration fuelled by fatty acids is higher during migration, reflecting greater capacities for ATP synthesis and fatty acid oxidation per mitochondrion (Rhodes et al., 2024). Together, these data indicate that the flight muscle mitochondrial phenotype is seasonally flexible to support greater fatty acid oxidation

during migration. However, seasonal flexibility in mitochondrial ROS dynamics remains unexplored. Mitochondrial function has also been shown to be sensitive to evolved variation in migratory strategy. Specifically, migratory subspecies have greater mitochondrial abundance than residents in goldcrests (*Regulus regulus*), great tits (*Parus major*), yellowhammers (*Emberiza citronella*) (Lundgren, 1988) and white-crowned sparrows (*Zonotrichia leucophrys*) (Rhodes et al., 2024), but not yellow-rumped warblers, though these birds were not sampled during migration (Toews et al., 2014). Furthermore, mitochondrial respiration is more tightly coupled to ATP synthesis in migratory compared to non-migratory subspecies (Rhodes et al., 2024; Toews et al., 2014). These evolved patterns are like those observed in seasonal flexibility, where the evolution of migration is associated with elevated capacities of fatty acid oxidation and ATP synthesis. The effects of migration strategy on mitochondrial ROS dynamics also remain unexplored. Furthermore, it remains unclear how mitochondrial physiology varies with migratory strategy when compared among migratory species (i.e. long-distance vs. short-distance migrants).

Little work has been done on investigating the effects of physiological damage incurred during flight on mitochondrial physiology. Mitochondrial abundance is unaffected by 6- and 4-hour wind tunnel flights in yellow-rumped warblers (Dick, 2017) and European starlings, respectively (Price et al., 2022). The only study to date to assess mitochondrial function following endurance flight found reduced pyruvate-fuelled phosphorylating respiration following c. 3 hour wind tunnel endurance flight in European starlings, but fatty acid-fuelled respiration was unaffected (Gerson, 2012).

1.7 Research summary

The overall objective of my thesis is to characterize mitochondrial physiology in relation to meeting the demands of migratory flight in songbirds. My findings will broaden the understanding of evolved metabolic strategies used by migratory birds to meet the physiological challenges associated with extreme endurance locomotion.

In Chapter 2, I tested the hypothesis that seasonal flexibility in mitochondrial function contributes to improved flight performance, predicting greater capacity for fatty acid oxidation and lower ROS emission in a migratory compared to a non-migratory phenotype. I tested this hypothesis using photoperiod manipulations in yellow-rumped warblers. I compared warblers freshly caught during migration to warblers on a non-migratory phenotype induced by a short-day photoperiod. Surprisingly, I found that pectoralis mass was lower in the migratory phenotype. However, mitochondrial abundance per unit mass and per whole organ was greater in the migratory phenotype, as were the activities of two mitochondrial enzymes used for fat catabolism. In addition, I found greater phosphorylating and non-phosphorylating respiration fuelled by fatty acids in the migratory phenotype. As a result, mitochondrial respiration was more uncoupled from ATP synthesis in the migratory phenotype, which likely contributed to lower rates of ROS emission. These findings show that flight muscle mitochondria are seasonally flexible in migratory songbirds and that this flexibility contributes to increasing oxidative capacity while mitigating the risk of oxidative stress.

In Chapter 3, I tested the hypothesis that endurance flight induces mitochondrial dysfunction in migratory songbirds, where I predicted that endurance flight would reduce OXPHOS capacity by decreasing fatty acid-fuelled phosphorylating respiration or increasing non-phosphorylating respiration and increasing ROS emission. I also tested the hypothesis that migratory songbirds selectively atrophy lean tissues during endurance flight, predicting that digestive tissues would be more catabolized than tissues essential for flight. I tested these hypotheses using a wind tunnel to simulate overnight migratory flight in blackpoll warblers. I compared birds following an eight hour wind tunnel flight, an eight hour fast and in resting conditions. In each group, I assessed flight muscle mitochondrial physiology, muscle ultrastructure and masses of several tissues in the flight apparatus (pectoralis, heart, lungs) and digestive system (liver, gizzard and proventriculus, digestive tract). I found that mitochondrial respiration and ROS emission were generally unaffected by endurance flight. While some respiration metrics (e.g. ETS complex II-IV flux) were lower in flown than fasted birds, flown birds were similar to rested controls. Similarly, muscle ultrastructure was unaffected by flight. I also found that endurance flight was associated with marginal reductions in masses of tissues in the digestive system (liver,

gizzard and proventriculus), while tissue mass in the flight apparatus (heart, pectoralis, lungs) was unaffected. These data suggest that in migratory songbirds, the flight muscle is robust against damaging aspects of endurance flight and that lean tissue may be selectively catabolized to protect tissues vital to maintaining flight performance.

In Chapter 4, I tested the hypothesis that evolved variation in mitochondrial function and pectoralis metabolism contribute to increased oxidative capacity in long-distance migrants. I predicted that long-distance migrants would have the highest pectoralis oxidative capacity, due to large pectoralis size, high mitochondrial abundance and capacity for phosphorylating respiration, while minimizing ROS emission. I used a comparative design that included 19 songbird species of varying migratory strategies, including resident (i.e. non-migratory), short-distance and long-distance migrants and that varied in migration distance between ~800 to ~7400 km. In contrast to my prediction, I found that pectoralis size was lowest in long-distance migrants, and mitochondrial respiration and ROS emission were generally similar among migratory strategies. Within migratory species, mitochondrial phosphorylating and ETS capacity declined with estimated migration distance. Together, these data indicate that mitochondrial function varies with migration strategy, but not to increase oxidative capacity in long-distance migrants. Instead, long-distance migrants have evolved a reduced flight muscle oxidative capacity, which may reflect a migration strategy centered on minimizing energy expenditure.

Together, my findings expand the understanding of migration physiology by highlighting the important role of mitochondria in supporting endurance locomotion. Flight muscle mitochondrial abundance and function are modulated by seasonal flexibility to increase tissue oxidative capacity and are resilient against the damaging aspects of endurance flight. In contrast, the evolution of long-distance migration is associated with reductions in flight muscle oxidative capacity, which may reflect lower flight costs experienced by long-distance migrants.

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Chapter 2

2 Migration increases mitochondrial oxidative capacity without increasing reactive oxygen species emission in a songbird

2.1 Introduction

The mitochondrion is a dynamic organelle that is plastic in form and function in response to changes in cellular energy demands. This mode of plasticity allows animals to modulate cellular energy supply to maintain performance despite variation in energy demand. One activity of high energy demand is locomotion, and performance in this activity has a strong determining effect on predator evasion and foraging success. Chronic increases in locomotory activity elicit profound changes in mitochondrial physiology. For example, endurance exercise training increases muscle mitochondrial abundance in humans (Fernström et al., 2004; Meinild Lundby et al., 2018; Tonkonogi et al., 2000), rats (Davies et al., 1981; Galbès et al., 2008; Krieger et al., 1980; Zoll et al., 2003) (but see Farhat et al., 2015) and mice (Beyfuss et al., 2018; Caffin et al., 2013; Heo et al., 2021; Pastore and Hood, 2013; Southern et al., 2017). These increases in mitochondrial abundance typically result in greater OXPHOS capacity when measured in permeabilized muscle fibers of humans (Meinild Lundby et al., 2018; Pesta et al., 2011), rats (Zoll et al., 2003) and mice (Heo et al., 2021). These changes in whole-tissue metabolism are partly explained by changes in function of individual mitochondria. Specifically, endurance exercise training increases phosphorylating respiration in isolated muscle mitochondria in humans (Fernström et al., 2004; Tonkonogi et al., 2000) and mice (Southern et al., 2017), but not in rats (Davies et al., 1981; Galbès et al., 2008). As a result, training can increase whole-tissue oxidative capacity through increases in mitochondrial abundance and improved mitochondrial performance, depending on the species. Together, these changes proximately improve the capacity for ATP synthesis via oxidative phosphorylation and ultimately improve endurance exercise performance.

While mitochondrial changes in response to endurance exercise training are well-defined, it remains poorly understood whether mitochondria respond similarly to increases in

locomotion in animals that have been selected for endurance locomotion over many generations. Artificial selection for high activity induces elevated activities of mitochondrial enzymes in locomotor muscles in captive lab mice (Guderley et al., 2006; Houle-Leroy et al., 2003; Templeman et al., 2012), although most of these differences may be explained by variation in training volume (Houle-Leroy et al., 2000). It remains unclear how natural selection for high locomotory activity may affect mitochondrial physiology in wild animals, or in animals where locomotory activity changes seasonally.

Migratory birds provide an excellent model to study adaptations for endurance locomotion, due to the use of long-distance movements between spatially disparate breeding and non-breeding grounds as part of their annual life-history cycle. These movements are performed via flight and allow birds to exploit spatiotemporal variation in resource availability. Aspects of migratory performance including timing and speed of migration are directly linked to reproductive performance via access to high-quality breeding territories, as shown in American redstarts (*Setophaga ruticilla*) (Smith and Moore, 2005). As a result, migratory performance, and its underlying physiological determinants, are under natural selection in songbirds. For all its potential benefits, migration is a significant challenge for birds because migratory flight is associated with the greatest movement requirements across the annual cycle, as shown in red-backed shrikes (*Lanius collurio*) (Macías-Torres et al., 2022). Furthermore, migratory flight is a highly demanding mode of locomotion that requires metabolic rates approximately 12-fold higher than at rest and may be sustained continuously for several hours to days at a time (reviewed by Guglielmo, 2018). These high rates of oxidative metabolism are also associated with increased reactive oxygen species (ROS) formation during flight, which may oxidatively damage tissues and potentially compromise tissue function if ROS formation exceeds antioxidant capacity (Jenni-Eiermann et al., 2014). However, migratory birds have acquired a suite of physiological adaptations in the flight muscle to overcome these demands, including high capacities for fatty acid oxidation (reviewed by Guglielmo, 2018), made possible by elevated oxidative capacity (Lundgren, 1988) and a higher degree of coupling between mitochondrial respiration and oxidative phosphorylation (Toews et al., 2014) relative to non-migratory birds.

Daily energy expenditure during migration may be the highest across the annual cycle and two-fold greater than in the wintering (i.e. non-breeding) season, as shown in lesser black-backed gulls (*Larus fuscus*) (Brown et al., 2023). In response to this seasonal variation in energy demands, migratory birds have evolved an impressive degree of seasonal flexibility in flight muscle energy metabolism. Before migration, in the absence of training (Price et al., 2011), birds remodel the flight muscle to improve endurance flight performance via increased capacities for O_2 and fuel delivery to mitochondria and consumption by mitochondria. These changes are achieved via reduced fiber diameter and increased capillary density (Ivy and Guglielmo, 2023), and increased expression of fatty acid binding protein and fatty acid translocase (Zajac et al., 2011; Zhang et al., 2015). At the mitochondrial level, flight muscle activities of β-hydroxyacyl-CoA dehydrogenase, citrate synthase, and carnitine palmitoyl transferase can increase by 20-150%, 25-50% and 50-100%, respectively (Banerjee and Chaturvedi, 2016; Lundgren and Kiessling, 1986; Marsh, 1981; McFarlan et al., 2009; Sharma et al., 2021; Zajac et al., 2011), but see (DeMoranville et al., 2019; Price et al., 2010). However, it is not clear if these changes simply reflect an increase in mitochondrial abundance or changes to mitochondrial function. Moreover, much less is known about seasonal flexibility in ROS management in the flight muscle of migratory songbirds, except to note that protein expression of Mn and Cu/Zn superoxide dismutases increase ~1.2-fold prior to migration in red-headed buntings (Banerjee and Chaturvedi, 2016). Elevated capacities for ATP synthesis and ROS quenching may be beneficial for other phases of the annual life-history cycle beyond migration, however birds transition out of the migratory phenotype following completion of migration. It is currently unclear why birds transition out of the migratory phenotype, but one potential explanation is a high physiological cost of maintaining the elevated total mitochondrial volume in flight muscle.

Migratory flight is fueled largely by oxidation of fatty acids by flight muscle mitochondria (reviewed by Guglielmo, 2018), so we wanted to better understand how mitochondria respond seasonally in songbirds. In addition, the mitochondrial electron transport system (ETS) is a source of intracellular ROS production (reviewed by Hernansanz-Agustín and Enríquez, 2021). To our knowledge, no previous studies have examined seasonal variation in mitochondrial function in migratory birds. However, previous work has shown mixed

evidence of seasonal mitochondrial plasticity in non-migratory songbirds that experience high wintering energy demands. Specifically, cold acclimation (which increases basal metabolic rate) has no effect on mitochondrial respiration in permeabilized pectoralis fibers from captive black-capped chickadees (*Poecile atricapillus*) (Milbergue et al., 2022), while winter acclimatization increases leak respiration in red blood cell mitochondria in three species of Palearctic tits (Nord et al., 2021).

2.1.1 Objective and hypothesis

The objective of this study was to investigate how seasonal plasticity of flight muscle metabolism is mediated by changes in mitochondrial physiology in migratory songbirds. I hypothesized that mitochondria are seasonally plastic, and that their function would be altered in the migratory phenotype to support the physiological challenges of migratory flight. There are two non-mutually exclusive strategies to achieve this transition: 1) an increase in mitochondrial quantity, and 2) an increase in mitochondrial quality. Under strategy 1, I predicted a higher pectoralis mitochondrial abundance in the migratory phenotype. Under strategy 2, I predicted that the migratory mitochondrial phenotype would include elevated capacity for phosphorylating respiration and lower or similar rates of ROS formation, compared with the non-migratory phenotype. I tested these hypotheses by comparing the mitochondrial abundance and *ex vivo* function of flight muscle mitochondria from captive yellow-rumped warblers (*Setophaga coronata*) between migratory and non-migratory phenotypes. To my knowledge, this study is the first to characterize the seasonal transition between migratory and non-migratory phenotypes in songbird mitochondrial function.

2.2 Methods

2.2.1 Experimental animals

All procedures followed guidelines set by the Canadian Council on Animal Care and were approved by the Western University Animal Care Sub-Committee (AUP 2018-092) and the Canadian Wildlife Service (SC-OR-2018-0256). I used a Nearctic migratory songbird, the myrtle subspecies of the yellow-rumped warbler (*Setophaga c. coronata*, Linnaeus, 1766). The myrtle warbler is a 10-15 g wood warbler that breeds in the boreal forest

throughout northern Canada and the northeastern United States and migrates to wintering grounds throughout the southern United States to as far south as Panama (Hunt and Flaspohler, 1998). The high spatial variation in migration phenology is likely the result of facultative migration extension (Terrill and Ohmart, 1984) and generalist habitat requirements (Parnell, 1969).

Warblers were captured during the autumn migration season (2020) in mist nets at Long Point, ON, Canada, 42.58°N, 80.40°W), a major stopover site for migrating songbirds. The age class and sex of each bird was determined from plumage and other characters, and wing chord was measured as a structural index of body size, as used previously in this and other songbird species (Kissner et al., 2003). I then transferred the birds into captivity at the Advanced Facility for Avian Research at Western University (London, ON, Canada) and housed them in aviaries (122 x 132 x 213cm) at room temperature with *ad libitum* access to diet made in-house (Guglielmo et al., 2017) supplemented with mealworms (*Telebrio molitor*) and water. I kept the birds on a long day photoperiod similar to natural conditions at Long Point (12.5L:11.5D) to preserve the migratory phenotype and randomly sampled half of the birds (n=8) within 2-4 weeks. I then manipulated the photoperiod to induce a non-migratory phenotype, as used previously in our lab for this species (Guglielmo et al., 2017) and other songbirds (Ivy and Guglielmo, 2023; Price et al., 2010; Price et al., 2011). I progressively shortened the photoperiod for the remaining birds (n=8) by one hour every 3 days until reaching a short-day photoperiod (9L:15D), then sampled the birds after 10-12 weeks. All birds were hatch-year, with similar sex ratios for each phenotype (migratory: 6 male, 2 unknown; non-migratory: 7 male, 1 unknown). These birds were also used for seasonal comparisons in pectoralis histology in a separate study (Ivy and Guglielmo, 2023). My measurements in the non-migratory phenotype may be confounded by captivity-induced reduction in movement, however, the migratory phenotype in songbirds is largely determined by environmental factors (e.g. photoperiod), and the seasonal transition may be observed without accompanying changes in movement (Price et al., 2010; Price et al., 2011; Zajac et al., 2011).

I humanely euthanized the birds using full anaesthesia under inhaled isofluorane followed by decapitation within ten seconds. I weighed the bird, and then quickly removed and

weighed the pectoralis major. A small piece (~100mg) from the middle of one side of the pectoralis was removed and snap-frozen in liquid nitrogen then stored at -80°C for enzyme analysis. I used the medial pectoralis to limit variation in enzyme activity across the pectoralis (Scott et al., 2009). I then lightly minced the remaining pectoralis in ice-cold biopsy preservation solution (Letellier et al., 1992; Veksler et al., 1987) (in mM: 2.77 CaK_2Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 15 Na_2phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 4-morpholineethanesulfonic acid, pH 7.1) and transferred the sample to the lab for mitochondrial isolation within 45 minutes.

2.2.2 Mitochondrial isolation

I isolated pectoralis mitochondria using differential centrifugation methods described previously, with modifications (Gerson, 2012; Muleme et al., 2006). All procedures were performed at 4°C. I first washed the pectoralis twice with 10 mL of homogenization buffer (HB) (in mM: 100 sucrose, 50 tris, 10 EDTA, 100 KCl, pH 7.4) to remove BIOPS. I then finely minced and digested the pectoralis in 10 mL of HB with protease (Sigma P8038; 0.2 mg/mL) for 3 minutes, stirring approximately once each minute. I decanted the HB and rinsed the pectoralis 3 times with 10 mL of HB. I homogenized the pectoralis using a loose-fitting Teflon pestle in a glass homogenizer at 100 rpm for 5-6 passes, or until no large pieces were visible. I filtered the homogenate through 4 layers of cheesecloth into a polycarbonate tube and centrifuged the filtrate at 1000 g for 10 minutes. I aspirated any lipids floating at the surface of the resulting supernatant and filtered the remaining supernatant through 4 layers of cheesecloth. I spun this filtrate at 8700 g for 10 minutes and re-suspended the resulting pellet in HB. I repeated the 8700 g spin and re-suspended the resulting pellet in a small volume of HB (100-200 µL) as the crude mitochondrial extract. I determined mitochondrial protein concentration using a Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) standards.

2.2.3 Mitochondrial fluoro-respirometry

I measured mitochondrial respiration and ROS emission rates via high resolution fluororespirometry using Clark-type polarographic oxygen electrodes and fluorometric

sensors, respectively (Oxygraph-2k; Oroboros, Austria). Mitochondria were assayed at 39°C in 2 mL of respiratory buffer (Gnaiger et al., 2000) (in mM: 0.5 EGTA, 3 $MgCl_2$, 20 taurine, 10 KH_2PO_4, 20 HEPES, 110 sucrose, 60 lactobionic acid; 0.1% (w/v) BSA, pH 7.1). O_2 electrodes were calibrated daily using respiratory buffer at air equilibration and in anoxia using a yeast suspension. To measure ROS emission rates, I used the fluorescence module (O2k-Fluo LED2) and tracked H_2O_2 emission via fluorescence linked to Amplex Ultra-Red (Makrecka-Kuka et al., 2015). Fluorometric sensors were calibrated for each sample after mitochondrial addition using 2 additions of freshly prepared 0.1 µM H_2O_2 standards before and after each run. For all measurements, I waited for the O_2 consumption rate to stabilize for at least one minute.

I simultaneously measured respiration, ROS emission rates, and O_2 concentration during oxidation of pyruvate and palmitoyl-carnitine in parallel. I first added 10 µM Amplex Ultra-Red (Invitrogen), 1 U mL^{-1} horseradish peroxidase, 15 µM diethylenetriamine-N,N,N',N,N-pentaacetic acid (DTPA) and 5 U mL^{-1} superoxide dismutase to each chamber before addition of mitochondria (100 µg of mitochondrial protein). I added DTPA to reduce background fluorescence from contaminating iron, as reported previously (Komlódi et al., 2018). In one chamber, I stimulated oxidative phosphorylation with the addition of 1 mM malate, 25 µM palmitoyl-carnitine and 200 µM ADP to measure state 3 respiration. After, I added 25 nM oligomycin to inhibit oxidative phosphorylation to measure state 4 respiration. I repeated these measurements in a separate chamber using 1 mM pyruvate instead of palmitoyl-carnitine. Respiration and ROS emission rates were corrected for background activity.

In a separate run, I measured mitochondrial respiration coupled to oxidative phosphorylation in response to stimulation by different electron sources. In duplicate, I added mitochondria and 0.5 mM ADP. To assess the NADH (N) pathway (complex I-IV flux), I stimulated respiration by adding 1mM pyruvate and 1mM malate. Next, I inhibited complex I with 0.5 µM rotenone and assessed the succinate (S) pathway (complex II-IV flux) by stimulating respiration with 5 mM succinate. Finally, I measured complex IV-mediated respiration by inhibiting complex III with 25 nM antimycin A and stimulating respiration with 0.5 mM TMPD and 2 mM ascorbate to limit TMPD auto-oxidation. To

assess TMPD auto-oxidation, I re-oxygenated the chambers and measured respiration in the presence of 0.5 mM KCN. I measured the difference in respiration between stimulation and inhibition of each complex as the complex-specific respiration. The remaining isolated mitochondrial preparations were frozen at -80°C for later analyses.

2.2.4 Enzyme activity assays

I assessed the activities of four metabolic enzymes in the pectoralis to assess seasonal variation in fatty acid and glycolytic metabolism using methods described previously (Price et al., 2010). First, I finely minced the pectoralis with a razor blade on ice, then diluted the tissue 10-fold in homogenizing buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% BSA, 50% glycerol, 0.1% Triton X-100, 50 µg/mL aprotinin). I mechanically homogenized the pectoralis on low power for 3 bouts of 10 seconds, separated by 30 seconds rest on ice (Polytron PT 10-35; Kinematica, Bohemia, NY). I then sonicated the homogenate on low power for 3 bouts of 10 seconds, separated by 30 seconds on ice (VirSonic 100; VirTis, Gardiner, NY). Within an hour of homogenization, I measured the apparent maximal activities of carnitine palmitoyltransferase (CPT), citrate synthase (CS), lactate dehydrogenase (LDH) and β-hydroxyacyl-CoA dehydrogenase (HOAD) in pectoralis homogenate using a UV/Vis spectrophotometer at 39°C (Cary 100 Bio; Varian, Palo Alto, CA). I assayed CPT and CS activities by measuring 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) absorbance at 412 nm, and assayed HOAD and LDH activities by measuring NADH absorbance at 340 nm. I used the following assay conditions for each enzyme, with ± denoting the metabolite omitted for background activity measurement: (in mM) CPT: ±5 carnitine, 0.15 DTNB, 0.035 palmitoyl-CoA, 50 Tris (pH 8.0); CS: ±0.5 oxaloacetate, 0.15 DTNB, 0.15 acetyl-CoA, 50 Tris (pH 8.0); HOAD: ±0.1 acetoacetyl-CoA, 0.2 NADH, 1 EDTA, 50 imidazole (pH 7.4); LDH: ±4 pyruvate, 0.15 NADH, 5 dithiothreitol, 50 imidazole (pH 7.4). I assayed all enzymes in triplicate using 1.5 mL cuvettes at a final volume of 1 mL, corrected for background activity and calculated enzyme activity using extinction coefficients 1.36×10^4 M^{-1} (DTNB) and 6.22×10^3 M^{-1} (NADH). I measured pectoralis CS activity to infer variation in mitochondrial abundance. I also measured CS activity in the isolated mitochondrial preparations following one freeze-thaw cycle and sonication, using methods described above. In addition, I measured total antioxidant

capacity in the isolated mitochondrial preparations using a commercially available kit that assesses enzymatic and non-enzymatic antioxidants via Cu^{2+} reduction (Abcam ab65329). Due to equipment failure on the day of measurement, I was only able to measure total antioxidant capacity in half (n=4) of the birds from each phenotype.

2.2.5 Western blots

I used Western blotting to assess variation in protein expression of ETS protein complexes. First, I isolated membrane proteins from the mitochondrial preparations used for respirometry. I pelleted the mitochondria at 15,000 g for 2 min at 4°C and incubated the re-suspended pellet in mitochondrial extraction buffer (in mM: 750 aminocaproic acid, 50 Bis Tris, pH 7.0) with 3.5 g maltoside per gram protein on ice for 15 minutes. I then centrifuged these samples at 13,000 g for 30 minutes at 4°C and discarded the pellet. I quantified protein concentration in these extracts using a commercially available bicinchoninic acid assay (Thermo Scientific 23225). I diluted the samples in Laemmli buffer (10% glycerol, 40 mM Tris, 2% sodium dodecyl sulfate (SDS), 0.005% bromophenol blue, 1% β-mercaptoethanol) and boiled them for 5 minutes using a hot water bath.

I then performed SDS-PAGE using pre-cast 10% polyacrylamide gels in the Bio-Rad mini-PROTEAN system (Bio-Rad, Hercules, CA, USA). Samples and protein ladder (Bio-Rad 161-0373) were loaded into the gel with running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS; pH 8.3) at 50 V for 10 minutes, followed by 120 V for 55 minutes. I then 'wet-transferred' the samples onto polyvinylidene difluoride membrane in transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris) at 4°C by running at 100 V for 2 hours. I then washed the membrane for five minutes three times with TBS-T (0.05% TWEEN-20, 20 mM Tris, 144 mM NaCl) and blocked the membrane overnight in TBS-T with 5% BSA at 4°C. I then immersed the membrane in 5% BSA with a cocktail of mouse primary antibodies for each of the ETS protein complexes (Abcam ab110413) diluted 1:1000 followed by incubation for 2 hours at room temperature. I then washed the membranes in TBS-T for 10 minutes repeated 3 times, immersed the membranes in donkey anti-mouse secondary antibody (Abcam ab6820) diluted 1:2000 in TBS-T and incubated at room

temperature for 1 hour. I repeated the membrane washes in TBS-T and imaged the blots using the Bio-Rad Clarity Western ECL substrate system in a Bio-Rad Chemi-Doc Touch.

2.2.6 Statistical analyses

All analyses were performed on R (Version 4.3.1; R Core Development Team, 2022) using the *car* package for general linear modelling (Fox and Weisberg, 2019). I conducted general linear modeling to assess differences between phenotypes while controlling for covariation with body mass, except for pectoralis mass, which was compared after controlling for covariation with wing chord as a structural size measure. I took a similar approach to assess mitochondrial ROS emission but included O_2 concentration as an additional covariate (Li Puma et al., 2020). All covariates with $p < 0.1$ were included in the final model. When no covariates were included, we used two sample, unpaired t-tests. I calculated free radical leak (FRL; 100 * (ROS emission / (2 * respiration)) to assess electron leak from the ETS as previously described (Brown et al., 2009). I also assessed the coupling of mitochondrial respiration to oxidative phosphorylation by calculating OXPHOS control efficiency (1 – state 4 respiration/state 3 respiration) and respiratory control ratio (RCR; state 3/state 4 respiration) (Divakaruni and Jastroch, 2022). To assess respiratory capacity independent of H^+ leak, I calculated net OXPHOS capacity (state 3 respiration – state 4 respiration) (Gnaiger, 2012). Since both FRL and OXPHOS control efficiency are proportions, I arcsine square root-transformed them prior to statistical comparisons to ensure normality in our dataset. When linear modelling detected significant or nearly significant covariation, I calculated estimated marginal means using the *emmeans* package (Lenth, 2023) and plotted these values to better show between-phenotype variation.

2.3 Results

2.3.1 Body and tissue mass

The body mass of migratory warblers was ~14% higher than non-migratory warblers ($t_{8.65}$ = 2.83; p = 0.02; Figure 2.1A). The estimated marginal mean for pectoralis mass was ~8% higher in the non-migratory warblers (Figure 2.1B; $F_{1,13}$ = 6.1, p = 0.03) with a nearly significant effect of wing chord ($F_{1,13}$ = 3.9; p = 0.07). Wing chord was similar between phenotypes (t_{14} = 0.98; p = 0.34; Figure 2.1C). In contrast, heart mass and liver mass did not correlate with wing chord; both heart mass ($t_{13.8}$ = 0.0, p = 1.00) and liver mass ($t_{8.0}$ = -0.5, p = 0.62) were similar between phenotypes (Figure A1.1).

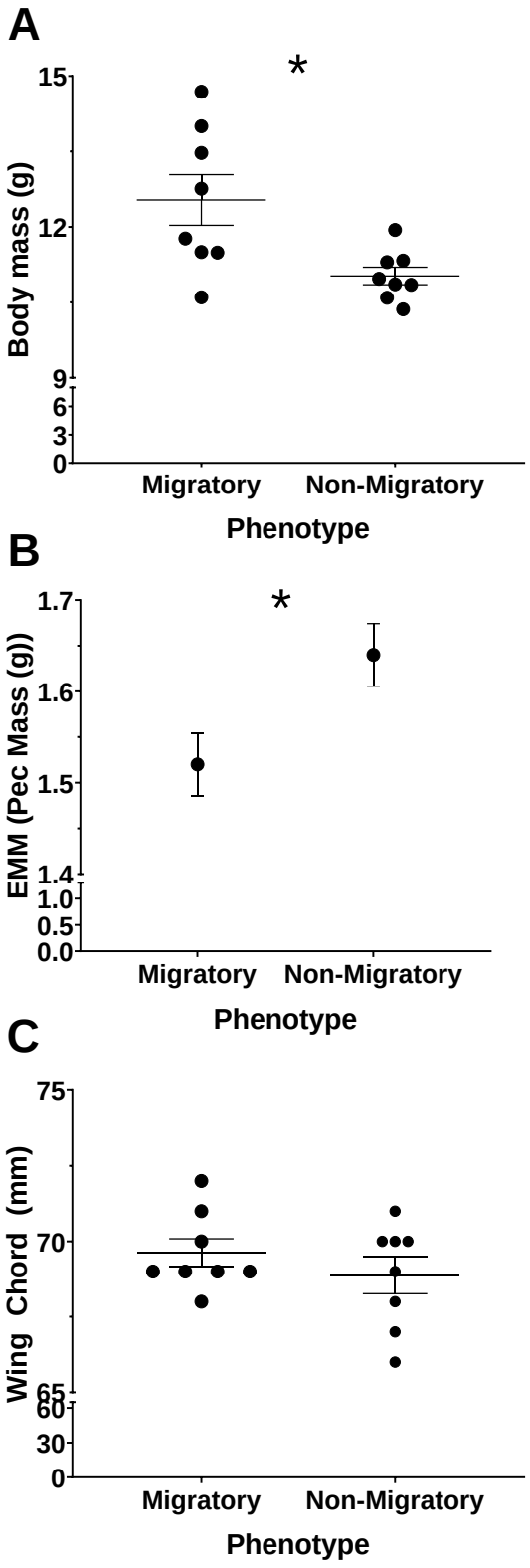
A
*
15
12
9
6
3
0
Body mass (g)
Migratory
Non-Migratory
Phenotype
B
*
1.7
1.6
1.5
1.4
1.0
0.5
0.0
EMM (Pec Mass (g))
Migratory
Non-Migratory
Phenotype
C
75
70
65
60
30
0
Wing Chord (mm)
Migratory
Non-Migratory
Phenotype

Figure 2.1: Seasonal variation in body composition in yellow-rumped warblers. Total body mass (A), estimated marginal means (EMM) of wet pectoralis mass, taking covariation from wing chord into effect (B) and wing chord (C). Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8. * represents significant difference between phenotypes ($p<0.05$).

2.3.2 Enzyme activity assays

After accounting for variation in body mass ($F_{1,13}$ = 3.8; p = 0.07), I found 60% higher pectoralis CS activity in the migratory phenotype (Table 2.1; $F_{1,13}$ = 13.1; p < 0.01). Similarly, I found 46% and 71% higher activities in the migratory phenotype for CPT (Table 2.1; $t_{14.0}$ = 2.18; p < 0.05) and HOAD (Table 2.1; $t_{9.2}$ = 3.46, p < 0.01), respectively. In contrast, I found 39% lower LDH activity in the migratory phenotype (Table 2.1; $t_{14.0}$ = 2.18, p < 0.05). The above enzyme activity measurements are expressed per unit mass of pectoralis, but due to seasonal variation in pectoralis mass, I also calculated whole-organ activities (Table 2.1). I found ~30%, ~50% and ~60% higher whole-pectoralis activities for CS (t_{14} = 2.25; p = 0.04), CPT (t_{14} = 1.89; p = 0.08) and HOAD ($t_{9.0}$ = 2.85; p = 0.02), respectively, while whole-pectoralis LDH activity was ~30% lower in the migratory phenotype (t_{14} = 2.33; p = 0.03). CS activity of the isolated mitochondrial preparations (migratory: 1.48 ± 0.66, non-migratory: 1.56 ± 1.07 μmol min^{-1} mg $protein^{-1}$) was similar between phenotypes ($t_{8.6}$ = 1.24, p = 0.25).

Table 2.1: Seasonal variation in pectoralis enzyme activity in yellow-rumped warblers. Apparent V_{max} measured for citrate synthase (CS), carnitine palmitoyl-transferase (CPT), β-hydroxyacyl-CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH). Activity rates expressed per wet mass of pectoralis (μmol minute^{-1} g^{-1}) and as whole-organ (μmol minute^{-1}). Whole-organ activity calculated as product of wet mass and wet mass-specific activity. Mass-specific CS activity presented as estimated marginal mean (EMM), taking significant covariation from body mass into account. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8. Significant differences between phenotypes represented by * ($p < 0.05$) and ** ($p < 0.01$), differences approaching significance represented by # ($p < 0.10$).

Enzyme	Migratory	Non-Migratory
CS (per mass)**	202.0 ± 13.5	126.0 ± 13.5
CPT (per mass)*	4.4 ± 0.2	3.0 ± 0.1
HOAD (per mass)**	90.7 ± 3.6	53.0 ± 1.4
LDH (per mass)*	389.2 ± 14.5	539.2 ± 19.6
CS (whole tissue)*	291.5 ± 9.1	223.2 ± 5.8
CPT (whole tissue)#	6.7 ± 0.3	4.9 ± 0.2
HOAD (whole tissue)*	139.9 ± 6.2	86.3 ± 2.4
LDH (whole tissue)*	598.0 ± 24.2	881.1 ± 35.3

2.3.3 Mitochondrial substrate oxidation

I measured phosphorylating (state 3) and non-phosphorylating (state 4) respiration for isolated pectoralis mitochondria during oxidation of glycolytic or fatty acid-derived substrates (Figure 2.2). Because mitochondrial CS activity did not differ between phenotypes, I express respiration relative to CS activity, but respiration rates relative to mitochondrial protein are shown in Appendix A (Figure A1.2). I found similar state 3 pyruvate respiration between phenotypes (Figure 2.2A; $F_{1,13}$ = 2.2; p = 0.16) after accounting for the effects of body mass ($F_{1,13}$ = 3.8; p = 0.07). In contrast, state 3 palmitoyl-carnitine respiration was ~70% higher in the migratory phenotype (Figure 2.2B; $F_{1,13}$ = 5.7, p = 0.03) after correcting for body mass ($F_{1,13}$ = 7.0; p = 0.02). I also found ~95% higher state 4 pyruvate respiration in the migratory phenotype (Figure 2.2C; $F_{1,13}$ = 6.7; p = 0.02) after accounting for body mass ($F_{1,13}$ = 3.3; p = 0.09). Similarly, I found that state 4 palmitoyl-carnitine respiration was 110% higher in the migratory phenotype (Figure 2.2D; $F_{1,13}$ = 12.0; p < 0.01) after accounting for body mass ($F_{1,13}$ = 7.7; p = 0.02). Despite this increase in state 4 respiration, net OXPHOS capacity was ~65% higher in the migratory phenotype (Figure 2.2F; $F_{1,13}$ = 4.8; p < 0.05) when oxidizing palmitoyl-carnitine, and after accounting for body mass ($F_{1,13}$ = 6.7; p = 0.02). However, after accounting for body mass ($F_{1,13}$ = 3.7; p = 0.08), we found similar net OXPHOS capacity between phenotypes for pyruvate oxidation (Figure 2.2E; $F_{1,13}$ = 1.7; p = 0.21). In contrast, when we expressed respiration relative to mitochondrial protein, I found little variation between phenotypes in state 3 respiration ($t_{10.9}$ = -1.4, p = 0.20), state 4 respiration ($t_{12.5}$ < 0.1, p = 1.0) and net OXPHOS capacity ($t_{10.8}$ = -1.5, p = 0.16) when oxidizing pyruvate (Figure A1.2). Similarly, after expressing respiration relative to mitochondrial protein, I found little phenotypic variation in state 3 respiration (t_{14} = -0.6, p = 0.56), state 4 respiration ($t_{14.0}$ = 0.5, p = 0.63) and net OXPHOS capacity ($t_{14.0}$ = -0.76, p = 0.46) when oxidizing palmitoyl-carnitine.

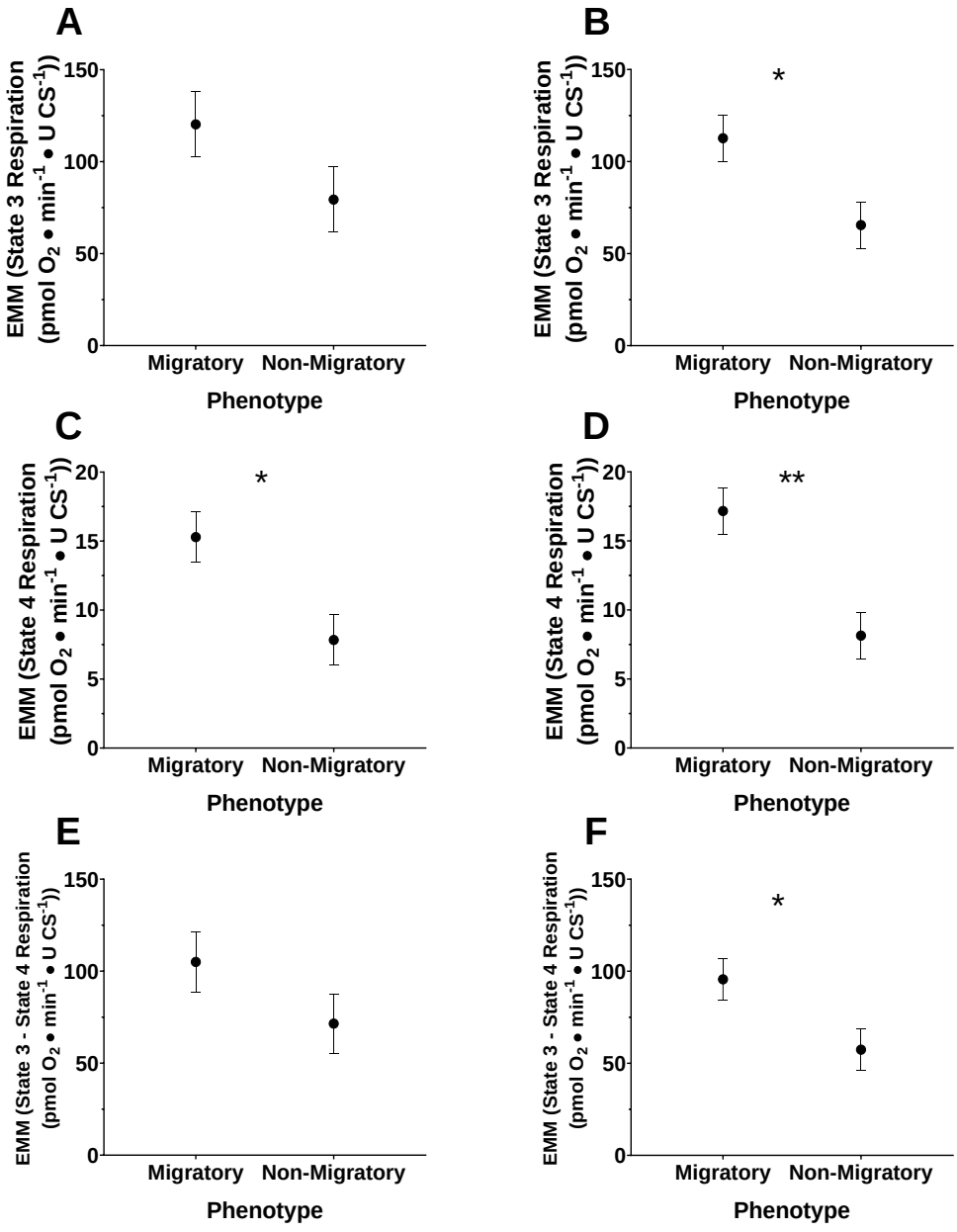
A
EMM (State 3 Respiration (pmol O2 • min-1 • U CS-1))
150
100
50
0
Migratory
Non-Migratory
Phenotype
B
*
C
*
D
**
E
F

Figure 2.2 Seasonal variation in substrate oxidation in yellow-rumped warbler pectoralis mitochondria. O_2 consumption rates of isolated pectoralis mitochondria were measured during oxidation of pyruvate (A, C, E) or palmitoyl-carnitine (B, D, F) substrate in phosphorylating (State 3; A, B) or non-phosphorylating conditions (State 4; C, D). Net OXPHOS capacity presented as difference between state 3 and state 4 respiration (E, F). O_2 consumption rates are expressed relative to citrate synthase (CS) activity (μmol min^{-1}; U) of individual mitochondrial preparations. Data presented as estimated marginal mean (EMM) after accounting for covariation from body mass. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8. Significant differences between phenotypes represented by * ($p < 0.05$) and ** ($p < 0.01$).

To better assess coupling between respiration and oxidative phosphorylation, I calculated OXPHOS control efficiency (Figure 2.3) and RCR (Figure A1.3). I found 5% and 4% lower OXPHOS control efficiency in the migratory phenotype for pyruvate (Figure 2.3A; $t_{10.5}$ = 4.0; $p < 0.01$) and palmitoyl-carnitine (Figure 2.3B; $t_{14.0}$ = 2.9; $p = 0.01$), respectively. Similarly, I found 27.5% and 18.5% lower RCR in the migratory phenotype for pyruvate ($t_{12.4}$ = 3.3, $p < 0.01$) and palmitoyl-carnitine ($t_{13.6}$ = 3.0, $p = 0.01$), respectively.

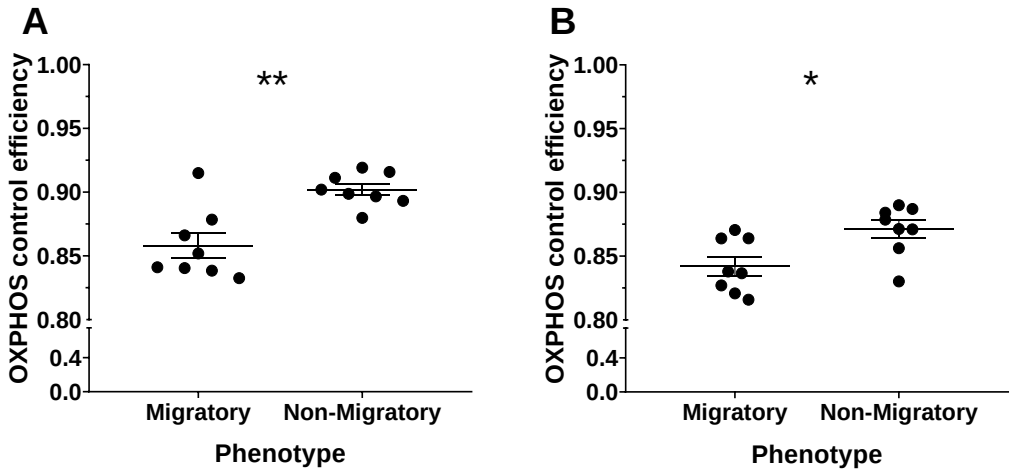

Figure 2.3 Seasonal variation in coupling of mitochondrial respiration to oxidative phosphorylation in yellow-rumped warblers. O_2 consumption rates of isolated pectoralis mitochondria were measured during oxidation of pyruvate (A) or palmitoyl-carnitine (B) substrate in phosphorylating and non-phosphorylating conditions. OXPHOS control efficiency was calculated as the proportion of state 3 O_2 consumption linked to oxidative phosphorylation. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8. Significant differences between phenotypes indicated by * ($p < 0.05$) and ** ($p < 0.01$).

2.3.4 N and S pathway flux

To better understand variation in state 3 respiration, I measured phosphorylating respiration mediated by the N (NADH) and S (succinate) pathways, mediated by complexes I-IV and II-IV, respectively and complex IV capacity (Figure 2.4). I found similar respiration rates between phenotypes for the N pathway (Figure 2.4A; $t_{14} < 0.1$; $p = 0.99$), S pathway (Figure 2.4B; $t_{14} = 1.5$; $p = 0.15$) and complex IV capacity (Figure 2.4C; $t_{14} = 1.29$; $p = 0.22$). Similarly, when expressed per mitochondrial protein (Figure A1.4), respiration rates were similar between phenotypes for the N pathway ($t_{13.5} = 1.4$, $p = 0.18$), S pathway ($t_{12.8} = 0.23$, $p = 0.82$) and complex IV capacity ($t_{12.0} = 0.38$, $p = 0.71$).

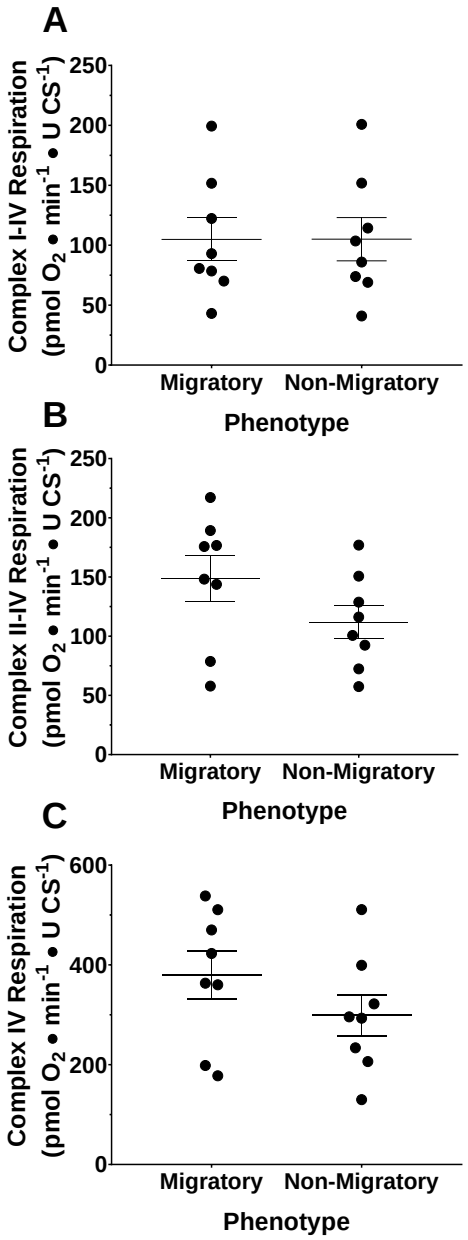

Figure 2.4 Seasonal variation in electron transport system complex-specific flux in isolated pectoralis mitochondria from yellow-rumped warblers. Respiration rates specific to A) complex I-IV, B) complex II-IV and and C) complex IV. Respiration rates are expressed relative to citrate synthase (CS) activity (μmol min^{-1}; U) of individual mitochondrial preparations. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8.

2.3.5 Mitochondrial ROS emission

I removed data from one non-migratory bird from our dataset as an outlier for each ROS measurement. I found similar state 3 FRL between phenotypes for pyruvate (Figure 2.5A; $F_{1,12} < 0.01$, $p = 0.96$), which positively correlated with assay O_2 concentration ($F_{1,12} = 5.14$, $p = 0.04$). State 3 FRL was also similar between phenotypes for palmitoyl-carnitine (Figure 2.5B; $F_{1,12} = 1.29$; $p = 0.28$), with nearly significant covariation with body mass ($F_{1,12} = 3.59$; $p = 0.08$). In contrast, I found that state 4 FRL was ~16% lower in the migratory phenotype for pyruvate (Figure 2.5C; $F_{1,12} = 13.35$; $p < 0.01$) after accounting for assay O_2 concentration ($F_{1,12} = 7.29$; $p = 0.02$). Similarly, state 4 FRL was ~20% lower for palmitoyl-carnitine in the migratory phenotype (Figure 2.5D; $t_{14} = 2.96$; $p = 0.01$). I also found similar mitochondrial total antioxidant capacities (migratory: 0.62 ± 0.15, non-migratory: 0.50 ± 0.15 pmol Trolox unit CS^{-1}) between phenotypes ($t_6 = 0.61$; $p = 0.57$). I also present absolute ROS emission rates standardized to CS activity and mitochondrial protein in Table 2.2. When standardized to CS activity, absolute ROS emission rates were similar between phenotypes for palmitoyl-carnitine oxidation in state 3 ($t_{13} = 0.19$, $p = 0.85$) and state 4 respiration ($t_{13} = 0.19$, $p = 0.85$). ROS emission rates per CS activity were also similar between phenotypes for pyruvate in state 3 respiration ($F_{1,11} = 0.87$, $p = 0.37$), but covaried with body mass ($F_{1,11} = 4.2$, $p = 0.07$) and O_2 concentration ($F_{1,11} = 5.3$, $p = 0.04$). Similarly, state 4 ROS emission for pyruvate was similar between phenotypes ($F_{1,11} = 1.85$, $p = 0.20$) but covaried with body mass ($F_{1,11} = 4.6$, $p = 0.06$) and O_2 concentration ($F_{1,11} = 5.0$, $p < 0.05$). In contrast, ROS emission rates standardized to mitochondrial protein were ~35% and ~30% lower in the migratory phenotype for state 3 ($t_{13} = 3.14$, $p < 0.01$) and state 4 respiration ($t_{13} = 3.38$, $p < 0.01$) for pyruvate, respectively. ROS emission rates per mitochondrial protein for palmitoyl-carnitine were similar between phenotypes in state 3 respiration ($t_{9.0} = 0.96$, $p = 0.36$) but were ~25% lower in the migratory phenotype in state 4 respiration, although this difference approached significance ($t_{13} = 1.88$, $p = 0.08$).

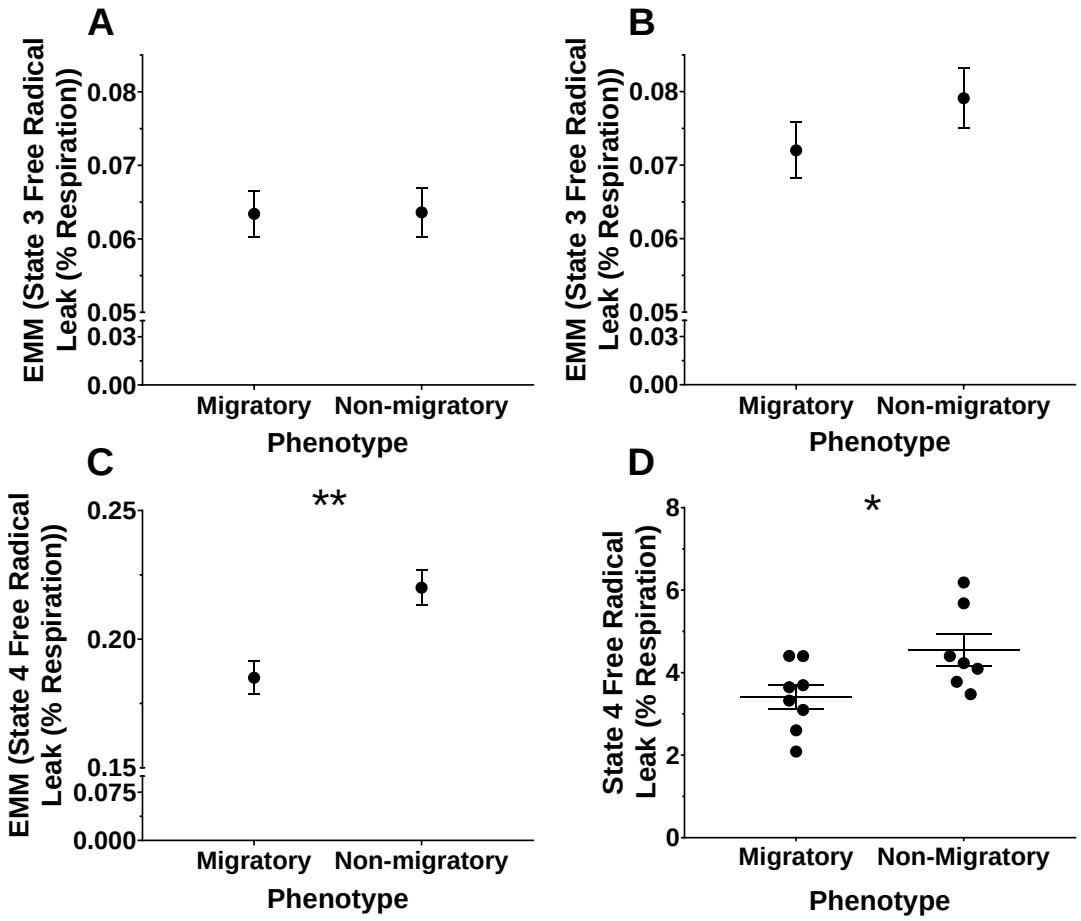

Figure 2.5 Seasonal variation in mitochondrial free radical leak (FRL) in yellow-rumped warbler pectoralis mitochondria. FRL was calculated as H_2O_2 emission rate standardized to atoms of oxygen consumed and expressed as a percentage of oxygen consumed. FRL was measured during oxidation of pyruvate (A, C) or palmitoyl-carnitine (B, D) substrate in phosphorylating (State 3; A, B) or non-phosphorylating (State 4; C, D) conditions. State 3 pyruvate FRL presented as estimated marginal means (EMM), taking covariation from assay O_2 concentration (A, C) or body mass (B) into effect. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8 (migratory), 7 (non-migratory). Significant differences between phenotypes indicated by * ($p < 0.05$), ** ($p < 0.01$).

Table 2.2: Seasonal variation in mitochondrial reactive oxygen species (ROS) emission from migratory yellow-rumped warblers. ROS emission rates were measured during oxidation of pyruvate or palmitoyl-carnitine substrate in phosphorylating (State 3) or non-phosphorylating conditions (State 4). ROS emission rates expressed standardized to citrate synthase (CS) activity (nmol H_2O_2 min^{-1} µmol min CS^{-1}) or to protein content of each isolated mitochondrial preparation (nmol H_2O_2 min^{-1} mg^{-1}). CS activity-standardized ROS emission rates for pyruvate oxidation presented as estimated marginal means, taking covariation from body mass and/or assay O_2 concentration into effect. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8 (migratory), 7 (non-migratory). Significant differences between phenotypes within measurement represented by * ($p < 0.05$) and ** ($p < 0.01$), differences approaching significance represented by # ($p < 0.10$).

	Pyruvate		Palmitoyl-carnitine	
	Migratory	Non-Migratory	Migratory	Non-Migratory
State 3 (per CS)	0.89 ± 0.13	0.68 ± 0.14	1.07 ± 0.06	1.01 ± 0.09
State 4 (per CS)	1.12 ± 0.17	0.71 ± 0.19	0.98 ± 0.05	1.03 ± 0.09
State 3 (per mg)	0.99 ± 0.04**	1.37 ± 0.02	1.42 ± 0.08	1.66 ± 0.03
State 4 (per mg)	1.15 ± 0.04**	1.66 ± 0.03	1.32 ± 0.06#	1.72 ± 0.04

2.3.6 Western blots

To assess seasonal potential variation in ETS protein complex abundance as a mechanistic explanation for variation in state 3 respiration, I conducted Western blotting using a cocktail containing one primary antibody that cross-reacts with one peptide from each of the ETS complexes in mice and other rodents, as previously used in our lab (Mathers and Staples, 2019). Preliminary experiments revealed that this antibody cocktail produced five bands in proteins extracted from rat gastrocnemius mitochondria, but only two bands in flight muscle mitochondria isolated from blackpoll warblers (*Setophaga striata*, Forster, 1772), ruffs (*Calidris pugnax*, Linnaeus, 1758) and yellow-rumped warblers (Figure A1.5). These bands were approximately 54 and 34 kDa, which we interpret to correspond to peptides from complexes V and II, respectively, based on similarity in mass with bands from rat mitochondria. Bands corresponding with complex I, III and IV peptides were not visible in any gels from the three bird species, suggesting that avian epitopes do not cross-react with these mammalian-derived antibodies. Nonetheless we found greater densities of bands consistent with complexes V (migratory: 4.1 A.U. µg protein^{-1}; non-migratory: 2.31 A.U. µg protein^{-1}) and II (migratory: 2.96 A.U. µg protein^{-1}; non-migratory: 1.89 A.U. µg protein^{-1}) in the migratory phenotype.

2.4 Discussion

I tested the hypotheses that flight muscle mitochondria are seasonally plastic, and that mitochondrial function is altered in the migratory phenotype to better meet the challenges of migratory flight. In support of both hypotheses, in the migratory phenotype I found higher pectoralis CS activity indicating a greater mitochondrial abundance. In isolated mitochondria I also found higher state 3 respiration fuelled by palmitoyl-carnitine and lower state 4 FRL. Taken together, these results indicate that yellow-rumped warblers seasonally modulate mitochondrial quantity and mitochondrial function to increase the capacity for energy provisioning while reducing oxidative costs of ROS during migration.

The two groups of birds used in our study were held in captivity for different durations, and it is possible that this contributed to the differences we found, perhaps through captivity stress. However, we believe this is unlikely because all birds in this study were healthy and

exhibited normal behaviour, and previous work in this species has shown limited effects of time in captivity on body composition (Dick and Guglielmo, 2019). Furthermore, seasonal flight muscle plasticity follows similar patterns in birds sampled in the wild in different seasons (McFarlan et al., 2009), compared to those sampled in captivity under different photoperiods (Zajac et al., 2011), as shown in in white-throated sparrows. I believe, therefore, that my results represent a real effect of the migratory phenotype.

My data indicate a high degree of seasonal plasticity in flight muscle metabolism in migratory birds. I found higher mass-specific activities of mitochondrial enzymes (CS, HOAD, CPT) and lower activity of the glycolytic enzyme, LDH, in the migratory phenotype, indicating that the migratory pectoralis phenotype has an elevated capacity for aerobic metabolism, in accord with previous seasonal comparisons in this species (Dick, 2017) and other songbirds (Banerjee and Chaturvedi, 2016; Lundgren and Kiessling, 1986; Marsh, 1981; McFarlan et al., 2009; Sharma et al., 2021; Zajac et al., 2011). Surprisingly, I found that the pectoralis was smaller in the migratory phenotype, in contrast to previous findings of pectoralis hypertrophy during migration in songbirds (DeMoranville et al., 2019; Price et al., 2011). The lower pectoralis mass in the migratory phenotype may be partly explained by lower muscle fiber diameter in these birds (Ivy and Guglielmo, 2023) and by a lower proportion of fast-glycolytic muscle fibers (Chang et al., 2024). These data reflect a lower capacity for mechanical power output in the migratory phenotype for yellow-rumped warblers. Nonetheless, when I expand the mass-specific enzyme activities to the whole-organ I still find higher activities of CS, HOAD and CPT in the migratory phenotype, reflecting an overall greater oxidative capacity which would help power endurance flight. Furthermore, maintaining a high cellular oxidative capacity may allow yellow-rumped warblers to minimize pectoralis size and reduce flight costs via reduced body mass.

In mammals, a muscle phenotype similar to migratory yellow-rumped warblers has been identified in ‘mini mice’ that have evolved smaller locomotory muscles with higher mass-specific mitochondrial enzyme activities in response to artificial selection for high running activity (Guderley et al., 2006; Houle-Leroy et al., 2003; Templeman et al., 2012). While these mice required ~11-12 weeks of wheel access to attain this phenotype, yellow-rumped

warblers attain similar results following cessation of breeding, potentially without increasing locomotory activity. However, total muscle activity of mitochondrial enzymes is similar between mini-mice and control mice lines (Houle-Leroy et al., 2003; Templeman et al., 2012), while total flight muscle activity is greater in migratory than non-migratory warblers. As a result, yellow-rumped warblers may be able to simultaneously modulate muscle size and oxidative capacity in response to increase locomotory demands, while lab mice may not share this plasticity.

I infer higher mitochondrial abundance in the migratory phenotype from higher CS activity, but I recognize that CS activity has not been validated as a proxy for mitochondrial volume by microscopy in birds, as has been done with endurance exercise training in humans (Meinild Lundby et al., 2018). However, transmission electron microscopy has revealed increases in flight muscle mitochondrial volume during migration in shorebirds (Evans et al., 1992). A seasonal increase in pectoralis mitochondrial volume could result from upregulation in signalling from perixosome-proliferator-activated receptor γ co-activator-1α (PGC-1α) and downstream increased expression of nuclear receptor factors 1 and 2 (reviewed by Jornayvaz and Shulman, 2010). Seasonal variation in PGC-1α signalling is poorly understood in migratory songbirds, but mRNA expression of the functionally similar PGC-1β increases 2.2-fold during migration in yellow-rumped warblers (Dick, 2017). In contrast, PGC-1α mRNA expression is ~70% higher during autumn migration than during breeding season, with few changes in PGC-1β in gray catbirds, despite the absence of seasonal changes in mitochondrial abundance (DeMoranville et al., 2019). Future investigations should explore the physiological processes underlying the seasonal changes in pectoralis mitochondrial abundance.

These whole-tissue changes are supported by seasonal remodelling of individual mitochondria, as state 3 respiration and net OXPHOS capacity for palmitoyl-carnitine oxidation were higher during migration. This greater capacity for fatty acid oxidation may be required to offset the potential costs of greater uncoupling of substrate oxidation from ADP phosphorylation during migration (Figure 2.3; Figure A1.3), which may reduce the efficiency of ATP synthesis. In addition, an increase in total mitochondrial abundance may further offset greater uncoupling by increasing tissue oxidative capacity. Future studies in

seasonal variation in mitochondrial function of migratory birds should directly assess mitochondrial ATP synthesis relative to O_2 consumption and ROS emission to provide a more holistic view of mitochondrial performance.

I found lower state 4 FRL in mitochondria from the migratory phenotype, suggesting that these mitochondria emit fewer ROS at submaximal workloads. These conditions correspond to high membrane potential, where ROS formation is likely near maximal. Nonetheless, absolute ROS emission rates (per mg protein) were lower in the migratory phenotype for both state 3 and state 4 respiration. I also found similar total mitochondrial antioxidant capacity between phenotypes, suggesting that the lower ROS emission in migration is not due to higher rates of ROS quenching, and instead may be the result of lower ETS electron leak (Murphy, 2009). However, to better understand *in vivo* mitochondrial ROS dynamics, future studies should address seasonal variation in the kinetics of mitochondrial ROS emission and quenching, as has been performed in rat skeletal muscle (Treberg et al., 2019). A lower ETS electron leak can be explained by a relatively more oxidized ETS, particularly at complexes I or III, where electrons can 'leak' from the ETS onto O_2 to form $O_2^{\bullet-}$ (Hernansanz-Agustín and Enríquez, 2021). Net decreases in ETS reduction state may be caused by increases in 'spare capacity' of the ETS via higher activity/abundance of ETS complexes, or by uncoupling ETS flux from OXPHOS (Brown et al., 2009; Lane, 2005). Both mechanisms divert electrons away from the principal sources of electron leak (Murphy, 2009). It remains unclear if migratory birds increase ETS 'spare capacity' during migration, but my findings of higher state 4 respiration and lower OXPHOS coupling efficiency during migration both indicate an increase in uncoupling of respiration from ATP synthesis. ETS electron leak may also be reduced by the increased formation of ETS supercomplexes, which may decrease reduction state by increasing electron flux among ETS complexes (Maranzana et al., 2013). While such supercomplexes increase in formation with exercise training in humans (Greggio et al., 2017), it is presently unclear whether supercomplex abundance is seasonally plastic in migratory birds. A lower ETS electron leak is likely beneficial for migrating birds by reducing the potential for oxidative damage to intracellular proteins, membranes, and free fatty acids.

My observations of higher state 4 respiration rates in the migratory phenotype suggest higher H^+ permeability across the inner mitochondrial membrane, however this should be interpreted cautiously, as my study did not control for membrane potential, which partly determines H^+ conductance (Divakaruni and Brand, 2011). A higher H^+ permeability may be explained by increased inner mitochondrial membrane phospholipid fatty acyl polyunsaturation (Brand et al., 2003) and/or by the increased abundance of anion carrier proteins, such as adenine nucleotide translocase and uncoupling proteins (reviewed by Divakaruni and Brand, 2011). It is presently unclear if mitochondrial membrane composition is seasonally altered in migratory birds, but polyunsaturated phospholipid composition of whole flight muscle does change seasonally in white-throated sparrows (Klaiman et al., 2009), though not in white-crowned sparrows (Price et al., 2010). Flight muscle phospholipid fatty acyl polyunsaturation is sensitive to dietary changes in songbirds, with turnover of most fatty acids taking ~2-3 weeks (Carter et al., 2019; Dick and Guglielmo, 2019; Price and Guglielmo, 2009). My migratory birds were sampled within 2-4 weeks of capture, potentially before complete membrane turnover would occur. However, this confounding effect is likely negligible because a majority of H^+ conductance across the inner mitochondrial membrane can be explained by expression of adenine nucleotide translocase (Brand et al., 2005). Seasonal expression patterns of adenine nucleotide translocase and avian uncoupling protein in migratory songbirds are presently unknown and are thus exciting avenues for future research.

Despite apparent advantages of the migratory phenotype (greater fatty acid oxidation, reduced ROS emission), this phenotype is not expressed year-round in songbirds. Our findings of an increased state 4 respiration in tandem with higher flight muscle mitochondrial abundance suggest an elevated energetic burden of maintaining the migratory phenotype. Leak respiration of liver and skeletal muscle mitochondria is estimated to account for ~15-20% of whole-animal standard metabolic rate in mammals (Rolfe and Brand, 1996; Rolfe et al., 1999) but unfortunately, comparable data for birds do not exist, to my knowledge. Moreover, data on seasonal flexibility in basal metabolic rate in migratory birds are limited and contradictory (McKechnie, 2008). However, the lower pectoralis mass in the migratory phenotype in our study may compensate for a higher per-unit mass metabolic cost of maintaining this tissue. Nonetheless, our observations of

increased state 4 respiration in the migratory phenotype agree with other observations. For example, whole-animal maximal metabolic rate induced by cold in black-capped chickadees (Milbergue et al., 2022) and exercise in brown trout (Salin et al., 2016), correlate positively with mitochondrial state 4 respiration, although mechanistic explanations underlying this relationship are unclear. As a result, elevated state 4 respiration may accompany the elevated whole-animal maximal metabolic rates previously observed during migration in songbirds (DeMoranville et al., 2019; Swanson, 1995; Swanson and Dean, 1999).

2.4.1 Conclusions

Together, these data suggest that flight muscle mitochondria are seasonally remodelled to increase cellular oxidative capacity while reducing the potential for oxidative stress. Both seasonal changes are likely to benefit migrating songbirds by proximately increasing ATP supply while reducing oxidative damage to the flight muscle which may ultimately translate to improved migration speed by avoiding compromised flight performance and prolonged recovery times at stopover (McWilliams et al., 2021).

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Chapter 3

3 Flight muscle mitochondria are robust against endurance flight damage in blackpoll warblers

3.1 Introduction

Exercising animals can pay a high energetic cost to support locomotory muscle contractions, sometimes requiring muscle ATP turnover rates that are >100-fold higher than at rest (Hochachka and Matheson, 1992). In addition, exercising animals must contend with increased production of cellular oxidants in contracting muscle, including reactive oxygen species (ROS) (reviewed by Finaud et al., 2006; Powers and Jackson, 2008), though our understanding of the consequences of exercise-induced ROS production is confined largely to mammals. ROS production increases with exercise intensity (Lovlin et al., 1987), so during high-intensity exercise, ROS production may overwhelm the ROS-quenching capacity of cellular antioxidants, resulting in oxidative stress (Davies et al., 1982; reviewed by Sies et al., 2017). Oxidative stress is accompanied by disturbed cellular redox balance, peroxidation of lipids, carbonylation of proteins and oxidative modifications to nucleic acids (Niess et al., 1996; Poulsen et al., 1996). In mammals, oxidative stress is linked to decreased muscle contractility (Andrade et al., 1998; Reid et al., 1993) and fatigue (Moopanar and Allen, 2005; Reid et al., 1992; Supinski et al., 1997) due to disruption of several processes, including ion pumping, myofilament interaction and fuel metabolism (reviewed by Reid, 2008). In wild animals, such losses in exercise performance could compromise predator evasion, foraging, mating, and migrating leading to severe fitness consequences. Therefore, animals that depend highly on locomotion may be under selective pressure to maintain exercise performance by balancing mechanical output of muscles with the energetic and oxidative costs of increased ATP production.

Mitochondrial physiology is an important determinant of contractile performance in exercising muscle. Mitochondria are simultaneously the primary site of cellular ATP synthesis and a major source of intracellular ROS. As a result, the mitochondrial phenotype is tightly regulated to ensure adequate energy supply, without incurring excess oxidative damage. However, exercise acutely challenges mitochondria, but again these effects are

best characterized in mammalian taxa. At high exercise intensities, mitochondria can become dysfunctional, and incapable of meeting cellular energy demands (Ostojic, 2016). Exercise-induced mitochondrial dysfunction is induced by oxidative stress associated with high-intensity muscle contractions, as shown in mammals (Ding et al., 2010; Sahlin et al., 2010) and fish (Kamunde et al., 2023). Excess ROS inhibit the activity of several mitochondrial matrix enzymes, ultimately impairing capacities of fatty acid β-oxidation and the Krebs cycle in humans (Larsen et al., 2016; Rasmussen et al., 2001). These exercise-induced impairments in substrate oxidation culminate in decreased respiratory capacity (Chen and Gollnick, 1994; Gollnick et al., 1990; Larsen et al., 2016; Layec et al., 2018; Lewis et al., 2021). In addition, exercise can increase respiration required to offset incipient proton leak across the inner mitochondrial membrane, which reduces the scope of the electron transport system (ETS) to support ATP synthesis (Davies et al., 1982; Fernström et al., 2007; Tonkonogi et al., 1998). However, the acute mitochondrial response to exercise is variable (Caillaud et al., 1999; Madsen et al., 1996; Rasmussen et al., 2001; Tonkonogi et al., 1999; Venditti et al., 2005), depending in part on exercise intensity (Chen and Gollnick, 1994), training level and antioxidant nutritional status (reviewed by Finaud et al., 2006). While the functional consequences of exercise on mitochondria are well-defined in humans and lab rodents, the effects of exercise in wild animals with differing exercise capabilities are poorly understood.

Migratory birds undergo extreme endurance locomotion as part of their annual life-history cycle. Many species fly continuously for hours to days, requiring metabolic rates over ten-fold greater than at rest (Guglielmo, 2018). In addition to high energetic requirements, migrating birds must contend with oxidative stress incurred during flight (Cooper-Mullin and McWilliams, 2016; Costantini et al., 2007; Dick and Guglielmo, 2019a; Eikenaar et al., 2020; Jenni-Eiermann et al., 2014; McWilliams et al., 2021; Skrip et al., 2015). Due to these high physiological costs and severe fitness consequences of unsuccessful migration, migratory birds are under selection to maintain energy balance and manage oxidative stress during endurance locomotion. These challenges are likely more pressing in birds such as the Nearctic-Neotropical wood-warbler, the blackpoll warbler (*Setophaga striata*, Forster 1772), which performs a 2-3 day continuous trans-Atlantic flight as part of its autumn migration (DeLuca et al., 2015; DeLuca et al., 2019). Nearly all of this flight is over open

water, so warblers have few opportunities to stop and resume flight. As a result, the migratory flight strategy used by blackpoll warblers may damage mitochondria.

Previous work has shown mixed evidence for changes in mitochondrial function resulting from flight in migratory songbirds. For example, transcription of genes linked to mitochondrial substrate oxidation and transport are upregulated, while genes linked to mitochondrial biogenesis are downregulated following 4 hours of wind tunnel flight in the pectoralis of yellow-rumped warblers (*Setophaga coronata*) (Dick, 2017). However, activity of mitochondrial enzymes carnitine palmitoyl-transferase, citrate synthase and β-hydroxyacyl-CoA dehydrogenase in the pectoralis are unaffected by flight in the same yellow-rumped warblers (Dick, 2017) and following up to 4 hours of flight in European starlings (*Sturnus vulgaris*) (Price et al., 2022). Similarly, fatty acid-fuelled phosphorylating and non-phosphorylating respiration plus ROS emission rates are unaffected by 3 hour wind tunnel flights in European starlings (Gerson, 2012). It remains unclear if mitochondria are robust against oxidative stress induced by endurance flight or if flight protocols from previous studies were insufficient in inducing mitochondrial dysfunction. Flight muscle protein carbonyl accumulation correlates directly with energy use in yellow-rumped warblers (Dick and Guglielmo, 2019a), so increasing total flight costs (e.g. through extending flight duration) may be necessary to induce mitochondrial dysfunction. Blackpoll warblers routinely fly for long durations in captive conditions (i.e. up to 28 hours) (Elowe et al., 2023), so this species is a strong candidate for performing wind tunnel flights of sufficient length to test hypotheses about flight-induced mitochondrial dysfunction.

In addition to mitochondrial changes, migratory flight is associated with changes at higher physiological levels of organization. Much of the energy consumed during migratory flight is fuelled by fat tissue catabolism (reviewed by Guglielmo, 2018), with the remaining energy supplied from lean tissue catabolism. Although the energy contribution of protein catabolism to endurance flight is small (<20%) (Dick and Guglielmo, 2019a; Dick and Guglielmo, 2019b), it can be important for metabolite homeostasis and water balance (Dohm, 1986; Gerson and Guglielmo, 2011). Since protein cannot be stored, protein catabolism is accomplished via atrophy of lean tissues. For example, migratory flight is

associated with substantial reductions in the size of muscles and particularly digestive organs, as shown with trans-Pacific flight in great knots (*Calidris tenuirostris*) (Battley et al., 2000), and flights across the Mediterranean sea and Sahara desert in garden warblers (*Sylvia borin*) (Bauchinger and Biebach, 2001; Bauchinger et al., 2005; Biebach, 1998) plus three additional passerine species (Schwilch et al., 2002). Transoceanic flights of blackpoll warblers result in reductions of visually-scored subcutaneous fat stores and flight muscle size, reflecting the high degrees of fat and lean mass catabolism required for ultra-endurance flight (Bayly et al., 2021). In wind tunnel endurance flights, songbirds (including blackpoll warblers; Elowe et al., 2023) lose whole-body lean mass measured non-invasively by quantitative magnetic resonance (Dick and Guglielmo, 2019a; Dick and Guglielmo, 2019b; Gerson and Guglielmo, 2011; Gerson and Guglielmo, 2013; Gerson et al., 2020; Groom et al., 2019; Groom et al., 2023; Guglielmo et al., 2017), but how this lean mass loss is partitioned between flight muscle and other body components has not been measured. Furthermore, the consequences of endurance flight-induced catabolism on flight muscle fiber ultrastructure remains unexplored. Seasonal hypertrophy of flight muscle is associated with increased fiber diameter, as shown in gray catbirds (*Dumetella carolinensis*) (Marsh, 1984) and 3 species of shorebirds (*Calidris* spp.) (Evans et al., 1992), so flight-induced atrophy is presumably associated with reductions in fiber size. Flight muscle atrophy has previously been shown to be associated with decreased fiber diameter in eared grebes (*Podiceps nigricollis*) during moult-induced flightlessness on stopover (Gaunt et al., 1990), but whether this or other ultrastructural changes to muscle occur during flight in migratory birds is unknown.

The objectives of this study were to determine the effects of simulated migratory flight on flight muscle mitochondrial function, the dynamics of lean mass catabolism, and flight muscle ultrastructure in blackpoll warblers. I hypothesized that migratory flight induces mitochondrial dysfunction in the pectoralis as evidenced by lower phosphorylating respiration and greater ROS emission. I further hypothesized that even under controlled wind tunnel flight conditions, the catabolism of lean mass would primarily result from reductions of digestive organs. If flight muscle mass was reduced, I expected to see decreased fiber size. My findings will broaden our understanding of the acute effects of endurance locomotion in a migratory bird.

3.2 Methods

3.2.1 Experimental animals

All procedures followed guidelines set by the Canadian Council on Animal Care and were approved by the Western University Animal Care Committee (AUP 2018-092) and the Canadian Wildlife Service (SC-OR-2018-0256). I used a Nearctic-Neotropical migratory songbird, the blackpoll warbler (*Setophaga striata*, Forster 1772). The blackpoll warbler is a 10-20 g wood warbler that breeds in the boreal forest throughout northern Canada and Alaska, USA, and migrates to wintering grounds in northern South America (DeLuca et al., 2020).

I used similar bird capture and housing methods as described previously (Chapter 2). Briefly, migrating warblers were captured in September 2020 (n = 16) in mist nets at Long Point, ON, Canada (42.58°N, 80.40°W). Seven additional warblers were captured in September 2021 with the intent to increase sample sizes in my different flight groups (defined below). The age class and sex of each bird was determined from plumage and other characters, and wing chord was measured as a structural index of body size, as used previously in other songbird species (Kissner et al., 2003). I then transferred the birds to the Advanced Facility for Avian Research at Western University (London, ON, Canada) and housed them in free-flight aviaries (122 x 132 x 213cm) at room temperature with *ad libitum* access to water and diet made in-house (Guglielmo et al., 2017) supplemented with mealworms (*Telebrio molitor*). We kept the birds on a long day photoperiod (12.5L:11.5D) to preserve the migratory phenotype. In 2020, I conducted my experiments following the conclusion of a separate study on the same birds, which included wind tunnel flights and non-terminal blood sampling. Flight experiments in 2020 were conducted after 7-13 weeks in captivity, while in 2021 flights were completed within 6-8 weeks of capture.

3.2.2 Flight experiments

I conducted simulated endurance flights using the bird wind tunnel at the Advanced Facility for Avian Research, as described previously (Dick and Guglielmo, 2019a; Elowe et al., 2023; Gerson and Guglielmo, 2011). The design of the wind tunnel allows birds to stop flying without risk of injury, so all flights in this system are volitional and birds are

monitored at all times during flight. I set the environmental conditions in the wind tunnel and antechamber to 15°C and 70% relative humidity for all flights, and only one bird was flown at a time. I first conducted test flights - birds that flew a minimum of 10 minutes at 8 m s^{-1} without stopping were selected as candidates for endurance flights. The remaining birds were randomly divided into a fasted control group and a pre-flight control group.

On the sampling day, I fasted the birds from each group for one hour in housing conditions, then assessed body composition using quantitative magnetic resonance (QMR; EchoMRI, Echo Medical Systems, Houston, TX, USA) (Guglielmo et al., 2011). For the flown group (n = 8), birds flew at 8 m s^{-1} for 7.5-8 hours non-stop. At this point I stopped the flight and repeated QMR measurements. I used the change in fat and lean masses between timepoints to calculate flight energy use with conversion factors of 39.6 kJ g^{-1} fat mass and 5.3 kJ g^{-1} lean mass, as described previously (Dick and Guglielmo, 2019a). From these measurements, I calculated total energy expenditure and its partitioning into fat and lean mass-derived energy. I also calculated average power as total energy expenditure divided by flight duration. Immediately after the post-flight QMR, I euthanized and dissected each bird (see next paragraph). The flown birds could not eat or drink while flying, so I repeated this QMR procedure with a fasted control group (n = 8) to account for the effects of food deprivation, which affects lean mass as shown in garden warblers (*Sylvia borin*) (Biebach, 1998). I placed birds from the fasted group in a small pet carrier without food or water for 7.5-8 hours in the wind tunnel antechamber and was set to the same temperature and humidity conditions as for the flown birds. I repeated the QMR measurements at the same time points as for the flown group. My third group was a pre-flight control (n = 7), which I sampled following the initial one hour fast and QMR measurement. This group served as a control for the effects of extended (8 hour) food deprivation in the non-flight group.

3.2.3 Tissue sampling

I quickly and humanely euthanized birds via complete isofluorane anaesthesia followed by decapitation. Next, I removed and weighed the pectoralis. For the birds sampled in 2020, I removed a small piece (~1 cm^3) from the middle of one side of the pectoralis, mounted it on cork, covered it in mounting medium (Cryomatrix; Thermo Fisher Scientific) and froze it in liquid N_2-chilled isopentane. These samples were stored at -80°C until used for muscle

ultrastructural measurements (see below). For the birds sampled in 2021, I removed a small piece of pectoralis, but directly snap-froze the pectoralis in liquid N_2 for oxidative damage marker assessment. For each bird sampled, the remaining pectoralis was transferred to ice-cold biopsy preservation solution (Letellier et al., 1992; Veksler et al., 1987) (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 15 Na_2phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 MES hydrate, pH 7.1) for the remainder of the dissection (c. 20 minutes). In addition to the pectoralis, I removed and weighed the heart and lungs as part of the flight apparatus. The atria were cut away from the heart to remove residual blood prior to weighing. Afterwards, I removed and weighed tissues from the digestive system, including the liver, gizzard with proventriculus, large intestine and small intestine and present digestive tract mass as the combined mass of the gizzard, proventriculus, large intestine and small intestine. I then transferred the pectoralis to the lab for mitochondrial isolation following a 10 minute transit.

3.2.4 Mitochondrial experiments

I assessed mitochondrial function using high-resolution fluororespirometry with isolated pectoralis mitochondria as described in 2.2.2.

3.2.5 Pectoralis histology

I assessed pectoralis fiber ultrastructure using histological techniques, as described previously (Ivy and Guglielmo, 2023). All steps were performed at room temperature, unless indicated otherwise. I first sectioned the mounted samples above) at 12 µm transverse to muscle fiber length at -20°C using a cryostat. These sections were placed on slides, air-dried and stored at -80°C. Next, I fixed the slides in acetone, followed by rinsing with phosphate buffered saline (PBS; 0.01 M, pH 7.4) and air drying for 30 minutes. I then incubated the slides in 0.3% H_2O_2 for 30 minutes, followed by a PBS rinse, incubation in 5% normal goat serum and PBS containing Tween-20 (0.05%), followed by another PBS rinse. This was followed by a 15 minute incubation in Avidin D solution (SP-2001, Vector Laboratories, Burlingame, CA, USA), another PBS rinse and then a 15 minute incubation in biotin solution (P-2001, Vector Laboratories). I rinsed the slides with PBS and incubated them for 30 minutes in blocking solution (Carbo-Free Blocking Solution, SP-5040, Vector

Laboratories). I incubated the slides overnight in a humidified chamber in 20 µg mL^{-1} fluorescein-labelled *Griffonia simplicifolia* lectin 1 (FL-11015, Vector Laboratories) with primary antibodies against laminin (L-9393; Millipore, Billerica, MA, USA) diluted 1:200 (in mM: 10 HEPES, 150 NaCl, 0.1 $CaCl_2$, 1 N-acetylgalactosamine, pH 7.5). Following the incubation, I rinsed the slides in PBS and then incubated with secondary antibodies (Alexa Fluor 594, goat anti-rabbit IgG; A11037, Life Technologies, Mississauga, ON, Canada) diluted in TPBS for one hour. Finally, I rinsed sections with PBS and mounted them with Vectashield (Vector Laboratories), followed by imaging with a bright-field microscope (Leica CTR6500; Wetzlar, Germany) with Leica Application Suite Advanced Fluorescence imaging software (v3.2.0.9652). I captured images throughout the muscle cross-section, in accord with standard stereological techniques (Egginton, 1990). I manually analyzed all images in ImageJ (https://imagej.net/ij/index.html; v1.50i).

3.2.6 Statistical analyses

All analyses were performed using R (https://www.r-project.org; Version 4.3.1; R Core Development Team, 2022). I assessed the coupling of mitochondrial respiration to oxidative phosphorylation by calculating OXPHOS control efficiency (1 – state 4 respiration/state 3 respiration) (Divakaruni and Jastroch, 2022). To assess respiratory capacity independent of H^+ leak, I calculated net OXPHOS capacity as the difference between state 3 respiration and state 4 respiration (Gnaiger, 2012). I also calculated free radical leak (FRL) as the proportion of O_2 consumption explained by H_2O_2 emission as an assessment of electron leak from the ETS (Brown et al., 2009). I arcsine square root-transformed OXPHOS control efficiency and FRL prior to statistical comparisons to ensure normality in my dataset.

Initial examination of the body composition and mitochondrial data revealed unexpected but obvious differences between the 2020 and 2021 sampling years (e.g. Table 3.1; Figure 3.2), likely caused by the longer period in captivity of the birds in 2020 before testing. Since the 2021 sample sizes in each flight group were only 2-3, it was not possible to statistically evaluate year effects. Thus, I analyzed the 2020 data and present the 2021 data for visual comparisons. While the magnitudes of values in 2021 may be different, the patterns are similar to those revealed in 2020. I used general linear modeling to assess

differences among flight groups. I found that body mass and composition changed in each flight group, so I included a fixed effect of wing chord as a structural index of body size. I took a similar approach to assess variation in mitochondrial ROS emission and FRL but included O_2 concentration as an additional covariate (Li Puma et al., 2020). I repeated these analyses with the inclusion of fat energy use and interaction between fat energy use and flight condition as fixed effects, as done previously (Dick and Guglielmo, 2019a). All effects with $p < 0.1$ were included in the final model. Whenever I detected a significant ($p < 0.05$) main effect of flight group, I used post hoc testing with Bonferroni correction to compare among flight groups.

3.3 Results

3.3.1 Initial body composition

I found that prior to each flight or non-flight experiment warblers from each flight group were similar in body composition (Table 3.1). There was no significant main effect of wing chord for any body composition metric. Body mass ($F_{2,13} = 1.09$, $p = 0.36$), fat mass ($F_{2,13} = 1.59$, $p = 0.24$), lean mass ($F_{2,10} = 2.91$, $p = 0.10$) and wing chord ($F_{2,13} = 1.08$, $p = 0.37$) were all similar among flight groups. However, I found an interaction between flight group and wing chord for lean mass ($F_{3,10} = 4.09$, $p = 0.04$), where lean mass increased with wing chord in all groups, but the slope of this relationship was greater in pre-flight birds than fasted or flown birds, which were similar to each other. The initial body mass and fat mass were generally higher in 2020 than in 2021, though this difference varied by flight group and by metric of body composition. Inter-annual differences were greater for body mass than for fat mass, while lean mass was generally similar between sampling years. Wing chord was also similar between sampling years.

Table 3.1: Body composition prior to simulated migratory flight in blackpoll warblers. Body composition assessed using quantitative magnetic resonance imaging following a one hour fast. Age class (HY: hatch year; AHY: after hatch year) and sex (U: unknown; M: male) inferred from plumage characteristics. Data presented as mean ± s.e.m., with sample sizes in parentheses.

Flight group	Pre-Flight		Fasted		Flown	
Sampling year	2020	2021	2020	2021	2020	2021
Initial body mass (g)	18.20 ± 0.72 (4)	15.93 ± 0.97 (2)	19.11 ± 0.81 (6)	15.42 ± 3.38 (2)	17.51 ± 0.83 (6)	12.93 ± 0.83 (2)
Initial fat mass (g)	7.12 ± 0.04 (4)	6.17 ± 0.82 (2)	8.50 ± 0.76 (6)	4.97 ± 4.10 (2)	6.81 ± 0.84 (6)	2.50 ± 0.85 (2)
Initial lean mass (g)	10.33 ± 0.71 (4)	9.16 ± 0.05 (2)	9.80 ± 0.26 (6)	9.59 ± 0.62 (2)	9.94 ± 0.17 (6)	9.76 ± 0.33 (2)
Wing chord (mm)	72 ± 1 (4)	74 ± 1 (3)	71 ± 1 (6)	74 ± 1 (2)	73 ± 1 (6)	71 ± 2 (2)
Age class	4 HY	3 HY	6 HY	2 HY	3 HY, 3 AHY	2 HY
Sex	4 U	3 U	6 U	2 U	5 U, 1 M	2 U

3.3.2 Whole animal energetics

I next assessed variation in whole-animal energy use for each flight group (Figure 3.1), as inferred by changes in body composition. The QMR measurements from one fasted bird sampled in 2021 returned erroneous data (i.e. negative lean energy use and fat energy use that was ~15 standard deviations above the mean), so this bird was excluded from all plots that compared mitochondrial measurements against fat energy use. In addition, one pre-flight bird sampled in 2021 was not imaged using QMR. I found no significant covariation with wing chord, so this was dropped from all models. I found that fat energy use was 3-fold higher in flown birds than in fasted birds (Figure 3.1A; $t_{7.96} = 7.4$, $p < 0.001$). In contrast, lean energy use was highly variable among individuals and was marginally higher in flown than in fasted birds (Figure 3.1B; $t_{7.04} = 1.93$, $p = 0.09$). I found that the proportion of total energy provided by fat mass catabolism was similar between fasted and flown birds (Figure 3.1C; $t_{8.78} = 1.19$, $p = 0.27$). Lastly, I found that average power was 3-fold greater in flown than in fasted birds (Figure 3.1D; $t_{7.60} = 7.63$, $p < 0.001$). Fat energy use was greater and lean energy use was lower in 2020 than in 2021. Similarly, relative fat energy use and power was greater in 2020 than 2021.

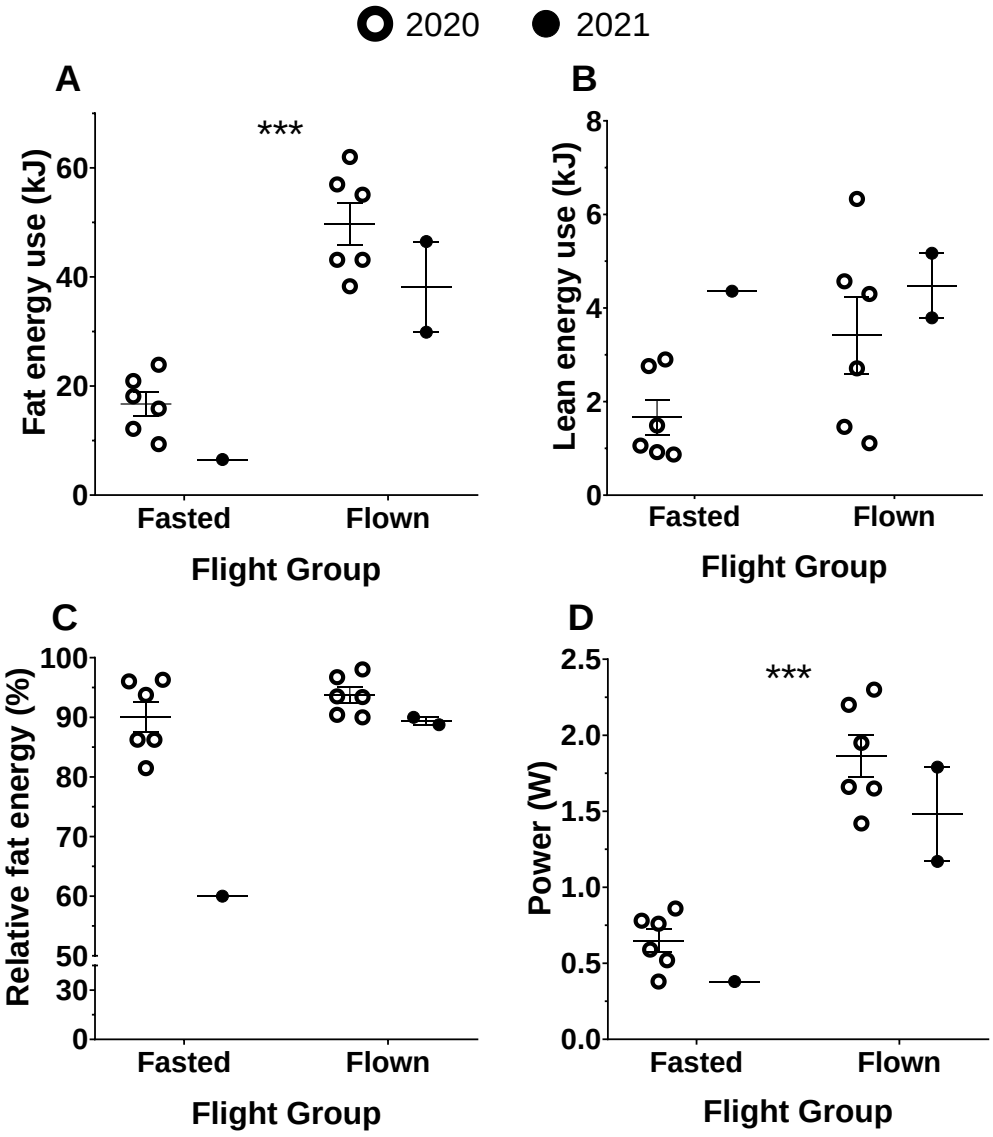

Figure 3.1: Effects of simulated migratory flight on energetics in blackpoll warblers. Warblers were sampled following an 8 hour fast without flight (fasted) or 8 hour wind tunnel flight without eating (flown). Changes in whole-animal fat and lean mass were used to calculate energy consumed from catabolism of fat mass (A) and lean mass (B). Proportion of fat mass catabolism (C) and total energy consumed standardized to time (D). Warblers were sampled in 2020 (empty) or 2021 (filled). N (2020, 2021) = 6,1 (fasted) and 6,2 (flown). Significant differences between flight groups within 2020 indicated by *** ($p < 0.001$). Data presented as mean ± s.e.m.

3.3.3 Mitochondrial fatty acid oxidation

I first compared mitochondrial fatty acid oxidation among flight groups and with fat energy use (Figure 3.2). I found that variation in state 3 respiration among flight groups approached significance (Figure 3.2A; $F_{2,11} = 3.57$, $p = 0.06$). In contrast, after adding fat energy to the model, I found that state 3 respiration did not vary between fasted and flown birds but found a negative relationship with fat energy use (Figure 3.2B; $F_{1,8} = 5.55$, $p < 0.05$; $\beta = -1.26$). In contrast, state 4 respiration was similar among flight groups (Figure 3.2C; $F_{2,11} = 1.15$, $p = 0.35$). However, when I included fat energy use in the model, I found that state 4 respiration was negatively associated with fat energy use (Figure 3.2D; $F_{1,7} = 9.20$, $p = 0.02$; $\beta = -0.38$), and state 4 respiration varied among flight groups ($F_{1,7} = 6.22$, $p = 0.04$). After accounting for covariation with fat energy use, the estimated marginal mean state 4 respiration was ~2.5-fold greater in the flown birds compared to fasted birds. I also found near-significant variation in net OXPHOS capacity among flight groups (Figure 3.2E; $F_{2,11} = 3.70$, $p = 0.06$). With the inclusion of fat energy use, I still found no variation between flown and fasted birds, or variation with fat energy use (Figure 3.2F; $F_{1,7} = 2.84$, $p = 0.14$), but I found a near-significant effect of wing chord ($F_{1,7} = 3.61$, $p < 0.10$). State 3, state 4 respiration and net OXPHOS capacity were higher in 2021 birds than in 2020.

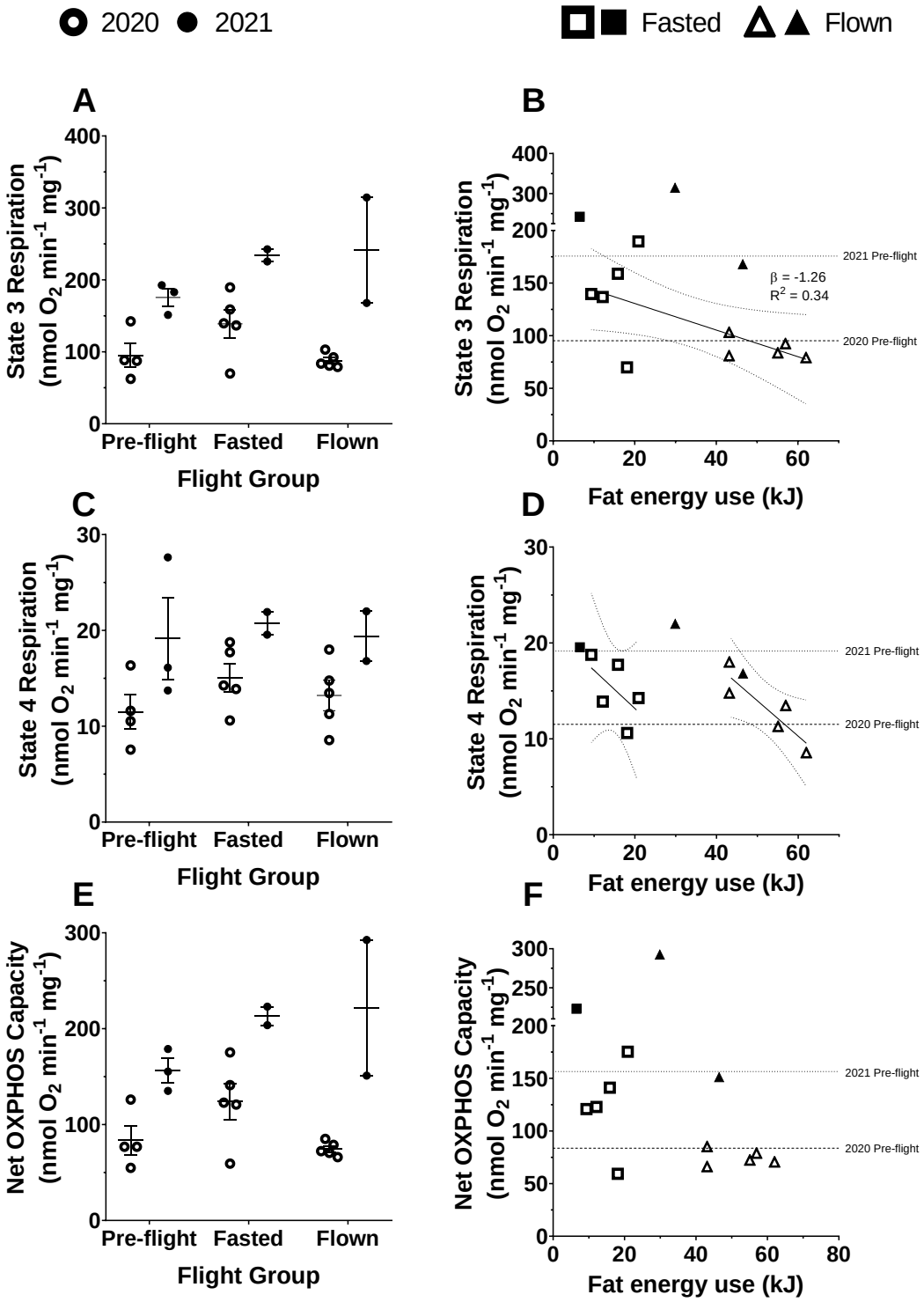
2020
2021
Fasted
Flown
A
State 3 Respiration (nmol O2 min-1 mg-1)
Pre-flight
Fasted
Flown
Flight Group
B
State 3 Respiration (nmol O2 min-1 mg-1)
2021 Pre-flight
2020 Pre-flight
β = -1.26
R2 = 0.34
Fat energy use (kJ)
C
State 4 Respiration (nmol O2 min-1 mg-1)
Pre-flight
Fasted
Flown
Flight Group
D
State 4 Respiration (nmol O2 min-1 mg-1)
2021 Pre-flight
2020 Pre-flight
Fat energy use (kJ)
E
Net OXPHOS Capacity (nmol O2 min-1 mg-1)
Pre-flight
Fasted
Flown
Flight Group
F
Net OXPHOS Capacity (nmol O2 min-1 mg-1)
2021 Pre-flight
2020 Pre-flight
Fat energy use (kJ)

Figure 3.2: Effects of simulated migratory flight on fatty acid oxidation in blackpoll warbler pectoralis mitochondria. Mitochondria were sampled in resting conditions (pre-flight), following an 8 hour fast without flight (fasted), or following an 8 hour wind tunnel flight without eating (flown). Warblers were sampled in 2020 (empty) or 2021 (filled). Changes in whole-animal fat mass in each group were used to calculate energy use derived from catabolism of fat mass (B, D, F). O_2 consumption rates of isolated pectoralis mitochondria were measured during palmitoyl-carnitine oxidation in phosphorylating (State 3; A, B) and non-phosphorylating conditions (State 4; C, D). Net OXPHOS capacity was calculated as the difference between state 3 respiration and state 4 respiration (E, F). O_2 consumption rates are expressed relative to protein content of individual mitochondrial preparations. Dotted lines correspond to mean pre-flight values for 2020 and 2021 (B, D, F). N (2020, 2021) = 4,3 (pre-flight) 5,2 (fasted) and 5,2 (flown). Solid lines indicate significant ($p < 0.05$) relationship, with 95% confidence bands.

I next assessed the coupling of mitochondrial respiration to OXPHOS during fatty acid oxidation (Figure 3.3). I found a significant negative relationship with wing chord (Figure 3.3A; $F_{1,10} = 16.65$, $p < 0.01$; $\beta = -0.02$), but no effect of flight group ($F_{2,10} = 0.57$, $p = 0.58$) on OXPHOS coupling efficiency. When I included fat energy in the model, I still found no effects of flight group or fat energy use (Figure 3.3B; $F_{1,7} = 0.14$, $p = 0.72$), but I found significant negative covariation with wing chord ($F_{1,7} = 57.65$; $\beta = -0.02$). OXPHOS coupling efficiency was higher in 2021 than 2020 for each flight group, although the inter-annual difference was greater in the flown group than in the pre-flight or fasted groups.

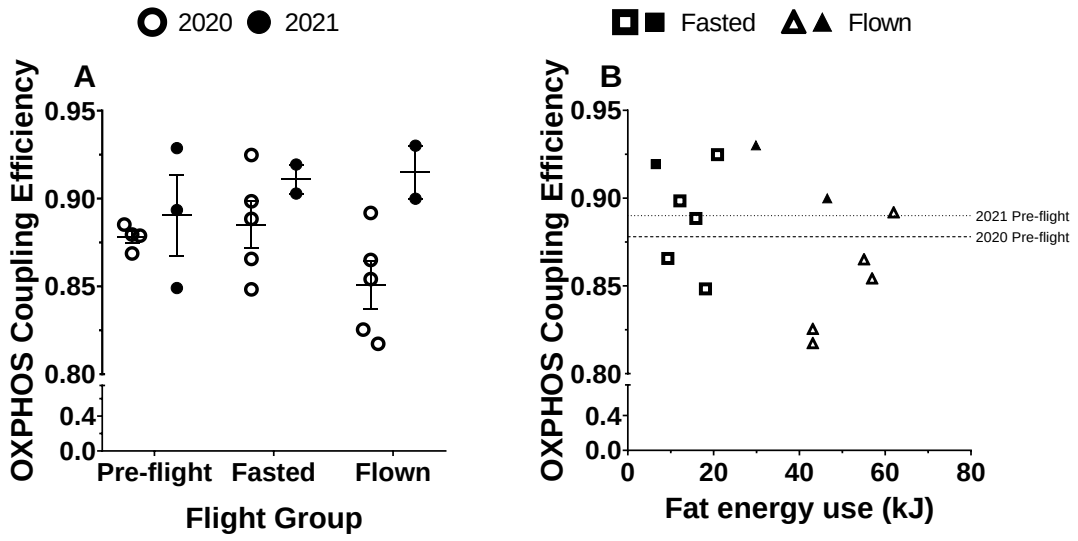

Figure 3.3: Effects of simulated migratory flight on coupling between mitochondrial respiration and oxidative phosphorylation in blackpoll warblers. Mitochondria were sampled in resting conditions (pre-flight), following an 8 hour fast (fasted), or following an 8 hour wind tunnel flight (flown). Warblers were sampled in 2020 (empty) or 2021 (filled). Changes in whole-animal fat mass in each group were used to calculate energy use derived from catabolism of fat mass (B). O_2 consumption rates of isolated pectoralis mitochondria were measured during palmitoyl-carnitine oxidation in phosphorylating (State 3) and non-phosphorylating conditions. OXPHOS coupling efficiency was calculated as the proportion of state 3 O_2 consumption linked to oxidative phosphorylation. Dotted lines correspond to mean pre-flight values for 2020 and 2021 (B). N (2020, 2021) = 4,3 (pre-flight) 5,2 (fasted) and 5,2 (flown).

3.3.4 Mitochondrial ETS flux capacities

I next assessed variation in ETS flux across flight groups (Figure 3.4). I found no significant covariation with wing chord for any metric, so this was dropped from all models. I found marginally significant variation among flight groups in complex I-IV flux (Figure 3.4A; $F_{2,13} = 3.45$, $p = 0.06$) and complex IV capacity (Figure 3.4E; $F_{2,13} = 3.25$, $p = 0.07$) while variation in complex II-IV flux (Figure 3.4C; $F_{2,13} = 5.16$, $p = 0.02$) was significant. Specifically, complex II-IV flux was higher in fasted than flown birds ($p = 0.03$), but no other pairwise differences were significant. After including fat energy use in the model, I found no main effects of flight group or fat energy use on complex I-IV flux, but I found a significant interaction between flight group and fat energy use (Figure 3.4B; $F_{2,9} = 5.81$, $p = 0.02$). Complex I-IV flux was positively associated with fat energy use in fasted birds, but this association was weakly negative in flown birds. In contrast, complex II-IV flux was negatively associated with fat energy use (Figure 3.4D; $F_{1,10} = 11.00$, $p < 0.01$; $\beta = -1.59$), but did not vary by flight group. I found no main effects of flight group or fat energy use in complex IV capacity, but I found a significant interaction between flight group and fat energy use (Figure 3.4F; $F_{2,9} = 4.78$, $p = 0.04$). While complex IV capacity was weakly positively related to fat energy use in fasted birds, complex IV capacity was negatively related to fat energy use in flown birds. ETS flux measurements were generally higher in 2021 than in 2020.

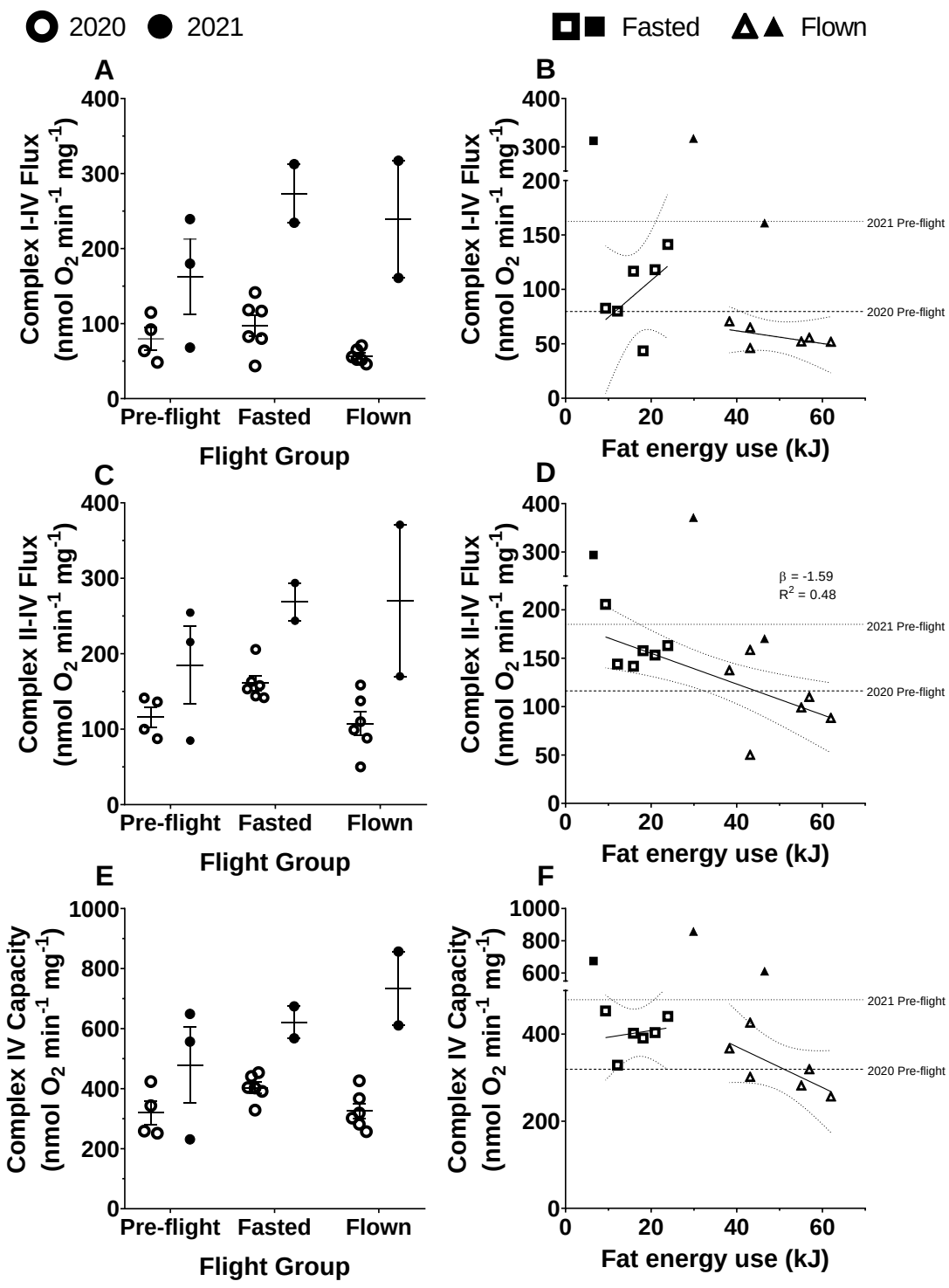
2020
2021
Fasted
Flown
A
B
C
D
E
F
Complex I-IV Flux (nmol O2 min-1 mg-1)
Complex II-IV Flux (nmol O2 min-1 mg-1)
Complex IV Capacity (nmol O2 min-1 mg-1)
Pre-flight
Fasted
Flown
Flight Group
Fat energy use (kJ)
2021 Pre-flight
2020 Pre-flight
β = -1.59
R2 = 0.48

Figure 3.4: Effects of simulated migratory flight on mitochondrial electron transport system complex-specific flux in blackpoll warblers. Mitochondria were sampled in resting conditions (pre-flight), following an 8 hour fast (fasted), or following an 8 hour wind tunnel flight (flown). Warblers were sampled in 2020 (empty) or 2021 (filled). Changes in whole-animal fat mass in each group were used to calculate energy use derived from catabolism of fat mass (B, D, F). Respiration rates specific to complexes I-IV (A, B), complexes II-IV (C, D) and complex IV (E, F). O_2 consumption rates are expressed relative to protein content of individual mitochondrial preparations. Dotted lines correspond to mean pre-flight values for 2020 and 2021 (B, D, F). Solid lines indicate significant ($p < 0.05$) relationship, with 95% confidence bands. N (2020, 2021) = 4,3 (pre-flight) 6,2 (fasted) and 6,2 (flown).

3.3.5 Mitochondrial ROS emission

I found little variation in state 3 ROS emission across flight groups (Figure 3.5A; $F_{2,11}$ = 1.81, p = 0.21). However, when comparing between fasted and flown birds, I found a marginally significant effect of flight group (Figure 3.5B; $F_{1,4}$ = 5.44, p = 0.08) plus significant effects of fat energy use ($F_{1,4}$ = 30.44, p < 0.01; β = -0.21), wing chord ($F_{1,4}$ = 17.74, p = 0.01; β = -0.41), O_2 concentration ($F_{1,4}$ = 20.44, p = 0.01; β = 0.04) and a significant interaction between flight group and fat energy use ($F_{1,4}$ = 18.78, p = 0.01). ROS emission was negatively associated with fat energy use in fasted birds, but positively associated with fat energy use in flown birds. In contrast, for state 4 ROS emission, I found marginally significant covariation with O_2 concentration (Figure 3.5C; $F_{1,9}$ = 4.36, p = 0.07), significant covariation with wing chord ($F_{1,9}$ = 6.65, p = 0.03; β = 0.08) and near-significant variation among flight groups ($F_{2,9}$ = 3.87, p = 0.06). When I included fat energy use in the model (Figure 3.5D; $F_{1,6}$ = 19.58, p < 0.01; β = -0.07), I found no effect of flight group, a near-significant effect of wing chord ($F_{1,6}$ = 4.31, p = 0.08; β = -0.07) and a significant interaction between flight group and fat energy use ($F_{1,6}$ = 15.04, p < 0.01). State 4 ROS emission was negatively associated with fat energy use in fasted and flown birds, but the slope of this relationship was steeper in the fasted birds. State 3 FRL (ROS emitted per oxygen atom consumed) varied marginally with wing chord (Figure 3.5E; $F_{1,10}$ = 4.19, p = 0.07), but did not vary with flight group ($F_{2,10}$ = 0.81, p = 0.47). Similarly, when I added fat energy use to the model, I again found no effects of flight group or fat energy use (Figure 3.5F; $F_{1,8}$ = 0.05, p = 0.83) on state 3 FRL. Similarly, I found that state 4 FRL did not vary among flight groups (Figure 3.5G; $F_{2,11}$ = 2.59, p = 0.12). State 4 FRL also did not vary among flight groups with the inclusion of fat energy use (Figure 3.5H; $F_{1,8}$ = 0.04, p = 0.85). All ROS emission and FRL measurements yielded lower values in 2021 birds compared to 2020 birds.

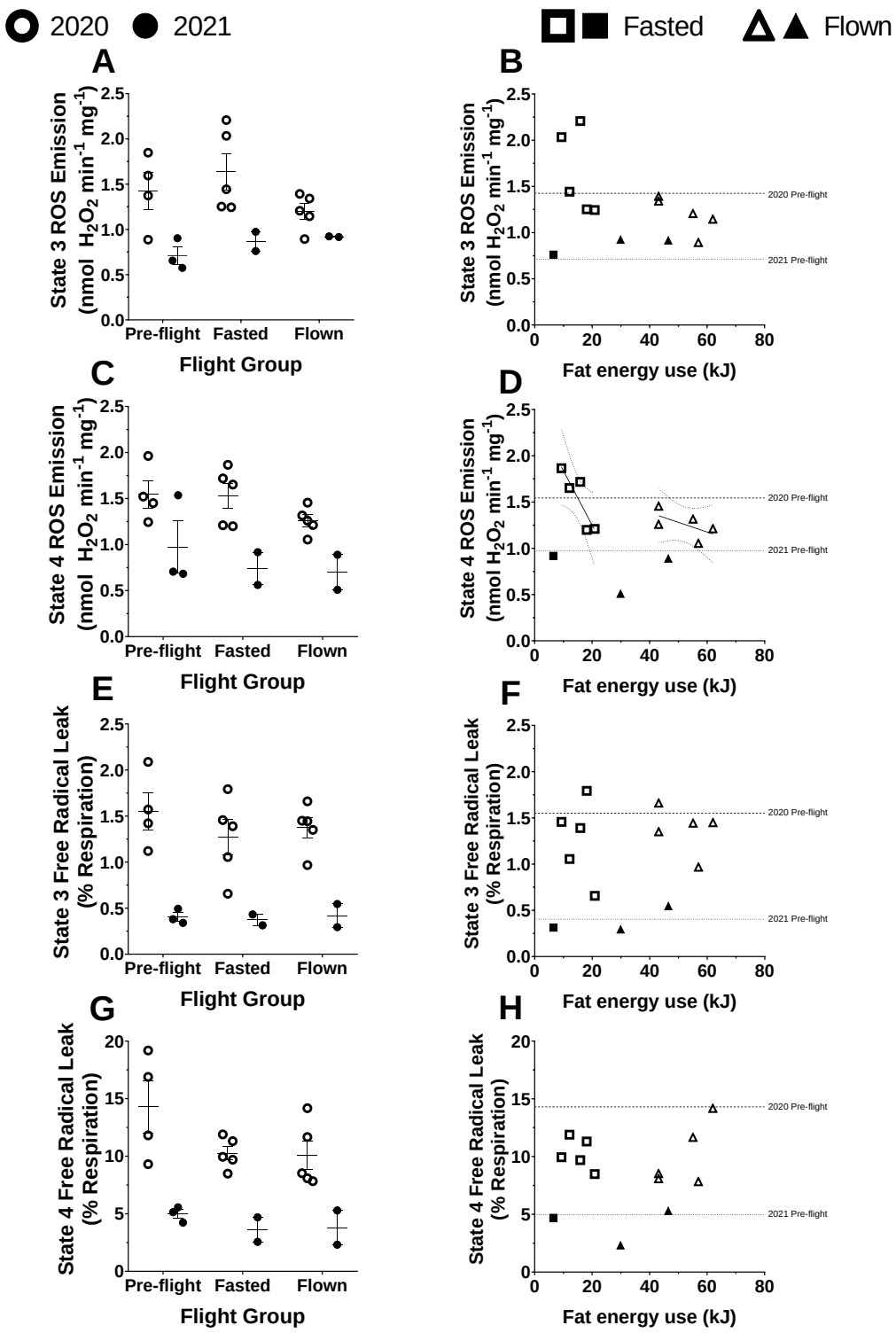
2020
2021
Fasted
Flown
A
B
C
D
E
F
G
H
State 3 ROS Emission (nmol H_2O_2 min^{-1} mg^{-1})
State 4 ROS Emission (nmol H_2O_2 min^{-1} mg^{-1})
State 3 Free Radical Leak (% Respiration)
State 4 Free Radical Leak (% Respiration)
Pre-flight
Fasted
Flown
Flight Group
Fat energy use (kJ)
2020 Pre-flight
2021 Pre-flight

Figure 3.5: Effects of simulated migratory flight on mitochondrial reactive oxygen species emission in blackpoll warblers. Mitochondria were sampled in resting conditions (pre-flight), following an 8 hour fast (fasted), or following an 8 hour wind tunnel flight (flown) in 2020 (empty) or 2021 (filled). Fat energy use derived from catabolism of fat mass (B, D, F, H). H_2O_2 emission rates of isolated pectoralis mitochondria were measured during palmitoyl-carnitine oxidation in phosphorylating (State 3; A, B, E, F) and non-phosphorylating (State 4; C, D, G, H) conditions. H_2O_2 emission rates are expressed relative to protein content of individual mitochondrial preparations (A-D) or relative to O_2 consumption rate as free radical leak (E-H). Dotted lines correspond to mean pre-flight values for 2020 and 2021 (B, D, F, H). N (2020, 2021) = 4,3 (pre-flight) 5,2 (fasted) and 5,2 (flown). Data presented as means ± s.e.m.

3.3.6 Tissue masses and histology

I assessed variation in the size of multiple tissues with simulated migratory flight (Figure 3.6). Only pectoralis mass was measured for one pre-flight bird sampled in 2021, due to the use of fixative following pectoralis excision for another study. I found no variation among flight groups in the wet mass of pectoralis (Figure 3.6A; $F_{2,13} = 1.43$, $p = 0.27$), heart (Figure 3.6B; $F_{2,13} = 0.18$, $p = 0.84$) and lungs (Figure 3.6C; $F_{2,12} = 1.60$, $p = 0.24$), which was the only tissue significantly related to wing chord ($F_{1,12} = 8.80$, $p = 0.01$; $\beta = 6.75$). In contrast, I found significant variation in the wet mass of liver (Figure 3.6D; $F_{2,13} = 6.23$, $p = 0.01$), digestive tract (Figure 3.6E; $F_{2,13} = 26.40$, $p < 0.001$) and gizzard plus proventriculus (Figure 3.6F; $F_{2,13} = 10.90$, $p < 0.01$). For these three tissues, mass was similar between fasted and flown birds, but both were significantly lower than in pre-flight birds. When we repeated these analyses using fat energy use as a fixed effect, we found no relationship with fat energy for pectoralis mass ($F_{1,10} = 0.20$, $p = 0.66$) or heart mass ($F_{1,10} < 0.01$, $p = 0.98$). In contrast, we found a positive relationship between lung mass and wing chord ($F_{1,7} = 5.91$, $p < 0.05$; $\beta = 5.54$), a significant main effect of flight group ($F_{1,7} = 7.15$, $p = 0.03$) and a near-significant interaction between fat energy use and flight group ($F_{2,7} = 3.48$, $p = 0.09$. In contrast, we found near-significant effects of flight group ($F_{1,9} = 4.19$, $p = 0.07$) and fat energy use ($F_{1,9} = 4.99$, $p = 0.05$) on liver mass. After taking fat energy use into account, the estimated marginal mean liver mass in flown birds was ~25% lower than in fasted birds. After controlling for fat energy use ($F_{1,9} = 2.44$, $p = 0.15$), gizzard and proventriculus mass marginally varied between flown and fasted birds ($F_{1,9} = 4.14$, $p = 0.07$). In contrast, digestive tract mass did not vary with fat energy use ($F_{1,10} = 1.25$, $p = 0.29$) or flight group. The effects of sampling year on lean tissue mass varied by tissue and flight group. Pectoralis mass was lower in 2021 than in 2020 for all flight groups, with a similar trend only in the pre-flight group for digestive tract and gizzard and proventriculus. All other tissues were similar between sampling years.

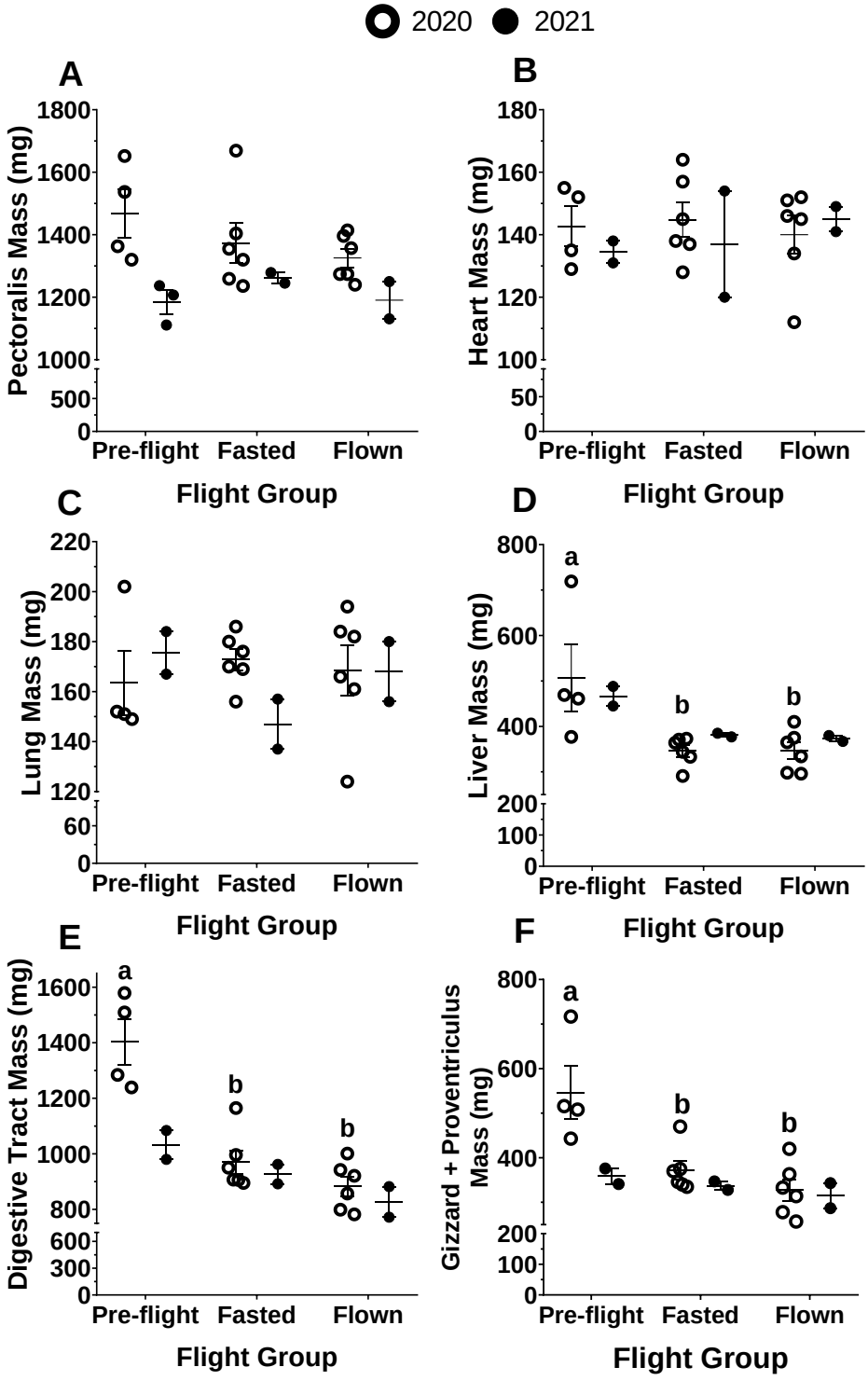
2020
2021
A
Pectoralis Mass (mg)
1800
1600
1400
1200
1000
500
0
Pre-flight
Fasted
Flown
Flight Group
B
Heart Mass (mg)
180
160
140
120
100
50
0
Pre-flight
Fasted
Flown
Flight Group
C
Lung Mass (mg)
220
200
180
160
140
120
60
0
Pre-flight
Fasted
Flown
Flight Group
D
Liver Mass (mg)
800
600
400
200
100
0
a
b
b
Pre-flight
Fasted
Flown
Flight Group
E
Digestive Tract Mass (mg)
1600
1400
1200
1000
800
600
300
0
a
b
b
Pre-flight
Fasted
Flown
Flight Group
F
Gizzard + Proventriculus Mass (mg)
800
600
400
200
100
0
a
b
b
Pre-flight
Fasted
Flown
Flight Group

Figure 3.6: Effects of simulated migratory flight on tissue size in blackpoll warblers. Wet masses presented for pectoralis (A), heart (B), lungs (C), liver (D), whole digestive tract (E) and combined masses of gizzard and proventriculus (F). Tissues were sampled in resting conditions (pre-flight), following an 8 hour fast (fasted), or following an 8 hour wind tunnel flight (flown). Birds sampled in 2020 (empty) and 2021 (filled). N (2020, 2021) = 4,2-3 (pre-flight) 6,2 (fasted) and 6,2 (flown). Data presented as means ± s.e.m. Different letters indicate significant difference ($p < 0.05$) between flight groups within 2020.

I found no variation in pectoralis fiber morphology among flight groups (Figure 3.7). After accounting for near-significant covariation from wing chord (Figure 3.7A; $F_{1,12} = 4.59$, $p = 0.05$), fiber density did not vary among flight groups ($F_{2,12} = 1.79$, $p = 0.21$). Similarly, fiber transverse area did not vary with flight group (Figure 3.7B; $F_{2,12} = 1.93$, $p = 0.19$), but covariation with wing chord was nearly significant ($F_{1,12} = 4.17$, $p = 0.06$). When I repeated these analyses with fat energy use in the model, I found significant main effects of flight group ($F_{1,7} = 13.40$, $p < 0.01$) and wing chord ($F_{1,7} = 8.99$, $p = 0.02$; $\beta = -82.07$), a significant interaction between fat energy use and flight group ($F_{2,7} = 5.13$, $p = 0.04$), but no significant main effect of fat energy use. Fat energy use was positively and negatively related to fiber density in fasted and flown birds, respectively. Similarly, fiber transverse area varied with flight group ($F_{1,7} = 10.10$, $p = 0.02$), but had marginally significant covariation with wing chord ($F_{1,7} = 5.23$, $p = 0.06$), and a marginally significant interaction between fat energy use and flight group ($F_{2,7} = 3.61$, $p = 0.08$), but no main effect of fat energy use.

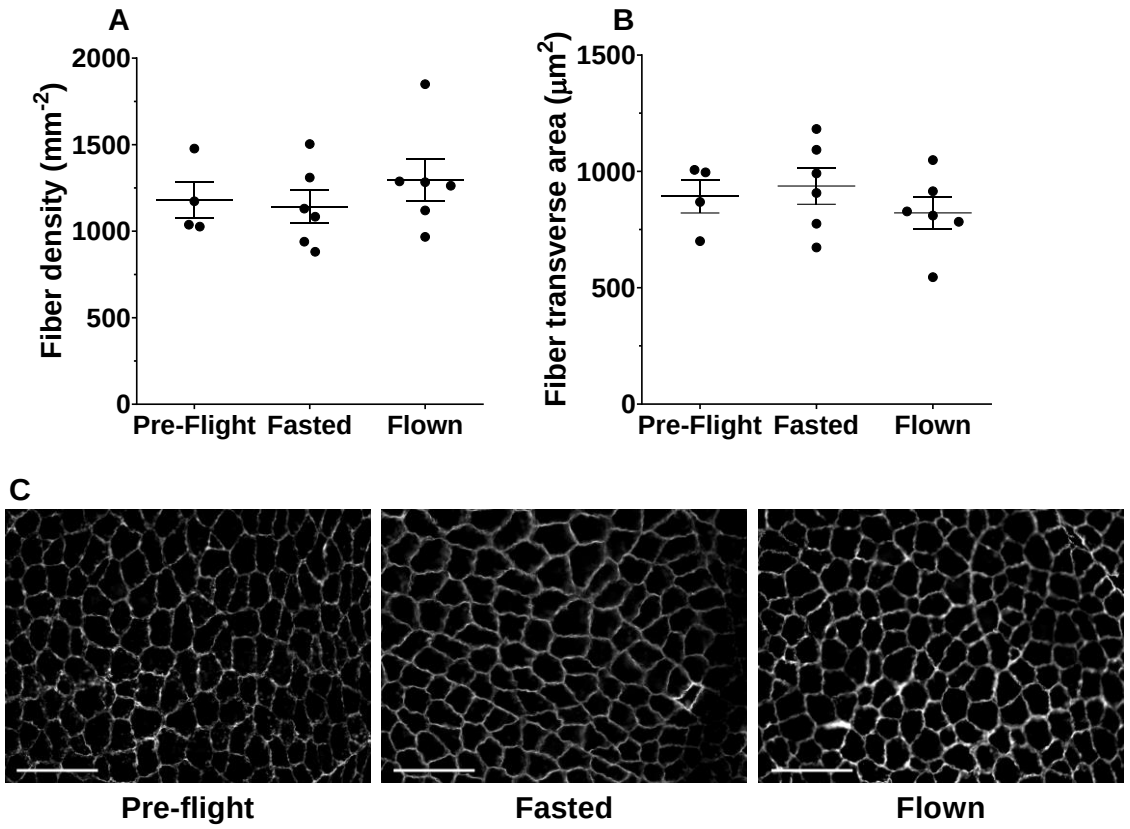

Figure 3.7: Effects of simulated migratory flight on pectoralis ultrastructure in blackpoll warblers. Pectoralis fiber density (A), fiber transverse area (B) and representative images (C). Tissues were sampled in resting conditions (pre-flight), following an 8 hour fast (fasted), or following an 8 hour wind tunnel flight (flown). N = 4 (pre-flight) 6 (fasted) and 6 (flown). Scale bar on representative images equal to 100 µm. Data presented as means ± s.e.m.

3.4 Discussion

My primary objective was to understand whether endurance flight induces mitochondrial dysfunction in blackpoll warblers, a species highly adapted for non-stop travel. However, I found little evidence in support of my hypothesis. While I found higher state 4 respiration and lower ETS complex II-IV flux in flown than fasted birds, supporting my prediction, both metrics were similar between flown and pre-flight birds. Furthermore, I found that state 3 respiration, net OXPHOS capacity and OXPHOS coupling efficiency were unaffected by simulated migratory flight, as were ETS complex I-IV flux, complex IV capacity, ROS emission and FRL rates. Together, these data indicate that the capacity for OXPHOS and respiratory chain function are robust against damage induced by simulated migratory flight in blackpoll warblers. These findings are consistent with previous wind tunnel experiments using European starlings (*Sturnus vulgaris*) (Gerson, 2012). I also assessed the hypotheses that lean mass catabolism during migratory flight is primarily associated with atrophy of digestive organs and that reductions in flight muscle mass are accompanied by decreased fiber size. In support of my hypothesis, I found that liver and combined gizzard and proventriculus mass were marginally reduced in flown birds, while the digestive tract and tissues associated with flight (pectoralis, heart, lungs) were similar between fasted and flown birds. I found no changes in pectoralis ultrastructure, but because pectoralis mass was unchanged by migratory flight, I cannot conclude that reduced fiber size is associated with reduced pectoralis mass.

3.4.1 Mitochondrial function during flight

My data indicate that flight muscle mitochondrial function is not compromised on the time scale of an overnight migratory flight in blackpoll warblers. By maintaining phosphorylating and non-phosphorylating respiration, ATP synthesis rates are unlikely to decline during flight. Furthermore, mitochondrial ROS emission rates and FRL were unaffected by flight, implying that electron leak rates are maintained during flight. As a result, endurance flight does not induce mitochondrial dysfunction. Mitochondrial resilience against exercise-induced damage is likely adaptive in blackpoll warblers because any disruption to contractile performance during endurance flight is likely to result in mortality. This resilience may also be under selection in other long-distance migrants that

must use long duration flights to cross ecological barriers, such as large water bodies (Gill et al., 2005), mountain ranges (Hawkes et al., 2011) or deserts (Schmaljohann et al., 2012).

In my study, all but one of the flown birds was flying well at the eight hour time period, indicating that blackpoll warblers are not exhausted by eight hours of flight. This is in accord with previous observations of significantly longer flights: blackpoll warblers fly 62 ± 10 hours non-stop in the wild (DeLuca et al., 2015), or up to 28 hours in a wind tunnel (Elowe et al., 2023). Mitochondrial dysfunction becomes apparent following exhaustive exercise where researchers 'push' animals to exercise past the point of volitional stoppage, as shown in horses (Chen and Gollnick, 1994; Gollnick et al., 1990) and rainbow trout (Kamunde et al., 2023), so the apparent lack of mitochondrial dysfunction observed in flown birds in my study may be explained by a lack of exhaustion. However, during wind tunnel flight, the closest birds get to exhaustion is volitional stoppage, wherein birds will not continue flying despite being in a physiological condition suitable for endurance flight. Volitional stoppage in wind tunnel flight may not be analogous to exhaustive exercise, because flying European starlings to volitional stoppage has no effect on mitochondrial fatty acid oxidation (Gerson, 2012). Furthermore, wind tunnel flights to volitional stoppage are often significantly shorter than flights used in the wild: blackpoll warblers and European starlings may fly in the wind tunnel for 28 hours (Elowe et al., 2023) and 3 hours (Gerson, 2012), respectively, until volitional stoppage, but fly 52-72 hours (DeLuca et al., 2015) and ~10 hours (Vīgants et al., 2023), respectively, in the wild. Therefore, flying migratory birds to exhaustion may not be feasible using a wind tunnel due to behavioural limitations in flight performance. An alternative experimental design to test mitochondrial dysfunction induced by endurance flight in migratory birds is to capture and assay birds in the wild before and after crossing an ecological barrier.

3.4.2 Changes to lean body components

I found that eight hours of simulated migratory flight was sufficient to decrease whole-animal lean mass, with differential changes in the masses of several lean tissues. I found that endurance flight did not change the masses of lean tissues associated with flight (pectoralis, heart, lungs), but the masses of some tissues associated with digestion (liver, gizzard and proventriculus) were reduced in flown birds. Blackpoll warblers do not eat or

drink while flying, so flight-induced reductions in digestive tissue masses are likely adaptive, due to reductions in flight costs and provisioning of metabolic H_2O. This digestive system atrophy may have only limited functional consequences, because blackpoll warblers maintain a high capacity to assimilate nutrients following transoceanic flight (Bayly et al., 2021). By preferentially atrophying digestive tissue first during flight, blackpoll warblers avoid compromising flight performance by catabolizing lean tissue essential for flight. The lower masses of the digestive tissues in fasted relative to pre-flight birds indicates that the size of these tissues is modulated in response to energy availability. One caveat to my findings is that tissues were not cleaned prior to weighing, so the large disparity in digestive tract and gizzard and proventriculus masses between pre-flight and fasted or flown birds may be partly explained by the presence of food material in the pre-flight birds. My findings corroborate previous observations of gastrointestinal atrophy during migratory flight in the wild (Bauchinger et al., 2005; Biebach, 1998; Schwilch et al., 2002).

I also assessed flight muscle ultrastructure in response to endurance flight. Pectoralis ultrastructure was unaffected by flight, indicating the absence of flight muscle atrophy. These data are in accord with pectoralis mass, which was similarly unaffected by flight. Since pectoralis mass did not decrease with flight, I am unable to conclude if decreased pectoralis size during flight is mediated by decreased fiber size. My findings contrast with previous observations of reduced muscle fiber size following an intense 10-day training regime (Fitts et al., 1989) or a running race in humans (Kayar et al., 1986). Part of this discrepancy may be explained by a high reliance on intramuscular glycogen in exercising mammals. Intramuscular glycogen depletion is accompanied by significant losses in H_2O that was ‘trapped’ by glycogen, ultimately reducing fiber diameter. In contrast, migrating birds have low reliance on intramuscular glycogen to fuel flight.

3.4.3 Captivity effects

The basis of variation between sampling years observed in my study remains unclear but may be at least partly explained by variation in time in captivity, where 2020 birds were in captivity longer than 2021 birds, due to their use in a separate study. While the effects of captivity on mitochondrial metabolism in wild birds are poorly understood, two weeks of

captivity has been shown to increase phosphorylating and non-phosphorylating respiration and decrease coupling in liver mitochondria from house finches (Koch et al., 2024). In contrast, I found that phosphorylating and non-phosphorylating respiration rates are lower and ROS emission rates are higher with greater time in captivity. This disparity is unlikely the result of differences in activity, because wind tunnel training (Gerson, 2012) and cage size (Koch et al., 2024) have limited effects on mitochondrial physiology in birds. Furthermore, 2020 warblers received more wind tunnel training, yet patterns in mitochondrial physiology were opposite of what is typically seen in endurance exercise training in mammals (Walsh et al., 2001). Seasonal phenotypic flexibility (i.e. the 2020 birds were in a non-migratory phenotype) is also an unlikely explanation because fat mass in the 2020 warblers was similar to or higher than in the 2021 warblers and both had greater fat mass than non-migratory blackpoll warblers (Ivy and Guglielmo, 2023). Furthermore, completion of an 8 hour flight implies that the warblers were in a migratory phenotype, although this assumption has yet to be tested. Lastly, flight muscle fiber ultrastructure is not seasonally flexible in blackpoll warblers (Ivy and Guglielmo, 2023). A final unresolved explanation is that the 2020 warblers spent a greater amount of time on my prepared diet than the 2021 warblers, which may influence mitochondrial membrane composition (Carter et al., 2019). The mechanisms underlying captivity effects on mitochondrial physiology in wild birds is deserving of attention, which may inform more robust experimental design in future studies.

3.4.4 Conclusions

My findings show that the flight apparatus is robust against the challenges of simulated migratory flight in an ultra long-distance migratory songbird. This protection may be facilitated by preferential catabolism of lean tissues in the digestive system, which grants the benefits of sparing the flight apparatus while reducing flight costs and gaining metabolic H_2O during flight. Together, my findings show that blackpoll warblers protect the flight apparatus at the cost of digestive system catabolism, which ultimately contributes to maintaining contractile performance during trans-oceanic flight.

3.5 References

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Chapter 4

4 Less is more: Long-distance migratory songbirds have small flight muscles and lower oxidative capacity

4.1 Introduction

Trade-offs between energy expenditure and locomotory activity are mediated by variation in the form and function of skeletal muscle, which is the primary tissue responsible for generating movement in animals. Skeletal muscle phenotype is dynamic and can vary within and among individuals or species in response to variation in physiological demands. Muscle plasticity in response to exercise training is well-explored in mammals and includes changes at all levels of physiological organization (reviewed by Hoppeler et al., 2011). Previous work has found evolved differences in muscle fiber type proportions and other physiological characteristics that match the typical locomotion animals performs, such as in primates (O'Neill et al., 2017), lizards (Bonine et al., 2001; Scales et al., 2009), fish (Cediel et al., 2008) and frogs (Astley, 2016; Chadwell et al., 2002). However, it remains unclear if similar patterns of evolved variation occur in muscle mitochondrial phenotypes in relation to endurance locomotion capacity, and this is the focus of my study.

Migratory animals provide a powerful model to test hypotheses about the evolution of muscle phenotypes and endurance locomotion. Migration is a naturally selected syndrome of traits that facilitate long-distance movement capacity, and has evolved independently in many animal taxa (Alerstam et al., 2003). In small non-soaring birds, migratory movements are performed using active flapping flight, which allows exceptionally high migration speeds relative to other modes of locomotion (Hedenström, 2003). Despite these benefits, migratory flight is associated with significant energetic and oxidative burdens. First, migratory flight requires greatly elevated whole-animal metabolic rates (i.e. ~12-fold higher than resting values) for many hours or days at a time (reviewed by Guglielmo, 2018). Second, reactive oxygen species (ROS) formation is high during migratory flight which can overwhelm antioxidant capacity, resulting in oxidative stress and compromised tissue function (reviewed by McWilliams et al., 2021). Together, these physiological challenges could contribute to the elevated mortality rates generally experienced by birds

during migration, though the increase in mortality is highly variable among species (i.e. 2-fold to 15-fold higher than non-migratory seasons) (Newton, 2024). Migratory birds are therefore under selection for traits that increase endurance locomotory performance by managing the energetic and oxidative challenges of migratory flight.

The energy requirements of avian migration vary with migration strategy, and so does the capacity for endurance locomotion. Migratory birds vary in migration distance (i.e. the distance travelled between breeding and non-breeding grounds), which is hypothesized to be positively correlated with the total energetic and temporal costs of migration. Despite the challenges of long-distance migration, some evidence suggests that long-distance migrants have similar annual energy expenditure (Brown et al., 2023), rates of mortality during migration (Newton, 2024) but greater annual survival (Winger and Pegan, 2021) compared to short-distance migrants. These findings suggest that long-distance migrants have acquired adaptations to overcome the challenges of long-distance migration, for example by increasing endurance exercise performance. Indeed, when exercising at speeds that elicit maximal metabolic rate, long-distance migrants have 60% longer times to exhaustion than short-distance migrants, despite having similar aerobic capacities ($\dot{V}O_2max$), as shown in four species of Palearctic-breeding songbirds (Hahn et al., 2022). This higher endurance capacity is supported by physiological adaptations in the flight muscle, which are predicted to increase endurance exercise performance as per mammalian exercise physiology models (van der Zwaard et al., 2021). Pectoralis size is apparently unaffected by migration distance in 55 Palearctic-breeding songbird species (Vágási et al., 2016), but these measurements were made outside of the migration season when pectoralis size typically increases (King et al., 2015; Marsh, 1984; Price et al., 2011), which may limit interspecific variation. Long-distance migrants have pectoralis muscle fibers with smaller transverse area and greater capillary densities than short-distance migrants and resident species, allowing higher delivery rates of fuel and O_2 when compared across 15 songbird species (Lundgren and Kiessling, 1988).

Little is known about how migration distance affects flight muscle metabolism. Our current understanding is based on intraspecific comparisons between migratory and non-migratory (resident) birds and between migratory and non-migratory phenotypes within a population.

For example, migratory birds have higher activities of mitochondrial enzymes (cytochrome c oxidase, citrate synthase and 2-hydroxyacyl-CoA dehydrogenase) in pectoralis muscle than non-migratory conspecifics, indicating a greater tissue oxidative capacity associated with migration (Lundgren, 1988; Rhodes et al., 2024). In addition, migrants have greater circulating non-enzymatic antioxidant capacity, relative to resident populations, as shown in common blackbirds (*Turdus merula*) (Eikenaar et al., 2017). Furthermore, pectoralis oxidative capacity is seasonally elevated during migration in songbirds (Banerjee and Chaturvedi, 2016; Chapter 2; Lundgren, 1987; Lundgren and Kiessling, 1985; Lundgren and Kiessling, 1986; McFarlan et al., 2009; Rhodes et al., 2024; Sharma et al., 2021; Zajac et al., 2011). Similarly, pectoralis antioxidant capacity is increased during migration, by increasing glutathione peroxidase activity in common quail (*Coturnix coturnix*) (Marasco et al., 2021) and transcription of superoxide dismutase in red-headed bunting (*Emberiza bruniceps*) (Banerjee and Chaturvedi, 2016). Together, these adaptations support the high reliance on abundant circulating fatty acids to fuel flight (Guglielmo, 2018) and minimize the reliance on limited fuel sources, particularly carbohydrates or proteins. Furthermore, the magnitude of flight-induced oxidative stress during flight is lowered. However, it is presently unclear how flight muscle oxidative capacity and ROS metabolism vary with migration distance among species. If pectoralis oxidative capacity and ROS metabolism are determinants of endurance exercise performance, then high pectoralis oxidative capacity and low potential for oxidative stress would allow long-distance migration.

Endurance locomotion in migratory birds is also supported by adaptations that lower the costs of migratory flight. For example, it is well-accepted that migration is associated with altered wing morphology. Specifically, migratory birds have more pointed wings with higher aspect ratio than non-migratory birds when compared across populations (Carvalho Provinciato et al., 2018), subspecies (Milá et al., 2008) and species (Lockwood et al., 1998), which lowers flight costs, as shown in Swainson's thrush (*Catharus ustulatus*) (Bowlin and Wikelski, 2008). A similar pattern of wing morphology emerges when long-distance and short-distance migrants are compared among populations (Baldwin et al., 2010; Fiedler, 2005), subspecies (Arizaga et al., 2006) or species (Chu et al., 2022; Marchetti et al., 1995; Minias et al., 2015; Mönkkönen, 1995; Vágási et al., 2016). Notably, long-distance migrants have lower flight costs than short-distance migrants, even after

controlling for wing shape, as shown in thrush nightingale (*Luscinia luscinia*) (Klaassen et al., 2000). This greater apparent flight efficiency in long-distance migrants reflects additional energy-saving adaptations, such as lower lean body mass compared to short-distance migrants (Kelsey et al., 2021). This adaptation reduces flight costs by lowering both the amount of tissue that must be kept aloft during flight and metabolic costs of tissue maintenance, though it remains unclear if all lean tissues are smaller in long-distance migrants, or tissue size is differentially reduced. Together, these morphological adaptations may require lower metabolic energy inputs during flight in long-distant migrants.

Mitochondrial physiology could be a critical determinant of migration performance, as mitochondria synthesize most of the ATP used during flight from the oxidation of fatty acids, the main fuel used during migration. However, mitochondria also emit reactive oxygen species (ROS) (Hernansanz-Agustín and Enríquez, 2021) and are energetically expensive to maintain (Rolfe and Brown, 1997). As a result, mitochondrial function is likely under selection in migratory birds to promote migratory performance. However, little is known about how mitochondrial physiology varies with migratory strategy, other than that flight muscle mitochondrial abundance (Lundgren, 1988; Rhodes et al., 2024) and coupling between respiration and ATP synthesis appear to be greater in migratory compared to resident subspecies in songbirds (Rhodes et al., 2024; Toews et al., 2014). Mitochondria are also seasonally flexible in migratory songbirds: mitochondrial abundance and capacities for oxidative phosphorylation and fatty acid oxidation are higher in migratory than non-migratory phenotypes within a population (Chapter 2; Rhodes et al., 2024). In contrast, the effects of migration strategy on mitochondrial ROS production have yet to be explored. All that is known is that mitochondrial ROS emission is seasonally flexible and is lower in the migratory phenotype (Chapter 2). Presumably, inter-specific, adaptational trends in mitochondrial physiology are similar to trends in intra-specific, seasonally plastic variation, where long-distance migrants have greater mitochondrial respiratory capacity and lower ROS emission than short-distance migrants. Such adaptations would further improve flight muscle oxidative capacity and lower the risk of oxidative stress during migratory flight.

4.1.1 Objective and hypotheses

The objective of this study was to assess the effects of migratory strategy on flight muscle metabolism in songbirds. I hypothesized that flight muscle oxidative capacity varies with migratory strategy and predicted that long-distance migration would be associated with a greater flight muscle oxidative capacity. I further predicted that long-distance migrants could evolve elevated flight muscle oxidative capacity through three non-mutually exclusive mechanisms: greater muscle size, greater mitochondrial abundance, greater mitochondrial fatty acid oxidation capacity. I also hypothesized that mitochondrial ROS production varies with migratory strategy and predicted that long-distance migration would be associated with lower mitochondrial ROS production. I tested these hypotheses using a comparative framework, by assessing flight muscle mitochondrial physiology in 19 Nearctic-breeding songbird species that vary in migratory strategy, including residents, short-distance migrants (overwinter in North America) and long-distance migrants (overwinter in South America). To my knowledge, this study is the first to assess a potential adaptive link between mitochondrial physiology and endurance locomotion in wild birds. The results of this study will be useful in revealing mechanisms of evolved variation in muscle metabolism across multiple levels of physiological organization in relation to endurance locomotion.

4.2 Methods

4.2.1 Experimental animals

All procedures followed guidelines set by the Canadian Council on Animal Care and were approved by the Western University Animal Care Committee (AUP 2018-092, 2022-028) and the Canadian Wildlife Service (SC-OR-2018-0256). I assessed mitochondrial function in 19 Nearctic-breeding songbird species that vary in migratory strategy, including 14 migratory and 5 resident species (Table 4.1). To control for seasonal variation (Chapter 2; Rhodes et al., 2024), I assessed mitochondrial physiology in the seasons predicted to elicit the highest energetic requirements of the flight muscle. I sampled migratory and resident species during the migration and non-migratory (winter) seasons, respectively. I captured migratory species at Long Point, ON (42.59°N, 80.40°W) in mist nets in autumn 2020,

2021, 2022 and spring 2022. I captured resident species at Western University campus in London, ON (43.01°N, 81.27°W) in winter 2022, 2023 using baited Potter traps, except for *Cardinalis cardinalis*, which was captured using mist-netting with breeding call playback. All migratory birds were held in short-term captivity (1-89 days) prior to sampling. All resident species were sampled on the day of capture, except *Passer domesticus*, which were also briefly held in captivity (2-8 days). Captive birds were housed at the Advanced Facility for Avian Research at Western University. Migratory species were group-housed in free-flight aviaries or individually in cages in controlled ambient conditions and long-day photoperiod (12.5L:11.5D), while *Passer domesticus* were housed in an outdoor aviary. All birds were provided food and water *ad libitum*. Frugivorous or insectivorous species were provided an agar-based diet prepared in-house (Chapter 2; Chapter 3), while granivorous species were provided a commercially available seed mixture with nutritional supplements (Mazuri Exotic Animal Nutrition, St. Louis, MO). All birds were supplemented daily with mealworms (*Telebrio molitor*).

Immediately prior to sampling, I measured wing chord of all species, except those sampled in autumn migration 2020 (*Catharus guttatus*, *C. ustulatus*, *Setophaga coronata*, *S. striata*), which were measured at time of capture. At the time of sampling, I humanely euthanized the birds using full anaesthesia under inhaled isofluorane for 5-10 seconds, followed by decapitation. I weighed the bird, then quickly removed and weighed the *pectoralis major*, whole heart, liver and lungs. A small piece from the middle of one side of the pectoralis was removed and snap-frozen in liquid nitrogen, then stored at -80℃ for enzyme analyses. The remaining pectoralis was lightly minced in ice-cold biopsy preservation solution (Letellier et al., 1992; Veksler et al., 1987) (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 15 Na_2phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 MES hydrate, pH 7.1) and transferred to the lab for mitochondrial isolation within 45 minutes.

4.2.2 Mitochondrial isolation

I conducted high-resolution fluro-respirometry on isolated mitochondria isolated from the pectoralis as described previously (Chapter 2; Chapter 3).

4.2.3 Mitochondrial fluoro-respirometry

I measured mitochondrial respiration and ROS emission rates using high resolution fluororespirometry, as described previously (Chapter 3; Coulson et al., 2024). Mitochondria were assayed at 39°C. I simultaneously measured respiration, ROS emission rates, and O_2 concentration during oxidation of palmitoyl-carnitine. ROS emission was measured via fluorescence using Amplex Ultra-Red. I stimulated oxidative phosphorylation with the addition of saturating amounts of malate, palmitoyl-carnitine and ADP to measure state 3 respiration and afterwards added oligomycin to inhibit oxidative phosphorylation to measure state 4 respiration.

In a separate run, I measured mitochondrial respiration in response to stimulation by different electron sources, as described previously (Chapter 3; Coulson et al., 2024). I assessed complex I-IV flux by stimulation with saturating concentrations of ADP, pyruvate and malate followed by inhibition with rotenone. Next, I assessed complex II-IV flux with the addition of succinate, followed by antimycin A. Finally, I measured complex IV capacity by first stimulating with N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and ascorbate followed by inhibition with KCN. I measured the difference in respiration between stimulation and inhibition of each complex as the complex-specific respiration. The remaining isolated mitochondrial preparations were frozen at -80°C for later analyses.

4.2.4 Citrate synthase activity

I measured the apparent maximal activity (V_{max}) of citrate synthase (CS) in pectoralis as a proxy for mitochondrial abundance using methods described in Chapter 2 with modification. I mechanically homogenized frozen pectoralis in ice-cold buffer, followed by snap-freezing in liquid N_2 and sonication 100; VirTis, Gardiner, NY, USA). Within an hour of sonication, I measured CS activity in triplicate in a 96-well plate using a spectrophotometer at 39°C.

4.2.5 Migration characterization

I estimated migration distances for each of the migratory species assayed, using methods previously described, with modification (Chu et al., 2022; Vágási et al., 2016; Winger and Pegan, 2021). I used 2021 range map data from eBird.org that were prepared from abundance measurements for each migratory species at a precision of 9 km x 9 km grid cell size (Fink et al., 2022). I used non-breeding ranges to categorize migratory species into short- to medium-distance migrants and long-distance migrants. Species with non-breeding range abundance only in North America were classified as short-distance migrants, while species with non-breeding abundance in South America were classified as long-distance migrants (Figure 4.2). I also calculated species-specific estimates of total migration distance as the great-circle distance between the geometric centroids of the breeding and non-breeding ranges using the R packages *sf* (Pebesma, 2018) and *geosphere* (Hijmans, 2022) (Table 4.1).

4.2.6 Statistical analyses

All analyses were performed using R (Version 4.3.1; R Core Development Team, 2022). I assessed the coupling of mitochondrial respiration to oxidative phosphorylation by calculating OXPHOS control efficiency (1 – state 4 respiration/state 3 respiration) and net OXPHOS capacity (state 3 respiration – state 4 respiration). I arcsine square root-transformed OXPHOS control efficiency prior to statistical comparisons to ensure normality in our dataset.

I prepared a phylogeny of our 19 species as a subset of one previously constructed using genomic DNA (Ericson et al., 2006) (Figure 4.1). For all comparisons, I used phylogenetic generalized least squares modelling via the *caper* package (Orme et al., 2023) to control for the effects of phylogeny. I used a maximum likelihood approach to estimate Pagel's λ (Pagel, 1997) as an estimate for strength of phylogenetic signal for each trait. λ ranges between 0 (no phylogenetic signal) and 1 (strong phylogenetic signal). For traits that had an undetectable phylogenetic signal ($\lambda < 0.001$), I repeated my analyses using general linear modelling via the *car* package (Fox and Weisberg, 2019) to increase statistical power. I included $\log_{10}$-transformed body mass as a covariate for all analyses, plus assay O_2

concentration for comparisons of ROS emission rates as used previously (Chapter 3; Coulson et al., 2024; Li Puma et al., 2020). All covariates with $p < 0.1$ were included in the final model. To better show interspecific variation with migratory strategy or migration distance, I plotted the residuals for each trait after accounting for all covariates. I used Tukey HSD as a post hoc test to investigate differences among migratory strategies. To assess the contribution of seasonal flexibility, I included data collected from *Setophaga coronata* in both migratory and non-migratory phenotypes (Coulson et al., 2024). I did not include the non-migratory phenotype data when fitting statistical models and instead used visual comparisons to assess the direction and magnitude of seasonal flexibility for each trait relative to the interspecific relationship with migration distance. I used the models fitted to all species to calculate the residual for non-migratory *Setophaga coronata*.

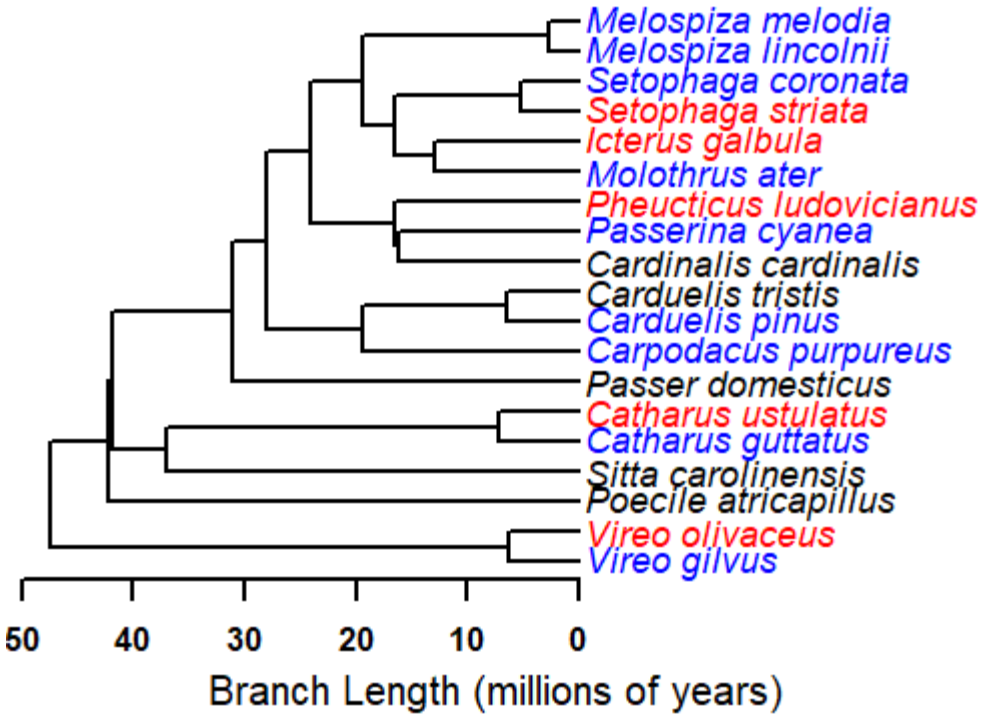

Figure 4.1: Cladogram of songbird species assayed. Cladogram of species used in this study constructed using phylogeny from Ericson et al., (2006) which is based on genomic DNA. Long-distance migrants are blue, short-distance migrants are red, resident species are black. Axis is in millions of years since the present.

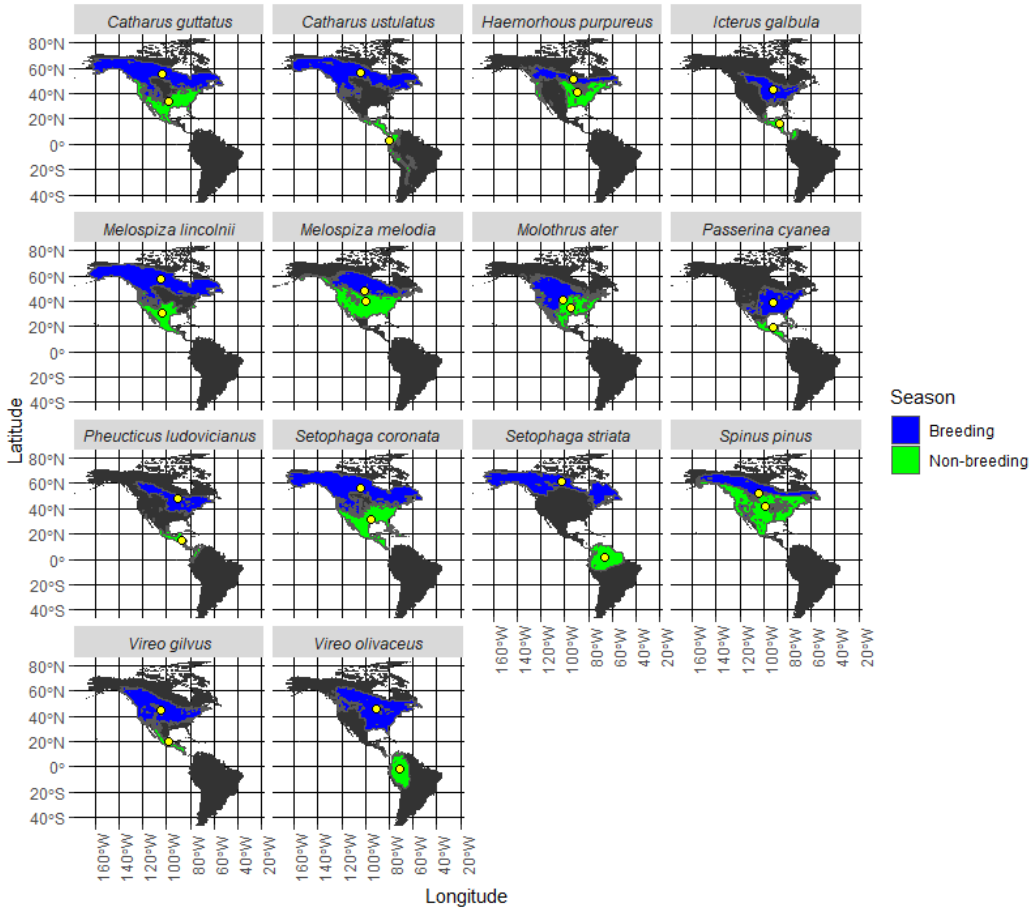

Figure 4.2: Songbird species range maps used to estimate migration distances. Maps prepared using 2021 eBird abundance data (eBird.org). Breeding ranges and non-breeding ranges are indicated by blue and green, respectively. Centroids for each range indicated by yellow circle.

Table 4.1: Sampling characteristics of songbird species used in this study. Migratory strategy determined as resident (do not migrate), short-distance migrant (overwinter in North America) or long-distance migrant (overwinter in South America). Migration distance estimated per species using great-circle distance between centroids of breeding and non-breeding ranges (Fig. 2). Sex presented as number of individuals identified as male (M), female (F) or unknown (U). Age class presented as number of individuals identified as hatch year (HY) or after hatch year (AHY). Values presented as mean ± s.e.m.

Family	Species	Migration strategy	Migration distance (km)	Body mass (g)	Sex (M,F,U)	Age class (HY, AHY)	Tarsus length (mm)	Wing chord (mm)
Cardinalidae	*Passerina cyanea*	Short-distance	2222	15.0±0.4	1,3,0	0,4	19.2±0.4	64.3±0.9
Cardinalidae	*Cardinalis cardinalis*	Resident	N/A	46.6	1;0;0	0;1	23.5	96
Cardinalidae	*Pheucticus ludovicianus*	Long-distance	3702	55.4±1.0	3,4,0	7,0	26.0±0.3	99.3±0.9
Fringillidae	*Spinus tristis*	Resident	N/A	13.5±0.2	1,1,0	0,2	14.2±0.7	68.5±1.5
Fringillidae	*Spinus pinus*	Short-distance	1144	13.8±0.3	4,1,0	0,4	15.2±0.3	72.0±1.4
Fringillidae	*Haemorhous purpureus*	Short-distance	1167	25.5±0.9	5,2,1	4,2	18.4±0.6	79.1±0.5
Icteridae	*Icterus galbula*	Long-distance	3097	42.6±1.9	2,5,1	5,2	25.2±0.7	90.3±1.5
Icteridae	*Molothrus ater*	Short-distance	821	48.1±1.6	6,2,0	0,8	26.1±0.8	104.4±2.1
Paridae	*Poecile atricapillus*	Resident	N/A	10.8±0.3	4,3,0	0,7	16.2±0.3	62.6±0.7
Parulidae	*Setophaga striata*	Long-distance	7388	16.5±1.1	0,0,6	8,0	21.9±0.6	72.0±0.8
Parulidae	*Setophaga coronata*	Short-distance	2832	12.5±0.5	6;0;2	8;0	n.d.	69.6±0.5
Passerellidae	*Melospiza lincolnii*	Short-distance	2931	17.2±1.1	0,0,3	3,0	23.2±0.3	61.0±1.0
Passerellidae	*Melospiza melodia*	Short-distance	912	20.3±0.4	1,2,5	6,2	22.9±0.7	62.8±0.6
Passeridae	*Passer domesticus*	Resident	N/A	26.9±1.1	2,2,0	0,4	20.4±0.3	76.0±2.0
Sittidae	*Sitta carolinensis*	Resident	N/A	20.4±0.5	2;2;0	0;4	18.2±0.7	86.8±1.0
Turdidae	*Catharus guttatus*	Short-distance	2346	31.8±0.7	0,0,8	8,0	n.d.	89.4±0.7
Turdidae	*Catharus ustulatus*	Long-distance	6303	33.5±0.7	0,1,8	6,3	n.d.	95.2±0.9
Vireonidae	*Vireo olivaceus*	Long-distance	5627	18.5±0.7	2,4,4	10,0	21.1±0.4	76.5±0.7

4.3 Results

4.3.1 Tissue masses

I found no significant effect of body mass for any trait except masses of pectoralis, heart, lung, and liver, so this covariate was removed from all other models. Similarly, I found no phylogenetic signal ($\lambda < 0.001$) for body mass-adjusted pectoralis mass in any comparison, and for wing chord-adjusted pectoralis mass when comparing among migratory strategies, so phylogeny was not considered in these models. Pectoralis mass covaried with body mass ($F_{1,15} = 308.4$, $p < 0.001$; $\beta = 1.12$), and varied with migration strategy (Figure 4.3A; $F_{2,15} = 15.6$, $p < 0.001$). Pectoralis mass was greatest in resident species, followed by short-distance migrants and then long-distance migrants. Within migratory species only, pectoralis mass covaried with body mass ($F_{1,11} = 168.6$, $p < 0.001$; $\beta = 1.00$) and was negatively related with migration distance (Figure 4.3B; $F_{1,11} = 10.6$, $p < 0.01$; $\beta = -2.71 \times 10^{-5}$). Migratory species have varying amounts of fat mass (Guglielmo et al., 2022; Vincze et al., 2019), which may confound my use of body mass as an index of body size, so I repeated these analyses using wing chord as a structural index of body size. After accounting for significant covariation from wing chord ($F_{1,10} = 31.7$, $p < 0.001$; $\beta = 3.35$), pectoralis mass was similar among migratory strategies and exhibited a strong phylogenetic signal (Figure 4.3C; $F_{2,10} = 1.1$, $p = 0.36$, $\lambda = 1.0$). In contrast, when examining only migratory species, I found similar covariation between pectoralis mass and wing chord ($F_{1,11} = 85.7$, $p < 0.001$; $\beta = 2.79$) with a negative relationship with migration distance (Figure 4.3D; $F_{1,11} = 13.3$, $p < 0.01$; $\beta = -4.15 \times 10^{-5}$).

I also investigated variation in the masses of heart, liver and lungs associated with migration strategy (Figure 4.4; Figure 4.5). When corrected for body mass, a strong phylogenetic signal was only detected for lung mass ($\lambda = 1.0$). I found that body mass was positively associated with the masses of heart ($F_{1,15} = 254.3$, $p < 0.001$, $\beta = 0.90$), liver ($F_{1,15} = 85.6$, $p < 0.001$, $\beta = 0.89$) and lungs ($F_{1,10} = 210.2$, $p < 0.001$, $\beta = 0.95$) across all species. Within migratory species, I found similar trends, where body mass was positively associated with masses of heart ($F_{1,11} = 146.3$, $p < 0.001$, $\beta = 0.83$), liver ($F_{1,11} = 84.3$, $p < 0.001$, $\beta = 0.89$) and lungs ($F_{1,7} = 90.0$, $p < 0.001$, $\beta = 0.88$). After adjusting for body mass,

the masses of heart (Figure 4.4A; $F_{2,15} = 9.7$, $p < 0.01$) and lungs (Figure 4.4E; $F_{2,10} = 5.9$, $p = 0.02$) varied with migration strategy, while liver mass was consistent among migration strategies (Figure 4.4C; $F_{2,15} = 1.0$, $p = 0.39$). After adjusting for body mass, heart mass was lowest in long-distance migrants, followed by short-distance migrants and then residents. Body mass-adjusted lung mass was similar between short- and long-distance migrants but was greater in short-distance migrants than in resident species. Within migratory species, body mass-adjusted heart mass declined with migration distance (Figure 4.4B; $F_{1,11} = 6.4$, $p = 0.03$, $\beta = -1.86 \times 10^{-5}$), while body mass-adjusted liver (Figure 4.4D; $F_{1,11} = 0.3$, $p = 0.60$) and lung mass (Figure 4.4F; $F_{1,7} = 0.7$, $p = 0.44$) were unaffected by migration distance.

I found positive associations with wing chord and strong phylogenetic signals for masses of heart ($F_{1,10} = 32.2$, $p < 0.001$, $\beta = 2.63$; $\lambda = 1.0$), liver ($F_{1,10} = 17.1$, $\beta = 2.45$; $\lambda = 0.93$) and lungs ($F_{1,10} = 46.0$, $p < 0.001$, $\beta = 2.68$; $\lambda = 1.0$) after adjusting for wing chord in all species. After adjusting for wing chord, migration strategy had no effect on masses of heart (Figure 4.5A; $F_{2,10} = 0.6$, $p = 0.54$), liver (Figure 4.5C; $F_{2,10} = 0.3$, $p = 0.73$) or lungs (Figure 4.5E; $F_{2,10} = 2.8$, $p = 0.11$). I found no phylogenetic signal for wing chord-adjusted tissue masses in migratory species only. I again found positive associations between wing chord and masses of heart ($F_{1,11} = 82.8$, $p < 0.001$, $\beta = 2.31$), liver ($F_{1,11} = 16.8$, $p < 0.001$, $\beta = 2.11$) and lungs ($F_{1,7} = 32.5$, $p < 0.001$, $\beta = 2.53$). Wing chord-adjusted heart mass was negatively associated with migration distance (Figure 4.5B; $F_{1,11} = 10.1$, $p < 0.01$, $\beta = -3.05 \times 10^{-5}$), but liver (Figure 4.5D; $F_{1,11} < 0.1$, $p = 0.77$) and lung (Figure 4.5F; $F_{1,7} = 3.5$, $p = 0.10$) masses were unaffected by migration distance.

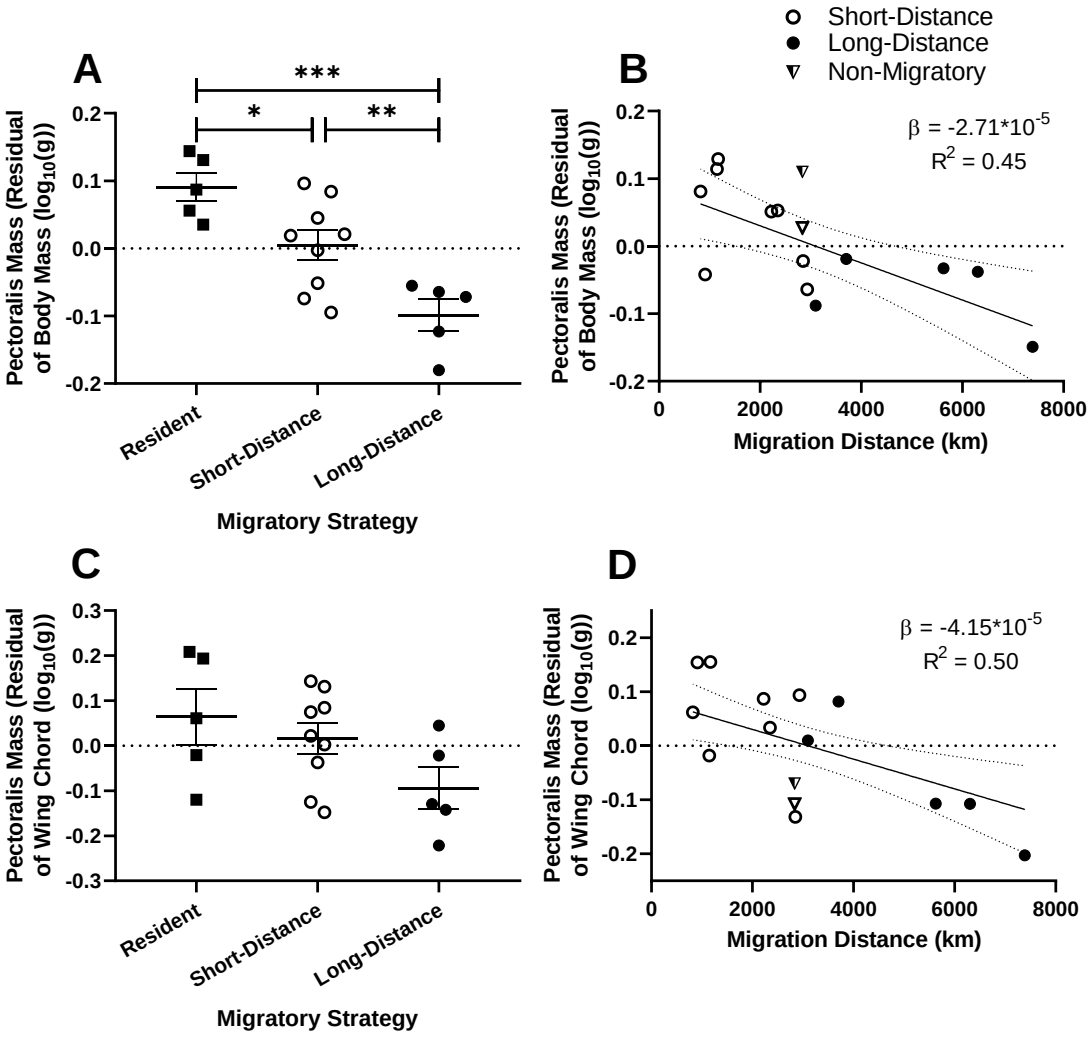
A
***
*
**
Pectoralis Mass (Residual of Body Mass (log10(g))
0.2
0.1
0.0
-0.1
-0.2
Resident
Short-Distance
Long-Distance
Migratory Strategy
B
Short-Distance
Long-Distance
Non-Migratory
β = -2.71*10-5
R2 = 0.45
Pectoralis Mass (Residual of Body Mass (log10(g))
0
2000
4000
6000
8000
Migration Distance (km)
C
Pectoralis Mass (Residual of Wing Chord (log10(g))
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
Resident
Short-Distance
Long-Distance
Migratory Strategy
D
β = -4.15*10-5
R2 = 0.50
Pectoralis Mass (Residual of Wing Chord (log10(g))
0.2
0.1
0.0
-0.1
-0.2
0
2000
4000
6000
8000
Migration Distance (km)

Figure 4.3: Effects of migration strategy on pectoralis size in songbirds. Pectoralis wet mass compared among species of different migratory strategy (A, C) or migration distance (B, D). Masses are presented as residuals after accounting for covariation from body mass (A, B) or wing chord (C, D). Migratory strategy determined as resident (do not migrate – filled squares), short-distance migrant (overwinter in North America – empty circles) or long-distance migrant (overwinter in South America – filled circles). For *Setophaga coronata*, data collected in the migratory phenotype are plotted in empty triangle (migratory phenotype) and data collected in the non-migratory phenotype (Chapter 2) plotted in half-filled triangle. Migration distance estimated as great-circle distance between centroids of breeding and non-breeding ranges of migratory species. Data presented as species means, calculated from 1-10 individuals. Significant differences among migratory strategies indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). Significant relationships ($p < 0.05$) shown as solid line with 95% confidence bands.

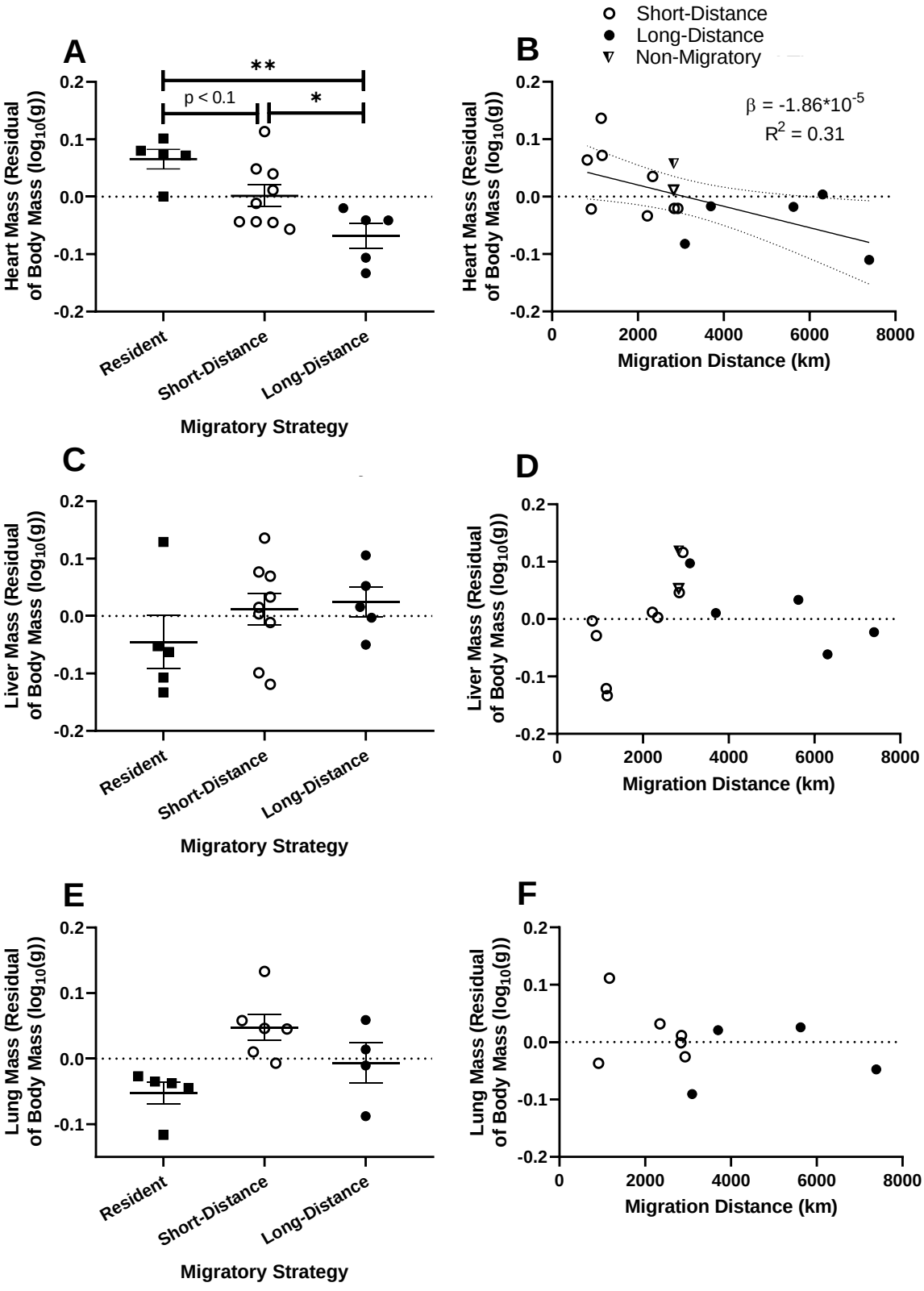
A
B
C
D
E
F
Short-Distance
Long-Distance
Non-Migratory
**
*
p < 0.1
β = -1.86*10-5
R2 = 0.31
Heart Mass (Residual of Body Mass (log10(g))
Liver Mass (Residual of Body Mass (log10(g))
Lung Mass (Residual of Body Mass (log10(g))
Resident
Short-Distance
Long-Distance
Migratory Strategy
Migration Distance (km)
0.2
0.1
0.0
-0.1
-0.2
0
2000
4000
6000
8000

Figure 4.4: Effects of migration strategy on lean tissue size in songbirds. Wet masses of heart (A, B), liver (C, D) and lungs (E, F) compared among species of different migratory strategy (A, C, E) or migration distance (B, D, F; see Figure 4.3 for description). Masses are presented as residuals after accounting for covariation from body mass. Data presented as species means ± s.e.m., calculated from 1-10 individuals. Significant differences among migratory strategies indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). Significant relationships ($p < 0.05$) shown as solid line with 95% confidence bands.

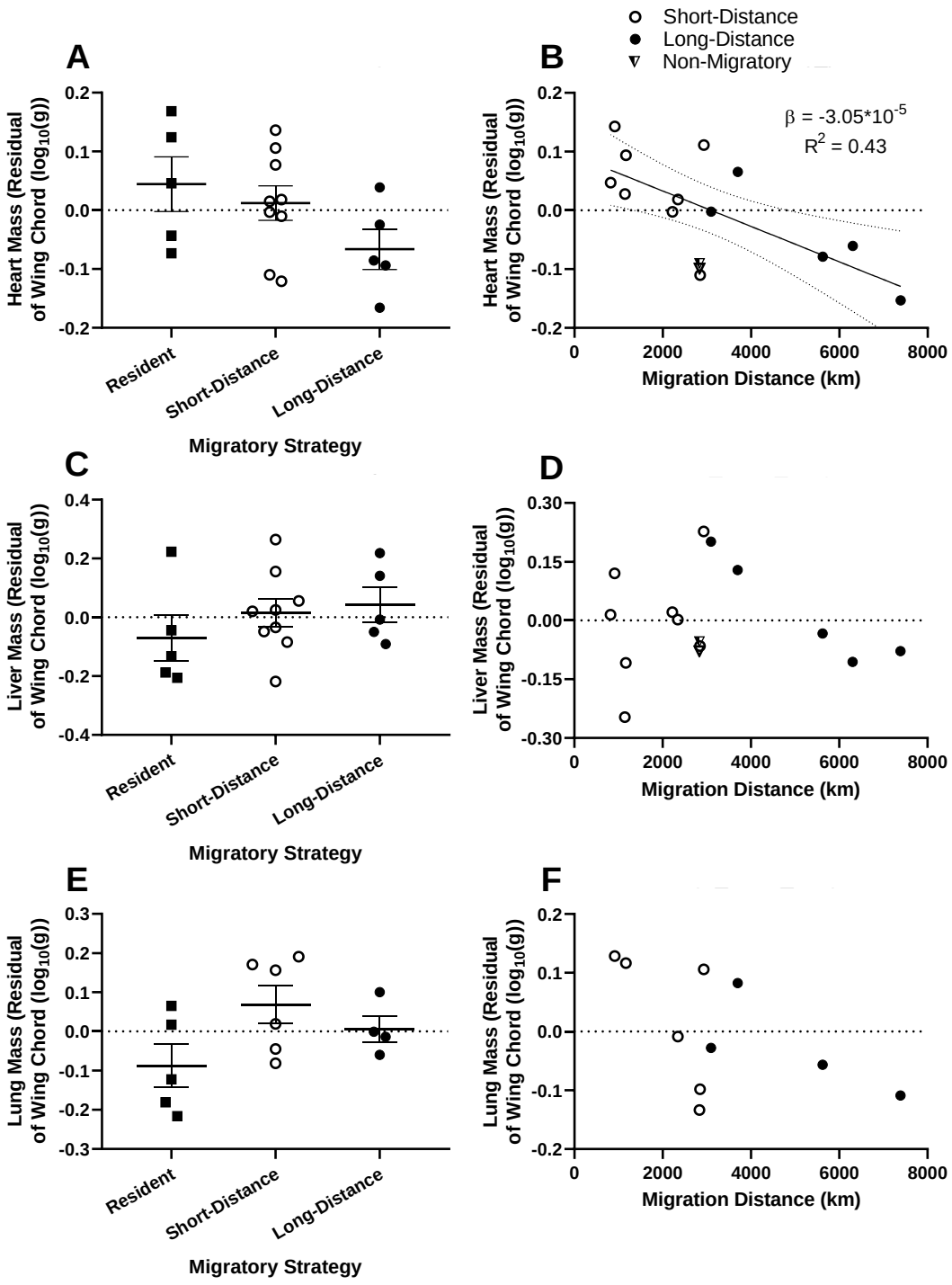
A
B
C
D
E
F
Short-Distance
Long-Distance
Non-Migratory
β = -3.05*10-5
R2 = 0.43
Heart Mass (Residual of Wing Chord (log10(g))
Liver Mass (Residual of Wing Chord (log10(g))
Lung Mass (Residual of Wing Chord (log10(g))
Resident
Short-Distance
Long-Distance
Migratory Strategy
Migration Distance (km)

Figure 4.5: Effects of migration strategy on lean tissue size in songbirds. Wet masses of heart (A, B), liver (C, D) and lungs (E, F) compared among species of different migratory strategy (A, C, E) or migration distance (B, D, F; see Figure 4.3 for description). Masses are presented as residuals after accounting for covariation from wing chord. Data presented as species means ± s.e.m., calculated from 1-10 individuals. Significant differences among migratory strategies indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). Significant relationships ($p < 0.05$) shown as solid line with 95% confidence bands.

4.3.2 Citrate synthase activity

For logistical reasons, I could not measure CS activity in one short-distance migrant species (*Melospiza lincolnii*) and one long-distance migrant species (*Setophaga striata*). I found no phylogenetic signal ($\lambda < 0.001$) for CS activity. Pectoralis CS activity did not vary with migratory strategy (Figure 4.6A; $F_{2,9} < 0.1$, $p = 0.97$), or with migration distance (Figure 4.6B; $F_{1,6} = 1.6$, $p = 0.99$).

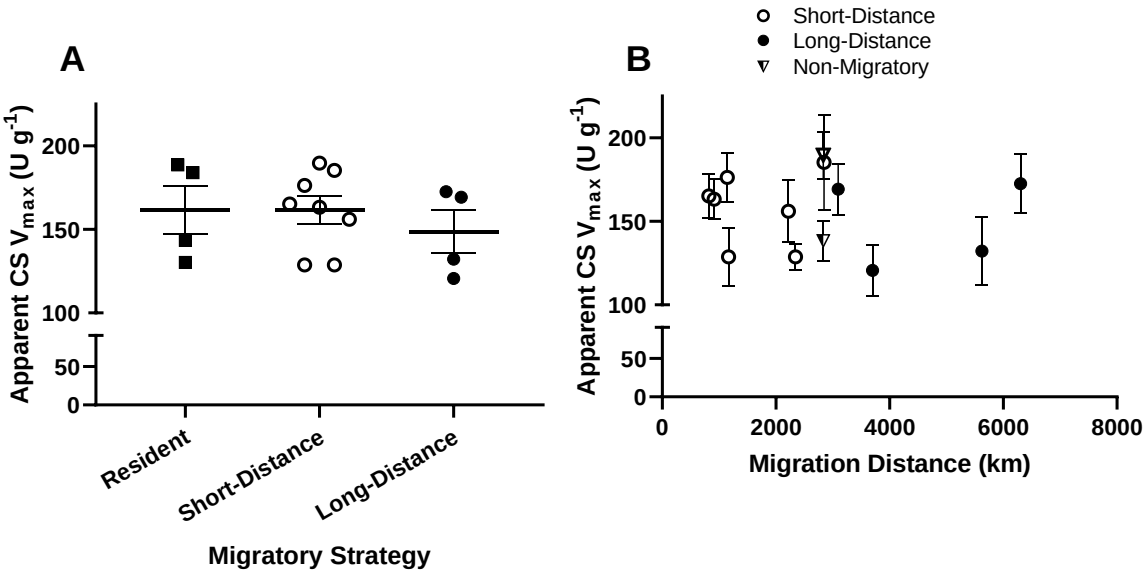

Figure 4.6: Effects of migration strategy on pectoralis citrate synthase (CS) activity in songbirds. Apparent maximal activity (V_{max}) of CS (standardized to pectoralis wet mass) compared among species of different migratory strategy (A) or migration distance (B; see Figure 4.3 for description). Data presented as species means ± s.e.m., calculated from 1-10 individuals.

4.3.3 Mitochondrial fatty acid oxidation

I was unable to acquire stable state 3 respiration measurements in one resident species (*Pinus tristis*) and therefore did not estimate OXPHOS coupling efficiency or net OXPHOS capacity. I found no phylogenetic signal for any measurements of mitochondrial respiration involving palmitoyl-carnitine oxidation ($\lambda < 0.001$). I found that migratory strategy was marginally associated with state 3 respiration (Figure 4.7A; $F_{2,15} = 3.5$, $p = 0.06$) and net OXPHOS capacity (state 3 – state 4 respiration) (Figure 4.7E; $F_{2,15} = 3.7$, $p = 0.05$) but did not vary with state 4 respiration (Figure 4.7C; $F_{2,16} = 1.6$, $p = 0.22$) or OXPHOS coupling efficiency (Figure 4.7G; $F_{2,15} = 0.79$, $p = 0.47$). Post-hoc testing revealed that short-distance migrant species had marginally greater ($p = 0.06$) state 3 respiration and 50% greater net OXPHOS capacity ($p < 0.05$) than resident species. Without consideration of phylogeny, I found that migration distance was significantly negatively associated with state 3 respiration (Figure 4.7B; $F_{1,12} = 7.8$, $p = 0.02$; $\beta = -0.02$) and net OXPHOS capacity (Figure 4.7F; $F_{1,12} = 6.6$, $p = 0.02$; $\beta = -0.02$), nearly significantly associated with state 4 respiration (Figure 4.7D; $F_{1,12} = 4.3$, $p = 0.06$), but not associated with OXPHOS coupling efficiency (Figure 4.7H; $F_{1,12} = 0.01$, $p = 0.91$).

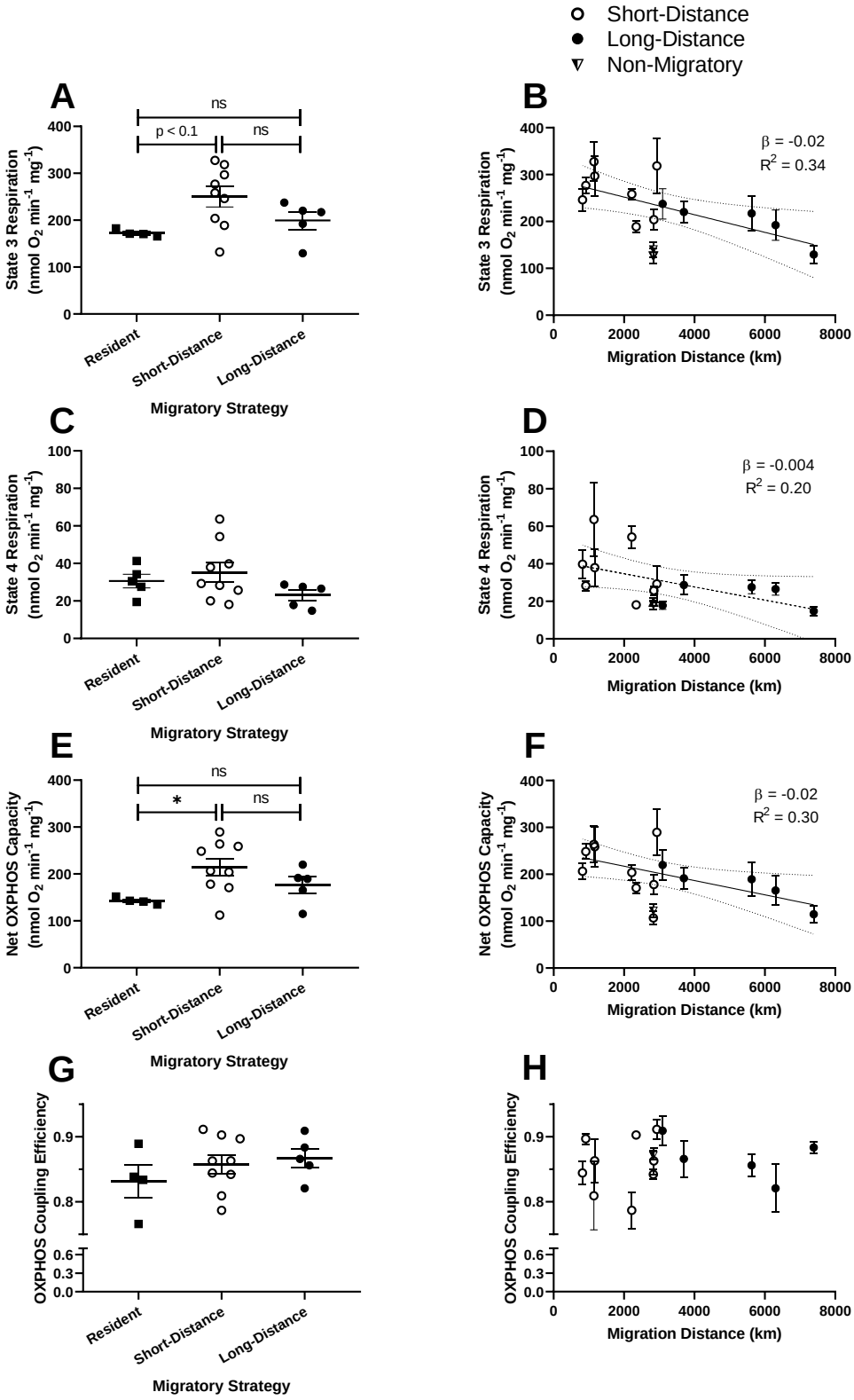
Short-Distance
Long-Distance
Non-Migratory
A
ns
p < 0.1
ns
State 3 Respiration (nmol O2 min-1 mg-1)
Resident
Short-Distance
Long-Distance
Migratory Strategy
B
β = -0.02
R2 = 0.34
Migration Distance (km)
C
State 4 Respiration (nmol O2 min-1 mg-1)
D
β = -0.004
R2 = 0.20
E
*
Net OXPHOS Capacity (nmol O2 min-1 mg-1)
F
β = -0.02
R2 = 0.30
G
OXPHOS Coupling Efficiency
H

Figure 4.7: Effects of migration strategy on fatty acid oxidation in songbird pectoralis mitochondria. O_2 consumption rates of isolated pectoralis mitochondria were measured during oxidation of palmitoyl-carnitine in phosphorylating (State 3; A, B) and non-phosphorylating conditions (State 4; C, D). Net OXPHOS capacity was calculated as the difference between state 3 respiration and state 4 respiration (E, F). OXPHOS coupling efficiency was calculated as the proportion of state 3 respiration linked to oxidative phosphorylation (G, H; 1 – state 3/state 4). O_2 consumption rates are expressed relative to protein content of individual mitochondrial preparations. Respiration compared among species of different migratory strategy (A, C, E, G) or migration distance (B, D, F, H; see Figure 4.3 for description). Data presented as species means ± s.e.m., calculated from 1-10 individuals. Significant differences among migratory strategies indicated by * ($p < 0.05$). Significant ($p < 0.05$) and nearly significant ($p < 0.10$) correlations shown as solid line or dotted line, respectively, with 95% confidence bands.

I also assessed mitochondrial ROS emission during palmitoyl-carnitine oxidation. I found a strong phylogenetic signal ($\lambda = 1$) for all ROS emission rate comparisons, so I accounted for phylogeny in all comparisons. State 3 ROS emission varied significantly with O_2 concentration across all species ($F_{1,10} = 26.6$, $p < 0.001$; $\beta = 7.8 \times 10^{-3}$) and within migratory species ($F_{1,8} = 20.9$, $p < 0.01$; $\beta = 7.81 \times 10^{-3}$), so I included O_2 concentration as a covariate. State 3 ROS emission did not vary with migratory strategy (Figure 4.8A; $F_{2,10} < 0.1$, $p = 0.97$), or migration distance (Figure 4.8B; $F_{1,8} = 0.3$, $p = 0.63$). Similarly, state 4 ROS emission significantly varied with O_2 concentration across all species ($F_{1,10} = 58.4$, $p < 0.001$; $\beta = 8.0 \times 10^{-3}$) and in migratory species only ($F_{1,8} = 55.4$, $p < 0.001$; $\beta = 7.70 \times 10^{-3}$), so O_2 concentration was included as a covariate. However, State 4 ROS emission was unaffected by migratory strategy (Figure 4.8C; $F_{2,10} = 1.5$, $p = 0.26$), and migration distance (Figure 4.8D; $F_{1,8} = 1.6$, $p = 0.25$).

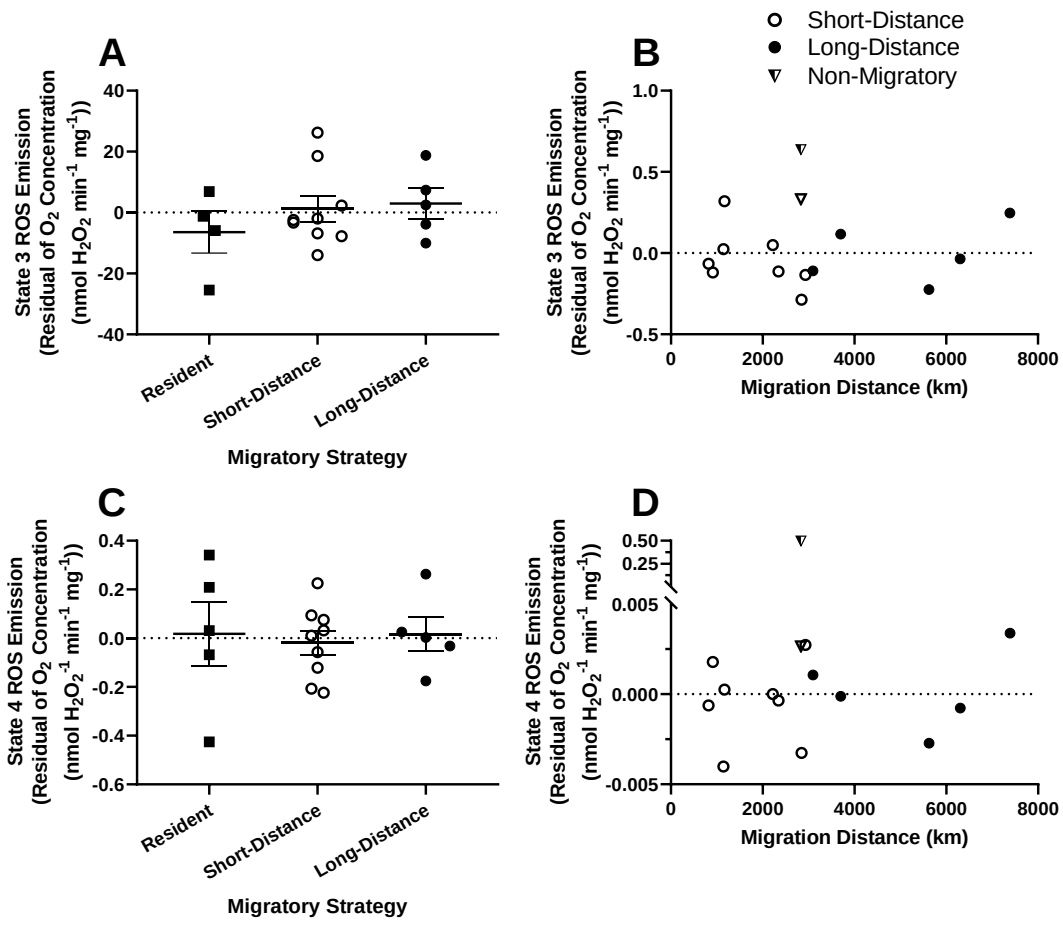

Figure 4.8: Effects of migration strategy on reactive oxygen species (ROS) emission during fatty acid oxidation in songbird pectoralis mitochondria. H_2O_2 emission rates of isolated pectoralis mitochondria were measured during oxidation of palmitoyl-carnitine in phosphorylating (State 3; A, B) or non-phosphorylating conditions (State 4; C, D). H_2O_2 emission rates compared among species of different migratory strategy (A, C) or migration distance (B, D; see Figure 4.3 for description). H_2O_2 emission rates standardized to protein content of individual mitochondrial preparations and expressed as residuals, after accounting for covariation from assay O_2 concentration. Data presented as species means, calculated from 1-10 individuals.

4.3.4 Electron transport system flux

I next assessed flux capacities through the ETS from varying electron sources. When comparing among migratory strategies, I found strong phylogenetic signals ($\lambda > 0.9$) for all flux measurements. In contrast, I found no phylogenetic signal ($\lambda < 0.001$) when comparing flux measurements with migration distance. I found near-significant variation among migratory strategies in oxidative flux through ETS complexes I-IV (Figure 4.9A; $F_{2,13} = 3.1$, $p = 0.08$, $\lambda = 0.98$), but no variation in complex II-IV flux (Figure 4.9C; $F_{2,13} = 2.5$, $p = 0.12$, $\lambda = 1.0$) or complex IV capacity (Figure 4.9E; $F_{2,13} = 1.3$, $p = 0.30$, $\lambda = 1.0$). In addition, I found that migration distance was negatively associated with complex I-IV flux (Figure 4.9B; $F_{1,12} = 4.8$, $p < 0.05$, $\beta = -0.02$), complex II-IV flux (Figure 4.9D; $F_{1,12} = 7.0$, $p = 0.02$, $\beta = -0.03$) and complex IV capacity (Figure 4.9F; $F_{1,12} = 5.9$, $p = 0.03$, $\beta = -0.07$).

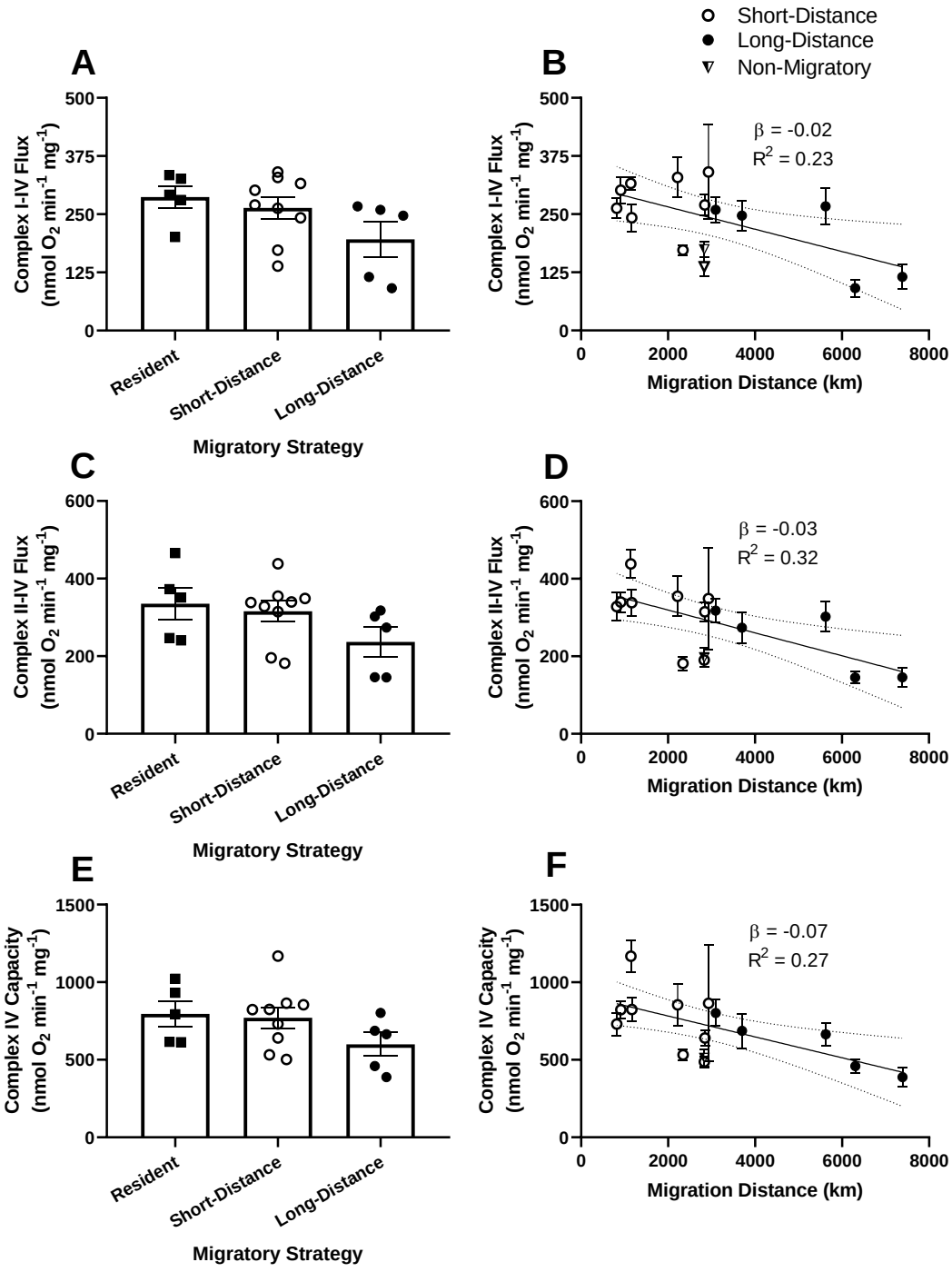
A
B
C
D
E
F
Short-Distance
Long-Distance
Non-Migratory
Complex I-IV Flux (nmol O2 min-1 mg-1)
Complex II-IV Flux (nmol O2 min-1 mg-1)
Complex IV Capacity (nmol O2 min-1 mg-1)
Resident
Short-Distance
Long-Distance
Migratory Strategy
Migration Distance (km)
β = -0.02
R2 = 0.23
β = -0.03
R2 = 0.32
β = -0.07
R2 = 0.27

Figure 4.9: Effects of migration strategy on electron transport system complex-specific flux in songbird isolated pectoralis mitochondria. Respiration rates specific to A) complexes I-IV, B) complexes II-IV and C) complex IV. Respiration compared among species of different migratory strategy (A, C, E) or migration distance (B, D, F; see Figure 4.3 for description). O_2 consumption rates are expressed relative to protein content of individual mitochondrial preparations. Data presented as species means ± s.e.m., calculated from 1-10 individuals. Significant ($p < 0.05$) correlations shown as solid line with 95% confidence bands.

4.4 Discussion

In this study, I tested the hypotheses that flight muscle oxidative capacity and potential for oxidative stress vary with migratory strategy in songbirds and predicted greater flight muscle size, mitochondrial abundance, fatty acid oxidation capacity and lower ROS emission in long-distance migratory species. However, my findings provided no support for my hypothesis, as pectoralis size, state 3 mitochondrial respiration, and net OXPHOS capacity decreased with migration distance, while mitochondrial abundance (as inferred by CS activity) was similar among species. Lower state 3 respiration was consistent with negative relationships for ETS complex I-IV, II-IV flux and complex IV capacity with migration distance, reflecting a lower respiratory capacity, while ROS emission was unaffected by migration distance. Together, my results show that pectoralis metabolism is altered in long-distance migratory songbirds, but not for the benefits of increased fatty acid oxidation capacity or lower ROS emission. Instead, my findings indicate that long-distance migration in songbirds is associated with reduced pectoralis oxidative capacity, which may reflect evolved reductions in physiological demands of endurance flight.

4.4.1 Reduced exercise capacity in long-distance migrants

I predicted that long-distance migratory songbirds would meet the energetic challenges of extended migration by evolving an enhanced capacity for endurance exercise by increasing flight muscle oxidative capacity. Contrary to my prediction, pectoralis oxidative capacity and heart size were reduced in long-distance migrants, suggesting a lower capacity for endurance exercise, as per the mammalian model (van der Zwaard et al., 2021). Despite this apparent reduction in exercise capacity, long-distance migrants make some of the most impressive long-distance movements in the world, such as a 2-3 day non-stop transoceanic flight by *Setophaga striata* (DeLuca et al., 2015). Clearly, the current model based on mammalian exercise physiology is insufficient to explain interspecific variation in avian migration physiology. I propose that the apparent negative relationship between endurance exercise capacity and migration distance observed in this study is adaptive and reflects how prioritization of time versus energy minimization during flight varies with migration distance. Under this new model, longer-distance migrants minimize energy expenditure by maintaining a sufficient capacity for endurance exercise with limited excess capacity. By

reducing flight costs, long-distance migrants increase maximal flight range for a given fuel load and may be less constrained by limited and/or unpredictable energy availability during migration, which is a greater challenge compared to short-distance migrants. Evidence for reduced flight costs in long-distance migrants currently exists at multiple levels of organization. Morphological adaptations include more pointed wings which increase aerodynamic efficiency (e.g. Chu et al., 2022; Minias et al., 2015; Vágási et al., 2016) and lower lean mass, which reduces total body mass (Kelsey et al., 2021). Behavioural adaptations include lower flight speed (Nilsson et al., 2014) and flying at lower altitude to access favourable winds (Schmaljohann et al., 2009), which both reduce cost of transport. Lower flight costs enable reduced musculature, which further reduces flight costs due to lower body mass. By saving energy during flight, long-distance migratory songbirds may in turn reduce time requirements for fuel replenishment at stopover. Indeed, stopover times are shorter in long-distance than short-distance migrants (Beauchamp, 2024), contributing to greater overall speed of migration in long-distance songbirds (Ellegren, 1993; Schmaljohann, 2019).

Pectoralis size is modulated by birds in response to flight demands. For example, pectoralis mass changes in concert with body mass throughout migration in red knots (*Calidris canutus*) (Lindström et al., 2000). By adjusting pectoralis size in response to flight demands, migratory birds can meet the mechanical workload of flight without maintaining unnecessary amounts of musculature. I hypothesize that the low pectoralis size in long-distance migratory songbirds is the result of adaptations that lower pectoralis demands during flight. While this mechanism is yet to be empirically tested, previous work has shown that increasing flight demands via manipulations of wing morphology are linked to greater pectoralis size relative to body size. For example, greater pectoralis mass is associated with moult in Eurasian tree sparrows (*Passer montanus*) (Lind and Jakobsson, 2001) and feather clipping in black-capped chickadees (*Poecile atricapillus*) (Petit and Vézina, 2014). Alternatively, some species maintain pectoralis size but reduce body mass in response to experimentally reduced wing area in common crossbills (*Loxia curvirostra*) (Fernández-Eslava et al., 2022) and great tits (*Parus major*) (Senar et al., 2002). If true, this mechanism would suggest that flight muscle phenotypic plasticity in response to flight demands is a determinant of interspecific variation of pectoralis metabolism.

4.4.2 Oxidative capacity

Despite having lower pectoralis oxidative capacity, long-distance migrants have a similar whole-animal aerobic capacity ($\dot{V}O_2max$) during exercise as short-distance migrants (Hahn et al., 2022). This disparity may be the result of underlying physiological mechanisms that allow long-distance migrants to maintain aerobic capacity despite a lower pectoralis oxidative capacity. I speculate that long-distance migrants have high rates of O_2 delivery to mitochondria, allowing full use of mitochondrial capacity at $\dot{V}O_2max$, while short-distance migrants have lower rates of O2 delivery, which limits $\dot{V}O_2max$ to a rate that is lower than predicted by pectoralis oxidative capacity. This mechanism has not been tested in migratory birds, but previous work in humans has shown that $\dot{V}O_2max$ can be lower than what is predicted by state 3 respiration (Boushel et al., 2011). Indeed, long-distance migrants have greater capacities for fuel and O_2 delivery to pectoralis mitochondria due to lower diffusion distance (Lundgren and Kiessling, 1988). Furthermore, migratory birds have greater hemoglobin concentrations and hematocrit than non-migratory subspecies (Rhodes et al., 2024) and species (Yap et al., 2019), which may translate to greater O_2 delivery to mitochondria in long-distance migrants than short-distance migrants.

The most well-documented model system similar to long-distance migratory birds is the ‘mini mouse’ phenotype generated by at least 14 generations of artificial selection for high running activity in lab mice (*Mus domesticus*) and is characterized by 50% reductions in locomotory muscle size (Houle-Leroy et al., 2003). Mini mice have greater endurance exercise performance than normal-muscled mice, as inferred by increased running speeds during endurance exercise (Schwartz et al., 2023; Syme et al., 2005) and greater reliance on lipids at submaximal exercise intensities (Templeman et al., 2012), but at the cost of lower sprint speeds (Dlugosz et al., 2009). This greater endurance exercise performance is determined by a suite of physiological changes in the locomotory muscle, similar to what is observed in migratory birds, including greater oxidative enzyme activity (Houle-Leroy et al., 2003), muscle capillary density (Wong et al., 2009), capacity for fatty acid delivery to mitochondria (Templeman et al., 2012) plus reduced expression of fast glycolytic fiber types (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008; McGillivray et al., 2009). Together, these traits increase the oxidative capacity of locomotor muscles

despite the lower size. At the whole-muscle level, mini mice have reduced muscle shortening velocity, but higher endurance during isolated contractions (Castro et al., 2022; Syme et al., 2005). These changes in muscle function are apparently sufficient to increase whole-animal endurance exercise performance without changes in $\dot{V}O_2max$, which has been reported as higher than (Schwartz et al., 2023; Templeman et al., 2012) or similar to (Rezende et al., 2006) normal-muscled mice. Surprisingly, mini mice may be less efficient than normal-muscled mice during endurance running, due to lower efficiency of muscle contractions (McGillivray et al., 2009) and higher cost of transport, which is partially explained by greater postural costs (Dlugosz et al., 2009). It remains unclear if natural selection acting on long-distance migrating birds has produced a similar phenotype as artificial selection for high running activity in mini mice. Notably, migratory birds may not have the same high postural cost that increases cost of transport in mini mice due to different biomechanical requirements between running and flight. All mice used in these selection experiments were provided *ad libitum* access to food, so adaptations that promote energy efficiency during locomotion may not be selected, while this selective pressure is likely much greater in migratory birds. Regardless, the physiological traits that are altered in mini mice may be opportunities to explore in migratory birds to test hypotheses about convergent evolution in energetics of endurance locomotion.

4.4.3 Contributions of seasonality and selective pressure

Interspecific variation in mitochondrial metabolism is determined by contributions of plasticity and selective pressure. Mitochondrial metabolism is seasonally flexible in migratory songbirds (Chapter 2; Rhodes et al., 2024) and varies with migration distance (this study). However, the magnitude of the effect caused by seasonal flexibility relative to selective pressure appears to vary among traits, as inferred by comparing migratory and non-migratory phenotypes in *Setophaga coronata* (Chapter 2). Specifically, seasonal flexibility in body size-adjusted pectoralis, heart and liver masses, pectoralis mitochondrial abundance and O_2 concentration-corrected ROS emission had a much greater apparent effect size than in all mitochondrial respiration measurements. Underlying reasons for why traits vary in degree of seasonal flexibility are unclear. I speculate that the degree of

seasonal flexibility in flight muscle energetic traits is determined by the capacity for seasonal flexibility and the resulting effect size on whole-animal flight energetics.

I compared short and long-distance migrants to winter-acclimatized resident songbirds to assess how songbirds adjust flight muscle metabolism in response to high energy demand, induced by migratory flight and low ambient temperature, respectively. I predicted greater pectoralis size, oxidative capacity and lower ROS emission in migratory species than residents. In partial support of my predictions, net OXPHOS capacity was greater in short-distance migrants than residents but was similar between residents and long-distance migrants. In contrast, all other metrics of mitochondrial respiration, ROS emission, and tissue sizes were similar between resident and migratory species, except for pectoralis, which was largest in resident species. This large pectoralis size is beneficial for small songbirds in the winter due to a greater capacity for shivering thermogenesis (Milbergue et al., 2018). Together, these findings indicate that songbirds respond to increased energy demands with evolved changes in pectoralis size and/or mitochondrial net OXPHOS capacity.

4.4.4 Limitations

Centroid-based migration approaches may be the best approach available to estimate migration distances among species because directly measuring migration distance (e.g. with radio-tracking) and assaying mitochondrial function in individual birds is presently not feasible. Similarly, stable isotope approaches (Hobson, 2005) would not be possible in this study due to resolution that is insufficient for short-distance migration and this approach would not apply to hatch-year autumn migrants. One limitation of the centroid-based approach to estimating migration distance is the oversimplification of the inherent variation in avian migration. I also acknowledge that my sampling design of capturing birds in southern Ontario likely results in population biases within my data. I therefore encourage future investigations to assess the magnitude of inter-population heterogeneity in migration physiology.

4.4.5 Conclusions and future directions

My findings show, for the first time, that long-distance migration in songbirds is associated with reduced oxidative capacity, which may reflect evolved reductions in endurance flight costs. A lower pectoralis oxidative capacity may benefit long-distance migrants by decreasing whole-animal basal metabolic rate and improving muscle contraction efficiency (i.e. energy consumed per mechanical output) (Ross and Wakeling, 2021). Notably, both reduced basal metabolic rate and greater muscle efficiency are hypothesized to explain the disparity between flight times predicted by allometry- or aerodynamic-based models and observed flight times in long-distance migrants (Piersma et al., 2022). The new model proposed in this study suggests that the energetics underlying long-distance migration is more complex than previously thought and that integrative approaches are required to identify energy-saving adaptations that allow ultra-endurance locomotion in migratory birds. Under this new model, selective pressure may have improved fuel delivery and utilization, while minimizing tissue size in long-distance migrants, in line with the theory of symmorphosis (Weibel et al., 1991).

Future studies should expand on previous comparative studies of endurance flight costs in migratory birds (Klaassen et al., 2000) and assess interspecific variation in flight efficiency with migration distance using wind tunnel experiments. Future investigations should also address potential functional consequences (e.g. on ATP synthesis capacity) and mechanisms underlying lower respiratory capacity in long-distance migratory songbirds, such as variation in inner mitochondrial membrane density (Heine et al., 2023). Interspecific variation in mitochondrial genome sequences appear to be unrelated to migration distance (Burskaia et al., 2021; Claramunt and Haddrath, 2023; Toews et al., 2014), but interspecific variation in flight muscle transcriptomics and proteomics are promising avenues of future research that may reveal mechanisms underlying known traits in pectoralis physiology in addition to novel traits associated with long-distance migration. My findings challenge assumptions that mitochondrial physiology is essentially the same across species as in migration simulation models (Pennycuick, 2008). A fuller understanding of how mitochondrial function changes with the types of flights birds engage in or the distances they typically migrate will improve these models. Like elite human

marathon runners, long-distance migratory songbirds are built for light and efficient travel to optimize endurance locomotion performance.

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Chapter 5

5 General discussion

5.1 Thesis summary

In this dissertation, I investigated variation in mitochondrial physiology in the context of songbird migration. I first assessed the degree of seasonal flexibility in mitochondrial physiology to test the hypothesis that flight muscle mitochondria are seasonally remodeled to support the demands of migratory flight (Chapter 2). I found greater total mitochondrial abundance and fatty acid-fuelled phosphorylating respiration in the migratory phenotype. I also found that non-phosphorylating respiration was greater in the migratory phenotype, which contributed to a lower degree of coupling between respiration and ATP synthesis, but with the benefit of lower reactive oxygen species (ROS) emission. Together, my findings indicate that mitochondrial quantity and quality are seasonally modified to improve endurance flight performance by increasing the capacity for fatty acid oxidation, while lowering the risk for oxidative stress. I conclude that seasonal modulation of mitochondrial function is used by migratory songbirds as part of the better meet the physiological demands of migration.

I next tested the effects of simulated migratory flight on mitochondrial physiology and lean mass dynamics to test the hypotheses that endurance flight causes flight muscle mitochondrial dysfunction and selective atrophy of lean tissues (Chapter 3). I found little evidence of mitochondrial dysfunction: after accounting for whole-animal fat use, leak respiration was greater in flown than fasted control birds, but phosphorylating respiration, coupling, and ROS emission were similar between flown and fasted birds. Furthermore, all metrics of mitochondrial metabolism were similar between flown birds and a pre-flight control group. In contrast, I found that after accounting for fat use, flight resulted in lower wet masses of some digestive system tissues (liver and gizzard and proventriculus), while masses of flight apparatus tissues (pectoralis, heart, lungs) did not change with flight, nor did pectoralis ultrastructure. My findings indicate that flight muscle mitochondria are generally resilient against the physiological challenges associated with endurance flight and contribute to sustained ATP synthesis throughout flight. In addition, migratory

songbirds selectively atrophy tissues of the digestive system which yields metabolic water and Krebs cycle intermediates but without compromising flight performance. My findings indicate that migratory birds preferentially protect flight apparatus function during endurance flight.

For my last chapter, I investigated the effects of evolved variation in migration strategy on mitochondrial physiology to test the hypothesis that long-distance migration is associated with elevated flight muscle oxidative capacity. Surprisingly, I found that body mass-adjusted pectoralis mass was lowest in long-distance migrants, followed by short-distance migrants and then resident species. I also found that mitochondrial abundance, respiration and O_2 concentration-corrected ROS emission rates were generally similar among resident, short-distance migrant and long-distance migrants. However, I found that in migratory species, migration distance was negatively related to body size-adjusted pectoralis mass, fatty acid-fuelled phosphorylating respiration and mitochondrial respiratory capacity, while mitochondrial abundance and ROS emission were unaffected by migration distance. I also visually compared these data to those collected in the non-migratory phenotype for Chapter 2 to assess the relative contributions of seasonal flexibility and interspecific variation. I found a large effect of seasonal flexibility on body mass-adjusted tissue mass for pectoralis, heart and liver and mitochondrial ROS emission. In contrast, I found a small effect of seasonal flexibility for all other measurements of mitochondrial function. In conclusion, long-distance migrants have evolved a low pectoralis oxidative capacity, which may be permitted by lower flight energy demands imparted by a suite of morphological and behavioural adaptations.

5.2 Mitochondrial efficiency and flight

As the predominant source of muscle ATP synthesis, mitochondrial function is integral to whole-animal energy metabolism during exercise. An unexpected and counter-intuitive finding in my dissertation was that higher leak respiration is associated with lower whole-animal flight costs in blackpoll warblers (Chapter 3). All birds flew the same total distance, so higher flight costs translate directly to higher cost of transport (energy expended per distance travelled), which suggests lower flight efficiency (Jahn and Seebacher, 2022). Therefore, blackpoll warblers with greater mitochondrial leak respiration have greater

flight efficiency, which is a significant benefit due to the increased maximal flight range for a given fuel load. This finding is in direct contrast to previous work, which has shown that mitochondrial uncoupling decreases exercise efficiency (power output per O_2 consumed) in cycling humans (Conley et al., 2013). It is unclear if greater flight efficiency is directly caused by increased leak respiration, or by another physiological process that is linked to leak respiration.

Notably, leak respiration is increased in the migratory phenotype in yellow-rumped warblers (Chapter 2) and white-crowned sparrows (Rhodes et al., 2024). If greater leak respiration is associated with lower flight costs (i.e. greater apparent flight efficiency), then perhaps seasonal flexibility in mitochondrial metabolism is tied to seasonal flexibility in flight efficiency. One potential mechanism for this phenomenon is that the density of the inner mitochondrial membrane (IMM) is increased in the migratory phenotype. A greater IMM surface area increases H^+ leak rates (Porter et al., 1996), which in turn increases leak respiration. By increasing IMM density, the volume of intermembrane space between cristae (intracristal volume) decreases, thus reducing the volume for ETS protein complexes to pump H^+ into, as previously hypothesized (Heine et al., 2023). Lower intracristal volume brings the benefit of lower required workload of the ETS to maintain the proton-motive force as previously hypothesized (Davies et al., 2011; Lee, 2020). This lower ETS workload may translate to improved mitochondrial efficiency, such as a greater rate of ATP synthesis per O_2 consumed, which improves whole-animal flight efficiency. Greater IMM density is also linked to greater total expression of ETS complexes (Heine et al., 2023) and lower ROS production (Quintana-Cabrera et al., 2021), which are both in line with my observations of greater state 3 respiration and lower ROS emission, respectively, in the migratory phenotype (Chapter 2).

An alternative approach to addressing the link between mitochondrial leak respiration and endurance flight efficiency in migratory birds is through diet manipulation. Some shorebirds consume high amounts of n-3 polyunsaturated fatty acids (PUFAs) during migration, which is linked to increased flight muscle oxidative capacity and has been hypothesized as a natural ‘doping strategy’ to improve endurance flight performance as shown in semipalmated sandpipers (*Calidris pusilla*) (Maillet and Weber, 2007), but not

in western sandpipers (*C. mauri*) (Dick et al., 2024). n-3 PUFAs may be incorporated into cellular membranes, increasing fluidity of membranes such as the IMM. At the cellular level, culture with n-3 PUFAs increases mitochondrial leak respiration in Sanderling myotubes (Young et al., 2021). Similarly, dietary supplementation of n-3 PUFAs in Western sandpipers results in increased leak respiration of flight muscle mitochondria (Young et al., *in prep*) and decreased whole-animal endurance flight costs (Dick et al., *in prep*). While these findings were from separate studies that used two different cohorts of birds, I speculate that the relationship between mitochondrial leak respiration and flight efficiency observed in blackpoll warblers in my dissertation (Chapter 3) is also present in Western sandpipers. If true, this would suggest that migratory songbirds and shorebirds use different mechanisms (seasonality, diet, respectively) to modulate leak respiration and in turn improve flight efficiency.

To address these questions, future studies should first confirm the negative relationship between leak respiration and flight efficiency using the same experimental design as in Chapter 3, with greater sample size and repeated with additional migratory songbird species. If this pattern is repeatable and expressed in multiple studies, the focus of future experiments should shift to underlying physiological mechanisms. Mitochondrial efficiency should be evaluated, which can be measured as ATP synthesis per O_2 consumption. IMM density can directly assessed using transmission electron microscopy. Empirically testing seasonal flexibility in endurance flight efficiency may not be possible, as birds in a non-migratory phenotype may not have the motivation for endurance wind tunnel flights. However, this hypothesis can be tested by evaluating seasonal flexibility in mitochondrial efficiency and IMM density, using the above methods with an experimental design similar to Chapter 2. Future studies may also test for convergent mechanisms with dietary intake of n-3 PUFAsin western sandpipers, by including diets of different n-3 PUFA content and repeating the above experiments in an experimental design similar to Chapter 3.

5.3 Mitochondrial morphology

Our understanding of the mitochondrial phenotype in migratory birds is incomplete without the study of mitochondrial morphology. In myocytes, mitochondria may be divided into two subpopulations that are classified based on intracellular location: subsarcolemmal (SS) and intermyofibrillar (IMF). In my dissertation, I used whole-muscle citrate synthase activity as a proxy for total mitochondrial abundance, but this assay is not informative as to the relative abundance of each mitochondrial subpopulation. Furthermore, I used crude mitochondrial fractions of muscle tissue to assess mitochondrial function, which were likely biased towards IMF mitochondria due to their greater abundance in myocytes. Therefore, my measurements may have masked interesting variation in SS mitochondrial abundance and function. Investigations of function in each subpopulation would yield novel insights into flight muscle metabolism, as mitochondrial subpopulations vary in function and degree of phenotypic plasticity, as shown with endurance exercise training in rats (Bizeau et al., 1998). This variation includes capacity for fatty acid oxidation, as SS mitochondria have a greater capacity to consume fatty acids than IMF, plus SS mitochondria increase this capacity to a greater extent in response to exercise training (Koves et al., 2005). Furthermore, the close proximity of SS mitochondria to the sarcolemma (and therefore capillaries) is ideal for accessing extracellular substrates and O_2. Therefore, I hypothesize that migratory birds modulate SS mitochondrial abundance and function in the flight muscle to increase fat oxidation capacity during endurance flight. This hypothesis may be tested using a seasonal comparison design similar to Chapter 2. Mitochondrial subpopulation abundance and function may be investigated using transmission electron microscopy (Mahalingam et al., 2017) and high-resolution respirometry combined with isolation via differential centrifugation (Lai et al., 2019), respectively.

In muscle, mitochondria form a reticulated network whose ultimate shape is determined by the equilibrium between two competing processes: fusion and fission. Both processes are dynamic and vary in response to cellular energy demand. For example, exercise training increases the expression of fusion proteins to a greater extent than fission proteins, which ultimately reshapes mitochondria into a fusion phenotype characterized by few, large

mitochondria (Axelrod et al., 2019; Ruegsegger et al., 2023). In turn, the fusion phenotype in skeletal muscle is associated with greater substrate oxidation (Axelrod et al., 2019), and fat oxidation (Huertas et al., 2019; Lee et al., 2014). Therefore, I hypothesize that variation in mitochondrial dynamics also contributes to high fatty acid use during migratory flight. This hypothesis could be tested with the use of transmission electron microscopy to directly quantify the fusion/fission phenotype of flight muscle mitochondria, while qPCR may be used to measure transcription of genetic factors that regulate mitochondrial dynamics, such as dynamin-related protein 1, optic atrophy 1, mitofusins 1 and 2 (Tilokani et al., 2018). I speculate that seasonal changes in the fusion/fission phenotype may improve flight muscle oxidative capacity in the absence of increased total mitochondrial abundance seen in some migratory songbirds, such as the gray catbird (DeMoranville et al., 2019).

5.4 New framework in adaptations in long-distance migration

5.4.1 Seasonal flexibility and migration distance interaction

Pectoralis size is determined by contributions of seasonal flexibility and selective pressure in migratory birds. In songbirds, pectoralis size outside of the migratory season is unaffected by migration distance (Vágási et al., 2016). Since pectoralis size during the migratory season declines with migration distance (Chapter 4), I speculate that the magnitude of seasonal hypertrophy in the pectoralis also declines with migration distance. If true, a lower degree of seasonal hypertrophy may be beneficial for long-distance migrants due to lower time requirements for seasonal transitions in the pectoralis phenotype. As a result, long-distance migrants may be able to extend their breeding season. Long-distance migrants have less time available for breeding compared to short-distance migrants due to the high time requirements of long-distance migration and the high seasonality of resource abundance associated with high latitude environments. I further speculate that a similar relationship between migration distance and magnitude of seasonal flexibility exists with lower levels of organization in the pectoralis, such as the mitochondrial phenotype, which may also yield benefits of reduced time requirements for pre-migratory preparation.

5.4.2 Migration distance and flight efficiency

In my dissertation, I proposed a new framework for migration physiology, where long-distance migrants are adapted to minimize energy expenditure during flight, as a potential mechanism to mitigate the potentially lethal consequences of unpredictable energy availability (Chapter 4). Reductions in flight costs also has the benefit of reducing requirements for fuel replenishment in long-distance migrants at stopover, ultimately reducing total stopover duration (Beauchamp, 2024). However, shortened stopover duration results in the consequence of less time available for repair of oxidative damage incurred during flight (Eikenaar et al., 2020). Despite the challenge of reduced opportunity for repair, long-distance migrants show no signs of compromised flight performance. I hypothesize that adaptations in long-distance migrants allow for lower time requirements associated with flight recovery. To this end, I propose two non-mutually exclusive mechanisms. First, long-distance migrants sustain less oxidative damage during flight, due to lower flight costs which lower oxidative stress (Dick and Guglielmo, 2019) and/or greater total antioxidant capacity (Eikenaar et al., 2017). Second, long-distance migrants recover oxidative damage more quickly, due to greater expression of repair enzymes. For example, early-arriving males may express more heat shock protein (HSP)90 than late-arriving males (Lobato et al., 2010). Furthermore, HSP expression is upregulated during migration (Jones et al., 2008; Sharma et al., 2021) and following migratory flight (Bounas et al., 2024), however interspecific comparisons in HSP expression have yet to be conducted. This hypothesis could be tested with a comparative design that uses short- and long-distance migratory species, as in Chapter 4, paired with comparisons of whole-animal energetics with post-flight oxidative damage markers and antioxidant capacity, as used previously (Dick and Guglielmo, 2019). To assess variation in rate of recovery, oxidative damage markers could be assessed at multiple time points post-flight.

5.5 Conclusion

The findings of my dissertation show that mitochondrial function is modulated by migratory birds to better meet the demands of endurance locomotion. My findings from Chapter 2 contribute to the growing literature base that the migratory phenotype is associated with elevated oxidative capacity, but for the first time show that this phenotype

also includes lower mitochondrial ROS emission. In contrast, my findings from Chapters 3 and 4 provide evidence that physiological processes are modified in long-distance migrants to improve endurance flight performance by increasing flight efficiency. Evolved variation in flight efficiency and its underlying mechanisms, plus associated consequences for oxidative stress, flight speed and stopover duration should be the focus of future investigations. These findings have the potential to reshape our collective understanding of physiological adaptations in endurance exercise.

5.6 References

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Tilokani, L., Nagashima, S., Paupe, V. and Prudent, J. (2018). Mitochondrial dynamics: overview of molecular mechanisms. *Essays in Biochemistry* **62**, 341–360.

Vágási, C. I., Pap, P. L., Vincze, O., Osváth, G., Erritzøe, J. and Møller, A. P. (2016). Morphological Adaptations to Migration in Birds. *Evolutionary Biology* **43**, 48–59.

Young, K. G., Vanderboor, C. M., Regnault, T. R. H. and Guglielmo, C. G. (2021). Species-specific metabolic responses of songbird, shorebird, and murine cultured myotubes to n-3 polyunsaturated fatty acids. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **320**, R362–R376.

Appendices

Appendix 1: Supplementary materials for Chapter 2

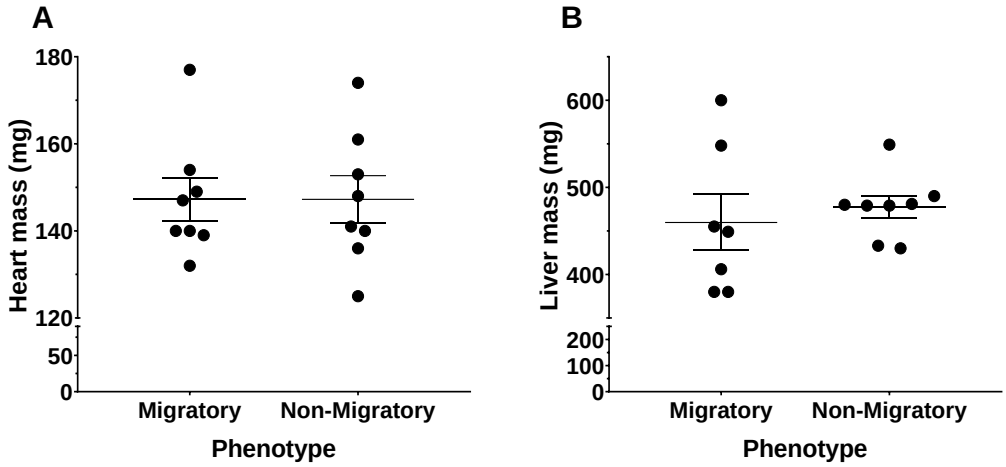


Figure A1.1: Seasonal variation in organ mass in yellow-rumped warblers. Wet masses of heart (A) and liver (B). Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 7-8.

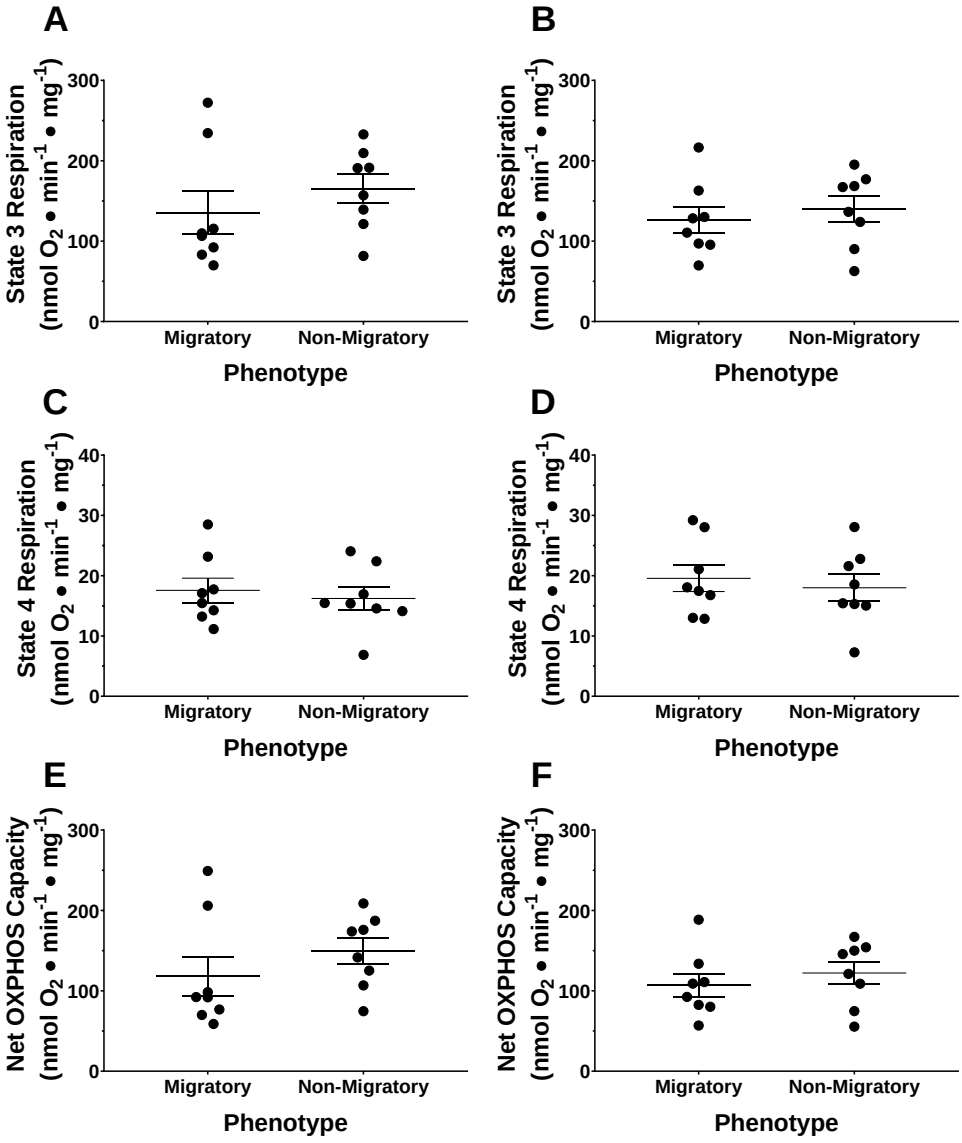


Figure A1.2: Seasonal variation in substrate oxidation in yellow-rumped warbler pectoralis mitochondria. Respiration rates of isolated pectoralis mitochondria were measured during oxidation of pyruvate (A, C, E) or palmitoyl-carnitine (B, D, F) substrate in phosphorylating (State 3; A, B) or non-phosphorylating conditions (State 4; C, D). Net OXPHOS capacity calculated as difference between state 3 and state 4 respiration rates. Respiration rates are expressed relative to protein content of individual mitochondrial preparations. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8.

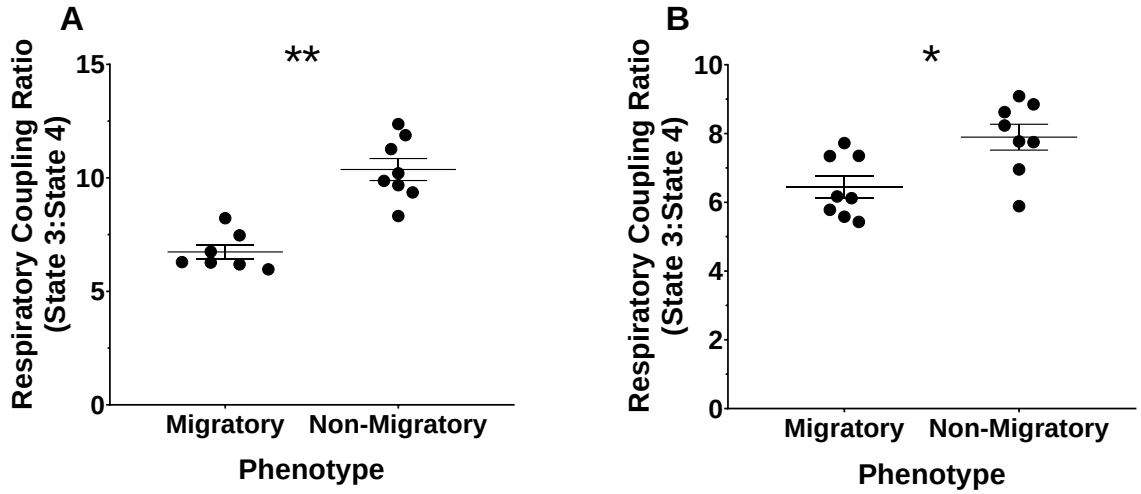


Figure A1.3: Seasonal variation in coupling of mitochondrial respiration to oxidative phosphorylation in yellow-rumped warblers. Respiration rates of isolated pectoralis mitochondria were measured during oxidation of pyruvate (A) or palmitoyl-carnitine (B) substrate in phosphorylating (state 3) and non-phosphorylating (state 4) conditions. Respiratory control ratio was calculated as the ratio of state 3 respiration to state 4 respiration. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 7 (migratory), 8 (non-migratory). Significant differences between phenotypes indicated by * ($p < 0.05$) and ** ($p < 0.01$).

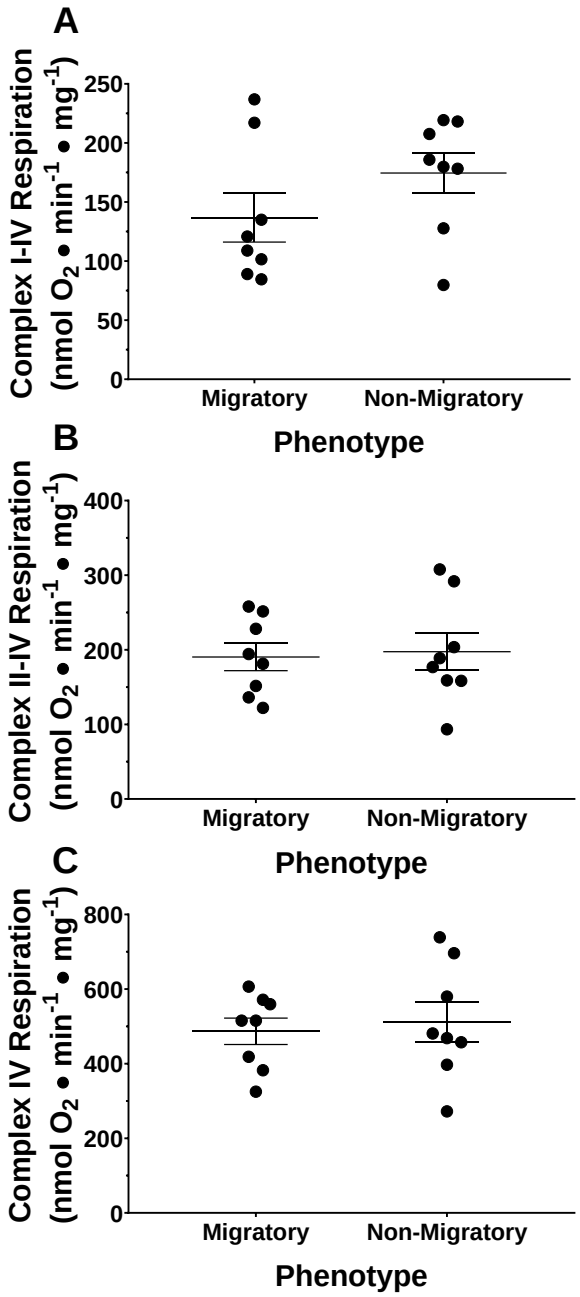


Figure A1.4: Seasonal variation in electron transport system complex-specific flux in isolated pectoralis mitochondria from yellow-rumped warblers. Respiration rates specific to A) complex I-IV, B) complex II-IV and C) complex IV. Respiration rates expressed relative to protein content of individual mitochondrial preparations. Migratory warblers sampled during autumn migration, non-migratory warblers sampled following short-day photoperiod acclimation. Data presented as mean ± s.e.m. N = 8.

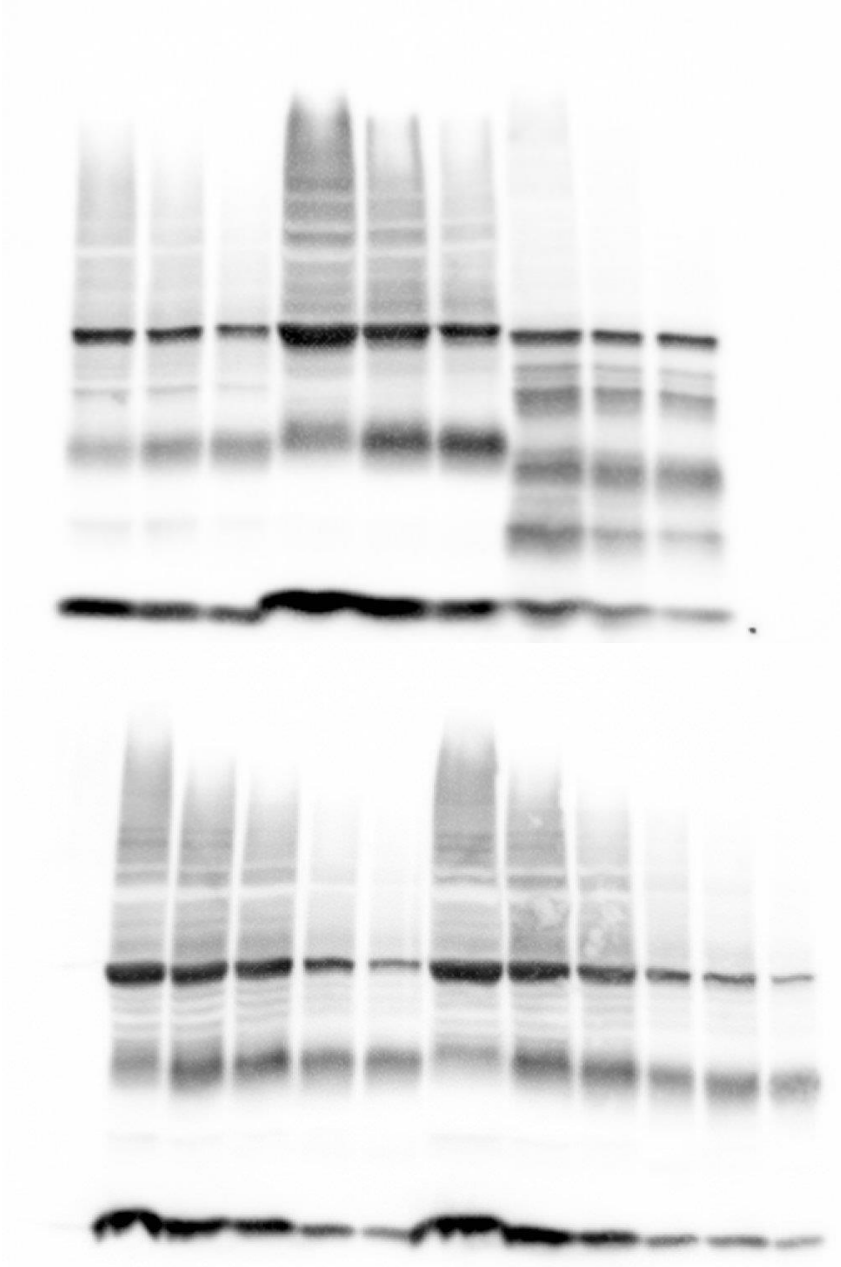


Figure A1.5: Western blotting of mitochondrial electron transport system complexes. Blots for three concentrations from two-fold serial dilutions of blackpoll warblers (BLPW), ruffs (RUFF) and rat muscle mitochondria (A) and for 5-6 concentrations of yellow-rumped warbler (YRWA) flight muscle mitochondria from migratory and non-migratory phenotypes (B). Bands are labelled by corresponding putative electron transport system complex.

Appendix 2: Regulatory approval for animal work

Environment and Climate Change Canada | Environnement et Changement climatique Canada

Scientific Permit
issued under sections 4 and 19 of the *Migratory Birds Regulations*, C.R.C. c.1035 pursuant to section 12 of the *Migratory Birds Convention Act, 1994*, S.C. 1994, c.22

Permit number:

SC-OR-2018-0256 Amended 4

Valid From: *(yyyy/mm/dd)*	**Expiry date:** *(yyyy/mm/dd)*
2022/03/01	2022/06/01

Report due annually on: *(mm/dd)*

01/31

Permit holder:

Christopher G. Guglielmo
University of Western Ontario

Address:

Dept. of Biology, University of Western Ontario, 1151 Richmond St. N.
London Ontario
N6A 5B7 Canada

Telephone number	**Email:**
[redacted]	[redacted]

Under this permit, the permit holder is authorized to:

Canada

Permit number:

SC-OR-2018-0256 Amended 4

Activities	Species	Applicable locations
Kill	American Goldfinch (Spinus tristis), American Redstart (Setophaga ruticilla), American Robin (Turdus migratorius), Baltimore Oriole (Icterus galbula), Black-capped Chickadee (Poecile atricapillus), Blackpoll Warbler (Setophaga striata), Black-throated Blue Warbler (Setophaga caerulescens), Common Yellowthroat (Geothlypis trichas), Dark-eyed Junco (Junco hyemalis), Hermit Thrush (Catharus guttatus), House Finch (Haemorhous mexicanus), Indigo Bunting (Passerina cyanea), Killdeer (Charadrius vociferus), Lincoln's Sparrow (Melospiza lincolnii), Magnolia Warbler (Setophaga magnolia), Northern Cardinal (Cardinalis cardinalis), Pine Siskin (Spinus pinus), Purple Finch (Haemorhous purpureus), Red-breasted Nuthatch (Sitta canadensis), Red-eyed Vireo (Vireo olivaceus), Rose-breasted Grosbeak (Pheucticus ludovicianus), Ruby-throated Hummingbird (Archilochus colubris), Song Sparrow (Melospiza melodia), Swainson's Thrush (Catharus ustulatus), Warbling Vireo (Vireo gilvus), White-breasted Nuthatch (Sitta carolinensis), Yellow Warbler (Setophaga petechia), Yellow-rumped Warbler (Setophaga coronata)	1

Permit number:

SC-OR-2018-0256 Amended 4

Take	American Goldfinch (Spinus tristis), American Redstart (Setophaga ruticilla), American Robin (Turdus migratorius), Baltimore Oriole (Icterus galbula), Blackpoll Warbler (Setophaga striata), Black-throated Blue Warbler (Setophaga caerulescens), Common Yellowthroat (Geothlypis trichas), Dark-eyed Junco (Junco hyemalis), Hermit Thrush (Catharus guttatus), House Finch (Haemorhous mexicanus), Killdeer (Charadrius vociferus), Lincoln's Sparrow (Melospiza lincolnii), Magnolia Warbler (Setophaga magnolia), Northern Cardinal (Cardinalis cardinalis), Pine Siskin (Spinus pinus), Purple Finch (Haemorhous purpureus), Red-eyed Vireo (Vireo olivaceus), Rose-breasted Grosbeak (Pheucticus ludovicianus), Ruby-throated Hummingbird (Archilochus colubris), Song Sparrow (Melospiza melodia), Swainson's Thrush (Catharus ustulatus), Warbling Vireo (Vireo gilvus), Yellow Warbler (Setophaga petechia), Yellow-rumped Warbler (Setophaga coronata)	1

Location(s):

1. Ontario (Southwestern Ontario locations as mentioned in permit application items)

General Terms and Conditions:

1. Prior to any use of this permit the permittee will notify the Ontario Ministry of Natural Resources relative to collecting procedures, times and localities of collection. Landowner's permission must be obtained prior to collecting on private property.
2. Permit or a copy of the permit to be carried in the field by all collectors.
3. The permit holder is authorized to collect and to possess for scientific research purposes, migratory birds - to wit: Yellow-rumped Warbler (Setophaga coronate-take limit 80 per year)

Swainson's Thrush (Catharus ustulatus- take limit 40 per year)
Blackpoll Warbler (Setophaga striata- take limit 40 per year)
Hermit Thrush (Catharus guttatus- take limit 40 per year)
Yellow Warbler (Setophaga peechia- take limit 30 per year)
Magnolia Warbler (Setophaga mangolia- take limit 30 per year)
Common Yellowthroat (Geothlypis trichas- take limit 30 per year)
Ruby-throated Hummingbird (Archilochus colubris- take limit 20 per year)

Permit number:

SC-OR-2018-0256 Amended 4

American Redstart (Setophaga ruticilla- take limit 30 per year)
Black-throated blue Warbler (Setophaga caerulescens- take limit 30 per year)
Killdeer (Charadrius vociferus- captively bred, take limit 20 per year)
Baltimore Oriole (Icterus galibula- take limit 8 per year)
Red-eyed Vireo (Vireo olivaceus- take limit 24 per year)
Warbling Vireo (Vireo gilvus- take limit 24 per year)
American Robin (Turdus migratorius- take limit 16 per year)
Dark-eyed Junco (Junco hyematlis- take limit 24 per year)
Rose-breasted Grosbeak (Pheucticus ludovicianus- take limit 8 per year)
Pine Siskin (Spinus pinus- take limit 8 per year)
American Goldfinch (Spinus tristis- take limit 8 per year)
House Finch (Haemorthous mexicanus - take limit 8 per year)
Purple Finch (Haemorthous purpureus- take limit 8 per year)
Song Sparrow (Melospiza melodia- take limit 8 per year)
Lincoln's Sparrow (Melospiza lincolnii- take limit 8 per year)
Northern Cardinal (Cardinalis cardinalis- take limit 8 per year)
Indigo Bunting (Passerina cyanea- take limit 8 per year)
Black-capped Chickadee (Poecile atricapillus- take limit 8 per year)
White-breasted Nuthatch (Sitta carolinensis- take limit 8 per year)
Red-breasted Nuthatch (Sitta canadensis- take limit 8 per year)
from locations in SW Ontario (Perth, Elgin, Middlesex, Haldimand, and Bruce Counties).

4. Capture, handling and housing procedures are to be performed according to the Animal Care Committee protocols of the University of Western Ontario and AUP #2018-092. Samples not to be retained are to be disposed of by the approved laboratory waste management system of the University of Western Ontario.
5. All birds are to be released into the wild by the conclusion of the study or otherwise be humanely euthanized.
6. Permit holder shall submit a written report, by January 31, of each year following, indicating the results of the study to the Canadian Wildlife Service, 335 River Road, Ottawa, ON, K1V 1C7.
7. Nominees to this permit are: Department of Biology faculty/staff acting under the direction of the permittee.

Nominee(s):	
Name	**Organization**
Employees , students and volunteers acting under the direction of the permit holder, of	University of Western Ontario

I declare that I have read and understand this permit, including all of the terms and conditions, and understand that I (permit holder) may be subject to prosecution for any violations.

Page 4 of 5

Figure A2.1: Scientific permit for collection and killing of birds protected under the *Migratory Birds Convention Act*.

Environment and Climate Change Canada | Environnement et Changement climatique Canada

Scientific Permit
issued under sections 4 and 19 of the *Migratory Birds Regulations*, C.R.C. c.1035 pursuant to section 12 of the *Migratory Birds Convention Act, 1994*, S.C. 1994, c.22

Permit number:

SC-OR-2022-0256

Valid From: *(yyyy/mm/dd)*	**Expiry date:** *(yyyy/mm/dd)*
2022/06/01	2026/01/01

Report due annually on: *(mm/dd)*

01/31

Permit holder:

Christopher G. Guglielmo
University of Western Ontario

Address:

Dept. of Biology, University of Western Ontario, 1151 Richmond St. N.
London Ontario
N6A 5B7 Canada

Telephone number	**Email:**
[redacted]	[redacted]

Under this permit, the permit holder is authorized to:

Canada

Permit number:

SC-OR-2022-0256

Activities	Species	Applicable locations
Kill	American Goldfinch (Spinus tristis), American Redstart (Setophaga ruticilla), American Robin (Turdus migratorius), Baltimore Oriole (Icterus galbula), Black-capped Chickadee (Poecile atricapillus), Blackpoll Warbler (Setophaga striata), Black-throated Blue Warbler (Setophaga caerulescens), Common Yellowthroat (Geothlypis trichas), Dark-eyed Junco (Junco hyemalis), Hermit Thrush (Catharus guttatus), House Finch (Haemorhous mexicanus), Indigo Bunting (Passerina cyanea), Lincoln's Sparrow (Melospiza lincolnii), Magnolia Warbler (Setophaga magnolia), Northern Cardinal (Cardinalis cardinalis), Pine Siskin (Spinus pinus), Purple Finch (Haemorhous purpureus), Red-breasted Nuthatch (Sitta canadensis), Red-eyed Vireo (Vireo olivaceus), Ruby-throated Hummingbird (Archilochus colubris), Song Sparrow (Melospiza melodia), Swainson's Thrush (Catharus ustulatus), Warbling Vireo (Vireo gilvus), White-breasted Nuthatch (Sitta carolinensis), Yellow Warbler (Setophaga petechia), Yellow-rumped Warbler (Setophaga coronata)	1

Permit number:

SC-OR-2022-0256

Take	American Goldfinch (Spinus tristis), American Redstart (Setophaga ruticilla), American Robin (Turdus migratorius), Baltimore Oriole (Icterus galbula), Blackpoll Warbler (Setophaga striata), Black-throated Blue Warbler (Setophaga caerulescens), Common Yellowthroat (Geothlypis trichas), Dark-eyed Junco (Junco hyemalis), Hermit Thrush (Catharus guttatus), House Finch (Haemorhous mexicanus), Lincoln's Sparrow (Melospiza lincolnii), Magnolia Warbler (Setophaga magnolia), Northern Cardinal (Cardinalis cardinalis), Pine Siskin (Spinus pinus), Purple Finch (Haemorhous purpureus), Red-eyed Vireo (Vireo olivaceus), Ruby-throated Hummingbird (Archilochus colubris), Song Sparrow (Melospiza melodia), Swainson's Thrush (Catharus ustulatus), Warbling Vireo (Vireo gilvus), Yellow Warbler (Setophaga petechia), Yellow-rumped Warbler (Setophaga coronata)	1

Location(s):

1. Ontario (Southwestern Ontario locations as mentioned in permit application items)

General Terms and Conditions:

1. This permit is valid only if it is signed by the permit holder.
2. This permit is non-transferable and is not valid if altered, other than by the Minister.
3. The permit holder is responsible for ensuring that all nominees comply with the permit terms and conditions.
4. The permit holder is responsible for informing Environment and Climate Change Canada's regional Canadian Wildlife Service permitting office immediately of any changes to nominees, personnel or project activities authorized under the permit.
5. The permit holder must comply with all applicable Federal, Provincial, Territorial and Municipal laws, bylaws and regulations and must acquire any other necessary permits or permissions.
6. This permit is not valid in any federal or provincial game preserve or bird sanctuary or national or provincial park, National Wildlife Area, Wildlife Management Unit or other protected areas without proper authorization.
7. The permit holder and nominees must carry a signed copy of the permit on their person when conducting the activities authorized by the permit. A copy of the permit must be shown to any Game Officer, or other authorized officer, immediately upon request.

Figure A2.2: Scientific permit for collection and killing of birds protected under the *Migratory Birds Convention Act*.

Christopher G. Guglielmo

From: eSirius3GWebServer [redacted]
Sent: June-11-18 1:06 PM
To: Christopher G. Guglielmo; Animal Care Committee
Cc: [redacted]
Subject: eSirius3G Notification -- 2018-092 New Protocol Approved

AUP Number: 2018-092
PI Name: Guglielmo, Christopher
AUP Title: Energetics, fuel metabolism, and water balance during exercise in migrating birds
Approval Date: 06/01/2018

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2018-092:1: entitled " Energetics, fuel metabolism, and water balance during exercise in migrating birds"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
   http://www.uwo.ca/univsec/policies_procedures/research.html

   b) University Council on Animal Care Policies and related Animal Care Committee procedures

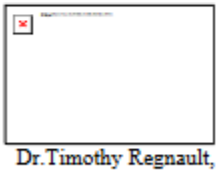
   http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
   e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:

i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to
hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care



Dr.Timothy Regnault,
Animal Care Committee Chair

The University
of Western Ontario
Animal Care Committee /
University Council on Animal Care
London,
Ontario Canada N6A 5C1
[redacted]
[redacted]
auspc@uwo.ca ï¿½
http://www.uwo.ca/research/services/animalethics/index.html

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***

2

Figure A2.3: Approval for Animal use protocol 2018-092.

Christopher G. Guglielmo

From: eSirius3GWebServer [redacted]
Sent: February 24, 2022 2:13 PM
To: Christopher G. Guglielmo; acc
Cc: ACC Office; mgrsmtgs
Subject: eSirius3G Notification -- 2022-028 New Protocol Approved

AUP Number: 2022-028
PI Name: Guglielmo, Christopher
AUP Title: Energetics, fuel metabolism, and water balance during exercise in migrating birds
Approval Date: 02/01/2022

Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2022-028:1: entitled " Energetics, fuel metabolism, and water balance during exercise in migrating birds" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
   - Western's Senate MAPP 7.12 [PDF]; and
   - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
   - Completed the appropriate institutional OH&S training;
   - Completed the appropriate facility-level training; and
   - Reviewed related (M)SDS Sheets.

Submitted by: Copeman, Laura on behalf of the Animal Care Committee

Dr. Rob Gros,
Animal Care Committee Chair

1

Figure A2.4: Approval for Animal use protocol 2022-028.

Curriculum Vitae

Name: Soren Coulson

Post-secondary Education and Degrees:

The University of Western Ontario
London, ON, Canada
Ph.D. Biology
2019-2024

McMaster University
Hamilton, ON, Canada
M.Sc. Biology
2017-2019

McMaster University
Hamilton, ON, Canada
B.Sc. (Hons) Biology – Physiology Specialization
2013-2017

Honours and Awards:

Dr. David Sherry AFAR Graduate Award
University of Western Ontario
2024

Western Sustainability Impact Fund
University of Western Ontario
2024

QEII Graduate Scholarship in Science and Technology
2021-2023

W.S. Hoar Award for best student oral presentation
Canadian Society of Zoologists
2023

Journal of Experimental Biology Travelling Fellowship
2022

Student/PDF Research Grant
Canadian Society of Zoologists
2022

G.F. Holeton Prize for best CPB student poster presentation
Canadian Society of Zoologists
2022

H. I. Battle Award for best student poster presentation Finalist
Canadian Society of Zoologists
2017

Poster Award
Canadian Society of Zoologists Satellite Symposium – 50 Years of Comparative Biochemistry: The Legacy of Peter Hochachka
2017

Related Work Experience

Teaching Assistant
The University of Western Ontario
2019-2024

Teaching Assistant
McMaster University
2017-2019

Publications:

Coulson, S. Z., Ivy, C. M., Staples, J. F., & Guglielmo, C. G. Flight muscle mitochondria are robust against endurance flight damage in blackpoll warblers (*Setophaga striata*). *Journal of Avian Biology*. *In review*.

Coulson, S. Z., Lyons, S. A., Robertson, C. E., Fabello, B., Dessureault, L. M., McClelland, G. B. Regulation of muscle pyruvate dehydrogenase activity and exercise fuel use in high-altitude deer mice. *Journal of Experimental Biology*. *227(*16), jeb246890.

Coulson, S. Z., Guglielmo, C. G., & Staples, J. F. (2024). Migration increases mitochondrial oxidative capacity without increasing reactive oxygen species emission in a songbird. *Journal of Experimental Biology*, *227*(9).

Sanita Lima, M., Lubbe, F. C., Dias dos Santos, S. H., Saruhashi, S., Maglov, J. M., Moreira do Nascimento, J., & **Coulson, S. Z.** (2024). Ecology, ethology, and evolution in the Anthropocene. *Biology Open*, *13*(3), bio060175.

Coulson, S. Z.*, Duffy, B. M., & Staples, J. F. (2024). Mitochondrial techniques for physiologists. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *271*, 110947.

Mahalingam, S., **Coulson, S. Z.***, Scott, G. R., & McClelland, G. B. (2023). Function of left ventricle mitochondria in highland deer mice and lowland mice. *Journal of Comparative Physiology B*, *193*(2), 207-217. https://doi.org/10.1007/s00360-023-01476-7

Coulson, S. Z.*, Robertson, C. E., Mahalingam, S., & McClelland, G. B. (2021). Plasticity of non-shivering thermogenesis and brown adipose tissue in high-altitude deer mice. *Journal of Experimental Biology*, *224*(10), jeb242279.

*joint first author