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Environmental Physiology of Flight in Migratory Birds

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Abstract

Migratory birds complete amazing journeys between their breeding and wintering grounds. Each journey comprises a series of flights that last hours to days, followed by stopovers where fuel stores are replenished. Despite the long flights undertaken by migratory birds, where respiratory water losses are high for extended periods of time, birds are not dehydrated after flight. My studies demonstrate that birds maintain hydration by modulating rates of endogenous water production in response to rates of water loss. In resting, water restricted house sparrows (*Passer domesticus*) I used quantitative magnetic resonance body composition analysis (QMR) and hygrometry to demonstrate that stressed resting birds increase the rate of lean mass (protein) catabolism to liberate water and maintain osmotic homeostasis. I then flew Swainson's thrushes (*Catharus ustulatus*) in a climatic wind tunnel under high- and low-humidity conditions for up to 5 hours. Flight under dry conditions increased the rate of lean mass loss, endogenous water production and plasma uric acid concentrations. This demonstrated that atmospheric humidity influences fuel composition in flight and suggest that protein deposition and catabolism during migration are a metabolic strategy to maintain osmotic homeostasis during flight. Next, I investigated the metabolic response to flight in the American robin (*Turdus migratorius*). These birds have high rates of endogenous water production early in flight due to a high contribution of carbohydrate and protein to energy during the transition to fat oxidation, and do not require additional protein catabolism to maintain water balance. Migratory birds may reduce excretory water losses to avoid dehydration in flight. I investigated kidney function in fed, rested and flown Swainson's thrushes and found no decrease in glomerular filtration rate during flight, however they rely on increased water reabsorption to reduce excretory water losses in flight and at rest. Finally, the effect of diet on mitochondrial metabolism was investigated. I demonstrated that the performance increases often attributed to high dietary polyunsaturated fatty acids are likely due to reduced rates of production of reactive oxygen species by mitochondria. Together, these studies advance our knowledge of the metabolic response to the environment in the context of bird migration.

Keywords

Avian flight, dehydration, protein catabolism, exercise metabolism, bird migration, kidney function, glomerular filtration rate, mitochondrial metabolism, polyunsaturated fatty acids, Swainson's thrush, American robin, house sparrow, plasma metabolites

Co-Authorship Statement

A version of Chapter 2 is published in the *American Journal of Physiology Regulatory, integrative and comparative physiology* with Christopher G. Guglielmo as a co-author. Dr. Guglielmo contributed to the design of the experiment, and provided funding and access to the necessary equipment. He also provided editorial comments on the manuscript.

A version of Chapter 3 is published in *Science* with Christopher G. Guglielmo as a co-author. Dr. Guglielmo contributed to the design of the experiment, provided funding and access to the wind tunnel and QMR, and aided with the use of general linear mixed models. He also helped interpret the data and provided editorial comments on the manuscript.

Chapters 4 and 5 are co-authored with Christopher G. Guglielmo, but are not yet published. Dr. Guglielmo helped by providing funding and access to equipment, and aided in the development of the technique used to measure GFR in Chapter 5. He also provided comments on earlier versions of the manuscript.

Chapter 6 is co-authored with Dr. Ulf Bauchinger, Dr. Edwin R. Price, Michelle Boyles, Lillie Langlois, Dr. Barbara Pierce, Dr. James F. Staples, Dr. Christopher G. Guglielmo and Dr. Scott R. McWilliams. As this chapter was part of a much larger project, Dr. Bauchinger was the postdoctoral fellow involved with all aspects of the larger project, and helped with everything from the coordination of animal care through to the design and execution of the project. Dr. Price provided help troubleshooting techniques in the lab and helped with many other aspects of the project. Michelle Boyles and Lillie Langlois were primarily responsible for animal care and flight training. Dr. Pierce and Dr. McWilliams were the principal investigators on the project, and were responsible for the procurement of funds and

experimental design. Dr. Staples helped with the development of the techniques required for mitochondrial isolation as well as access to laboratory equipment. Dr. Guglielmo aided by providing funding and access to the wind tunnel and to laboratory equipment, and by providing editorial comments on the manuscript.

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My wife has been on this journey with me since the very beginning. We met early on during our undergraduate degrees in biology and graduated together. Without her support through undergrad, then a master's degree, and now a PhD, I don't think I would have seen the success that I have. She has supported me in every way she can. Our undergraduate mentor, and good friend Guy was right when he said meeting her was the best thing I ever did. Thank you.

My parents have always supported me in all my endeavors. They have provided encouragement and advice throughout my life, and have always stressed the importance of being independent and capable. Having both received doctoral degrees themselves, they understand the challenges of research, but they also understand the joy it can bring. Without their influence, I certainly would not have had so much success. Thank you.

Now that I have been in graduate school for a long time (some might say too long), I can say with some authority that choosing a supervisor is, without contest, the most important decision a student makes. Without hesitation, I can say that I made a great decision when I joined Chris' lab. I am grateful to Chris for his mentorship and friendship over the years. He has taught me a great deal that will help me move forward as a scientist and be successful. Chris teaches by example. As a person, Chris is generous, friendly and understanding. He always has the wellbeing of his students in mind, and he treats everyone with respect. These actions may seem trivial to some, but they create an atmosphere of mutual respect in the lab, which seems to translate directly into productivity. As a scientist Chris is critical, and insightful and his breadth of knowledge is amazing. He is always aware of the latest technology or cutting edge techniques that will open new doors in our field. I have benefited a great deal from Chris' vision. The facility and resources that he provided me can be found nowhere else in the world, but I have also learned that without creativity, sound background knowledge, and a complete understanding of what is important in the field, even the best equipment will not guarantee success. Chris has provided me the freedom to pursue my interests, and his style of mentorship has translated into great success for me.

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My committee, Dr. Beth MacDougall-Shakleton and Dr. Brent Sinclair have provided lots of helpful advise over the years, and I am happy that I picked such a good committee. I would also like to thank Dr. Jim Staples, who gave me my start in graduate school. I learned a lot from Jim, much of which has shaped my actions and decisions throughout my PhD. Most importantly, Jim was cool with me crossing the hall to move to Chris' lab once I finished my Masters. Overall, I am very pleased with my decision to stay at Western for my doctorate. I have received help from many faculty members, always without hesitation. The department is open, and the graduate program is a very good one. Thanks to all.

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List of Abbreviations

AB- Assay Buffer

$A_{i(0)}$ - zero time intercept of the exponential decay curve fitted to the concentration of marker in the plasma over time

AVT - Arginine vasotocin

BUTY- ß-OH-Butyrate

CCCP- Cyanide m-chlorophenylhydrazone

CPT- Carnitine-palmitoyl transferase

CT - Control

ΔP – Proton motive force

$\Delta\Psi_m$ – Mitochondrial membrane potential

FITC- Fluorescein isothiocyanate

FWR – Fractional water reabsorption

GFR – glomerular filtration rate

GLM- General linear model

GLUC- Glucose

GLYC- Glycogen

HB- Homogenization buffer

HEWL- High evaporative water loss

$J_c^C{}_x$ – control coefficient of X over substrate oxidation

$J_c^{\Delta P}{}_x$ – control coefficient of X over ΔP

$J_c^L{}_x$ – control coefficient of X over proton leak

$J_c^P{}_x$ – control coefficient of X over phosphorylation

k - the exponent of the exponential decay curve fitted to the concentration of marker in plasma over time

LEWL – Low evaporative water loss

Mb – Body mass

MP – Malate Pyruvate

MPC – Malate palmitoyl carnitine

MUFA – Monounsaturated fatty acid

NEFA- Non-esterified fatty acid

PL - Phopsholipid

P_m – Concentration of marker in plasma

PPAR – Peroxisome proliferator-activated receptor

PUFA – Polyunsaturated fatty acid

Qi - Total quantity of marker injected

QMR – Quantitative magnetic resonance

RMR – Resting metabolic rate

ROS – Reactive oxygen species

RQ – Respiratory quotient

RS – Rotenone succinate

S3 – State 3 respiration

S4 – State 4 respiration

SFA – Saturated fatty acid

SHMR – Shivering metabolic rate

Sp – Dilution space

TCA – Tricarboxylic acid cycle

TPP^+ - Tetraphenylphosphonium ion

TRIG - Triglyceride

UA – Uric Acid

UFA – Unsaturated fatty acid

U_m - Concentration of marker in urine

VCO_2 – Volumetric carbon dioxide production rate

VH_2O – Volumetric water release rate

V_{MP} – Minimum power velocity

V_{MR} – Maximum range velocity

VO_2 – Volumetric oxygen consumption rate

WR – Water restricted

1 INTRODUCTION - PHYSIOLOGICAL LIMITATIONS TO MIGRATION

1.1 What is Migration?

The conspicuous seasonal passages of migratory animals, particularly birds, insects and fish, have inspired much curiosity and received much attention from generations of biologists from many sub-disciplines. Migrants face unique challenges during their long journeys, and their distinctive life histories have had a dramatic influence on how these animals have evolved to overcome these challenges.

Migratory species are especially common in high latitude regions where the seasonally-predictable deterioration of habitats in winter favours the evolution of migration (Dingle, 1996). Each year in Canada, Pacific salmon (*Oncorhynchus spp.*) return to their natal waters to spawn, sometimes migrating thousands of kilometers upstream after living for 3 - 5 years in the open ocean (Groot and Margolis, 1991). Monarch butterflies (*Danaus plexippus*) arrive each summer and then make spectacular migrations south in fall. Limited by their short lifespan, Monarchs migrate north through a series of generations with the offspring of each generation continuing the journey. The last Monarchs produced in the summer then migrate back to wintering grounds in Mexico where they have never been before (Wassenar and Hobson, 1998). Migratory birds can fly non-stop for many hours or even days at a time during their annual migration to high latitude areas where they take advantage of ephemerally abundant food sources to breed. They then fly back to their wintering grounds to avoid the harsh winter climate of the

temperate and polar regions (Berthold, 1990). Although the migratory strategies of fish, insects and birds may seem vastly different, many aspects of their behaviour and physiology are similar, allowing for a precise yet generalizable definition of migration to be developed.

After much debate in the literature, Kennedy's (1961) definition of a migrant, modified slightly by Dingle (1996), has become the most widely accepted, and I paraphrase it below as it relates to migration in this thesis:

> *"Migratory behaviour is persistent and straightened-out movement effected by the animal's own locomotory exertions. It depends on the temporary inhibition of station-keeping behaviours but promotes their eventual disinhibition ..."*

Dingle (Dingle, 1996) presents a much more detailed explanation of the meaning and justification for this definition. When applied to migratory birds in the context of this thesis, the simplest interpretation is that birds depart from an established home range in one habitat and fly directly to another, in a persistent and straightened-out journey. During their journey these birds suppress typical station-keeping behaviours, which could include foraging, territory defense, or competing for mates. Once birds arrive on the breeding grounds, they suppress the migratory behaviours, and resume the station keeping behaviours necessary for breeding (Dingle, 1996).

Typically, bird migration comprises a series of long duration flights, each separated by a period of stopover where birds replenish their endogenous stores in preparation for the next leg of the journey. For example, the Bar-tailed godwit (*Limosa*

lapponica) winters in Australia and New Zealand and breeds in the high arctic regions of Alaska and eastern Russia. During the northward journey, these birds make a non-stop flight of about 5000 km from Australia to the Korean peninsula, where they refuel for 3 - 5 weeks before completing a second non-stop flight (6000 km) to breeding areas. After breeding, the godwits make a single non-stop flight from Alaska to New Zealand, a distance of 11,000 km (Gill et al., 2009). Most songbirds (Order *Passeriformes*) complete their migrations through a series of nocturnal flights, each lasting 6 hours or more (Wikelski et al., 2003), although some are known to complete crossings of large ecological barriers such as the Sahara Desert or Gulf of Mexico in a single flight (Biebach, 1990; Stutchbury et al., 2009).

The ability to travel great distances by 'persistent movement' is a characteristic of any migratory animal, and like the behaviours that constitute the migratory phenotype, the physiology that allows these animals to complete such journeys also shares many commonalities among taxa. Due to the shared evolutionary history of all animal taxa, migratory animals do not possess unique biochemical processes for metabolism. Instead, biochemical processes common to all vertebrates have been optimized through evolution to allow migratory animals to complete their migrations (Hochachka and Somero, 1984).

1.2 Metabolic Fuels for Migration

Most migratory animals use fat as the primary metabolic fuel for long-duration locomotion. Fat is the ideal metabolic fuel for animals travelling long distances for a number of reasons. First, fat has a very high energy density, meaning that per unit mass

it provides far more energy than either of the other two potential metabolic fuels: carbohydrate or protein (Jenni and Jenni-Eiermann, 1998). The high energy density of fat results primarily from the highly chemically reduced aliphatic chains of fatty acids, but also because fat is stored anhydrously. Both carbohydrate and protein bind significant amounts of water, reducing their energy density dramatically (Jenni and Jenni-Eiermann, 1998). Second, fat can readily be stored outside of muscles in large adipose stores until it is needed, meaning that large amounts of energy can be stored without impeding muscle performance. Migratory birds may double their mass in preparation for a migratory flight, and the majority of the mass gain is fat (Piersma and Jukema, 1990). Birds caught after very long flights have notably depleted fat stores, which they replenish during stopover before continuing on their journey (Battley et al., 2000; Schwilch et al., 2002). Salmon caught early in migration have high levels of subcutaneous fat, which is subsequently depleted after a migration to the spawning grounds (Idler and Bitners, 1958). Monarch butterflies have fat bodies exceeding 100% of their lean body mass during the migratory season (Beall, 1948; Gibo and McCurdy, 1993). Because of the obvious advantages to storing fat, the quantity of fat an animal has prior to migration has often been used as a proxy for migration distance, condition of an individual, or quality of the habitat in which it is re-fuelling (Battley et al., 2004; Gill et al., 2005; Guglielmo et al., 2005; Seewagen et al., 2011).

Despite the advantages of fat as a metabolic fuel, the storage and catabolism of fat has drawbacks and limitations. First, the additional mass from fat increases the cost of transport and may make animals more susceptible to predation, particularly for flying animals (Witter and Cuthill, 1993). Moreover, since fat is not soluble in the aqueous

medium of the blood or cytoplasm, it must be bound to proteins like albumin while in circulation, and must be actively transported across cell and mitochondrial membranes (McClelland, 2004; McWilliams et al., 2004). The up-regulation of all the proteins associated with fat transport and catabolism represents a substantial metabolic cost, and could limit exercise intensity in many animals. As a result, it is not possible for most animals to transport and catabolize fat rapidly enough to maintain intense exercise (McClelland, 2004). In many animals as exercise intensity increases, the relative contribution of fat to the total energy expenditure decreases, and carbohydrates become the dominant fuel. Carbohydrates are ideal for fuelling high intensity exercise because they are soluble in the blood and cytoplasm, they can quickly be mobilized from glycogen stores, and they can be rapidly oxidized by working muscles. However, carbohydrates have low energy density due to their high water content (Jenni and Jenni-Eiermann, 1998). As a result, carbohydrate stores are relatively heavy, precluding the accumulation of stores large enough for long distance travel. Due to these biochemical limitations, most mammals do not have the capacity to fuel high intensity exercise for long durations. In order to rely on fat for long duration activity, exercise intensity must be low (40 - 60% of $\dot{V}O_2$-max; Weber, 2011)

Migratory birds have overcome the limitations imposed by the transport of fat, and possess the biochemical machinery to mobilize, transport and catabolize fat to fuel high intensity exercise (Guglielmo, 2010; Guglielmo et al., 2002; Guglielmo et al., 1998; Guglielmo and McFarlan, 2007; Weber, 2011). For swimming, walking, or running animals, reducing the rate of locomotion results in a reduction in metabolic rate. A running animal can walk to reduce exercise metabolism, increasing the relative

proportion of fat used for energy. A swimming animal can simply swim slower if exercise intensity is too high to be fuelled by endogenous fat reserves. Flying, on the other hand, is more expensive at lower speeds. For flight, the relationship between energy expenditure and speed follows a U-shaped curve (Hendenström, 2002; Tobalske et al., 2003; Figure 1-1). A hovering animal must exercise at high intensity to counteract the forces of gravity to stay aloft. Similarly, an animal flying fast or against a headwind must operate at just as high an exercise intensity as a bird hovering in order to counteract the exponential increase in drag as speed increases (Hendenström, 2002; Tobalske et al., 2003). In between hovering and traveling at high speed, the metabolic cost of flight is lower. The bottom of the U-shaped curve represents the minimum power speed, which still requires a bird to exercise at high exercise intensity, about twice the maximal aerobic performance of mammals (Butler, 1991; Butler and Woakes, 1990; Ward et al., 2001). Thus, flying birds, unlike running mammals or a swimming fish, cannot simply slow down to reduce metabolic rate to a range where the demands of exercise can be met primarily by the catabolism of fat.

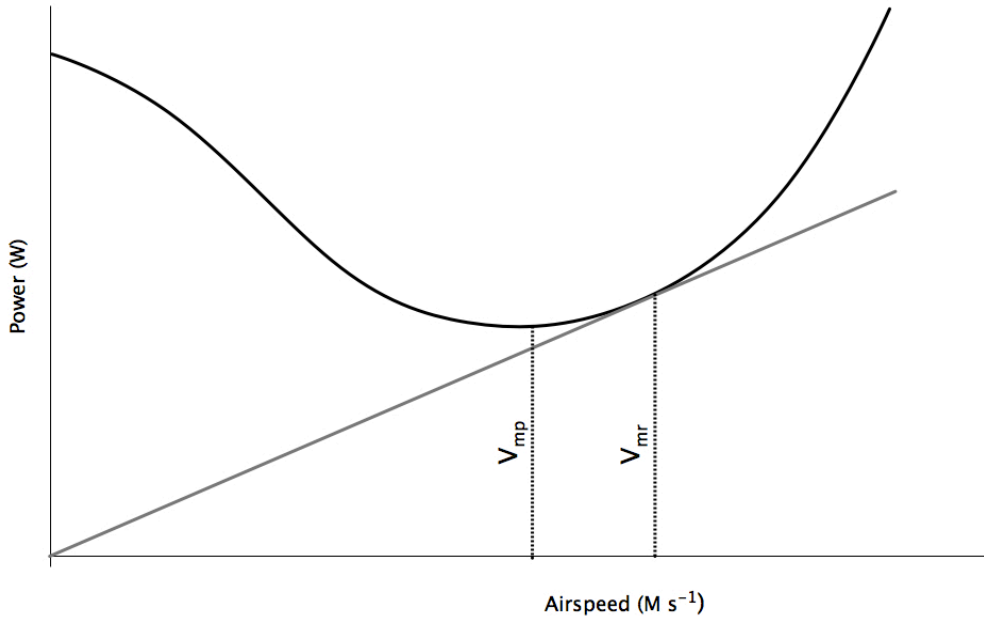

Figure 1-1. Flight costs (Power in Watts) as they relate to flight speed (airspeed). At low airspeeds, hovering flight is costly, and at high airspeed, flight costs are just as high in order to counteract drag. At moderate flight speeds, animals can minimize the power requirement (minimum power velocity: V_{mp}) for flight, or maximize the range relative to energy expenditure (maximum range velocity: V_{mr}). Adapted from (Hedenström, 2002).

Birds have evolved biochemical and physiological mechanisms to meet the high rate of fat catabolism required for flight. Migratory birds seasonally up-regulate rate limiting enzymes and transporters involved in fatty acid metabolism (Guglielmo, 2010). This, combined with endogenously controlled increases in adiposity that correspond to the migratory seasons has allowed migratory birds to fly for exceedingly long durations (Gill et al., 2009; Piersma and Davidson, 1992). Thus, it seems that migratory birds have overcome one of the most apparent limitations to flight duration, fuel limitation. However, the ability of migratory birds to mobilize and catabolize fat at such high rates can, itself, be limited by environmental factors, as we will see, and it may also make other metabolic limitations more apparent.

1.3 Limitations on Migration Imposed by Diet

The unique metabolism of birds has made them ideal candidates to investigate factors that influence fat metabolism during exercise. Due to the quantity of fat that is stored prior to departure on a migratory flight, and the rate at which birds are able to catabolize fat, birds are ideal for testing hypotheses about how the composition of fat can influence exercise metabolism. There has been a great deal of research into the factors that influence migratory performance, and what limits migration distance. One of the factors that has been shown to affect flight performance is the fatty acid composition of the diet of migratory birds (Pierce et al., 2002; Price, 2010; Price and Guglielmo, 2009; Price and Guglielmo, 2005). Diets high in poly-unsaturated fatty acids (PUFA) result in increased peak metabolic rate (Pierce et al., 2005; Price and Guglielmo, 2009), and greater metabolic efficiency in flight (McWilliams et al., 2007). Due to these gains in

performance, diet choice based on fatty acid composition may play a large role in migration success.

1.4 Limitations on Migration Imposed by Protein Catabolism

It was long thought that migratory birds used fat for 100 % of their energy in flight (Odum, 1964). This was convenient for the estimation of flight costs and flight range, but it turned out to be incorrect. It was not until Piersma and Jukema (1990) showed that gains in mass during stopover "were more than fat alone" that this paradigm began to shift. From this it was inferred that birds may catabolize protein during flight. Shortly after, other researchers started to document the use of protein during migratory flight (Biebach, 1990; Biebach, 1998; Jenni-Eiermann and Jenni, 1991). Protein, unlike carbohydrate and fat has no storage tissue, so it must be catabolized directly from muscles and organs. It has long been assumed that this results in functional losses of these tissues, but this has not been empirically confirmed. Biebach (1998), found that trans-Saharan migrants had reduced pectoralis muscle mass and internal organ mass after long flights. These reductions in organ and muscle size have since been shown under a number of conditions and in many species (Battley et al., 2000; Bauchinger and Biebach, 2001; Bauchinger et al., 2005; Lindström et al., 2000). It has also been shown that these reductions result in functional losses of internal organs, but functional losses to skeletal muscle have not been investigated (Karasov and Pinshow, 1998; Karasov et al., 2004).

Initially it was thought that the use of protein as a fuel for migratory flight was similar to its use during the final phase, phase III, of prolonged fasting; as a last resort,

once all fat and carbohydrate has been depleted (Cherel and Le Maho, 1985). Phase I of fasting occurs shortly after the initiation of fasting, and stored carbohydrate is used as the primary source of energy. Phase II of fasting occurs upon the depletion of carbohydrate stores, and during this phase fat becomes the primarily energy source. This phase is the longest phase of fasting. Once fat stores are depleted, an animal then enters Phase III of fasting. Here protein, the only fuel remaining, is catabolized (Cherel and Le Maho, 1985). However, it has recently become clear that the protein catabolized in flight is supplemental to fat catabolism (during phase II), and perhaps is being catabolized for a metabolic necessity other than energy (Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann and Jenni, 1991; Klaassen et al., 2000). If protein is catabolized at a high rate, excessive catabolism of organs and muscles may result in functional losses that limit flight duration. Thus, understanding the metabolic role of protein catabolism in flight will provide insight into the possibility that lean mass catabolism could be limiting to flight duration, independent of the quantity of fat stores available.

The catabolism of protein is a complex process, and proteins can be broken down by multiple pathways. Proteins can be broken down through the ATP-dependent proteolytic system of ubiquitination, or through the lysosomal or non-lysosomal proteolytic processes involving calcium-activated cysteine proteases (Hershko and Ciechanover, 1982). Regardless of the pathway involved, the catabolism of proteins yields amino acids that are further trans-aminated or de-aminated, allowing the carbon chains to enter the Krebs cycle to be oxidized for energy or used for processes such as gluconeogenesis (Dohm, 1986). Thus, one possible role for the catabolism of protein by migratory birds in flight could be to replenish the Krebs cycle intermediates that become

depleted during sustained fat catabolism (Jenni and Jenni-Eiermann, 1998). Although this is almost certainly a metabolic necessity during long duration flight, this should result in a constant rate of protein catabolism relative to the rate of fat catabolism, and be unaffected by external stimuli, such as temperature or humidity. On the other hand, because of its high water content, protein (or lean mass as I refer to it in this thesis) could be an important source of water, particularly under dehydrating flight conditions, as I describe below.

1.5 Limitations on Migration Imposed by Water Loss

The risk of dehydration places a potential limit on migration performance (Carmi et al., 1992; Klaassen, 1996; Klaassen, 2004). Birds have extraordinarily efficient lungs that allow oxygen to be delivered to the muscles during high intensity activities such as flight (Butler, 1991). Although this allows birds to fly for extended periods of time, it creates new problems not typically encountered by other groups of animals. One problem is that the high ventilation required for flight results in extremely high rates of respiratory water loss. In one of the only studies measuring respiratory water loss in flight, Engel et al. (2006) showed that European starlings (*Sturnus vulgaris*) have a breathing frequency of approximately 4 Hz, and respiratory water loss as high as 3.0 mL h^{-1}. For comparison, birds approximately the same size as a Starling (Northern Cardinal, *Cardinalis cardinalis* and White-winged Doves, *Zenaida asiatica*) exposed to temperatures between 35 °C and 45 °C in dry air have similar rates of water loss (Dawson, 1958; McKechnie and Wolf, 2004). Thus, while flying, even at moderate temperatures, respiratory water losses can be

similar to those experienced in desert-like conditions at rest. Yet, most migratory birds fly for hours or days at a time, sometimes at temperatures exceeding 30 °C (Schmaljohann et al., 2008) with no opportunity to drink.

Numerous studies have shown that migratory birds are not dehydrated after long-distance flights (Biebach, 1990; Giladi and Pinshow, 1999; Landys et al., 2000). Many hypotheses have been proposed to explain the maintenance of water balance during flight in migratory birds. It was suggested that birds increase water stores prior to flight (Torre-Bueno, 1978). The dehydration incurred during flight would then result in birds arriving at their destination at 'normal' hydration states. There is no evidence for this, and the additional water weight could add substantially to flight costs. In fact, many departing migratory birds were found to be lower in relative total body water (see Biebach, 1990; Torre-Bueno, 1978 for discussions). This reduction in relative total body water was simply a product of body mass being increased due to large fat reserves, rather than water content being reduced (Ellis and Jehl Jr, 1991). It was also hypothesized that birds would modulate water losses behaviourally, choosing altitudes where the temperature, and consequently the water vapor deficit, is lower (Biebach, 1990). This strategy may not always be available, or optimal however. In a recent study investigating the tradeoff between choosing altitude to minimize water loss, or tailwinds to minimize travel time, it was found that trans-Saharan migrants fly at altitudes below 1.5 km in order to take advantage of favourable tail winds. Ambient temperatures in this range of altitudes were in excess of 30 °C, and relative humidity was below 40 % (Schmaljohann et al., 2009), whereas higher altitudes offered cooler and more humid conditions.

Another hypothesis for how birds maintain hydration in flight, now dubbed the protein-for-water hypothesis, predicted that birds might increase the relative contribution of protein to their fuel mixture because protein has a much higher yield of water per unit energy (0.155 g H_2O kJ^{-1}) than fat (0.029 g H_2O kJ^{-1}) (Carmi et al., 1992; Carmi et al., 1995; Klaassen, 1995; Klaassen, 1996; Klaassen, 2004). This type of novel metabolic strategy has not been previously documented, and the influence of ambient conditions (temperature and humidity) on the rate of protein catabolism could have profound implications to the strategies employed by migratory birds, both during flight and stopover. This hypothesis provided a framework that would explain both the unusually high rates of protein catabolism found in migrants, while also explaining how migrant birds maintain water balance in flight.

1.6 Research Summary

The overall goal of my thesis is to investigate the influence of external factors, specifically diet and ambient environmental conditions on metabolism and water balance of migratory birds in flight. Most of the chapters (Chapters 2-5) of this thesis investigate the metabolic strategies employed by migratory birds to minimize or offset water losses. One chapter (Chapter 6) investigates the effect of dietary polyunsaturated fatty acids on exercise performance. It is my hope that the findings of these investigations advance the current understanding of the metabolic and whole-animal strategies used by migratory birds to overcome the physiological challenges imposed by long-duration flights.

In Chapter 2, I tested the protein-for-water hypothesis in resting, non-migratory House sparrows (*Passer domesticus*) using a quantitative magnetic resonance body composition analyzer (QMR), which rapidly and non-invasively measures fat mass, lean mass and total body water of live birds. With this new technology I was able to measure changes in lean mass over a 16 - 18 h water restriction within individuals. I clearly show that water restricted birds increase the rate of lean mass catabolism compared to control birds, which had free access to water. Additionally, using hygrometry, I was able to show that the additional lean mass catabolized in the water-restricted birds resulted in sufficient increases in endogenous water production to offset respiratory losses. Birds were also exposed to a metabolic challenge (shivering) in order to assess the effect of increased metabolic rate on protein catabolism. In this case, there was no difference in the rate of lean mass catabolism between water restricted and control birds, and water gains from increased fat catabolism were sufficient to offset losses during the metabolic challenge. This chapter showed, for the first time, that birds do use a protein-for-water strategy when faced with mild dehydration at rest. It did not confirm the use of such a strategy when metabolic rate was increased by shivering because substantial quantities of endogenous water were produced from the catabolism of fat.

In Chapter 3, I flew migratory Swainson's thrushes (*Catharus ustulatus*) in a wind tunnel under carefully chosen humidity regimes in order to test the protein-for-water hypothesis in free flying migratory birds exposed to ecologically relevant conditions. I manipulated only humidity in order to achieve high or low evaporative water loss conditions in flight, while minimizing the possible effects differences in heat load could introduce. Since flight duration has a dramatic effect on the relative proportion of lean

mass catabolized, and because there could be substantial variation in the use of lean mass among individuals, I used a repeated measured approach. Each individual bird flew flights matched in duration, but under opposite evaporative water loss conditions. Birds achieved flights in excess of 5 hours, which is similar to what has been measured in the wild for this species. The data clearly showed that birds flown under high evaporative water loss conditions had higher rates of lean mass catabolism, and increased plasma uric acid concentrations, providing clear support for the protein-for-water hypothesis.

In Chapter 4, I investigated the metabolic response to flight under two different humidity regimes in a short duration migrant, the American robin (*Turdus migratorious*). As a result of their relatively large size, I was able to collect blood before and after flight, and investigate plasma metabolite profiles as flight duration increased up to 1 hour. Here, I found little effect of water loss on metabolism, but it is one of the only studies to date to investigate the metabolic response to short duration flight in a migratory bird. These birds showed a classic transition in fuel mixture as flight duration increased. Early in flight, energy was derived primarily from carbohydrate and protein, but as flight progressed, the relative contribution from fat increased. Thus, early in flight, rates of endogenous water production are high, and these birds did not rely on a protein-for-water strategy.

In Chapter 5, I developed a new technique using a fluorescent marker to measure glomerular filtration rate (GFR) in Swainson's thrushes (*Catharus ustulatus*) at rest, during feeding, and in flight. Since flying and resting birds had no access to water, I expected GFR to be reduced in flight and at rest relative to feeding. However, the additional protein catabolized under high evaporative water loss conditions (Chapter 3)

may limit a bird's ability to restrict GFR, since uric acid must be removed from the blood. I found that GFR was not different between fed, rested and flown birds, and that migratory birds rely instead on increased fractional water reabsorption to minimize excretory water losses.

Chapter 6 was part of a large collaborative project investigating the effects of polyunsaturated fatty acids on migratory flight performance. My contribution to this project was to investigate the effects of polyunsaturated fatty acids on mitochondrial metabolism and rates of reactive oxygen release after flight and training. Birds were fed one of four isocaloric diets, high in polyunsaturated fatty acids (PUFA), or high in monounsaturated fatty acids (MUFA). Each of these diets was divided into either a high or low vitamin E diet. Birds were either untrained, trained in the wind tunnel for 15 days and then given two days to recover before sampling, or exercised, where they were sampled immediately after the final flight. The data indicate that reactive oxygen species (ROS) production is reduced by diets high in polyunsaturated fatty acids. Birds fed a monounsaturated fatty acid diet had reduced mitochondrial oxidative capacity, presumably due to higher ROS damage, which possibly limited flight duration.

Collectively, these studies provide new insights into the metabolic and physiological strategies used by migrants to extend flight duration. Migratory birds overcome the limitation imposed by dehydration by using a unique metabolic strategy (protein-for-water) in order to increase endogenous water production to offset high rates of water loss. Also, these birds may have evolved to prefer diets high in PUFA during stopover in order to minimize the rate of reactive oxygen species production in flight, thus overcoming a potential limitation imposed on flight duration by the accumulation of

oxidative damage. These two strategies likely contribute to the success of migratory birds in completing their annual journeys.

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2 HOUSE SPARROWS (*PASSER DOMESTICUS)* INCREASE PROTEIN CATABOLISM IN RESPONSE TO WATER RESTRICTION[1]

2.1 Introduction

Birds have an exceptional ability to rapidly mobilize and catabolize fat to fuel metabolically demanding activities such as flight or thermogenesis (Guglielmo, 2010; Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004; Swanson, 1991). Supplemental to fat catabolism, it has become apparent that protein in lean tissue is also catabolized during flight, thermogenesis, and at rest (Battley et al., 2000; Biebach, 1998; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002; Karasov and Pinshow, 1998; Klaassen et al., 1997; Schwilch et al., 2002; Swain, 1992). Protein is primarily catabolized for energy during phase III of fasting when fat and glycogen stores have been depleted (Cherel and Le Maho, 1985), but protein catabolism during phase I of fasting, while an animal still has sufficient energy stores remaining, may be in response to other physiological factors.

Catabolism of protein during flight in birds has been documented through gravimetric changes in muscles and organs (Battley et al., 2000; Bauchinger et al., 2005;

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Landys et al., 2005; Schwilch et al., 2002), and through changes in plasma metabolites such as uric acid (George and John, 1993; Guglielmo et al., 2001; Jenni-Eiermann and Jenni, 1991; Jenni-Eiermann et al., 2002; Schwilch et al., 1996; Tsahar et al., 2006). Since there is no storage tissue for protein as there is for fat (adipocytes) or carbohydrates (liver and muscle glycogen), protein is used directly from muscles and organs with possible negative consequences to flight performance in the case of muscle catabolism, or nutrient absorption and processing in the case of organ catabolism.

There has been much discussion about the possible role for this seemingly maladaptive phenomenon (Battley et al., 2000; Biebach, 1998; Carmi et al., 1992; Carmi et al., 1995; Dohm, 1986; Jenni and Jenni-Eiermann, 1998; Klaassen, 2004; Klaassen et al., 1997; Landys et al., 2005; Landys et al., 2000; Pennycuick and Battley, 2003; Schwilch et al., 2002). Protein catabolism may be necessary for gluconeogenesis or for the anaplerosis of tricarboxcylic acid (TCA) cycle intermediates, both of which may be necessary during sustained fat catabolism (Dohm, 1986; Jenni and Jenni-Eiermann, 1998). The breakdown of protein could also aid in the maintenance of water balance under dehydrating conditions especially in uricotelic animals, where the excretion of nitrogenous wastes requires less water than in ureotelic animals (Jenni and Jenni-Eiermann, 1998; Klaassen, 2004; Wright, 1995). For the same amount of energy released, catabolism of wet protein results in the release and production of 0.155 g H_2O kJ^{-1}, approximately five times more bound and metabolic water than the catabolism of fat (0.029 g H_2O kJ^{-1}) (Jenni and Jenni-Eiermann, 1998). Thus, protein may serve as a source of endogenous water to offset water losses, while additionally providing the

metabolites necessary for gluconeogenesis and anapleurosis of TCA cycle intermediates (Klaassen, 2004).

Many of the studies documenting substantial lean mass losses of birds were performed on trans-Saharan migrants, or after multi-day non-stop flights in shorebirds, where water stress is possible (Battley et al., 2000; Bauchinger and Biebach, 2001; Biebach, 1998; Carmi et al., 1992; Karasov and Pinshow, 1998; Klaassen, 2004; Landys et al., 2000). Although there is evidence that long-term dehydration can increase protein catabolism in humans and Richardson's ground squirrels (*Spermophilus richardsonii*) (Berneis et al., 1999; Bintz and Strand, 1983; Haussinger, 1996), to date there are no studies showing a direct reduction in the total lean mass of an animal due to acute dehydration. Thus, we postulate that the amount of lean mass catabolized may not necessarily depend on energetic demands, and may instead be a response to water deficit or other stressors. If this is the case, it is expected that water-restriction will increase lean mass catabolism, ultimately resulting in maintenance of water balance due to endogenous water gains.

Until recently, it has proven difficult to accurately measure the effects of water restriction on body composition over time within an individual (Lindström and Piersma, 1993; Piersma and Klaassen, 1999). Techniques such as heavy water dilution for estimating lean mass rely on total body water and can be confounded by manipulation of water balance (Speakman, 2001). Catabolism of protein in a uricotelic animal results in respiratory quotients (CO_2 produced ÷ O_2 consumed) very similar to those of fat catabolism (Klaassen et al., 1997), precluding the use of respirometry for the determination of fuel mixture including a protein component. Destructive body

composition analysis requires a large number of individuals, and would lack the power of a repeated measures design. Therefore, in this study we used a quantitative magnetic resonance body composition analyzer (QMR), which accurately and non-invasively measures lean mass, fat mass, and total body water in un-anaesthetized animals (McGuire and Guglielmo, 2010; Taicher et al., 2003; Tinsley et al., 2004). Changes in body composition were monitored longitudinally in individual birds throughout the course of an 18 h water restriction at rest, followed by a 4 h period of elevated metabolic rate (shivering). Our goal was to investigate whether water balance status affects the rate of lean mass catabolism during rest and simulated endurance exercise in house sparrows (*Passer domesticus*). In order for birds to maintain water balance during the resting phase of this experiment, additional endogenous water production should be necessary from lean mass catabolism. However, during the shivering phase of the experiment high metabolic rates will result in elevated metabolic water production primarily from fat. Depending on the rate of ventilatory water loss during shivering net metabolic water gains could reduce or preclude water required from lean mass catabolism.

2.2 Materials and Methods

2.2.1 *Animal Care*

Male house sparrows were caught using mist nets during March of 2008 near the University of Western Ontario campus (London, Ontario, Canada). Birds were moved to the animal care facility within 1 h of capture where they were weighed, individually colour banded, and placed in individual 40 cm X 45 cm X 45 cm cages with water and a diet consisting of a mixture of millet seed and Mazuri® Small Bird diet. Birds were

maintained on a 12L:12D (lights on at 06:00) cycle at 24°C for the duration of the experiment. Birds were kept for a minimum of two weeks before the experiment began. All animal care protocols followed the Canadian Council on Animal Care guidelines and were approved by the University of Western Ontario Council on Animal Care and the Animal Use Subcommittee (Protocol # 2006-011-04).

2.2.2 *Experimental Protocol*

Sparrows were randomly assigned to either a water-restricted (WR; n = 8) or control (CT; n = 7) group, which were housed individually in adjacent cages, thus controlling for minor fluctuations in light and disturbance in the housing facility. These pairs of birds followed identical routines throughout the experimental period. Each experimental day between 15:30 and 17:30, one CT bird and one WR bird were weighed on a digital scale, scanned in duplicate using QMR (Initial scan) and returned to their cages. Each replicate QMR scan lasted approximately 90 s, and the mean values were used. After the initial scan, water was removed from the WR bird. At 07:00 the following morning, food was removed from the WR and CT birds. At 10:00 birds were assumed to be post-absorptive, and were placed in identical adjacent 1 L respirometry chambers maintained at 24°C for a period of two hours to determine resting metabolic rate (RMR) and resting rates of total evaporative water loss (see below), at this time the WR birds had experienced 16-18 h without access to free water. After two hours at 24°C, the birds were scanned in duplicate using QMR (before shivering), blood sampled from the right brachial vein (rest), and then placed in identical individual 1 L respirometry chambers that had been pre cooled to initiate a four-hour shivering trial at 5 °C. Shivering metabolic rate (SHMR) and rates of total evaporative water loss were measured

(see below). At the conclusion of the shivering trial, birds were scanned in duplicate a third time using QMR (post shivering) and a final blood sample was taken from the left brachial vein (shivering). The bottom of each metabolic chamber was lined with aluminum foil and all droppings were collected and immediately frozen at -30 °C after the resting period and the shivering trial. Each individual bird was only used once during the course of the experiment.

2.2.3 *Quantitative Magnetic Resonance*

Quantitative magnetic resonance body composition analysis has been shown to be extremely accurate and precise, and the principle of the methods has been described elsewhere (McGuire and Guglielmo, 2010; Taicher et al., 2003; Tinsley et al., 2004). The instrument we used was specifically designed for use with small birds (model Echo-MRI-B, Echo Medical Systems, Houston, TX, USA). Our validation studies with house sparrows indicate that fat, wet lean, and total body water are measured with precisions (CV) of 3 %, 0.5 %, and 3 %, respectively, and relative accuracies of ± 11 %, ± 1 % and ± 2 %, respectively (Guglielmo, Gerson, McGuire and Seewagen unpublished data). Overall changes in body mass, lean mass, and fat mass were compared between WR and CT groups using repeated measures general linear model (GLM) with initial mass as a covariate. Between treatment effects for each experimental interval (Initial to before shivering and before shivering to post shivering) were assessed by comparing the differential in body mass, lean mass and fat mass for each interval using GLM (SPSS v. 17.0).

2.2.4 *Respirometry, Hygrometry, and Estimated Water Budgets*

Flow through respirometry was used to measure resting (RMR) and shivering

metabolic rate (SHMR) simultaneously with rates of evaporative water loss. Incurrent air was scrubbed of CO_2 and water vapor using soda lime and Drierite®, respectively. All four respirometry chambers were well sealed and received constant flow of approximately 600 ml min^{-1} (measured after the chambers with a Sierra Instruments 840-L mass flow meter, Monterey, CA, USA). Excurrent air was sub-sampled at a rate of 150 ml min^{-1} through a H_2O analyzer (Licor LI-7000) after which air passed through a Drierite column to the CO_2 (Sable Systems CA-2A, Las Vegas, NV, USA) and oxygen gas analyzers (Sable Systems FC-1B) with CO_2 and H_2O scrubbing between the two gas analyzers. Gas analyzers were calibrated with a certified standard (20.9% O_2, 2.0% CO_2 balanced with N_2; Praxair, London, Ontario, Canada). Multiplexing allowed measurement of each chamber in 30-minute intervals, with a 10-minute baseline measurement every hour. Two respirometry chambers were placed in a temperature controlled cabinet maintained at 24°C (PTC-1, Sable Systems), while another two chambers were maintained at 2-5°C in a Styrofoam cooler lined with copper tubing that was connected to a water bath (Lauda E100) circulating -8.0°C propylene glycol. This circulating temperature was most effective at maintaining chamber temperature between 2°C and 5°C during the shivering trial. All instruments were connected to an analog to digital converter (UI-2, Sable Systems), which was connected to a laptop computer. Data collection and analysis was done using Expedata software (Sable Systems). Fractional concentrations of O_2 and CO_2 were lag corrected and $\dot{V}O_2$ (mL min^{-1}), $\dot{V}CO_2$ (mL min^{-1}) and $\dot{V}H_2O$ (mg H_2O h^{-1}) were calculated from the mean for the final 20 minutes of each 30-minute sampling period for each channel using equations 11.1, 11.6, and 11.9 respectively from (Lighton, 2008). Calculations assume that 1 mL of water vapor is

equivalent to 0.803 mg H_2O (Lighton, 2008). $\dot{V}H_2O$ at rest and during shivering was extrapolated over time to estimate total water lost during those periods. Endogenous water production (sum of metabolic water and water liberated from catabolism of lean mass) was calculated from the values in (Jenni and Jenni-Eiermann, 1998), and the change in lean and fat mass measured by QMR. Estimated total evaporative water loss and endogenous water production were compared between treatments using paired Student's t-test. Upon analysis of the respirometry data, the RQ values were unrealistically low, and it was determined that the fuel cell in the oxygen analyzer had expired, thus $\dot{V}O_2$ data was discarded. $\dot{V}CO_2$ and $\dot{V}H_2O$ during resting and shivering trials were compared between treatment groups using Student's t-test.

2.2.5 *Uric Acid and Osmolality Determination*

Uric acid was determined by endpoint assay (Wako Uric Acid 20R/30R kit) as in (Tsahar et al., 2006) for both plasma and droppings. Droppings were weighed and dried to constant mass at 45°C. Dried excreta was then ground using a small glass mortar and Teflon pestle and dissolved 120-fold (w/v) in 0.1 M glycine buffer, pH 9.3 for analysis; plasma was analyzed undiluted. Excreted uric acid was only compared between treatments during the resting period due to a low number of dropping samples during the shivering trial. Plasma osmolality was measured in 10 μL of plasma using a Wescor Vapro 5520 vapor pressure osmometer calibrated as per the manufacturers instructions. Plasma uric acid and osmolality from rest and shivering blood samples were compared between water restricted and control birds using repeated measures GLM. Excreted uric acid was compared between treatments using t-test (SPSS v.17).

2.3 Results

2.3.1 *Body Composition*

All birds lost mass throughout the experiment (Figure 2-1a $F_{2,20} = 124.769$, P=0.001), but WR birds lost on average 1.01 g (4.3 %) more than CT birds (Figure 2-1a $F_{1,10} = 6.785$, P=0.026). There was no significant difference in initial mass between treatments (t-test: t = 1.150, DF = 13, P = 0.271) and most mass loss occurred during the resting period for both WR and CT birds. Although mass loss during this time was 0.73 g (3.0 %) greater in water-restricted animals, mass loss at rest was not significantly different between treatments ($F_{1,13} = 2.811$, P=0.117). The greater mass loss in water-restricted animals was a result of an additional 0.85 g (4.3 %) of lean mass loss overall ($F_{1,12} = 21.372$, P=0.001). The majority (74.02 %) of the lean mass loss occurred between the initial and before shivering time points, and during this time WR birds lost significantly more lean mass than CT birds (Figure 2-1b $F_{1,13} = 5.435$, P = 0.036); there was no significant difference between treatments in lean mass loss during shivering ($F_{1,13} = 1.410$, P=0.256). No significant differences in fat mass losses were evident between water restricted and control birds for either the overnight interval ($F_{1,13} = 0.230$, P=0.639) or during the shivering trial ($F_{1,12} = 2.98$, P = 0.108) (Figure 2-1c).

2.3.2 *Respirometry*

No significant differences were found between water restricted and control groups in $\dot{V}CO_2$ (t = 0.320, DF = 13, P = 0.754) or $\dot{V}H_2O$ (t = 0.675, DF = 13, P = 0.512) at rest or during shivering ($\dot{V}CO_2$ t = -0.495, DF = 14, P = 0.628; $\dot{V}H_2O$ t = -1.421, DF = 14, P = 0.177). Shivering resulted in a significant increase in $\dot{V}CO_2$ over resting in both

treatments (WR: $t = -7.169$, $DF = 14$, $P<0.001$; CT: $t = -2.259$, $DF = 14$, $P<0.001$; Table 2-1).

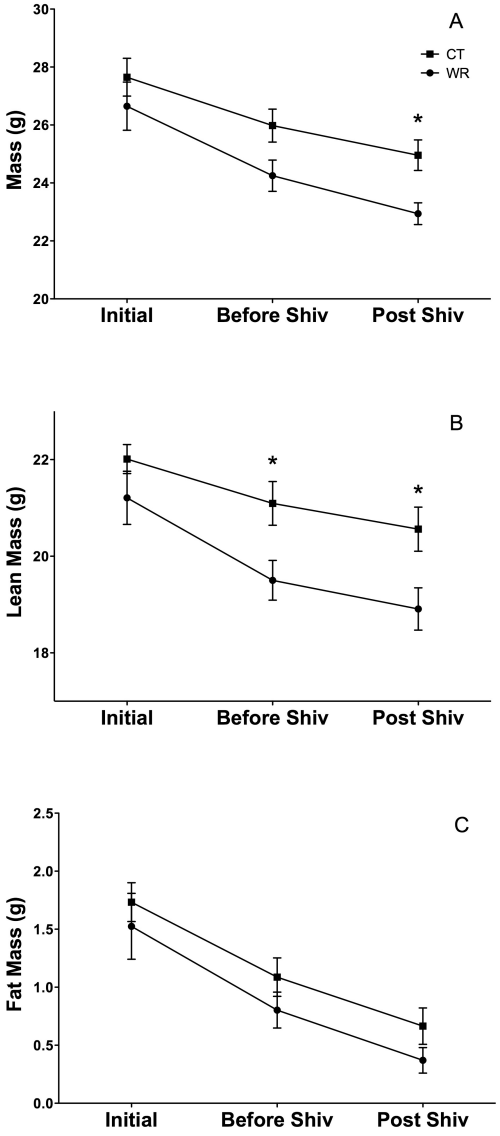

Figure 2-1. Changes in mass, lean mass and fat mass during water restriction. a) Water restriction resulted in greater mass loss overall. b) Lean mass loss in WR birds was greater during the resting period and overall. c) No significant differences in fat mass losses were evident between water restricted and control birds. WR: n = 8, CT: n = 7. Values are means ± SEM. * Indicates significant difference between WR and CT at $P < 0.05$. Values published in manuscript are Means ± SD.

	WR		CT	
	Rest	**Shivering**	**Rest**	**Shivering**
$\dot{V}CO_2$ (mL CO_2 min^{-1} g^{-1})	2.00 (0.28)	5.02 (1.68)*	1.88 (1.35)	4.54 (2.21)*
$\dot{V}H_2O$ (mg H_2O min^{-1} g^{-1})	3.89 (0.77)	5.40 (1.22)	4.33 (1.64)	4.85 (1.01)

Table 2-1. $\dot{V}CO_2$ and $\dot{V}H_2O$ at rest and during shivering in WR and CT birds. No significant differences were found between WR and CT. Shivering resulted in significantly elevated $\dot{V}CO_2$ over resting values within each treatment group. * Indicates $P < 0.05$. Values are means (SEM).

2.3.3 *Plasma Osmolality and Uric Acid*

Plasma osmolality at rest was 7.3% higher in water-restricted birds (Figure 2-2: $F_{1,11} = 20.080$, P = 0.001), and was 4.97 % higher in WR birds after the shivering trial (Figure 2-2: $F_{1,11} = 9.639$, P = 0.010). Plasma concentrations of uric acid were also elevated in the water-restricted group at rest (Figure 2-3: $F_{1,11} = 15.384$, P = 0.002). However, post-shivering, there was no significant difference in plasma uric acid between the water restricted and control samples (Figure 2-3: $F_{1,11} = 1.963$, P = 0.192). Both water-restricted and control animals experienced an increase in plasma uric acid with shivering (Figure 2-3: WR: t = -3.984, DF = 7, P = 0.005, CT: t = -3.736, DF = 5, P = 0.013). Treatment did not affect excreta uric acid concentration (t = -1.60, DF = 6, P =0.080), water content (t = 0.832, DF = 7, P = 0.432), or the total amount of uric acid lost in excreta during the resting period (t = -1.379, DF = 6, P = 0.108), although the uric acid concentration of the excreta as well as the total amount of uric acid lost tended to be higher in WR birds (Table 2-2).

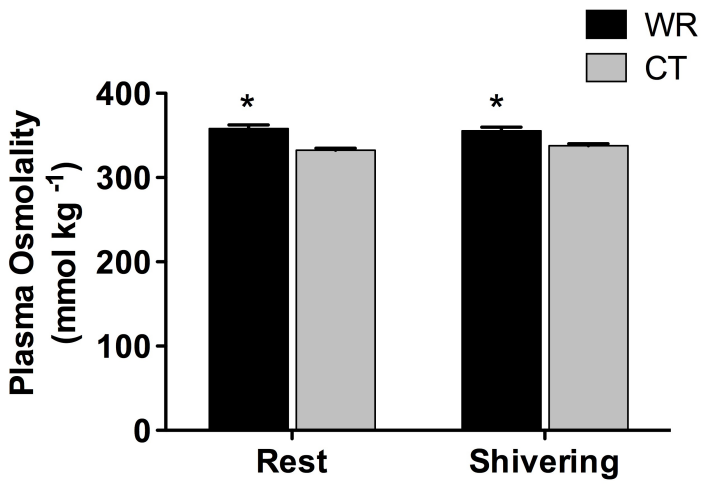

Figure 2-2. Plasma osmolality was significantly elevated by WR both at rest, and post-shivering (WR: n = 8, CT: n = 7). Values are means ± SEM. * Indicates significant differences between treatments at $P < 0.05$. Values published in manuscript are Means ± SD.

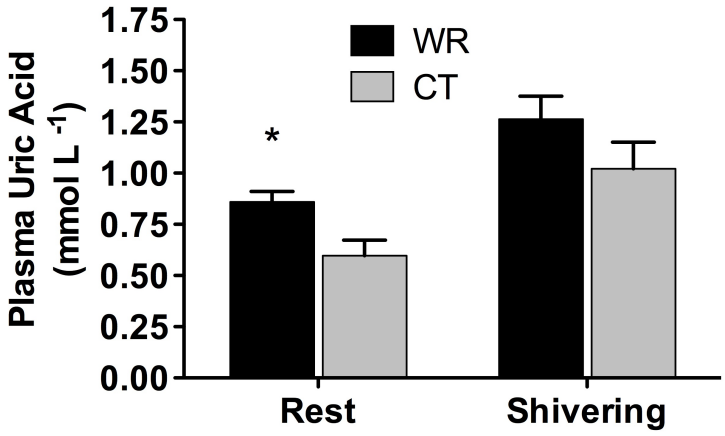

Figure 2-3. Plasma concentrations of uric acid were elevated in the water-restricted group at rest, but not after shivering. Both treatments had increased plasma uric acid post-shivering (WR: P = 0.005, CT: P = 0.013). Values are means ± SEM. * Indicates significant differences between treatments at P <0.05. Values published in manuscript are Means ± SD.

2.3.4 *Water Budgets*

Water restricted sparrows produced significantly more endogenous water from the catabolism of lean mass at rest ($t = -2.489$, $DF = 14$, $P=0.026$), but not during the shivering trial ($t = 0.93$, $DF = 14$, $P=0.927$) and they tended to produce more water during the entire experiment from lean mass catabolism ($t = -2.089$, $DF = 13$, $P=0.057$). As a result WR birds produced more total endogenous water (water from lean and fat) at rest ($t = -1.94$, $DF = 14$, $P = 0.036$) than control birds. During shivering, CT birds had greater total endogenous water production ($t = 2.27$, $DF = 12$, $P = 0.020$). There were no significant differences in estimated total evaporative water loss between treatments for either the resting ($t = 1.033$, $DF = 13$, $P = 0.320$) or shivering ($t = -0.69$, $DF = 13$, $P = 0.946$) periods. At rest, WR birds maintained positive net water balance, which was significantly higher than CT birds, where net water balance was negative ($t = -2.129$, $DF = 9$, $P = 0.033$). During shivering, metabolic water production exceeded water losses regardless of treatment, but CT birds had greater gains than WR birds ($t = 2.20$, $DF = 13$, $P = 0.023$). Total body water as a percent of body mass did not change significantly between resting and shivering periods (Rest: $t = -0.045$, $DF = 11$, $P = 0.482$; Shivering: $t = -0.876$, $DF = 7$, $P = 0.204$) (Table 2-3).

REST	Uric Acid (mg/g)	% Moisture	Uric Acid lost (mg)
Water Restricted	418.01 (29.79)	5.97 (2.73)	344.01 (29.41)
Control	309.91 (60.46)	9.74 (3.60)	261.62 (52.00)
P-value	0.080	0.147	0.108

Table 2-2. Uric acid concentration, moisture and total uric acid lost in excreta at rest during respirometry from WR and CT birds. No significant differences were evident between treatments. Values are means (SEM).

	WR		CT	
	Rest	**Shivering**	**Rest**	**Shivering**
Total Evaporative water loss (mL)	1.85 (0.15)	0.554 (0.03)	2.20 (0.32)	0.550 (0.043)
Endogenous water production Lean (g)	1.40 (0.15)*	0.487 (0.038)	0.769 (0.20)*	0.567 (056)
Total Endogenous water production (g)	2.19 (0.28)*	0.964 (0.05)†	1.48 (0.23)*	1.14 (0.06)†
Net water balance (g)	0.348 (0.19)*	0.409 (0.06)	-0.443 (0.54)*	0.598 (0.06)
Body water (% of M_b)	63.07 (0.01)	64.41 (0.01)	63.00 (0.01)	62.93 (0.01)

Table 2-3. Estimated water budgets for WR and CT treatment groups throughout an 18 – 20 h dehydration period, followed by a 4 hour shivering trial. Total endogenous water production was calculated from changes in body composition, assuming 0.155 g H_2O kJ^{-1} for lean mass and 0.029 g H_2O kJ^{-1} for fat as in (Jenni and Jenni-Eiermann, 1998). Net water balance is the difference between total evaporative water loss and total endogenous water production, CT birds had access to water during the resting period. Body water % is the QMR value for total body water divided by total body mass for the before and after shivering time points. * indicates significant differences ($P < 0.05$) between treatment groups, but within metabolic states. Values are means (SEM).

2.4 Discussion

This is the first study, to our knowledge, to directly test the hypothesis that birds preferentially catabolize protein as a means to liberate endogenous water under conditions of water stress. The elevated lean mass loss as a result of acute water-restriction as shown by the direct measurement of body composition over time within individuals represents clear support for this hypothesis. Although this hypothesis has been proposed mainly as a strategy for long distance migratory flight (Jenni and Jenni-Eiermann, 1998; Klaassen, 2004), this experiment serves as a proof of concept that a physiological mechanism exists whereby water balance status can influence fuel mixture. Whereas a greater reduction in lean mass occurred only at rest in the water restricted group, it is important to note that during shivering, metabolic water production far exceeded water losses, consequently additional water production from lean mass was unnecessary.

The strongest evidence for accelerated lean mass catabolism as a result of water restriction comes from the QMR data, which was corroborated by the elevated plasma uric acid levels. Changes in body composition coupled with $\dot{V}H_2O$ allowed the estimation of water budgets for each phase of this experiment, which indicate that higher rates of lean mass catabolism in the WR birds resulted in endogenous free water gains sufficient enough to offset evaporative losses at rest. During shivering, metabolic rate was elevated approximately 2.5-fold regardless of treatment. This elevation in metabolic rate was primarily fuelled by fat catabolism, and the relative contribution from lean mass was similar for each treatment. Since water balance was maintained during the resting period,

at the expense of lean mass in the WR birds, elevated lean mass catabolism during shivering was unnecessary for the WR birds.

Birds typically maintain plasma volume during water restriction (Carmi et al., 1994), discounting the possibility that elevated osmolality and uric acid were simply a product of reduced plasma volume, rather than an increase in the actual metabolites responsible. In fact, elevated plasma osmolality may be a response to dehydration that facilitates the maintenance of plasma volume by favouring the movement of intracellular water to blood vessels, down the osmotic gradient thus resulting in cellular dehydration (Arad et al., 1989). There is evidence indicating hyperosmolality alone may influence cellular metabolism in mammals, ultimately resulting in elevated protein catabolism (Berneis et al., 1999; Haussinger, 1996). Whether this mechanism exists in birds has yet to be explored. The resulting endogenous water production may alleviate cellular dehydration while amino acids and peptides may bring intracellular osmolality towards equilibrium with the interstitial fluid and plasma. This mechanism would not only liberate water, but would reduce the osmotic gradient between the intra- and inter-cellular compartments. Taking this into account, the decrease in plasma osmolality during the shivering trial in the WR birds could be a product of water gains due to higher metabolism overall, and thus greater metabolic water production, resulting in the expansion of plasma volume during the shivering period.

Responses of birds to extended dehydration typically include reduced glomerular filtration rate and greater tubular water re-absorption, resulting in production of more concentrated urine (Goldstein and Braun, 1988; Goldstein and Skadhauge, 2000). Although no significant differences were found between treatments, the trends in uric

acid excretion are consistent with this response. However, the degree of the response is subtle, perhaps because the period of water restriction in the current experiment was relatively short. Birds are uricotelic and use extrarenal water reabsorption in the colon to minimize excretory water losses (Goldstein and Skadhauge, 2000). For this reason, birds may be different from mammals in their ability to benefit from a protein-for-water strategy so long as urine does not reach a concentration where extrarenal absorption is repressed (Goldstein and Braun, 1988). However, highly concentrated urine is unlikely in the current study due to the relatively short water restriction and the maintenance of water balance from elevated protein catabolism. Body water as a percent of body mass did not change throughout the experiment, but it has been noted that the amount of water relative to body mass is not a good indicator of water stress or dehydration (Klaassen, 2004).

Due to the limited amount of plasma available, other analyses were not possible in this study. The response of arginine vasotocin (AVT), prolactin, aldosterone, or corticosterone to water restriction could have provided insight into possible control of fuel selection during water restriction. However, this study did not differ substantially in terms of magnitude or duration of water restriction from many other studies that have thoroughly investigated the hormonal response to acute water restriction in birds at rest and during exercise (Arnason et al., 1986; Giladi et al., 1997; Goldstein and Braun, 1988; Saito and Grossmann, 1998). Corticosterone does affect fuel use in birds, and would be the likely hormone responsible for elevated lean mass catabolism. However, experimental design must incorporate careful control of handling and other stressors during the experiment for measurements of corticosterone to be meaningful.

The protein-for-water phenomenon could have broad implications during many life history stages of birds including breeding and migration, especially in light of projected changes in climate (Houghton et al., 2001). The use of protein for water may help explain recent evidence that birds actually fly under conditions unfavourable to water balance (Schmaljohann et al., 2008), taking advantage of favourable winds, and possibly maintaining water balance at the expense of protein. If water stress during migratory flight or stopover refueling results in additional lean mass catabolism, refueling rates may be reduced (Sapir et al., 2004) leading to increased stopover duration, delaying arrival on the breeding grounds (Both and Visser, 2001).

2.4.1 *Perspectives and Significance:*

Since much of the discussion surrounding the hypothesis that protein can be stored and used for water production has focused on migratory flight, it would seem logical to design experiments in order to test the possibility that water balance may influence the proportions of fat and lean mass utilized during flight. To this end, we feel that the present study uses a simple yet informative suite of minimally invasive techniques that could be implemented to study the possible effects of environmental conditions on fuel use in avian flight. It would then be interesting to modify existing fuel use models for bird flight to examine the possible consequences of dehydration to flight range in terms of changing fuel mixture (Carmi et al., 1992; Carmi et al., 1995; Klaassen, 1996; Klaassen, 2004). It should be noted that due to the very high metabolic rates experienced during flight, a vast amount of metabolic water is produced from the catabolism of fat alone. Whether this water production is balanced by water loss depends entirely on the ambient conditions experienced during flight. Rates of water loss are constant across

temperatures during flight, until a threshold temperature is reached above which evaporative water loss increases with temperature (Engel et al., 2006; Giladi and Pinshow, 1999). This threshold temperature has been identified to be around 20°C for both rose-coloured starlings (*Sturnus roseus)* and pigeons (*Columbia livia*) (Engel et al., 2006; Giladi and Pinshow, 1999). Flying at temperatures above this threshold may result in greater lean mass utilization for water in flight. Future studies should investigate this phenomenon in resting and exercising animals in order to more fully understand the mechanisms involved in the control of lean mass catabolism as well as the osmoregulatory consequences of lean mass catabolism during water restriction at both the cellular and whole animal levels.

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3 FLIGHT AT LOW AMBIENT HUMIDITY INCREASES PROTEIN CATABOLISM IN MIGRATORY BIRDS[2]

3.1 Introduction

During migration, birds may travel thousands of kilometers between breeding and wintering grounds, stopping periodically to replenish fuel stores. The energy for flight is derived primarily from the oxidation of fatty acids stored in subcutaneous, abdominal, and intramuscular fat depots (Jenni and Jenni-Eiermann, 1998). Lean mass (mainly protein) is also catabolized, even while substantial fat stores remain, resulting in reductions in the sizes of muscles and organs during flight (Battley et al., 2000). This use of lean mass can be explained by a variety of factors including 1) a beneficial reduction in mass in order to minimize energy costs, and increase flight range, 2) the requirement for gluconeogenesis and anaplerosis of Kreb's cycle intermediates, 3) endogenous protein turnover, and 4) the production and liberation of endogenous water for the maintenance of water balance (Battley et al., 2000; Bauchinger and McWilliams, 2009; Jenni and Jenni-Eiermann, 1998; Lindström et al., 2000; Pennycuick, 1998; Schwilch et al., 2002).

During flight, influx of water arises solely from endogenous sources (Jenni and Jenni-Eiermann, 1998; Klaassen, 2004). High ventilation rates result in elevated rates of respiratory water loss (Engel et al., 2006; Giladi and Pinshow, 1999), and it has been suggested that dehydration, not fuel supply, may limit flight range under some conditions

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(Carmi et al., 1992; Klaassen, 2004). Non-stop flights last upwards of eight hours in songbirds, and can last for several days in some shorebirds (Gill et al., 2005; Stutchbury et al., 2009), yet birds show no signs of post-flight dehydration in the wild (Biebach, 1990; Landys et al., 2000). Since the catabolism of fat yields little endogenous water per unit energy (0.029 g H_2O kJ^{-1}) relative to protein catabolism (0.155 g H_2O kJ^{-1}), protein may be a crucial source of water for long distance flights (Jenni and Jenni-Eiermann, 1998; Klaassen, 2004). Therefore, increasing the relative contribution of protein to the fuel mixture would represent a functional metabolic strategy whereby substrates are catabolized to avoid water stress, rather than to simply meet energy requirements.

3.2 Hypothesis, Materials and Methods

Here we test the protein-for-water hypothesis in a migratory bird during flight. Swainson's thrushes (*Catharus ustulatus*), nearctic-neotropical migrants, were flown in a temperature and humidity controlled wind tunnel under conditions of high evaporative water loss (HEWL: 2 g H_2O m^{-3}; 13% relative humidity, RH) and low evaporative water loss (LEWL: 12 g H_2O m^{-3}; 80% RH) at 18 °C and 10 m s^{-1} true wind speed. These conditions were chosen because they are ecologically relevant for this species, and resulted in a minimum water vapor density deficit of 13.2 g m^{-3} for the HEWL flights, and 3.2 g m^{-3} for LEWL flights (assuming breath was cooled to ambient temperature upon exhalation, and was saturated to 15.2 g m^{-3}). The HEWL treatment represents moderately dry conditions comparable to what birds may encounter during desert crossings (Schmaljohann et al., 2008).

Five individuals flew a total of 20 flights, where each individual was flown two to eight times in paired flights of matching durations. Each pair of flights consisted of one flight under each humidity treatment, and the initial humidity conditions were determined randomly. Total body mass changes were measured with a balance (0.001 g). Dry fat, wet lean, and total body water masses were measured immediately before and after flights using quantitative magnetic resonance (QMR) body composition analysis (Guglielmo et al., 2011). Blood samples were taken after the final QMR scan, within 5 minutes of the end of the flight. General linear mixed models (Proc Mixed; SAS 9.2) were used for comparisons between humidity treatments while taking into account flight duration and repeated measures on individuals. For further description of the wind tunnel, detailed experimental methods, and statistical analyses please see appendix A.

3.3 Results

Initial voluntary flight durations ranged from 30.3 min to 315.8 min (mean ± SEM: 158.9 ± 19.6 min). Each flight under the initial humidity condition was matched in duration (within 29 ± 7 sec) under the alternate humidity condition. Flight costs, as estimated by changes in body composition assuming 39.6 kJ/g for dry fat and 5.3 kJ/g for wet lean mass (Jenni and Jenni-Eiermann, 1998), were 4.1 ± 0.2 W (HEWL) and 4.2 ± 0.2 W (LEWL), which represents an approximately 9-fold increase in metabolic rate over resting assuming an RQ of 0.71 at rest (Holmes and Sawyer, 1975; Lighton, 2008), and were not significantly different between treatments ($F_{1,8} = 0.09$, $P = 0.777$). These flight costs are similar to those estimated in wild free-flying Swainson's thrushes by the

doubly-labeled water technique (4.3 W; Wikelski et al., 2003); however, a direct comparison should be made cautiously due to differences in experimental conditions between studies.

During flight, the rate of total mass loss differed between humidity treatments (flight duration*treatment interaction: $F_{1,7} = 13.11$, $P = 0.0085$; Figure 3-1a). Under HEWL conditions, birds lost 10.23 ± 0.81 mg min^{-1} (95% CI 8.36 – 12.10) whereas birds lost 7.27 ± 1.4 mg min^{-1} (95% CI 4.04 – 10.50) under LEWL conditions. The rate of lean mass loss also differed between humidity treatments (flight duration*treatment interaction: $F_{1,7} = 7.40$, $P = 0.0297$; Figure 3-1b), where flight under HEWL conditions resulted in a 3.55 ± 0.91 mg min^{-1} rate of lean mass loss (95% CI 1.45 – 5.65). There was no significant relationship between amount of lean mass lost and flight duration under LEWL conditions (95% CI -2.00 – 4.11). The rate of fat mass loss did not differ between humidity treatments (flight duration*treatment interaction $F_{1,7} = 0.31$, $P = 0.597$; Figure 3-1c) and was 5.7 ± 0.31 mg min^{-1} (95% CI 4.98 – 6.41) for both treatments combined. Lean mass contributed on average 16.8 ± 3.2 % of the energy under HEWL conditions and 10.6 ± 3.4 % under LEWL conditions, yet due to the low energy density of lean mass this had little effect on total energy expenditure. All birds had substantial fat stores (pre-flight fat was 16.8 ± 1.1 % of initial mass), and there were no differences in initial fat between treatments ($F_{1,9} = 0.27$, $P = 0.614$). Therefore, it is unlikely that differences in lean mass catabolism between humidity treatments were due to differences in fat availability, flight costs, or energy demand (Schwilch et al., 2002).

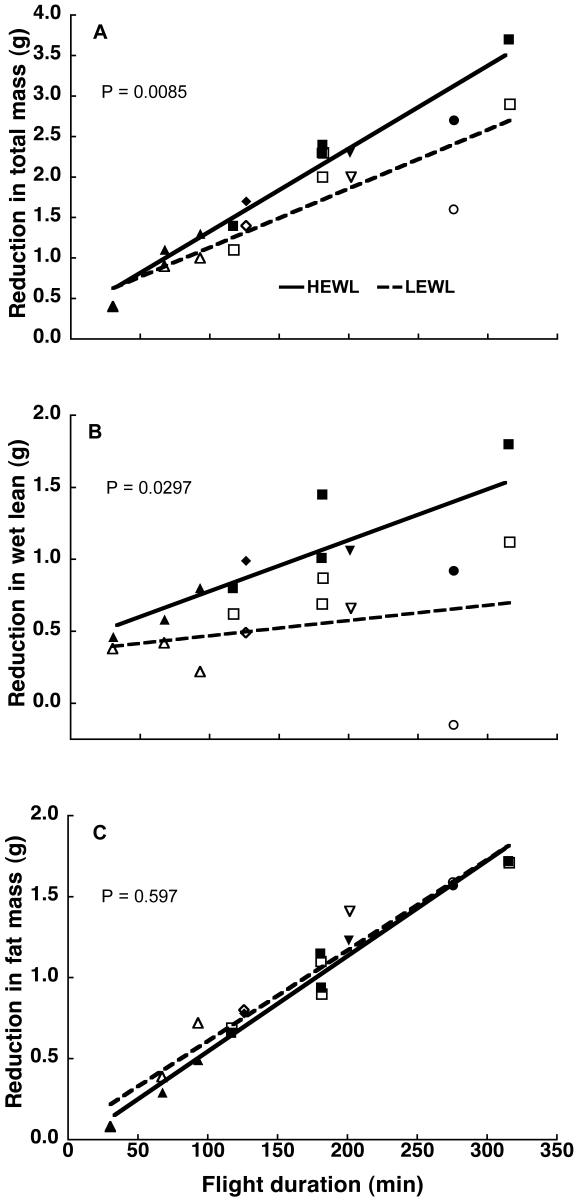

Figure 3-1. A) Mass loss and B) wet lean mass loss were significantly higher during flights at HEWL conditions (closed symbols and solid lines) relative to LEWL flights (open symbols and dashed lines). C) Fat mass losses were similar between treatments. Symbol shapes are specific to individual birds. See text for detailed statistics.

There were no significant differences between humidity treatments in relative body water content post-flight (relative to total mass: 54.7 ± 0.01 %, $F_{1,9} = 1.09$, P = 0.325; relative to lean mass: 78.7 ± 0.002 %, $F_{1,9} = 0.22$, P = 0.647). Plasma osmolality was not affected by humidity treatment ($F_{1,5} = 1.88$, P = 0.229; Figure 3-2a) or flight duration ($F_{1,5} = 0.22$, P = 0.656), indicating that the birds were not dehydrated (Giladi et al., 1997) even after prolonged flight under HEWL conditions. Plasma uric acid concentration was not significantly related to flight duration ($F_{1,6} = 3.76$, P = 0.10), but was higher after HEWL flights ($F_{1,6} = 6.23$ P = 0.046), indicating elevated protein catabolism (Figure 3-2b). The additional lean mass catabolism during HEWL flights resulted in a 21.7 ± 4.9 % increase in endogenous water production relative to energy expenditure ($F_{1,8} = 23.09$, P = 0.001; Figure 3-2c), offsetting evaporative water losses experienced during flight.

3.4 Discussion

We have previously shown a protein-for-water metabolic strategy in water-restricted house sparrows (*Passer domesticus*) at rest, but not when metabolic rate was elevated by shivering (Chapter 2, Gerson and Guglielmo, 2011). Therefore, it was not known if this strategy would be evident in flight due to high water gains from fat catabolism alone. Our findings show that under moderately dry conditions, water loss in flight is sufficient to induce water production through increased protein catabolism. Moreover, the strong influence of rate of water loss on relative use of fat and protein indicates that the fuel composition in flight is influenced by factors other than energy

demand, and that a physiological mechanism must exist by which lean mass catabolism is responsive to ambient humidity and/or water stress.

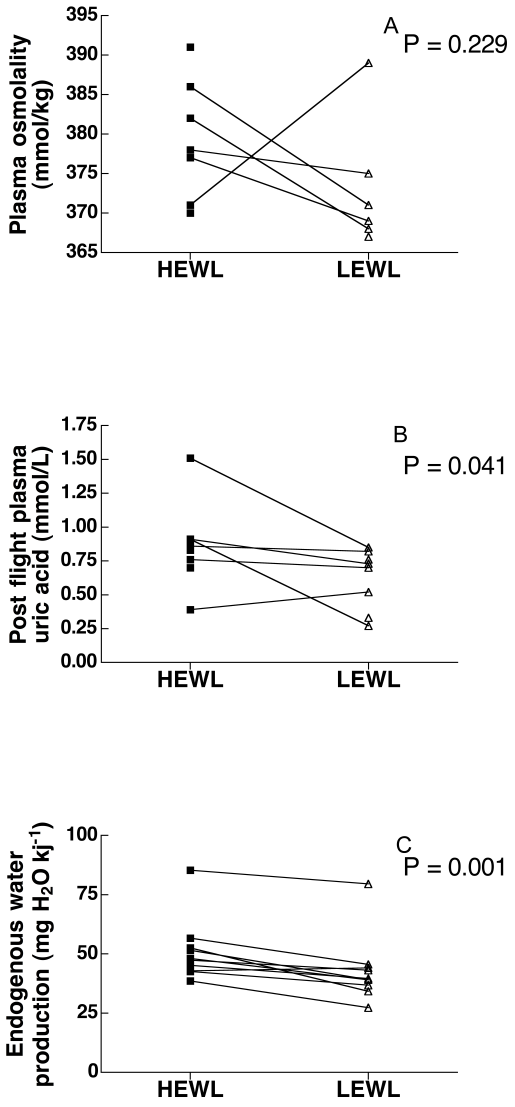

Figure 3-2. Plasma osmolality of Swainson's thrushes was unaffected by ambient conditions experienced during flight in a wind tunnel (A), yet plasma uric acid was higher in birds flown under HEWL conditions (B). Greater lean mass catabolism during HEWL flights resulted in significantly higher endogenous water production relative to energy expenditure (C). Lines connect paired flights within individuals.

A basal rate of protein catabolism likely occurs to produce metabolites necessary for sustained fatty acid oxidation and gluconeogenesis, while also reducing body mass and producing some water. High rates of water loss result in an elevation of lean mass catabolism above this basal level. An added benefit to elevated lean mass loss may be a concomitant reduction in body mass, which over the course of a long flight could result in energy savings (Pennycuick, 1998), although this was not evident in our experiment. The benefits of lean mass catabolism in flight must be weighed against potential costs of reduced size and functional capacity of muscles and organs. Most importantly, nutrient processing tissue lost from the alimentary tract, liver and other organs must be rebuilt upon arrival at stopover sites before high rates of refueling are possible (McWilliams and Karasov, 2001). Therefore, protein sparing should be most important for species that need to refuel quickly during frequent, short refueling bouts, such as most migratory songbirds.

Many studies have documented lean mass deposition and catabolism during the migratory period (Battley et al., 2000; Bauchinger et al., 2005; Klaassen, 1996; Klaassen et al., 2000; Seewagen and Guglielmo, 2011), yet the significance of these processes for the maintenance of physiological homeostasis has previously only been explored theoretically (Jenni and Jenni-Eiermann, 1998). Thrushes in our experiment were able to maintain osmotic homeostasis even in the face of a 4-fold difference in water vapor density deficit. Humidity treatment did not affect plasma osmolality or relative body water content, suggesting the maintenance of cellular water homeostasis at the expense of protein. By maintaining cellular hydration status, perturbations of ion gradients important to cellular metabolism, and in particular muscle contraction, may be avoided.

The protein-for-water strategy has clear functional significance for bird migration. The maintenance of water balance is an immediate necessity during migratory flight, and the use of protein to this end, within limits, allows birds to complete migratory flights in the face of unfavourable environmental conditions. For example, recent observations indicate that trans-Saharan migrants will fly under conditions unfavourable to water balance in order to take advantage of favourable winds (Schmaljohann et al., 2009). It may be that these birds are able to optimize flight range despite high rates of evaporative water loss by increasing lean mass catabolism. When compared to the conditions of this experiment, trans-Saharan passerine migrants seem to be exposed to much more extreme water deficits (estimated minimum water vapor density deficit of 22.6 g m^{-3} in Schmaljohann et al., 2008), which may account for the dramatic reductions in lean mass that have been recorded in trans-Saharan migrants (Bauchinger et al., 2005; Biebach, 1998)

When considered from the perspective of water balance, the oft-reported hypertrophy of guts and muscles during migration seasons could in part reflect the only mechanism available to birds to provision water for flight. Flying for long periods under dehydrating conditions may require storage of water in a low energy density, high water content form such as glycogen or protein, thus causing additional increases in the sizes of muscles and organs beyond those associated with powering flight at higher body mass or processing more food. We hypothesize, therefore, that birds preparing to cross major ecological barriers, particularly those with low atmospheric humidity (deserts), should store excess lean mass in preparation for departure. Moreover, the protein-for-water strategy may be unique to uricotelic animals, which dispose of nitrogenous wastes with

minimal associated water losses. Migrating bats, for example, may show very different responses to water stress than birds.

Our findings will be useful for the modification of existing bird flight range models by incorporating the effects of atmospheric conditions on fuel mixture, providing new insight regarding the limits to migratory flight. We have shown that ambient conditions experienced aloft can affect the composition of metabolic fuels used in flight, which may then influence flight range or overall pace of migration. Higher average temperatures in flight could potentially increase water losses, requiring birds to stop prematurely, or catabolize more protein. Additional time at stopover could then be required to replenish excess protein catabolized in flight.

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4 THE METABOLIC RESPONSE TO SHORT DURATION FLIGHT IN AMERICAN ROBINS (*TURDUS MIGRATORIUS)*

4.1 Introduction

Flight is one of the most energetically demanding forms of locomotion (Schmidt-Nielson, 1972) and migratory birds are unique among vertebrates in their ability to fuel long-duration high-intensity exercise predominantly with fat (Jenni and Jenni-Eiermann, 1998). Many migratory species of bird increase adiposity and body mass coinciding with migratory periods (i.e. spring and fall) indicating endogenous control of fat stores in preparation for migration (Blem, 1976; Jehl Jr, 1997). After long duration flights in the wild, fat stores are depleted but are replenished quickly during stopover (Battley et al., 2000; Guglielmo et al., 2005; Jehl Jr, 1997). Additionally, measurements of high levels of plasma free fatty acids, respiratory quotients near 0.7, and depletion of fat using quantitative magnetic resonance have confirmed exceptionally high rates of fat catabolism in flight (Jenni-Eiermann and Jenni, 1991; Jenni-Eiermann et al., 2002; Landys et al., 2005; Rothe et al., 1987; Gerson and Guglielmo, 2011a; Gerson and Guglielmo, 2011b; Chapter 2 & 3).

Of the three metabolic fuels, fat has the highest energy density compared to protein and carbohydrate, making it an ideal source of energy for flying animals, where mass minimization is paramount (Jenni and Jenni-Eiermann, 1998). Most of the fat that birds accumulate for migration is stored extra-muscularly in adipose tissue, which has a benefit of allowing the storage of large quantities of fat. However, in order for these

extra-muscular fatty acids to be catabolized by the flight muscles they must be transported through the circulation and into muscles at very high rates (Guglielmo, 2010). The low solubility of fatty acids in water makes their transport in blood and cytosol very slow, and thus migrant birds require enhanced protein-mediated transport systems. This is facilitated by a number of fatty acid transporters and oxidative enzymes, which are upregulated during migration in birds (Guglielmo, 2010; Guglielmo et al., 2002b; Guglielmo et al., 1998; McFarlan et al., 2009; Price, 2010).

Although fat is the predominant fuel during long duration flight, the early phase of flight is fuelled by carbohydrate and protein. As the duration of flight (or exercise) progresses, fuel mixture gradually switches over to a steady state where oxidative demand is met primarily by fat (Brackenbury and El-Sayed, 1984; Rothe et al., 1987; Weber, 2011). During switch over, the mixture of fuels changes over time, and in pigeons (*Columba livia*) it stabilizes after about one to two hours of flight (George and John, 1993; Rothe et al., 1987). However, this switchover may be much faster in migratory birds due to their enhanced ability to mobilize and catabolize fat (Jenni-Eiermann et al., 2002). Fuel use has not been investigated during the switchover period in any migrant passerine bird.

The catabolism of glucose, although rapid, releases relatively little energy for its mass, and due to storage and weight constraints carbohydrates do not contribute substantially to the energetics of long duration exercise in birds (Brackenbury and El-Sayed, 1984). Protein has not historically been considered a fuel for endurance exercise, but it is now apparent that considerable protein is catabolized by birds in flight (Battley et al., 2000; Bauchinger and Biebach, 1998; Bauchinger et al., 2005; Biebach, 1998; Gerson

and Guglielmo, 2011a; Gerson and Guglielmo, 2011b; Chapters 2 & 3). This protein catabolism occurs supplemental to fat catabolism, rather than in response to depletion of fat reserves as in phase III of starvation (Cherel and Le Maho, 1985). That is, protein is catabolized while fat is still abundant, and for this reason protein catabolism likely plays a functional role important to exercise metabolism.

A number of non-mutually exclusive hypotheses have been proposed to explain the role of protein catabolism in endurance flight. First, it has been proposed that protein catabolism could provide metabolites necessary for the replenishment of Kreb's cycle intermediates, which would be depleted during sustained fat catabolism due to processes such as gluconeogenesis (Dohm, 1986; Jenni and Jenni-Eiermann, 1998; Klaassen, 1996). This hypothesis does not have much empirical support from studies conducted on birds, but this role of protein catabolism almost certainly is responsible for baseline rates of protein catabolism in vertebrates (Dohm, 1986).

Since protein catabolism yields five times more endogenous water than the catabolism of fat (Jenni and Jenni-Eiermann, 1998), a second hypothesis posits that rates of protein catabolism could be increased to offset high rates of water loss. Previous experiments show that birds flown under high evaporative water loss conditions do, in fact, increase the rate of protein catabolism (Gerson and Guglielmo, 2011a). This protein-for-water metabolic strategy maintains the energy density of the fuel mixture, but increases the endogenous water production rate resulting in the maintenance of water balance in flight under high evaporative water loss conditions. Additionally, the lean body mass of the animal is decreased, reducing the future water requirement.

Plasma metabolite analysis has been used to provide insight into exercise metabolism of birds (Bordel and Haase, 1993; George and John, 1993; Gerson and Guglielmo, 2011a; Guglielmo et al., 2001; Jenni-Eiermann et al., 2002). High plasma concentrations of ß-OH-butyrate, non-esterified fatty acids (NEFA), and glycerol are indicative of high rates of fat mobilization during exercise. Also, it has been proposed that triglycerides may be utilized by small passerines in order to increase the concentration of circulating fatty acids in the plasma, once albumin has been saturated with NEFA (Jenni-Eiermann and Jenni, 1992). This alternate strategy has been documented only in wild small passerine birds caught mid-flight during their annual migration (Jenni-Eiermann and Jenni, 1991; Jenni-Eiermann and Jenni, 1992). ß-OH-butyrate, a ketone body, is synthesized in the liver in response to high circulating concentrations of NEFA and low carbohydrate availability. Under fasting conditions, ß-OH-butyrate aids in the sparing of glucose by reducing muscle uptake of glucose and can cross the blood brain-barrier, thus reducing the need for glucose by the brain during periods of high fat utilization (Robinson and Williamson, 1980). NEFA are mobilized during exercise from triglycerides stored in adipocytes, releasing glycerol into circulation through the action of hormone sensitive lipase (Jenni and Jenni-Eiermann, 1998; Price, 2010). Uric acid is the final end product of protein catabolism in birds, and is increased in circulation when rates of protein catabolism are high (Gerson and Guglielmo, 2011a). In order to assess the metabolic response to flight, each of these metabolites was measured to determine the relative contribution of fat, protein and carbohydrate to metabolism, as well as to gain insight into the dynamics of plasma metabolites during early flight.

Many studies have investigated the metabolic response to flight, but surprisingly few of these studies have focused on migratory passerine birds. The initial goal of this study was to investigate fuel use and the metabolic response to flight during the switch-over period in a passerine migrant, the American robin (*Turdus migratorius)*. Fuel use was determined using quantitative magnetic resonance body composition analysis (QMR), and the metabolic response to flight was assessed by measuring plasma metabolite concentrations before and after flight. Since it has been shown that the protein-for-water metabolic strategy is utilized during long flights under high evaporative water loss (HEWL) conditions, a secondary goal of this study was to characterize the metabolic response to water loss during the switchover period. It was predicted that high rates of evaporative water loss would result in a higher relative contribution to energy from protein and carbohydrates, and thus cause differences in circulating concentrations of uric acid and glucose.

4.2 Materials and Methods

4.2.1 *Animal Care*

Twenty-four American robins (*Turdus migratorius*) were caught in and around London, Ontario, Canada (43°04’28” N, 81°20’14”W) from July through September 2009. Eight of these birds were brought into captivity as nestlings early in summer and hand-raised, which seemed to have little benefit for flight propensity, or calmness in captivity. Four birds were caught at Long Point, Ontario, Canada as adults during fall migration, and the rest were caught as hatch year birds in the summer. Birds were

maintained in smooth walled aviaries (3.7 m x 2.4 m x 3.1 m) on natural light cycle until March 2010. At this time they were switched to long light cycle (16L:8D) in order to induce migratory disposition, as indicated by mass gain. Birds were fed ground Mazuri® (Purina Mills LLC) Small Bird Diet supplemented with berries and mealworms.

4.2.2 *Wind Tunnel*

Birds were flown in the wind tunnel at the Advanced Facility for Avian Research at Western University. This wind tunnel was specifically designed for bird flight and allows the researcher to independently control humidity and temperature within the range of 0 - 90% RH at any temperature from -15°C to 30°C, while also controlling wind speed between 0 and 20 m s^{-1}. For a technical description of the wind tunnel see (Gerson and Guglielmo, 2011a, Appendix A).

4.2.3 *Experimental Design and Changes in Body Composition*

Birds were initially trained in the wind tunnel for a period of two weeks starting March 1, 2010 in order to identify birds with a natural inclination to fly in the wind tunnel, and to try to increase flight durations of birds that were initially less inclined to fly. Birds were trained to sit on a perch held by the researcher. Once perched, birds were moved into the test section of the wind tunnel, which was set at a wind speed of 3 - 5 m s^{-1}. Wind speed was gradually increased until birds started to take voluntary "test" flights, returning to the perch in between flights. As birds voluntarily increased the duration of these test flights, the perch was removed to encourage sustained flight. The perch was placed back in the tunnel once the birds started looking for a place to land. With this strategy, the birds would learn not to land on the bottom of the test section of the tunnel. Total training time would not exceed 30 minutes in a day. Birds were trained

individually or in pairs. Over the course of a few training sessions, flight durations increased in some birds. Once training was completed, birds were given 1 - 5 days to recover before the start of the experiment in order to replenish fat reserves. Of the 24 birds in captivity, 12 were used for flight experiments.

Birds were housed in pairs in rolling flight cages during the experiment. This facilitated catching the birds for weighing and blood sampling. Food was removed 3 h prior to flight, which commenced either at 13:00, or at 18:00 each day. Immediately prior to the flight, birds were weighed, and scanned using QMR (Guglielmo et al., 2011) and were hand released into the wind stream. Birds flew at a wind speed of 12 m s^{-1}, at 18°C, and absolute humidity was either 12 g H_2O m^{-3} (80% RH; LEWL) or 2 g H_2O m^{-3} (13% RH; HEWL). In order to determine the metabolic response to humidity, we attempted to fly each individual bird once under each humidity condition, where the initial flight condition was determined randomly. All flights by an individual bird commenced at the same time of day. Two days prior to a scheduled flight, birds were food restricted for 3 h, exactly as on the day of the flight, and blood samples were taken by brachial puncture. Thus, these blood samples were taken at the same time of day as the scheduled commencement of the flight, and the metabolite levels should approximate those of birds on flight days. A maximum of 2 capillary tubes (75 μl each) was attempted, but some birds yielded more blood. After the completion of a flight, birds were scanned a second time using QMR, weighed, and bled within 5 minutes. Birds were given six days to recover before the repeated experimental flight under the other humidity treatment. We attempted to match the initial flight duration during the second flight, but few birds successfully completed a flight of matching duration. Flight costs were calculated from

changes in QMR fat and lean mass assuming 39.6 kJ g^{-1} for fat, and 5.3 kJ g^{-1} for lean mass (Jenni and Jenni-Eiermann, 1998).

4.2.4 *Plasma Metabolite Analysis*

All blood samples were centrifuged at 13,000 x g (IEC MicroCL 17, Thermo-scientific hematocrit centrifuge), hematocrit was determined and the plasma fraction was drawn off and stored at -80°C until analysis. All plasma metabolite assays were performed using standard clear 96 well plates and a microplate spectrophotometer (Biotec Powerwave X340). Uric acid was analyzed in undiluted plasma. For all other metabolites, plasma was diluted 3-fold in 0.9% saline. Glycerol, and triglyceride concentrations were measured by endpoint assay and ß-OH-butyrate was measured by kinetic assay as in Guglielmo et al. (2002a) and Guglielmo et al. (2005). Uric acid was determined using a commercially available kit as in Gerson and Guglielmo (2011a), Gerson and Guglielmo, (2011b), and Tsahar et al., (2006). Plasma NEFA, glucose and phospholipid (PL) concentrations were determined by endpoint assay using commercially available kits (NEFA-HR(2), Autokit Glucose, and Phospholipid C; Wako Diagnostics) as in Guglielmo et al. (2005).

4.2.5 *Statistical Analysis*

Since the dataset contained repeated measures in an unbalanced design, general linear mixed models were used with individual as a random factor for all analysis (Zuur et al., 2009). Models were determined using backward stepwise selection where non-significant ($P > 0.05$) terms were dropped sequentially until only significant terms remained. All analyses initially included initial body mass and flight duration as covariates. Statistical comparisons between pre- and post-flight levels of plasma

metabolites were made using paired t-tests. Humidity treatments were pooled if no significant differences existed. Of the 10 completed flights, we were able to acquire seven blood samples post-flight. Due to the low sample size, general linear mixed models would sometimes not converge. In these cases, due to the low number of repeated measures on a single individual, we used general linear models.

4.3 Results

4.3.1 *Changes in Body Composition and Flight Energetics*

During the experimental timeframe, 10 flights were completed by 6 birds where body composition and plasma samples were collected. Of these flights, 4 were completed under the HEWL conditions, and 6 under the LEWL conditions. Only 3 birds completed a flight under both conditions, but flight durations were not matched. Birds flew an average of 37.23 min (range: 17.88 – 54.46 min). There were trends toward a significant interaction between humidity treatment and flight duration on change in mass and change in lean mass (Change in mass $F_{1,5.94} = 4.53$, $P = 0.078$; Change in lean mass $F_{1,6} = 4.653$, $P = 0.074$; Figure 4-1 a and c). However, these nearly significant interactions were driven by small sample size and a single point high in the LEWL treatment, and so were not considered further. Longer flight durations resulted in greater reduction in mass ($F_{1,5.031} = 59.893$, $P = 0.001$), fat mass ($F_{1,8} = 100.217$, $P < 0.001$) and lean mass ($F_{1,8} = 10.605$, $P = 0.012$; Figure 4-1), but no significant effect of humidity treatments were evident (mass: $F_{1,3.751} = 0.064$, $P = 0.813$; fat mass: : $F_{1,3.698} = 0.560$, $P = 0.499$; lean mass: $F_{1,7} = 0.296$, $P = 0.603$).

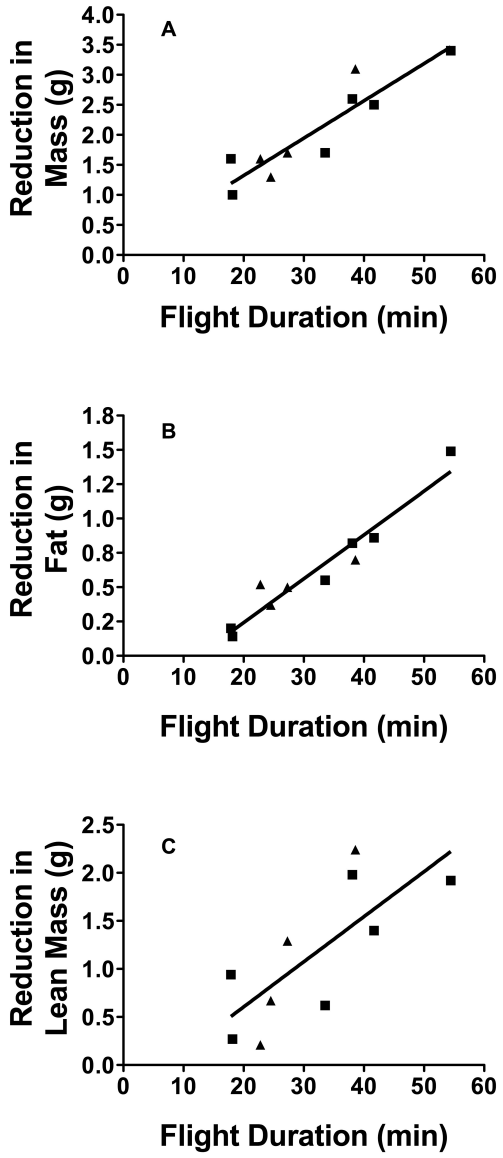

Figure 4-1. The reduction in mass, fat mass and lean mass in American robins as a function of flight duration. Flight resulted in significant reductions in mass (A), fat mass (B), and lean mass (C), although there was no effect of humidity treatment on the rate of body composition change. See text for statistical details. HEWL: squares; LEWL: triangles.

Birds catabolized a fuel mixture consisting primarily of fat, but a considerable proportion of the energy required for flight was derived from lean mass (Figure 4-2a). As flight progressed the relative proportion of energy from fat increased ($F_{1,7.712} = 112.631$, $P < 0.001$, Figure 4-2a), while the relative proportion of energy from lean decreased ($F_{1,8} = 10.605$, $P = 0.012$; Figure 4-2a). Overall mean flight costs were determined to be 14.90 W and, unexpectedly, there was a significant increase in estimated flight costs with flight duration (Figure 4-2b; $F_{1,7.221} = 12.337$, $P = 0.009$). Although the proportion of energy from lean mass was around 20%, catabolism of lean mass accounted for over 50% of the mass loss. There was no effect of pre-flight mass on flight costs ($F_{1,5.334} = 0.55$, $P = 0.823$), and thus mass was not included as a covariate in the model testing the effect of flight duration on flight costs.

4.3.2 *Plasma Metabolites*

There was a linear increase in post flight uric acid levels with flight duration ($F_{1,6} = 16.204$, $P = 0.007$; Figure 4-3). NEFA tended to decrease slightly with flight duration, but this trend was not significant ($F_{1,3.485} = 3.632$, $P = 0.140$). ß-OH-Butyrate and hematocrit increased quickly after the initiation of flight, and remained constant throughout the flight (ß-OH-Butyrate: $F_{1,5} = 2.186$, $P = 0.199$; Hematocrit: $F_{1,5} = 1.378$, $P = 0.293$). Triglycerides decreased quickly early in flight, and then seemed to increase to a plateau that remained constant up to 1 h, but there was no effect of flight duration ($F_{1,5} = 1.378$, $P = 0.293$; Figure 4-3). No other metabolites showed obvious relationships with flight duration (phospholipid: $F_{1,5} = 0.393$, $P = 0.558$; glucose: $F_{1,5} = 0.569$, $P = 0.485$; glycerol: $F_{1,5} = 0.543$, $P = 0.494$).

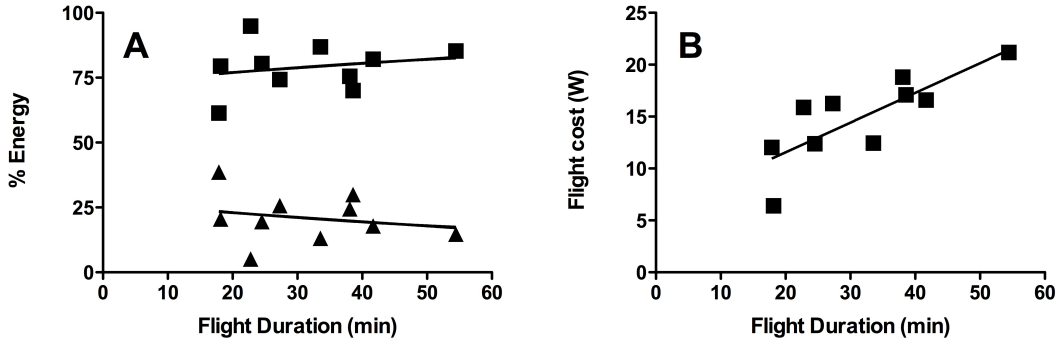

Figure 4-2. The relative contribution of fat and lean mass to total flight costs in American robins. A) As flight duration increased, there was a significant reduction in the relative proportion of energy derived from lean mass (triangles), and a corresponding increase in the relative proportion of energy from fat (squares). B) As flight progressed flight costs increased. See text for further explanation and statistical details.

Comparisons between pre- and post-flight metabolite levels using paired t-tests indicated significant increases in glycerol ($t = -5.707$, $DF = 6$, $P = 0.001$), ß-OH-butyrate ($t = -5.843$, $DF = 6$, $P = 0.001$), NEFA ($t = -23.193$, $DF = 6$, $P < 0.001$), and uric acid ($t = -1.535$, $DF = 7$, $P = 0.05$) after flight. Phospholipids ($t = 4.113$, $DF = 5$, $P = 0.009$) and glucose ($t = 9.898$, $DF = 6$, $P < 0.001$) were significantly reduced post flight (Figure 4-4).

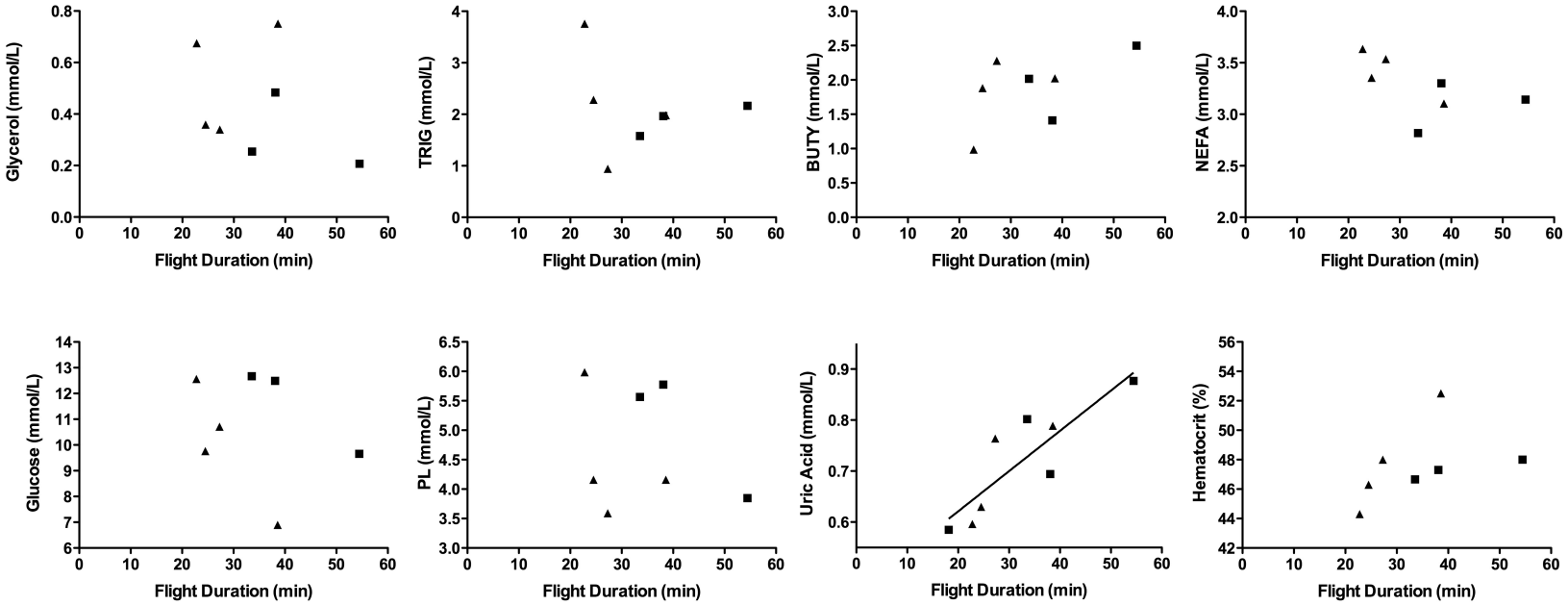

Figure 4-3. Plasma metabolite concentrations in response to flight duration in American robins. There was no effect of humidity on any of the plasma metabolites, but treatments are shown. LEWL: Triangles, HEWL: squares. Only uric acid increased with flight duration as indicated by the regression line. See text for statistical details.

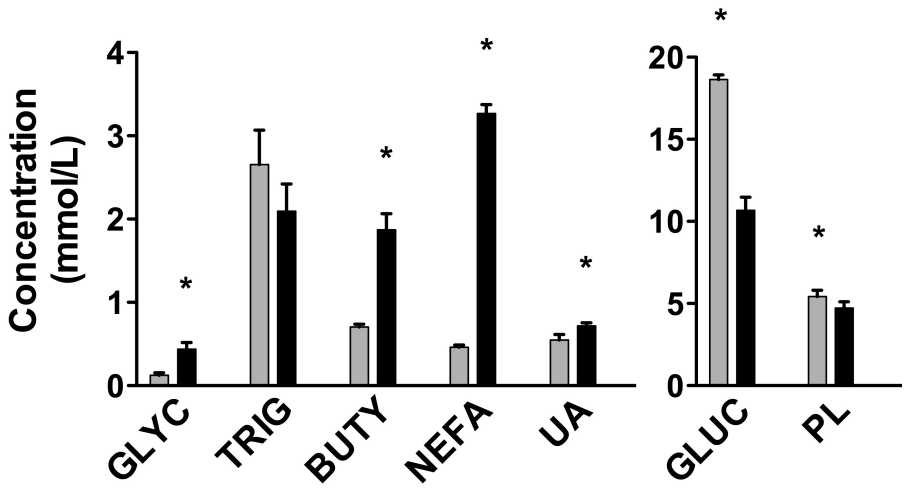

Figure 4-4. Plasma concentrations of metabolites measured before (grey bars) and after (black bars) flight. GLYC: Glycerol; TRIG: Triglyceride; BUTY: ß-OH-butyrate; NEFA: non-esterified fatty acids: UA: uric acid; GLUC: glucose: PL: phospholipids. * indicates a significant difference between pre- and post-flight levels within a metabolite ($P < 0.05$)

4.4 Discussion

In a previous study of Swainson's thrushes (*Catharus ustulatus*; Chapter 3) we found that the rate of lean mass catabolism was increased when birds flew for one to five hours under the same low humidity conditions as used in the current experiment. The present results show that early in flight there is no detectable effect of humidity on the rate of lean mass catabolism in American robins. During short flights the large contribution of lean mass, and likely carbohydrate, to fuel during the switchover period produces substantial quantities of water, irrespective of the environmental conditions. Thus, these birds may in fact be in water surplus early in flight and likely rely on excretory water losses as a means of reducing mass.

The birds lost a surprisingly large amount of mass even during short flights, some more than three grams in 55 minutes, yet flight costs were not reduced in birds that experienced greater mass loss. Instead, flight costs increased with flight duration, even when flight costs were expressed relative to body mass (data not shown). Calculating flight costs for short duration flights has long been a problem in the study of flight physiology and many techniques have been used (Engel et al., 2006; Ward et al., 2004). The use of the doubly labeled water method is accurate for long flights, while mask respirometry is commonly used for shorter flights even though the drag produced by a mask and tether affect flight costs. Heart rate as a proxy for metabolic rate, and heat transfer modeling have also been used to calculate flight costs during short duration flights, but require specialized equipment and validation within a species (Ward et al., 2004). The use of QMR to calculate flight costs has not been directly validated against more established methods, yet it does provide comparable flight costs for long duration

flights (up to 5 h) in small songbirds (Gerson and Guglielmo, 2011a). It seems likely that the costs of short duration flights may be misrepresented by QMR because of the measurement error of the instrument and the small changes in mass (~1 g for shortest flights). It is also possible that the flight costs early in flight are simply not reflected well by changes in body composition. Anaerobic metabolism or the aerobic catabolism of plasma or even intramuscular glucose may not be detected with QMR. For these reasons we interpret this increase in flight costs with flight duration cautiously.

With the exception of uric acid, there was no effect of flight duration on post-flight concentrations of any of the measured plasma metabolites. The increase in uric acid up to one hour is similar to what has been observed early in flight in red knots (*Calidris canutus*) flown in a wind tunnel for up to 12 h (Jenni-Eiermann et al., 2002). In red knots, uric acid concentrations reached a plateau after the first hour, and then remained stable for the remainder of the flight. ß-OH-Butyrate increased quickly upon the initiation of flight, again reaching a plateau after about 20 min, which was maintained for the duration of the flight. This is in agreement with studies on metabolite profiles of migrant birds flown in a wind tunnel, although the present study does provide increased resolution of the rapid response of ß-OH-Butyrate to exercise in birds (Jenni-Eiermann et al., 2002). Surprisingly, NEFA showed a decreasing trend with flight duration, but the magnitude of the in-flight concentration was substantially higher than resting values, reaching levels as high as 3.6 mmol L^{-1}. This value is higher than what has been previously documented for long distance migrants. Jenni-Eiermann et al. (2002) flew red knots in a wind tunnel for up to 12 hours, but plasma NEFA concentrations were maintained around 1.0 mmol L^{-1} in most birds. Similarly, birds caught in flight during

migration had plasma NEFA concentrations between of 1.0-2.0 mmol L^{-1} (Jenni-Eiermann and Jenni, 1992). This may indicate a very high initial lipolytic rate in robins that overshoots demand early on, before steady state is achieved (Vaillancourt et al., 2005). Alternatively, this could be a product of the time between the end of the flight and when the blood sample is collected, as NEFA concentrations could peak due to the sudden stop of exercise. Birds were weighed and scanned in the QMR before blood was taken, but this entire process was completed within 5 minutes of ending the flight.

When compared to pre-flight levels there were significant increases in glycerol, NEFA, and ß-OH-butyrate, all of which are indicative of high rates of fat catabolism. As discussed previously, uric acid increased post-flight as well. Glucose was reduced in response to flight indicating carbohydrate metabolism initially, but due to the high levels of NEFA and ß-OH-butyrate, reduced reliance on glucose is likely for the duration of flight. This initial hypoglycemia has not been previously documented in migratory birds in flight. Previous studies have shown no change in blood glucose with flight (Jenni-Eiermann et al., 2002). Blood glucose levels may recover once steady state flight is reached, but the apparent inability to maintain blood glucose levels could also indicate a greater reliance on glucose in flight for these birds. If this reliance on glucose is not reduced as flight progresses, it could result in greater lean mass catabolism to sustain gluconeogenesis. This metabolic constraint may contribute to the short-hop migratory strategy of American robins.

Interestingly, there was a slight decrease in triglyceride concentration post-flight. It has previously been suggested that during migratory flight small passerine birds may use very low-density lipoproteins high in triglycerides to transport fatty acids in high

concentration through the blood, and that this augments the NEFA-bound-to-albumin transport pathway (Jenni-Eiermann and Jenni, 1992; Price, 2010). The evidence for such a strategy has been mixed, where larger birds and birds flown in wind tunnels show reduced or stable plasma triglyceride in response to flight, and wild-caught small migrants show increased triglyceride (Jenni-Eiermann et al., 2002). The current study, using relatively large American robins (~70 g) provides more evidence that triglycerides, and therefore the lipoprotein transport pathway, are not up-regulated during flight in larger passerine birds. Interestingly, levels of NEFA are very high in American robins post-flight, suggesting that transport by NEFA is sufficient to fuel flight. Future studies could use constant infusion techniques to more fully understand the flux of NEFA in flight in these birds.

Our study shows the value of QMR as a tool for the rapid and accurate measurement of changes in body composition of animals, as well as the determination of flight energetics. It appears that costs of short flights may not be accurately represented by changes in QMR body composition, illustrating a limitation to the use of this technology. This should be more thoroughly investigated using birds of various body masses, and flight durations. The plasma metabolite profiles of American robins in flight fit closely with previous published values, yet this study provides increased resolution to the metabolic response in the early stages of flight.

Although metabolic switchover has been thoroughly documented in many species of exercising mammals (Weber, 2011), there are few studies investigating this phenomenon in birds. Changes in respiratory quotient have been shown in pigeons (Rothe et al., 1987), but, to our knowledge, this is the only study investigating switch-

over in a migratory bird. The metabolic response early in flight may be substantially different between long and short-distance migrants, and further investigation of this possibility may provide insight into the metabolic adaptations that allow extreme long duration flight in migratory birds.

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5 MEASUREMENT OF GLOMERULAR FILTRATION RATE AT REST AND DURING FLIGHT IN A MIGRATORY BIRD USING A SINGLE BOLUS INJECTION OF FITC-INULIN

5.1 Introduction

Birds are distributed throughout the world and have evolved physiological adaptations that allow them to maintain homeostasis in a wide variety of environments. With regard to osmoregulation and water balance, considerable attention has been paid to the physiological adaptations of desert dwelling birds to reduce water losses, focusing on cutaneous water losses (Tieleman and Williams, 2002; Wolf and Walsberg, 1996), respiratory water losses (McKechnie and Wolf, 2004; Tieleman et al., 1999; Wolf and Walsberg, 1996), hormonal control of osmoregulation (Giladi et al., 1997; Goecke and Goldstein, 1997; Goldstein, 2006), renal function including rates of glomerular filtration and fractional water reabsorption (FWR) (Goldstein and Bradshaw, 1998; Goldstein and Braun, 1989; Goldstein and Rothschild, 1993; Hartman Bakken et al., 2008; Hartman Bakken et al., 2004; McWhorter et al., 2004), as well as the post-renal modification of urine (Dawson et al., 1985; Goldstein and Skadhauge, 2000).

Glomerular filtration rate (GFR) is the rate at which plasma is filtered across the capillaries of the glomerulus into the urinary space of the Bowman's capsule (Goldstein and Skadhauge, 2000). GFR is primarily controlled by arginine vasotocin (AVT), which reduces arterial flow to the glomerulus via constriction of the afferent arterioles, and GFR is typically reduced during dehydration in many species of birds (Goldstein and

Skadhauge, 2000; Skadhauge, 1981). Fractional water reabsorption (FWR) occurs in the collecting duct, where a large proportion of the water filtered through the glomerulus is reabsorbed. Resting values of FWR range from 70 - 90% and FWR is typically increased during dehydration, acting as a secondary point of regulation over excretory water losses (Goldstein and Skadhauge, 2000; Skadhauge, 1981).

Several studies have examined physiological mechanisms that aid nectar-feeding birds in osmoregulation when excessive quantities of water are consumed and must be eliminated without concomitant losses of osmolites. These studies have focused primarily on kidney function, and have identified many species of birds that have dynamic control over GFR and FWR. During feeding, nectarivores must eliminate large quantities of water and rates of GFR are high, yet at rest and through the night GFR is reduced to minimize water losses (Hartman Bakken et al., 2008; Hartman Bakken et al., 2004; Hartman Bakken and Sabat, 2008; McWhorter et al., 2004). Typically, nectar feeding birds are small (< 10 g), thus researchers have been forced to develop innovative methodologies for the accurate measurement of GFR, minimizing both the volume of blood as well as the number of blood samples required (Hartman Bakken et al., 2008; Hartman Bakken et al., 2004; Hartman Bakken and Sabat, 2008; McWhorter et al., 2004).

Osmoregulation and water balance are crucial to long distance migration in birds, because while in flight birds may have high rates of water loss and no opportunity to drink. Small passerine migrants typically complete migration through a series of non-stop nocturnal flights, each followed by a period of stopover to replenish fat reserves and rebuild lean tissue that was catabolized during the previous flight (Gerson and Guglielmo, 2011a; McWilliams and Karasov, 2001). Flights usually last 6 - 12 hours

(Wikelski et al., 2003), but sometimes may last for days (Gill et al., 2009). Flights are fuelled primarily by fat, although considerable catabolism of protein also occurs (Chapters 2 & 3;Gerson and Guglielmo, 2011a; Guglielmo et al., 2001; Guglielmo, 2010; Guglielmo et al., 2005; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002; Klaassen et al., 2000; McWilliams et al., 2004; Schwilch et al., 2002). Rates of respiratory water loss are extremely high (Engel et al., 2006) as a result of the high breathing frequencies and tidal volumes required to sustain this long duration aerobic exercise. Yet, these birds do not typically stop in order to prevent dehydration. Although thousands of migratory birds cross ecological barriers such as the Sahara desert (Schmaljohann et al., 2009) or the Gulf of Mexico (Stutchbury et al., 2009), no studies have convincingly documented dehydration in migratory birds after long flights in the wild (Biebach, 1990; Skadhauge, 1981).

In order to maintain water balance in flight, migratory birds increase the endogenous production of water through a shift in the fuel mixture to include a greater proportion of protein, offsetting the high rates of respiratory water loss (Gerson and Guglielmo, 2011a; Gerson and Guglielmo, 2011b; Chapters 2 & 3). Although fat has the highest energy density, protein catabolism yields approximately five times more bound and metabolic water (Jenni and Jenni-Eiermann, 1998). Quantitative magnetic resonance analysis of body composition and analysis of uric acid concentrations in plasma have shown that migratory birds flown under high evaporative water loss conditions have higher rates of protein catabolism, which results in an increase in endogenous water production that is sufficient to offset respiratory water losses (Gerson and Guglielmo,

2011a; Chapter 3). Although this protein-for-water strategy has been well documented in birds, the osmoregulatrory consequences of this strategy have not been investigated.

Once arriving at a stopover (Alerstam and Linström, 1990), migratory birds become hyperphagic and consume large quantities of insects and fruit (Smith, 2007), both of which may have a high water content (Tsurim et al., 2008). Thus, migratory birds seem to face an osmoregulatory challenge during refueling, similar to that faced by avian nectarivores; they must reduce water losses during long duration flight, but must retain the ability to excrete large quantities of water while maintaining osmotic balance during stopover refueling. It has been shown that GFR is reduced slightly during flight in pigeons (Giladi et al., 1997), but the renal response to flight has not been assessed in any migratory bird.

The determination of GFR in small birds utilizes either a single bolus injection of a marker, or surgical implantation of an osmotic minipump to deliver a marker, usually ^{14}C-inulin, or [1-^{14}C]-L-glucose. These are ideal markers for the determination of GFR as they typically remain unbound in the plasma, are not metabolized, are freely filtered at the glomerulus, and are not reabsorbed or secreted by the kidney (Giladi et al., 1997; Goldstein and Bradshaw, 1998; Hartman Bakken et al., 2004; McWhorter et al., 2004). In the case of a single bolus injection, the rate of elimination of the marker from the plasma can be used to calculate GFR. The rate of inulin clearance, traditionally using constant infusion, and more recently using a single bolus injection followed by serial blood sampling, has long been held as the gold standard for the determination of GFR (Qi et al., 2004). This technique poses several methodological challenges, especially when applied to small animals. Quantification of inulin was initially difficult, and thus the use

of radio-labeled inulin has become widespread. Recently, fluorescein-labeled inulin (FITC-Inulin) has been used to measure GFR which eliminates typical concerns associated with using radio-isotopes (Qi et al., 2004).

After a single bolus injection of a marker, the modified slope intercept method is commonly used for the determination of GFR. This method requires only a single blood sample in conjunction with serial urine collection and has been particularly successful in small animals (Florijn et al., 1994). This technique assumes the rate of disappearance of the marker is matched by the rate of appearance in the urine. As long as the clearance of inulin follows first-order exponential decay, this technique yields an accurate determination of GFR in very small (~5 g) birds and mammals (Hartman Bakken, 2006; Hartman Bakken et al., 2008; Hartman Bakken et al., 2004; McWhorter et al., 2004).

In many cases, inulin kinetics appear to follow a second order exponential decay model (Qi et al., 2004; Sturgeon et al., 1998), where the initial rapid decay phase represents distribution of the marker throughout the plasma volume and interstitial space, and the slow phase of the curve represents clearance of the marker from the plasma at the glomerulus (Figure 5-1). It has been shown that the slope intercept method tends to overestimate GFR (Qi et al., 2004; Sturgeon et al., 1998), but in small animals such as hummingbirds, the rapid-phase of the second-order decay curve appears to be so rapid that it is probably negligible, yielding accurate determinations of GFR using only first order kinetics.

Here we present novel methodology for the accurate determination of GFR using a single bolus injection of FITC-Inulin followed by two blood samples in a medium sized

(~30 g) bird. However, we determined that first order kinetics do not give an accurate determination of GFR in these birds. Therefore, we use single-pool models for the determination of GFR for comparisons within this study. We also use two-pool models with an experimentally-derived constant estimating the initial rapid phase of the model to yield a more accurate and globally comparable measure of GFR in a medium sized bird.

Our primary goal was to use our new method to measure GFR and FWR of a migratory bird in flight, at rest, and during feeding in order to test the hypothesis that renal function is dynamically regulated by these different phases of migration. We expected that due to the high rates of respiratory water losses experienced in flight, GFR would be reduced as a means to conserve water. During active feeding GFR should be increased and FWR should be reduced in order to eliminate excess water. Our secondary goal was to investigate the renal response to elevated rates of protein catabolism, and associated increases in plasma uric acid, that result from flight under low humidity conditions.

5.2 Materials and Methods

5.2.1 *Animal Care*

Twenty-four Swainsons thrushes (*Catharus ustulatus)* were caught at Long Point, Ontario, Canada during the spring and fall of 2011, and kept in two large indoor aviaries (2.3 m x 2.4 m x 3.5 m). Four birds were in captivity from a previous study (Gerson and Guglielmo, 2011a) and were included in this study. Birds captured in spring were used for validation of the techniques and were maintained on a 16L:8D light cycle throughout

the summer. Additional birds were captured in early fall of 2011 and were also maintained on the 16L:8D light cycle until December, when birds were placed on a 12L:12D winter cycle. In March 2012 birds were again placed on a 16L: 8D light cycle to induce migratory fattening and restlessness as in Owen and Moore (2008). Thus, all data were collected under the same light cycle, while birds were in migratory disposition. Birds were fed a semi-synthetic maintenance banana mash diet as described in Gerson and Guglielmo (2011a). All birds maintained healthy weight while in captivity.

5.2.2 *Wind Tunnel*

Birds were flown in the wind tunnel located at the Advanced Facility for Avian Research at Western University. This wind tunnel was specifically designed for bird flight and allows the researcher to independently control humidity and temperature within the range of 0 - 90% RH at any temperature from -15°C to 30°C, while also controlling wind speed between 0 and 20 m s^{-1}. The wind tunnel has a plenum (4.0 m x 5.0 m) surrounding the working section of the tunnel. Resting birds were held in a rolling flight cage (dimensions 1.2 m x 0.7 m x 1.8 m) within the plenum, beside the working section, and were exposed to the same temperature and humidity conditions as the bird in flight. For a complete technical description of the tunnel please see Gerson and Guglielmo (2011a; Appendix A).

5.2.3 *Preparation and Measurement of FITC-Inulin*

100 mg FITC-Inulin (Sigma, F-3272) was dissolved in 2 mL of 0.9% NaCl by heating the solution in a boiling water bath. Once cooled to room temperature, the

solution was dialyzed against 0.9% saline in well-rinsed benzoylated dialysis tubing with a nominal molecular weight cutoff of 2000 kDa (Sigma D7884) at room temperature in the dark for 24 h to remove any unbound FITC from solution. The dialyzed solution was then sterile filtered through a 0.22 μm filter into a sterile vacutainer and stored in the dark at room temperature until use (Qi et al., 2004).

Since the fluorescence of FITC is significantly affected by pH (Qi et al., 2004), 3 μL of plasma, urine, or 100-fold diluted stock solution was pipetted into 147 μl of 500 mM HEPES buffer, pH 7.4 at room temperature, and vortexed for 5 sec. Then, 50 μL of this solution was loaded onto a 96-well black plate in duplicate. Plates were read at an excitation wavelength of 485 nm, and an emission wavelength of 538 nm using a Spectramax M2e plate reader (Molecular Devices, USA) at room temperature as in Qi et al. (2004).

5.2.4 *Experimental Design*

To ensure that birds did not live in the rolling flight cages for too long, increasing the risk of feather damage that might preclude them from the study, birds were organized into two cohorts. Birds in the first cohort were maintained in six rolling flight cages for approximately three weeks in pairs while they participated in the study, after which they were moved back to the large aviary. A second cohort was then moved from the large aviary into the rolling flight cages. Each bird was tested in the wind tunnel to identify birds with the natural inclination to fly in the wind tunnel. Each "flying" bird was then paired up with a "resting" bird. Each experimental day, a flying/resting pair of birds had food removed at 11:00, and at 13:00 were moved into the wind tunnel, which was pre-set

to either high evaporative water loss (HEWL: 18°C, 2.0 g H_2O m^{-1}) or low evaporative water loss conditions (LEWL: 18°C, 12.0 g H_2O m^{-1}) as in Gerson and Guglielmo (2011a). At this time each bird was weighed and injected with a pre-weighed syringe containing ~2.0 mg inulin/g M_b of a ~3% solution of FITC-inulin in the left pectoralis muscle. A small aliquot of the injected solution was saved each experimental day for the calculation of the initial fluorescence for the calculation of Q_i (below). The empty syringe was weighed, and the difference in weight was recorded as the amount injected. Birds were then allowed a minimum of 45 min for equilibration of the inulin to occur. Birds were weighed and scanned using a quantitative magnetic resonance body composition analyzer (QMR; Echo Medical Systems , USA) to quantify fat mass, lean mass and total body water (Guglielmo et al., 2011), and a small blood (5-50 μL) sample was taken via brachial puncture in resting birds or from a toe-nail clip in flying birds as pre-flight brachial puncture seemed to dramatically reduce flight propensity in these birds. This equilibration period was determined to be sufficient during preliminary validations. Once this blood sample was taken, resting birds were placed in a covered rolling flight cage located in the plenum of the wind tunnel where they sat for 2 h. Flying birds were released directly into the wind tunnel and flew for a maximum duration of 2 h. This duration was pre-determined to be short enough to avoid complete washout of the FITC-inulin marker while still having the bird reach a physiologically relevant steady state. After the flight or resting period, birds were immediately bled (< 75 μL) from the opposite brachial vein, scanned using QMR and weighed. All blood samples were immediately spun at 2,000 x g (Galaxy Mini Microcentrifuge, VWR International) and plasma was stored on ice in the dark until the experiment was complete (< 2.5 h), then all

samples were stored at -80°C in the dark until analysis. All samples were analyzed within two weeks. A uretal urine sample was collected by inserting a blind-ended polyethylene sampling tube (Intramedic PE 160, Becton Dickinson, USA) into the cloaca as described by (Goldstein and Braun, 1988). Urine was spun at 2,000 x g to remove precipitated uric acid. To measure GFR and FWR in fed birds we used the same protocol, except that we left birds in their rolling flight cages in the animal room with normal access to food and water, and we did not scan using QMR. All fed birds were bled via a brachial puncture.

5.2.5 *Calculation of GFR and FWR*

GFR for experimental data was estimated by fitting a first-order exponential decay curve to the concentration of marker in the plasma over time plot, similar to (Hartman Bakken et al., 2004), except that since two blood samples were taken the decay curve was determined directly. GFR was calculated using equation (1).

$$\text{GFR} = k \bullet Sp \tag{1}$$

Where k is the exponent of the exponential decay curve fitted to the concentration of marker in plasma over time, and Sp is the dilution space of the marker and was calculated using equation (2).

$$Sp = Q_i \bullet A_{i(0)}^{-1} \tag{2}$$

Where Q_i is the total quantity of marker injected, and $A_{i(0)}$ is the zero time intercept of the exponential decay curve fitted to the concentration of marker in the plasma over time.

Units were converted to ml h^{-1}. FWR was calculated as in (Hartman Bakken et al., 2004) using equation (3).

$$FWR = 1-(P_m \bullet U_m^{-1}) \quad (3)$$

Where P_m and U_m are the simultaneous concentrations of the marker in plasma and urine, respectively.

Upon analysis using the single pool model we discovered that estimated $A_{i(0)}$ was low, resulting in unrealistically high values for the dilution space for inulin ($Sp > M_b$) indicating inulin did not follow first order exponential decay, and a second order decay curve would more accurately describe the relationship. In order to determine the degree to which using first order decay models overestimated GFR, and to develop a correction factor so that the values calculated for GFR using the first-order decay kinetics could be comparable outside of this study, the decay of FITC-inulin was determined using IV-jugular injection followed by serial bleeding as in (Qi et al., 2004). IV injection minimizes equilibration time and allows an accurate determination of the entire decay curve (Figure 5-1).

Five birds were successfully injected IV in the jugular with the same dose as described above. Injection volumes were determined gravimetrically as above. Birds were then bled from the brachial vein at 2, 5, 10, 20, 30, 50 and 70 minutes post injection using a 28 gauge needle to minimize blood loss at each collection. Not all birds were bled at all intervals. Once a total of 300 μl of blood was taken, the bird was returned to its cage with food and water. All blood samples were centrifuged as described above, and plasma was saved until analysis. Concentrations of FITC-inulin in plasma were

plotted against time since injection and a two-phase exponential decay model was fit using Prism 5 (Figure 5-1; Graphpad Software, USA (Qi et al., 2004)). GFR using these two-phase models was calculated using equation 4.

$$GFR = Q_i \div [(A_{1(0)}/k_1) + (A_{2(0)}/k_2)] \quad (4)$$

Where Q_i is the total quantity of marker injected, $A_{1(0)}$ is the zero time intercept for the rapid phase of the decay curve, k_1 is the exponent of the rapid phase of the decay curve, and $A_{2(0)}$ and k_2 are the zero time intercept and the exponent of the slow phase of the decay curve respectively (Figure 5-1).

5.2.6 *GFR Calculation Using Two-Phase Exponential Decay.*

Using experimental data collected from IV-injections and serial bleeds, GFR during rest, flight, and feeding was re-calculated using equation (4) as described above. The slow phase of the second-order exponential decay curve describes elimination of the marker through filtration, similar to the parameters obtained using the slope intercept method (Figure 5-1). Thus, a 'corrected' GFR was estimated from the data by estimating the rapid phase of the curve ($A_{1(0)}/k_1$), and using the experimentally determined values for the slow phase of the curve. These two values of GFR were compared using linear regression. Inclusion of unity within the confidence intervals of the slope of this relationship as well as r^2 values were used to determine the accuracy of the estimated parameter. The experimentally derived term for the rapid phase of the curve was then used to correct experimental values of GFR for fed, resting and flying birds in order to more accurately determine GFR.

The mean value for the rapid phase ($A_{1(0)}/k_1$) of the two-phase exponential decay model (equation 4) determined during the serial determination of GFR was 7.43. Applying this value as an estimation of the rapid decay term in equation (4) yielded an accurate estimation of GFR (Figure 5-2A). When plotted against the measured values of GFR, the slope of this relationship was 0.95 ± 0.299 with an R^2-value of 0.77. As the confidence intervals include the line of unity, the corrected values closely approximate the measured values. Although sample size was small due to the difficulty of IV-injections in a small bird, this provided a satisfactory correction factor to be applied to experimental data. This correction factor was then applied to the experimental data. The relationship of single pool uncorrected GFR values to the corrected ones are shown in Figure 5-2B. The mean coefficient of variation between these values was 15.34% and the uncorrected single-pool determination of GFR overestimated GFR by an average of 19.3%. GFR values calculated using 2-phase exponential decay were compared to allometrically predicted values using the equation presented in Bennett and Hughes, (2003).

5.2.7 *Statistics*

To increase statistical power and control for individual variation in fuel mixture in flight (as seen in Gerson and Guglielmo, 2011a), we attempted to measure GFR in flight in the same individual under both HEWL and LEWL conditions. However, many birds would not repeat a flight within the experimental timeframe. Thus, the dataset contains unbalanced repeated measures and general linear mixed models were used with individual as a random factor for all analysis. Models were determined using backward stepwise selection where non-significant terms were dropped sequentially until only

significant ($P < 0.05$) terms remained. All analyses initially included initial body mass as a covariate, but it was not significant and was dropped from all models. Statistical comparisons of GFR were made using the values calculated using first order exponential decay and equation (1) because even though these are relative measures of GFR, they do not require estimates of decay curve parameters ($A_{1(0)}/k_1$). Although these values may not be directly comparable to other studies, this GFR determination makes no assumptions and therefore comparisons within this study are most accurate.

In order to investigate the effect of state (Fed, Resting or Flying) on GFR and FWR, the HEWL and LEWL treatment groups were pooled. Thus, only state (Fed, Resting, Flying) was a factor in this analysis. To investigate the effect of humidity on GFR and FWR, the fed group was excluded from this analysis as this group was not exposed to humidity treatments. Thus state (Rest or Flight) and humidity (HEWL or LEWL) were both factors in the model. Flight duration was initially included as a covariate but was not significant, and was removed.

Energy expenditure was calculated from the changes in fat mass and lean mass as in Gerson and Guglielmo (2011a) using the energy equivalents presented in Jenni and Jenni-Eiermann (1998). Energy expenditure was compared among states, and between humidity treatments. All statistical analyses were performed using SPSS 19.0.

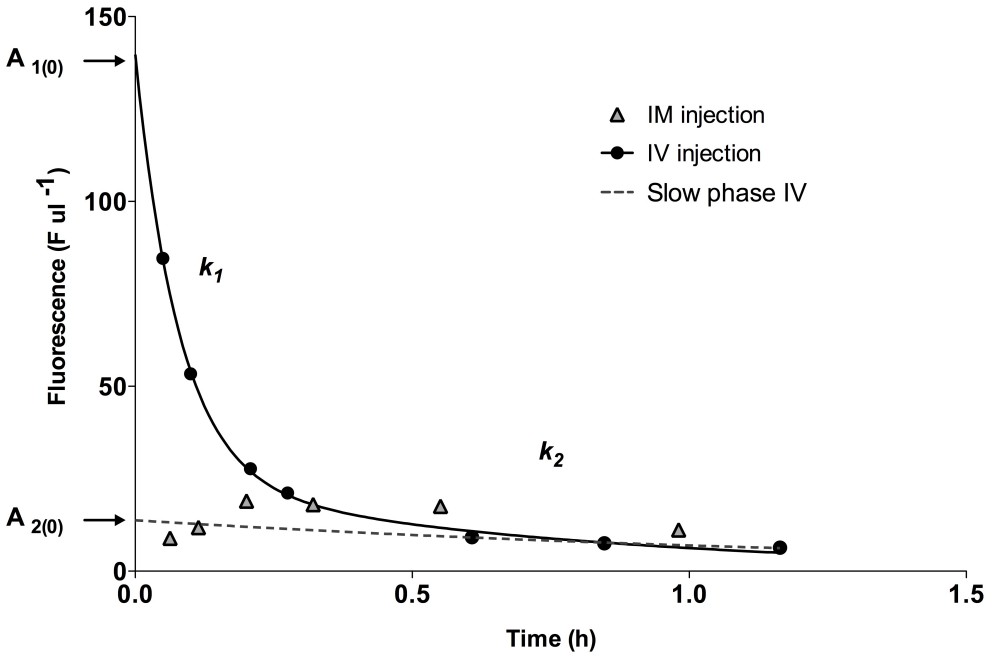

Figure 5-1. A second order decay curve fit to the concentration of FITC-inulin over time after IV-injection. $A_{1(0)}$ and k_1 represent the intercept and slope of the rapid phase of the curve. $A_{2(0)}$ and k_2 represent the intercept and slope of the slow phase of the curve. The dotted line represents the slow phase of the curve, which approximates the fit of a first order decay curve to the experimental data. Triangles show the equilibration time and decay of the marker when injected intramuscularly.

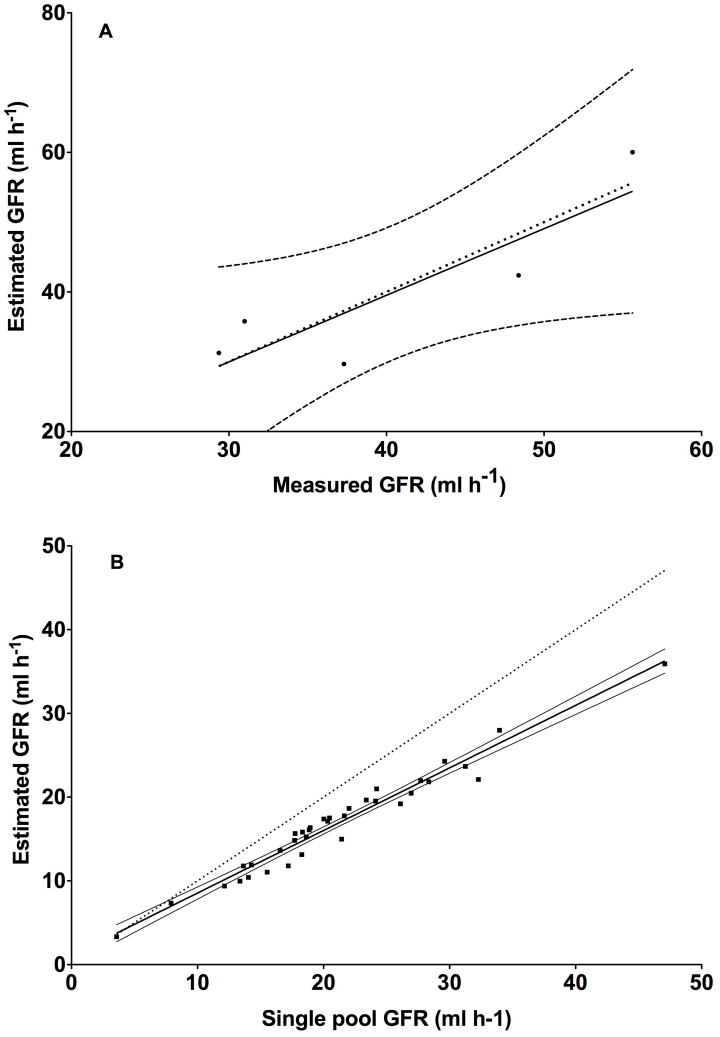

Figure 5-2. A) Linear regression of estimated GFR against calculated GFR. Estimated GFR was determined using second order exponential decay parameters and equation (4) where the rapid phase of the decay curve was estimated from experimentally derived parameters from IV injections. Measured GFR represents GFR calculated using equation (4) and all experimentally derived parameters. Dotted line represents line of unity. B) Comparison of GFR as estimated as in A using 2-pool model, and the calculation of GFR using a single pool model. Curved dotted lines around lines of best fit indicate 95 % confidence intervals in both panels.

5.3 Results

5.3.1 *Feeding, Resting, Flying*

No significant differences in GFR were evident among fed, rest, and flown birds ($F_{2,29.245} = 2.163$, $P = 0.133$; Figure 5-3A), but there were significant differences among these groups in FWR ($F_{2,9.975} = 9.659$, $P = 0.005$; Figure 5-3B). Post hoc analysis indicated that fed birds had a significantly lower FWR when compared to both flight birds ($P = 0.003$), and rest birds ($P = 0.003$). However, flight and rest birds did not differ in FWR ($P = 0.932$).

5.3.2 *Humidity*

There were no significant differences in GFR between humidity treatments or between flight and rest birds (State: $F_{1,21.791} = 0.002$, $P = 0.966$, Humidity: $F_{1,12.44} = 0.851$, $P = 0.374$; Figure 5-4A). No significant differences were evident in FWR between flight and rest or between humidity regimes (State: $F_{1,10} = 0.159$, $P = 0.698$, Humidity: $F_{1,10} = 0.417$, $P = 0.533$; Figure 5-4B).

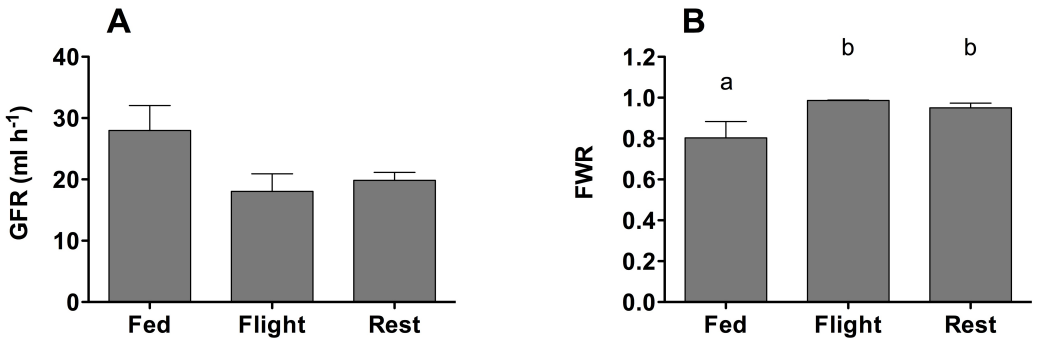

Figure 5-3. A) No differences in glomerular filtration rate (GFR) were evident among Fed, Flight and Rest groups ($P > 0.05$). B) Fractional water reabsorption (FWR) was significantly reduced in Fed birds. Different letters indicate significant differences at the $P < 0.05$ level. Bars represent Means ± SEM.

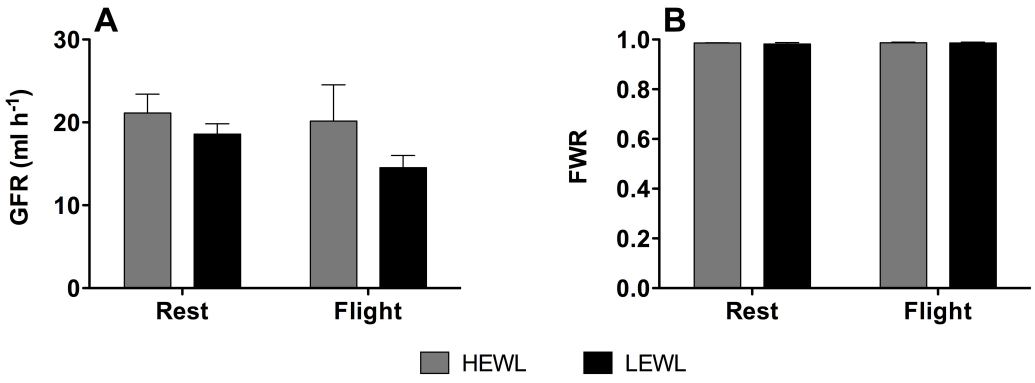

Figure 5-4. No differences in glomerular filtration rate (GFR; A) or fractional water reabsorption (FWR; B) were evident between flight and rest, or between high evaporative water loss (HEWL) and low evaporative water loss (LEWL) treatments ($P > 0.05$). Bars represent Means ± SEM.

5.3.3 *Body Composition and Energetics*

When comparing flight to rest birds, flown birds lost significantly more mass ($F_{1,13.393} = 10.316$, $P = 0.007$), but not lean mass ($F_{1,27} = 0.002$, $P = 0.963$) and there was no effect of humidity treatment (Mass: $F_{1,17.579} = 0.405$, $P = 0.532$; Lean Mass: $F_{1,27} = 0.050$, $P = 0.826$). There were significant reductions in fat mass with flight ($F_{1,20.138} = 60.015$, $P < 0.001$), but there was no effect of humidity on fat mass loss ($F_{1,21.483} = 1.066$, $P = 0.313$; Table 5-1).

When comparing energy expenditure between humidity treatments and between flight and rest birds there was a significant interaction ($F_{1,25.687} = 13.504$, $P = 0.001$; Figure 5-5). Flight birds had significantly higher energy expenditure compared to resting birds ($F_{1,13.83} = 181.395$, $P < 0.001$), and within the flight group, birds flown under the high evaporative water loss conditions had significantly higher flight costs ($F_{1,6} = 8.651$, $P = 0.026$). Within resting birds, there was no significant effect of humidity on energy expenditure ($F_{1,16.861} = 1.285$, $P = 0.273$).

5.3.4 *GFR Determination Using Two-Phase Exponential Decay and Comparisons to Other Species*

Estimating the rapid phase of the second order exponential decay of inulin yielded GFR measurements that were 26% lower than what was estimated using first order kinetics (Figure 5-2). GFR calculated using first and second order equations were 54% and 27% higher, respectively, than allometrically predicted values (Table 5-2).

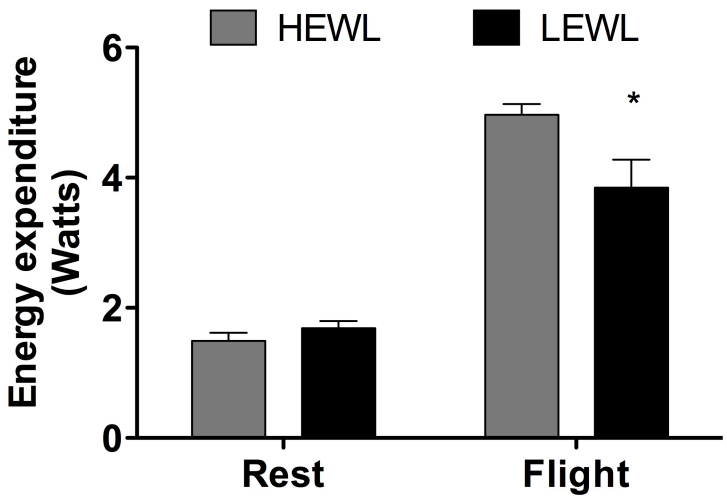

Figure 5-5. Flight birds had significantly higher energy expenditure compared to Rest birds ($P < 0.001$). Within Flight birds, birds flown under high evaporative water loss conditions (HEWL) had significantly higher energy expenditure than birds flown under low evaporative water loss conditions (LEWL). * indicates a significant difference within flight birds ($P < 0.05$). Bars represent Means ± SEM.

State	Humidity	% Energy from Lean	% Energy from Fat	Δ Mass (g)	Δ Lean Mass (g)	Δ Fat Mass (g)
Flight	HEWL (5)	14.94±2.93	85.062±2.925	-1.33 ± 0.10[a]	-0.73 ± 0.04	-0.62 ± 0.09[a]
	LEWL (3)	11.64 ± 3.57	88.36 ± 6.18	-1.10 ± 0.03[a]	-0.57 ± 0.18	-0.63 ± 0.16[a]
Rest	HEWL (9)	31.89 ± 4.34	68.10 ± 4.34	-0.81 ± 0.12[b]	-0.64 ± 0.07	-0.20 ± 0.29[b]
	LEWL (13)	28.96 ± 3.23	71.04 ± 3.23	-0.77 ± 0.09[b]	-0.67 ± 0.21	-0.24 ± 0.02[b]

Table 5-1. Relative contributions of changes in fat and lean mass to the energetics of flight and resting periods. Different letters indicate significant differences within a column.

5.4 Discussion

Many studies on the renal function of birds have shown dynamic control of GFR in response to water loading or restriction (Dawson et al., 1985; Goldstein and Braun, 1988; Goldstein and Bradshaw, 1998; Hartman Bakken et al., 2008; Hartman Bakken et al., 2004; McWhorter et al., 2004; Skadhauge, 1981). The renal function of Swainson's thrushes does not seem to respond dramatically to water restriction, flight, or feeding, but the trends identified in this study do agree with published studies. Previous studies investigating GFR in flight have shown reductions in the GFR of flown birds compared to rested birds, but these differences were slight (Giladi and Pinshow, 1999). It does seem that Swainson's thrushes initially regulate FWR, rather than GFR, in order to minimize water losses during resting and flight, while increasing the amount of water voided during feeding.

5.4.1 *Feeding, Resting, Flying*

Feeding resulted in a significant reduction in FWR relative to both resting and flight, indicating increased water reabsorption is primarily relied upon during periods of water restriction (resting and flying treatments). The diet of these birds in captivity has a high water content of approximately 45%, and the efficient elimination of water would be paramount during feeding. There was also a trend toward decreased GFR with water restriction. The GFR of fed birds was ~60% higher than that of the flight and rested birds, yet no significant differences were evident. Typically, an increase in GFR is associated with decreased FWR (Goldstein and Bradshaw, 1998; Hartman Bakken et al., 2004; McWhorter et al., 2004; Stallone and Braun, 1985; Williams et al., 1991). Since

both FWR and GFR are primarily controlled by AVT (Stallone and Braun, 1985), it is unlikely that only FWR would respond to feeding or periods of water restriction.

It is clear from our findings that the magnitude of the response of FWR is much greater than that of GFR, as FWR increased significantly from 0.8 to 0.98 within hours of removing water. The conditions experienced during flight and rest were not extreme, and were designed to mimic those experienced by wild migrants as in Gerson and Guglielmo (2011a). Thus, it is possible that flight under hotter, drier conditions would elicit a more dramatic reduction in GFR, although it does appear that the upper limit of FWR was reached.

Method	GFR (ml h^{-1})
Slope intercept method assuming first order kinetics	21.08 ± 1.36
Corrected two-pool determination	16.82 ± 1.04
Allometrically predicted	11.03 ± 0.19

Table 5-2. Comparison of the methods for the determination of GFR in birds. Single pool determination utilizes the slope intercept method and equation (1). Two-pool determination utilizes the slope intercept method and equation (4), where the rapid phase of the decay curve is estimated. Allometrically predicted values use the equation GFR = $0.013 M_b^{0.76}$ from Bennett and Hughes (2003).

5.4.2 *Humidity*

There were no significant differences in GFR or FWR between flight and rested birds, nor were any differences evident between humidity treatments. Previous to flight and resting measurements, birds were food and water restricted for three hours. This was done so that all birds were in a post-absorptive state, allowing for more accurate determination of changes in body mass during flight, while also ensuring no exogenous water input through digestion or absorption of water through the gastrointestinal tract. It seems that this period of water restriction was sufficient for birds to respond by increasing FWR above 0.98.

There were interesting trends in the response of GFR to humidity. GFR for the HEWL flight group was higher than the LEWL flight group. This trend may indicate an obligatory level of GFR required during HEWL flights where it has been shown that protein catabolic rates and plasma levels of uric acid are increased. The resulting higher rates of uric acid release into the bloodstream may preclude the reduction of GFR, and birds in flight under high evaporative water loss conditions may rely on FWR to reduce excretory water losses, while still filtering waste products. Interestingly, pigeons flown in the desert had higher GFRs in summer relative to winter, and only during the summer were GFRs reduced relative to resting levels, perhaps providing evidence indicating a tradeoff between water conservation and uric acid elimination as is suspected in the current study (Giladi and Pinshow, 1999).

5.4.3 *Body Composition and Energetics*

One of the more surprising findings was the significant effect of humidity on flight costs. It is likely that these birds were not maximizing fat catabolism as the mean

percent energy from fat was only 85-88 %, rather than upwards of 90-95%, indicating these birds were either still switching over to fat oxidation, or possibly, the stress of handling resulted in increased protein catabolism due to high corticosterone levels (Landys et al., 2006). Early in flight, birds catabolize a fuel mixture substantially higher in lean mass (Rothe et al., 1987). This releases large quantities of endogenous water, presumably resulting in rapid mass loss and reducing flight costs during a long flight. This process could be slowed under LEWL conditions contributing to the difference in flight costs. Endogenous water production during early flight may exceed respiratory losses under moderate atmospheric conditions, necessitating excretory elimination and precluding full reduction of GFR in flight. It is also possible that the difference in flight costs may be a result of slight differences in the lean mass contribution to flight as a result of the humidity treatments (Gerson and Guglielmo, 2011a).

5.4.4 *GFR Determination Using Two-Phase Exponential Decay.*

It has been previously shown that the calculation of GFR from first order decay parameters can result in overestimation of GFR by as much as 50% (Qi et al., 2004; Sturgeon et al., 1998). The determination of GFR using a 2-pool model has been shown to approximate constant infusion within 10% (Qi et al., 2004). The use of a correction factor is legitimate if the parameters of the entire second-order decay curve have been experimentally determined in the species in question. The rapid phase of the decay curve represents the distribution of the marker throughout the body and the distribution in the interstitial fluid, both of which are independent of the experimental manipulation, and likely scale with body mass and/or metabolic rate. Small animals should quickly distribute the marker. Therefore, the total contribution of the rapid distribution phase of

the decay curve would contribute less to the overall calculation of GFR. The overestimation of GFR by the slope intercept method assuming first order kinetics likely increases with body mass.

Most studies utilizing the slope intercept method and first order exponential decay for the determination of GFR in hummingbirds (Hartman Bakken et al., 2004), small bats (Hartman Bakken et al., 2008), or other small (1-10 g) animals (McWhorter et al., 2004) report a reasonable marker distribution space of around 20% M_b for inulin. Interestingly, the dilution space calculated in the current study from $A_{1(0)}$ of the IV injected birds was 18.4% of M_b, but the dilution space calculated from $A_{i(0)}$ using first order was in excess of 100% of M_b. We recommend that the marker dilution space be reported in future studies as a diagnostic for the appropriate use of first-order kinetics, and whether a correction factor should be determined for the experimental species.

5.4.5 *Comparisons to Other Species*

GFR calculated in this study are higher than allometrically predicted values for this species (Table 5-2). However, the list of species used by Bennett and Hughes (2003) to determine this predictive equation is far from complete. Due to interest in the renal function of desert dwelling animals this group of birds are over-represented in the dataset used to determine this allometric relationship. Desert dwelling birds likely have a greater ability to reduce GFR compared to migratory species due to their evolutionary history. Thus, the predictive equation may underestimate GFR for many species, increasing the likelihood that the corrected GFR values presented in Table 5-2 are fairly accurate.

5.4.6 *The Role of Kidneys in Osmoregulation of Migratory Birds*

Overall it does not seem that the reduction of GFR is the primary mechanism for the reduction of excretory water losses in flight. In both this study and Giladi et al. (1997), a modest reduction in GFR was observed in flight, while FWR was not different from resting values. During flight, cardiac output is increased 5-fold (Butler et al., 1977), which without reflexive action in the renal arterioles would lead to an increase in glomerular filtration pressure. A lack of increase in GFR indicates sufficient autoregulation of mean afferent arteriole pressure in response to exercise (Goldstein and Skadhauge, 2000).

Given the available data, it seems the role of the kidney in the conservation of water in flight is not as central as originally proposed. Reductions in GFR are modest, and FWR is higher after flight, but only marginally so. Also, urine osmolality does not seem to be increased post flight (Giladi et al., 1997). Urine flow rate is reduced in flight (Giladi et al., 1997), but no information exists on the role of post-renal urine modification in water conservation during flight, which may play a substantial role in water conservation in these birds. Many species rely on post-renal modification of urine in order to reabsorb water. In this process, urine is refluxed in the lower intestine, where water and ions can be reabsorbed (Goldstein and Braun, 1986).

Migratory birds show dramatic reductions in the sizes of many organs after long duration flights (Battley et al., 2000; Bauchinger and Biebach, 2001; Bauchinger and McWilliams, 2009; Bauchinger et al., 2005; Biebach, 1998; Karasov and Pinshow, 1998). Of particular interest are the large reductions in both the kidney and intestine. Garden warblers (*Sylvia borin*) crossing the Sahara have kidneys that are reduced in size by up to

40%, and small intestines are typically reduced by as much as 50% (Bauchinger and McWilliams, 2009; Bauchinger et al., 2005). Given these reductions in the kidney, it would be interesting to investigate renal function immediately after a long duration flight. If urine concentrating ability and water reabsorption are compromised after flight, there may be increased reliance on post-renal modification. Few investigations have documented the plasticity of the lower intestine (colon) after migratory flight, but evidence suggests it too is substantially catabolized (Bauchinger et al., 2005) indicating that migratory birds may face severe osmoregulatory challenges during stopover.

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6 DIETARY POLYUNSATURATED FATTY ACIDS, VITAMIN E SUPPLEMENTATION, AND ENDURANCE FLIGHT: EFFECTS ON MITOCHONDRIAL FUNCTION IN EUROPEAN STARLINGS (*STURNUS VULGARIS*)

6.1 Introduction

During their annual cycle, migratory birds travel long distances between their breeding and wintering grounds. This annual migration is usually completed through a series of endurance flights, each lasting many hours, which are followed by periods of stopover where fuel stores are replenished. Flight is more energy efficient (energy per unit distance covered) than walking/running, and faster than either swimming or running. However, flying requires the greatest instantaneous rate of energy expenditure (energy per unit time) of any form of sustainable aerobic locomotion (Schmidt-Nielson, 1972). Flying birds exercise at approximately 10 times their basal metabolic rate (BMR) and at up to ~90% of $\dot{V}O_2$-max for extended periods of time (Butler et al., 1977; Guglielmo, 2010; McWilliams et al., 2004; Rothe et al., 1987). Since the ability to migrate is a prerequisite for breeding success, and these birds cover long distances at great metabolic cost, it is likely that natural selection has favoured metabolic strategies that maximize efficiency in flight.

Birds have evolved a tremendous capacity to store, mobilize and catabolize fat as a fuel for high intensity exercise (Blem, 1976; Butler, 1991; Guglielmo et al., 2002a; Price, 2010; Price et al., 2008). Migratory flight of birds is fuelled almost exclusively by fat (Jenni and Jenni-Eiermann, 1998). Mammals, by comparison, reduce the relative

contribution of fat to exercise as intensity increases and fuel high intensity exercise primarily with carbohydrates. This is primarily because the mobilization and catabolism of fat is limited by the requirement for protein-mediated transport of insoluble fatty acids in circulation and across membranes (McClelland, 2004). However, birds are able to overcome this limitation by seasonally up-regulating the fatty acid transporters necessary to move fatty acids quickly from the adipocytes to the mitochondrion of the muscle cell (Guglielmo et al., 2002a; McFarlan et al., 2009).

Since birds conspicuously store large quantities of fat (the fat can be seen quite clearly under the skin) and rapidly mobilize these stores to fuel flight, investigation of the composition of fat stores and membranes has been a topic of interest for decades (Blem, 1976; Bower and Helms, 1968; Conway et al., 1994; Hicks, 1967; Odum, 1964; Price, 2010). It has more recently been revealed that the composition of fatty acids in the diet can have substantial effects on exercise performance in birds (Pierce et al., 2005; Price and Guglielmo, 2009). Thus, the composition of the fatty acids in the diet, which is reflected in the tissues, could have ramifications for migration success. For this reason, it is expected that migratory birds may readily exploit diets rich in fatty acids that promote exercise performance (Blem, 1976; Guglielmo et al., 2002b; Klaiman, 2009; Maillet and Weber, 2006; Price, 2010).

The fatty acids found in the tissues of vertebrates are typically un-branched with an even number of carbons ranging in size from 12-24 carbons. These fatty acids can be either unsaturated (UFA) or saturated (SFA), as determined by the presence or absence of double bonds in the aliphatic carbon chain. Unsaturated fatty acids may contain a single (monounsaturated; MUFA) or multiple (polyunsaturated; PUFA) double bond(s). These

double bonds can be found, starting from the methyl end of the fatty acid, on either the 3rd or the 6th carbon. Usually multiple double bonds are separated by three carbons. Thus, fatty acids where the first double bond is found on the third carbon are termed ω-3, and those with the first double bond located at the sixth carbon, ω-6. The double bonds present in unsaturated fatty acids change the physical structure and the chemical properties (i.e. melting point) of the molecule, which have broad implications on metabolism. When incorporated into phospholipid bilayers, unsaturated fatty acids alter the membrane fluidity and activity of membrane bound enzymes (Hulbert and Else, 1999; Hulbert et al., 2005) and when catabolized as a fuel, the addition of double bonds reduces the energy density of a fatty acid, but results in increased relative mobilization, potentially reducing the cost of transport (Price, 2010).

A number of studies of birds indicate that the fatty acid composition of the diet is reflected in the tissues of the animal (Egeler et al., 2003; Klaiman, 2009; Maillet and Weber, 2006), and the fatty acid profile may influence metabolism (Brand et al., 2003). With respect to exercise performance in birds, Pierce etal. (2005) showed that feeding red-eyed vireos (*Vireo olivaceus*) a diet composed of 58% unsaturated fatty acids but high in linoleic acid (18:2ω-6; 18 carbons, 2 double bonds, first double bond is on the 6th carbon) resulted in a 32.8% increase in peak metabolic rate (MR) over birds fed a diet higher in total unsaturates, but lower in PUFA. Price and Guglielmo (2009) found a similar increase in peak MR in birds fed a diet high in ω-6 fatty acids. Additionally, McWilliams et al. (2007) showed that a diet high in PUFA resulted in more efficient long duration flight in a wind tunnel.

Although the benefits of unsaturated fatty acids to exercise have been shown in a number of studies, the exact fatty acid(s), including the length and degree of unsaturation, or the overall fatty acid profile responsible, as well as the mechanisms responsible for gains in exercise performance are still not clear. Recently, a framework has been put forward that should aid in disentangling this complex phenomenon (Price, 2010).

Two dominant hypotheses, each with support in the literature, that have been proposed to explain the gains in exercise performance realized through high UFA diets. The fuel hypothesis postulates that the properties of the fuel alone are responsible for increases in exercise performance. As mentioned previously, unsaturation and chain length of fatty acids will affect both energy density and mobilization rates. In general, as fats become more unsaturated, the energy density (mmol ATP g^{-1}) goes down slightly, but the fats are more readily mobilized from adipocytes and used by muscle carnitine acyl-transferase (Price et al., 2011). Thus, catabolism of unsaturated fatty acids may be more rapid, assuming that fuel supply is limiting in exercise (Price et al., 2008).

The second hypothesis, termed the phospholipid hypothesis, attributes increases in exercise performance to the changes in the physical properties of membranes and membrane bound enzymes, as unsaturated fatty acids are incorporated into the fatty acid chains of membrane phospholipids. There is considerable evidence that membrane fatty acid composition can alter proton leak in intact mitochondria (Brand et al., 2003; Hulbert and Else, 1999). Proton leak is a 'futile cycle' that is estimated to account for as much as 20% of BMR in rats (Rolfe and Brown, 1997). Briefly, as protons are pumped into the inter-membrane space of the mitochondria during substrate oxidation, an electrochemical gradient is established that then is alleviated by F_0/F_1-Atpase during phosphorylation,

resulting in the production of ATP. Alternatively, the electrochemical gradient can be alleviated via proton leak, where protons leak back into the matrix of the mitochondria without the production of ATP. Fatty-acyl composition of membrane phospholipids may also alter the activity of membrane bound enzymes, including the Na^+, K^+-ATPase, or complexes of the electron transport chain (Hulbert and Else, 1999). Additionally, there is evidence that fatty acid composition of mitochondria are responsible for well documented allometric relationships between body mass and proton leak, and body mass and mitochondrial respiration rates across species in both mammals and birds (Brand et al., 2003; Hulbert, 2007; Porter et al., 1996). Due to the complexity of the fatty acid profiles, it is not clear which aspect of membrane composition (total unsaturation, total PUFA, ω-6/ω-3, etc.) is most important to increasing exercise efficiency, but proton leak certainly can be altered by the composition of the membrane (Hulbert, 2007). Thus, gains in exercise efficiency, or performance associated with diets high in unsaturated fatty acids may be due to reduced proton leak, thus increasing the efficiency of mitochondrial oxidative phosphorylation.

Although proton leak is an inherent metabolic inefficiency that is extremely costly, it is ubiquitous and therefore may confer some benefit (Brand, 2000). Mitochondria are responsible for the majority of ATP production in the cell through the process of oxidative phosphorylation. However, one by-product of oxidative metabolism is the production of reactive oxygen species (ROS). Superoxide is produced at complexes I and III of the electron transport chain, and ROS production tends to increase as the mitochondrial proton gradient increases (Droge, 2002; Korshunov et al., 1997; Votyakova and Reynolds, 2001). Thus, it has been proposed that mitochondrial proton

leak acts to reduce the magnitude of the proton gradient as a means to modulate ROS production (Brand, 2000). ROS are responsible for intracellular damage to a number of classes of molecules including proteins, DNA, and fatty acids. The double bonds of MUFA and PUFA are particularly susceptible to ROS damage, and the attack by superoxide radicals could result in the cross-linking of fatty acids in phospholipids, or in the disruption of normal catabolism of fat. Thus, migratory birds may experience a tradeoff between increased exercise performance due to reduced proton leak, and higher rates of ROS production and damage. Antioxidants act to scavenge ROS, and if in sufficient supply, could reduce the impact of this potential tradeoff.

Due to the previous findings that diets high in polyunsaturated fatty acids increase flight efficiency in migratory European starlings (*Sturnus vulgaris*; McWilliams and Pierce, 2006), it was the goal of this study to investigate mitochondrial energetics of European starlings. Here we test of one aspect of the phospholipid hypothesis: that more unsaturated mitochondrial membranes result in more efficient phosphorylation and have lower leak. Birds were studied at rest with no flight training, at rest after two weeks of daily flight training in a wind tunnel, and immediately after flight following two weeks of daily flight training, on one of four diets differing in fatty acid composition as well as vitamin E concentration. To investigate the effects of polyunsaturated fatty acids on mitochondrial function, birds were fed either a diet high in PUFA or high in MUFA. To assess the potential tradeoff between increased flight efficiency and ROS damage, each of these diet groups was further divided into a high or low vitamin E treatments since vitamin E is an antioxidant that may reduce the effects of ROS. Thus, four diets were administered: PUFA high vitamin E, PUFA low vitamin E, MUFA high vitamin E,

PUFA low vitamin E. Mitochondria were isolated from the pectoralis muscle, and substrate preferences and respiration rates, proton leak kinetics, phosphorylation kinetics, and ROS production rates were measured. We predicted that birds on the high PUFA diet would have higher mitochondrial respiration rates and lower proton leak, and the high dietary vitamin E would mitigate the trade-off between ROS production and reduced proton leak in the PUFA diet.

6.2 Materials and Methods

6.2.1 *Study Species, Diet Groups, and Care and Maintenance of Birds*

As this study represents only one aspect of a much larger investigation, the following sections describing the experimental design and details of diets and flights in the wind tunnel have been adapted from (Bauchinger et al., submitted) and (Nebel et al., 2011).

One hundred and twenty adult European starlings (*Sturnus vulgaris)* were caught near London, Ontario, Canada, during the last two weeks of July 2009 using mist nets. European starlings have been trained to fly in wind tunnels for long duration in other studies (Engel et al., 2006), and their energy expenditure during long distance flight has been shown to be affected by dietary fatty acid composition (McWilliams and Pierce, 2006). Thus, this species was ideal for use in this study.

Captive birds were held at the Advanced Facility for Avian Research at Western University under constant ambient temperature of 20°C under natural day length, which was adjusted once each week to the natural changes in day length. From September 21

onwards all starlings were maintained at 13 hrs 9 min light and 10 hrs 51 min dark for the remainder of the experiment.

We randomly assigned starlings to one of four diet groups. We fed each group one of four isocaloric agar-based semi-synthetic diets that had the same macronutrient composition (41% carbohydrate: 13% protein: 30% fat), and only differed in the relative amounts of plant oils and the amount of supplementary vitamin E (see below). Although the total mass of plant oil added to the diets was identical, the relative quantities of olive oil and sunflower oil were adjusted to produce diets that contained primarily monounsaturated fatty acids (MUFA) or primarily polyunsaturated fatty acids (PUFA). We supplemented these diets with either low or high vitamin E (5 or 30 International Units per kg diet) to produce four experimental diets: MUFA high vitamin E (n = 40 birds), PUFA high vitamin E (n = 40), MUFA low vitamin E (n = 20), and PUFA low vitamin E (n = 20). Birds in the high vitamin E groups were maintained in two separate aviaries in groups of 40 (each 3.7 m x 2.4 m x 3.1 m) while birds in the low vitamin E groups were maintained in two other aviaries in groups of 20 (each 2.3 m x 2.4 m x 3.5 m). These amounts of vitamin E correspond to levels recommended for poultry eating diets with low or high PUFA (5 and 30 IUs, repectively (Klasing, 1998; Scott et al., 1982). Aviaries had natural branches and *ad libitum* food and water were supplied daily when the lights turned on.

6.2.2 *Food Contents and Food Preparation*

We produced fresh diet each week. All four diets were prepared identically except that the addition of plant oil and vitamin E was adjusted as described below. Agar (60 g, 10906 USB Corp.) was dissolved into 3000 g of boiling water. This agar-water mixture

was then transferred into a commercial grade stainless steel blender with 738 g D-glucose (D 16-10, Fisher Scientific), 360 g casein (12845 USB Corp.), 93.6 g cellulose (Celufil 13292, USB Corp.), and 90 g salt (Salt Mixture Briggs, 902834 MP Biomedicals). The mixture was then cooled in a refrigerator for 1 hour and then 50.4 g amino acid mix (Langlois and McWilliams, 2010; Murphy and King, 1982) 7.2 g vitamin mix (AIN Vitamin Mixture 76, 905454, MP Biomedicals, Inc.), 116 g crushed freeze-dried mealworms (Exotic Nutrition Co., Newport News, Virginia), and olive and sunflower oil (amounts differed by diet) were added and the mixture was blended a second time. To make MUFA diets we added 356 g olive oil and 4 g sunflower oil and for PUFA diets we added 270 g olive oil and 90 g sunflower oil. To produce high vitamin E diets, we added 0.18 g of vitamin E (DL-alpha Tocopherol Acetate, 100559, MP Biomedicals, Inc.) with the respective oils before the final mixing. The mixture was blended for three minutes, poured into a plastic storage container, and stored at 4°C until solidification was complete. Each day we mashed the food, placed it in large trays, and offered it to the birds.

6.2.3 *Experimental Groups*

We had three flight treatment groups. *Untrained* birds (n = 44) were housed in the large aviaries and never flew in the windtunnel. *Trained* birds (n = 40) and *exercised* birds (n = 38) were flown in a windtunnel designed for bird study at the Advanced Facility for Avian Research at Western University (see Gerson and Guglielmo, 2011) for details). We trained birds in groups of three to fly voluntarily from their transport cage (1.2 m x 0.7 m x 1.8 m) into the wind tunnel at which point the 0.5 m opening to the flight section was closed. Birds then flew for the prescribed lengths of time based on their

day of training. The training was conducted for a given group of three birds over 15 consecutive days according to the following schedule: day 1 = 10 min, day 2 = 10 min, day 3 = 20 min, day 4 = 30 min, day 5 = 30 min, day 6 = 45 min, day 7 = 60 min, day 8 = 90 min, day 9 = 30 min, day 10 = 120 min, day 11 = 180 min, day 12 = rest, day 13 = 15 min, day 14 = 15 min. On Day 15, birds were flown until they voluntarily stopped and perched on the bottom of the working section, and could not be coaxed to continue flying. After this ultimate flight birds in the *exercised* group were immediately euthanized by decapitation under isoflurane anesthesia. In order to maintain adequate sample size, only birds consuming the high vitamin E diet were placed in the *exercised* group. Birds in the *trained* group were allowed to recover for two days following the ultimate flight and then were euthanized at the same time of day as birds in the *exercised* group. We recorded the actual flight time for each individual and summed the total time spent flying for the entire 15-day period for each bird. We staggered the start of each 15-day training period by three days so that we could train a total of 20 groups of three birds over 58 days. At the start of the 15-day flight-training period, each group of three birds was moved from their aviaries into transport cages that were then wheeled into the windtunnel during flight training.

6.2.4 *Tissue Sampling*

Given that the two-week flight training was scheduled in groups of three birds over time, we sampled tissues from 1-2 *trained* birds in each group, and 1-2 *exercised* birds in each group, along with 1-2 randomly selected *untrained* birds. Due to the staggering of groups, tissues were sampled every day from 19 Oct to 16 Dec 2009. *Trained* birds were sampled two days after their final flights (= day 18) allowing the

comparison of both the effects of flight training, as well as the immediate effects of a given long-duration flight. Birds in the *trained* group represent individuals during migration that have been resting between long-distance migratory flights for at least 2 days.

Randomly-selected *untrained* birds were moved from their aviaries and individually housed for one to two days in cages (0.6 m x 0.5 m x 0.5 m) so that we could control their access to food and water on the day of tissue sampling. Only birds that had completed molt were selected. Birds did not receive food on the day of tissue sampling, but had access to drinking water for 15 minutes immediately after lights on. Sampling occurred 5 - 7 hours after lights on. A blood sample was collected, M_b was recorded to the nearest 0.01 g, and then birds were euthanized and tissues were collected.

6.2.5 *Mitochondrial Isolation, Substrate Preference, and Membrane Potential*

Immediately after euthanasia, 4 - 5 g of the left pectoralis muscle was quickly excised and placed in ice-cold homogenization buffer (HB; 100 mM Sucrose, 50 mM Tris base, 10 mM EDTA, 100 mM KCl, BSA 0.1%, pH 7.4 at 0°C). Mitochondria were isolated using a protocol modified from Muleme et al. (2006). Immediately after being excised, muscle was finely minced in ice-cold HB and held on ice for 10 - 20 minutes while being transferred to the laboratory. Typically, two animals were sampled per day. Mitochondrial isolation occurred simultaneously for both preparations, and animal sampling was typically staggered by 15 minutes except in the case of *exercised* birds, where birds were sampled when voluntary flight ended.

Once in the lab, minced muscle was rinsed with ice-cold HB, and then incubated in HB with Nagarse (Sigma, 0.2 mg ml^{-1}) for 3 minutes, after which the tissue was rinsed three times with HB. Tissue was then homogenized in ~20 ml HB at approximately 100 rpm with 5-6 passes using a loose fitting Teflon pestle (~0.4 mm clearance) in a 30 ml glass mortar. Homogenization was stopped once no large pieces of muscle were visible. The homogenate was cooled on ice, and filtered through 4 layers of cheesecloth into polycarbonate tubes and centrifuged at 1000 x *g* at 4°C for 10 min. Floating lipids were aspirated from the surface of the supernatant, which was then filtered through four layers of cheesecloth and spun at 8,700 x *g* at 4°C for 10 min. The resulting pellet was carefully re-suspended in HB and centrifuged a second time at 8,700 x *g* at 4°C for 10 min, after which the resulting crude mitochondrial pellet was re-suspended in 1.0 ml of HB.

Mitochondrial respiration rates were determined at 39°C using a temperature controlled polarographic O_2 analyzer (Dual digital model 20, Rank Brothers, Bottisham, UK) in 2.0 mL (substrate preference) or 3.0 mL (membrane potential) of assay buffer (AB: 230 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4, 10 mM Tris HCL, 0.5% BSA, pH 7.4 at 39°C.) similar to Suarez et al. (1986). The oxygen analyzer was calibrated to ambient air, using O_2 concentrations previously reported (Gerson et al., 2008; Muleme et al., 2006), and corrected for assay temperature and barometric pressure. For substrate preference assays, mitochondria were added to a final concentration of 0.1±0.003 mg protein mL^{-1} and maintained at 39°C. Maximal state 3 (phosphorylating, addition of 0.2 mM ADP) and state 4 (non-phosphorylating, addition of oligomycin: 10 mg/mL dissolved in ethanol once ADP had been depleted) respiration rates were determined for three different substrates. Oxidation of succinate in the presence of rotenone, to inhibit

complex I of the electron transport chain (RS: 6 mM succinate, 2 μg mL^{-1} Rotenone dissolved in ethanol), oxidation of pyruvate in the presence of malate (MP: 1 mM malate, 10 mM pyruvate), and oxidation of palmitoyl carnitine in the presence of malate (MPC: 1 mM malate, 0.05 mM palmitoyl carnitine) were determined.

Kinetics of mitochondrial proton leak and phosphorylation were measured as in Brown et al. (2007) and Gerson et al. (2008) where rates of oxygen consumption and proton motive force were recorded simultaneously. Oxygen consumption was measured as during the substrate preference assays. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured as an approximation of ΔP as in Brown et al. (2007). To measure $\Delta\Psi_m$, tetraphenylphosphonium (TPP+), a lipophilic anion, was added and measured with a TPP+ selective electrode (World Precision instruments, Sarasota, Florida, USA). To calibrate the electrode, five additions of TPP+ (500 μM) were made to the chamber, each of which resulted in an increase of [TPP+] in the chamber by 1 μM. Calibration was performed for each replicate of each mitochondrial preparation after mitochondria were added to the chamber, but before substrate was added (see Brown et al., 2007; Gerson et al., 2008). For both proton leak and phosphorylation kinetics, mitochondria (0.385±0.01 mg mL^{-1}) were added to 3 mL AB at 39°C, after which calibration of the TPP+ electrode was performed followed by addition of succinate (6 mM) for proton leak kinetics or succinate (6 mM) and ADP (1.0 mM) for phosphorylation kinetics. For proton leak kinetics, oligomycin (10 mg/mL dissolved in ethanol) was also added to inhibit the phosphorylation component of oxidative phosphorylation. Once maximal respiration rates were evident, respiration was titrated using 5 sequential additions of malonate (500 μM), each raising the overall concentration by 1 μM. Malonate is a competitive inhibitor

of complex II, and thus the titration resulted in a stepwise reduction in respiration rate and, concomitantly, membrane potential. Between malonate additions, stable rates of oxygen consumption and the corresponding TPP^+ concentration were recorded. Finally, CCCP (cyanide m-chlorophenylhydrazone; 0.1 μM) was added to completely depolarize the mitochondrial membrane and allow for drift correction of the TPP+ electrode. Membrane potential was calculated from TPP^+ concentrations using the modified Nerst equation as in Brown et al. (2007) and Gerson et al. (2008).

6.2.6 *ROS Release*

Rates of reactive oxygen species (ROS) release were measured as in Brown et al., (2007) and Brown et al. (2009). Briefly, mitochondria (0.1±0.003 mg protein mL^{-1}) were incubated at room temperature in a 1.7 mL centrifuge tube with buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% bovine serum albumin, pH 7.4 at 37 °C), horseradish peroxidase (4 U/mL), and homovanillic acid (4 mM). Succinate and rotenone were added to achieve state 2 respiration rates (approximately equal to state 4 respiration) and basal rates of H_2O_2 release were measured. Substrates were added in the same concentrations used for substrate preference assays (above). After 0 and 10 min of incubation, the reaction was stopped by immersing the centrifuge tubes in ice and a 225 μL sample from the centrifuge tube was added to 75 μL of glycine buffer (0.1 M glycine, 25 mM EDTA, pH = 12 at room temperature) in 96-well black plates, and fluorescence was immediately measured at 310 nm excitation and 420 nm emission (SpectraMax M2e, Molecular Devices, Sunnyvale CA). A standard curve was generated using the known rate of H_2O_2 production by glucose oxidase with glucose as a substrate as in Brown et al. (2009).

6.2.7 *Data Analysis*

Mitochondrial respiration rates from all substrates and ROS release rates were compared between *trained* and *untrained* groups among all four diet treatments using diet (MUFA and PUFA) and vitamin E (high and low) as factors in a multifactor general linear model (GLM). ROS release rates were transformed by the natural logarithium + 1 to achieve a normal distribution, but untransformed data are plotted. The *exercised* group was composed only of birds fed high vitamin E in both diets (MUFA and PUFA). Therefore, the effect of diet, exercise, and training on mitochondrial respiration rates and ROS release rates was investigated only among birds receiving the high vitamin E diets. In all models, all main factors (Diet, vitamin E, training) as well as all two-way interactions were included in the initial model. Non-significant terms were sequentially removed until only significant terms ($P < 0.05$) remained. All statistics were performed using R (version 2.14).

Top down elasticity analysis was used to characterize control over each of the three components of oxidative phosphorylation: substrate oxidation, proton leak, and phosphorylation as in Brown et al. (2007) using equations from Hafner et al. (1990). Briefly, a second order polynomial was fit to the relationship between oxygen consumption and membrane potential for both proton leak, and phosphorylation assays for each mitochondrial preparation. The first derivative of this relationship was calculated and used to calculate elasticities using equations (Hafner et al., 1990) as described by Brown et al. (2007). All control coefficients were calculated using equations 1-12 in Hafner et al. (1990). Elasticities and control coefficients were calculated for both maximal state 3 and maximal state 4 respiration rates. The kinetics of substrate oxidation

was determined as the slope of the linear relationship between membrane potential at maximal state 3 oxygen consumption and membrane potential at maximal state 4 oxygen as in Brown et al. (2007).

6.3 Results

6.3.1 *Flights and Training*

During the 15 day training period, birds flew an average of 717.68 ± 188.95 min in the tunnel, and *Flight* birds flew an average of 180.19 ± 98.16 min during the ultimate flight. Flight and training duration breakdown by diet and experimental group can be found in Table 6-1.

6.3.2 *Mitochondrial Substrate Preference and ROS Release Rates*

Mitochondria were isolated from 87 of the 120 starlings in the experiment (Table 6-1). There were significant interactions between vitamin E treatment and *exercised* treatment for rotenone succinate state 3 respiration ($F_{1,68} = 12.05$, $P < 0.001$; Figure 6-1 A), rotenone succinate state 4 respiration ($F_{1,68} = 9.26$, $P = 0.003$; Figure 6-1 B), malate pyruvate state 3 respiration ($F_{1,61} = 8.209$, $P = 0.006$; Figure 6-1 C), malate pyruvate state 4 respiration ($F_{1,61} = 4.241$, $P = 0.044$; Figure 6-1 D), and malate palmitoyl canitine state 4 respiration ($F_{1,57} = 4.65$, $P = 0.0348$; Figure 6-1 F). Mitochondrial respiration rates increased with training only in the low vitamin E groups. Additionally, there was a significant effect of training alone on malate palmitoyl carnitine state 3 respiration ($F_{1,59} = 5.14$, $P = 0.027$; Figure 6-1 E). For all variables where a significant interaction term existed, the dataset was split by vitamin E treatment in order to determine if there was a

training effect within each vitamin E treatment. Within the high vitamin E treatment, a training effect was evident only in the oxidation of malate pyruvate under state 3 conditions ($F_{1,34} = 6.858$, $P = 0.013$). Within the low vitamin E treatment, a significant training effect was evident in rotenone succinate state 3 ($F_{1,28} = 34.195$, $P < 0.001$) and state 4 ($F_{1,28} = 30.224$, $P < 0.001$) oxidation, malate pyruvate state 3 ($F_{1,27} = 34.795$, $P < 0.001$) and state 4 ($F_{1,27} = 12.107$, $P = 0.002$) oxidation, as well as palmitoyl carnitine oxidation under state 4 conditions ($F_{1,25} = 4.723$, $P = 0.039$). There were no effects of fatty acid composition of the diet (MUFA or PUFA) on any of the above mentioned respiration rates. Mitochondrial reactive oxygen species release rates were significantly higher in the MUFA diet ($F_{1,64} = 5.88$, $P = 0.018$; Figure 6-1 G), but there was no effect of exercise or vitamin E.

Diet	Vitamin. E	Training	Mitochondrial preparations (n)	Training Duration (min)	Ultimate flight duration (min)
MUFA	High	*Untrained*	12	NA	NA
		Exercised	7	746.63 ± 38.46	181.75 ± 38.75
		Trained	9	739.70 ± 34.13	108.36 ± 20.065
	Low	*Untrained*	9	NA	NA
		Trained	6	827.44 ± 35.68	190.25 ± 30.41
PUFA	High	*Untrained*	13	NA	NA
		Exercised	8	837.13 ± 22.74	178.63 ± 17.92
		Trained	8	674.70 ± 37.66	81.10 ± 16.36
	Low	*Untrained*	9	NA	NA
		Trained	6	714.89 ± 24.93	132.33 ± 15.55

Table 6-1. Sample sizes and flight durations for each training and diet group. Values are means ± SEM.

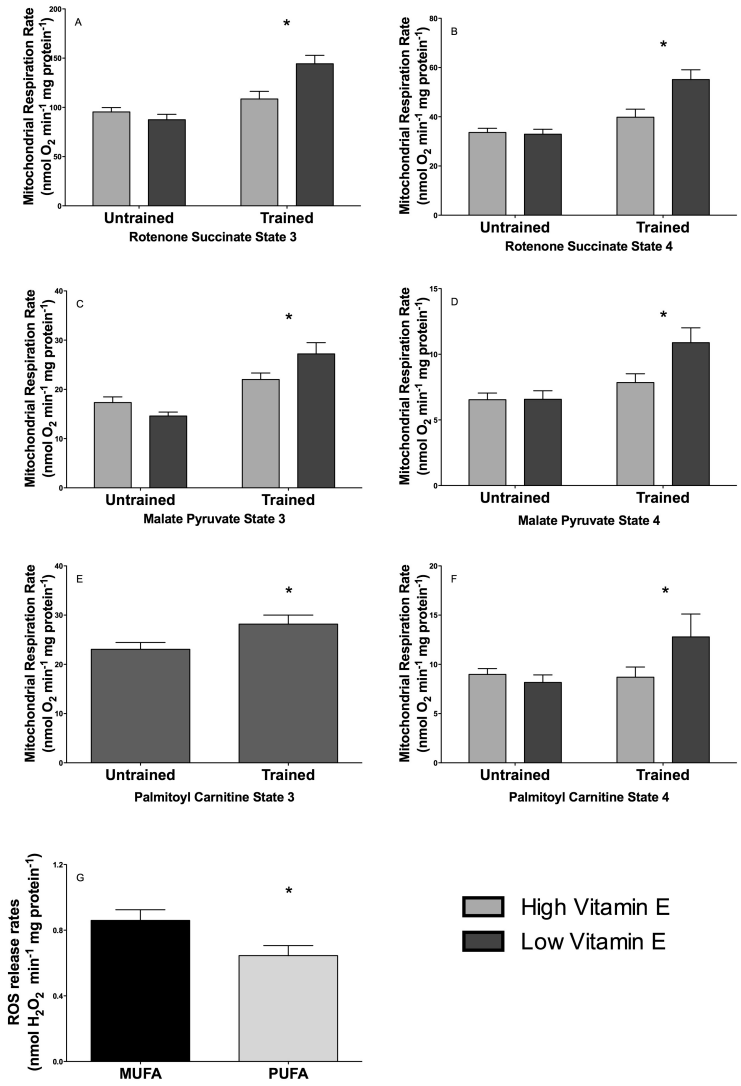

Figure 6-1. Mitochondrial respiration rates while in the presence of ADP (state 3) or once ADP supply has been depleted (state 4) with succinate and rotenone (A, B), malate and pyruvate (C, D), or palmitoyl-carnitine (E, F) as substrate. * indicates a significant interaction between vitamin E treatment and training in A, B, C, D, & F) where a training effect was evident only in the low vitamin E diet groups. Fatty Acid composition did not have any effect on these respiration rates, but ROS release rates were lower in birds fed the PUFA diet * indicates significance at $P < 0.05$. See text for complete statistical details. Bars represent means ± SEM.

There was a significant effect of treatment on malate pyruvate state 3 respiration rates ($F_{2,48}$ = 3.85, P = 0.028; Figure 6-2) where training resulted in a significant increase in respiration rates over *untrained* (P = 0.044) and over *exercise* (P = 0.049). Malate pyruvate state 4 respiration rates showed a similar trend ($F_{2,48}$ = 2.69, P = 0.07, Figure 6-2). Although not significant, post-hoc analysis indicated this trend was a result of increased respiration rates in *trained* birds compared to *untrained* and *exercise groups.* Also, there was a trend towards a significant effect of diet on malate palmitoyl-carnitine oxidation rates (State 3: $F_{1,47}$ = 3.41, P = 0.071; State 4: $F_{1,47}$ = 3.66, P = 0.062), where birds fed the PUFA diet had higher respiration rates, but there was no effect of training.

Since mitochondrial ROS production rate is highly influenced by membrane potential, we tested the effect of diet and state 4 membrane potential on rates of ROS release. There was no relationship between state 4 membrane potential and ROS release rate in control birds. However, there was a significant direct relationship between state 4 membrane potential and rates of ROS release ($F_{1,6}$ = 6.382, P = 0.045) in *exercised* birds, but there was no effect of diet. In *trained* birds, there was a significant effect of diet ($F_{1,8}$ = 21.341, P = 0.002) and state 4 membrane potential ($F_{1,8}$ = 12.526, P = 0.002) on rates of ROS release, but no effect of vitamin E. To further investigate this in *trained* birds, the data set was split by diet. There was a negative relationship between ROS release and state 4 membrane potential within the PUFA diet ($F_{1,6}$ = 31.826, P = 0.001; Figure 6-3c), and this relationship was significant only within the high vitamin E group ($F_{1,3}$ = 19.971 P = 0.021).

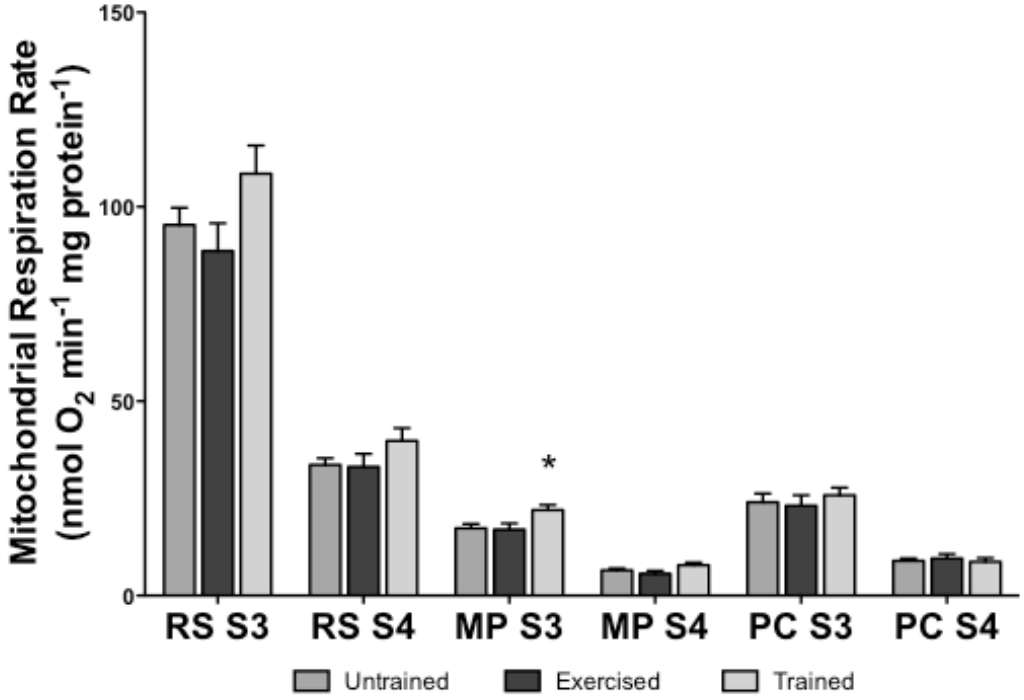

Figure 6-2. State 3 (S3; phosphorylating) and state 4 (S4; non-phosphorylating) respiration rates of mitochondria isolated from the skeletal muscle of *untrained*, *exercised* and *trained* European starlings oxidizing various substrates. RS: Rotenone succinate, MP: Malate pyruvate, PC: palmitoyl carnitine. Within the high vitamin E groups there was a training effect only when mitochondria were provided malate pyruvate as a substrate, and there was no overall effect of fatty acid composition of the diet. Bars represent means ± SEM.

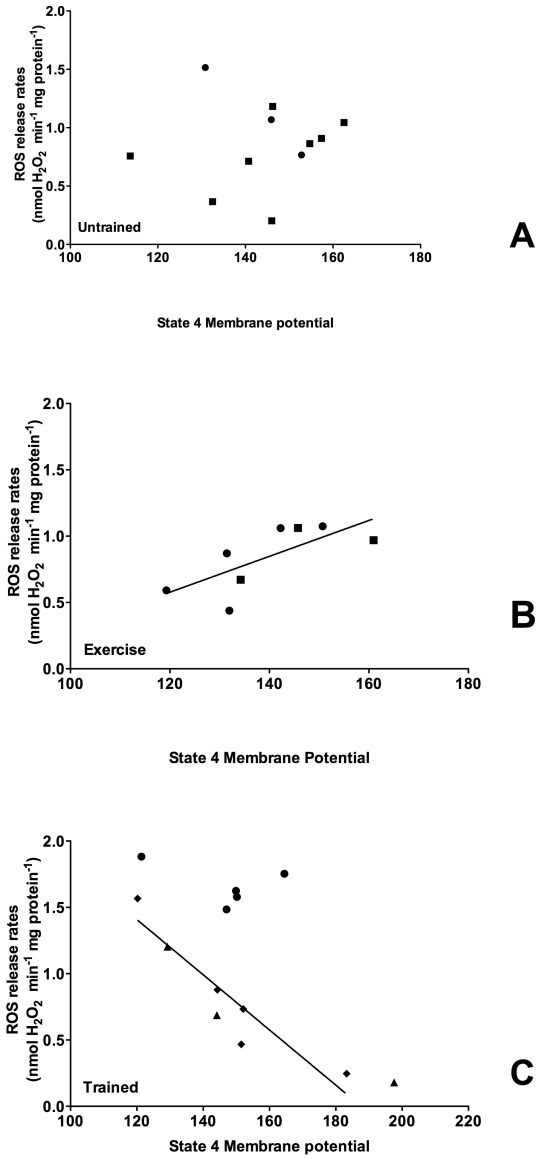

Figure 6-3. Mitochondrial ROS release rates as a function of state 4 membrane potential in *Untrained* (A), *Exercised* (B) and *Trained* (C) birds. **(A)** There was no relationship between ROS release rates and state 4 membrane potential in control birds. **(B)** Immediately after flight, there was a significant positive correlation between state 4 membrane potential and ROS release rates. **(C)** After training, there was a very strong negative relationship between state 4 membrane potential and ROS release rates, but only in the birds fed PUFA diet. Circles: MUFA; Squares PUFA; Diamonds: PUFA high vitamin E; Triangles: PUFA low vitamin E.

There were significant correlations among a number of mitochondrial respiration parameters, and flight or training duration (Table 6-2). Within the MUFA diets, mitochondrial respiration rates tended to correlate negatively with both duration of the ultimate flight and training duration, with the exception of malate pyruvate state 3 respiration which correlated positively with training duration in the high vitamin E group. Within the PUFA diets, on the other hand, most mitochondrial respiration rates correlated positively with flight duration and training duration in the *trained* birds, except in the low vitamin E group, where malate-pyruvate state 4 respiration rate correlated negatively with training duration. Respiration rates from *exercised* birds did not correlate with either flight duration or flight training (Table 6-2).

6.3.3 *Kinetics of Mitochondrial Substrate Oxidation, Proton Leak, and Phosphorylation*

In order to more thoroughly understand the mitochondrial response to diet, vitamin E, and training we examined each of the components involved in oxidative phosphorylation; substrate oxidation, proton leak, and phosphorylation kinetics.

Substrate kinetics (Figure 6-4), showed some differences between diets, where in the MUFA high vitamin E diet there was an obvious increase in the rate of oxidation with little or no increase in $\Delta\Psi$ under both state 3 (upper left) and state 4 (lower right) sections of the curve. The increase in mitochondrial respiration rate was more dramatic in the MUFA low vitamin E group, while there was a substantial increase in $\Delta\Psi$ with training under state 4 conditions. Few differences were evident in either of the PUFA diets.

The kinetics of proton leak were only weakly affected by diet. The MUFA high vitamin E diet increased maximal state 4 respiration rates with no change in membrane

potential with training (high vitamin E only; Figure 6-5 A). This shift indicates an increase in proton leak as higher oxygen consumption is required to maintain a common membrane potential with training, this pattern was not evident in the PUFA diets. The opposite pattern was evident in the MUFA low vitamin E diet (Figure 6-5 C), where proton leak decreased with training. Again, few substantial differences were evident within the PUFA diets (Figure 6-5 B, D). Differentiating patterns in proton leak in the PUFA low vitamin E group was not possible, due to large variance in membrane potential measurements.

Phosphorlyation kinetics did not differ with training within either PUFA diet (Figure 6-6 B, D), but training had substantial effects in both MUFA diets (Figure 6-6 A, C). Training resulted in an upward shift in the phosphorylation curve in the MUFA low vitamin E diet, showing increased respiration rate without a corresponding increase in membrane potential. Exercise resulted in a rightward shift of the phosphorylation curve in the MUFA high vitamin E diet, which shows that increased oxygen consumption would result in increased membrane potential as well in this diet group. These shifts indicate that the response of the phosphorylation system to training differs based on diet and vitamin E regime.

Diet	Vitamin. E	Training	Variable 1	Variable 2	Pearson Corrleation	N	P-Value
MUFA	High	*Exercised*	Flight Dur.	MP-4	-0.777	7	0.040
		Trained	Flight Dur.	State 3 ΔΨ	-0.991	4	0.009
		Trained	Training Dur.	MP-3	0.735	7	0.060
	Low	*Trained*	Flight Dur	RS-3	-0.947	5	0.015
		Trained	Flight Dur.	RS-4	-0.976	5	0.004
		Trained	Flight Dur.	MPC-3	-0.905	5	0.035
		Trained	Flight Dur.	MPC-4	-0.926	5	0.024
PUFA	High	*Trained*	Flight Dur.	RS-3	0.862	7	0.013
		Trained	Flight Dur.	RS-4	0.863	7	0.012
		Trained	Flght Dur.	MP-3	0.967	6	0.002
		Trained	Training Dur.	RS-4	0.864	7	0.012
		Trained	Training Dur.	MP-3	0.782	7	0.038
	Low	*Trained*	Training Dur.	MP-4	-0.986	6	0.001

Table 6-2. Correlation coefficents for all significant correlations of mitochondrial parameters against ultimate flight duration, and total training duration. RS: Rotenone succinate, MP: Malate pyruvate, PC: palmitoyl carnitine. -3 indicates state 3 respiration, -4 indicates state 4.

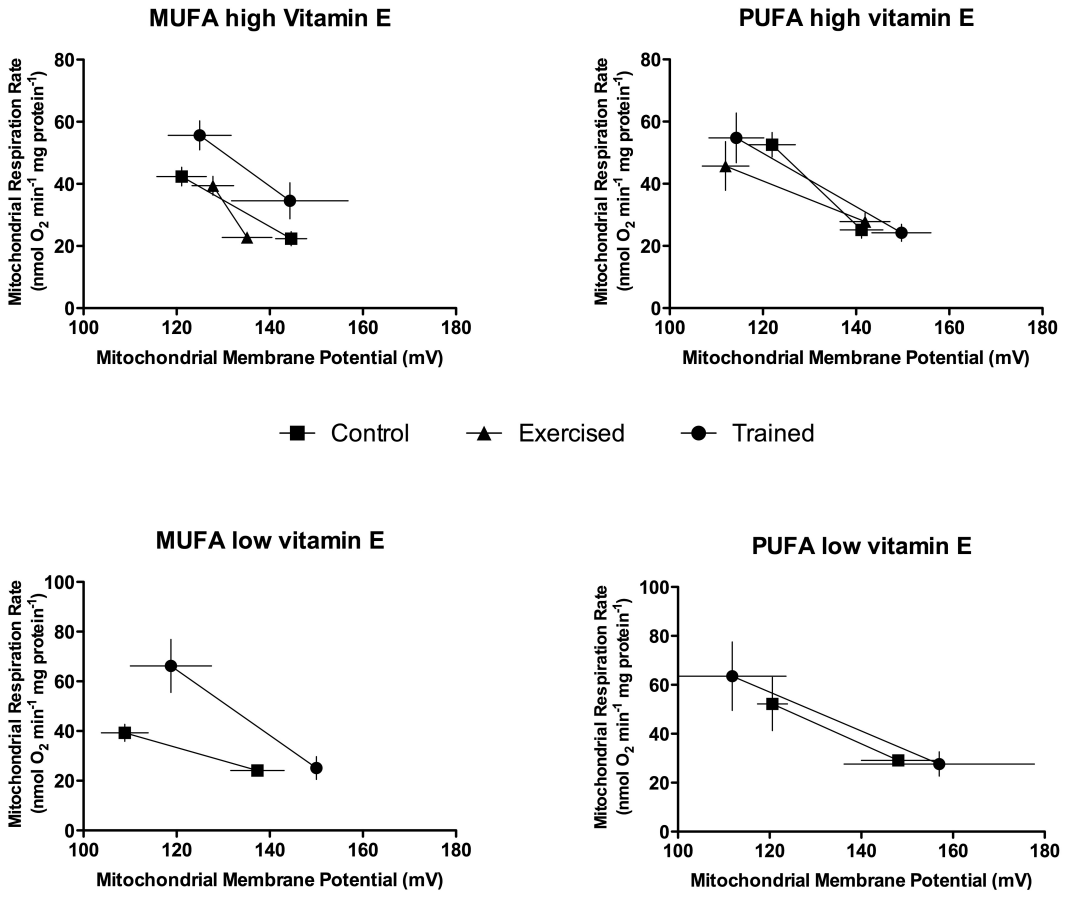

Figure 6-4. Kinetics of substrate oxidation for each of the diet groups (panels) and training groups (separate lines). Plots represent means ± SEM, where the upper left plot corresponds to state 3 respiration rates and membrane potential, and the lower right plot corresponds to state 4 respiration rates and membrane potential.

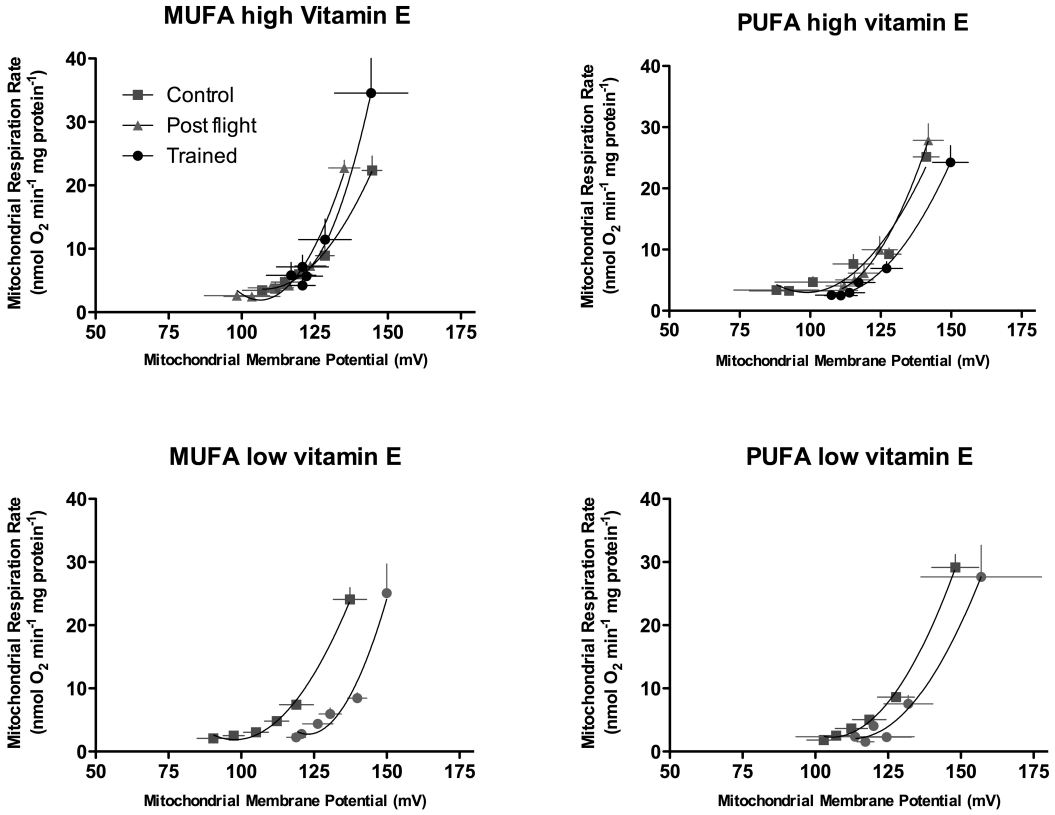

Figure 6-5. Kinetics of mitochondrial proton leak for each of the diet groups (panels) and training groups (separate lines). Upper right of each panel represents maximal state 4 respiration rate and membrane potential, which were then titrated with malonate in order to determine the kinetic curves. Plots represent means ± SEM.

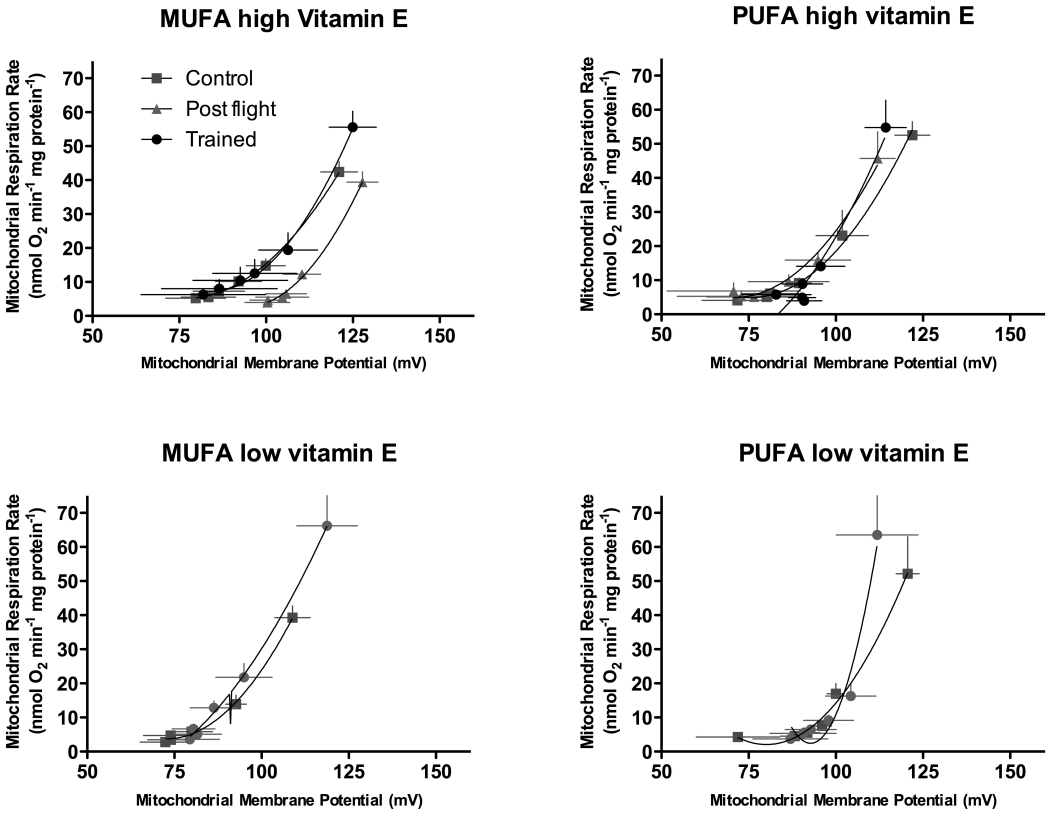

Figure 6-6. Kinetics of mitochondrial phosphorylation for each of the diet groups (panels) and training groups (separate lines). Upper right of each panel represents maximal state 3 respiration rate and membrane potential, which were then titrated with malonate in order to determine the kinetic curves. Plots represent means ± SEM.

6.3.4 *Top Down Elasticity Control Analysis*

Due to the complexity of the kinetic curves for proton leak, phosphorylation, and substrate oxidation, top down elasticity control analysis was employed in an attempt to better quantify the trends described above. This analysis partitions oxidative phosphorylation into three parts: substrate oxidation ($\Delta\Psi$ producer), proton leak ($\Delta\Psi$ consumer), and phosphorylation ($\Delta\Psi$ consumer). In this analysis, the $\Delta\Psi$ is the intermediate between the $\Delta\Psi$ producer, and the $\Delta\Psi$ consumers. This analysis results in the determination of a coefficient of control for each component of the system over the other components in the system, where 0 indicates no control and 1 indicates complete control. By plotting these coefficients, we can more readily assess how the experimental treatments changed the dynamics of the system.

In state 3 respiration, there were few differences among diet treatments, except within the *exercised* treatment. Exercise substantially increased control over substrate oxdation (Figure 6-7 A) and proton leak (Figure 6-7 B) by both substrate oxidation and by phosphorylation in the MUFA high vitamin E group relative to PUFA high vitamin E group. These trends indicate a more dominant role of substrate oxidation in determining the total flux through the system as a result of the MUFA high vitamin E diet and exercise. Surprisingly, these differences were no longer evident after two days of rest in the *trained* group. The effect of exercise was also evident during state 4 respiration (Figure 6-8), where leak had no control over substrate oxidation in the MUFA high vitamin E diet group, but had almost 50% of the control in the PUFA high vitamin E diet group (Figure 6-8 A, B).

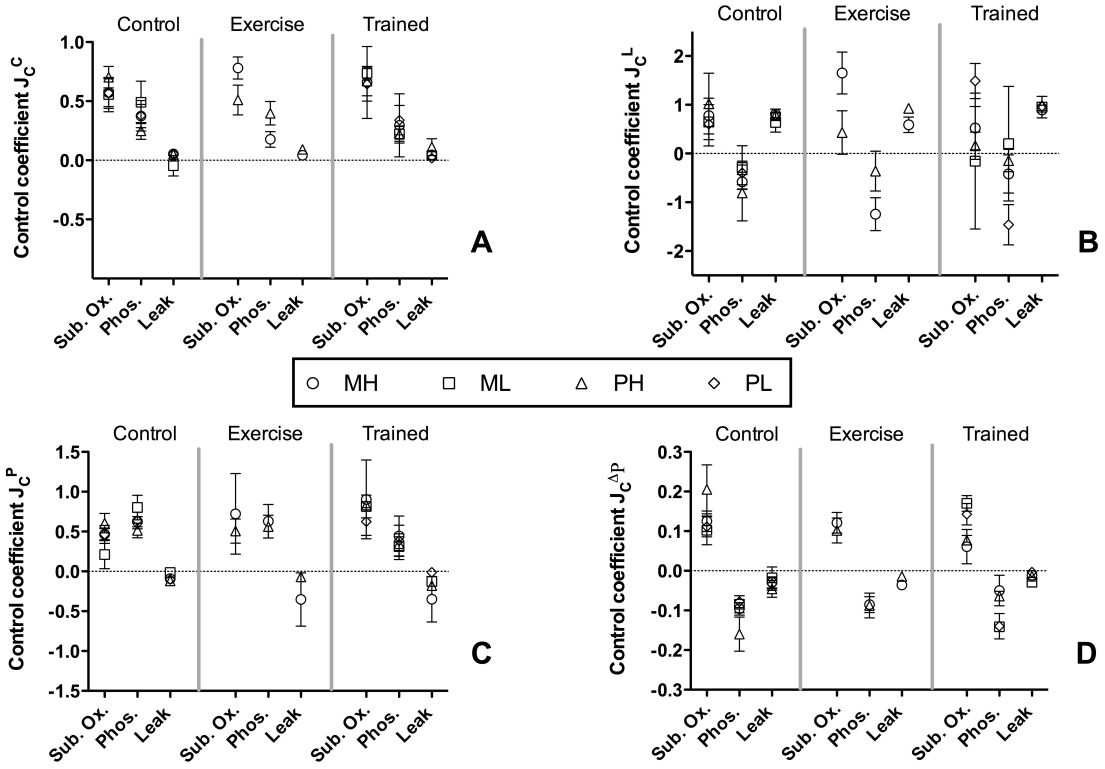

Figure 6-7. Control coefficients determined by top down elasticity analysis for state 3 respiration. Panel A (J_c^c) shows the control over substrate oxidation by the variables on the x-axis. Panel B (J_c^l) shows the control over proton leak, panel C (J_c^p), the control over phosphorylation and panel D ($J_{c^{\Delta p}}$), the control over ΔP by the three components of the system on the x-axis. Substrate oxidation (Sub. Ox.), phosphorylation (Phos), and proton leak (Leak). See text for details. Plots represent means ±SEM.

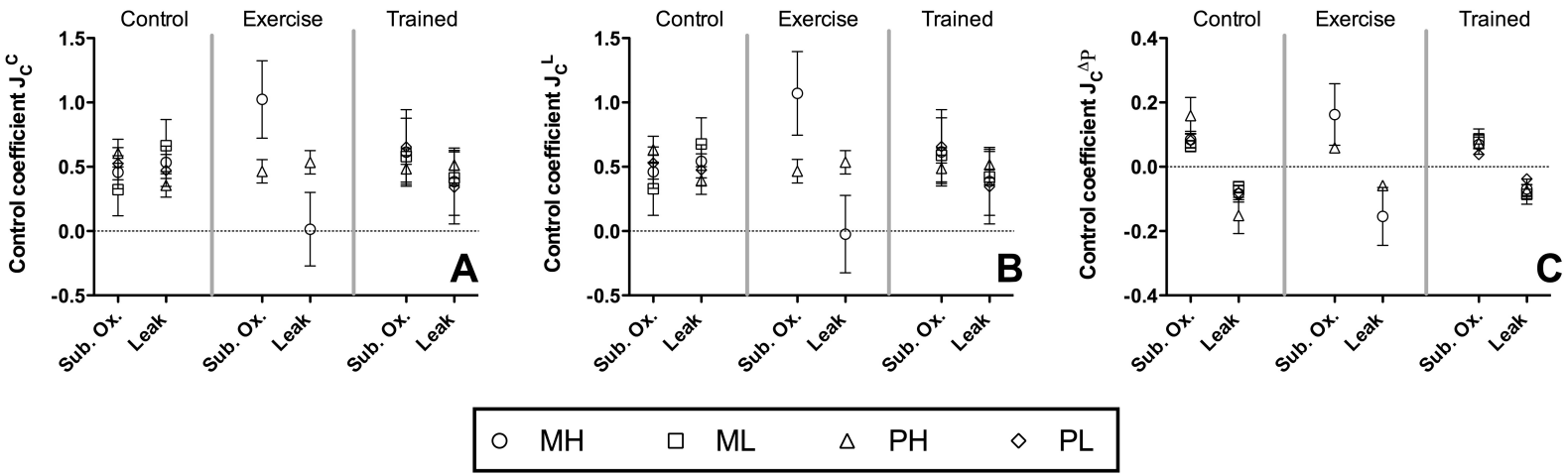

Figure 6-8. Control coefficients determined by top down elasticity analysis for state 4 respiration. Panel A (J_c^c) shows the control over substrate oxidation by the variables on the x-axis. Panel B (J_c^l) shows the control over proton leak, panel C ($J_c^{\Delta P}$), the control over ΔP by the three components of the system on the x-axis. Substrate oxidation (Sub. Ox.), and proton leak (Leak). See text for details. Plots represent means ±SEM.

6.4 Discussion

These data show that mitochondrial function is affected by both exercise and diet. It seems that the greatest perturbations in mitochondrial function are only evident immediately after flight implicating oxidative damage is responsible. These data to not provide strong support for greater capacity or efficiency of oxidative phosphorylation as a result of diets high in PUFA. Instead, it seems that high dietary PUFA helps reduce ROS release, which results in less oxidative damage during exercise. It is this damage that seems to alter mitochondrial function, possibly reducing whole animal exercise performance.

6.4.1 *Mitochondrial Respiration Rates and ROS Production*

Mitochondria from European starlings had the highest rates of oxygen consumption while oxidizing succinate in the presence of rotenone. Oxidation rates of malate-pyruvate were similar to those of palmitoyl-carnitine indicating respiration is limited by either the Krebs cycle or complex I, as electrons enter the electron transport chain at complex II (via $FADH_2$) during succinate oxidation. Since $FADH_2$ is a product of ß-oxidation during the breakdown of fats, $FADH_2$ linked respiration should be high to accommodate the very high rates of fat metabolism necessary for flight. However, the rate of oxidation of palmitoyl-carnitine was relatively slow. It could be that transport of palmitoyl-carnitine into the mitochondrion is limiting in preparation, but this could be overcome by increases in mitochondrial density *in vivo*, or aided by greater mobilization rates of polyunsaturated fatty acids by the carnitine-palmitoyl transferase (CPT; Price et al., 2011). There was no apparent effect of diet on respiration rates of mitochondria oxidizing any substrate, except for a trend towards higher respiration rates as a result of

the PUFA diet while oxidizing palmitoyl-carnitine in the high vitamin E groups. When pooled with low vitamin E diet groups, this trend persisted, which may indicate PPAR-mediated upregulation of CPT as a result of dietary PUFA (McClelland, 2004).

6.4.2 *Diet, Training, and Flight*

Although there was little effect of dietary fatty acids on mitochondrial respiration rates of all the substrates tested, birds fed low vitamin E seemed to have a more dramatic response to training. We did collect data on low vitamin E birds immediately after flight, but in high vitamin E fed birds, all training effects were attenuated immediately post-flight for both diets. Yet, flight duration two days prior to sampling (*trained* birds) correlated negatively with mitochondrial respiration rates, particularly in the MUFA low vitamin E group. Therefore, the low vitamin E fed birds may increase oxidative capacity during training in anticipation of reductions in oxidative capacity suffered due to high oxidative damage in flight (Table 6-2). This could indicate either a reduced capacity to dispose of ROS, or higher rates of ROS production in flight as a result of the MUFA diet. Within the high vitamin E diet group, there was a significant training effect, but only when oxidizing malate and pyruvate. This likely indicates the upregulation of citrate synthase in the Krebs cycle, as this has been shown to be upregulated during migration (Maillet and Weber, 2006; McFarlan et al., 2009), possibly as a result of a high PUFA diet, and may be upregulated in flight (Price et al., personal communication)

The most obvious effect of diet was an increase in ROS release rates in the MUFA fed birds, not including the *flight* birds (Figure 6-1 G). This was primarily a product of a reduction in ROS release during training in the birds fed the PUFA diet. Birds fed the PUFA diet, and especially those fed the PUFA high vitamin E diet, had a

substantial reduction in ROS release rates as membrane potential increased; they did not conform to a well established relationship between membrane potential and ROS production (Korshunov et al., 1997; Turrens, 2003; Votyakova and Reynolds, 2001); Figure 6-3). We did not control for any endogenous antioxidants present in the tissue. Thus, it may be that birds with higher resting membrane potentials due to training also have higher endogenous antioxidant capacity. Interestingly, this relationship was no longer apparent immediately after exercise, similar to the other training effects discussed above. After flight the PUFA high vitamin E group showed the expected positive relationship between membrane potential and ROS release rates, potentially indicating a depletion of antioxidants during flight. There was no relationship between membrane potential and ROS release rates in *untrained* birds, presumably due to adequate antioxidant capacity at rest.

There was a differential response of proton leak and substrate oxidation to exercise depending on diet. The kinetic curves and the control coefficients indicate birds on the PUFA high vitamin E diet displayed little disruption of mitochondrial function after exercise, as control of substrate oxidation was shared between the substrate oxidation system and the phosphorylation systems, similar to *trained* and *untrained* birds. On the other hand, MUFA high vitamin E fed birds dramatically shifted control of substrate oxidation to be almost completely controlled by the substrate oxidation system after exercise. This is likely a result of the reductions in respiration rates and phosphorylating membrane potential during exercise. The reduction in substrate oxidation would make the reactions that contribute to substrate oxidation rate limiting, controlling the flux through the rest of the system. Reductions in the substrate oxidation

system during flight could be a protective mechanism in response to high ROS production rates since high substrate oxidation rates have been implicated in the overproduction of ROS in heat stressed birds (Kikusato et al., 2010). Therefore, it is plausible that reductions in substrate oxidation may be a mechanism to alleviate the higher ROS production found in the MUFA fed birds, possibly at the cost of reduced maximal respiration and ATP production rates.

Rates of ROS production can be influenced by the phospholipid fatty acid composition of the mitochondrial membranes, where greater polyunsaturation, mainly through the incorporation of shorter chain unsaturated fatty acids (18:2 ω-6), may result in reduced rates of ROS production and damage (Pamplona et al., 1996). The reason for the lower ROS release rates with the PUFA diet could be a result of fewer radicals escaping electron transport, possibly as a result of alterations in membrane fluidity due to the fatty acid composition of the bi-layer, or due to conformational changes in membrane bound enzymes as a result of protein-lipid interactions (Yeagle, 1989). Alternatively, due to the greater susceptibility of PUFA to ROS damage, antioxidant systems could be maintained at a higher level in response to a high PUFA content in the cell or mitochondrion. Thus, more endogenous antioxidant capacity may also be available in birds fed the PUFA diet, regardless of the vitamin E present in the diet.

6.4.3 *Conclusions*

There are a number of findings in this study that help to explain the metabolic benefits provided by diets rich in PUFAs. Primarily, it seems there is a differential response to exercise contingent on diet fatty acid saturation. Mitochondrial respiration rates were reduced immediately post-flight in the MUFA diet, resulting in a disruption of

the oxidative phosphorylation system by a shift in metabolic control away from phosphorylation and toward substrate oxidation. PUFA fed birds had lower ROS release rates, and tended not to be as dramatically affected by flight (i.e. no correlation), or seemed to be able to increase mitochondrial respiration rates as flights progressed. Although evidence for reduced proton leak as a means to increase flight efficiency is not compelling, it does seem that a diet high in PUFAs reduces the production of ROS or impact of oxidative damage, preventing the perturbation of mitochondrial function. The disruption of mitochondrial function by ROS could result in less efficient production of ATP (due to the loss of oxygen and electrons from the system) or lower maximal respiration rates, accounting for the higher flight costs that have been previously shown in European starlings fed a MUFA diet and flown in a wind tunnel.

Birds fed the MUFA diet seemed to benefit more from vitamin E supplementation. Although we do not have flight performance data for these birds, nor do we have tissue fatty acid composition, these data will be available at a later date allowing useful comparisons to be made.

Our results provide evidence that fatty acid composition affects mitochondrial function of birds, but the effects are most evident immediately after flight. This indicates that accumulated oxidative damage may limit flight duration, raising the interesting possibility that long distance migrants may have evolved mechanisms to reduce ROS production, or enhance antioxidant capacity. Few differences were found within or between the *trained* or *untrained* birds indicating the perturbations in mitochondrial function are corrected quickly after flight, although some parameters were correlated with the duration of a flight made two days previous to sampling. As mentioned previously,

there is support for the fuel hypothesis (Price et al., 2008), but gains in performance simply as a result of fuel would not be realized if exercise-induced mitochondrial damage was too great. Therefore, maximum gains in exercise performance are likely to result from both the greater mobilization of PUFAs, and the maintenance of proper mitochondrial function, possibly through the mediation of ROS production.

6.5 References

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7 THESIS SUMMARY

7.1 Summary

The studies presented in this thesis provide new information on how migratory birds have evolved to overcome the physiological challenges of long duration flight. Although previous studies have focused on either the metabolic adaptations that allow birds to sustain flight for long durations, or the potential limitations on migration imposed by dehydration, never before has it been demonstrated that birds alter their metabolism in response to water loss. Increasing the rate of protein catabolism to balance water losses incurred during flight is a metabolic strategy that has been documented only in the studies contained in this thesis (Chapters 2 & 3). Following this novel finding, I tailored my subsequent studies (Chapters 4 and 5) to include humidity manipulations in an attempt to expand our knowledge of this metabolic strategy in other species of migrants, and to investigate possible whole-animal consequences of this strategy. Also, the metabolic and renal responses to relatively short duration flights had not previously been investigated, and new information from these studies may help to explain the metabolic differences between short distance and extreme long distance migrants, and the role of kidney function in flight. In chapter 5, I investigated biochemical responses to diet selection, and I found that oxidative damage could also limit flight performance or duration. Here I discuss some of the implications of the findings of this thesis for the flight and stopover phases of migration.

7.2 Flight

Although Chapters 2 and 3 are the first empirical studies of the role of protein catabolism in flight, there are numerous studies investigating the possible consequences of protein catabolism for long distance migration (Bauchinger et al., 2005; Bauchinger and Biebach, 2001; Biebach, 1990; Karasov and Pinshow, 1998; Pennycuick and Battley, 2003). Now that we have a better understanding of the role of protein catabolism in flight, we can make more informed predictions regarding the costs and benefits of this metabolic strategy.

It is not known if the protein catabolic pathways responsible for the protein-for-water mechanism are distinct from those involved in normal protein turnover, or other forms of exercise-induced muscle damage. Identifying the processes involved and the specific proteins being catabolized will be extremely important to our understanding of the impact of this strategy at the whole animal level by reducing the functional capacity of organs and muscles. Bauchinger and Biebach (2001) found that flight induced reductions in muscle mass in migratory garden warblers (*Sylvia borin*) was primarily the result of myofibrillar catabolism, decreasing the myofibrillar to sarcoplasmic protein ratio. Thus, the contractile components of the muscle were degraded to a greater degree than the rest of the muscle cell in flight. This change could influence the dynamics of calcium ion cycling in the cell, which is necessary for muscle contraction and is controlled by the sarcoplam. Whether this pattern of selective protein degradation is beneficial to flight energetics or muscle function in migratory birds has not been directly investigated, but presumably muscle function is not degraded substantially or these birds would not have arrived at the stopover location where they were sampled. However,

rebuilding myofibrillar protein during stopover may be costly (below). This study is among the only evidence for selective degradation of specific proteins during flight in migratory birds. It would be interesting to investigate which classes of proteins are catabolized within the organs that are vital to nutrient and waste processing (liver and kidney) during flight. As mentioned in Chapter 5, investigating whole animal processes such as GFR immediately after a long duration flight would provide insight into the effect the catabolism of these organs has on stopover (see below).

As an overall strategy for flight, increasing the relative contribution of protein to the fuel mixture in response to water restriction can affect flight range. Due to the higher rate of mass loss under high evaporative water loss conditions, both due to water losses and the catabolism of protein, flight costs will be lower and range may be extended. For example, the flight range of a 30 g thrush with 7.5 g of initial fat was estimated for birds flown under high or low evaporative water loss conditions using software specifically designed to calculate flight ranges of migratory birds from their body composition (FLIGHT v1.5; Pennycuick and Battley, 2003). In Chapter 3, I showed that under high evaporative water loss conditions, 17% of energy was derived from protein resulting in an estimated maximum flight range of 3580 km. Using identical criteria except for a 10% protein contribution, as found for birds flying under the low evaporative water loss conditions, maximum flight range is 2780 km, a reduction in flight range of 28%. Thus, the use of protein-for-water can extend flight range, allowing migrants to escape unfavourable conditions and seek better stopover habitat in addition to aiding in the maintenance of the water budget. Finding optimal stopover habitat may become more crucial under unfavourable conditions, given the additional need to replenish greater

amounts of protein. The cost of this strategy will mostly be realized during stopover, assuming that protein or fat stores are sufficient for a bird to reach suitable stopover habitat.

One drawback of this type of modeling is we do not know the threshold of protein degradation, after which functional losses are realized. For fat, it can be assumed that a very small fraction (< 1 %) of total fat is functional (comprises membranes etc.), so in these models all fat is considered fuel. For protein, we do not know when functional losses will occur due to in-flight protein catabolism for specific tissues. Investigating these physiological limitations will be difficult, but could provide valuable information helping improve our understanding of the consequences of protein catabolism in flight.

Shorter duration flights do not seem to require additional protein catabolism under high evaporative water loss conditions (Chapter 4), nor do the higher rates of water loss affect renal function in flight (Chapter 5). Due to the high contribution of carbohydrate and protein early in flight, it seems that birds early in flight are in water surplus. Therefore, it was unnecessary for these birds to catabolize more lean mass, or reduce GFR. In fact, allowing high rates of excretory water loss early in flight may be an adaptive mechanism to reduce mass quickly. Although birds may eventually run a water deficit, requiring protein catabolism as flight duration increases, the additional energy savings from this early mass loss could also increase flight range. Thus, it seems that the kidney may be important to the energetic costs of flight, in addition to its role in osmoregulation. Due to the water surplus early in flight, it is also possible that migratory birds switch to a short-hop migratory strategy in the face of high evaporative water loss conditions (Biebach, 1990).

Finally, my results indicate that diet can have a profound influence on the metabolism of birds in flight. Although the energetic benefits of PUFA have been shown many times (Pierce et al., 2005; Price, 2010; Price and Guglielmo, 2009), the mechanisms responsible for these benefits are still in question. Chapter 6 is the only study to date investigating mitochondrial energetics of a migratory bird. Although proton leak was hypothesized to be involved in the gains in exercise performance with high PUFA diets, my data show that ROS are more important to the effects of diet on mitochondrial function. Therefore, an animal's antioxidant capacity may be a determinant of flight duration. An assessment of the antioxidant systems of extreme long distance migrants may reveal unique biochemical adaptations to reduce or scavenge ROS. Currently, the high levels of polyunsaturated fatty acids in the tissues and membranes of some migrant birds are thought to activate peroxisome proliferator-activated receptors (PPAR) directly, which upregulate enzymes involved in fatty acid oxidation (Maillet and Weber, 2006). Instead, it may be that high levels of PUFA simply act to reduce rates of mitochondrial ROS production, allowing long duration, high intensity activity without excessive oxidative damage.

7.3 Stopover

Protein catabolism in flight due to high evaporative water loss likely increases stopover duration. Although flight is extremely expensive, the majority of energy spent during migration occurs at stopover (Wikelski et al., 2003). This is primarily a product of the time spent at stopover and the cost of basic metabolic processes such as thermoregulation and food assimilation, and behaviours such as foraging. After a

migratory flight over the Sahara, Garden warblers (*Sylvia borin*) have livers, intestines, kidneys and pectoralis muscles that are as much as 50% smaller than before flight (Bauchinger et al., 2005; Karasov and Pinshow, 1998). During the first few days of the subsequent stopover, these birds show low rates of mass gain (Gannes, 2002; Karasov and Pinshow, 2000). It is thought that during this initial period, food intake rates are limited by the capacity of the organs to digest and assimilate food (Karasov and Pinshow, 2000; Karasov et al., 2004). It is not until three days after arrival that these birds are able to resume feeding at high rates (Gannes, 2002), a timeframe that corresponds with the recovery of organ mass (Karasov et al., 2004). In order to rebuild lean tissue, dietary protein requirements are increased (Munoz-Garcia et al., 2012). Therefore, variation in the rate of evaporative water loss experienced in flight due to weather could affect the length of stopover, alter dietary requirements of during refueling, or change habitat requirements throughout a season (Shochat et al., 2002). If higher dietary protein is required to rebuild organs and tissues, this could affect water requirements, fattening rate, or the distribution of animals during stopover (Sapir et al., 2004; Shochat et al., 2002; Tsurim et al., 2008). Future studies could investigate the effect of ambient conditions experienced during flight on diet choice and habitat use, and stopover duration during migration.

One benefit of in-flight protein catabolism to stopover may be a reduction in basal metabolic rate after arrival (Battley et al., 2001). After a multi-day flight, Great knots (*Calidris tenuirostris)* reduced BMR by 42% when compared to pre-flight birds. The reduction in metabolism due to smaller organs likely contributes to rapid refueling, since less energy is spent on maintenance metabolism. Recent evidence suggests that songbirds

may use hypothermia at night during stopover (Wojciechowshi and Pinshow, 2009), resulting in accelerated mass gain. These strategies in birds are similar to what has been proposed for migratory bats (McGuire et al., 2011), indicating a universal advantage to reduced metabolic cost during stopover.

7.4 Climate change

Trans-Sarahan migrant birds have evolved migratory timing and strategies to deal with high rates of water loss in flight. These birds are able to overcome reductions in organ mass, and likely budget time to account for extended stopovers. In North America, average temperatures are expected to rise by as much as 3 °C each century, but there is much variability across the continent (Karl et al., 1996). Within the continental USA, the greatest increases in temperature will occur in the desert southwest, and along the east and west coast (Karl et al., 1996). These areas correspond to the Central, Atlantic, and Pacific migratory flyways (Lincoln et al., 1998). We do not know how Nearctic-Neotropical migrants, that have not historically had to cope with high rates of water loss in flight, will need to adapt to higher rates of protein catabolism as a result of higher temperatures experienced during migration.

As global temperatures rise, many migratory birds may be required to increase protein catabolism in flight to offset water losses, or change migration strategy. This may result in unanticipated delays during stopover increasing the energetic cost of migration, which may ultimately result in increased mortality during migration, delayed arrival at the breeding grounds, or impaired reproduction Moving forward, it will be important to

identify the consequences of in-flight protein catabolism in many species of migratory birds, with the goal of gaining a better understanding of both whole animal organ function after flight, and ecological implications of higher lean mass losses. Understanding the inter-specific variation in metabolic strategies may aid in predicting population level changes that will likely occur as a result of climate change.

7.5 Concluding remarks

The behaviour of migration is one that requires unique physiological strategies, ranging from metabolic adaptations that allow long duration activity, to adaptations that allow orientation, or the proper timing of life history events (molt, breeding etc.) among others. This thesis provides new insight into a small part of migration biology. It is my hope that these studies have impact in the field, and help to advance our understanding the physiology of migration.

7.6 References

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Appendix A: Chapter 3 Supplementary material

Materials and Methods

Animal Care:

Swainson's thrushes (*Catharus ustulatus*; n = 27) were captured on Long Point (42°34'57.70"N, 80°23'52.49"W, Ontario, Canada) during migration in spring and fall of 2010, and were transported to the University of Western Ontario Advanced Facility for Avian Research within 3 h of capture. Birds were kept in outdoor aviaries initially, and later moved to indoor aviaries until experimental flights began. Birds were fed a banana mash, agar-based diet and maintained on natural civil twilight light cycle from May through October after which they were maintained on 12L:12D. Birds were held for a minimum of 2 weeks before flights began to ensure sufficient acclimation to captivity. Birds were collected with permission from the Canadian Wildlife Service (Appendix B: permit # CA0256) and all animal care protocols followed the Canadian Council on Animal Care guidelines and were approved by the University of Western Ontario Council on Animal Care and the Animal Use Subcommittee (Appendix B: Protocol # 2010-216).

Flights:

Birds were habituated to the wind tunnel during a series of two to five 10-20 min flights. Birds that were willing to fly without additional encouragement were selected for the experiment (n = 5), and kept in groups of two or three in 70 cm x 123 cm x 185 cm high aviaries. Flights started either at 15:00 or at 18:00, and each individual always flew at the same time of day. Food and water were removed 3 h prior to the start of the flight. At

the beginning of the flight, birds were captured from their cage, weighed (± 0.01g, Acculab EC211), scanned once in the QMR, and released by hand into the wind stream. The initial flight was terminated once the bird showed signs of lack of motivation (attempted perching). Birds were removed from the tunnel, weighed, scanned in the QMR, and blood sampled (26 ga needle, 70 µl heparinized capillary tubes, approximately 150 µl) from a brachial vein within 5 minutes of the end of the flight. We failed to collect blood from one individual for both flights in a pair. Each individual bird was allowed a minimum of 3 days and a maximum of 11 days to recover before the next flight. The next flight was carried out exactly as the initial flight, except that the ambient humidity was opposite the initial flight (HEWL or LEWL), and the flight was terminated by the investigator at the same time as the initial flight. Five individuals completed at least one paired flight, and in some cases, up to 4 paired flights, resulting in 20 flights overall; the initial humidity conditions for each paired fight was determined randomly

QMR and Plasma Analysis:

Body composition was measured using a QMR customized for small birds and bats (model Echo-MRI-B, Echo Medical Systems, Houston, TX, USA). QMR has proven to be very accurate and precise for a variety of animals (Guglielmo et al., 2011; McGuire and Guglielmo, 2010; Taicher et al., 2003). Validation against oven drying and chemical extraction with petroleum ether shows that this instrument has precisions (CVs) of 2.4 %, 0.7 %, and 1.9 %, and relative accuracies of approximately ± 11 %, ± 1 % and ± 2 %, for fat, wet lean and total body water masses of small birds, respectively (Guglielmo et al., 2011). For analysis, awake birds were placed into a ventilated clear plastic holding tube (5 cm diameter) and scanned for 138 s.

Blood was centrifuged (IEC MicronCL 17 centrifuge, Thermo Scientific, Nepean, Ontario) at 13,000 x g for 10 minutes, and plasma was stored at -80 °C until analysis. Both uric acid and osmolality were determined in undiluted plasma. Uric acid was measured by endpoint assay (Wako Uric Acid 20R/30R kit). Plasma osmolality was measured in 10 μL of plasma using a Wescor Vapro 5520 vapor pressure osmometer calibrated as per the manufacturer's instructions as in Chapter 2 and Gerson and Guglielmo (2011)

Wind Tunnel:

The study was conducted using a wind tunnel designed for bird study at the Advanced Facility for Avian Research at the University of Western Ontario. The wind tunnel has an all-steel recirculating design with the test section enclosed in a plenum (approx 4 m x 5 m x 2.5 m high) that allows for simulation of altitudes up to 7000 m by regulating barometric pressure, temperature and humidity. A 0.5 m open test section allows birds and investigators easy entry and exit of the closed test section with minimal disturbance to the flow. The closed test section is 2 m long and octagonal in cross section with 1 m vertical and 1.5 m horizontal dimensions. Maximum true air speed is 20.7 m/sec, and temperature and humidity can be regulated precisely between -15 to 30 °C and 10 – 90 % RH. Outside of the wall boundary layer and without an upstream net, velocity uniformity (standard deviation of velocity) is 0.5 % and turbulence intensity ranges between 0.12 – 0.27 % (measured by single wire hot wire anemometry by the manufacturer, Aiolos Engineering, Toronto, ON, Canada). Birds in this study were flown with a net (2.5 cm x 2.5 cm mesh, with a 0.15 mm filament thickness) at the front of the test section.

Statistical Analysis:

Model Choice and Selection

We used general linear mixed models (Proc Mixed; SAS 9.2) for all statistical analyses to account for repeated measures of individuals in an unbalanced design (some individuals were involved in more than one paired flight). This approach alone allowed us to account for covariates, test for interactions and account for the repeated measures of individuals, without having to sacrifice data due to an unbalanced design. For each model we verified that the error structure of dependent variables followed a Gaussian (normal) distribution, and visual inspection of residual-quantile plots, residual-predicted mean plots, and plots of residuals against treatment and against flight duration confirmed that all data met the assumptions of a general linear mixed model (Zuur et al., 2009). We determined that the covariance structure was best-modeled using compound symmetry and subjects were nested by flight-pair. Since flights occurred during both spring and fall, but paired flights within an individual always occurred within 10 days of each other, nesting by flight pair controls temporally within individuals, and ensures more accurate comparison between flights within a pair (Littell et al., 1998; Zuur et al., 2009).

Models testing for the effects of humidity treatment on the reductions in mass, fat mass, and lean mass contained humidity treatment as a class variable, flight duration and initial body mass as covariates, and all two-way interactions. All non-significant terms were dropped from the models sequentially until only significant terms remained. Differences between humidity treatments in rates of mass loss, lean mass loss, and fat mass loss were determined by testing for significant treatment by flight duration interactions, indicating differences in slope. For both reduction in total mass and lean mass, the interaction of

humidity treatment and flight duration was significant (see below for statistical output from all models). For reduction in fat mass there was no interaction between humidity treatment and flight duration, and after dropping this term from the model, there was no effect of humidity treatment, but a significant effect of flight duration. When slopes differed between humidity treatments (mass and lean mass), parameter estimates were generated separately for each treatment. Rates of mass loss and lean mass loss (humidity treatments separated), and rate of fat mass loss (humidity treatments combined) were estimated using only flight duration as a factor in the model, and 95% confidence intervals were calculated from these estimates and standard errors. For mixed models testing for differences between humidity treatments with flight costs, relative body water, plasma osmolality, plasma uric acid, and endogenous water production rate as dependent variables, there was no significant interaction between flight duration and humidity treatment. Thus, humidity treatment and flight duration were tested only as main effects, and only significant effects are presented in the main text.

Additional Analysis

As a check on the robustness of the general linear mixed models against more well-established statistical methods, the effect of humidity treatment on each dependent variable was tested using paired t-tests where only one matched pair of flights from each individual was used per test (12 possible combinations). Due to the paired nature of the experiment, effects of flight duration and initial body mass on dependent variables are assumed to be accounted for experimentally, and are thus not controlled for statistically. We report mean, median, maximum, and minimum degrees of freedom, and associated P-values in Table S2. Our analysis shows that even when the variance due to different

flight durations is not accounted for, and significant amounts of data are excluded from each test, the results generally corroborate the findings of the general linear mixed models. One exception is plasma uric acid, which is no longer significant except when referencing the minimum P-value using this analysis. However, plasma uric acid concentration is affected by both the rate of production and excretion, and birds were observed to produce droppings in flight. Thus the plasma concentration is best used as an index, and does not wholly reflect uric acid production rate during flight.

Bird ID	Flight Pair	Condition	Pre flight mass (g)	Pre flight Fat (g)	Pre flight lean (g)	Pre flight total body water (g)	post flight mass (g)	post flight fat (g)	post flight lean mass (g)	Post flight total body water (g)	Flight duration (min)	Plasma uric acid (mmol/L)	Plasma osmolality (mmol/kg)
1	1	lewl	35.50	7.08	23.11	18.08	34.10	6.28	22.62	17.87	126.02		
1	1	hewl	36.90	8.90	22.96	17.61	35.20	8.12	21.97	17.18	126.22		
2	1	hewl	32.50	4.36	23.27	17.92	31.10	3.70	22.47	17.68	116.67	0.76	386.00
2	1	lewl	33.70	5.79	23.10	17.84	32.60	5.10	22.48	17.69	117.25	0.70	371.00
2	2	hewl	34.20	6.01	23.32	17.91	31.80	5.07	21.87	17.07	181.00	0.86	371.00
2	2	lewl	33.30	4.83	23.44	18.23	31.30	3.73	22.75	18.00	181.05	0.85	369.00
2	3	hewl	33.70	4.85	23.84	18.67	30.00	3.13	22.04	17.02	315.00	0.70	377.00
2	3	lewl	33.80	5.46	23.27	18.26	30.90	3.75	22.15	17.86	315.80	0.76	369.00
2	4	lewl	35.50	7.02	22.89	17.66	33.20	6.12	22.02	17.17	181.65	0.52	368.00

2	4	hewl	41.20	12.90	23.13	18.30	38.90	11.75	22.12	17.11	180.51	0.91	378.00
3	1	lewl	30.60	3.50	22.23	17.27	29.70	3.11	21.81	17.00	67.31	0.73	
3	1	hewl	31.10	3.78	22.23	17.58	30.00	3.49	21.65	17.30	67.68	0.91	370.00
3	2	lewl	31.30	4.39	22.02	19.03	30.30	3.67	21.80	17.08	93.13	0.82	389.00
3	2	hewl	29.90	3.30	21.57	17.06	28.60	2.81	20.77	16.70	93.25	1.51	377.00
3	3	lewl	30.90	3.73	22.22	17.44	30.50	3.65	21.84	17.36	30.32		
3	3	hewl	30.80	4.19	21.54	17.04	30.40	4.11	21.08	16.50	30.82		
4	1	hewl	32.60	5.94	21.82	17.01	30.30	4.71	20.76	16.42	200.93	0.83	391.00
4	1	lewl	35.20	8.17	22.48	17.28	33.20	6.76	21.82	16.84	201.70	0.33	367.00
5	1	hewl	31.60	4.87	21.62	16.94	28.90	3.30	20.70	16.31	275.68	0.39	382.00
5	1	lewl	31.70	5.36	21.38	16.67	30.10	3.77	21.53	17.28	275.41	0.27	375.00

Table S 1. Complete dataset for all measured variables. Please see text for details on calculated variables.

Supplemental Statistical Output:

Additional output from statistical models for mass loss, lean mass loss, and fat mass loss. All relevant statistics for other dependent variables are presented in the main body of the text.

The models for dependent variables (mass loss, lean mass loss, or fat mass loss) used to test for effects of treatment (HEWL or LEWL) and flight duration are below. Please see above for a complete description of statistical methodology.

Dependent Variable= humidity treatment + flight duration + treatment*flight duration

Total mass loss

Full Model

Effect	NDF	DDF	F Value	Pr>F
Humidity treatment	1	7	0.47	0.5171
Flight duration_	1	7	64.74	<.0001
Interaction (Humidity*Flight duration)	1	7	13.11	0.0085

Regression to estimate rate of mass loss (g min^{-1}) HEWL

Effect	Estimate	Error	DF	t Value	Pr > |t|
Intercept	0.3052	0.1451	8	2.10	0.0686
Flight duration	0.01023	0.000805	8	12.71	<.0001

Regression to estimate rate of mass loss (g min^{-1}) LEWL

Effect	Estimate	Error	DF	t Value	Pr > |t|
Intercept	0.4048	0.2590	8	1.56	0.1567
Flight duration	0.0073	0.0014	8	5.06	0.001

Lean mass loss

Full model

Effect	Num DF	Den DF	F Value	Pr > F
Humidity treatment	1	7	0.13	0.7312
Flight duration	1	7	4.93	0.0617

Interaction (Humidity*Flight duration)	1	7	7.40	0.0297

Regression to estimate rate of lean mass loss (g min^{-1}) HEWL

Effect	Estimate	Error	DF	t Value	Pr > |t|
Intercept	0.4238	0.1647	8	2.57	0.033
Flight duration	0.0036	0.0009	8	3.88	0.0047

Regression to estimate rate of mass loss (g min^{-1}) LEWL

Effect	Estimate	Error	DF	t Value	Pr > |t|
Intercept	0.3635	0.2392	8	1.52	0.1671
Flight duration	0.0010	0.001325	8	0.8	0.4467

Fat mass loss

Full Model

Effect	Num DF	Den DF	F Value	Pr > F
Humidity treatment	1	7	0.97	0.3575

Flight duration	1	7	336.38	<0.0001
Interaction (Humidity*Flight duration)	1	7	0.31	0.5972

No interaction term

Effect	Num DF	Den DF	F Value	Pr > F
Humidity treatment	1	8	1.20	0.3059
Flight duration	1	8	336.37	<0.0001

Regression to estimate rate of mass loss (g min^{-1}); combined treatments fat loss rate

Effect	Estimate	Error	DF	t Value	Pr > |t|
Intercept	0.0016	0.05643	8	0.03	0.9783
Flight duration	0.0057	0.00031	8	18.34	<0.0001

Dependent Variable	Max DF	Median DF	Min DF	Mean p-value	Median p-value	Max p-value	Min p-value
Change in Mass (g)	4	4	4	0.073	0.061	0.167	0.028
Change in Lean mass (g)	4	4	4	0.032	0.026	0.068	0.005
Change in Fat mass (g)	4	4	4	0.304	0.147	0.935	0.046
Uric Acid (mmol/L)	3	3	2	0.167	0.143	0.377	0.038
Plasma Osmolality (mmol/kg)	3	2	2	0.241	0.191	0.531	0.089
Endogenous water production (g/kJ)	4	4	4	0.015	0.005	0.054	0.001
Flight costs (W)	4	4	4	0.608	0.580	0.977	0.302

Table S 2. Mean, median, minimum and maximum degrees of freedom (DF), t-values, and associated P-values from paired t-tests run on each dependent variable in all combinations of individual flight pairs presented in the main text. Please see supplementary statistical analysis section for more details.

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Appendix B: Permits and Ethics approval

Western

April 2, 2006

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2010

Dear Dr. Guglielmo:

Your Animal Use Protocol form entitled:
The effects of diet, season, body fat, age and antioxidant defense on metabolic fuel selection during exercise in migratory birds.
Funding Agency NSERC Discovery - Grant #311901-05; UWO Small ADF - Grant #SG05-32; CFI LOF, ORF

has been approved by the University Council on Animal Care. This approval is valid from **April 2, 2006 to April 30, 2007.** The protocol number for this project is 2006-011-04.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Bird-Wild	White Throated Sparrow	Adult & fledged juveniles M/F	C	90
Bird-Wild	Yellow Rumped Warbler	Adult & fledged juveniles M/F	C	90
Bird-Wild	Hermit Thrush	Adult & fledged juveniles M/F	C	90
Bird-Wild	Magnolia Warbler	Adult & fledged juveniles M/F	C	60
Bird-Wild	American Redstart	Adult & fledged juveniles M/F	C	60
Bird-Wild	Red Eyed Vireo	Adult & fledged juveniles M/F	C	60
Bird-Wild	American Robin	Adult & fledged juveniles M/F	C	60
Bird-Wild	White Crowned Sparrow	Adult & fledged juveniles M/F	C	60
Bird-Wild	Gray Catbird	Adult & fledged juveniles M/F	C	60
Bird-Wild	Purple Finch	Adult & fledged juveniles M/F	C	60

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol C. Guglielmo, J. Wasylenko, D. Cheshuk
Approval Letter - J. Wasylenko, D. Cheshuk

University Council on Animal Care • The University of Western Ontario
Animal Use Subcommittee • Health Sciences Centre • London, Ontario • N6A 5C1 • Canada

Western

April 8, 2010

This is the Original Approval for this protocol

Dear Dr. Guiglielmo:

Your Animal Use Protocol form entitled:
Energetics, fuel use, water balance and immunocompetence during exercise in migrating birds
Funding Agency NSERC - 311901-05 and up for renewal at present

has been approved by the University Council on Animal Care. This approval is valid from **April 8, 2010 to April 30, 2011.** The protocol number for this project is **2010-216 which replaces 2006-011-04 which has expired.**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED

Species	4 Year Total Numbers Estimated as Required	List All Strain(s)	Age / Weight
Songbird	660	All bird species as permitted by CWS and USFWS	all

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - C. Guglielmo, S. Waring

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Environment Canada Environnement Canada

CANADIAN WILDLIFE SERVICE - PERMIT
PERMIS - SERVICE CANADIEN DE LA FAUNE

		Permit to/for Permis de/pour **SCIENTIFIC-CAPTURE**	Permit no. No de permis **CA 0256**
Organization Organization **University of Western Ontario**	Issued under section Délivré en vertu de l'article 19	of de MIGRATORY BIRD REGULATIONS	
Surname Nom de famille **Guglielmo**	Name Prénom **Christopher**	Department Département **Department of Biology**	

1151 Richmond Street North
London On
N6A 5B7

Date of issue Date d'émission **June 19, 2009**	Date of expiry Date d'expiration **June 19, 2012**
Signature of holder Signature du détenteur	For the minister Pour le ministre

Special Conditions - Conditions spéciales

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.
2. Landowner's permission must be obtained prior to collecting on private property.
3. Permit or a copy of the permit to be carried in the field by all collectors.
4. The permit holder is authorized to collect and to possess for scientific research purposes, migratory birds – to wit: Cedar Waxwing (Bombycilla cedrorum), Wood Thrush (Hylocichla mustelina), Tree Swallow (Tachycinetra bicolor), Barn Swallow (Hirundo rustica), Purple Martin (Progne subis), White-throated Sparrow (Zonotrichia albicollis), White-crowned Sparrow (Zonotrichia leucophrys), Yellow-rumped Warbler (Dendroica coronata), Hermit Thrush (Catharus guttatus), Swainson's Thrush (Catharus ustulatus), American Robin (Turdus migratorius), Gray Catbird (Dumetella carolinensis), Red-eyed Vireo (Vireo olivaceus), Ruby-throated Hummingbird (Archilocus colubris), Semipalmated Plover (Calidris pusilla), Sanderling (Calidris alba), Ruddy Turnstone (Arenaria interpres), Black-bellied Plover (Pluvialis squatarola) and Killdeer (Charadrius vociferus) – from locations as situated within Oxford, Perth, Elgin, Middlesex, Norfolk and Haldimand Counties, Ontario.
5. Take is limited to a maximum of 60 birds per species per year.
6. Additional permission is granted to receive from Simon Fraser University, Vancouver, BC, migratory birds – to wit: Ruff (Philomachus pugnax), Western Sandpiper (Calidris mauri) and Dunlin (Calidris alpina) – as currently held in a captive breeding facility. Take is limited to a maximum of 60 birds per species per year.
7. All specimens are to be taken to the Department of Biology, University of Western Ontario, London, Ontario, for further analysis.
8. Capture, handling and housing procedures are to be performed according to the Animal Care Committee protocols of the University of Western Ontario.
9. All birds are to be released into the wild by the conclusion of the study or otherwise be humanely euthanized. No birds are to be donated or loaned to another individual or institution without the prior consent of the Canadian Wildlife Service.
10. All specimens are to be retained at the University of Western Ontario for scientific study purposes.
11. Samples not to be retained are to be disposed of by the approved laboratory waste management system of the University of Western Ontario.
12. Permit holder shall submit a written report, by January 31, of each year following, indicating the results

Canada

Curriculum Vitae

Alexander R. Gerson

ACADEMIC TRAINING

University of Western Ontario
Ph.D. Biology Sept 2007 – August 2012
Advisor: Dr. Christopher G. Guglielmo
Thesis: Environmental physiology of flight in migratory birds

University of Western Ontario
M.Sc. Biology Sept 2004 - June 2007.
Advisor: Dr. James F. Staples
Thesis: The effect of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation.

State University of New York, College of Environmental Science and Forestry
B.Sc. Environmental and Forest Biology. Sept 2000 - May 2004.

GRANTS, FELLOWSHIPS AND AWARDS

Natural Sciences and Engineering Research Council of Canada, Postdoctoral fellowship (NSERC-PDF). 2012.

The Canadian Council of University Biology Chairs Graduate Student Research Prize. 2011.

W.S. Hoar Award finalist. 2011. Canadian Society of Zoologists annual meeting, Ottawa Ontario.

Ontario Graduate Scholarship (2011-2012).

EPCOR Water Ltd. Student Travel Award (2011).

Top Student Poster Award. American Physiological Society, Westminster CO, USA. 2010.

Michael Locke Graduate Travel Bursary in Zoology.

American Association for the Advancement of Science (AAAS): Science Program for Excellence in Science Award (2009).

American Museum of Natural History, Frank M. Chapman Memorial Scholarship. 2009.

Natural Sciences and Engineering Research Council of Canada, Alexander Graham Bell Canada Graduate Scholarship (NSERC-CGS-D). 2009.

Ontario Graduate Scholarship (2009-2010). Declined.

American Physiological Society Top Student Poster Award. 2006.

Maple Leaf Award (2004). State University of New York, College of Environmental Science and Forestry.

Academic All American (2003, 2004).

PUBLICATIONS:

Nebel, S., Bauchinger, U., Buehler, D.M., Langlois, L.A., Boyles, M., **Gerson, A.R.,** Price, E.R., McWilliams, S.R., Guglielmo, C.G. (2012). Constitutive immune function in European starlings *Sturnus vulgaris* decreased immediately after an endurance flight in a wind tunnel. **Journal of Experimental Biology.** 215, 272-278

Gerson, A.R., Guglielmo, C.G. (2011). Flight at low ambient humidity increases protein catabolism in migratory birds. **Science.** Vol. 333, no. 6048, pp.1434-1436.

Guglielmo, C.G., McGuire, L.P., **Gerson, A.R.,** Seewagen, C.L. (2011). Rapid, Non-invasive measurement of fat, lean, and total body water masses of live birds using quantitative magnetic resonance. **Journal of Ornithology**. 152, S75-S85.

Price, E.R., Bauchinger, U., Zajac, D.M., Cerasale, D.J., McFarlan, J.T., **Gerson, A.R.,** McWilliams, S.R., Guglielmo, C.G. (2011). Migration and exercise-induced changes to flight muscle size in migratory birds and association with IGF-1 and myostatin mRNA expression. **Journal of Experimental Biology**. 214, 2823-2831.

Gerson, A.R., Guglielmo, C.G. (2011). House sparrows (*Passer domesticus)* increase protein catabolism in response to water restriction. **American Journal of Physiology: Regulatory, Integrative and Comparative Physiology**. 300, R925-R930.

Gerson, A.R., Brown, J.C.L., Thomas, R., Bernards, M.A., Staples, J.F. (2008). Effects of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation. **The Journal of Experimental Biology**. 211, 2689-2699.

Staples, J. F., **Gerson, A. R.** and Brown, J. (2008). Mitochondrial respiration and proton leak in hibernation and daily torpor. In **Hypometabolism in animals: torpor, hibernation and cryobiology.** eds. B. G. Lovegrove and A. E. McKechnie. Pietermaritzburg: University of KwaZulu-Natal.

Brown, J. C. L., **Gerson, A. R.** and Staples, J. F. (2007). Mitochondrial metabolism during daily torpor in the dwarf Siberian hamster: the role of active regulated changes and passive thermal effects. **American Journal of Physiology: Regulatory, Integrative and Comparative Physiology**. 293: R1833-R1845.

Gerson, D. F., M. L. Bontius, M. A., Wojtyk, J. F., Lee, J. J., Johnson, D. C,. Lee, R. Kahn, and **A. R. Gerson** (2001). Measuring bioreactor mixing with mixmeter. **Genetic Engineering News**, 21(15): 68-69.