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The Long Haul: Migratory Flight Preparation and Performance in Songbirds

Morag F. Dick, *The University of Western Ontario*

Supervisor: Dr. Christopher Guglielmo, *The University of Western Ontario*

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Abstract

Migration requires birds to sustain high intensity endurance exercise for periods lasting from hours to days. Similar to athletes, preparation and nutrition is key to success. Birds seasonally prepare for migration, which includes increasing the capacity to oxidize fat in the flight muscles. Beyond fuelling migration, n-3 long-chain polyunsaturated fatty acids (PUFA) are hypothesized to be natural doping agents and increase endurance and fatty acid oxidative capacity. I examined how birds prepare for and sustain migratory flight and directly tested the role of n-3 PUFA. Using yellow-rumped warblers (*Setophaga coronata*) as a model species, I first examined the effects of migratory season and endurance flight on the flight muscle transcriptome. I compared the transcriptomes of fall migratory and wintering birds and fall migrants at rest and after a four-hour flight in a wind tunnel. During the migratory season there was a coordinated upregulation of lipid metabolic pathways. Flight altered the transcriptome more than season did, and was characterized by the upregulation cytoplasmic fatty acid transporter gene expression and downregulation of genes related to glucose metabolism. Additionally, during flight there was an upregulation of pathways related to muscle damage and inflammation, and for protein synthesis and growth. Next, I tested the role of n-3 PUFA by feeding warblers diets enriched in n-3 or n-6 PUFA and assessed endurance flight performance and muscle metabolism. Neither PUFA diet altered endurance capacity or energy costs. However, contrary to the hypothesis, n-3 PUFA decreased muscle oxidative enzyme activites. Finally, as PUFA are prone to

oxidative damage I investigated if diet and flight influenced antioxidants or oxidative stress. Endurance flight resulted in oxidative stress indicated by decreased glutathione: glutathione disulphide ratio and increased protein carbonyls, but no effect of PUFA was found. Protein oxidative damage was tightly related to the energy costs of flight, suggesting that optimizing flight efficiency reduces muscle damage. Overall, I found no strong positive or negative impacts of dietary PUFA. This suggests that seasonal preparation and maintenance of flight is not influenced by PUFA. Together these studies provide insight into how birds are adapated to meet the metabolic challenges of migration.

Keywords

Bird migration, avian flight, endurance exercise, flight muscle, lipid metabolism, polyunsaturated fatty acids, yellow-rumped warbler, oxidative stress, reactive oxygen species, transcriptomics, wind tunnel.

Co-Authorship Statement

A version of Chapter 2 will be submitted to BMC Genomics for publication with Dr. Christopher G. Guglielmo as a co-author. Chris provided the funding, lab space, and training in animal care, handling and sampling, and wind tunnel use. Additionally, Chris provided advice and input into the study design, and comments during the preparation of the chapter/manuscript.

I will submit a version of Chapter 3 to the American Journal of Physiology – Regulatory, Integrative and Comparative Physiology, with Chris as a co-author. Chris provided funding, lab space and access to the wind tunnel, and training in fatty acid analysis. Additionally, Chris provided advice and feedback in the development of the study, during the study, and in the interpretation and preparation of the manuscript

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List of Abbreviations

ABCD-1	ATP-binding cassette subfamily D-1
AFAR	Advanced Facility for Avian Research
ARA	Arachidonic acid, 20:4 n-6
Bmal1	Brain and muscle arnt-like protein 1
BMR	Basal metabolic rate
Clock	Circadian locomotor output cycles kaput
CPM	Counts per million
CPT	Carnitine palmitoyl transferase
CS	Citrate synthase
DHA	Docosahexaenoic acid, 22:6 n-3
DNPH	2,4-dinitrophenylhydrazine
EPA	Eicosapentaenoic acid, 20:5 n-3
ERR	Estrogen-related nuclear receptors
$FABP_{pm}$	Fatty acid binding protein plasma membrane
FAME	Fatty acid methyl ester
FAT/CD36	Fatty acid translocase
FDR	False detection rate
FOG	Fast oxidative glycolytic
GAGE	Generally Applicable Gene-set Enrichment
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione

GSSG	Glutathione disulphide
H-FABP	Heart-type fatty acid binding protein
HOAD	3-hydroxyacyl-CoA- dehydrogenase
IGF1	Insulin like growth factor 1
KEGG	Koyoto Encyclopaedia of Gene and Genomes
LCPUFA	Long chain polyunsaturated fatty acid
LDH	Lactate dehydrogenase
MAPK	MAP-kinase activated protein
mTOR	Rapamyocin
MUFA	Monounsaturated fatty acid
NEFA	Nonesterified fatty acids
NFκβ	Nuclear factor kappa beta
NrF2	Nuclear factor E2-r7elated factor 2
OS	Oxidative stress
PGC-1α	PPARγ coactivator 1α
PGC-1β	PPARγ coactivator 1β
PMR	Peak metabolic rate
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acids
QMR	Quantitative magnetic resonance
RER	Respiratory exchange ratio
RPC	Relative contribution of protein

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RQ	Respiratory quotient
RS	Reactive species
SFA	Saturated fatty acid
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TNF	Tumour necrosis factor
$\dot{V}CO_2$	Volumetric carbon dioxide production rate
$\dot{V}O_2$	Volumetric oxygen consumption rate

1 Introduction

1.1 Migration

Migration allows animals to maximize fitness in response to seasonal changes in resources, and is typically associated with movement between breeding areas and wintering/ non-breeding areas (Dingle, 2014). Animals across different taxa and body sizes complete migrations via water, air, or land movements, with the diverse list including whales, insects, crabs, fish, bats, ungulates, and birds (Dingle, 2014). Birds are arguably one of the best-studied migratory groups and 19% of known avian species are migratory (Kirby et al., 2008).

Bird migration is a feat of endurance, and there are numerous examples that demonstrate the extreme athletic ability needed to complete these journeys. For example, bar-tailed godwits (*Limosa lapponica*) make a single 11 700 km flight on their southbound migration from Alaska to New Zealand (Gill et al., 2005). Bar-headed geese (*Anser indicus*) prove no mountain is too high, and migrate through the Himalayan mountains despite the challenges of limited oxygen and thin air (Hawkes et al., 2011). Great snipes (*Gallinago media*) are one of the fastest known avian migrants, covering between 4300 and 6800 km in as little as 48 – 96 h (Klaassen et al., 2011). Blackpoll warblers (*Setophaga striata*), weighing only 16 g, complete one long flight and travel as far as 2540 km in 62 h during their south bound migration (DeLuca et al., 2015).

These feats of endurance require birds to push their limits and have been compared to humans travelling to the moon (Guglielmo, 2010). Birds must prepare for these journeys and ensure adequate fuel stores and hydration while travelling to an unknown destination. Typically, migration is a series of flights interspaced with periods of stopover, where birds recover and refuel in advance of their next journey. Migration is a risky endeavour and has the highest mortality rates of the annual cycle (Sillett and Holmes, 2002). The exact causes of mortality during migration are hard to determine, but birds may be at greater risk of starvation, which can also increase vulnerability to predation, inclement weather and competition for resources at stopover (Newton, 2006). Hence, preparation for migration is critical for success to ensure birds are ready to depart when conditions are appropriate. The migratory syndrome is a series of integrated physiological, morphological and behavioural traits that shift birds away from station keeping in favour of persistent movement and allocation of energy away from growth and reproduction towards fuel deposition (Dingle, 2014). In terms of endurance capacity, these shifts ensure that birds are able to store and oxidize fat adequately and this is accomplished through hyperphagia, fuel storage, and alterations to substrate metabolism (Bairlein, 2002; McWilliams et al., 2004; Ramenofsky and Wingfield, 2007; Guglielmo, 2010).

1.2 Fuelling migratory flight

Flapping flight is one of the most energetically costly forms of locomotion, greater than either running or swimming (Butler, 1991). However, the high velocity

achieved makes flight an overall more energy efficient form of transport (Butler, 1991). To maintain flight, birds need a high capacity to supply ATP for sustained muscle contractions. The fuel source used depends on a variety of factors including flight duration (Gerson and Guglielmo, 2013; Guglielmo et al., 2017) and feeding state (Welch et al., 2006). Initially, carbohydrates and protein are the primary fuels for flight (Rothe et al. 1987; Gerson and Guglielmo, 2103; Guglielmo et al., 2017). Fat becomes the dominant fuel source with increasing flight duration (Rothe et al., 1987; Gerson and Guglielmo, 2013), and contributes over 90% of the energy required with the remainder derived from lean mass catabolism (Klaassen and Kvist, 2000; Guglielmo et al., 2017).

Fat is the most energy dense fuel source due to highly chemically reduced aliphatic chains of fatty acids and because fat is stored without water (Jenni and Jenni-Eiermann, 1998). In comparison, sugar can be stored in the form of glycogen in the liver and muscles, but since it is stored with water this decreases the energy provided per unit mass stored (Jenni and Jenni-Eiermann, 1998). Unlike fat and sugar, there is no dedicated storage mechanism for protein in the body (Jenni and Jenni-Eiermann, 1998). Birds catabolize lean mass while fasting or flying (Jenni-Eiermann and Jenni, 1991; Biebach, 1998; Bauchinger and Biebach, 2001; Gerson and Guglielmo, 2011a;) and this has various potential benefits. Protein catabolism may replenish glucose, Krebs cycle intermediates, and metabolic water (Jenni and Jenni-Eiermann, 1998; Gerson and Guglielmo, 2011b). The initial stages of flight

may use a high proportion of lean mass to lower body mass, and flight muscle size decreases to match decreasing body mass (Schwilch et al., 2002).

Managing fuel stores and use during migration are likely key components of optimal performance. Fuelling flight with a high proportion of fat enables birds to use the most energy dense and thus lightest fuel load (Jenni and Jenni-Eiermann, 1998). In preparation for migration, birds accumulate fat stores that may account for up to 50% of their body mass (Battley et al., 2001; Bauchinger and Biebach, 2001; Piersma and Jukema, 2002). Depleted fat stores are replenished at stopover sites. If fat stores become limited during flight, protein catabolism may increase to meet energy demands (Schwilch et al., 2002). The transport of fuel throughout the body is a crucial element to ensuring sufficient substrates are available for the flight muscles and has been highlighted as a fundamental element of migratory physiology (Guglielmo, 2010). Furthermore, hypotheses about how dietary fatty acids influence the capacity or efficiency of fat use during migration have been proposed, but have not been empirically tested (Weber, 2009; Price, 2010; Pierce and McWilliams, 2014).

1.3 Seasonal changes in the flight muscle

The pectoralis and supracoracoideus muscles are collectively referred to as the “flight muscles”. The pectoralis muscle powers the down stroke during flight and accounts for 80-90 % of the flight muscle mass in most birds, while the supracoracoideus elevates the wing for the upstroke (Biewener, 2011). Fast

oxidative glycolytic fibres (FOG) are the dominant fibre type in passerine flight muscles (Lundgren and Kiessling, 1988), and exclusively FOG fibres are present in small passerines (< 15 g) (Welch and Altshuler, 2009). No seasonal changes are observed in small passerine flight muscle fibre type, but this may vary in larger passerines (Lundgren and Kiessling, 1988). Additionally, seasonal increases in flight muscle mass may occur during migration (Marsh, 1984; Dietz et al., 1999; Lindström et al., 2000).

The seasonal regulation of flight muscle size has been proposed to be under the control of myostatin, an inhibitor of growth, and insulin-like growth factor 1 (IGF1), a promoter of muscle growth (Swanson et al., 2009; Price et al., 2011; King et al., 2015; Zhang et al., 2015a). The role of myostatin in regulating muscle mass during migration has mixed support, and positive, negative, or no relationships have been observed between myostatin gene expression and flight muscle size (Swanson et al., 2009; Price et al., 2011a; King et al., 2015; Zhang et al., 2015a). However, changes in myostatin at the protein level support a regulatory role. Myostatin protein expression decreases during migration and exercise training (King et al., 2015; Zhang et al., 2015a). IGF1 is a growth stimulator produced in muscles during contraction (Adams, 1998) and its gene expression is elevated in migratory birds and during endurance flight (Price et al., 2011a).

Alteration in flight muscle oxidative capacity is crucial for ensuring energy supply for flight and to support the shift towards fat utilization, and starts with fatty acid transport into the muscles. Fatty acids are hydrophobic by nature and are

transported in the blood using albumin as a carrier protein (Weber, 1988). The diffusion of fatty acids across the cell membrane is likely insufficient to meet energy demands (McWilliams et al., 2004). In mammals as much as 80% of fatty acid cellular uptake is protein-mediated and is done via plasma membrane fatty acid binding protein ($FABP_{pm}$) and fatty acid translocase (FAT/CD36) (McArthur et al., 1999). Heart-type fatty acid binding protein (H-FABP) acts as a cytosolic chaperon and transports fatty acids within the cytoplasm; it serves as a cytoplasmic sink for fatty acids and maintains uptake across the cell membrane (McArthur et al., 1999). Seasonal upregulation of fatty acid transporters has been observed in multiple passerine and non-passerine migratory bird species (Guglielmo et al., 1998; Pelsers et al., 1999; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011; Zhang et al., 2015b; Banerjee and Chaturvedi, 2016). H-FABP in particular is 70% more abundant during migration in western sandpipers (*Calidris mauri*, Guglielmo et al., 1998; Guglielmo et al., 2002), suggesting its upregulation may be a key step supporting endurance flight.

Changes in metabolic enzymes support the increased reliance on fatty acid oxidation during flight. Long chain acyl-CoA synthetase on the outer mitochondrial membrane converts free fatty acids into acyl-CoA (McArthur et al., 1999). Carnitine palmitoyl transferase I (CPT I) catalyzes the transfer of the acyl group to carnitine, and the acyl-carntine is transferred across the inner mitochondrial membrane by a translocase and the acyl-carnitine can be converted back to acyl-CoA by CPT II in the mitochondrial matrix (McGarry and Brown, 1997). Once transported into the

mitochondria, the fatty acids can enter the β-oxidation pathways, with the acetyl-CoA produced entering the Krebs cycle and ultimately the production of ATP in the electron transport chain. The activity of 3-hydroxyacyl-CoA- dehydrogenase (HOAD) and citrate synthase (CS) are used as indicators of the capacity for β-oxidation, and tricarboxylic acid cycle capacity, respectively. The activities of CPT, CS, and HOAD increase during migration in many species, supporting the increased fatty acid oxidative capacity (Marsh, 1981; Driedzic et al., 1993; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011; DeMoranville, 2015). The degree of upregulation of fatty acid transporters and enzymes may depend on species and season in free-living wild birds (McFarlan et al., 2009; DeMoranville, 2015; Zhang et al., 2015b). Elevated fatty acid transport capacity may be a more universal marker of migration in the flight muscles (Zhang et al., 2015b).

The upregulation of oxidative capacity in the flight muscles is suggested to be controlled by peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), PGC-1β, and the peroxisome proliferator activated receptor family (PPAR)

(Weber, 2009; DeMoranville, 2015). As a transcriptional coactivator, PGC-1α regulates the expression of genes involved in substrate oxidation and mitochondrial oxidative phosphorylation and is considered to be a master regulator of mitochondrial biogenesis (Scarpulla, 2011; Camera et al., 2016). PGC-1α mRNA abundance is elevated in the muscles of trained mice and is associated with increased peak metabolic rate and endurance capacity (Calvo et al., 2008). The function of PGC-1β in skeletal muscles and exercise is less clear than PGC-1α and

likely has a similar but distinct role compared to PGC-1α (Schnyder et al., 2017). Similar to PGC-1α, increasing PGC-1β mRNA abundance improves exercise performance (Arany et al., 2007). PGC-1β is required for normal mitochondrial structure and function, and oxidative capacity (Zechner et al., 2010; Ramamoorthy et al., 2015). Knocking out either PGC-1α or PGC-1β gene expression reduces endurance capacity in mice, and lack of both PGC-1s drastically reduces endurance capacity further (Zechner et al., 2010).

PPARs are a family of nuclear hormone receptors, with only partial overlap among isomers in activation or suppression of gene expression among the three subtypes (Willson et al., 2000). PPARα and PPARβ (also referred to as PPARδ) support energy metabolism, and are found in higher levels in skeletal muscle (Grygiel-Górniak, 2014). Increasing PPARα and PPARβ gene and protein expression increases oxidative capacity in muscles and improves fatigue resistance (Perry et al., 2010). DeMoranville (2015) observed an increase in PGC-1α, PGC-1β and PPARα in the fall migratory period compared to the breeding season in gray catbirds (*Dumetella carolinensis*) suggesting they are key molecular regulators initiating the metabolic changes during migration.

1.4 Consequences of endurance flight

A consequence of sustaining exercise and elevated metabolic rate is the generation of reactive species, with reactive oxygen species (ROS) being the primary reactive species produced (Ji, 1999; Costantini et al., 2008). ROS can react

with proteins, DNA and lipids causing damage to cells and potentially leading to cell death (Sies and Jones, 2007). Mitochondrial oxidative phosphorylation is suggested to be the primary source of ROS production during exercise and superoxides are produced from the incomplete reduction of oxygen (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). ROS is also produced by NADPH oxidases during muscle contraction in the sarcoplasmic reticulum, sarcolemma and transverse tubules (Zuo et al., 2011; Sakellariou et al., 2013; Goncalves et al., 2015). There is evidence for flight muscle damage and impaired mitochondrial function during flight (Guglielmo et al., 2001; Gerson, 2012) and these could be functional consequences of high ROS production.

ROS production needs to be counteracted by the antioxidant system to maintain cellular function. The antioxidant system is composed of endogenous enzymatic and non-enzymatic antioxidants, and exogenous sources from the diet (Powers and Jackson, 2008). Endogenous non-enzymatic factors include uric acid, which is oxidized to allantoin in birds (Tsahar et al., 2006), and glutathione (GSH), which is oxidized to glutathione disulphide (GSSG) via glutathione peroxidase and recycled back to GSH via glutathione reductase (Powers and Jackson, 2008; Cooper-Mullin and McWilliams, 2016). Other antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the reaction of superoxide to hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and oxygen (Powers and Jackson, 2008). Diet can serve as an exogenous source of antioxidants, with essential nutrients, such as vitamin C and E, and pigment molecules, such as

carotenoids and flavonoids, contributing to antioxidant capacity (Skrip and McWilliams, 2016). When the production of ROS exceeds antioxidant defenses there is an alteration to redox balance. This can lead to oxidative stress (OS) and impact redox control, cell signalling, and mitochondrial and contractile function (Sies and Jones, 2007).

Changes in antioxidant capacity during endurance flight are not consistent and are potentially related to differences in study systems and methods of assessment. The OXY adsorbent test is a common method that measures non-enzymatic antioxidant capacity in serum, but does not include uric acid in its measurement (Beaulieu et al., 2011). During endurance flight (~200 km) non-enzymatic serum antioxidant capacity decreases in homing pigeons (*Columbia livia,* Costantini et al., 2008). However, no difference in non-enzymatic antioxidant capacity was detected in recently arrived garden warblers (*Sylvia borin*) at stopover (Skrip et al., 2015) and after flight in Northern bald ibis (*Geronticus eremita*, Bairlein et al., 2015). It is important to note that plasma uric acid levels increase during flight (Schwilch et al., 1996; Gerson and Guglielmo, 2013), and could contribute to the serum antioxidant capacity, but this is not accounted for with the OXY adsorbent test. As part of migratory preparation, birds increase their plasma non-enzymatic antioxidant capacity prior to departure (Skrip et al., 2015). Changes in antioxidant enzyme capacity also occur during migration (Jenni-Eiermann et al., 2014; Banerjee and Chaturvedi, 2016). European robins (*Erithacus rubecula*) increase blood cell glutathione peroxidase activity during a migratory flight (Jenni-Eiermann et al.,

2014). Recently, in a proteomic study, Mn SOD was identified as an upregulated protein during migration in the flight muscles of red-headed buntings (*Emberiza bruniceps,* Banerjee and Chaturvedi, 2016).

More consistently, endurance flight results in increased ROS leading to oxidative stress (Costantini et al., 2008; Skrip et al., 2015) and damage to blood cell proteins (Jenni-Eiermann et al., 2014). Although the majority of ROS during flight will be produced in the flight muscles, changes in oxidative stress and antioxidants in the flight muscle have not previously been assessed during migration or flight in birds. Atlantic salmon (*Salmo salar*) increase muscle SOD activity, but not catalase activity during migration (Bombardier et al., 2009). A similar pattern has been observed following acute exercise in humans (Powers et al., 1994; Wang et al., 2015). Thus, along with up-regulating aerobic capacity in flight muscle, birds may need to alter antioxidant enzymes to sustain endurance flight.

1.5 Studying migratory preparation and flight performance

Studies of migratory preparation in flight muscles originally focused on muscle size, enzyme activities, and gene and protein expression of candidate genes (Marsh, 1981; Marsh, 1984; Driedzic et al., 1993; Guglielmo et al., 1998; Ramenofsky et al., 1999; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011; Zhang et al., 2015b). Recent technological advances have created the 'omics revolution', which is changing the ways we approach comparative animal physiology, including songbird physiology (Stager et al., 2015; Banerjee and Chaturvedi, 2016; Fudickar et

al., 2016). Transcriptomics is the global analysis of thousands of transcript abundances simultaneously. This allows for examination of known genes and pathways of interest, as well as the identification of novel ones.

Low cost entry into transcriptomics was initially through commercially available microarrays for traditional model species, such as chickens (*Gallus gallus*), and has been successfully applied to study the flight muscles of penguins (Teulier et al., 2012). However, incomplete hybridization of RNA to the microarray probes due to species specificity may reduce the sensitivity of the method (Ranz and Machado, 2006). The development of low cost rapid high throughput sequencing generated the next wave of the revolution and led to the development of RNAseq (Wang et al., 2009). RNAseq sequences small fragments of transcripts (eg. 100 bp long) producing reads and uses the number of reads per transcript to estimate transcript abundance (Wang et al., 2009). A huge benefit of RNAseq is that is does not require any prior genomic information, and the sequence reads can be used to create a *de novo* transcriptome assembly, making it suited for studying species without sequenced genomes (Haas et al., 2013). This flexibility has created new opportunities for how we study migratory birds, and enabled the examination of seasonal changes in flight muscle gene expression in relation to migration (Fudickar et al., 2016) and over wintering physiology (Stager et al., 2015). These studies highlight the importance of seasonal changes in lipid metabolism, and test hypotheses surrounding muscle growth. The degree to which seasonal preparation equips birds for endurance flight versus alterations during flight is unknown. Acute

endurance exercise influences PPAR expression (Perry et al., 2010), suggesting that further alterations in fatty acid metabolism may occur during endurance flight. Additionally, the negative consequences of exercise such as muscle damage and inflammation (Cannon et al., 1991; Townsend et al., 2015; Camera et al., 2016) have not been characterized in migratory birds but could also contribute to the capacity to sustain flight.

1.6 Role of dietary fatty acids

Fatty acid nutrition has been highlighted as potentially important to migratory performance and success (Weber, 2009; Price, 2010; Guglielmo, 2010; Pierce and McWilliams, 2014). The effects of fatty acids on physiology depend on their chemical structure. Fatty acids can be saturated (SFA), monounsaturated (MUFA) with the insertion of a single double bond into the carbon chain, or polyunsaturated (PUFA) with two or more double bonds. The most common PUFA types are the n-3 PUFA and n-6 PUFA, reflecting the location of the first double bond relative to the methyl end. Animals are unable to synthesize n-3 PUFA and n-6 PUFA making them essential fatty acids in the diet, while SFA and MUFA can be synthesized *de novo* (Leeson and Summers, 2001).

The proportion of PUFA, particularly the long-chain PUFA (>20 carbons long), in animal tissues is a reflection of diet (Pierce et al., 2005; Wang et al., 2007; Price and Guglielmo, 2009; McCue et al., 2009). Marine ecosystems are dominated by n-3 long-chain PUFA that are synthesized primarily from microalgae, bacteria and thraustochytrids and are transferred up the food web (Nicols, 2003). As a

result, birds consuming marine invertebrates tend to have higher proportions of eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) in their cellular membranes and lipid stores (Maillet and Weber, 2006; Wang et al., 2007; Weber, 2009). In contrast, terrestrial insectivores, granivores, and fruigvores tend to have diets richer in the n-6 PUFA (Conway et al., 1994; Klaiman et al., 2009; Pierce and McWilliams, 2014; Andersson et al., 2015). The deposition of fatty acids into membranes and adipocytes is dependent on dietary intake, tissue and structure. PUFA are preferentially deposited in the heart and brain over liver and muscle, and in cell membranes over stored lipids (McCue et al., 2009). Preferential tissue deposition is best observed in ruby-throated hummingbirds (*Archilochus colubris*), which deposit high levels of DHA into their flight muscle membranes while eating a low-fat nectar-based diet (Infante et al., 2001).

Fatty acid composition may alter endurance flight performance through various mechanisms (Figure 1.1). First, fatty acid mobilization from adipose and muscle uptake and oxidation is influenced by fatty acid chain length and saturation (Price et al., 2008; Price and Guglielmo, 2009; Price et al., 2011b). Shorter and more unsaturated fatty acids are preferentially mobilized from adipose in white-crowned sparrows (*Zonotrichia leucophrys*) and ruffs (*Philomachus pugnax*) (Price et al., 2008; Price et al., 2011b). This pattern is reflected in the plasma fatty acids of fasting birds (Groscolas, 1990; Mustonen et al., 2009). Fatty acid uptake and oxidation rates in muscles are greater with more unsaturated and/or shorter fatty acids (Price et al., 2011b). If fatty acid mobilization and oxidation is a limiting factor

for endurance flight, the composition of the adipose tissue could be key for performance.

The second mechanism by which fatty acids could influence exercise performance involves membrane phospholipids. Phospholipid fatty acid composition influences membrane fluidity and permeability and modulates the activity of membrane-bound proteins and mitochondrial proton leak (Power et al., 1997; Infante et al., 2001; Stillwell and Wassall, 2003; Price 2010). Increasing amounts of unsaturated fatty acids, particularly long-chain PUFA, in the flight muscle is correlated with oxidative enzyme activities (Maillet and Weber, 2006; Maillet and Weber, 2007; Nagahuedi et al., 2009; Guglielmo, 2010).

Third, fatty acids and eicosanoid derivatives act as ligands for PPAR and thus, could alter oxidative capacity (Grygiel-Górniak, 2014). PPAR activation varies with fatty acid structure and cellular concentration, and is strongest with PUFA (Mochizuki et al., 2006). Therefore, increasing intake of PUFA may help increase the activation of PPARs and the expression of genes controlling lipid oxidation (Weber, 2009). Lastly, PUFA may influence oxidative balance and ROS production (Gerson 2012; Alan and McWilliams, 2013; Skrip and McWilliams, 2016). The high number of double bonds in PUFA makes them prone to oxidative damage (Alan and McWilliams, 2013; Skrip and McWilliams, 2016). However, increasing PUFA intake can lower net mitochondrial ROS production in flight muscles by altering the rate of production or increased antioxidant capacity (Gerson, 2012). Thus, increasing PUFA intake may lower ROS production, increase oxidative damage or require an increase

in antioxidant capacity. These mechanisms are not mutually exclusive and could interact to influence how fatty acids affect performance. Thus, depending on interpretation, multiple hypotheses can be formulated regarding the value of dietary PUFA for migration performance (Weber, 2009; Pierce and McWilliams, 2014).

Maillet and Weber (2006; 2007) proposed the natural doping hypothesis, which states that n-3 long-chain PUFA prime the flight muscles of birds for endurance flight. The hypothesis was developed by observing semipalmated sandpipers (*Calidris pusilla*) feeding on invertebrates rich in n-3 long-chain PUFA during stopover in the Bay of Fundy before departing on a three day nonstop migratory flight (Weber, 2009). Correlations between aerobic and oxidative enzymes and DHA and EPA in the flight muscle phospholipids suggested a relationship (Maillet and Weber, 2007). A controlled test of DHA and EPA as "doping agents" in bobwhite quail (*Collinus virginianus*) found a similar effect, suggesting that diet alone can increase aerobic capacity (Nagahuedi et al., 2009). However, this effect was not found with migratory white-throated sparrows (*Zonotrichia albicollis*) when comparing n-3 and n-6 PUFA diets, indicating that seasonal upregulation of aerobic capacity is not modulated by diet, or that n-3 and n-6 PUFA may have similar or no effects (Price and Guglielmo, 2009).

Currently, there is no compelling evidence for dietary fatty acids as doping agents in migratory birds or enhancing endurance flight performance. Burst exercise performance is elevated in birds consuming high proportions of alpha-

linoleic acid (18:2 n-6) (Pierce et al., 2005; Price and Guglielmo, 2009), but a link between burst and endurance performance is also lacking. In rats, diets enriched with n-6 PUFA increase endurance capacity (Ayre and Hulbert, 1997) and n-3 PUFA increases fatigue resistance in rats (Clavel et al., 2002; Henry et al., 2015). However, the 30 min time frame used in the studies limits the comparisons to endurance flight in birds. In humans, physiological indicators of performance suggest that PUFA can modify performance, such as improved heart rate and lipid metabolism during exercise (Raastad et al., 1997; Buckley et al., 2009). Human exercise studies have yielded mixed results, but overall very little evidence is available to suggest that PUFA enhance endurance performance at the organismal level (reviewed by Shei et al., 2014). Studies at the organismal level that focus on endurance flight performance are needed for birds to fully test the potential role of n-3 PUFA on flight and if it does have the potential to improve migratory performance.

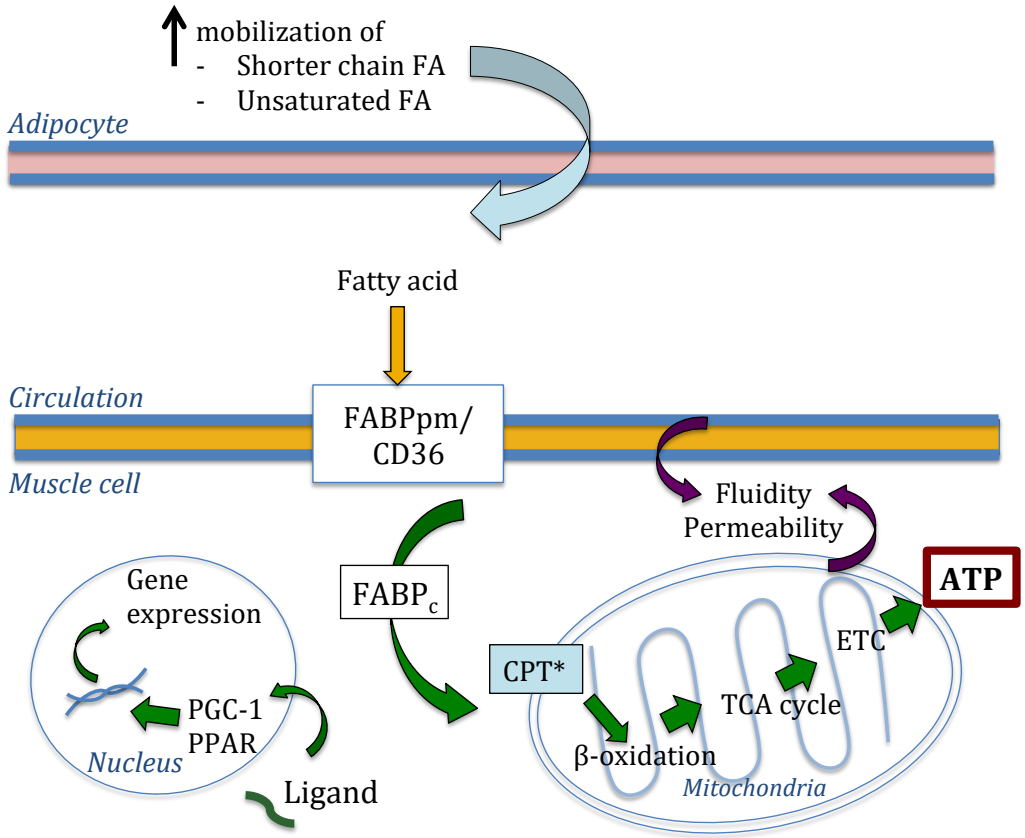

Figure 1.1 Potential mechanisms for fatty acids to influence flight muscle fatty acid oxidation. The light blue arrow and box indicates potential points for preferential fatty acid mobilization from adipose and oxidation in the mitochondria and favours shorter and/or more unsaturated fatty acids. Purple arrows indicate membranes that may increase in fluidity and permeability in response to increasing dietary polyunsaturated fatty acids (PUFA). Green arrows indicate the potential increasing availability of n-3 PUFA as ligands for peroxisome proliferator-activated receptor (PPAR), which would result in increased oxidative capacity. Carnitine palmitoyl transferase (CPT), cytosolic fatty acid binding protein ($FABP_c$), electron transport chain (ETC), fatty acid translocase (CD36), plasma membrane fatty acid binding protein ($FABP_{pm}$), tricarboxylic acid (TCA).

1.7 Yellow-rumped warblers

The yellow-rumped warbler (*Setophaga coronata*) is one of the most common warbler species in North America and is often used in field and captive studies (Afik et al., 1997; McWilliams et al., 2002; Dossman et al., 2016; Marshall et al., 2016). Debate exists if the two migratory subspecies of yellow-rumped warblers, myrtle warblers (ssp *coronata*) and Audubon's warblers (ssp *auduboni*) are separate species (Toews et al., 2016). For the purposes of this thesis, yellow-rumped warblers refer only to the myrtle warbler subspecies (Figure 1.2). Yellow-rumped warblers are intermediate distance nocturnal migrants, and migrate between the breeding grounds in the boreal forests of North America, and wintering areas in the mid to southern United States and Mexico (Milá et al., 2008; Figure 1.3). Stopover duration for rest and refuelling between migratory flights is 6.9 days on average, with fatter birds departing earlier (Dossman et al., 2016).

Yellow-rumped warblers are flexible foragers, consuming primarily fruit during the fall and winter and insects during the spring and summer (Parrish, 1997; Hunt and Flaspohler, 1998). When offered the chance, warblers prefer consuming diets rich in simple sugars, which can be absorbed passively in the small intestine in addition to protein mediated sugar transport and efficiently converted into fat (Afik et al., 1997; Marshall et al., 2016). Yellow-rumped warblers are also known for their higher capacity to assimilate long chain saturated fatty acids found in waxy berries

such as bay berries (Place and Stiles, 1992), but do show preferences for unsaturated fatty acids (McWilliams et al., 2002).

Yellow-rumped warblers make an ideal model species for captive studies for various reasons. First, they adjust well to captivity and can be maintained on various diets allowing for targeted nutritional studies (Afik et al., 1997; Marshall et al., 2016; Guglielmo et al., 2017). Second, the migratory condition of the warblers can be controlled by altering photoperiod, with migratory restlessness behaviour occurring within 20 to 25 days after switching to a long day photoperiod from a short day photoperiod (Moore and Simm, 1985). Finally, and most significant to my studies, yellow-rumped warblers are highly successful in wind tunnel studies simulating migratory endurance flight (Guglielmo et al., 2017).

Figure 1.2 Photo of a myrtle warbler, a sub-species of yellow-rumped warblers (*Setophaga coronata*).

Figure 1.3 Range map of the yellow-rumped warbler (*Setophaga coronata*) myrtle subspecies. Range map based on Milá et al. (2008). The purple star indicates Long Point, Ontario (42°32′51″N 80°3′33″W).

1.8 An introduction to the studies

The purpose of my research was to investigate how migratory birds prepare for and sustain migratory flight, and to understand how these processes may be influenced by dietary fatty acids. For this, I used the yellow-rumped warbler as a model for migratory flight and physiology.

Chapter Two examines changes in global gene expression in the flight muscles to determine what changes occur in preparation for and during a migratory flight. Using RNAseq, I examined seasonal changes between fall migratory and winter non-migratory birds at rest, and after a four hour simulated migratory flight. Overall, my results indicate that fatty acid metabolism is upregulated during migration, and is maintained during flight. However, flight had greater impact than season on the flight muscle transcriptome than season. During flight, the shift to fat as the fuel source led to a decrease in expression of genes related to glucose metabolism. Furthermore, protein synthesis and growth pathways as well as inflammation and degradation pathways were upregulated during flight. This suggests that flight muscle protein degradation and synthesis are required while flying to maintain muscle function.

In Chapters Three and Four, I directly test the natural doping hypothesis from the tissue level to whole animal performance. The study in Chapter Three tests whether enrichment of long chain n-3 or n-6 PUFA in muscle membranes improves endurance exercise performance and aerobic and oxidative capacity in the flight

muscles. Overall, I found no effect of PUFA on flight performance. However, supplementing birds with n-3 PUFA decreased PPARβ mRNA abundance and muscle aerobic enzymes, suggesting decreased aerobic and endurance capacity, and is in direct opposition to the natural doping hypothesis. Furthermore, I found a disconnect between measures of flight performance and muscle "indicators of performance". For instance, feeding an n-3 PUFA diet decreased flight muscle oxidative enzymes, but did not alter endurance capacity, energy costs, or fuel mixture. This suggests that decreasing muscle enzyme activites may not neccesarily influence performance.

In Chapter Four, I assess flight muscle endogenous antioxidant status, oxidative damage, and whether dietary PUFA influence the response to flight. The data indicate that endurance flight is a true oxidative challenge for birds, with protein damage increasing with flight duration and energy expenditure (independent of flight duration/distance). Dietary PUFA did not alter antioxidants or oxidative damage during flight.

Collectively these studies provide new insights into migration physiology and lay important groundwork for understanding muscle physiology during flight. Migratory birds maintain a high capacity to oxidize fatty acids in their flight muscles and enhance this capacity during flight. Endurance flight is demanding and the studies presented here provide some of the first examples of the negative aspects of flight on the flight muscles and how birds may manage them. Finally, I find no support for any positive or negative effects of PUFA on animal performance and

oxidative stress, suggesting that the migration syndrome is powerful and not easily influenced or enhanced by diet.

1.9 References

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2 Transcriptomic changes in the flight muscle of yellow-rumped warblers in response to migratory state and endurance flight.

2.1 Introduction

Migratory birds have an incredible capacity to perform high intensity endurance exercise under challenging conditions, with nonstop migratory flights lasting from hours to days (Gill et al., 2005; DeLuca et al., 2015; Stutchbury et al., 2016). Unlike mammals, birds are able to maintain high metabolic rates during flight while obtaining upwards of 90% of the required energy from fat, with the remainder contributed by protein from lean mass catabolism (Jenni and Jenni-Eiermann, 1998; McClelland, 2004). The ability of birds to use fatty acids as fuel depends on alterations to their physiology and biochemistry, particularly in the flight muscles (pectoralis and supracoracoideus muscles). Fat is the most energy-dense fuel source, and the capacity to store, transport and oxidize fatty acids is critical to supply the energy needed to maintain flight (Guglielmo, 2010). Compared to fat, the catabolism of lean mass as fuel contributes far less energy per gram, but it can provide more water, and replenishes glucose and citric acid cycle intermediates (Jenni and Jenni-Eiermann, 1998; Gerson and Guglielmo, 2011).

As part of seasonal migratory preparation, birds accumulate fat stores that account for 70 to 80% of mass gain (Lindström and Piersma, 1993; Bairlein, 2002). Muscles and organs that support exercise may also increase in size to help power flight, with enlargement of the heart and flight muscles being most prominent

(Marsh, 1984; Guglielmo and Williams, 2003). The control of muscle hypertrophy during migration is still unclear. However, insulin-like growth factor 1 (IGF1), a growth promoter, and myostatin, a growth inhibitor, may contribute to this regulation (Price et al. 2011; King et al., 2015).

During migration, there is increased expression of proteins in the flight muscle for transporting fatty acids across the cell membrane [fatty acid binding protein (FABPpm) and fatty acid translocase (FAT/CD36)], and within the cell [heart-type fatty acid binding protein (H-FABP)] (Guglielmo et al., 1998; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011). Increased capacity to oxidize fatty acids at high rates is enhanced in the migratory state as shown by increased activities of oxidative enzymes [citrate synthase (CS), carnitine palmitoyl transferase (CPT) and 3-hydroxyacyl-CoA dehydrogenase (HOAD)] (Marsh, 1984; Driedzic et al., 1993; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011). However, seasonal and species differences in response may be observed (Zhang et al., 2015). Recently, transcriptomic and proteomic analyses suggest that during migration there is a coordinated increase in many aspects of fatty acid metabolism including transport, fatty acid oxidation and peroxisomal activity (Banerjee and Chaturvedi, 2016; Fudickar et al., 2016). Moreover, migratory preparation also includes increasing antioxidant enzyme capacity to manage the high levels of reactive oxygen species (ROS) produced during aerobic metabolism(Banerjee and Chaturvedi, 2016).

Less is known about physiological and biochemical changes that may occur in the flight muscles of birds during endurance flights, when birds are in a catabolic state while exercising and fasting. During migratory flight, sustained high metabolic rate leads to oxidative damage to both blood proteins and lipids (Jenni and Jenni-Eiermann, 1998; Skrip et al., 2015) and there is evidence of post-flight muscle damage (Guglielmo et al., 2001). The negative effects of ROS damage in the muscle have not been directly characterized. Endurance flight in European starlings (*Sturnus vulgaris*) decreases mitochondrial ATP production rate and oxidative damage could be the cause of this (Gerson, 2012). Increased gene expression of IGF1 also suggests that endurance flight stimulates pathways related to muscle growth or maintenance (Price et al., 2011). Potentially, migratory preparation may be sufficient to support the initial stages of flight, but further upregulation of key genes is required to sustain flight for many hours, particularly with a shift in fuel from carbohydrates to stored fat (Gerson and Guglielmo, 2013).

Migratory flight in birds differs from traditional mammalian exercise in duration, metabolic intensity and fuel use (Guglielmo, 2010; Camera et al., 2016). Migratory birds fly at a relatively high exercise intensity using fat as the primary fuel source, and this differs from endurance exercise in mammals, which is at lower intensity compared to birds and uses a mixture of fat and carbohydrates for fuel (McClelland, 2004; Guglielmo, 2010). As such, it may share some but not all the physiological changes associated with a bout of exercise in mammals. Endurance exercise in animals leads to increased oscillations in cellular calcium concentration,

ROS production, and energy substrates that can trigger a variety of signalling cascades (Egan and Zierath, 2013; Ramamoorthy et al., 2015; Camera et al., 2016). For example, activation of peroxisome proliferator-activated receptor gamma isoforms (PGC-1α and PGC-1β) and peroxisome proliferator-activated receptors (PPAR) can lead to mitochondrial biogenesis and increased aerobic capacity (Wu et al., 1999; Scarpulla, 2011; Camera et al., 2016). Additionally, initiation of an inflammatory response may occur due to cellular damage mediated through pathways such as the tumour necrosis factor (TNF) and nuclear factor κβ (NFκβ) pathways (Cannon et al., 1991; Townsend et al., 2015). It is unknown exactly how migratory preparation and flight compares to mammalian endurance exercise or if it contains elements more associated with resistance exercise such as muscle hypertrophy via IGF1/rapamyocin (mTOR) pathway (Rommel et al., 2001; Camera et al., 2016).

I examined seasonal changes in the flight muscle transcriptome of yellow-rumped warblers (*Setophaga coronata*) to explore differences in the fall migratory and wintering phenotypes. Secondly, as migratory flight could provoke large alterations to the flight muscle transcriptome, I examined the effect of simulated migratory flights. I predict that seasonal preparation for migration will be associated with an upregulation of genes supporting aerobic capacity, fatty acid transport and oxidation, and muscle growth. Endurance flight is predicted to further upregulate genes supporting fatty acid metabolism, and those related to mediating cellular damage, initiating an inflammatory response, and muscle growth.

2.2 Materials and methods

2.2.1 Birds and experimental design

Animal capture and study procedures were approved by the University of Western Ontario Animal Care Sub-Committee (Protocol 2010-216) and the Canadian Wildlife Service (Permit CA-0256, Appendix A). Juvenile male yellow-rumped warblers were caught in September 2012 at Long Point, Ontario (Figure 1.3), a stopover point in their fall southbound migration. The warblers were housed at the Advanced Facility for Avian Research (AFAR) in cages (121 cm wide × 68 cm deep × 185 cm high) and fed a banana-based fruigivore diet (Gerson and Guglielmo, 2011) supplemented with mealworms (*Tenebrio molitor*). Initially, the birds were kept on a natural fall photoperiod (12 h light: 12 h dark), and this was changed in mid-November to a short day winter photoperiod (8 h light: 16 h dark) while the birds entered a non-migratory wintering condition. Nocturnal behaviour was video-recorded during the sampling periods to ensure that migratory birds, and not wintering birds, expressed migratory restlessness behaviour (Agatsuma and Ramenofsky, 2006).

To compare seasonal changes in the flight muscle transcriptome between fall migratory conditioned and wintering non-migratory conditioned birds, I sampled five warblers at lights out in October 2012 and January 2013. The birds were food-deprived for 2 h prior to sampling and immediately before sampling, wet lean mass and fat mass of the birds was measured using quantitative magnetic resonance (QMR, Guglielmo et al., 2011). The birds were anesthetised with isoflurane

(PrFlorane, Baxter, Mississaugua, ON) and immediately killed by decapitation. Approximately 1.5 g of the right pectoralis muscle was immediately flash frozen in liquid nitrogen and stored at -80 °C for later analysis. Seasonal changes in body mass and composition were analyzed using Student t tests in R (version 3.3.1 - R Development Core Team, 2014).

To assess changes in the flight muscle transcriptome that occurs during migratory flight compared to rest, five warblers were sampled immediately after a simulated nocturnal fall migratory flight. For the flights, the birds were food-deprived for 2 h prior to lights turning off, and at lights out the body composition was measured using QMR. The birds were flown for 4 h (~115 km) under controlled conditions (low light at 8 m/s, 15 °C and 70% RH) in a wind tunnel designed for birds at AFAR (for wind tunnel description see Gerson and Guglielmo, 2011). Following the flight, body composition was measured again by QMR, and the flight muscle was sampled as described above. Flight energy costs and fuel composition were calculated from fat and lean mass loss during the flight (Gerson and Guglielmo, 2011) and described in Chapter 3.

2.2.2 Muscle enzyme assays

Metabolic activities of CPT, HOAD, CS, and lactate dehydrogenase (LDH) were measured according to Price et al. (2010) to assess changes in aerobic and anaerobic capacity in the flight muscles. Briefly, approximately 100 mg of muscle was diluted in 9 volumes (10-fold dilution) of ice-cold homogenization buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% defatted BSA, 50% glycerol, 0.1% Triton X-100,

50 µg/ml), and homogenized 3 times for 10 s using a Polytron PT 10-35 homogenizer with a 7 mm generator (Kinetica, USA), waiting 30 s between each bout. The homogenates were then stored at -80 °C for up to 3 months until analysis. Enzyme assays were conducted using a Cary 100 UV/Vis Bio Spectrophotometer (Varian, Palo Alto, CA), with the temperature maintained at 39 °C using a Peltier temperature control module.

Activities were measured in a final reaction volume of 1 ml using disposable cuvettes. CPT activity was measured by the reaction of carnitine with palmitoyl CoA producing CoA and CoA reacts with DTNB yielding the yellow coloured TNB. CPT activity was assayed using 10 µl of a 1:10 dilution of the homogenate, 5 mM carnitine, 0.15 mM DTNB, and 0.035 mM palmitoyl CoA in a 50 mM Tris buffer (pH 8 at 39°C). CS activity was assayed through the reaction of oxaloacetic acid with acetyl CoA yielding citrate and CoA, and the rate of reaction monitored its reaction with DTNB. CS was measured using 10 µl of 1:20 diluted homogenate, 0.5 mM oxaloacetic acid, 0.15 mM DTNB, 0.15 mM acetyl CoA, and 50 mM Tris buffer. HOAD activity was measured by monitoring the decrease in NADH from the reduction of acetoacetyl CoA and was assayed with 10 µl of 1:10 homogenate with 0.2 mM NADH, 1 mM EDTA, 0.1 mM acetoacetyl CoA, and 50 mM imidazole buffer (pH 7.4 at 39 °C). LDH was assayed by monitoring the decrease in NADH from the production of lactate from pyruvate, and was measured using 10 µl of 1:100 homogenate, with 4 mM pyruvate, 0.15 mM NADH, 5 mM DTT, and 50 mM imidazole buffer. The maximum enzyme activity was calculated from the changes in absorbance at 412 nm for CS

and CPT and 340 nm for HOAD and LDH. Enzyme activity was analyzed using 1-way ANOVA and Tukey post hoc tests in R.

2.2.3 RNA sequencing and transcriptome assembly

Three birds from each condition were randomly selected for high-throughput mRNA sequencing (RNAseq, Wang et al., 2009). To isolate the RNA from the flight muscle, 70 mg of tissue was homogenized in 1 ml of TRIzol reagent (Life Technologies, Burlington, ON) in glass homogenizers according to the manufacture's protocol. The isolated RNA was further purified using the Qiagen RNeasy mini CleanUp kit (Qiagen Inc., Mississauga, ON). Total RNA integrity values (RIN) were between 8.1 - 9.7 as measured using the 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Illumina library preparation, sequencing, and *de novo* transcriptome assembly were performed at Genome Québec, McGill University following standard protocols. The cDNA libraries were constructed using the TruSeq RNA sample preparation kit (Illumina, San Diego, CA), which specifically targets only mRNA for the library generation. Each bird has its own library prepared with a unique barcode tag to identify the bird's sequences. Three birds were multiplexed per cell lane using 3 lanes in total. Sequencing was carried out on the Illumina HiSeq 2000 platform with 100 bp paired-end reads and a library insert size of approximately 200 bp.

The sequencing generated 65 to 88 million paired reads per library. The reads were trimmed from the 3' end and the sequencing adapters removed from the

reads using Trimmomatic software (Bolger et al., 2014). Reads had a minimum length of 32 bp and met the sequence quality score threshold (phred score > 30). The nine libraries were pooled for the *de novo* transcriptome assembly using the Trinity software suite (http://trinityrnaseq.sourceforge.net/, Haas et al., 2013). The reads were normalized using the Trinity normalization utility to increase assembly runtime efficiency and memory use (Titus et al., 2012), and the normalized reads were used for the assembly of continuous sequences, or contigs. The contig assembly generated 123 636 gene components and 227 136 transcript isoforms. The contigs had a minimum length of 201 bp and maximum length of 38 260 bp. The mean contig length was 1366 bp and the median length of 602 bp. Gene abundance for each library was calculated using RSEM (http://deweylab.biostat.wisc.edu/rsem/, Li and Dewey, 2011), and low quality contigs were removed resulting in 73 341 transcripts, with 68 577 unique genes. The assembled transcripts were aligned using BLASTX to the NCBI nr protein database (SWISS-PROT, TrEMBL similarity set at >30% and probability of a sequence match by chance set (E-value <1e-5)). Using the best hit, 42 754 (58%) genes were successfully identified. Transcripts with the same BLAST identifier were aggregated together, reducing the number of transcripts to 37 990 genes.

2.2.4 Differential gene expression

Differential gene expression was assessed using edgeR (version 3.4, Robinson et al., 2010) with migratory season (fall and winter) and flight (fall unflown and flown) analyzed separately. Transcripts were filtered for low

expression, requiring at least three of six individuals to have a minimum read count of 1 count per million (cpm). This filtering reduced the transcript number to 12 069 in the migratory comparison and 12 050 for the flight comparison. The libraries were normalized to control for differences in library size (Robinson and Oshlack, 2010). A false detection rate (FDR) of 0.05 was the cut-off for differential expression.

2.2.5 KEGG pathway enrichment analysis

To examine changes in metabolic and signalling pathways, I used the Generally Applicable Gene-set Enrichment (GAGE) method (Luo et al., 2009). GAGE tests for significant enrichment of Koyoto Encylcopaedia of Gene and Genomes (KEGG) pathways. KEGG identifiers for the transcriptome were obtained by using the KEGG automatic annotation server for annotation (Moriya et al., 2007). Using the tabulated counts for each KEGG term, differential testing was performed on the KEGG terms and used to test for pathway enrichments for migration and flight separately. The q-value, an adjusted p-value, of 0.15 was used as a cut-off for the identification of pathways that were significantly enriched. Pathview (Luo and Brouwer, 2013) was used to generate enrichment pathway maps for significant pathways to identify genes contributing to the pathway up or downregulation.

2.3 Results

2.3.1 Body composition and flight muscle metabolic enzymes

Body mass and composition data for the migratory unflown and pre-flight composition from the flown birds were combined to test for seasonal changes in

body composition. Body mass did not differ between migratory and winter condition birds ($t = 0.36$, $P = 0.72$), but body composition differed with migratory birds having 1.25 g more fat ($t = 3.81$ $P = 0.002$) and 1.02 g less lean mass ($t = 4.72$, $p < 0.001$, Table 2.1). There was no effect of flight on any metabolic enzyme activity when comparing unflown and flown fall migrants ($p > 0.05$), so the fall-flown and unflown groups were combined to test for seasonal differences (Figure 2.1). Birds in the migratory condition had 2.7-fold higher CPT activity ($t = 3.32$, $P = 0.005$), 1.27-fold higher CS activity ($t = 2.54$, $P = 0.02$), 1.7-fold higher HOAD activity ($t = 2.51$, $P = 0.005$), and 1.92-fold lower activity of LDH ($t = 9.70$, $P < 0.001$). Overall, birds in the fall had larger fat stores and higher aerobic and oxidative capacity in the flight muscles.

Table 2.1 Seasonal differences in body composition of yellow-rumped warblers (*Setophaga coronata*).The migratory group includes migratory unflown birds and the pre-flight condition.

	Migratory		Wintering	
Body mass (g)	12.93	±0.09	12.81	±0.09
Fat mass (g)	2.18	±0.08*	0.93	±0.08
Lean mass (g)	8.66	±0.04*	9.68	±0.03

* Indicate statistical differences between seasons ($P \leq 0.05$). n=10 for migratory, and n=5 for wintering birds.

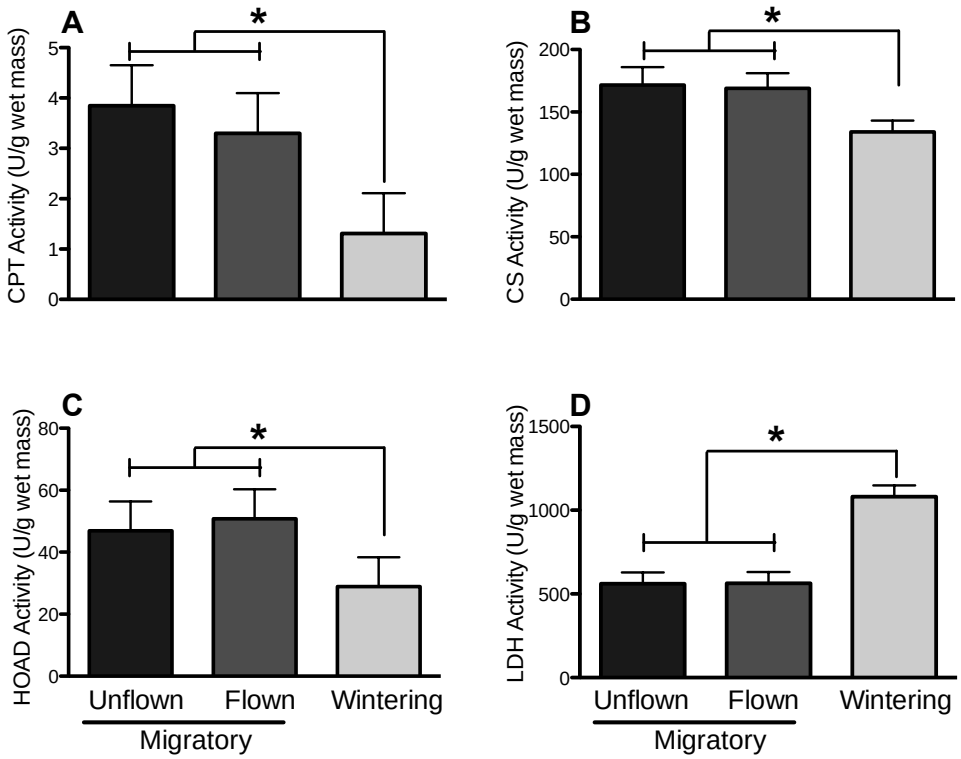

Figure 2.1 Seasonal differences in flight muscle enzyme activity. A) carnitine palmitoyl transferase (CPT), B) citrate synthase, C) 3-hydroxyacul-CoA dehydrogenase (HOAD), D) lactate dehydrogenase (LDH).* Indicates statistical difference between migratory unflown and flown combined, and wintering warblers ($P \leq 0.05$). n=5 per group.

2.3.2 Transcriptome alterations during migration

During the fall migratory period 455 genes (3.7 % of 12 069 genes) were differentially expressed compared to the wintering non-migratory condition, with 192 (1.6 %) having higher and 263 (2.1 %) having lower abundance during the fall (selected genes in Table 2.2, full list in Supplemental File 1). Of the differentially expressed transcripts, only 128 (67 %) of the upregulated and 173 (66 %) of the downregulated genes had a fold change of at least 2. Notable differentially expressed genes encoded proteins relating to fatty acid metabolism were regulators of mitochondrial biogenesis and fatty metabolism (PGC-1α, PGC-1β and PPAR), and components of lipid metabolism: FABP, acyl-COA dehydrogenase, and a peroxisome-specific fatty acid transporter, ATP-binding cassette subfamily D-1 (ABCD-1) (Vasiliou et al., 2009). Similar to the metabolic enzyme activity, there was increased aerobic capacity and decreased anaerobic capacity during migration, with lactate dehydrogenase gene expression being downregulated during migration. Genes involed in muscle hypertrophy were both up and downregulated. The growth hormone receptor was upregulated during migration, but there was a general downregulation of gene encoding the Wnt-5, a regulator of myogenesis and differentiation.

KEGG pathway enrichment analysis detected 4 significantly upregulated pathways during migration (Figure 2.2, Table 2.3), and three of these pathways were directly linked to fatty acid metabolism. This is in striking contrast to 45 downregulated pathways observed during migration (Figure 2.2), 20 of which are

essential and non-redundant pathways (Table 2.3). Many of the downregulated pathways can be functionally grouped into circadian rhythm, hormone signalling, inflammation and immune function, and muscle growth. It should be noted that many hormone and signalling pathways share common genes and could be part of a common signalling pathway.

Table 2.2 Selected differentially expressed genes during migration and proposed function. Fold change (FC) relative to the migratory season.

Gene accession	FC	P Value	FDR	Description	Proposed function
gi|498991207|ref|XP_004532043.1|	3.81	3.34E-06	3.20E-04	Acyl-CoA dehydrogenase fadE12-like [*Ceratitis capitata*]	Fatty acid metabolism
gi|350538451|ref|NP_001232098.1|	3.51	9.29E-08	1.42E-05	Putative fatty acid-binding protein [*Taeniopygia guttata*]	Fatty acid metabolism
gi|431892685|gb|ELK03118.1|	3.34	1.67E-04	7.64E-03	Long-chain-fatty-acid--CoA ligase 6 [*Pteropus alecto*]	Fatty acid metabolism
gi|224095830|ref|XP_002187790.1|	2.93	3.97E-11	1.37E-08	Peroxisome proliferator-activated receptor alpha [*Taeniopygia guttata*]	Fatty acid metabolism
gi|326931726|ref|XP_003211976.1|	2.20	3.69E-05	2.19E-03	Acetyl-coenzyme A synthetase	Aerboic metabolism
gi|449474754|ref|XP_002192758.2|	2.20	1.71E-03	4.67E-02	Peroxisome proliferator-activated receptor gamma coactivator 1-beta [*Taeniopygia guttata*]	Mitogenesis and fatty acid metabolism
gi|224052475|ref|XP_002198152.1|	2.16	8.53E-05	4.49E-03	Acyl-CoA desaturase [*Taeniopygia guttata*]	Fatty acid metabolism
gi|483498881|gb|EOA94206.1|	1.80	3.53E-04	1.45E-02	Long-chain-fatty-acid--CoA ligase 1	Fatty acid metabolism
gi|449514170|ref|XP_002193695.2|	1.61	5.61E-04	2.11E-02	Growth hormone receptor [*Taeniopygia guttata*]	Muscle growth
gi|224050848|ref|XP_002198647.1|	0.64	1.51E-03	4.34E-02	L-lactate dehydrogenase A chain [*Taeniopygia guttata*]	Anerobic metabolism
gi|310789275|gb|ADP24691.1|	0.44	4.33E-09	9.49E-07	Adipose triglyceride lipase [*Passer domesticus*]	Fatty acid metabolism
gi|82524637|ref|NP_001032346.1|	0.31	5.25E-08	9.06E-06	Wnt-5b precursor [*Gallus gallus*]	Muscle growth
gi|449509407|ref|XP_002188433.2|	3.81	1.33E-07	1.87E-05	Period circadian protein homolog 2-like [*Taeniopygia guttata*]	Circadian rhythm

FDR, false detection rate

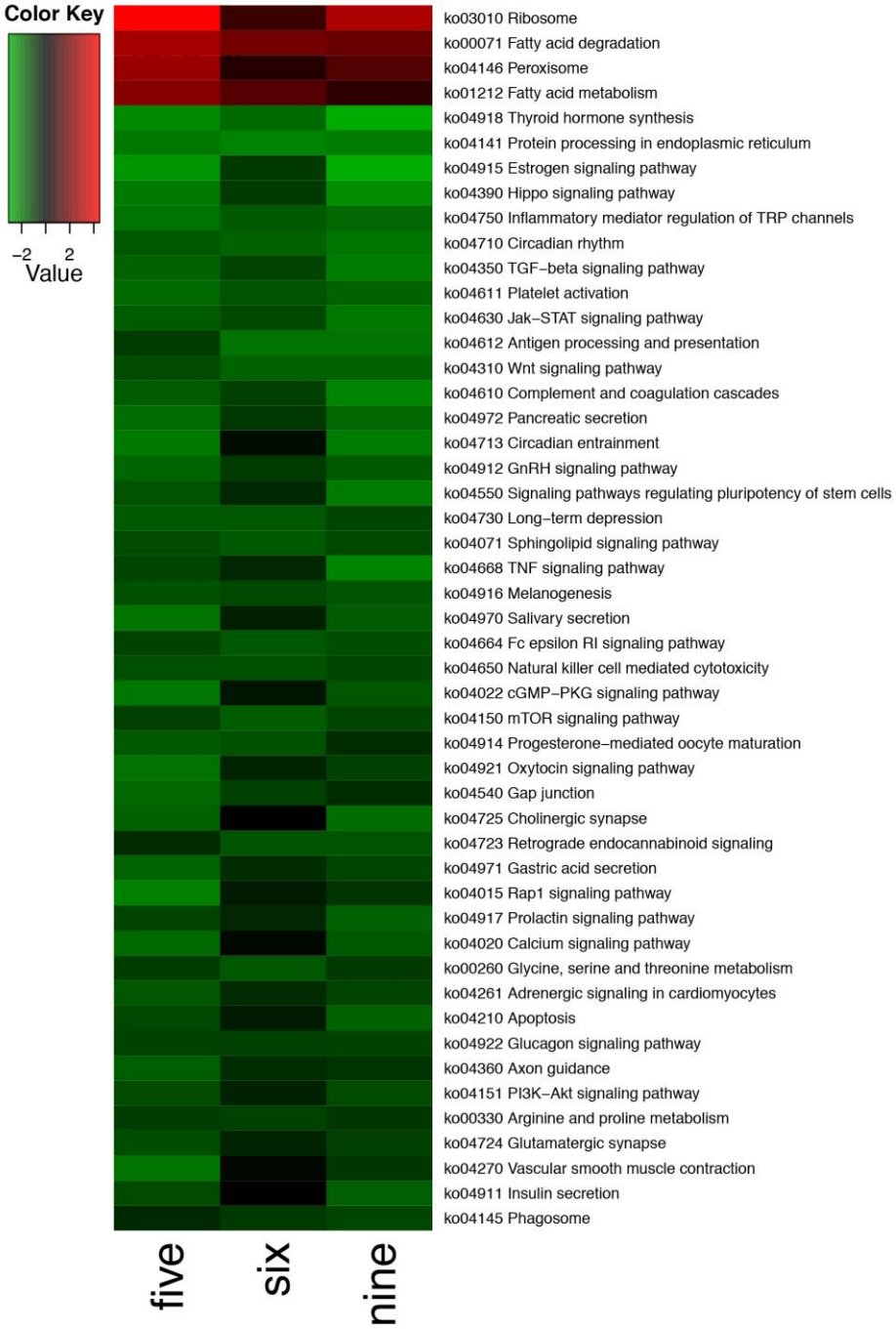

Figure 2.2 Heatmap of up- and downregulated pathways in the flight muscle during the migratory season. Red indicates upregulation and green downregulation during migration. Migratory bird IDs are indicated on the bottom.

Table 2.3 Significantly enriched KEGG pathways during the migratory season.

Migration upregulated pathway	Proposed function	p value	q value	Set size
ko03010 Ribosome	Protein translation	<0.001	0.00	114
ko00071 Fatty acid degradation	Fatty acid metabolism	<0.001	0.01	25
ko04146 Peroxisome	Fatty acid metabolism	0.003	0.14	55
ko01212 Fatty acid metabolism	Fatty acid metabolism	0.004	0.15	33
Migration downregulated pathway	**Proposed function**	**p value**	**q value**	**Set size**
ko04918 Thyroid hormone synthesis	Cell signalling	<0.001	0.01	23
ko04141 Protein processing in endoplasmic reticulum	Protein synthesis	<0.001	0.01	114
ko04915 Estrogen signaling pathway	Cell signalling	<0.001	0.01	35
ko04390 Hippo signaling pathway	Myogenesis	0.001	0.03	64
ko04750 Inflammatory mediator regulation of TRP channels	Response to heat	0.001	0.04	27
ko04710 Circadian rhythm	Circadian rhythm	0.001	0.04	18
ko04350 TGF-beta signaling pathway	Myogenesis	0.002	0.04	38
ko04610 Complement and coagulation cascades		0.002	0.04	27
ko04611 Platelet activation		0.002	0.04	47
ko04612 Antigen processing and presentation	Immune function	0.002	0.04	22
ko04310 Wnt signaling pathway	Myogenesis	0.003	0.05	56
ko04630 Jak-STAT signaling pathway	Cell signalling	0.003	0.05	49
ko04972 Pancreatic secretion	Cell signalling	0.004	0.05	28
ko04912 GnRH signaling pathway	Cell signalling	0.005	0.06	34
ko04713 Circadian entrainment	Circadian rhythm	0.005	0.06	32
ko04550 Signaling pathways regulating pluripotency of stem cells	Myogenesis	0.006	0.07	53
ko04730 Long-term depression	Cell signalling	0.007	0.07	21
ko04071 Sphingolipid signaling pathway	Extracellular signalling	0.007	0.07	53
ko04668 TNF signaling pathway	Inflammation	0.008	0.07	52
ko04916 Melanogenesis	Cell signalling	0.008	0.07	29

2.3.3 Transcriptome Response to Endurance Flight

During the 4 h simulated migratory flight, the metabolic rate rose to 1.75 ±0.03 J/s (~8 x basal metabolic rate; Chapter 3) and 97.3 ± 0.4 % of the estimated energy used was contributed from fat mass catabolism. Flight had a considerably larger effect on the flight muscle transcriptome than migratory condition itself. A total of 1778 genes (14.6% of 12 050 genes) were differentially expressed during flight. Of these genes, 925 were enriched during flight with 543 (59 %) of these having greater than 2-fold change (selected genes in Table 2.4, full list in Supplemental File 1). The remaining 853 genes were downregulated, and 566 (66 %) had more than a 2-fold changed in abundance. Many of the upregulated genes were related to fatty acid metabolism, including genes encoding the proteins for PPARβ, the low-density lipoprotein receptor, and 2 cytosolic FABPs. Intriguingly, downregulation of lipid metabolism regulators was observed, and decreased mRNA abundance of genes encoding the proteins for PGC-1α, PGC-1β, and sterol regulatory element binding protein 1, a regulator of carbohydrate and lipid metabolism (Guillet-Deniau et al. 2002), was observed. Carbohydrate metabolism genes were also downregulated during flight, including genes encoding proteins for glycogen synthase, 2 pyruvate kinases, and glucose 1,6-bisphospahte synthase. The gene expression of 2 MAP kinase-activated proteins (MAPK) increased, and the MAPK signalling pathway can lead to the initiation of other cell signals, including energy metabolism, growth, cellular stress response, inflammation and apoptosis (Zetser et al., 1999; Akimoto et al., 2005; Li, 2005). Differentially

expressed genes supporting growth included genes encoding proteins for 2 IGF1 receptor transcripts, and Wnt-5. Inflammatory pathway signalling and apoptosis included upregulation of genes encoding proteins for interleukin 1 and 6 receptors, TNF receptor 1, NFκβ inhibitor alpha and 2 transcripts for the apoptosis-stimulating p53 protein 1.

KEGG pathway enrichment analysis identified 16 enriched pathways (Figure 2.3, Table 2.5; Supplemental File 3), with 9 upregulated and 7 downregulated during flight. Interestingly, of the upregulated pathways during flight, 8 were found to be downregulated during the migratory season and were related to muscle growth, protein synthesis, and inflammation and cellular damage. Downregulated pathways during flight included those related to glucose metabolism and mitochondrial oxidative phosphorylation.

Table 2.4 Selected differentially expressed genes during flight and proposed function. Fold change (FC) relative to flight.

Gene annotation	FC	P Value	FDR	Description	Function
gi|224060501|ref|XP_002188146.1|	8.94	5.01E-13	7.28E-11	D-beta-hydroxybutyrate dehydrogenase, mitochondrial [*Taeniopygia guttata*]	Ketogenesis
gi|498991207|ref|XP_004532043.1|	3.92	9.03E-08	4.32E-06	Acyl-CoA dehydrogenase fadE12-like [*Ceratitis capitata*]	Fatty acid metabolism
gi|224046040|ref|XP_002187795.1|	3.51	1.63E-16	3.92E-14	Low-density lipoprotein receptor class A domain-containing protein 4 isoform 1 [*Taeniopygia guttata*]	Fatty acid metabolism
gi|224048217|ref|XP_002188863.1|	3.32	2.05E-03	1.88E-02	Fatty acid-binding protein, brain isoform 1 [*Taeniopygia guttata*]	Fatty acid metabolism
gi|345306526|ref|XP_001508767.2|	2.79	8.07E-04	9.10E-03	Insulin-like growth factor 1 receptor-like [*Ornithorhynchus anatinus*]	Muscle Growth
gi|113206140|ref|NP_001038140.1|	2.77	1.76E-08	1.02E-06	Interleukin 6 receptor alpha precursor [*Gallus gallus*]	Cytokine & Inflammation
gi|82524637|ref|NP_001032346.1|	3.20	3.01E-10	2.57E-08	Protein Wnt-5b precursor [*Gallus gallus*]	Muscle growth
gi|10720326|sp|O57429.1|UBP2_CHICK	2.64	3.30E-09	2.29E-07	Ubiquitin carboxyl-terminal hydrolase 2;	Protein degradation
gi|465971934|gb|EMP32900.1|	2.51	1.31E-03	1.34E-02	Apoptosis-stimulating of p53 protein 1 [*Chelonia mydas*]	Apoptosis
gi|291399762|ref|XP_002716270.1|	2.38	4.35E-06	1.25E-04	Glycerol-3-phosphate dehydrogenase 1-like [*Oryctolagus cuniculus*]	Energy metabolism
gi|449504530|ref|XP_002200620.2|	2.33	4.15E-08	2.11E-06	Apoptosis-stimulating of p53 protein 1 [*Taeniopygia guttata*]	Apoptosis
gi|449490414|ref|XP_002199092.2|	2.33	2.03E-06	6.47E-05	Peroxisome proliferator-activated receptor delta [*Taeniopygia guttata*]	Fatty acid metabolism
gi|224066687|ref|XP_002187461.1|	2.16	3.67E-04	4.76E-03	Interleukin-1 receptor-associated kinase-like 2-like [*Taeniopygia guttata*]	Cytokine signalling
gi|326929760|ref|XP_003211024.1|	1.66	3.25E-04	4.31E-03	Acetyl-CoA carboxylase 2-like [*Meleagris gallopavo*]	Fatty acid metabolism
gi|449473720|ref|XP_002189995.2|	1.79	4.12E-03	3.22E-02	MAP kinase-activated protein kinase 3 [*Taeniopygia guttata*]	Cell signalling
gi|57530631|ref|NP_001006346.1|	1.82	3.85E-05	7.66E-04	Fatty acid binding protein 5 (psoriasis-associated) [*Gallus gallus*]	Fatty acid metabolism

Table 2.3 (continued).

Gene Annotation	Log FC	P Value	FDR	Description	Function
gi|224090576|ref|XP_002187201.1|	1.82	1.88E-04	2.76E-03	Interleukin-6 receptor subunit beta [*Taeniopygia guttata*]	Cytokine signalling
gi|159895428|gb|ABX09998.1|	1.83	1.52E-03	1.50E-02	NF-kappa-B inhibitor alpha [*Gyps fulvus*]	Inflammation
gi|224062731|ref|XP_002199843.1|	3.32	2.90E-04	3.92E-03	Insulin-like growth factor 1 receptor-like [*Taeniopygia guttata*]	Muscle Growth
gi|401664564|ref|NP_001257909.1|	0.67	4.22E-03	3.28E-02	Glutathione peroxidase 3 (plasma) precursor [*Taeniopygia guttata*]	Antioxidant
gi|395852279|ref|XP_003798667.1|	0.64	3.22E-03	2.65E-02	Glycogen phosphorylase, muscle form isoform 1 [*Otolemur garnettii*]	Carbohydrate metabolism
gi|149474187|ref|XP_001505412.1|	0.62	8.17E-04	9.20E-03	Glycogen [starch] synthase, muscle isoform 1 [*Ornithorhynchus anatinus*]	Carbohydrate metabolism
gi|449269405|gb|EMC80178.1|	0.59	1.77E-04	2.63E-03	Pyruvate kinase muscle isozyme, partial [*Columba livia*]	Glycolysis
gi|224044133|ref|XP_002187700.1|	0.53	1.19E-04	1.94E-03	Glucose 1,6-bisphosphate synthase [*Taeniopygia guttata*]	Glycolysis
gi|489133935|ref|WP_003043720.1|	0.48	4.13E-06	1.21E-04	D-lactate dehydrogenase (cytochrome) [*Thermus aquaticus*]	Anerboic metabolism
gi|5702302|gb|AAD47248.1|	0.20	2.77E-08	1.49E-06	Pyruvate kinase M2 [*Homo sapiens*]	Glycolysis
gi|224064940|ref|XP_002189312.1|	0.67	9.32E+00	4.50E-03	Glucose-6-phosphate isomerase [*Taeniopygia guttata*]	Glycolysis
gi|449490243|ref|XP_002195870.2|	0.20	9.21E-07	3.27E-05	Myogenin [*Taeniopygia guttata*]	Muscle growth and repair
gi|45383858|ref|NP_989457.1|	0.26	1.29E-12	1.67E-10	Sterol regulatory element-binding protein 1 [*Gallus gallus*]	Energy metabolism
gi|224050054|ref|XP_002192636.1|	0.59	3.54E-03	2.86E-02	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha [*Taeniopygia guttata*]	Fatty acid metabolism and mitogenesis
gi|449474754|ref|XP_002192758.2|	0.30	1.11E-07	5.19E-06	Peroxisome proliferator-activated receptor gamma coactivator 1-beta [*Taeniopygia guttata*]	Fatty acid metabolism and mitogenesis

FDR, false detection rate

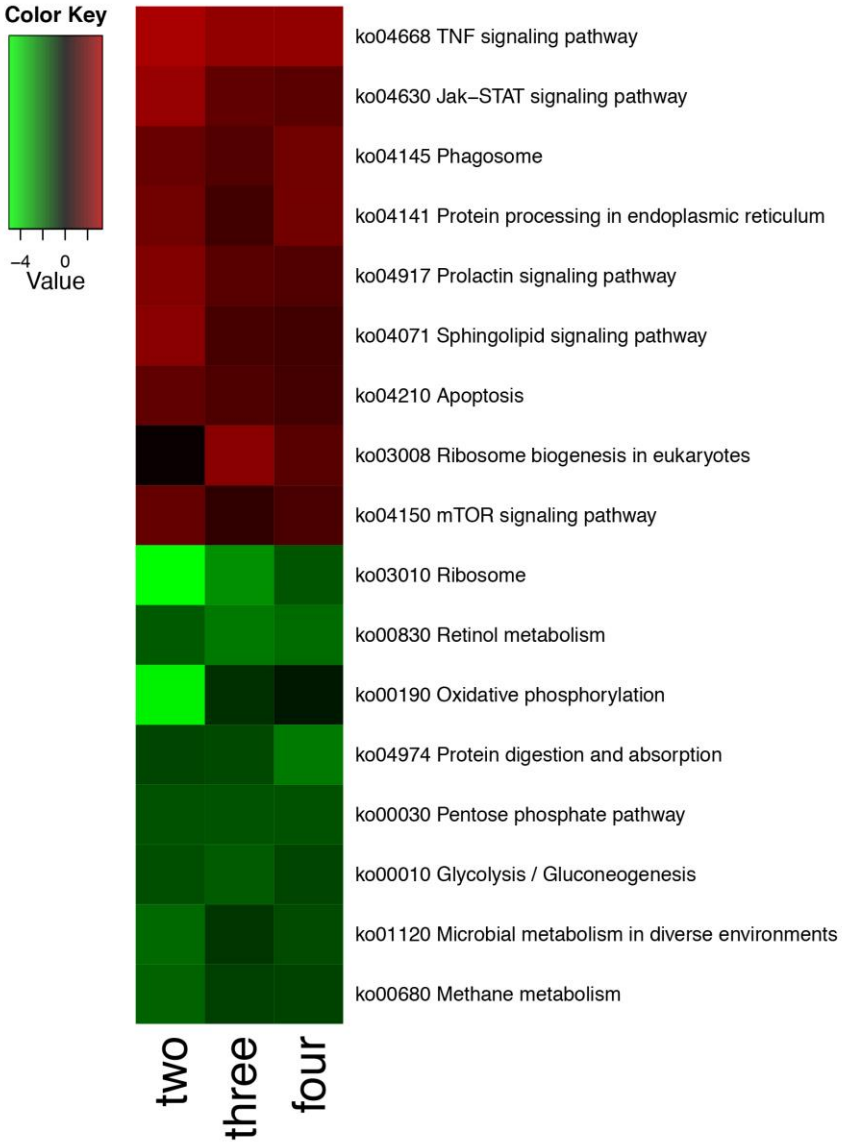

Figure 2.3 Heatmap of significantly up and downregulated pathways in the flight muscle during migration. Red indicates upregulation and green downregulation during migration. Migratory flown bird IDs are indicated on the bottom.

Table 2.5 Significantly enriched KEGG pathways during simulated migratory flight.

Flight upregulated pathway	Proposed function	p-vlaue	q value	Set size
ko04668 TNF signaling pathway	Inflammation	<0.001	<0.01	52
ko04630 Jak-STAT signaling pathway	Myogenesis	<0.001	0.01	52
ko04145 Phagosome	Degradation	0.001	0.02	61
ko04141 Protein processing in endoplasmic reticulum	Protein synthesis	0.002	0.02	114
ko04917 Prolactin signaling pathway	MAPK signalling	0.002	0.02	30
ko04071 Sphingolipid signaling pathway	Extracellular signalling	0.001	0.04	53
ko04210 Apoptosis	Cell death	0.003	0.09	70
ko03008 Ribosome biogenesis in eukaryotes	Protein synthesis	0.004	0.10	60
ko04150 mTOR signaling pathway	Protein synthesis	0.006	0.13	67

Flight downregulated pathway	Proposed function	p value	q value	Set size
ko03010 Ribosome	Protein translation	<0.001	<0.01	114
ko00830 Retinol metabolism		0.001	0.02	14
ko00190 Oxidative phosphorylation	Mitochondrial function	0.001	0.02	100
ko04974 Protein digestion and absorption	Amino acid uptake	0.002	0.09	18
ko00030 Pentose phosphate pathway	Sugar metabolism	0.004	0.11	15
ko00010 Glycolysis / Gluconeogenesis	Sugar metabolism	0.004	0.11	30
ko01120 Microbial metabolism in diverse environments	Sugar metabolism	0.004	0.11	98

2.4 Discussion

Similar to other migratory birds (Ramenofsky et al., 1999; Guglielmo et al., 2002; Zajac et al., 2011; Marshall et al., 2016), during the migratory season the yellow-rumped warblers exhibited an upregulation of oxidation enzymes, greater fat stores, and they expressed nocturnal migratory restlessness behaviour and hyperphagia (personal observation). Alterations in the transcriptome revealed coordinated upregulation of genes supporting fatty acid metabolism during migration. Furthermore, greater changes occurred in the transcriptome during flight than seasonally. This provides a view of how birds sustain energy production for muscle contraction and the effects of muscle damage. Here, I discuss seasonal changes, which are primarily related to fatty acid metabolism, and the effect of flight which influences energy metabolism and cellular response during the transition to a stable exercise state.

2.4.1 Transcriptome alteration during migratory season

Migratory preparation in the flight muscles ensures that birds are able to sustain high rates of ATP production for prolonged periods of time, and requires a coordinated increased in fatty acid oxidation pathways. PGC-1α, PGC-1β, and PPARs are major regulators of lipid metabolism and mitochondrial biogenesis, and have been suggested to be key regulators of lipid metabolism during migration (Weber 2009; DeMoranville, 2015). PGC-1α, PGC-1β, and PPARα gene expression was elevated during the migratory season (Table 2.3), supporting their association with increasing aerobic capacity observed in migratory gray catbirds (*Dumetella*

carolinesis, DeMoranville, 2015). Increased mitochondrial biogenesis during the migratory season is observed from the increase in CS activity, an indicator of mitochondrial density in skeletal muscle (Larsen et al., 2012). PGC-1α gene and protein expression increases during acute exercise and training (Perry et al., 2010), and this enables mitochondrial remodelling, which, shifts the fuel source from carbohydrate to fat as part of the training effect (Koves et al., 2005). Regarding exercise performance, PGC-1α increases whole body peak metabolic rate (Calvo et al., 2008), which also occurs during migration in yellow-rumped warblers (Swanson and Dean, 1999). Less is known about PGC-1β in terms of exercise performance (Schnyder et al., 2017). However, it likely plays a role in maintaining mitochondrial structure and increasing oxidative capacity in skeletal muscle (Zechner et al., 2010; Ramamoorthy et al., 2015). PPARα mRNA abundance has been reported to be similar or elevated in endurance-trained animals (Kannisto and Chibalin, 2006; Perry et al., 2010). PPARα and PPARβ regulate the expression of similar types of genes; however, PPARβ is the major PPAR found in skeletal muscle (Burri et al., 2010). PPARα supports fatty acid oxidation during fasting (Muoio et al., 2002), and helps control the expression of fatty acid transporters and β-oxidation genes (Finck et al., 2005). Estrogen-related nuclear receptors (ERR) are regulators of cellular metabolism, particularly oxidative and fatty acid metabolism (Huss et al., 2004; Alaynick, 2008), and have been suggested as potential regulators of lipid metabolism during migration (DeMoranville, 2015). Although the estrogen signalling pathway was downregulated during the migratory season, expression of ERRs was increased in the KEGG pathway analysis (Supplemental File 2). This

supports the role of ERRs in increasing lipid oxidative capacity and is unrelated to the traditional estrogen signalling pathway.

Activation of PGC-1α, PPARs and other potential regulators leads a coordinated response in the flight muscle similar to the increased aerobic capacity from endurance exercise training (Camera et al., 2016). Transporting fatty acids into and within cells is increased during the migratory season (Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011; Zhang et al., 2015; Banerjee and Chaturvedi, 2016). I found increased expression of a gene encoding the protein for a cytosolic FABP (Putative fatty acid-binding protein) during the migratory season. The capacity to oxidize fatty acids increased during the migratory season, which was indicated through enrichment of the fatty acid degradation pathway, and included increased mRNA abundances of genes encoding acyl-CoA dehydrogenase and acyl-CoA desaturase, and enzyme activity of CPT, HOAD and CS. Additionally, the increase in aerobic capacity was mirrored by a decrease in anaerobic metabolism and both LDH enzyme activity and mRNA abundance were decreased during the migratory season. This response is similar to the differences in the flight muscle transcriptome of migratory and resident dark-eyed juncos (*Junco hyemalis*, Fudickar et al., 2016), and proteomic changes during migration in red-headed buntings (*Emberiza bruniceps*, Banerjee and Chaturvedi, 2016). The coordinated change during the migratory season demonstrates the fundamental importance of the capacity to fuel flight with fat.

The breakdown of fatty acids through β-oxidation occurs in the mitochondria and peroxisomes (Poirier et al., 2006). Mitochondrial β-oxidation produces ATP more efficiently since $FADH_2$ and NADH production is coupled with oxidative phosphorylation (Speijer, 2011). However, polyunsaturated fatty acids are oxidized slowly in mitochondria compared to peroxisomes (Hiltunen et al., 1986), and peroxisomal oxidation of very long chain fatty acids prior to mitochondrial oxidation may lower free radical formation (Speijer, 2011). Similar to migratory juncos (Fudickar et al., 2016), I found that the peroxisome pathway was upregulated during the migratory season, and included increased gene expression of a peroxisome-specific fatty acid transporter, ABCD1. Along with increased capacity for mitochondrial β-oxidation during migration, peroxisomes also play an important role in supporting fatty acid oxidation, especially if birds are fuelling flight with a high proportion of polyunsaturated fatty acids.

The large number of downregulated pathways during the migratory season suggests that migratory preparation involves alterations to aspects of muscle function and maintenance other than increasing fatty acid oxidation. The downregulated pathways during the migratory season can be functionally grouped into circadian rhythm, cell signalling, muscle growth, and inflammation (Table 2.4), and share similarities to downregulated gene ontologies observed in migratory dark-eyed juncos (Fudickar et al., 2016). However, I propose that alterations to circadian rhythm and sampling at the start of the dark period may have further contribute to the large number of downregulated pathways observed in the migratory conditioned birds.

The circadian rhythm is regulated by brain and muscle arnt-like protein 1 (Bmal1) and circadian locomotor output cycles kaput (Clock), and their activation increases the gene expression of their inhibiting proteins, period 2 and cryptochrome 1 (Harfmann et al., 2015). Both photoperiod and stage of migration (premigratory, migratory, post-migratory) alter the peak amplitudes and oscillations in expression of these genes (Singh et al., 2015), and may explain the overall downregulation of the circadian rythym pathway during migratory season. Up to 2300 genes show a daily rhythm of expression in skeletal muscle, including PGC-1α, MyoD1 (a master regulator of muscle gene expression) and Wnt (a regulator of myogenesis and differentiation) (Harfmann et al., 2015). This suggests that time of day and seasonal differences in daily activity may influence the muscle transcriptome. The migratory and wintering birds in this study were sampled at the beginning of the dark period. During migration, songbirds enter a quiescent period of rest prior to departing on a nocturnal migratory flight (Ramenofsky et al., 2003; Agatsuma and Ramenofsky, 2006). The role of this period is still unclear, but one hypothesis is that it serves as a transitional state for metabolic and hormonal signalling from the anabolic daytime refuelling to the catabolic nocturnal flight (Ramenofsky et al., 2003). If the flight muscle is preparing for migration and shifting to a catabolic state, it could be reflected in the decreased cell signalling, muscle growth and inflammation that I observed. Seasonal changes in immune function and inflammation in the flight muscles have not been directly investigated during migration, but endurance training can have an anti-inflammatory effect on the body, as observed through lower plasma TNFα receptors levels (Conraads et al.,

2002). If seasonal migratory preparation and endurance training are similar, this could explain the decreased enrichement of inflammatory pathways, along with the potential effects of the quiescent period.

Muscle hypertrophy may occur during migration (Price et al., 2011; King et al., 2015; Fudickar et al., 2016). In my study, the decreased enrichment of the hippo and Wnt signalling pathways suggests that muscle growth is downregulated in the Migratory Unflown birds. These pathways control the development of stem cells, regeneration and organ size, and can be critical factors determining muscle fibre size (Polesskaya et al., 2003; Wackerhage et al., 2014; Watt et al., 2015). Furthermore, IGF1 mRNA abundance increases in the skeletal muscle during the migratory season and may increase muscle growth (Price et al., 2011), which I did not observe. A unique characteristic of my study is the time of day the birds were sampled compared to other studies. Price et al. (2011) sampled birds during the morning when the birds would be feeding and/or refuelling, which differs from my sampling point, where the birds were food-deprived and potentially during their quiescent period. It seems more likely that muscle growth would occur as part of the refuelling process during the day, rather than during the quiescent period before departure when birds are fasting. As such the expression of genes supporting muscle growth would also show a rhythmic expression. Potentially, the ideal time point for examining muscle growth was not captured in my study, but was in the study by Price et al. (2011). Combined with the lower lean mass during the fall migration, conclusions about muscle hypertrophy are difficult to make in my study,

but I do highlight that time of day and/or stage of migration should be explored in more detail.

2.4.2 Transcriptome alterations during migratory flight

Upon departure on a migratory flight, birds switch fuel sources from glucose and protein to predominantly fat (Gerson and Guglielmo, 2013). The 4 h simulated migratory flights did not alter metabolic enzyme activity, suggesting that migratory preparation increases enzyme activity to the levels needed to support fat fuelled endurance flight and these are maintained. Furthermore, no significant enrichment of pathways related to fatty acid metabolism was observed during flight. However, key regulators of lipid metabolism genes and transporters were differentially expressed. Interestingly, PGC-1α and PGC-1β decreased and PPARβ increased in mRNA abundance during flight. As a master regulator of the skeletal muscle response to exercise training (Camera et al., 2016), this decrease in PGC-1α and PGC-1β abundance was unexpected. A single bout of exercise in humans increases gene expression of PGC-1α, PGC-1β and PPARβ, and training results in a decrease in the amplitude of PGC-1α but not PPARβ gene expression in skeletal muscles (Perry et al., 2010). Additionally, the response during exercise can be highly variable (Camera et al., 2016). PGC-1α is required for the full activation of fatty acid oxidation by PPARβ in muscles (Kleiner et al., 2009). Muscle contraction and alteration to cellular energy availability (free fatty acids and glycogen stores) may increase the gene expression of PPARβ (Barrès et al., 2012; Philp et al., 2013), supporting its increased abundance during flight. PPARβ activation increases the expression of genes supporting fatty acid oxidation and may include the increased

expression of genes encoding for two cytosolic fatty acid binding proteins and acyl-CoA dehydrogenase. An increase in brain and epidermal associated cytosolic fatty acid transporters suggest that flight further stimulates cytosolic fatty acid transporter gene expression and this is in addition to the seasonal increases in expression. Furthermore, it also suggests that other cytosolic fatty acid transporters may also be present in the flight muscles.

Migratory flight requires high rates of mitochondrial ATP production for prolonged periods of time. The expression of genes in the oxidative phosphorylation pathway was decreased during flight, which is unexpected given the importance of ATP production. Endurance flight decreases mitochondrial ATP production capacity, and this could be a consequence of increased oxidative damage (Gerson, 2012). If oxidative phosphorylation proteins are damaged and not replaced this could contribute to the lower ATP production efficiency. Mitochondrial function returns to normal after recovery from flight (Gerson, 2012), and upregulation of the oxidative phosphorylation pathway would be expected during the post-flight recovery period. Further studies characterizing mitochondrial function during migration, flight, and the recovery period will help to elucidate if changes in the transcriptome have functional consequences to whole animal performance or if the downregulation is limited to the transcriptome level.

Fasting and the high dependence on fatty acid oxidation during flight dramatically reduced the reliance on carbohydrates for energy. This was reflected in the transcriptome with decreased expression of genes encoding glycogen synthase

and glycogen phosphorylase during flight. Furthermore, glycolysis and carbohydrate metabolism pathways were downregulated during flight, and included decreased mRNA abundances of of genes encoding for two pyruvate kinase genes, glucose 1,6-bisphosphatase, and glucose-6-phosphate isomerase. A similar downregulation of glucose metabolism has been noted with migratory preparation in juncos (Fudickar et al., 2016). Moreover, although LDH activity remained constant during flight, a decrease in LDH mRNA abundance suggests that lower levels of translation are required to maintain enzyme activity, or that the activity could decrease with longer duration flights.

During flight a coordinated increase in protein synthesis pathways was observed, with upregulation of expression of genes in the mTOR, ribosome biogenesis and protein processing in the endoplasmic reticulum pathways. The upregulation of these pathways may be mediated through the IGF1 receptor, which increased in gene abundance during flight and supports the increase of IGF1 observed in flying European starlings (*Sturnus vulgaris*, Price et al., 2011). Moreover, mRNA abundance of the gene encoding for Wnt5 precursor protein increased, which would also influence myogenesis and differentiation, through the Wnt pathway. A bout of endurance exercise is not typically associated with upregulation of the mTOR pathway in humans and mice (Williamson et al., 2006; Ogasawara et al., 2014;), and is generally associated with adaption to resistance exercise (Camera et al., 2016). However, upregulation of the mTOR pathway during endurance exercise has been observed and is suggested as a potential control point for mitogenesis (Edgett et al., 2013). Both the metabolic intensity and duration of

the endurance flights exceed those traditionally used in mammalian endurance models (Williamson et al., 2006; Edgett et al., 2013; Ogasawara et al., 2014). Migratory flight may require birds to upregulate protein synthesis to replace damaged proteins in order to maintain muscle function because of the increased length and intensity of exercise.

Endurance flight was also associated with the upregulation of other pathways related to cell damage and repair, which are initiated by changing cellular calcium concentrations, ROS production, and availability of energy substrates (Camera et al., 2016). Currently, our understanding of the damaging effects of migratory flight in birds is limited to increased plasma creatine kinase (Guglielmo et al., 2001), lower ATP production rate (Gerson, 2012), and increased blood markers of oxidative damage (Costantini et al., 2008; Jenni-Eiermann et al., 2014; Skrip et al., 2015). In terms of antioxidant capacity, the gene encoding the protein for nuclear factor E2-related factor 2(Nrf2), an activator of antioxidant gene expression (Gorrini et al., 2013; Ishii, 2004) was increased, suggests that increased antioxidant gene expression may occur during flight. However, no downstream effects of Nrf2 on antioxidant gene expression was observed at the transcriptome level, and the expression of the gene encoding glutathione peroxidase decreased during flight.

Tissue damage from extensive exercise triggers a cellular response to stress and inflammation (Townsend et al., 2015). The TNF pathway was upregulated during flight and this can initiate the development of an inflammatory response, and includes the NFκβ and ubiquitin-mediated proteolytic pathways in its pathway. A

single bout of endurance exercise is sufficient to increase plasma TNFα levels (Cannon et al., 1991). Activation of the TNF pathways can cause various cellular alterations including inhibition of IGF1/mTOR mediated protein synthesis and increased ubiquitin-mediated proteolysis via the NFκβ signalling pathway (García-Martínez et al., 1993; Fernández-Celemín et al., 2002). NFκβ is also associated with decreasing oxidative capacity and suppression of PGC-1α, PGC-1β and PPAR signalling (Remels et al., 2010). NFκβ pathway upregulation may thus explain the decreased enrichment of oxidative phosphorylation and PGC-1 expression. However, TNF and NFκβ pathway enrichment is at odds with the coordinated increase in IGF1/mTOR mediated protein synthesis I observed. Protein degradation of damage cellular components may supply the building blocks for new proteins while both flying and fasting, and thus require both anabolic and catabolic pathways.

Furthermore, NFκβ can initiate apoptosis, another upregulated pathway during flight. Markers of apoptosis were upregulated and included genes encoding the proteins for apoptosis-stimulating p53 protein, caspase-3, an initiator of apoptosis, and anti-apoptotic protein NR13 (inhibits caspase-3) (Moradi-Améli et al., 2002). In conjunction with the upregulation of the apoptosis pathway, the increased enrichment of the phagosome pathway would enable clearing of dead cells and cellular debris. Autophagy in muscles has recently received renewed interest in endurance exercise physiology and it may play an important role during exercise by removing damaged or unfit cellular components including organelles and proteins (Camera et al., 2016). In mice, ROS production during endurance

exercise increases autophagy, and blocking this process negatively impacts mitochondrial function (Qiao et al., 2015). The mTOR pathway may be involved in the autophagy process through initiation of phagosome formation (Kamada et al., 2000). Autophagy and protein degradation increases the available building blocks for new proteins (Camera et al., 2016), and as such would help support protein synthesis during flight.

The upregulation of genes involved in protein synthesis, inflammation, protein degradation and misfolding suggest that this is an integrative stress response. The integrative stress response is a coordinated cellular response to a stressor to restore homeostasis, which alters transcription, translation, post-translational modifications and protein stability (Pakos-Zebrucka et al., 2016). This response can aid in cell recovery and increase the transcription of genes that support immediate cell stability and function, but also lead to cell death if the stressor is severe enough in intensity or duration. The stress response can be initiated by the unfolded protein response (Korennykh and Walter, 2012), which was an upregulated component of the Protein Processing in the Endoplasmic Reticulum pathway. Additionally, changes to cellular energy, calcium balance, and redox balance may also initiate the response (Pakos-Zebrucka et al., 2016) and could occur during flight. Although the inflammatory and cell death pathways may be upregulated during flight, this could be aiding to maintaining homeostasis during flight and as such make it an intriguing avenue of research in the context of migration.

2.5 Summary: preparing for and sustaining migratory flight

My study emphasizes that migratory preparation in the flight muscle centers around augmenting aerobic capacity and the ability to transport and oxidize fatty acids, and that this is a fundamental element of migration. Additionally, few other changes may be are required. The upregulation of PGC-1α, PGC-1β and PPARα in the yellow-rumped warblers suggests that these genes are key regulators of lipid metabolism involved in the seasonal preparation, whereas the upregulation of PPARβ during flight appears to play a role in the maintenance of and transition to a stable exercising state. The transition to stable endurance flight involves further augmentation of cytosolic fatty acid transport and lower dependence on glycolysis, reflecting the switch in fuel from carbohydrates to fat. The similar expression levels of genes related to fatty acid oxidation while at rest, despite other pathway downregulation, suggests that yellow-rumped warblers maintain a highly aerobic phenotype to ensure readiness for departure.

Migratory flight, like other intense endurance feats causes muscle damage. Birds must manage the damage during flight in order to sustain muscle function. During the simulated migratory flights both inflammation and apoptotic pathways, and protein synthesis and growth pathways were upregulated. While these pathways may not normally be upregulated at the same time, it suggests that removal of damaged cellular components and their replacement may simultaneously occur during flight. Further studies at the protein level may help us understand if this is occurring during flight. The majority of the upregulated

pathways during flight were relatively downregulated during the migration season. Differences in the circadian rhythm between fall and wintering birds may partially explain the downregulation. Additionally, avoiding chronic upregulation of inflammatory pathways during the migratory season may be necessary for muscle health. Many muscle diseases and pathologies are associated with persistent inflammation and atrophy (Longo and Dalakas, 2015), which if occurring during migration would negatively impact performance and potentially even the capacity to migrate.

Overall, fatty acid oxidation appears to be maintained at high levels throughout the migratory season. However, the flight muscle is not static and alterations may occur in response to recent movements and stage of migration. Additionally, pathways relating to muscle growth, inflammation and damage that have been previously unexplored in the context of bird migration were identified; this data generates many questions about their role in sustaining and recovering from migratory flight. This study provides the stepping-stones to a new level of understanding the muscle physiology of birds and how they manage to complete such extreme migratory flights.

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3 Dietary polyunsaturated fatty acids influences oxidative enzymes, but not endurance flight performance in a migratory songbird

3.1 Introduction

The migratory flights of birds are extreme feats of endurance exercise that last from several hours to many days (Wikelski et al., 2003; Gill et al., 2009; DeLuca et al., 2015). Similar to human athletes and other animals, proper nutrition could be important to a bird's migration success. Fatty acid nutrition has been highlighted for its potential to enhance flight performance (Price and Guglielmo, 2009; Weber, 2009; Pierce and McWilliams, 2014). Mechanistically, there are several potential ways fatty acids can influence physiology and performance, yet these mechanisms have been explored only superficially in migratory birds.

Variation in dietary fatty acid composition and subsequent changes to tissues fatty acid composition could affect migratory flight performance. First, shorter and more unsaturated fatty acids may be better fuels since they are preferentially mobilized from adipose tissue (Price et al., 2008), and in the muscle more unsaturated fatty acids have higher rates of transport into mitochondria and subsequent oxidation (Price et al., 2011). Second, more unsaturated, and especially long-chain polyunsaturated fatty acids (PUFA), in phospholipids increase membrane fluidity and permeability (Stillwell and Wassall, 2003; Price, 2010), which may alter the activity of membrane-bound proteins and mitochondrial respiration (Power et al., 1997; Infante et al., 2001). Third, PUFA can regulate lipid metabolism by serving as ligands for peroxisome proliferator-activated receptors

(PPAR), a family of nuclear hormone receptors that are major regulators of lipid and glucose homeostasis (Weber, 2009; Grygiel-Górniak, 2014). Furthermore, the inflammatory response may be altered by certain fatty acids via their effects on eicosanoid synthesis (Price, 2010; Andersson et al., 2015). These mechanisms are not mutually exclusive and have been variously invoked to explain benefits of certain fatty acids found in the diets of birds (Price and Guglielmo, 2009; Weber, 2009; Pierce and McWilliams, 2014).

The natural doping hypothesis states that long chain n-3 PUFA, such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), help prime the flight muscles of birds for endurance flight (Maillet and Weber, 2006; Maillet and Weber, 2007; Weber, 2009). Increasing the proportion of DHA and EPA in membranes increases membrane fluidity, and could alter metabolic protein activity (Weber, 2009). Furthermore, EPA and DHA are high-affinity ligands for PPAR (Grygiel-Górniak, 2014). These mechanisms were proposed to increase flight muscle aerobic and fatty acid oxidizing capacity needed for endurance flight (Weber, 2009). Support for the natural doping hypothesis was observed in refuelling migratory semipalmated sandpipers (*Calidris pusilla*) that consumed a diet high in DHA and EPA, and the incorporation of these fatty acids into membranes was correlated with the oxidative enzyme activities (Maillet and Weber, 2006; Maillet and Weber, 2007). A follow up study of captive sedentary bobwhite quail (*Collinus virginianus*) found a similar effect of EPA and DHA supplementation on enzyme activities (Nagahuedi et al., 2009), providing evidence that dietary fatty acids can influence the regulation of metabolic enzyme activity.

The potential to enhance migratory performance may not be limited to n-3 PUFA. Other evidence suggests there are benefits of PUFA in general or of n-6 PUFA on peak metabolic rate (PMR) in red-eyed vireos (*Vireo olivaceous*, Pierce et al, 2005) and white-throated sparrows (*Zonotrichia albicollis*, Price and Guglielmo, 2009). In addition, Price and Guglielmo (2009) found no effect of dietary n-3 and n-6 PUFA on flight muscle oxidative capacity in sparrows, but did observe an increase in PMR in birds fed n-6 PUFA. Using a carefully designed feeding protocol, Price and Guglielmo (2009) were able to manipulate the phospholipid composition of the flight muscle but not the adipose fatty acid composition. This allowed them to attribute changes in PMR to the fatty acid composition of the fuel (Price and Guglielmo, 2009). However, the high proportion of DHA in the flight muscle phospholipids may limit conclusions about the effect of n-3 and n-6 PUFA on membrane phospholipids and PPAR signalling since DHA was enriched in the membranes of all the diet groups.

Studies from a wide variety of taxa suggest that both n-3 and n-6 PUFA alter exercise performance at the whole animal level. In rats, n-6 PUFA are associated with increased endurance (Ayre and Hulbert, 1997) and long chain n-3 PUFA increase resistance to fatigue (Peoples and McLennan, 2014). In mammals (Ruf et al., 2006) and Atlantic salmon (*Salmo salar,* McKenzie et al., 1998; Chatelier et al., 2006) running or swimming speed is positively associated with the n-6 PUFA, 18:2 n-6. On the other hand, in humans, endurance exercise training increases the proportion of DHA in muscle independent of diet (Helge et al., 2001).

If PUFA can modulate muscle metabolism, and in turn whole animal endurance exercise performance, this could alter the likelihood of a successful migration. Differences in their foraging ecologies among species provide different fatty acids to different species. Migratory shorebirds fuelling in marine environments may eat diets rich in n-3 PUFA (Maillet and Weber, 2007), while insects and berries consumed by songbirds tend to be richer in n-6 PUFA (Conway et al., 1994; Pierce and McWilliams, 2014; Andersson et al., 2015). If n-3 and n-6 PUFA differ in their effects on muscle metabolism, then the type of PUFA available could influence which birds can benefit and the resulting effect on migratory performance.

A key consideration in assessing performance benefits of different diets is ensuring the performance test is relevant to the animal's life history. In the case of avian migration, the best metrics of flight performance are arguably endurance capacity and energy efficiency. Both of these factors could impact the length of migratory flights, and the amount of fuel that is deposited and carried. Furthermore, when assessing if a fatty acid influences performance, the experimental diets need to be carefully formulated to ensure that only targeted fatty acids are manipulated. Otherwise there can be confusion about the source of the effect, which limits the interpretation of the results (Guglielmo, 2010).

The goal of this study was to experimentally test the natural doping hypothesis from the tissue level to whole animal performance in yellow-rumped warblers (*Setophaga coronata*). I created diets specifically enriched in an n-3 long-

chain PUFA (DHA), an n-6 long-chain PUFA (arachidonic acid; ARA, 20:4 n-6), or monounsaturated fatty acids (MUFA). Based on the natural doping hypothesis, I predicted that birds supplemented with n-3 long-chain PUFA would have increased oxidative capacity in the muscles, and improved endurance and flight performance compared to birds fed a low PUFA and n-6 PUFA diet. This study is the first to fully integrate tissue to whole animal performance level tests of the influence of fatty acids on migratory birds. I measured: 1) effects of diet on whole animal performance, 2) effects of diet on PPAR mRNA abundance and oxidative enzyme activity, 3) changes in muscle metabolism during flight, and 4) the correlation between changes in flight muscle metabolism and whole animal performance.

3.2 Materials and methods

3.2.1 Animals and experimental design

Yellow-rumped warblers were caught using mist nets in October 2013 at Long Point, Ontario and housed at the Advanced Facility for Avian Research at the University of Western Ontario (London, Ontario) in free flight aviaries (3.7 m long × 2.1 m wide × 3 m tall and 3.6 m long × 2.4 m wide × 2.7 m tall). The birds were initially kept on a fall photoperiod of 12 h light and 12 h dark (12L:12D) and switched to a short-day winter photoperiod (9L:15D) on November 29th, 2013 to allow the birds to enter a non-migratory wintering condition. The birds were fed a high carbohydrate diet throughout the study (Table 3.1), with canola oil used as the fat source until the start of the experiment. Animal capture, care and procedures followed Canadian Council on Animal Care guidelines and were approved by the

University of Western Ontario Animal Use Subcommittee (protocol 2010-216), and by the Canadian Wildlife Service (permit CA-0256, Appendix 1).

I divided the birds into three experimental diet groups, and started the experimental diets while they were still on the short-day photoperiod. After two weeks of acclimation to the diets, the birds were switched to a long day photoperiod (16 L:8D) to photo-stimulate them into a spring migratory condition. I blocked the starting date of treatments across 10 weeks to control the amount of time the birds consumed the diets and when they entered migratory condition. During the study, the birds were housed in pairs in cages measuring 121 cm wide × 68 cm deep × 185 cm high for weeks 1 and 2, and when on the long day photoperiod housed in cages measuring 70 cm wide × 50 cm deep × 60 cm high.

The three experimental diets each had a unique dietary oil blend used in the high carbohydrate diet (Table 3.1). The monounsaturated fatty acid diet (MUFA diet) oil blend was composed of olive and coconut oil (Table 3.2), which created a diet low in long-chain PUFA and higher in MUFA. The n-3 PUFA diet oil blend was composed of olive oil, coconut oil, and DHASCO, an algal oil high in DHA. The n-6 PUFA diet oil blend was composed of olive oil, coconut oil, and ARASCO, a fungal oil high in ARA. Four weeks after starting the diet treatments with the first block of birds, it became apparent that the n-3 PUFA diet birds were having health problems. The birds were weaker than normal, had reduced flight capacity, and appeared to have larger than normal fluxuations in daily food intake and body mass. Two birds died unexpectedly during this period. The n-3 and n-6 PUFA diets were both

reformulated, reducing the PUFA intake by half (Table 3.3). After the reformulation, there were no further health issues, and there were no indications of any differences in health between the diet groups. This problem influenced only the first 4 experimental blocks; with the first 2 blocks being the only birds to have detectable health issues and the two surviving individuals were not included in the study.

In week 5 of each treatment, basal metabolic rate (BMR) and peak metabolic rate (PMR) were measured for each bird (see below). In week six, wind tunnel flight assessments and tissue sampling occurred. The intention was for each block to have one bird undergo endurance flight assessment (Flown; see below), and one sampled at rest as an unflown Control. As flying in a wind tunnel is voluntarily, if neither bird flew in that block, they would both be sampled as control birds. I allowed 2-3 days recovery after an attempted flight before sampling as a Control bird.

Table 3.1 Composition of the diet fed to the yellow-rumped warblers (*Setophaga coronata*) during the feeding trial.

Ingredients	g	%
Dextrose[1]	450	16.3
Casein[2]	100	3.6
Agar[2]	45	1.6
Briggs-N Salt mix[3]	44	1.6
AIN-76 vitamin mix[3]	15	0.5
Water	2000	72.4
Oil blend	85	3.1
Cellulose[4]	24	0.9

Nutrient Composition	
Energy (kJ/g as fed)	16.4
Fat (% energy)	22.9
Carbohydrate (% energy)	64.1
Protein (% energy)	13.7

[1]ADM Corn Processing, Decatur, IL
[2]Affeymetric USB, Cleveland, OH
[3]MP Biomedicals, Solon, OH
[4]Sigma Aldrich, Oakville, ON

Table 3.2 Composition of the original experimental oil blends used in the experimental semisynthetic diets fed to the yellow-rumped warblers (*Setophaga coronata*).

	MUFA	n-3 PUFA	n-6 PUFA
	% of Dietary Oil		
Extra virgin olive oil[1]	72	40	46
Coconut oil[2]	28	0	27
ARASCO (20:4 n-6)[3]	0	0	27
DHASCO (22:6 n-3) [3]	0	60	0
	Net Fatty Acid Profile (g/ 100 g oil)		
SFA	39.4	41.9	40.2
MUFA	53.8	43.7	42.2
PUFA	6.74	14.1	17.0
n-3 PUFA	0.50	10.2	0.37
DHA (22:6 n-3)	0	9.92	0
n-6 PUFA	6.25	3.89	16.8
18:2 n-6	6.05	3.54	7.84
ARA (20:4 n-6)	0.15	0.35	8.96

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

[1]Loblaws Inc., Toronto, ON

[2]Spectrum Naturals, Delta, BC

[3]DSM Nutritional Products Ltd, Kaiseraugst, Switzerland

Table 3.3 Composition of the reformulated dietary oil blends used in the experimental semisynthetic diets fed to the yellow-rumped warblers (*Setophaga coronata*).

	MUFA	n-3 PUFA	n-6 PUFA
	% of Dietary Oil		
Olive oil[1]	72	57	58
Coconut oil[2]	28	15	30
ARASCO (20:4 n-6)[3]	0	0	12
DHASCO (22:6 n-3) [3]	0	28	0
	Net Fatty Acid Profile (g/ 100 g oil)		
SFA	39.4	39.8	39.8
MUFA	53.8	49.1	47.1
PUFA	6.74	10.2	11.2
n-3 PUFA	0.50	5.02	0.42
DHA (22:6 n-3)	0	4.62	0
n-6 PUFA	6.25	5.14	10.8
18:2 n-6	6.05	4.89	6.74
ARA (20:4 n-6)	0.15	0.25	4.06

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

[1]Loblaws Inc., Toronto, ON

[2]Spectrum Naturals, Delta, BC

[3]DSM Nutritional Products Ltd, Kaiseraugst, Switzerland

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

3.2.2 Respirometry

To measure BMR, warblers were food-deprived for 2 h prior to the start of the dark period and placed into stainless steel chambers (1.12 L) in an incubator at 31°C (Sanyo Incubator MIR-154, Sanyo Scientific, Japan). Inflow air into the chamber was dried using a gas drier (PC-4 Peltier Effect Dryer, Sable Systems International) and two dessicant columns (800 ml Drierite, W A Hammond Drierite, Zenia, OH). The dried air was split into 4 lines and passed individually through flow controllers (Flowbar 8, Sable Systems International, Las Vegas, NV) that regulated the flow to three 1.5 L respirometry chambers and a background line at a rate of 1 L/min. Outflow air lines from the chambers were attached to a multiplexer (MUX multiplexier, Sable Systems International), so that each line could be measured for 5 min before switching to the next line. The outflow air was sub-sampled at a rate of 200 ml/min before measuring water vapour (RH-300, Sable Systems International), CO_2 (CA-2A, Sable Systems International), and O_2 (FC-1B, Sable Systems International). The instruments were connected to an analog to digital converter (UI-2, Sable Systems International) to a Windows laptop computer. Using Expedata (Sable Systems International), both VO_2 and VCO_2 were lagged corrected, and were calculated in ml/min as:

$$VO_2 = FR_i \frac{F_{iO_2} - F'_{eO_2} - F'_{eO_2}(F'_{eCO_2} - F_{iCO_2})}{1 - F'_{eO_2}}$$

and

$$VCO_2 = FR_i \frac{F_{iCO_2} - F'_{eCO_2} - F'_{eCO_2}(F'_{eO_2} - F_{iO_2})}{1 - F'_{eCO_2}}$$

Where FR_i is the flow rate into the chamber, F_i is the fractional gas composition, and F'_e is the fractional composition adjusted for water vapour assuming 1 ml of water vapour is equivalent to 0.803 mg H_2O (Lighton, 2008). BMR was defined as the lowest rate of oxygen consumption during a 5 min sampling period. The BMR $\dot{V}O_2$ measurements were converted into watts (W, J/s) adjusting for respiratory coefficient (RQ) following Lighton (2008):

$$W = \frac{\dot{V}O_2 \times (16 + 5.164 \times RQ)}{60}$$

Two to three days after BMR measurements I used a hop/hover wheel to measure PMR (Chappell et al., 1999; Price and Guglielmo, 2009). The 24 cm diameter enclosed flight wheel (7.7 L) was continuously supplied with dried air at a rate of 3 L/min. The air exiting the chamber was subsampled at 200 ml/min and water vapour, CO_2, and O_2 were measured as described above. The birds were food-deprived for 3 h prior to the PMR measurement. Each bird was given a 2 min adjustment period after entering the flight wheel. The wheel was then manually spun, first slowly and then increasing in speed to maintain hovering flight. The birds were exercised until they began to pant and/or were unable to maintain hovering flight. PMR was calculated, similar to BMR, from the highest instantaneous $\dot{V}O_2$ averaged over a 1 min period (Chappell et al., 1999). PMR measurements were not collected from all birds because a PMR measurement was stopped if the bird's

behaviour in the flight wheel had the potential to damage their flight feathers or cause other bodily harm. I did not include a correction for instantaneous measurement of O_2 (z-correction, Bartholomew et al., 1981), which has been used in previous studies (Chappell et al., 1999; Pierce et al. 2005). The $\dot{V}O_2$ used for the PMR measurement was taken from a plateau where outflow gas concentrations were stable for several minutes and the z-correction does not influence these readings if the gas concentrations remained relatively stable. Aerobic scope was calculated as PMR/ BMR (Bishop, 1999).

3.2.3 Endurance flight assessments and sampling

Endurance flight performance was assessed using a wind tunnel designed for birds. Warblers were food-deprived for 2 h prior to the start of the dark period, to simulate the quiescent period that occurs prior to a migratory flight (Agatsuma and Ramenofsky 2006). After the lights turned off (19:00), the birds were weighed and scanned using quantitative magnetic resonance (QMR, Echo-MRI-B, Echo Medical Systems, Houston, TX, USA) to measure fat and wet lean mass (Guglielmo et al., 2011) and placed in a cotton bag for 5 min. The birds were then flown under minimal light conditions, at 8 m/s, 70% RH, and 15 °C. Endurance flights lasted for up to 360 min. Flights ended when the bird voluntarily stopped 3 times within a 5-min period, or reached the 360 min mark. The change in lean and fat mass were used to estimate the energy contribution from each fuel source using the conversion factors of 39.6 kJ/g for fat, and 5.3 kJ/g for lean mass (Jenni and Jenni-Eiermann, 1998; Gerson and Guglielmo, 2011). These values were used to calculate the total energy expended during flight, and to infer the relative protein contribution (RPC,

protein energy in kJ/total energy in kJ) to the fuel mixture. Although lean mass catabolism would include carbohydrates (glycogen in the muscle and liver), and lipids (triglycerides, phosopholipids, and free fatty acids) the bulk of the energy would be from proteins. Glycogen stores are minimal in birds, and are not quantitavely a major fuel source for endurance flight (Jenni and Jenni-Eiermann, 1998), especially after the 2 h food-deprivation prior to flight. Furthermore, total lipid content of the flight muscles were less than < 3.5 % and livers were under 5%, and with phospholipids being the dominant lipid class. As such, the catabolism of lean mass predominately provides protein as the fuel.

The Flown birds were sampled immediately after flight (~2-7 h after lights off). During sampling, a 60-70 µl blood sample was collected and centrifuged (2000 × g for 10 min) to isolate the plasma. Following this, the birds were weighed and the body composition measured using QMR. The bird was then anesthetised using isoflurane (^{Pr}Florane, Baxter, Mississaugua, ON), killed by decapitation, and adipose and flight muscle samples collected. The collected samples were flash frozen in liquid nitrogen and stored at -80 °C. Control birds were food-deprived for 2 h prior to sampling at lights out. Similar to the Flown birds, a 60-70 µl blood sample was collected, body mass and composition measured. The birds were anesthetised and killed, and muscle and adipose sampled collected and flash frozen. The sexes of the birds were identified post-mortem.

3.2.4 Fatty acid profiles

Fatty acid profiles of adipose, flight muscle phospholipids, and plasma were analyzed. Total lipids were extracted from 10 mg adipose, 50 mg flight muscle, and 10-15 µl of plasma, using methods modified from Price and Guglielmo (2009) and Thomas et al. (2012). Briefly, the tissue was homogenized in 2 ml chloroform/methanol/ 0.1% butylated hydroxytoluene (2:1:0.003 v/v/wt) and centrifuged at 20 000 × g for 15 min. This was followed by the addition of 1 ml of 0.25 % potassium chloride. The samples were then incubated at 70 °C for 10 min, and the bottom organic layer was removed and dried under a stream of nitrogen. For the flight muscle, the phospholipid fraction was isolated using Supelclean solid phase extraction columns (Supelclean, Sigma-Aldrich Co, Oakville, ON). Briefly, neutral lipids were eluted with 1.8 ml chloroform: isopropanol (2:1 v/v), followed by nonesterified fatty acids (NEFA) with 1.6 ml of isopropyl ether: acetic acid (49:1 v/v), and the phospholipid fraction eluted with 2 ml of methanol. The isolated fractions were dried under a nitrogen stream and re-suspended in 100 µl chloroform. I added heptadecanoic acid (17:0, 200 µl, 3 mg/10 ml in hexane, Sigma) to the re-suspended lipids as an internal standard. Fatty acid methyl esters (FAME) were generated by the addition of 200 µl 0.5 N methanoic-HCl (Sigma-Aldrich) to the dried lipids, and heated for 30 min at 90 °C. Afterwards, 800 µl water was added and the FAME were extracted 3 times with 500 µl hexane. The hexane extract was dried under nitrogen and suspended in 80 µl chloroform. The FAME were separated on a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA), with a flame ionization detector and a DB-225ms column (30 m long, 0.250 internal diameter,

0.25µm film; Agilent Technologies, Palo Alto, CA) using N_2 as the carrier gas. The temperature program was 80 °C for 2 min, followed by a 5 °C/min ramp to 180 °C and held for 5 min, then a 1 °C/min to 200 °C, followed by a 10 °C/min ramp to 240 °C which was held for 3 min. Fatty acid peaks identities were determined by comparing retention times to commercial standards (Supelco 37 mix, Supelco PUFA No. 3 Menhaden Oil, Supelco FAME mix C8-C24, Sigma-Aldrich). To calculate fatty acid mass proportion the peak area for each fatty acid was divided by the total peak area of identified peaks, and expressed as a proportion. With the exception of DHA, ARA, and eicosapentaenoic acid (EPA, 20:5 n-3), only fatty acids with at least one group with an average >1% of total fatty acids were included for statistical analysis.

3.2.5 Flight muscle lipid metabolism and PPAR expression

Expression of flight muscle PPARα, PPARβ, PPARγ, heart type fatty acid binding protein (H-FABP), and a validated housekeeping gene (GAPDH; P = 0.63) were measured using quantitative real-time PCR. RNA was isolated from 30 mg of flight muscle tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Mississauga, ON). Muscle samples were homogenized using a micropestle. All RNA samples had an A_{260}/A_{280} >1.9. cDNA synthesis was done using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Burlington, ON) , and the cDNA was then diluted 100 fold with water. Quantitative real-time PCR was run for each gene with a CFX384 Real-Time system instrument (Bio-Rad, Mississauga, ON). Primers were designed using transcripts from a de novo assembly of a yellow-rumped warbler flight muscle transcriptome (Chapter 2) and submitted to NCBI/Primer Blast tool (Table 3.4). The PCR reaction conditions were 2 µl of the diluted cDNA, 6 µl 2x master mix (SensiFast

SYBR and Fluorescein Mix, Bioline, Tauton MA), 0.33 µM of the primers in a total reaction volume of 12 µl. Primers and reaction conditions for H-FABP are as described by McFarlan et al. (2009). The cycling conditions for all other genes were as follows: 95 °C held for 10 min, followed by 45 cycles of 95 °C for 20 sec, 59 °C for 20 sec, 72 °C for 10 sec. All samples were run in triplicate. Amplification efficiency was within 96-102% for all the genes. The mRNA abundance was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each gene, the arithmetic mean expression of the MUFA Control group was set to 1.

Maximum enzyme activity in the flight muscle of carnitine palmitoyl transferase (CPT; EC 2.3.1.21), citrate synthase (CS; EC 2.3.3.1), 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35), and lactate dehydrogenase (LDH; EC 1.1.1.27) was measured following Price et al. (2010) and as described in Chapter 2.

Table 3.4 Primer sequences for qPCR for peroxisome proliferator activated receptors (PPAR) mRNA abundance in yellow-rumped warblers (Setophaga coronata).

Target	Nucleotide sequence 5' to 3'
GAPDH	Forward: TCCCGAAGCGGTAAAGATGG
	Reverse: CCGGAAGTGGCCATGAGTAG
PPARα	Forward: ATGGCTGGTTTGAGACACCC
	Reverse: TACCTGTGACACTTCCCCGA
PPARβ	Forward: GACTCTGCTCAAGTACGGGG
	Reverse: AGGATGATGGCAGCCACAAA
PPARγ	Forward: TCGTGCCCTCCATAACAAGG
	Reverse: ATGGCACCTGATTGCTCAGT

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase, PPARα, peroxisome proliferator activated receptor alpha, PPARβ, peroxisome proliferator activated receptor beta, PPARγ, peroxisome proliferator activated receptor gamma.

3.2.6 Statistical analysis

All statistics were performed using SAS (version 9.4), with significance set at $\alpha < 0.05$, and trends in the data were considered at $P < 0.1$. Normality and constant variance assumptions were tested and verified, and the data transformed when needed to meet these assumptions. By chance the sexes were evenly distributed across the diet treatments. Sex, tarsus length and wing cord were not significant factors or covariate ($P > 0.05$) and as such were not included in the statistical analysis. Body mass and the blocking factor were included as covariates when significant ($P \leq 0.05$). Weekly body mass was analysed using repeated measures to test for a effects of diet, time, and their interaction on body mass. Metabolic rates (BMR and PMR) and final body mass and body composition analyses were performed using 2-way ANOVA testing for an effect of diet. For these analyses the Flown and Control birds for each diet were combined as the birds were treated identically until the wind tunnel flight and there was no significant effect of flight ($P > 0.05$).

Fatty acid profiles were first analyzed by MANOVA using Wilks Lamda for each tissue to test for significant main effects and the diet and flight interaction. If the interaction was not significant in the MANOVA it was dropped from the model to simplify the analysis. Significant effects or interactions were then evaluated by univariate analysis on individual fatty acids and fatty acid classes using ANOVA.

The Kruskal-Wallis non-parametric test was used to test for differences in flight duration. Other flight performance parameters tested included initial body

mass and flight duration as covariates, and I initially tested for their interactions. Non-significant interactions were removed from the model ($P > 0.05$). Correlations between flight performance variables were determined using Pearson's correlation coefficient.

Muscle metabolic enzyme activities and gene expression were analyzed using 2-way ANOVA to test for the main effects and interaction of diet and flight treatment. When required to meet the assumptions of normality, the mRNA gene expression was log transformed. Pearson's correlation coefficients were used to test for relationships between muscle metabolic indicators and whole animal performance. For flight duration, all flights were included in the correlation analysis. To control for the effect of flight duration on energy cost and relative protein contribution, only the 360 min flights were used for those correlations.

3.3 Results

3.3.1 Fatty acid composition

The muscle phospholipids showed the greatest response to manipulation of dietary fatty acid composition (Table 3.5). The experimental diets altered the fatty acid composition of the muscle phospholipids (MANOVA: Wilks $\lambda = 0.03$: $F_{14,80} = 31.86$, $P < 0.0001$), but flight did not have any further effect (MANOVA: Wilks $\lambda = 0.85$: $F_{6,41} = 0.47$, $P = 0.83$) nor was there an interaction between diet and flight (MANOVA: Wilks $\lambda = 0.87$: $F_{12,82} = 0.47$, $P = 0.93$). The MUFA diet group had higher total MUFA and lower saturated fatty acids (SFA) in the muscle phospholipids compared to the n-3 and n-6 PUFA groups, and this was primarily the result of

higher proportions 18:1 n-9 and lower 16:0 and 18:0 in the MUFA group. No differences in total PUFA were observed between the diet groups. However, the MUFA group had less long-chain PUFA, but with both DHA and ARA present. The n-6 PUFA and n-3 PUFA diet groups had higher ARA and DHA levels, respectively in muscle phospholipids.

In adipose tissue there was an overall effect of diet on fatty acid composition (MANVOA: Wilks $\lambda < 0.001$: $F_{20,74} = 8.56$, $P < 0.0001$); however, flight did not have any effect (MANOVA: Wilks $\lambda = 0.09$: $F_{10,37} = 1.24$, $P = 0.29$), and there was no interaction between flight and diet (MANOVA: Wilks $\lambda = 0.80$: $F_{20,74} = 0.45$, $P = 0.97$). Patterns in the proportions of total SFA, MUFA and PUFA in the adipose were similar to those observed in the muscle phospholipids (Table 3.6). Differences in long-chain PUFA were present, but the levels were much lower compared to the muscle phospholipids, and none of the proportions of long-chain PUFA exceeded 1%.

In contrast to the flight muscle phospholipids and adipose, in the plasma there was an interaction between diet and flight (MANOVA: Wilks $\lambda = 0.44$: $F_{20,78} = 1.92$, $P = 0.02$), and main effects of flight (MANOVA: Wilks $\lambda = 0.53$: $F_{10,39} = 3.45$, $P = 0.002$) and diet (MANOVA: Wilks $\lambda = 0.02$: $F_{20,78} = 22.06$, $P < 0.0001$). The differences in the plasma fatty acids among diets were similar to those observed in the muscle phospholipid and adipose (Table 3.7). Additionally, the effect of flight on fatty acid composition was diet specific. In the MUFA group, 16:1 n-7, 18:1 n-9 and 18:2 n-6 increased during flight, and there was an overall increase in total MUFA

and a decrease in SFA. In the n-3 PUFA group, MUFA decreased and SFA increased during flight. No significant effect of flight was observed in the n-6 PUFA group.

Table 3.5 Fatty acid composition of the muscle phospholipid from yellow-rumped warblers (*Setophaga coronata)* in fed different dietary oil blends. Data are present as mean mass %. Fatty acid groups include minor fatty acids not listed above. Values with different superscript letters differ ($P \leq 0.05$).

Fatty acid	MUFA		n-3 PUFA		n-6 PUFA		F value	P value
16:0	27.86	±0.72^{b}	33.94	±0.70^{a}	29.50	±0.72^{b}	$F_{2,50}$=19.69	<0.0001
18:0	25.71	±0.61^{b}	28.18	±0.60^{a}	29.61	±0.61^{a}	$F_{2,50}$=10.18	0.0002
18:1 n-9 cis	18.14	±0.57^{a}	7.14	±0.56^{c}	10.12	±0.57^{b}	$F_{2,50}$=99.16	<0.0001
18:2 n-6 cis	6.58	±0.38^{a}	1.31	±0.37^{b}	1.65	±0.38^{b}	$F_{2,50}$=60.91	<0.0001
20:4 n-6	10.34	±1.01^{b}	0.67	±0.98^{c}	18.95	±1.01^{a}	$F_{2,50}$=88.73	<0.0001
20:5 n-3	0.06	±0.11^{b}	1.17	±0.11^{a}	0.09	±0.11^{b}	$F_{2,50}$=34.28	<0.0001
22:6 n-3	8.26	±1.40^{b}	25.00	±1.36^{a}	7.07	±1.39^{b}	$F_{2,50}$=53.16	<0.0001
SFA	54.00	±1.04^{c}	62.70	±1.02^{a}	59.58	±1.04^{b}	$F_{2,50}$=18.43	<0.0001
MUFA	19.72	±0.59^{a}	8.91	±0.57^{c}	11.67	±0.59^{b}	$F_{2,50}$=93.20	<0.0001
PUFA	26.29	±1.21	28.38	±1.18	28.34	±1.21	$F_{2,50}$=0.98	0.38
LCPUFA	19.19	±1.30^{b}	27.03	±1.27^{a}	26.53	±1.30^{a}	$F_{2,50}$=11.50	<0.0001
n-3 PUFA	8.90	±1.46^{b}	26.36	±1.42^{a}	7.60	±1.46^{b}	$F_{2,50}$=53.44	<0.0001
n-6 PUFA	17.09	±0.90^{b}	2.01	±0.87^{c}	20.71	±0.89^{a}	$F_{2,50}$=128.08	<0.0001
n-6: n-3	2.13	±0.86^{b}	0.10	±0.85^{c}	4.69	±0.86^{a}	$F_{2,50}$=7.17	0.002

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain PUFA.

Table 3.6 Fatty acid composition of the adipose from yellow-rumped warblers (*Setophaga coronata*) in fed different dietary oil blends. Data are present as mean mass %. Fatty acid groups include minor fatty acids not listed above. Values with different superscript letters differ ($P < 0.05$).

Fatty acid	MUFA		n-3 PUFA		n-6 PUFA		F value	P value
12:0	3.88	±0.21^{ab}	3.13	±0.21^{b}	4.39	±0.21^{a}	$F_{2,50}$=9.29	0.0004
14:0	3.09	±0.24^{b}	4.11	±0.23^{a}	3.73	±0.24^{b}	$F_{2,50}$=4.85	0.012
16:0	27.97	±0.86	29.09	±0.84	27.87	±0.86	$F_{2,50}$=0.64	0.53
16:1 n-7	3.75	±0.29	3.32	±0.28	3.78	±0.29	$F_{2,50}$=0.81	0.45
18:0	6.32	±0.37^{b}	8.38	±0.36^{a}	8.45	±0.37^{a}	$F_{2,50}$=10.72	0.0002
18:1 n-7	1.14	±0.08^{a}	0.93	±0.08^{ab}	0.72	±0.08^{b}	$F_{2,47}$=6.99	0.002
18:1 n-9 cis	49.66	±1.05^{a}	44.06	±1.03^{b}	45.75	±1.05^{b}	$F_{2,50}$=7.71	0.001
18:2 n-6 cis	3.48	±0.37	3.80	±0.36	3.53	±0.37	$F_{2,50}$=0.22	0.80
20:4 n-6	0.04	±0.05^{b}	0.04	±0.05^{b}	0.63	±0.05^{a}	$F_{2,50=}$50.47	<0.0001
20:5 n-3	0.002	±0.01^{b}	0.04	±0.01^{a}	0.002	±0.01^{b}	$F_{2,50}$=9.64	0.0003
22:6 n-3	0.09	±0.12^{b}	0.98	±0.11^{a}	0.001	±0.12^{b}	$F_{2,50}$=20.24	<0.0001
SFA	41.72	±0.87^{b}	45.14	±0.85^{a}	44.91	±0.87^{a}	$F_{2,50}$=4.87	0.012
MUFA	54.18	±0.85^{a}	49.41	±0.83^{b}	50.27	±0.85^{b}	$F_{2,50}$=9.11	0.0005
PUFA	3.85	±0.45	5.15	±0.44	4.58	±0.45	$F_{2,50}$=2.11	0.13
LCPUFA	0.15	±0.13^{c}	1.12	±0.13^{a}	0.65	±0.13^{b}	$F_{2,50}$=13.15	<0.0001
n-3 PUFA	0.26	±0.13^{b}	1.26	±0.13^{a}	0.22	±0.13^{b}	$F_{2,50}$=19.46	<0.0001
n-6 PUFA	3.54	±0.37	3.86	±0.36	4.29	±0.37	$F_{2,50}$=1.00	0.38
n-6: n-3	20.07	±2.07^{a}	5.38	±2.02^{b}	20.34	±2.07^{a}	$F_{2,50}$=17.62	<0.0001

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain PUFA.

Table 3.7 Fatty acid composition of the plasma from Control and Flown yellow-rumped warblers (*Setophaga coronata)* in fed different dietary oil blends. Data are present as mean mass % ± SEM. Fatty acid groups include minor fatty acids not listed above. Values with different superscript letters differ (P <0.05). For significant diet and flight interactions, letter comparisons are within Control and Flown groups, and * indicates significant effect of flight within a diet group.

Fatty acid	Flight	MUFA		n-3 PUFA		n-6 PUFA		F value	P value
12:0		1.07	±0.13^{a}	0.61	±0.13^{b}	0.79	±0.13^{b}	$F_{2,50}$=3.52	0.04
14:0		1.84	±0.21	1.42	±0.21	1.50	±0.22	$F_{2,50}$=1.11	0.34
15:0		1.57	±0.42	1.37	±0.42	0.53	±0.44	$F_{2,50}$=1.66	0.2
16:0		25.40	±0.50	26.24	±0.51	26.49	±0.50	$F_{2,50}$=1.27	0.29
16:1 n-7	C	1.85	±0.16^{a*}	2.00	±0.16^{a*}	1.32	±0.17^{b}	$F_{2,50}$=5.12	0.01
	F	2.37	±0.17^{a}	1.49	±0.18^{b}	1.56	±0.18^{b}		
18:0		15.32	±1.41	13.54	±1.45	17.82	±1.47	$F_{2,50}$=2.12	0.13
18:1 n-7		1.14	±0.08^{a}	0.93	±0.08^{ab}	0.72	±0.08^{b}	$F_{2,47}$=6.99	0.002
18:1 n-9 cis	C	32.00	±2.64*	37.46	±2.63	30.28	±2.78	$F_{2,50}$=4.62	0.01
	F	41.34	±2.78^{a}	30.58	±2.94^{b}	28.21	±2.94^{b}		
18:2 n-6 cis	C	4.32	±0.50*	5.19	±0.37*	3.83	±0.53	$F_{2,50}$=5.84	0.005
	F	6.20	±0.50^{a}	3.59	±0.56^{b}	3.35	±0.56^{b}		
20:3 n-6		0.57	±0.48	0.26	±0.49	1.28	±0.51	$F_{2,50}$=1.08	0.34
20:4 n-6		5.02	±0.80^{b}	0.71	±0.82^{c}	12.96	±0.80^{a}	$F_{2,50}$=55.06	<0.0001
20:5 n-3		0.21	±0.25^{b}	4.32	±0.25^{a}	0.10	±0.26^{b}	$F_{2,50}$=89.76	<0.0001
22:6 n-3		0.48	±0.36^{b}	6.96	±0.37^{a}	0.36	±0.37^{b}	$F_{2,50}$=105.69	<0.0001
SFA	C	47.91	±2.57^{a*}	39.08	±2.57^{b}	47.29	±2.71^{a}	$F_{2,50}$=3.33	0.04
	F	40.25	±2.71	45.29	±2.88	46.57	±2.88		
MUFA	C	37.13	±2.26^{a*}	41.64	±2.26^{b*}	32.88	±1.74^{a}	$F_{2,50}$=5.51	0.01
	F	45.50	±2.38^{a}	34.35	±2.53^{b}	33.04	±2.53^{b}		
PUFA		12.52	±1.07^{b}	17.66	±1.11^{a}	19.00	±1.13^{a}	$F_{2,50}$=9.82	0.0003
LCPUFA		5.82	±0.97^{b}	12.43	±1.00^{a}	13.59	±1.02^{a}	$F_{2,50}$=18.08	<0.0001
n-3 PUFA		1.18	±0.58^{b}	11.92	±0.60^{a}	0.93	±0.61^{b}	$F_{2,50}$=109.80	<0.0001
n-6 PUFA		10.90	±0.84^{b}	5.44	±0.87^{c}	17.88	±0.89^{a}	$F_{2,50}$=50.10	<0.0001
n-6: n-3		10.11	±1.75^{b}	0.50	±1.80^{c}	23.94	±1.85^{a}	$F_{2,50}$=41.35	<0.0001

C, control; F, Flight; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long-chain PUFA.

3.3.2 Body composition and metabolic rate

Warblers decreased in body mass during the first week of adjustment to the experimental diets and immediately following the switch to long-day photoperiod, but they increased in body mass as they entered into a migratory condition (Figure 3.1; week: $F_{6,371} = 13.14$, $P < 0.001$). There was no significant main effect of diet or interaction between diet and time (diet: $F_{2,371} = 2.70$, $P = 0.07$; diet × time: $F_{12,371} = 0.19$, $P = 0.99$), and the blocking factor (treatment start date) was significant, with the last two blocks being heavier overall ($F_{9,371} = 5.09$, $P < 0.001$). Final body composition at sampling in the Controls and pre-flight in the Flown birds did not differ among the diets for body mass ($F_{2,53} <0.01$, $P = 0.99$), wet lean mass ($F_{2,53} = 0.23$, $P = 0.79$) or fat mass ($F_{2,53} = 0.48$, $P = 0.62$) and there was no effect of the blocking factor ($P > 0.05$) (Table 3.8).

Diet did not significantly influence BMR ($F_{2,48} = 1.24$, $P = 0.29$), after controlling for the positive effect of the covariate body mass (slope = 0.008; $F_{1,47} = 34.79$, $P < 0.001$). Unlike BMR, not all PMR measurments were successful, and only 31 successful PMR were measured (16 in the Control group, with 15 in the Flown group, 7 of which flew for 360 min). Similarly to BMR, no diet effects were detected in PMR ($F_{2,26} = 2.38$, $P = 0.11$), after controlling for the effects of the covariates body mass (slope = 0.003, $F_{1,26} = 9.97$, $P = 0.04$) and the blocking factor ($F_{8,26} = 9.97$, $p < 0.001$). Correspondingly, there were no significant differences among diets in aerobic scope ($F_{2,27} = 2.02$, $P = 0.15$), with the blocking factor included as a covariate ($F_{8,27} = 4.48$, $P < 0.001$).

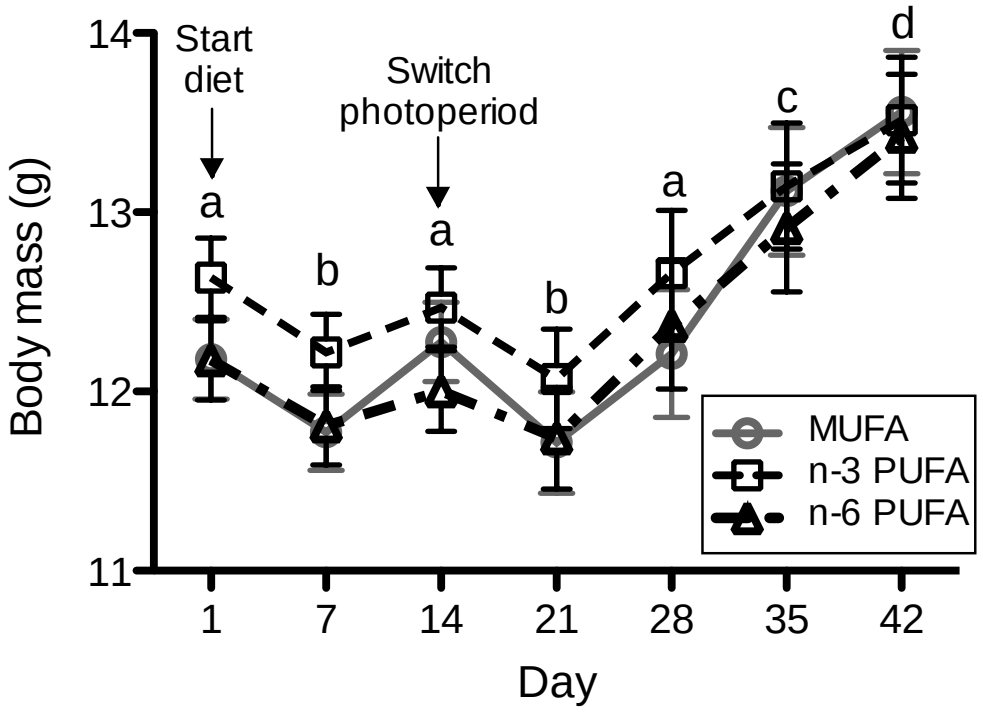

Figure 3.1 Body mass (mean ± SEM) of the yellow-rumped warblers (*Setophaga coronata)* fed experimental diets over the first 42 days. Arrow at day 1 indicates the start of the experimental diets. Arrow at day 14 indicates the shift from short day to long day photoperiod. Letters indicate overall significant differences among days ($P \leq 0.05$).

Table 3.8 Final body mass and composition at sampling (Controls) and pre-flight (Flown), basal metabolic rate (BMR) and peak metabolic rate (PMR) of yellow-rumped warblers (*Setophaga coronata*).

	MUFA		n-3 PUFA		n-6 PUFA	
Body mass (g)	13.55	±0.33	13.53	±0.36	13.54	±0.34
Fat mass (g)	2.85	±0.23	2.54	±0.24	2.80	±0.23
Lean mass (g)	8.53	±0.15	8.66	±0.16	8.52	±0.15
BMR (W)	0.220	±0.004	0.226	±0.004	0.228	±0.004
PMR (W)	2.00	±0.05	1.84	±0.05	1.92	±0.05
Aerobic Scope	9.00	±0.31	8.31	±0.32	8.29	±0.30

Body composition values reported are means ± SEM. BMR, PMR and aerobic scope are least square means ± SEM with body mass included as a covariate.

3.3.3 Endurance flight performance

Twenty-four warblers successfully completed flights that could be used to measure energy and fuel metabolism (Figure 3.2), with no difference in the number of flights among the diets ($\chi^2 = 0.42$, P = 0.81). The majority of flights were 360 min in length (N = 15, 5 in each diet group), and the remaining flights were typically 60-120 min in length. Birds that flew longer had lower flight power (J/s) and relative protein contribution used to fuel the flight (Table 3.9). The amount of fat catabolized was positively correlated with flight duration, but the amount of lean mass catabolized did not vary with flight duration (Table 3.9). Examining the birds that flew the full 360 min, body mass was positively correlated with flight costs, and negatively with relative protein contribution (Table 3.10). Additionally, the amount of fat catabolized was positively correlated to body mass (r = 0.74, P = 0.001), and there was a trend for less lean mass catabolism with increasing body mass (r = -0.49, P = 0.06).

Flight duration did not vary among the diet groups ($\chi^2 = 0.8296$, P = 0.67; Table 3.11). Additionally, diet did not alter the amount of mass catabolized during flight ($F_{2,19} = 0.43$, P = 0.65), with the included covariates: flight duration ($F_{1,19} = 67.8$, P < 0.0001) and pre-flight body mass ($F_{1,19} = 0.82$, P = 0.38). Similarly, no significant differences in lean mass catabolized (diet: $F_{2,19} = 0.48$, P = 0.62; duration: $F_{1,19} = 0.15$, P = 0.71; preflight body mass: $F_{1,19} = 4.17$, P = 0.55) or fat mass catabolized (diet: $F_{2,19} = 1.88$, P = 0.18; duration: $F_{1,19} = 198$, P < 0.0001; preflight body mass: $F_{1,19} = 14.16$, P = 0.001) were observed. Total energy used did not differ

between diets (diet: $F_{2,19} = 2.17$, $P = 0.14$; duration: $F_{1,19} = 270.14$, $P < 0.0001$; preflight body mass: $F_{1,19} = 13.89$, $P = 0.001$). The relative protein contribution to the fuel mixture did not differ between diets (diet: $F_{2,19} = 1.20$, $P = 0.33$; duration: $F_{1,19} = 28.79$, $P < 0.0001$; preflight body mass: $F_{1,19} = 4.18$, $P = 0.06$). Similar results were observed when testing the 360 min flights only (Table 3.11). Flight power (W) did not differ between the diets when examining 360 min flights only (diet: $F_{2,11} = 1.11$, $P = 0.36$; preflight body mass: $F_{1,11} = 11.67$; $P = 0.006$). Power is reported for the 360 min flights only because it is positively correlated with flight duration, and flight power decreases with increasing flight duration. This could create an issue with autocorrelation and yield misleading conclusions about the effect of diet, particularly give the uneven distribution of flights in this study (i.e.: short 60-120 min flights or 360 min flights).

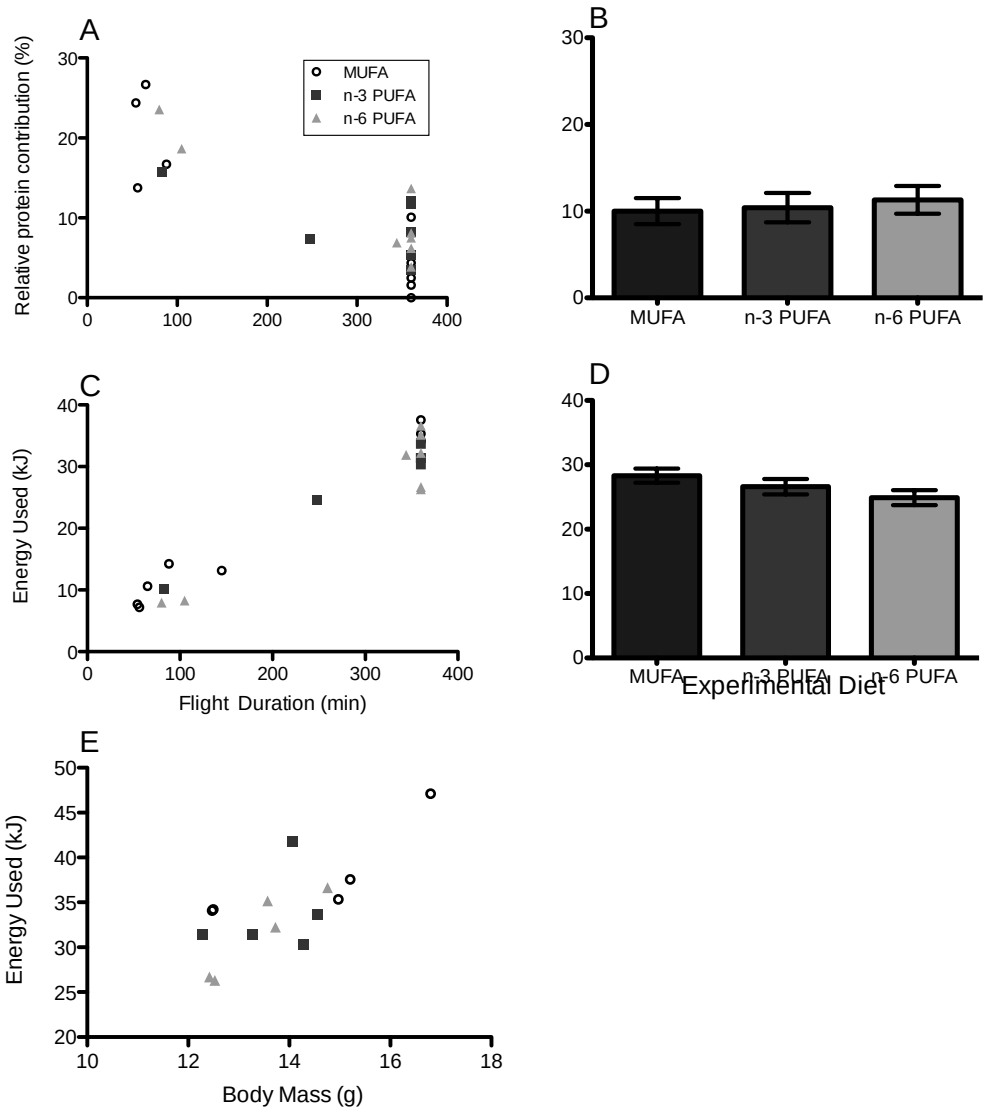

Figure 3.2 Effect of diet on relative protein contribution to fuel mixture and total energy used during endurance flight of yellow rumped warblers (*Setophaga coronata*). Warblers were fed diets enriched in monounsaturated (MUFA, black open circles), n-3 polyunsaturated (n-3 PUFA, grey squares), or n-6 polyunsaturated (n-6 PUFA, light grey triangles) fatty acids and flown in a wind tunnel at 8 m/s, 15 °C, and 70% relative humidity. A) Distribution of relative protein contribution with flight duration, B) relative protein contribution accounting for the effect of body mass and flight duration (LSMEAN ± SEM), C) distribution of total energy used and flight duration D) total energy used accounting for flight duration and body mass (LSMEAN ± SEM), E) distribution of energy cost and body mass for the 360 min flights only.

Table 3.9 Correlations between flight duration and flight performance, PPAR mRNA abundance, oxidative enzymes, and metabolic rate.

		Flight Duration, min	
		r	P-value
Flight Performance	Power (J/s)	**-0.56**	**0.003**
	RPC (%)	**-0.80**	**<0.0001**
	Fat catabolized (kJ)	**0.90**	**<0.0001**
	Lean mass catabolized (kJ)	-	0.46
Body Composition	Body mass (g)	-	0.53
	% Body fat	-	0.70
	Body fat (g)	-	0.72
	Lean mass (g)	-	0.98
PPAR and Oxidative Enzymes	PPARβ	-	0.34
	PPARα	-	0.88
	FABP	-	0.10
	CPT (U/mg)	-	0.85
	HOAD (U/mg)	-	0.51
	CS (U/mg)	-	0.07
	LDH (U/mg)	-	0.53
Metabolic Rate	PMR (W, n=15)	-	0.45
	BMR (W)	-	0.57
	Aerobic scope (n=15)	-	0.58

Bold values are significantly correlated with flight duration ($p<0.05$). RPC, relative protein contribution.

Table 3.10 Flight cost and relative protein contribution (RPC) correlations with body composition, PPAR mRNA abundance and oxidative enzymes, and metabolic rate of the 360 min flights only.

		Flight cost, kJ		RPC	
		r	P-value	r	P-value
Flight Performance	RPC (%)	**-0.73**	**0.0019**	1.00	-
	Energy from fat (kJ)	**0.98**	**<0.0001**	**-0.82**	**0.0002**
	Energy from lean mass (kJ)	**-0.64**	**0.01**	**0.98**	**<0.0001**
Body Composition	Body mass (g)	**0.75**	**0.0012**	**-0.53**	**0.04**
	% Body fat	-	0.09	**-0.56**	**0.03**
	Body fat (g)	**0.60**	**0.02**	**-0.59**	**0.02**
	Lean mass (g)	**0.68**	**0.005**	-	0.33
PPAR and Oxidative Enzymes	PPARβ	-	0.20	-	0.31
	PPARα	-	0.54	-	0.41
	FABP	-	0.85	-	0.41
	CPT (U/mg)	-	0.60	-	0.34
	HOAD (U/mg)	-	0.54	**-0.56**	**0.03**
	CS (U/mg)	-	0.57	-	0.27
Metabolic Rate	LDH (U/mg)	-	0.47	-	0.27
	PMR (W, n=7)	-	0.72	-	0.31
	BMR (W)	**0.58**	**0.03**	-	0.22
	Aerobic scope (n=7)	-	0.30	**0.82**	**0.02**

Bold values are significantly correlated with flight costs or RPC ($p<0.05$).

Table 3.11 Flight performance metrics and body composition changes for yellow-rumped warblers (*Setophaga coronata*) fed the experimental diets for all flights or 360 min flights only.

	MUFA	n-3 PUFA	n-6 PUFA
	All Flights		
	n = 9	n = 7	n = 8
Duration (min)	241.9 ±70.7	304.4 ±78.1	301.2 ±75.3
Mass loss (g)	1.06 ±0.06	1.13 ±0.06	1.05 ±0.06
Fat loss (g)	0.67 ±0.03	0.61 ±0.04	0.58 ±0.03
Lean loss (g)	0.34 ±0.06	0.44 ±0.07	0.39 ±0.07
	360 min Flights		
	n = 5	n = 5	n = 5
Mass loss (g)	1.23 ±0.08	1.31 ±0.08	1.27 ±0.08
Fat loss (g)	0.87 ±0.04	0.79 ±0.04	0.76 ±0.04
Lean loss (g)	0.28 ±0.09	0.49 ±0.09	0.42 ±0.09
Energy used (kJ)	36.1 ±1.6	34.1 ±1.6	32.5 ±1.7
Power (W)	1.67 ±0.08	1.58 ±0.08	1.51 ±0.08
RPC (%)	4.43 ±1.6	8.01 ±1.6	7.33 ±1.6

Duration is mean ±SEM. All other values are least squared means ±SEM, with body mass and flight duration included as covariates All Flights, and body mass included as a covariate for the 360 min flights. RPC: relative protein contribution.

3.3.4 PPAR mRNA abundance and metabolic enzymes

Neither diet nor flight influenced PPARα mRNA abundance in flight muscles (diet: $F_{2,50} = 1.5$, $P = 0.23$; flight: $F_{1,50} = 2.3$, $P = 0.14$, diet × flight: $F_{2,50} = 0.16$, $P = 0.85$, Figure 3.3). In contrast, PPARβ mRNA abundance differed among diets ($F_{2,50} = 3.77$, $P = 0.03$) and increased during flight ($F_{1,50} = 21.44$, $P < 0.001$), but there was no interaction (diet × flight: $F_{2,50} = 1.43$, $P = 0.25$). PPARβ mRNA abundance was lower in the n-3 PUFA diet group. For PPARγ, an interaction between diet and flight was observed ($F_{2,50} = 3.26$, $P = 0.047$), where expression decreased in both the MUFA and n-3 PUFA group during flight, but not n-6 PUFA, which had intermediate mRNA abundance levels.

Metabolic enzyme activities were also influenced by diet, but not by flight, body mass, or the blocking factors (Figure 3.4). The activity of CPT was not related to diet or flight ($P > 0.05$). However, when the two PUFA groups were combined they had lower CPT activity than the MUFA group ($F_{1,50} = 4.58$, $P = 0.037$). Activity of CS was lower in the n-3 PUFA group compared to the n-6 PUFA group (diet: $F_{2,50} = 6.81$, $P = 0.002$; flight: $F_{1,50} = 0.09$, $P = 0.76$, diet × flight: $F_{2,50} = 1.24$, $P = 0.30$). The n-3 PUFA group also had lower HOAD activity compared to the MUFA diet groups (diet: $F_{2,50} = 3.28$, $P = 0.046$; flight: $F_{1,50} = 1.02$, $P = 0.32$, diet × flight: $F_{2,50} = 2.21$, $P = 0.12$). In contrast, LDH activity was elevated in the n-3 PUFA group compared to the n-6 PUFA group (diet: $F_{2,50} = 5.91$, $P = 0.008$; flight: $F_{1,50} < 0.01$, $P = 0.97$, diet × flight: $F_{2,50} = 0.64$, $P = 0.53$). H-FABP mRNA abundance was not influenced by diet, but a trend for increased expression during flight was observed (diet: $F_{2,50} = 0.48$, $P = 0.62$; flight: $F_{1,50} = 3.63$, $P = 0.06$, diet × flight: $F_{2,50} = 0.15$, $P = 0.86$, Figure 3.5).

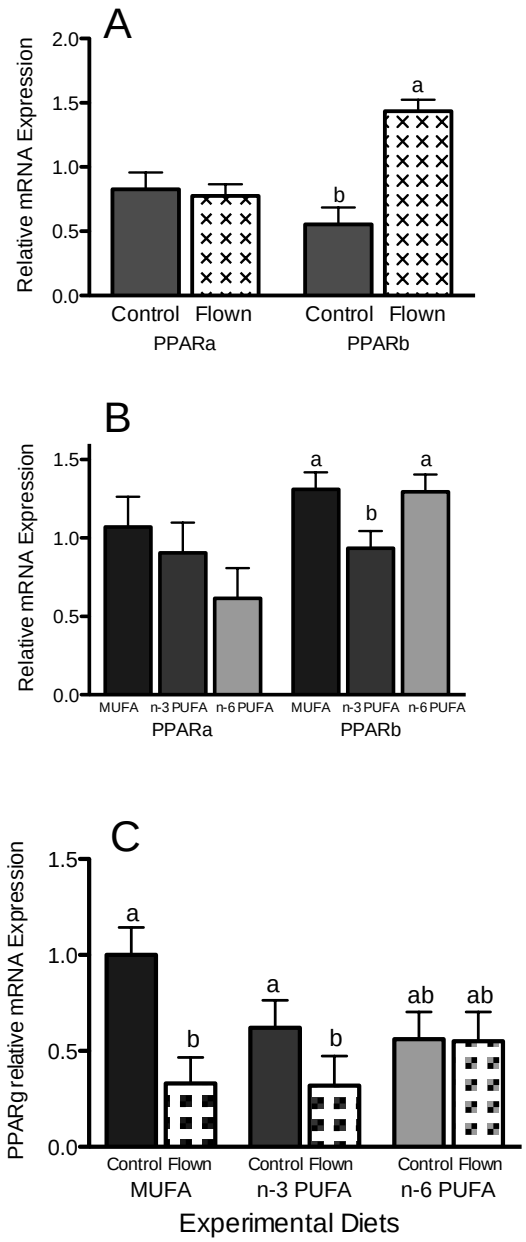

Figure 3.3 Effect of diet and flight on PPAR mRNA abundance in yellow-rumped warblers (*Setophaga coronata*). A) Effect of flight on PPARα and PPARβ mRNA abundance. B) Effect of diet on PPARα and PPARβ expression. C) Effect of diet and flight on PPARγ mRNA abundance. Values are means ± SEM. Values that do not share a common letter differ ($P \leq 0.05$).

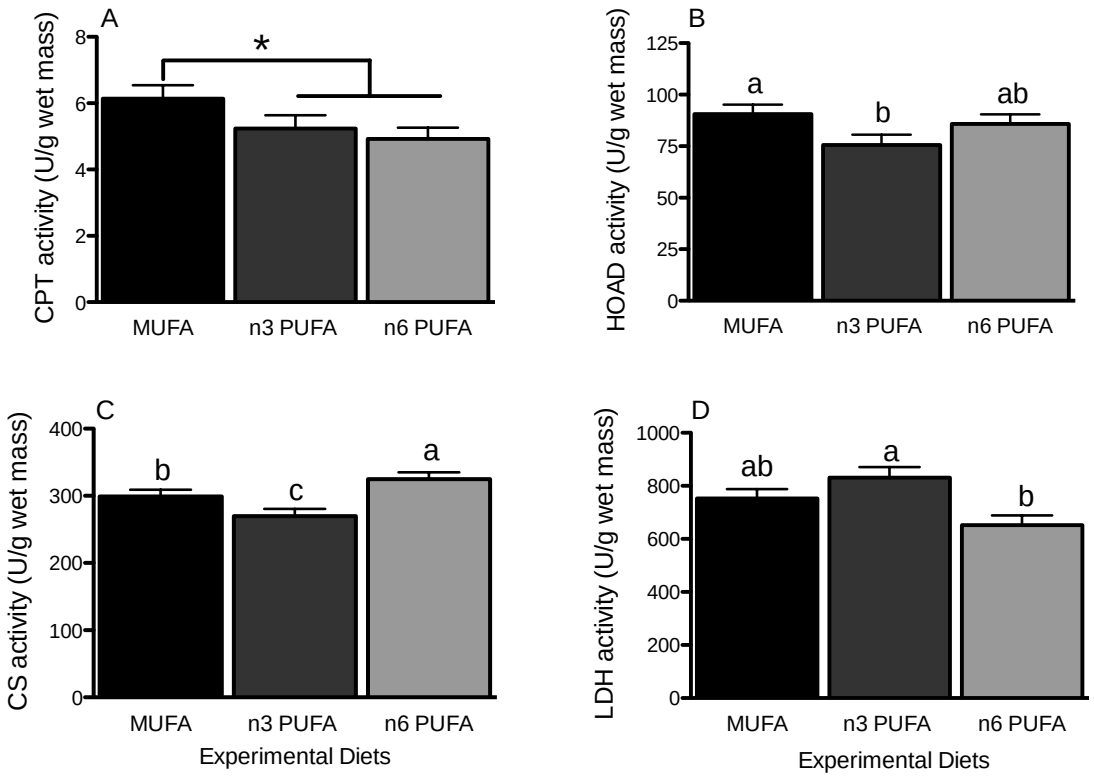

Figure 3.4 Effect of dietary PUFA on flight muscle enzyme activities. A) carnitine palmitoyl transferase (CPT), B) 3-hydroxyacyl-CoA dehydrogenase (HOAD), C) citrate synthase (CS), and D) lactate dehydrogenase (LDH) of yellow-rumped warbler (*Setophaga* coronata) fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA), or n-6 polyunsaturated (n-6 PUFA) fatty acids. Values are means ± SEM. Values that do not a share a letter differ ($P \leq 0.05$). * Indicates a significant difference between MUFA and the n-3 and n-6 PUFA diets combined.

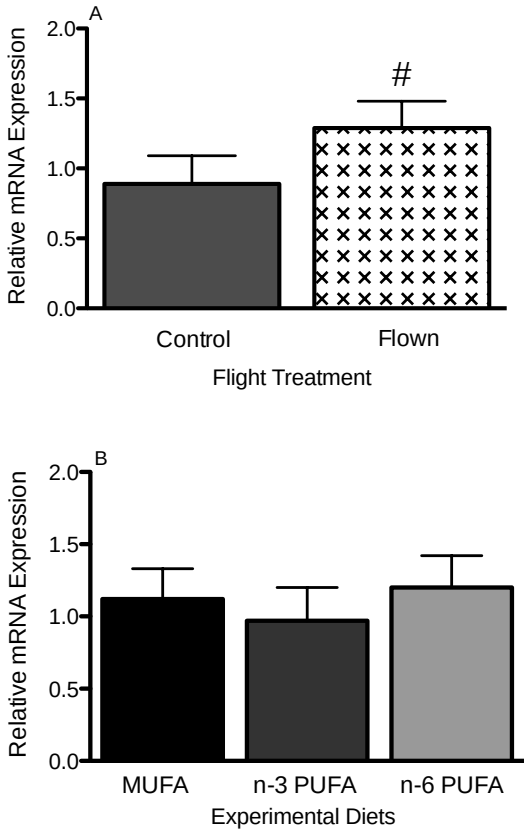

Figure 3.5 Effect of A) flight and B) diet on heart type fatty acid binding protein (H-FABP) mRNA abundance of yellow rumped warblers (*Setophaga coronata)*. Warblers were fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA), or n-6 polyunsaturated (n-6 PUFA) fatty acids and Flown or unflown (Control) in a wind tunnel. # Indicates a trend (P = 0.06).

3.3.5 Correlations between flight performance and muscle fatty acid oxidation, and metabolic rate

There were no relationships between flight duration and muscle enzyme activities, BMR, PMR or aerobic scope (Table 3.9). Since flight performance (power and relative protein contribution) change greatly with duration, I focused solely on the 360 min flight for correlations between metabolic rate and muscle metabolism with flight performance (Table 3.10). BMR was significantly correlated with flight power, likely as a result of body mass influencing both factors. Although, neither BMR nor PMR were correlated with relative protein contribution, aerobic scope was positively correlated with the relative protein contribution. In terms of the muscle metabolism, the only significant correlation with flight performance was a negative correlation between HOAD and relative protein contribution.

3.4 Discussion

The natural doping hypothesis proposes that DHA and EPA increase flight muscle oxidative capacity, thereby enabling prolonged endurance flight of migratory birds (Maillet and Weber, 2006; Maillet and Weber, 2007; Weber, 2009). In the current study, I found no support for the natural doping hypothesis. PUFA did influence muscle lipid metabolism, but feeding diets enriched with n-3 PUFA caused a coordinated decrease in oxidative capacity. However, dietary differences in muscle metabolism were not reflected in exercise performance, and no treatment difference was observed at the whole animal level.

A similar, but distinct, hypothesis suggests that PUFA and/or 18:2 n-6 benefit migratory birds by increasing metabolic flight efficiency, PMR, and aerobic scope

(Price and Guglielmo, 2009; Pierce and McWilliams, 2014). Unlike previous studies, I formulated experimental diets to manipulate only the intake of the DHA and ARA to avoid potential confusion in the interpretation of the effects of dietary fatty acid composition on exercise and metabolism (Guglielmo, 2010). The proportion of 18:2 n-6 was similar among all diets to help ensure that only long-chain PUFA were manipulated. Other dietary differences were present but fairly minor, such as lower SFA in the MUFA diet and lower PUFA and 18:2 n-6 in the n-3 PUFA compared to n-6 PUFA.

3.4.1 Effects of dietary fatty acids and flight on tissue fatty acid composition

The effect of dietary fatty acids on fatty acid composition was most apparent in the muscle phospholipids. Birds fed the n-3 and n-6 PUFA diets had the highest levels of long-chain PUFA in their muscles, which reflected the long-chain PUFA present in their diets. Birds fed the MUFA diet had the lowest proportion of long-chain PUFA in phospholipids, with a mix of ARA and DHA, presumably from elongation and desaturation of 18:2 n-6 and 18:3 n-3 respectively (Stevens, 2009). Many birds have high levels of long-chain PUFA in their membranes, despite these fatty acids not being present in their diets (Klaiman et al., 2009; Ben-Hamo et al., 2011). This suggests that birds actively synthesized long-chain PUFA from precursor fatty acids for incorporation into muscle membranes, and do so with any available precursor fatty acids.

Differences among dietary treatments in the adipose fatty acid composition of birds were much smaller compared to the muscle phospholipids. Although

significant differences were observed in the long-chain PUFA, they contributed < 1.5% of the total fatty acids and as such would likely not influence overall fuel composition. The proportion of PUFA was lower than reported in other studies (Pierce et al., 2005; Price and Guglielmo, 2009; McCue et al., 2009). Excess essential fatty acids, such as ARA and DHA, may be stored into the neutral lipids, including adipose (McCue et al., 2009). In my study, long-chain PUFA were likely not consumed in great enough quantities for this to occur at levels observed in other studies. Rather than being stored, ARA and DHA appeared to be preferentially incorporated into more metabolically active tissues such as the flight muscles. The greatest differences in fatty acid composition in the adipose were in the proportions of SFA and MUFA. The MUFA diet group had higher proportions of MUFA and lower proportions of SFA compared to birds fed PUFA diets. However, this difference was small (< 5%). As such, it is unlikely that the fatty acid composition varied enough among diets to result in large differences in mobilization from the adipose during flight (Price et al., 2008). Since the differences in the adipose fatty acid composition among diets were much smaller than in the muscle phospholipids, my study primarily tests the effects of phospholipids, which is most relevant to the natural doping hypothesis.

Similar to the adipose and muscle phospholipids, the expected differences in the plasma fatty acid composition from the dietary treatments were observed. Only a few fatty acids changed during flight (16:1n-7, 18:1 n-9, 18:2 n-6), and these changes were only observed in the MUFA and n-3 PUFA diet groups. In the MUFA diet group, MUFA increased and SFA decreased in the plasma during flight,

reflecting the major fatty acid components of adipose tissue. In the n-3 PUFA group, flown individuals had lower total MUFA during flight. Due to the small plasma volumes available, I was not able to measure fatty acid composition in the individual lipid classes (non-esterified fatty acids [NEFA], polar and neutral lipids). This would enable a more direct examination of changes in plasma NEFA composition during flight for differential and preferential fatty acid mobilization, which was not observed in the adipose tissue.

3.4.2 Effect of diet on whole animal performance

3.4.2.1 Body composition and metabolic rate

Overall, no significant differences in body mass or composition were observed among diet birds between the experimental diet, suggesting that the chronic increased intake of DHA and ARA, at the levels used in the current study, does not cause overt systematic toxicity. This is in contrast to Andersson et al. (2015), who found finding that higher plasma ARA concentration are negatively associated with body condition in urban great tits (*Parus major*). BMR was not affected by diet, and was comparable with other measurements of migratory yellow-rumped warblers (Swanson and Dean, 1999; Guglielmo et al., 2017). PMR, was also not affected by diet. Both Pierce et al. (2005) and Price and Guglielmo (2009) found that increased 18:2 n-6 intake increased PMR. My experimental diets contained similar proportions of 18:2 n-6 in each diet, which could explain why no difference was found. Furthermore, Price and Guglielmo (2009) attributed the increase in PMR from high n-6 PUFA diet to changes in fuel composition, which differed more in their study than in my study. The fold difference between PMR and

BMR (aerobic scope) is ~8.5 fold × BMR. This scope is higher than previous measurements with yellow-rumped warblers (~4.6 × BMR, Guglielmo et al., 2017), but lower than studies of other passerines (~10.4 × BMR, red-eyed vireos, Pierce et al., 2005) and ~9.6 × BMR house sparrows (*Passer domesticus,* Chappell et al., 1999). Variation in methodology (the intensity and duration of spinning before the PMR reading) could contribute to differences among studies and potentially influence whether a significant effect was detected.

3.4.2.2 Effect of individual variation and flight duration on flight performance

Overall, the estimated flight costs were approximately ~1.59 J/s and 7.2 × BMR for the 360 min flights, similar to values previously measured in yellow-rumped warblers (Guglielmo et al., 2017). The warblers in my study were flying at 8 m/s. This is slower than wild migrating blackpoll warblers (*Setophaga striata*) who have estimated flight speeds of 10 – 13 m/s (DeLuca et al., 2015). This suggests that the yellow-rumped warblers may have been flying slower than they would be in nature. However, the mechanical output for flight as a function of velocity is U-shaped and the curve is relatively flat at intermediate velocities (Tobalske et al., 2003). As such, the wind tunnel flights are likely representative of the energy demands of real migratory flights.

Characterization of how energy costs and fuel mixture changes during flight has been described in previous studies (Gerson and Guglielmo, 2013; Guglielmo et al., 2017). Similar to these studies, the relative protein contribution and flight cost decreased over time. Flight duration did not influence the absolute amount of lean

mass catabolized, unlike fat catabolism, which was tightly correlated with flight duration. Lean mass use during flight does not linearly increase overtime, and its use during the initial stage of endurance flight is greater while the transition to fatty acid oxidation occurs (Gerson and Guglielmo, 2013).

The large number of 360 min flights I obtained allows for exploration of individual variation in flight performance traits. Flight fuel mixture was correlated to body mass and composition, with heavier and fatter individuals having an inferred lower relative protein contribution. This shift was driven by both an absolute increase in fat catabolism and a decrease in lean mass catabolism. A similar relationship was described with resting and active birds and mammals (Jenni and Jenni-Eiermann, 1998). This differs from other wind tunnel studies. A wind tunnel study of red knots (*Calidris canutus*) found that the relative protein contribution remains constant with increasing energy costs (Jenni-Eiermann et al., 2002). However, their conclusion was based on body mass loss and plasma metabolite profiles to determine fuel mixture, and not direct measurements. A recent wind tunnel study using yellow-rumped warblers found that body mass or composition was not significantly related to fuel mixture when examining flight of varying durations that also influence fuel mixture (Guglielmo et al., 2017). My study provides evidence that body mass and percent body fat alters fuel mixture and is independent of flight duration (Table 3.11). The consequences or benefits of this fuel shift are unclear. All birds had a minimum of body fat of 16 % at the start and 11 % at the end of the flight. It is unlikely that the increase in relative protein contribution is related to insufficient fat stores (5 - 10% body fat) during flight

(Schwilch et al., 2002). The growing number of wind tunnel studies being completed may allow for a meta-analysis of factors influencing flight performance and fuel mixture to produce an aerial view of fuel use and management during flight.

3.4.2.3 Effect of diet on flight performance

Voluntary flight duration was similar among all diets, suggesting that within a 360 min timeframe DHA and ARA do not influence endurance capacity or fatigue. This 360 min duration is ecologically relevant for nocturnal migratory songbirds. The metabolic energy cost of flight was also not reduced by dietary PUFA, which was suggested from preliminary results from a study conducted by McWilliams et al. (2006). The natural doping hypothesis focuses on priming muscle for increased fatty acid oxidation needed for flight, which could lower relative protein contribution. However, I found no difference in inferred fuel mixture among birds fed the experimental diets, suggesting that long chain n-3 PUFA are not as critical for oxidizing fatty acids at high rates during flight. In some animal studies, dietary PUFA can influence exercise performance, but others find no effect. For example in fish, long chain n-3 PUFA may increase (Wagner et al., 2004), decrease (McKenzie et al., 1998; Chatelier et al., 2006) or not influence (Wilson et al., 2007; Regan et al., 2010) athletic performance. Reduced fatigue resistance was observed in rats fed long chain n-3 PUFA (Clavel et al., 2002; Henry et al., 2015). Conversely, diets higher in n-6 PUFA are associated with increased endurance capacity (Ayre and Hulbert, 1997). Shei et al. (2014) reviewed 13 human studies on the effects of DHA and EPA supplementation on performance, and found no overall enhancements in direct measures of athletic performance, but physiological indicators of performance were

altered. For example, supplementing trained athletes with n-3 PUFA reduced plasma triacylglycerides but no detectable enhancement of aerobic and running performance was observed (Raastad et al., 1997). Additionally, supplementation with n-3 PUFA in elite athletes improves heart rate during exercise but does not translate to any effect on endurance performance or recovery (Buckley et al., 2009). The direct response of exercise performance to dietary n-3 PUFA appears to be complex and not robust, and other physiological factors may influence the effect. Migratory birds may be a special case in terms of exercise performance, where endogenous upregulation of aerobic and fatty acid oxidation capacities during the migratory period increases performance (Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011), reducing any potential effect of diet on performance.

3.4.3 Effects of long-chain PUFA on fatty acid metabolism

3.4.3.1 Effects of diet on fatty acid metabolism

Generally, it has been suggested that increasing n-3 long-chain PUFA increase PPAR activation, resulting in increased expression of fatty acid metabolism genes (McClelland, 2004; Grygiel-Górniak, 2014). This is beneficial for endurance exercise, as increased oxidative capacity in muscle increases fatigue resistance (Clavel et al., 2002). On the other hand, there is evidence that n-3 long-chain PUFA increase anaerobic metabolism in skeletal muscles, rather than aerobic metabolism (Higuchi et al., 2008). PPARβ mRNA abundance was lower in the birds from the n-3 PUFA diet treatment. This change was coordinated with an overall decrease in aerobic and oxidative enzyme activities (CPT, HOAD, CS), and increased anaerobic capacity (LDH). This is opposite to the predictions of the natural doping hypothesis

and supporting evidence in semipalmated sandpipers and quail (Maillet et al., 2007; Nagahuedi et al., 2009), and consistent with another study in which no changes in enzyme activity were observed with variation in dietary and tissue PUFA in white-throated sparrows (Price and Guglielmo, 2009). Both studies used similar manipulations, comparing n-3 long-chain PUFA with 18:2 n-6. Differences among the results of these studies and my study hint at the potential that the response to PUFA is species-specific.

The role of long-chain PUFA on the activity of membrane bound proteins may explain the decreased CPT activity in both PUFA diets. The fatty acid composition of the cellular, mitochondrial, and sarcoplasmic reticulum membranes are similar and all respond to dietary PUFA manipulations (Nagahuedi et al., 2009). As such, the increase in long-chain PUFA phospholipids in the PUFA groups would likely enrich the mitochondrial membranes comparably. Increasing the proportion of n-3 long-chain PUFA in the mitochondirial membranes increases the activity of CPT in mitochondrial preparations (Power et al., 1997). However, the homogenization used the CPT enzyme assay in my study would disrupt membrane (eg. freeze thaw cycles, and Triton-X 100 in the homogenization buffer). As such, the potential influence of membrane fatty acids on protein activity may not be accounted for in this study and others (Maillet and Weber, 2007; Nagahuedi et al., 2009). Potentially, if PUFA increase CPT activity, the activity in the PUFA diet groups may have been higher when assayed using a method that keeps the membranes intact.

3.4.3.2 Effect of flight on muscle metabolism

In my study, PPARα mRNA abundance was not influenced by diet or flight. In contrast, PPARβ mRNA abundance increased during flight, and the abundance was overall lower in the n-3 PUFA group. Exercise training influences the gene and protein expression of PPAR in a time-dependent manner. One exercise bout can increase the gene expression of PPARβ, which then returns to baseline following recovery, while PPARα gene expression is not altered by exercise (Perry et al., 2010). However, successive training increases both PPARα and PPARβ protein content at similar rates (Perry et al., 2010). During exercise, muscle contraction, and/or increasing cellular NEFA and decreasing glycogen stores may result in PPARβ activation and increase its mRNA abundance (Barrès et al., 2012; Philp et al., 2013). Glycogen stores would be depleted during the initial stages of flight, and cellular uptake of NEFA increased as the major fuel (Jenni and Jenni-Eiermann, 1998). These patterns suggest that while both PPARs show changes in preparation for migration in birds (DeMoranville, 2015) and exercise training in humans (Perry et al., 2010), PPARβ gene expression during flight may play an important role in the transition to and maintenance of ATP production during flight. PPARγ decreased in mRNA abundance in birds fed the MUFA and n-3 PUFA diets. PPARγ is most associated with adipose tissue and increasing its lipid storage preventing lipotoxicity in non-adipose tissue (Grygiel-Górniak, 2014). The function of PPARγ in skeletal muscle is less understood than the other PPAR, but a role in maintaining insulin sensitivity, glucose homeostasis, and preventing lipid accumulation in skeletal muscle has been identified (Amin et al., 2010). Decreased mRNA abundance

suggests that PPARγ mRNA abundance may be less important for sustaining muscle function during flight, particulairly if PPARγ increases lipid storage as it does in adipose tissue. The trend for increased H-FABP mRNA abundance during flight suggests that maintaining or increasing H-FABP may be important for maintaining adequate fatty acid transport to the mitochondria. Endurance flight did not alter metabolic enzyme activities, but increased mRNA abundance of these genes, via PPAR, may be needed to maintain their activity.

3.4.4 Correlations of flight performance with metabolic rate, PPAR and metabolic enzymes

Directly measuring flight performance under controlled conditions provides a realistic metric of how animals may perform in the wild. Understanding the relationship between flight performance and muscle physiology and biochemistry is needed to grasp how alterations at the muscle level may be reflected in whole animal performance. The only significant factor affecting flight cost was body mass or related factors (fat and lean mass, and BMR). A positive correlation between flight power and BMR is likely driven by body mass, as heavier birds have higher BMR and higher flight costs. Two significant correlations were identified for fuel mixture. First, the inferred relative protein contribution was positively correlated to aerobic scope, but not PMR and BMR or respective RER. Due to the difficulties in PMR measurements, the aerobic scope relationship was limited to only 7 individuals. Potentially, birds that have a greater relative protein contribution during flight also have a greater protein contribution while exercising in a flight wheel. The exact contribution of protein, carbohydrates and lipids to the fuel

mixture during the PMR measurements cannot be determined from the RER measurements (~0.74). After 3 h of fasting, glucose supplies may be limited, and an increased ability to use amino acids as fuel could boost PMR. The second relationship was a negative correlation between relative protein contribution and HOAD activity, an enzyme in fatty acid β-oxidation pathway. Lower dependence on fatty acids for fuel may be reflected by lower HOAD activity in these individuals.

Given the few significant correlations with flight performance, whether metabolic rate and metabolic enzymes are predictors of performance in a wind tunnel or migration is unclear. Similarly, Swanson et al. (2013) found that muscle size but not metabolic enzyme activity could predict thermogenic capacity. Flight metabolic rate (power) was lower than PMR in my study and in Guglielmo et al. (2017). As noted by Conway et al. (1994) and Price (2010), inclement weather and anthropogenic factors, may increase the flight energy costs. This would require birds to sustain a metabolic rate closer to PMR levels, and/or rely on increased fatty acid oxidation capacity for energy production. The question of if and how modulating enzyme activity influences flight performance remains, but the answer is necessary to understanding how dietary fatty acids and other factors may influence migration.

3.4.5 Do n-3 PUFA and n-6 PUFA improve migratory performance*?*

Overall, my study did not provide support for the natural doping hypothesis, or for the benefits of PUFA on migratory performance. Another controlled study also found no performance benefits of n-3 PUFA in migratory birds (Price and

Guglielmo, 2009). However, other factors could alter the effect of dietary PUFA on muscle metabolism and performance and should be considered when evaluating the effects of dietary PUFA. The first is the possibility that migratory state influences the effect of PUFA. During migration the flight muscle has higher aerobic and fatty acid oxidative capacity (Guglielmo et al., 2002; McFarlan et al., 2009), and photoperiod appears to be a sufficient cue for priming the flight muscle for migration (Zajac et al., 2011). If the migratory state already primes birds for migration, further potential priming by fatty acids may be limited. Furthermore, it is important to note that the differences in metabolic enzymes in my study are small compared to seasonal upregulation during migration (McFarlan et al., 2009; Zajac et al., 2011; Chapter 2).

Second, muscle fibre type may also influence the response to PUFA. Small birds, including passerines and sandpipers, have exclusively fast-oxidative glycolytic fibres in their flights muscles (Lundgren and Kiessling, 1988; Evans et al., 1992). Larger or more sedentary birds have additional fibre types in their muscles. For example, European robins (*Erithacus rubeula*) also have intermediate fibres, and quail and blackbirds (*Turdus merula*) also have fast glycolytic fibres in their flight muscles (Lundgren and Kiessling, 1988; Choi et al., 2013). Increasing oxidative capacity from n-3 PUFA could be the result of the shifting of muscle fibres to a more oxidative fibre type (Hashimoto et al., 2016). If fibre type switching is important for the effect of n-3 PUFA, this may limit the scope or magnitude of the effect of n-3 PUFA in smaller passerines and sandpipers that already have highly oxidative muscle fibres and explain why quail responded dramatically to n-3 PUFA supplementation (Nagahuedi et al. 2009).

Third, there are large differences in the intake and ratio of n-3 and n-6 PUFA among migratory birds. Sandpipers and other shorebirds consume a diet high in n-3 PUFA, compared to the high in n-6 PUFA diets of passerines (Conway et al., 1994; Weber, 2009; Pierce and McWilliams, 2014; Andersson et al., 2015). The yellow-rumped warblers used in my study fall into the latter group, and this may have influenced their response to PUFA. Species may also differ in their tolerances. The warbler's health was seriously affected when provided with the original n-3 PUFA diet, suggesting that there may be a safe upper limit for n-3 long-chain PUFA or DHASCO for warblers. The DHASCO inclusion level was originally chosen to be an estimated size-adjusted match to the intake of DHA used by Nagaheudi et al. (2009) and was based off the daily dose given and estimated body size of bobwhite quail and average diet consumption of captive yellow-rumped warblers during migration. It is not possible to determine if the effect was a result of high DHA intake or DHASCO itself. DHASCO is generally recognized as safe and the highest doses tested in toxicity studies find no observable adverse effects (Hadley et al. 2010), but these are likely a bit lower than the ones used in the current study. Future work should incorporate a comparative aspect and include species for which ecological or observational studies have suggested a direct role for n-3 PUFA, such as sandpipers (Maillet and Weber, 2007), and hummingbirds (Infante et al., 2001).

Finally, the inclusion of ARA did not appear to cause any detrimental effects to yellow-rumped warblers. ARA is a precursor molecule to pro-inflammatory prostaglandins (Calder, 2015). Andersson et al. (2015) proposed that increasing ARA could increase inflammation in birds, especially those exposed to high

environmental pollutants. The effect of n-3 and n-6 PUFA intake and ratio on inflammation is still not fully clear, but the physiological effect of PUFA is more complex than simple categorizations of pro-inflammatory for ARA, and anti-inflammatory for EPA and DHA, and there are multiple levels of regulation (Calder, 2015). Since I found no evidence for ARA negatively impacting health or performance, higher proportions of ARA are likely not a strong indicator of poorer condition in songbirds.

3.5 Conclusion

The natural doping hypothesis was directly tested using carefully formulated diets to test the effect of long-chain PUFA on animal physiology from the tissue to whole animal level. No direct support for the natural doping hypothesis, or for any benefit or consequence of feeding diets low or high in n-3 or n-6 long-chain PUFA on endurance exercise performance was observed. Alterations in the flight muscle suggest decreased aerobic and oxidative capacity in the flight muscle with feeding n-3 PUFA. However, since this was not associated with any flight performance parameter (endurance capacity, energy costs, and fuel mixture), conclusions about the direct effects of small modulations in flight muscle metabolism on exercise performance are limited. The effect of dietary PUFA on performance may be dependent on other factors such as species and migratory condition.

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4 Management of oxidative stress during endurance flight in yellow-rumped warblers (*Setophaga coronata*) is not influenced by long-chain PUFA intake

4.1 Introduction

Migratory flight requires birds to sustain high metabolic rates for prolonged periods of time. Seasonal changes in the flight muscle that enable birds to accomplish these feats of endurance has generally focused on increasing fatty acid transport and oxidation capacity (Guglielmo, 2010). However, migratory flights may also pose an oxidative challenge that may shape migration (Jenni-Eiermann et al., 2014). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are a consequence of exercise (Ji, 1999; Costantini et al., 2008). Mitochondrial respiration is one source of reactive species, with approximately 0.15% of oxygen consumed being converted into superoxides (St-Pierre et al., 2002). Additional ROS is produced during muscle contraction from NADPH oxidases in the sarcoplasmic reticulum, sarcolemma, and transverse tubules (Zuo et al., 2011; Sakellariou et al., 2013; Goncalves et al., 2015). ROS produced during exercise can be important regulators of contractile force, cell signalling pathways, and gene expression (Powers and Jackson, 2008; McClung et al., 2010; Powers et al., 2010). However, excessive ROS production can lead to oxidative stress (OS), where the pro-oxidant: antioxidant ratio favours pro-oxidants, causing damage to macromolecules, impaired redox control, and changes in cell signalling (Sies and Jones, 2007). Birds must manage ROS produced during flight in order to make non-stop travel lasting

up to several days possible. Failure to do so could impair the functioning of contractile machinery and mitochondrial function needed to sustain flight, impacting flight efficiency and endurance capacity.

Birds can use endogenous antioxidant enzymes, such as catalase, superoxide dismutase (SOD), and glutathione peroxidase, to defend against ROS (Powers and Jackson, 2008). They may also limit the production of ROS from the mitochondria (Brand, 2000), or produce antioxidants such as glutathione or uric acid, a metabolic by-product produced during flight (Tsahar et al., 2006). Dietary antioxidants can also contribute to defenses and include anthocyanins found in berries (Schaefer et al., 2008) or excess protein intake, which may increase plasma uric acid (Smith and McWilliams, 2009; Alan and McWilliams, 2013; Eikenaar et al., 2016). Birds likely use a mixture of these mechanisms to manage ROS in preparation for and during migratory flight.

Similar to the seasonal upregulation of aerobic and oxidative capacity in the muscle during migration (McFarlan et al., 2009; Zajac et al., 2011), increasing antioxidant capacity may be a key element to migratory preparation. In the flight muscle, Mn-SOD protein content increases during migration (Banerjee and Chaturvedi, 2016). At stopover, birds increase circulating antioxidants in preparation for the next migratory flight as part of the refuelling process (Skrip et al., 2015; Eikenaar et al., 2016). Furthermore, frugivorous birds may preferentially select berries rich in antioxidants (Schaefer et al., 2008). During a migratory flight, glutathione peroxidase activity increases in blood cells (Jenni-Eiermann et al., 2014), and plasma uric acid concentrations may increase in some birds (Gerson and

Guglielmo, 2011; Guglielmo et al., 2017). Despite the increase in antioxidant capacity in preparation for flight and further alterations during flight, an overall decrease in plasma antioxidant capacity occurs during flight (Costantini et al., 2007; Costantini et al., 2008). The effects of OS during flight are observed from increased protein damage in blood cells (Jenni-Eiermann et al., 2014) and increased concentration of plasma creatine kinase, a marker of muscle damage (Guglielmo et al., 2001). Perturbations to flight muscle mitochondrial function also occur and decrease ATP production capacity (Gerson, 2012). After flight, birds repair the damage accrued to their mitochondria (Gerson, 2012), replenish their fuel stores and may increase circulating non-enzymatic antioxidant capacity (Eikenaar et al., 2016). Thus, the refuelling process may concurrently aid in the repair of oxidative damage from the previous flight (Skrip et al., 2015).

Molecules differ in susceptibility to oxidative damage, depending on their structure and location within the cell. Although polyunsaturated fatty acids (PUFA) have been highlighted for their potential to enhance migratory performance (Price and Guglielmo, 2009; Weber, 2009; Pierce and McWilliams, 2014), they are also prone to oxidative damage. The high susceptibility to oxidative attack is because hydrogen atoms located near double carbon bonds require less chemical energy to remove than hydrogens on carbons with single bonds (Skrip and McWilliams, 2016). Once a fatty acid loses an electron to a free radical, it becomes a lipid radical and can form a lipid peroxyl radical with oxygen, which can then react with another PUFA and increasing damage further (Skrip and McWilliams, 2016). Additionally,

PUFA are incorporated into mitochondrial membranes, which puts them in close proximity to the site of ROS production (Price, 2010; Skrip and McWilliams, 2016).

Feeding a high PUFA diet to white-throated sparrows (*Zonotrichia albicollis)* increases oxidative damage to plasma metabolites, however it also increases circulating antioxidant capacity (Alan and McWilliams, 2013). In the flight muscle, PUFA may also decrease the net mitochondrial production of ROS or increase antioxidant capacity resulting in less damage during endruance flight (Gerson, 2012). So, despite increased susceptibility to ROS, PUFA may be protective against ROS by increasing antixodiant capacity. This is an example of a hormetic effect, where sustained exposure to a mild oxidative stressor increases antioxidant defenses, which also increases the capacity to manage greater stressors. This effect has been demonstrated in rats, where n-3 PUFA supplementation increases basal lipid oxidative damage and antioxidant enzyme capacity in hearts (Abdukeyum et al., 2016). The increased antioxidant capacity in the supplemented rats at rest meant that during an acute oxidative challenge the rats were better able to manage ROS production and accrued less damage (Abdukeyum et al., 2016). Potentially, increasing dietary PUFA may also increase antioxidant capacity and lower oxidative damage during endurance flight.

The type of PUFA may also influence this response; n-3 PUFA (docosahexaenoic acid, DHA, 22:6 n-3) increases the mRNA abundance of glutathione peroxidase and SOD in skeletal muscle compared to the n-6 PUFA, arachidonic acid (ARA, 20:4 n-6) (Hashimoto et al., 2016). Interestingly, despite

differences in enzyme mRNA abundance, no difference was detected in lipid peroxidation compared with the control (Hashimoto et al., 2016). During migration, increasing dietary PUFA may elicit a complex response. At rest, birds fed high PUFA diets may have higher oxidative damage, but also higher antioxidant capacity. However, since ROS production is elevated during endurance flight, PUFA fed birds may have lower net damage because of the higher antioxidant capacity.

The majority of studies have assessed oxidative balance in migratory birds using blood samples. These changes may not fully represent how birds manage ROS during flight, and the response could differ among tissues. In this study I examined antioxidant enzymes and oxidative damage in the flight muscles of migratory yellow-rumped warblers (*Setophaga coronata*) after simulated migratory flights and determined if dietary DHA and ARA influence the management of ROS. I predicted that long-chain PUFA would increase antioxidant enzyme activity and damage at rest, but this would help lower oxidative damage during endurance flight.

4.2 Materials and Methods

4.2.1 Birds and experimental design

Yellow-rumped warblers were fed one of three diets as described previously (Section 3.2.1). Briefly, I carefully formulated diets enriched in n-3 PUFA (DHA, a long chain n-3 PUFA), n-6 PUFA (ARA, a long chain n-6 PUFA), or monounsaturated fatty acids (MUFA). The birds began eating each diet in a wintering non-migratory condition elicited by long-term exposure to short daylight (9L:15D). After two weeks they were switched to long day photoperiod (16L:8D) to trigger a spring

migratory condition. The birds were grouped into 10 blocks by start date to control the amount of time the birds consumed the diets and when they entered a migratory condition. After 4 weeks on long days, half the birds (Control) were food-deprived for 2 h before the dark period and sampled at the start of the dark period (19:00 h). The other half (Flown) was sampled after a voluntary nocturnal flight in a wind tunnel of up to 360 min. The birds were anesthetised using isoflurane (^{PR}Florane), and killed by decapitation. Samples of the flight muscle were flash frozen in liquid nitrogen and stored at -80 °C.

4.2.2 Antioxidant enzyme activity

For the catalase and SOD assays, approximately 100 mg of flight muscle tissue was homogenized in 1 ml of cold 20 mM HEPES buffer (pH 7.2, with 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). The homogenate was centrifuged at 10 000 × g for 15 min at 4 °C, and the supernatant was split into 2 centrifuge tubes and stored at -80 °C until assayed. For the catalase assay, homogenates were diluted with the buffer to approximately 0.1 mg/ml on the day of assay. Protein content of the homogenate was measured using the Bradford assay (BioRad Laboratories, Mississauga, ON) with a microplate spectrophotometer (Bio-Tek Powerwave X340; Winooski, VT) using bovine serum albumin standards. Catalase was measured in duplicate using a method modified from Weydert and Cullen (2009), which measures the rate hydrogen peroxide consumption. Briefly, 10 µl of homogenate was added to 810 µl phosphate buffer and a background scan at 240 nm for 2 min (Cary 100, Varian, Inc. Palo Alto, CA). The reaction was initiated by the addition of 180 µl 30 mM hydrogen peroxide and scanned for a further 2 min. Catalase activity,

in U/mg protein, was calculated from the molar extinction of hydrogen peroxide (34.9 $M^{-1}cm^{-1}$) and corrected for background hydrogen peroxide degradation levels (intra-assay coefficient of variability (CV) 4.26 %).

Superoxide dismutase activity was measured using a colourimetric superoxide dismutase assay kit (706002, Cayman Chemical Co, Ann Arbour, MI). The SOD activity assay utilizes a tetrazolium salt to detect the capacity of SOD to dismutate superoxide radicals produced by xanthine oxidase, which also results in the secondary conversion of the terrazolium salt into formazan dye. The sample homogenates were diluted with the sample buffer (50 mM Tris-HCl, pH 8.0) to approximately 0.2-0.25 mg/ml in concentration for the assay, and 10 µl of the diluted homogenate was used per reaction with the samples run in duplicate (inter-assay CV 2.95 %, intra-assay CV 2.90 %). The prepared plates were incubated with the assay buffer (50 mM Tris-HCl, pH 8, with 0.1 mM diethylenetriaminepentaacetic acid and 0.1 mM hypozanthine), the tetrazolium salt solution, and xanthine oxidase on a shaker for 30 min at room temperature (~ 20 °C) and then read at 460 nm to detect the formazan dye. SOD activity was calculated using the linear regression of the standard curve, and expressed per mg of protein.

4.2.3 Glutathione

The reduced form of glutathione (GSH), and the oxidized disulphide dimer (GSSG) were measured using a Glutathione Assay kit (Cayman Chemical Co., 703002). Here, total glutathione is measured by the reaction of GSH with DTNB, yielding TNB and GSTNB and GSTNB is recycled back to GSH by glutathione

reductase producing another TNB. The inclusion of glutathione reductase also reduces GSSG to GSH resulting the measurement of total glutathione. To measure GSSG only, 2-vinylpyridine is added to the samples to derivatized GSH preventing it from reacting with DTNB and this allows only GSH from GSSG to be measured. Approximately, 60-65 mg of flight muscle tissue were homogenized in 9 volumes of 50 mM PBS with 1 mM EDTA (pH 7.4), and centrifuged at 10 000 × g for 15 min at 4 °C. 500 µl of the supernatant was removed, and deproteinated with the addition of 500 µl of 10% metaphophoric acid (MPA). The mixture was allowed to sit at room temperature for 5 min, and then centrifuged for 2 min at 2000 × g. The deproteinated supernatant was collected and stored at -20 °C and assayed the next day. Prior to assaying, 50 µl of a 4 M triethanolamine was added to the sample. To quantify total glutathione concentration, 50 µl of the deproteinated sample was diluted in 950 µl MES buffer (pH 6.0, 0.2 M 2-(N-morpholino)ethanesulponic acid, 0.1 M phosphate, and 2 mM EDTA). For GSSG, 500 µl of the deprotinated sample was diluted with 250 µl of MES buffer, and incubated with 7.5 µl of 1 M 2-vinylpyridine in ethanol for 60 min to derivatize the GSH in the sample, and glutathione standards treated in the same manner. Samples were run in duplicate according to the kit instructions, after the addition of the glutathione reductase and glucose-6-phosphate dehydrogenase and enzyme co-factors the microplates were incubated in the dark on a shaker for 25 min, and the microplate was scanned at 405 nm to measure the absorbance of TNB. Total glutathione and GSSG concentrations were calculated using standard curves. GSH concentration was calculated from the difference in total glutathione and GSSG concentrations. The

intra-assay CV and inter-assay CV were 2.21 % and 3.98 % for total glutathione, and 1.11 % and 3.07 % for GSSG.

4.2.4 Protein carbonyl quantification

Protein carbonyl concentration was measured using a modified method from Levine et al. (1990) with the addition of a chromaphore removal step (Barreiro et al., 2005). Briefly, 60 mg of tissue was homogenized in 600 µl of buffer (50 mM PB buffer with 10 mM EDTA, pH 7). The homogenate was centrifuged at 10 000 × g, 10 min, at 4 °C, and 500 µl supernatant collected and stored at -80 °C until assay the next day. To remove chromaphores in the homogenate, the 500 µl supernatant was added to 1.4 ml HCl-acetone (3:100 v/v) and the protein precipitated. The precipitate was washed twice with 1.6 ml of HCl-acetone, followed by 2 washes of 1.8 ml 10% trichloroacetic acid (TCA). Between washes the samples were centrifuged at 10 000 × g for 10 min at 4 °C. The pellet was resuspended in 500 µl of 50 mM phosphate buffer with 6 M urea. Next, the dechromaphored homogenate was divided into two 200 µl aliquots, and 500 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl was added to one aliquot and 500 µl 2.5 M HCl to the other aliquot to serve as a blank. The samples were incubated in the dark at room temperature for 45 min, and vortexed every 15 min. Following incubation, 1 ml of 20% TCA solution was added and they were placed on ice for 10 min before centrifuging at 10 000 × g for 10 min at 4 °C. The samples were then washed with 1 ml of 10% TCA and placed on ice for 5 min, and centrifuged again with the supernatant discarded. The pellet was then washed twice with 1 ml of ethanol-ethyl acetate (1:1 v/v) to remove any residual DNPH, and centrifuged at 10 000 × g for 10

min at 4 °C, discarding the supernatant each time. Finally, the pellet was resolubilized in 500 µl of 6 M guanidine hydrochloride and placed in a 37 °C water bath for 20 min, and vortexed. The samples were centrifuged at 700 × g for 10 min to remove any debris. The absorption of the samples was measured at 380 nm, in duplicate, with 200 µl of the labelled or blank sample. The carbonyl content was measured using an absorbance coefficient of 22 000 $M^{-1}cm^{-1}$. Protein content of the final samples was calculated from the absorbance of a BSA standard curve in 6 M guanidine hydrochloride at 280 nm.

4.2.5 Statistics

To establish if any of the measured variables changed during flight, I first examined if flight duration influenced antioxidants or damage as either a main effect or interaction with diet. If flight duration influenced a variable, Control and Flown birds were analyzed separately. Control birds were analyzed using 2-way ANOVA to test for an effect of diet. The Flown birds were analyzed with flight duration as main effect and interaction with diet. Body mass, sex, and the start date blocking factor were tested as potential covariates but were not significant factors for $P \leq 0.05$ any variable and were not included in the analyses ($P > 0.05$). If duration did not influence values in the Flown group, Flown and Control birds were analyzed using a 2-way ANOVA. Correlations between flight performance (duration, energy used, and relative protein contribution (RPC) to fuel mixture) and antioxidants and protein carbonyls were determined using Pearson's Correlation coefficients. For duration, all flights were used. However, as duration influences flight costs and RPC (see Chapter 3), only the 360 min flights were used for these correlation analyses.

Statistical analysis was performed using SAS (v 9.4). Values are presented as means ± SEM, except when flight duration was a significant factor, in which case least squares means are presented instead for the Flown groups. Significance was accepted at $\alpha < 0.05$.

4.3 Results

4.3.1 Antioxidant enzymes

Within the Flown birds, there was no effect of flight duration or interaction between duration and diet on catalase activity (duration: $F_{1,18} = 0.65$, P = 0.65; diet × duration: $F_{2,18} = 1.83$, P = 0.19). Combining the analysis with the Control birds, there was no effect of diet, flight or their interaction (diet: $F_{2,50} = 0.07$, P = 0.94; flight: $F_{1,50} = 1.10$, P = 0.30; diet × flight: $F_{2,50} = 0.53$, P = 0.59, Figure 4.1A). SOD activity in the Flown birds was not influenced by flight duration or its interaction with diet (duration: $F_{1,18} = 0.33$, P = 0.57, diet × duration: $F_{2,18} = 2.22$, P = 0.13). When combined with Control birds, there was an effect of flight ($F_{1,50} = 5.05$, P = 0.03) and SOD activity was 17% higher in the Flown birds compared to Control birds (Figure 4.1B). Total SOD activity was not influenced by diet in either the Control or Flown birds nor was there a diets by flight interaction (diet: $F_{2,50} = 0.71$, P = 0.50; diet × flight: $F_{2,50} = 2.57$, P = 0.09). The higher SOD activity during flight may have been a result of the lower SOD activity in the Control n-6 PUFA diet group. Although the interaction between diet and flight was not significant (P = 0.09), within the Control birds the n-6 PUFA group had lower activity than the MUFA group, with the n-3 PUFA group being intermediate in activity. Only the n-6 PUFA diet group had

significantly higher expression during flight when examining the diet and flight interaction.

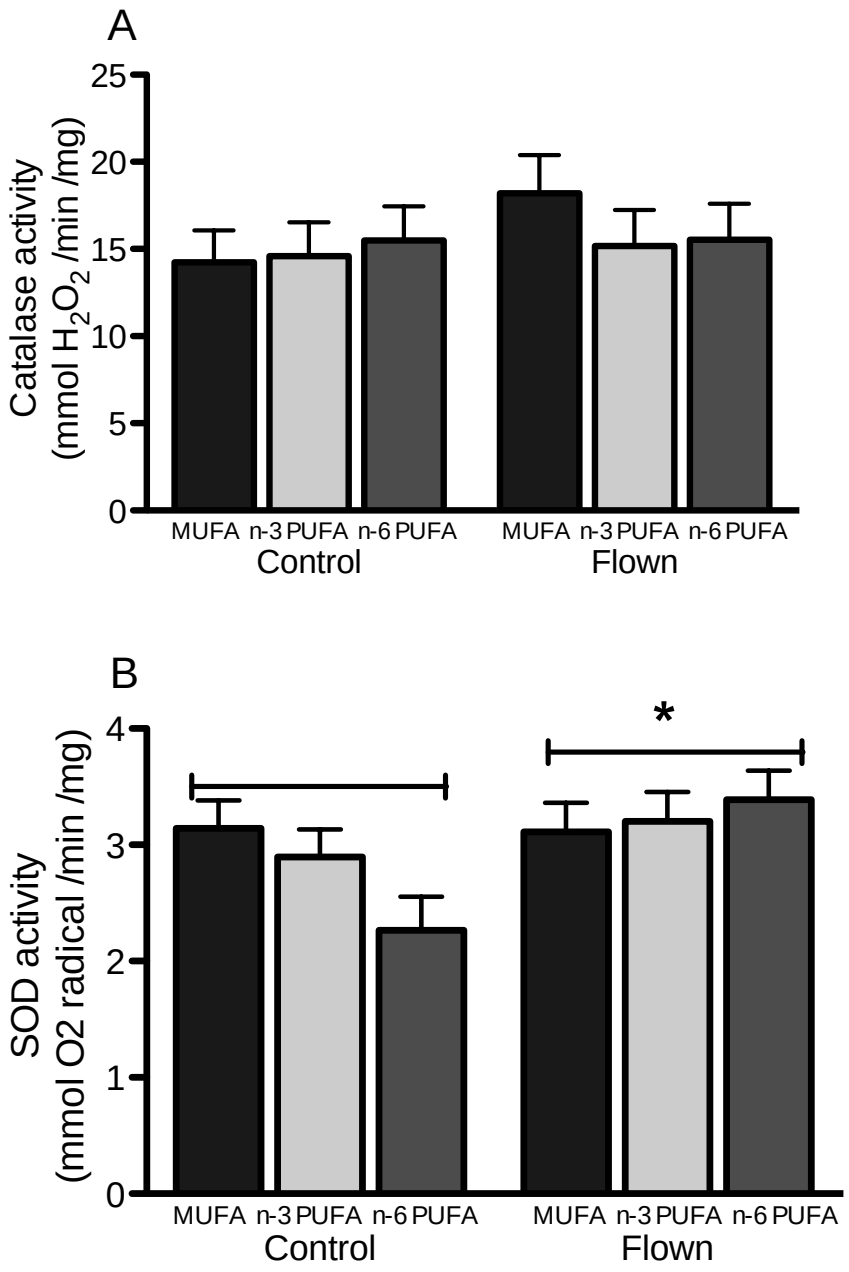

Figure 4.1 Flight muscle A) catalase and B) superoxide dismutase activity per mg protein in yellow-rumped warblers (*Setophaga coronata*) fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA), or n-6 polyunsaturated (n-6 PUFA) fatty acids. Values are means ± SEM. * Indicates a significant difference between Control and Flown birds ($P \leq 0.05$).

4.3.2 Glutathione

Total glutathione concentrations were $\log_{10}$ transformed to meet the normality assumption. Total glutathione concentrations were not influence by flight duration or an interaction between flight and diet (duration: $F_{1,18} = 0.95$, $P = 0.34$, duration × diet: $F_{2,18} = 0.63$, $P = 0.44$). Total glutathione concentration did not differ between Control and Flown birds, nor between dietary treatments (diet: $F_{2,50} = 2.41$, $P = 0.10$, flight: $F_{1,50} < 0.01$, $P = 0.96$; diet × flight: $F_{2,50} < 0.01$, $P = 0.99$, Figure 4.2A). GSH in the flown birds was not influenced by flight duration ($F_{1,18} = 0.68$, $P = 0.42$) or its interaction with diet ($F_{2,18} = 0.02$, $P = 0.98$). There was no difference in GSH concentrations between the Control and Flown birds, among diets, or its interaction (Flight: $F_{1,50} = 0.18$, $P = 0.68$; diet: $F_{2,50} = 2.29$, $P = 0.11$, diet × flight: $F_{2,50} = 0.14$, $P = 0.87$, Figure 4.2B). GSSG concentration in the Flown birds was not influenced by flight duration ($F_{1,18} = 2.21$, $P = 0.16$) or its interaction with diet ($F_{2,18} = 2.05$, $P = 0.16$). GSSG concentrations significantly increased by 27% during flight, and diet did not affect GSSG concentrations (diet: $F_{2,50} = 1.48$, $P = 0.24$, flight: $F_{1,50} = 17.52$, $P = 0.0001$; diet × flight: $F_{2,50} = 0.36$, $P = 0.70$, Figure 4.2C). The GSH: GSSG ratio was not influenced by flight duration ($F_{1,18} = 1.00$, $P = 0.33$), or its interaction with diet ($F_{2,18} = 2.05$, $P = 0.16$). Compared to the Control birds, Flown birds had a lower GSH: GSSG ratio (diet: $F_{2,50} = 3.04$, $P = 0.06$, flight: $F_{1,50} = 24.05$, $P < 0.0001$, diet × flight: $F_{2,50} = 0.80$, $P = 0.45$, Figure 4.2D), indicating that Flown birds were under oxidative stress.

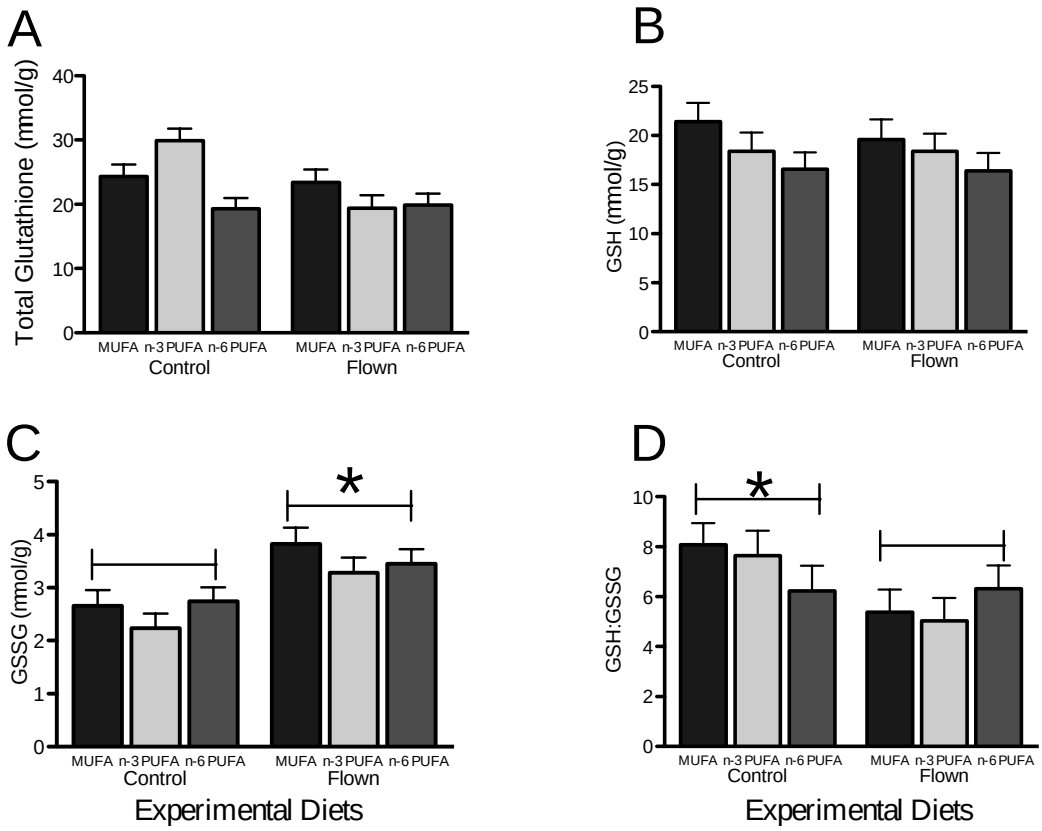

Figure 4.2 Flight muscle glutathione conent per g wet tissue of yellow-rumped warblers (*Setophaga coronata*). A) total glutathione, B) reduced glutathione (GSH), C) gluthione disulphide (GSSG), and D) GSH: GSSG ratio in warblers fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA), or n-6 polyunsaturated (n-6 PUFA) fatty acids. Values are means ± SEM. * Indicates a significant difference between Control and Flown birds ($P \leq 0.05$).

4.3.3 Protein Carbonyls

In the Control birds, diet did not influence protein carbonyls ($F_{2,29}$ = 0.28, P = 0.76). Within the Flown birds, flight duration increased protein carbonyls, but there was no effect of diet or the diet × duration interaction (duration: $F_{1,18}$ = 4.73, P = 0.04; diet: $F_{2,18}$ = 0.18, P = 0.84; duration x diet: $F_{2,18}$ = 0.88, P = 0.43, Figure 4.3).

4.3.4 Flight performance, antioxidant defence and oxidative damage

No significant correlations between the antioxidants and flight performance (duration, energy costs, inferred relative protein contribution) or body mass were observed when testing all flights combined (Table 4.1), or in the 360 min flights alone (Table 4.2). Protein carbonyls were significantly correlated to flight duration (r = 0.40, P = 0.05, Figure 4.3B); however, the relationship between protein carbonyls and total energy costs was stronger (r = 0.58, P = 0.003). The effect of flight duration was removed by examining the 360 min flights only, and this made the correlation between energy use and protein carbonyls even stronger (r = 0.86, P = 0.0002, Figure 4.3C). Furthermore, in the 360 min flights protein damage was also correlated to the fuel mixture used, with protein damage decreasing with increasing relative protein contribution (r = -0.53, P = 0.05, Figure 4.3D).

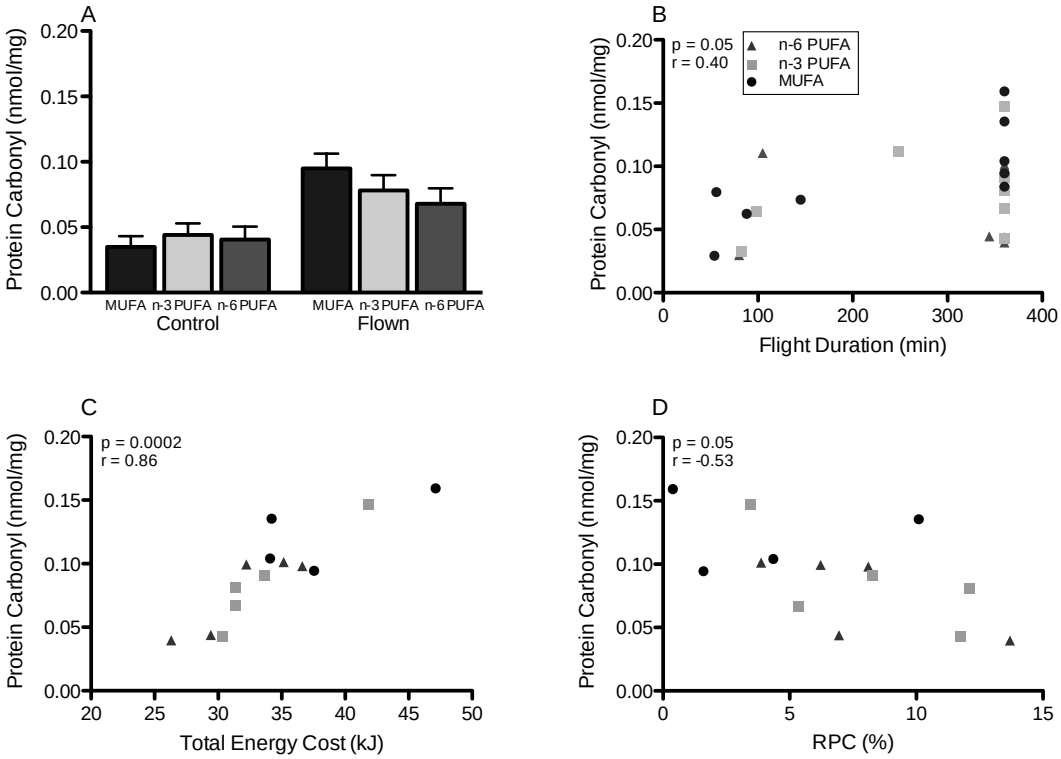

Figure 4.3 Factors affecting flight muscle protein carbonyls in yellow-rumped warblers (*Setophaga coronata*). A) Control (MEAN ±SEM) and Flown (flight duration included as a covariate, LSMEAN ±SEM). B) Effect of flight duration and diet on protein carbonyls. C) Relationship between protein carbonyls and total energy cost for the 360 min flights. D) Relationship between protein carbonyls and relative protein contribution (RPC) in the 360 min flights only.

Table 4.1 Correlations between measures of flight performance and measures of antioxidant status and protein damage for all flights (56 – 360 min) made by yellow-rumped warblers. Bolded values indicate significant correlations.

	Duration, min		Total energy		Body mass	
	r	P-value	r	P-value	r	P-value
SOD	-	0.24	-	0.77	-	0.76
Catalase	-	0.30	-	0.20	-	0.53
Total GSH	-	0.52	-	0.47	-	0.39
GSH	-	0.54	-	0.44	-	0.62
GSSG	-	0.14	-	0.08	-	0.09
GSH: GSSG	-	0.79	-	0.97	-	0.26
Protein Carbonyls	**0.40**	**0.05**	**0.58**	**0.003**	0.28	0.17

Table 4.2 Correlations between measures of flight performance and measures of antioxidant status and protein damage in 360 min flights made by yellow-rumped warblers. Bolded values indicate significant correlations ($P \leq 0.05$).

	Body mass, min		Total energy, kJ		RPC, %	
	r	P-value	r	P-value	r	P-value
SOD	-	0.99	-	0.18	-	0.08
Catalase	-	0.60	-	0.14	-	0.48
Total GSH	-	0.81	-	0.48	-	0.68
GSH	-	0.55	-	0.38	-	0.52
GSSG	-	0.07	-	0.44	-	0.34
GSH: GSSG	-	0.11	-	0.30	-	0.33
Protein Carbonyls	-	0.17	**0.86**	**0.0002**	**-0.53**	**0.05**

4.4 Discussion

Managing the production and effects of ROS during endurance flight is likely to play an important role in sustaining flight. High levels of ROS can impair muscle function and lead to muscle weakness and fatigue (Sies and Jones, 2007). Migratory flight poses an oxidative challenge due to the increased production of reactive oxidative species (Jenni-Eiermann et al., 2014). However, small levels of ROS are needed for optimal muscle contraction (Powers and Jackson, 2008; Powers et al., 2010; McClung et al., 2010), and could therefore be beneficial or required at certain levels for endurance flight. I found potential evidence for increased antioxidant capacity during flight, but ROS production overwhelmed antioxidant defenses leading to oxidative damage. Furthermore, protein damage was strongly linked to energy expenditure. To my knowledge, this is the first study to examine directly oxidative stress in the flight muscle of birds after simulated migratory flights.

Changes in antioxidant enzyme activity during flight differed between catalase and superoxide dismutase. Catalase activity did not change during flight. It is currently not clear if catalase activity changes with acute exercise, as the response does not appear to be uniform across studies in human models (Powers and Jackson, 2008). During exercise, catalase activity may decrease during the initial stage before returning to initial activity level in mice (Wang et al., 2015). I found no effect of flight duration on catalase activity, suggesting that catalase activity remains constant throughout the flight in yellow-rumped warblers. In contrast, SOD activity is modified by physical activity; and high intensity and long duration exercise

results in the largest increases in activity in rats (Powers et al., 1994). Overall, in my study SOD activity significantly increased during flight, but this is could be driven by lower activity in the Control n-6 PUFA group. In red-headed buntings (*Emberiza bruniceps*), the Mn-SOD (a mitochondria-specific SOD isomer) increases in abundance during the migratory season (Banerjee and Chaturvedi, 2016). Similar patterns have been observed in migrating Atlantic salmon (*Salmo salar*) during spawning, where SOD activity increases but catalase activity remains unchanged (Bombardier et al., 2010). These findings suggest that SOD may be an important and dynamic antioxidant in the muscles of migrating animals.

Total glutathione, and GSH did not change during flight, suggesting that there was no major increase the transport of glutathione into the muscle. GSH can react directly with radicals to donate a pair of electrons or it can serve as the substrate for glutathione peroxidase, with both yielding GSSG (Powers and Jackson, 2008). GSSG concentrations increased and drove the decrease in the GSH: GSSG ratio. Although total glutathione, and GSH did not change during flight, the relatively lower levels of GSSG means that it can increase without significantly influencing total glutathione or GSH. Because flight duration did not influence the GSH: GSSG ratio, flying birds may have a different redox homeostasis balance than at rest, and this could be beneficial for optimizing muscle force contraction or for cell signalling.

Endurance flight may cause oxidative stress that is apparent in recently arrived birds at stopover (Costantini, 2007; Costantini 2008; Jenni-Eiermann et al., 2014). For example, flight significantly increased flight muscle protein carbonyl

concentrations in yellow-rumped warblers. The damage accrued during flight was correlated with not only the duration of flight, but was also strongly correlated with the energy costs of flight. This relationship suggests that birds that have increased flight costs as a result of either longer flight duration or higher energy costs will have greater oxidative damage to proteins. This is especially true with the 360 min flights, where 74% of the variability in protein damage could be explained by energy cost of flight and suggests that the differences in protein damage could be attributed directly to the metabolic cost of flight. Conversely, it could also be that birds that accrue more protein damage may be metabolically less efficient flyers.

Protein oxidation during flight was also correlated to the fuel mixture used as increasing RPC correlated with lower protein oxidation. This relationship could be an artefact of the negative relationship between flight power and RPC (Chapter 3). However, catabolizing more protein could also convey benefits in terms of managing oxidative stress. First, uric acid (the waste product of amino acid catabolism) is a potent antioxidant (Powers and Jackson, 2008). During flight, an increase in plasma uric acid may occur (George and John, 1993; Schwilch et al., 1996), and the oxidization of uric acid to allantoin can be used as a plasma marker of oxidative stress (Tsahar et al., 2006). Increasing RPC may also increase the production of uric acid and increase the antioxidant capacity during flight. Alternatively, birds that have a higher RPC during flight may have higher protein turnover during flight. Protein carbonyls can serve as a tag for degradation (Radak et al., 2013). If protein turnover is higher due do a higher RPC, the net accumulation of carbonyls may potentially be lower. Regardless of the cause, it is not just how far

a bird flies that predicts oxidative damage. The amount of fuel and the composition of the fuel influence the amount of oxidative damage to the flight muscles.

PUFA are vulnerable to ROS due to their structure, and proximity to radical production in mitochondrial membranes. Once damaged, PUFA can form lipid peroxyl radicals, which can react with other PUFA, perpetuating the damage (Ng et al., 2005; Skrip and McWilliams, 2016). High PUFA diets may act as a chronic low dose oxidative stressor, which can result in increased antioxidant capacity and better protection during an acute oxidative challenge (Abdukeyum et al., 2016). This may potentially explain why feeding PUFA reduces ROS production and helps preserve mitochondrial function during endurance flight (Gerson, 2012). My study showed no significant effect of dietary PUFA on antioxidant enzymes or oxidative damage at rest or after endurance flight. This suggests that dietary intake of DHA and ARA, and their incorporation into flight muscle membranes does not alter ROS defenses or net damage in yellow-rumped warblers. This differs from white-throated sparrows (*Zonotrichia albicollis*), in which dietary PUFA increased damage to plasma metabolites but also increased antioxidant capacity (Alan and McWilliams, 2013). Furthermore, the type of PUFA also did not influence antioxidant enzyme activity or damage. DHA increases the mRNA abundance of antioxidant enzymes in muscle compared to ARA, but this did not translate to any difference in muscle damage in rats (Hashimoto et al., 2016). Due to logistical and tissue constraints, I was unable to measure lipid oxidative damage but this should be included in future studies. The PUFA groups could potentially have higher lipid damage because of the increased proportion of long-chain PUFA in their

membranes (Chapter 3). The increased vulnerability of PUFA to oxidative damage has been highlighted as a concern in migratory birds (Price, 2010; Alan and McWilliams, 2013; Skrip and McWilliams, 2016). However, my study suggests that a prolonged high intake of PUFA is not a large oxidative burden, nor do PUFA alter the antioxidant management or damage during migratory flight.

Overall, endurance flight may increase SOD activity. However, the flight muscle is also in a temporary state of oxidative stress and oxidative damage is an unavoidable hazard. The timeframe for resolving ROS damage occurring in flight appears to be relatively rapid. Within a few days, the redox balance in the blood returns to normal (Skrip et al., 2015), and mitochondrial function returns to pre-flight function (Gerson, 2012). The strong correlation between oxidative damage and flight energy costs suggests that factors that influence energetics will also influence oxidative damage, such as body mass, feather condition and flight mechanics. Thus, optimizing flight performance should not only reduce the energy costs but also limit flight-related damage. It is currently unknown if the severity oxidative damage limits migration. If an oxidative stressor is great enough it could negatively influence metabolic flight efficiency, flight duration, or recovery time at stopover. All of these factors influence the migratory performance and should be included in assessments of migratory performance.

4.5 References

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5 General Discussion

5.1 Thesis summary

Migratory birds have the capacity to complete large seasonal movements that enable them to exploit changes in resource availability. The ability to undertake these journeys requires a wide range of capabilities that we are still trying to comprehend. The capacity to become some of the best ultra endurance marathoners in the world is just one of these attributes. The studies presented in this thesis deepen our understanding of how birds accomplish their migratory flights and illuminate the regulation of fuel metabolism and the consequences of flight, and test important ideas about the role of diet on flight performance. Collectively, my studies indicate that changes in muscle gene expression and biochemistry are required for flight and that oxidative damage is a cost that must be mitigated.

Lipid metabolism has been a long-standing theme in studies of bird migration physiology due to the importance of fat as a fuel source (Guglielmo et al., 1998; Ramenofsky et al., 1999; Guglielmo et al., 2002; McFarlan et al., 2009; Zajac et al., 2011; Zhang et al., 2015). My transcriptomic analysis revealed coordinated increases in the expression of genes and pathways supporting fatty acid oxidation during the migration season and during endurance flight (Chapter 2). Astonishingly, the major difference between the winter and fall migratory season were pathways related to lipid metabolism. This suggests that no other potential major pathway has been overlooked in previous targetted studies. During endurance flight, birds maintained the upregulation of these pathways and increased oxidative enzyme

activity, and further augmented cytosolic fatty acid binding proteins to increase transport capacity (Chapters 2 and 3). Thus, my research provides new insights into the importance of lipid metabolic pathways for bird migration.

Muscle damage is a consequence of flight, and during endurance flight protein degradation, inflammation, and apoptosis pathways were upregulated (Chapters 2 and 4). Flight also caused the upregulation of muscle growth and protein synthesis pathways (Chapter 2). Combined, these findings suggest that birds must cope with muscle damage and replace lost proteins to maintain muscle function during flight. Flight alters the redox balance in the flight muscle leading to oxidative stress and damage, as was observed through a decrease in the glutathione (GSH): glutathione disulphide (GSSG) ratio and increased protein carbonyl concentration (Chapter 4). One of my most interesting findings was that individual variation in oxidative damage to the flight muscle was strongly correlated to the energy expended by individuals during flight (Chapter 4). This suggests that high quality individuals that are energetically efficient flyers will also minimize muscle damage.

The results of my experimental test of the natural doping hypothesis were contrary to its predictions. Feeding n-3 long chain polyunsaturated fatty acids (PUFA) decreased peroxisome proliferator activated receptor β (PPARβ) mRNA abundance and oxidative enzyme activities, but the modulations were smaller than the seasonal changes (Chapter 3). There was no effect of dietary long-chain PUFA on

peak metabolic rate (Chapter 3), nor was there any evidence for long-chain PUFA altering endogenous antioxidants or oxidative damage (Chapter 4).

My results suggest that endogenous upregulation of fatty acid metabolism during migration is robust and sufficient to support endurance flight. Modulations of gene expression and membrane composition caused by dietary PUFA do not appear to alter migratory flight performance. Additionally, my work highlights the consequences of endurance flight in terms of muscle damage and inflammation that have not been directly assessed before, but may have implications on how we analyze migratory strategies. Below I elaborate on some of the implications of my findings for migration and discuss future research directions.

5.2 Fatty acid oxidation

Simiarl to previous studies (Marsh, 1981; Driedzic et al., 1993; Guglielmo et al., 2002; Ramenofsky et al., 1999; McFarlan et al., 2009; Zajac et al., 2011; Zhang et al., 2015; Fudickar et al., 2016), I found that seasonal upregulation of fatty acid oxidation is a key element to migratory preparation. The scale of the upregulation and coordination in the flight muscles of yellow-rumped warblers (*Setophaga coronata*) is similar to that in migratory dark-eyed Juncos (*Junco hyemalis*, Fudickar et al., 2016). The molecular initiator or regulator of this shift in energy metabolism may be controlled by PPARγ-coactivator 1α (PGC-1α), PGC-1β and PPARα. An upregulation of PPARβ occurs during flight and may further promote increases in lipid transport and oxidative capacity. Future studies utilizing PPAR antagonists could help elucidate if blocking PPARα and PPARβ have negative impacts on

seasonal preparation and endurance capacity, as different PPAR likely contribute differently to the regulation of muscle physiology.

The stimuli that initiate increased expression of genes encoding the proteins for PGC-1α and PPARα during migration are not known. Initiating migratory condition requires the integration of environmental cues (eg. photoperiod and temperature) that alter endogenous rhythms via endocrine signals (Roenneberg and Merrow, 2005). Photoperiod can trigger birds to enter a migratory condition, and the resulting upregulation of lipid metabolism and behavioural traits (Chapter 3; Ramenofsky et al., 1999; Zajac et al., 2011; Vandermeer, 2013). Endurance capacity is crucial to the ability to migrate and needs to be coordinated with the other physiological and behavioural changes. Training can influence aerobic capacity and increase PGC-1α and PPARα expression (Calvo et al., 2008; Camera et al., 2016). However, birds are able to increase flight capacity without endurance training (Chapter 3, Dietz et al., 1999), suggesting that this change is under hormonal control rather than a result of physical training.

Our understanding of the hormonal controls of both migratory behaviour and muscle metabolism is limited to testosterone in males birds during the spring migration (Vandermeer, 2013). Testosterone modulates but does not fully control the seasonal upregulation oxidative enzymes (Vandermeer, 2013). However, fall migrants with low circulating gonadal hormones and spring female migrants with low testosterone but high estrogen levels all migrate (Wingfield et al., 1990). Although, I did not examine sex differences directly, I did not detect any effect of sex

on flight performance or muscle physiology and biochemistry during the initial statistical analyses. I did observe enlarge testes during dissections suggesting elevated testosterone in the males during the spring in Chapter 3. This suggests that elevated testosterone in males during the spring migration may not elevate oxidative enzymes in yellow-rumped warblers when compared to females. The major hormonal control of the seasonal upregulation of fatty acid oxidation in the flight muscles is likely under the control of a hormone associated with both fall and spring migration and not sex specific.

Thyroid hormones are candidates for coordinating behaviour and muscle metabolism. Circulating levels of thyroid hormones are higher in migratory birds and change with photoperiod, and thyroid hormones are required for the expression of migratory restlessness behaviour and body mass gain (Wingfield et al., 1996; Pérez et al., 2016). Thyroid hormones are particularly interesting regarding regulation of muscle lipid metabolism because the active form, triiodothyronine (T_3), can directly increase PGC-1α mRNA abundance in skeletal tissue (Wulf et al., 2008; Bocco et al., 2016). Thus, T_3 may provide a link between photoperiod, migratory behaviour, and muscle metabolism during spring and fall migration of birds.

What is striking is the robust nature of the upregulation of fatty acid metabolism and the number of downregulated pathways observed during migration (Chapter 2). There are seasonal changes in the maintenance of circadian rhythm (Singh et al., 2015) and this enables preparation for predictable events. Migration

changes the daily schedule of birds. This change is most notable for birds that fly nonstop for days nonstop or switch from being diurnal to daytime refuelling and nocturnal migratory flights. There is some evidence to suggest flight muscle enzyme activities are altered throughout the day during migration (Ramenofsky et al., 1999). The full circadian rhythm of the flight muscle is not known, let alone circannual changes in the circadian rhythm, but these should be considered in future study designs. The circadian rhythm has a large impact on gene expression in the muscle and upwards of 2300 genes are differentially regulated through the day (Harfman et al., 2015). Additionally, in my study endurance flight caused a larger number of differentially expressed genes than seasonal change, and the majority of the upregulated pathways during flight were found to be downregulated during the migratory season When seasonal changes, stage of migration, and effect of migratory flight are combined, the assumption that one time point can be used to study aspects of migratory physiology is likely incorrect. We need to understand how and when physiological and biochemical changes occur in order to test hypotheses. For example, myostatin has received mixed support for its role in regulating muscle size at the mRNA level (Swanson et al., 2009; Price et al., 2011; King et al., 2015). Potentially, myostatin mRNA abundance is decreased in the intial stages of the migratory season when birds are increasing flight muscle size prior to departing from their wintering or breeding grounds, and also decreased in the post migratory flight recovery period if regeneration of the flight muscle is required.

5.3 Migration damage control

Endurance flight can negatively impact the flight muscles in many ways, including decreasing muscle size (Bauchinger and Biebach, 2001) and increasing muscle damage and mitochondrial dysfunction (Guglielmo et al., 2001; Gerson, 2012). I characterized flight-related protein damage and inflammation in the muscle of birds for the first time (Chapters 2 and 4). The full scale of flight-induced damage and subsequent recovery time in the flight muscles is unknown, but mitochondrial function and circulating antioxidants return to normal within a few days (Gerson, 2012; Skrip et al., 2015). The effect of the severity of muscle and oxidative damage on stopover duration is not known, but would provide insight into how birds manage the oxidative and physical challenges of migration in combination with limited time and energy.

Interest in antioxidants and oxidative stress is growing in migration physiology (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). Previous studies tended to focus on blood markers of antioxidant capacity and reactive oxygen metabolites to assess oxidative stress during flight (Costantini et al., 2007; Costantini et al., 2008; Jenni-Eiermann et al., 2014; Bairlein et al., 2015; Skrip et al., 2015) rather than the muscle-focused approach used in Chapter 4. I found that oxidative damage in the flight muscles is strongly correlated with energy expenditure (Chapter 4). Thus, improving fuel use during migration by optimizing the amount of fuel carried and metabolic efficiency not only influences the energetics of migration but also the damaging effects of migration. This may be one

mechanism birds can use to manage reactive oxygen species (ROS) production during migration.

How birds manage the oxidative challenge of migration is likely to be multifaceted. I documented a potential increase in superoxide dismutase (SOD) activity and a decrease in the GSH: GSSG ratio during flight. Seasonal increases in SOD expression help in the preparation for ROS during flight (Banerjee and Chaturvedi, 2016). Other studies have pointed to the importance of dietary sources of antioxidants during migration and how birds replenish them at stopovers (Skrip et al., 2015; Eikenaar et al., 2016). My study focused on endogenous antioxidants in the flight muscle only. Other studies have examined circulating antioxidants, which combines endogenous and exogenous (dietary) antioxidants into one value (Costantini et al., 2007; Costantini et al., 2008; Bairlein et al., 2015; Skrip et al., 2015; Eikenaar et al., 2016). The interplay between endogenous and exogenous antioxidant capacity has not been addressed and may depend on species and migratory strategy. If birds rely heavily on circulating antioxidants that are replenished at stopover, this would favour migrants that make short movements enabling regular replenishment. In contrast, species that make large migratory movements may rely more heavily on endogenous antioxidant enzymes due to limited opportunities to replenish exogenous antioxidants. Furthermore, insectivores, fruigivores, granivores and nectivores may also differ in type and amount of antioxidants consumed (Beaulieu et al., 2011; Bolser et al., 2013; Catoni et al., 2008; Cooper-Mullin and McWilliams, 2016; Eeva et al., 2010) and in how they

contribute to ROS management. Thus, a general mechanism to manage ROS during migration may not exist.

5.4 Natural doping and fatty acid nutrition

The natural doping hypothesis posits that n-3 polyunsaturated fatty acids (PUFA) prime the flight muscles of semiplamated sandpipers (*Calidris pusilla*) for endurance flight by increasing aerobic and oxidative metabolism (Maillet and Weber, 2006; Maillet and Weber, 2007; Weber, 2009). Chapter 3 is the most comprehensive test of the natural doping hypothesis to date and, overall, I found no evidence to support it (Figure 5.1). With the exception of relatively small changes in metabolic enzymes, there was no positive or negative impact of increasing intake of either n-3 PUFA (docosahexaenoic acid, DHA) or n-6 PUFA (arachidonic acid, ARA). The only controlled studies examining n-3 PUFA nutrition in migratory birds have found no compelling benefits of supplementation with n-3 PUFA (Chapter 3, Price and Guglielmo, 2009). Support for a beneficial role of n-3 PUFA for flight performance relied on correlations (Infante et al., 2001; Maillet and Weber, 2007) or examined non-migratory birds that are not capable of endurance flight (Nagahuedi et al., 2009). Furthermore, ARA may unfairly be classified as a harmful fatty acid due to perceived, but untested, pro-inflammatory effects in urban great tits (*Parus major*, Andersson et al., 2015).

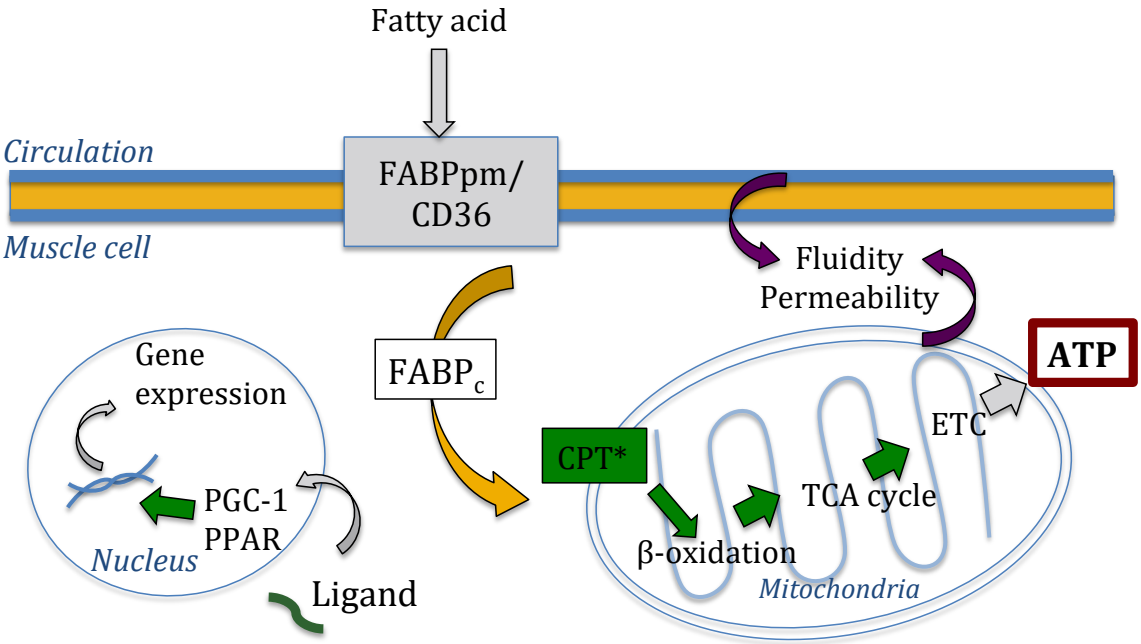

Figure 5.1 Summary of the natural doping hypothesis predictions for enhanced oxidative capacity. Green arrows indicate decreased oxidative enzyme activity and lower peroxisome proliferator-active receptor β (PPARβ) expression in the n-3 PUFA diet group. Purple arrows indicate the enrichment of cellular membranes with n-3 long-chain PUFAs. Grey arrows and boxes indicate potential points where n-3 PUFA may increase expression or activity supporting lipid metabolism but were not evaluated. Carnitine palmitoyl transferase (CPT), cytosolic fatty acid binding protein ($FABP_c$), electron transport chain (ETC), fatty acid translocase (CD36), peroxisome proliferator-activated receptor (PPAR), plasma membrane fatty acid binding protein ($FABP_{pm}$), tricarboxylic acid (TCA).

I found no correlations between individual endurance flight performance and previous measures of performance used in migratory birds: peak metabolic rate and muscle metabolic enzymes. The experimental diets did cause small modulations in fuel metabolism at the muscle level. Whether these modulations alter flight performance is not known. It has been shown that the thermogenic capacity in American goldfinches (*Spinus tristis*) is correlated with flight muscle size but not metabolic enzymes (Swanson et al., 2013). As such, there may be limitations to our interpretation of metabolic enzyme activities. Additionally, increased enzyme activity could be interpreted to correlate with better performance or decreased enzyme efficiency (i.e. requireding more enzyme protein to accomplish the same outcome) depending on one's viewpoint. In this thesis, I use the standard viewpoint that increasing activity equals increase capacity or performance. But, I do acknowledge that we do not undersand the effects of small modulations in enzyme activity on whole animal performance, and thus making conclusions about their benefits or consequences is limited. Assessing whether alterations in physiology and biochemistry predict exercise performance is difficult and is a limitation in the field of exercise performance (Camera et al., 2016). In terms of understanding the role of fatty acid nutrition on migratory birds, robust and integrative evidence is still needed for the capacity of PUFA to modulate migratory performance. Current evidence suggest that do not, but references to their potential are still made in reviews (Weber, 2009; Pierce and McWilliams, 2014). Without empirical evidence of enhanced performance or health, correlations involving fatty acids need to be treated cautiously as there is direct evidence for no effect.

One area that has not been fully addressed in avian fatty acid nutrition is the potential for species differences in metabolism. The activity of delta-5 and delta-6 desaturases determines if the rate of synthesis rate long-chain PUFA meets an animal's requirement or if long-chain PUFA are essential nutrients (Leeson and Summers, 2001). Mammals vary in their capacity to synthesize long-chain PUFA from their precursors. For example, hyper-carnivores have almost no capacity to synthesize n-3 and n-6 long-chain PUFA (Rivers et al. 1975). In comparison, herbivores can synthesis long-chain PUFA and require a dietary source of n-3 and n-6 PUFA, but not necessarily long-chain PUFA (Castro et al., 2012). Numerous birds have demonstrated the capacity to synthesize long-chain PUFA, including domestic chickens (*Gallus gallus*, Lopez-Ferrer et al., 2001), yellow-rumped warblers (Chapter 3), Monk parrots (*Myiopsitta monachus*, Petzinger et al., 2013), and Japanese quail (*Coturnix japonica*, Ben-Hamo et al., 2011). However, whether species differences in fatty acid metabolism occur in birds, as has been observed in mammals (Rivers et al., 1975; Castro et al., 2012), is not known.

It can be hypothesized that the capacity to synthesize long-chain PUFA can be lost if a dependable dietary source is available (Martinez del Rio and McWilliams, 2016). Thus, species variation may exist. Additionally, the capacity or requirements for long-chain PUFA can also be dependent on life stage and diet (Twining et al., 2016; Zheng et al., 2005). In the case of yellow-rumped warblers, their natural diet likely contains mostly 18:2 n-6 and 18:3 n-3 (Conway et al., 1994; Pierce and McWilliams, 2014; Andersson et al., 2015), and the capacity to synthesize ARA, EPA (eicosapentaenoic acid) and DHA from these fatty acids was observed in Chapter 3.

Whether this may also make these birds more sensitive to higher than normal levels of long-chain PUFA is unknown, but it may have contributed to the health issues I observed when birds were fed a diet that was apparently too enriched in n-3 PUFA (Chapter 3). On the other hand, sandpipers consume diets rich in DHA and EPA, and likely not by choice (Weber, 2009). Potentially, their capacity to synthesize these fatty acids could have been lost or is insufficient to meet requirements, but this has not been directly studied. If this is the case consuming only 18 carbon PUFA would have negative health effects. Recently, it was demonstrated that tree swallow (*Tachycineta bicolor*) nestlings have lower immunity and poor body condition when provided with 18:3 n-3 as their primary n-3 PUFA source versus DHA and EPA (Twining et al., 2016). If swallows are consuming fewer aquatic and more terrestrial insects this may lower their intake of EPA and DHA and, in turn, impair nestling health and survival (Hixson et al., 2015; Twining et al. 2016). Thus, understanding species differences in fatty acid metabolism may be crucial to our ability to identify when and where fatty acid nutrition may be a concern.

I proposed in Chapter 1 that the dietary flexibility of yellow-rumped warblers made them an ideal model species for the study of migration in captivity. This flexibility may have limitations when comparing yellow-rumped warblers to other species that do not share the same flexibilities. For example, if sandpipers require a dietary source of n-3 long-chain PUFA because they are not capable of synthesizing at a high enough rate, then natural doping may occur. However, it would be related to meeting nutritional requirements rather than to doping. Finally, it should also be noted that the interest in the benefits of n-3 PUFA is heavily

influenced by the largest model species in the world, humans. The “western diet” has created a mismatch between the n-6: n-3 PUFA ratio that humans evolved with and the one we now consume (Simopoulos, 2003). This has led to a wealth of studies on the health benefits of n-3 PUFA and negative consequences of n-6 PUFA. This bias needs to be acknowledged when we approach comparative nutrition as it could lead to an undue focus on n-3 PUFA that is not ecologically relevant to many bird species.

5.5 Concluding remarks

Migration is remarkable in many ways. Birds have evolved to become extreme endurance athletes, navigate towards the unknown, and suppress the urge to stop, all while maintaining a tight schedule. My thesis focuses on the mechanisms that enable birds to sustain endurance flight and the physiological consequences of flight. The most novel and intriguing findings in this thesis were derived from my ability to simulate migratory flight under controlled conditions and thus examine birds during a migratory event. It is my hope that these studies provide some fodder for future research and challenge existing ideas and approaches to studying migration physiology.

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Appendix A Permits and Ethics Approval

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	Permit no. No de permis
SCIENTIFIC	CA 0256

Organization Organization	Issued under section Délivré en vertu de l'article	of de
University of Western Ontario	19	MIGRATORY BIRD REGULATIONS

Surname Nom de famille	Name Prénom	Department Département
Guglielmo	Christopher	Department of Biology

1151 Richmond Street North
London On
N6A 5B7

Date of issue Date d'émission	Date of expiry Date d'expiration
June 27, 2013	June 30, 2015

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. Landowner's permission must be obtained prior to collecting on private property.
2. Permit or a copy of the permit to be carried in the field by all collectors.
3. The permit holder is authorized to collect and to possess for scientific research purposes, migratory birds – to wit: Cedar Waxwing (Bombycilla cedrorum), Tree Swallow (Tachycinetra bicolor), Purple Martin (Progne subis), White-throated Sparrow (Zonotrichia albicollis), Yellow-rumped Warbler (Setophaga coronate), Hermit Thrush (Catharus guttatus), Swainson's Thrush (Catharus ustulatus), American Robin (Turdus migratorius) and Ruby-throated Hummingbird (Archilocus colubris) – from locations as situated within Oxford, Perth, Elgin, Middlesex, Norfolk and Haldimand Counties within Ontario. Take is limited to a maximum of 60 birds per species per year.
4. Additional permission is granted to receive from Simon Fraser University, Vancouver, BC, migratory birds – to wit: Western Sandpiper (Calidris mauri) – as currently held in a captive breeding facility. Take is limited to a maximum 100 birds per year. Upon completion of this study a number of the Western Sandpiper (Calidris mauril) will be returned to the captive colony at Simon Fraser University for further study or released.
5. Further permission is given to send specimens of Western Sandpiper to one or more of the following; (Canada) University of Western Ontario, ON; University of Guelph, ON; (USA) Miller School of Mecine, Miami, FL; University of Nevada, Reno; Citrus Heights, CA; University of Maryland; Kansas State University; USDA National Wildlife Research Center, Fort Collins, CO, Unika Wildlife Pathology, Mukilteo, WA; Deepwater Horizon NRDAR; or the University of Florida, Gainesville, FL for toxicological and biochemical analysis.
   It is the responsibility of the permittee to apply for all scientific collection and export permits and authorities as required under United States of America federal or state laws.
6. Capture, handling and housing procedures are to be performed according to the Animal Care Committee protocols of the University of Western Ontario. Samples not to be retained are to be disposed of by the approved laboratory waste management system of the University of Western Ontario.
7. All other birds are to be released into the wild by the conclusion of the study or otherwise be humanely euthanized.
8. Permit holder shall submit a written report, by January 31, of each year following, indicating the results of the study to the Canadian Wildlife Service, 867 Lakeshore Road, Burlington, ON., L7R 4A6.
9. Nominees to this permit are: Department of Biology faculty/staff as acting under the direction of the permittee.

Canada

Curriculum Vitae

Morag Dick

Academic Training

Ph.D. Biology 2011- Present
University of Western Ontario, London, ON
Thesis Title: The Long Haul: Migratory Flight Preparation and Performance in Songbirds
Supervisor: Dr. Christopher Guglielmo

M.Sc. Agriculture 2007-2010
Dalhousie University and Nova Scotia Agricultural College, Truro, NS
Thesis Title: Fatty Liver Syndrome in Mink- Causes and Metabolic Consequences
Supervisor: Dr. Kirsti Rouvinen-Watt

B.Sc. Animal Biology 2003-2007
University of Guelph, Guelph, ON

Honours and Awards

Best Student Presentation, Comparative Nutrition Society	2016
Duane Ullrey International Student Award, Comparative Nutrition Society	2016
Roy McClements Student-Keeper Award, AZA Nutritional Advisory Group	2015
Travel Award, Department of Biology, UWO	2015
Best Poster, Biology Graduate Research Forum, UWO	2013
Canadian Bioinformatics Workshop Award, bioinformatics.ca	2012
Susan D. Crissey Memorial Scholarship, Comparative Nutrition Society	2010
Hon. Dr. Roger S. Bacon Scholarship in Agriculture, NSAC	2009
Susan D. Crissey Memorial Scholarship, Comparative Nutrition Society	2008
Arlen Kerr Memorial Scholarship, Canada Mink Breeders	2008
Graduate Entrance Scholarship, NSAC	2007

Related Work Experience

Teaching assistant, University of Western Ontario	2011-2015
Organic Cropping Systems Technician, University of Manitoba	2010
Comparative Nutrition Intern, Lincoln Park Zoo, Chicago, IL	2009
Teaching Assistant, Nova Scotia Agricultural College	2008-2009
Ruminant Nutrition Research Assistant, University of Guelph	2006-2007

Publications

Marshall TJ, Dick MF, Guglielmo CG. 2015. Seasonal dietary shifting in songbirds is not caused by changing nutritional targets. *Comparative Biochemistry and Physiology: part A* 192:75

Dick MF, Hurford J, Lei S, Mustonen A-M, Nieminen P, Rouvinen-Watt K. 2014. High feeding intensity increases the severity of fatty liver in the American mink (*Neovison vison*) with potential ameliorating role for long-chain n-3 polyunsaturated fatty acids. *Acta Veterinaria Scandinavica* 56:5

Rouvinen-Watt K, Harris L, Dick M, Pal C, Lei S, Mustonen A-M, Nieminen P. 2012. Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the American mink (*Neovison vison*). *British Journal of Nutrition* 108:1360