THESIS

# TISSUE-SPECIFIC SEASONAL CHANGES IN MITOCHONDRIAL RESPIRATORY FUNCTION AND MEMBRANE COMPOSITION IN THE GOLDEN-MANTLED GROUND SQUIRREL

Submitted by

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# ABSTRACT

## TISSUE-SPECIFIC SEASONAL CHANGES IN MITOCHONDRIAL RESPIRATORY FUNCTION AND MEMBRANE COMPOSITION IN THE GOLDEN-MANTLED GROUND SQUIRREL

Mammals that hibernate, such as the golden-mantled ground squirrel (*Callospermophilus lateralis*; GMGS), cease to feed, reduce metabolic rate, and lower body temperature (Tb) during the winter months, surviving almost exclusively on the oxidation of lipids from endogenous fat stores. Whether mitochondria, the cellular sites of oxidative metabolism, undergo changes in response to low Tb, hypometabolism, and decreasing ambient temperature (Ta) to facilitate this remarkable phenotype is unclear. It has been postulated that changes in mitochondrial membrane composition reported in response to cold exposure in some species may facilitate maintenance of respiratory enzyme function with decreasing Tb. However, no studies to date have investigated the function and membrane fatty acid (FA) composition of mitochondria from different tissues across seasons in a hibernating mammal. We hypothesize that tissue-specific differences in mitochondrial respiration occur across seasons in the GMGS that may parallel distinct changes in mitochondrial membrane composition. We compared the respiration, substrate preference, and membrane composition of GMGS mitochondria isolated from liver, heart, skeletal muscle, and brown adipose tissue (BAT) from summer, fall (prehibernating), winter (hibernating), and spring (posthibernating) seasons. Maximal mitochondrial oxidative phosphorylation (OXPHOS)-supported respiration was determined by high-resolution respirometry at 37$^{o}$C in the presence of saturating concentrations of ADP and respiratory substrates.

Mitochondrial membrane FA composition was determined by gas chromatography on phospholipid fractions obtained from isolated mitochondria. Maximal OXPHOS assayed at 37$^{o}$C tended to increase from summer to winter in liver, heart and BAT, and decrease in skeletal muscle, with tissue- and season-specific changes in pyruvate versus FA (palmitoylcarnitine) oxidation capacity. OXPHOS capacity was uniformly suppressed by decreasing Ta in all tissue mitochondria from torpid GMGS in the winter, despite widely variable changes in mitochondrial membrane composition across tissues and seasons. Taken together, these findings argue against a consistent relationship between changes in mitochondrial membrane composition and respiratory function across seasons in GMGS, but highlight distinct tissue- and season-specific differences that may have important biological effects that remain to be elucidated.

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# DEDICATION

*I would like to dedicate this thesis to all of my friends and family back home in New Hampshire, here in Colorado, and everywhere else in the world.*

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# CHAPTER I: BACKGROUND

## I.A. Introduction

Mammals that hibernate, such as the golden-mantled ground squirrel (*Callospermophilus lateralis*; GMGS), cease to feed, reduce metabolic rate, and lower body temperature during the winter months, surviving almost exclusively on the oxidation of lipids from endogenous fat stores. Hibernating mammals exhibit remarkable metabolic plasticity that enables them to survive these seasonal changes in ambient temperature and food availability; however, whether mitochondria, the cellular sites of oxidative metabolism, undergo adaptive changes in response to hypometabolism and decreasing ambient temperature to facilitate this remarkable phenotype is unclear. To my knowledge, this thesis project is the first study which comprehensively investigates mitochondrial respiratory function and membrane FA composition in heart, muscle, liver, and BAT from the warm summer months, to fall and winter in a hibernator. Results highlight distinct tissue- and season-specific differences that may have important biological effects, but argue against a consistent relationship between changes in mitochondrial membrane composition and respiratory function across seasons in hibernating mammals.

## I.B. Hibernation Physiology

Hibernating mammals such as the golden-mantled ground squirrel (*Callospermophilus lateralis*, GMGS) fast during the winter months, a period of time during which individuals enter a drastically reduced metabolic state known as torpor, which can last from a few days to several weeks depending on the amount of fat stored, the ambient temperature of the burrow, and a number of other physiological and environmental factors. Staples (2016) specifically defines

torpor as a process during which metabolic rate is reversibly suppressed to less than 10% of resting values, with exceptions for certain species. Throughout hibernation, torpor bouts are separated by periods known as interbout arousals (IBAs), during which internal body temperature (Tb) of the animal increases from ambient temperature (4˚C) to near euthermic (i.e. normal internal) levels (37˚C), and metabolic processes are no longer suppressed. The oscillatory cycle of torpor bouts and IBAs during hibernation can collectively last up to eight months in certain hibernating mammals (Buck and Barnes 2000; Otis et al. 2011). Hibernators exhibit circannual rhythms of body mass and food intake, increasing food intake in the summer and fall (i.e. hyperphagia) while simultaneously decreasing energy demands; this amounts to an approximate doubling of body mass primarily in the form of white adipose tissue (WAT) in the months preceding hibernation (Florant et al. 2010). The rapid increase in fat storage generally results in a shift toward lipids as the primary oxidative substrates, indicated by a respiratory quotient of 0.7 in torpid ground squirrels (South and House 1967; Buck and Barnes 2000).

Food intake decreases (i.e. hypophagia) and eventually ceases prior to hibernation in late fall, and GMGS will continue to fast for nearly seven months as they undergo bouts of torpor. During this time, hibernators must rely primarily on the oxidation of lipids from endogenous fat stores to fuel any metabolic processes as their Tb reaches a minimum of nearly 4˚C, often near ambient temperature (Ta); these fat stores are often high in the essential FAs linoleic acid (18:2n6) and linolenic acid (18:3n3), both which strongly influence the depth and duration of torpor bouts (Florant 1998) and can also be incorporated into such membrane phospholipids as the inner mitochondrial membrane (IMM). While most mammals increase metabolic rate in response to decreasing temperatures, hibernating mammals are unique in that they suppress metabolic processes by nearly 95% as Tb and Ta drop (Carey et al. 2003). Geiser (2004) and

Heldmaier et al. (2004) suggest that passive cooling due to decreasing Tb (and Ta) may contribute less than 30% to the reduction of mass-specific oxygen consumption, while Toien et al. (2011) suggest that in black bears, metabolic suppression experienced during torpor is completely independent from Tb. Although the mechanisms responsible for active suppression of metabolic rate during hibernation remain unclear, mitochondria are an ideal experimental target as the site of oxidative phosphorylation, overall adenosine triphosphate (ATP) production, and temperature-sensitive membrane fluidity and enzyme function.

### I.C. Basic Mitochondrial Physiology

Prior to discussing tissue-specific seasonal changes in mitochondrial respiration and membrane composition of hibernating mammals, it is important to first review basic mitochondrial physiology. This section will focus on substrate utilization of mitochondria, describing sources of both carbohydrates (pyruvate) and FAs for the tricarboxylic acid (TCA) cycle, as well as the components and processes which comprise the TCA or Krebs cycle and electron transport system (ETS; see Figure 1.1 below for a review).

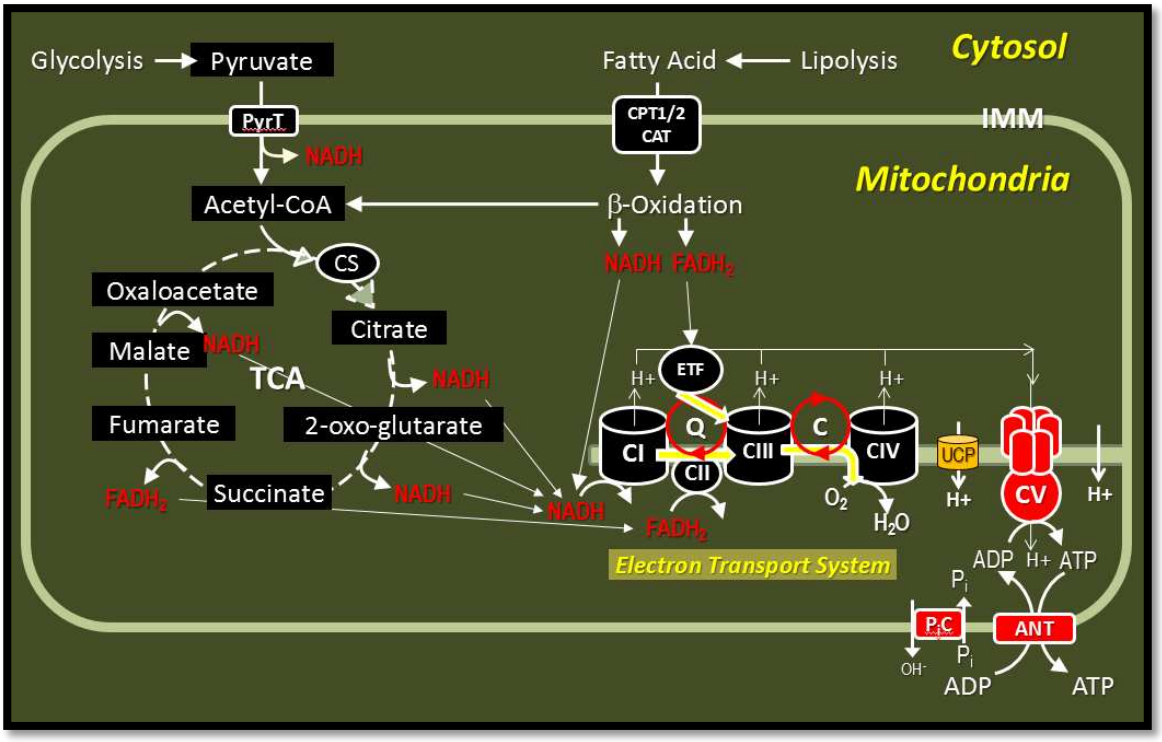


**Figure 1.1. A summary of the TCA Cycle and ETS.** Carbohydrates can enter the TCA cycle through the glycolytic pathway, while FAs can enter the TCA cycle via beta-oxidation. The primary purpose of the TCA cycle is to generate reducing equivalents such as NADH and $FADH_2$, in addition to $CO_2$ and guanosine triphosphate (GTP), through the oxidation of pyruvate and amino acids. These reducing equivalents, produced from a number of chemical reactions, ultimately power the ETS and drive ATP production.

### I.C.1. *Carbohydrates and FAs as Substrates for the TCA Cycle*

Once exogenous glucose has been transported into the cytosol via glucose transporters, it is phosphorylated and can either be stored as glycogen or converted into glucose-6-phosphate (G6P). G6P principally enters the glycolytic pathway; the process of glycolysis converts one molecule of glucose to two molecules of pyruvate, while simultaneously generating reduced nicotinamide adenine dinucleotide (NADH) and a small amount of ATP (Cappellini and Fiorelli 2008). Pyruvate can either be converted to lactate via lactate dehydrogenase (LDH) within the cytosol or transported to the mitochondrial matrix, where pyruvate dehydrogenase (PDH) will

oxidize and decarboxylate pyruvate into acetyl-CoA. The two-carbon acetyl group from acetyl-CoA then transfers to oxaloacetate to form citrate via citrate synthase, and thus the TCA cycle begins (Williamson and Cooper 1980; Fernie et al. 2004).

FAs can also enter the TCA cycle through acetyl-CoA. Once free FAs are released via lipolysis within the cytosol, those FAs can be esterified to fatty acyl-coenzyme A (CoA), which is then converted to fatty acyl carnitine via carnitine palmitoyltransferase (CPT) I in the outer mitochondrial membrane. CPT II converts the acyl carnitine back to fatty acyl-CoA once it has been transported into the mitochondrial matrix, and the fatty acyl-CoA can subsequently enter the beta-oxidation pathway (Drynan et al. 1996). Acetyl-CoA, NADH, and flavin adenine dinucleotide in its hydroquinone form ($FADH_2$) are generated during this process; while NADH and $FADH_2$ enter the ETS as reducing equivalents, acetyl-CoA again serves as a starting substrate for the TCA cycle, combining with oxaloacetate to form citrate (Williamson and Cooper 1980; Fernie et al. 2004).

### I.C.2. *The TCA Cycle and Generated Reducing Equivalents*

The primary purpose of the TCA cycle is to generate reducing equivalents such as NADH and $FADH_2$, in addition to $CO_2$ and guanosine triphosphate (GTP), through the oxidation of pyruvate and amino acids; these reducing equivalents, produced from a number of chemical reactions, ultimately power the ETS and drive ATP production. Carrier proteins transport substrates into the mitochondrial matrix, where they can then enter the TCA cycle at various steps to be oxidized (Kibbey et al. 2007). The oxidation of pyruvate, citrate, 2-oxo-glutarate, and malate all feed electrons into Complex I of the ETS by means of reducing NAD+ to NADH (Fig. 1.1); beta-oxidation of fatty acyl-CoA within the mitochondrial matrix also produces NADH that

is subsequently fed into Complex I. The oxidation of succinate is unique in that it feeds electrons into Complex II, rather than Complex I, of the ETS; this reaction occurs via the reduction of FAD+ to $FADH_2$. Beta-oxidation of fatty acyl-CoA also produces $FADH_2$ that can provide electrons to the electron transferring protein (ETF). Additionally, the conversion of glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP) via G3P-dehydrogenase within the inner mitochondrial membrane can generate $FADH_2$, yet another source for donating electrons to the ETS (Williamson and Cooper 1980; Fernie et al. 2004).

### I.C.3. *The ETS*

As shown in Figure 1.1, there are four locations within the mitochondria where electrons enter the ETS: G3PDH, Complex I, Complex II, and the ETF (Chance and Williams 1955). Electrons at all four sites ultimately serve to reduce the carrier ubiquinone (Coenzyme Q) to ubiquinol ($QH_2$). This reduction occurs at Complex I via a flavin mononucleotide (FMN); simultaneously, four protons are translocated from the matrix to the intermembrane space, generating a proton gradient. Electrons from ubiquinol are then transferred to Complex III, comprised of ubiquinol-cytochrome *c* oxidoreductase; two electrons are directed to cytochrome *c*, while two other electrons reduce the quinone of ubiquinone to quinol (Scheffler 2007). Electrons continue to flow into Complex IV, comprised of cytochrome *c* oxidase, via mobile electron carrier cytochrome *c*. These electrons are transferred to molecular oxygen and subsequently generate two molecules of water. The pumping of protons from the matrix to the intermembrane space at all three of the aforementioned complexes produces the proton gradient necessary to generate ATP via ATPase (Complex V); this process is driven by the flow of

electrons through the ETS. (Although Complex II does deliver electrons to quinone via succinate dehydrogenase, it does not contribute to the proton gradient.)

In coupled mitochondria from such tissues as heart, muscle, and liver, Complex V phosphorylates ADP to produce ATP by means of the proton gradient generated by the flow of electrons removed from metabolic substrates (i.e. the production of ATP is *coupled* with the flow of electrons through the ETS), which ultimately reduce oxygen and H+ at Complex IV to form water in the process known as oxidative phosphorylation (OXPHOS). Though mitochondrial respiration in these tissues is principally coupled, the degree of reliance on OXPHOS, glycolysis, FA oxidation, and the TCA cycle as energy sources varies between heart, muscle, and liver. For example, under normal conditions, the majority of ATP produced in the heart is via mitochondrial OXPHOS using a mix of carbohydrate and lipid substrates, although in a fasted state, beta-oxidation of FAs is the principal source of electrons (Opie 2004). In Type I skeletal muscle (i.e. the muscle type from which all of our mitochondrial samples were isolated), OXPHOS again acts as the major energy source, while Type II skeletal muscle relies mostly on glycolysis. In liver, alpha-ketoacids degraded from amino acids are a significant energy source, particularly since the enzyme needed to convert acetoacetate to acetyl-CoA is nearly absent in this tissue (Berg et al. 2002).

However, in contrast to coupled OXPHOS, mitochondria isolated from brown adipose tissue (BAT) are almost completely uncoupled through the activity of uncoupling proteins (UCPs), the most prevalent in mammals being UCP-1. These UCPs dissipate the proton gradient generated by the ETS as heat rather than harnessing the gradient to generate ATP, contributing to a process known as non-shivering thermogenesis (NST) in which non-phosphorylating respiration occurs (often referred to as “leak”). The main fuel sources of BAT include glucose

and FAs, particularly those derived from lipid droplets in the brown adipocytes (Townsend and Tseng 2014). BAT is most prevalent in human infants and hibernating mammals, although it was recently found to be concentrated, albeit in small amounts, in the supraclavicular region of human adults (Sacks and Symonds, 2013). Additionally, a recent trend in the literature has focused on the "beiging" of WAT, or rather the recruitment of brown fat-like adipocytes in WAT (Brestoff et al. 2015; Stanford et al. 2015). These "beige cells" are distinct from WAT in that they express UCP1, and similar to BAT, the process of beiging can be activated by a number of stimuli including cold exposure, β3-selective adrenergic receptors, and exercise (Brestoff et al. 2015; Stanford et al. 2015).

**I.D. Fatty Acid Composition of the Mitochondrial Membrane**

Phospholipids within the mitochondrial membrane are essential in maintaining structural integrity of the organelle; for transporting necessary proteins into the mitochondria; and in ensuring the activation of certain respiratory enzymes. Similar to membranes of other organelles, phospholipid classes such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are most prevalent within the mitochondrial membrane, collectively comprising approximately 70% of total mitochondrial phospholipids (Schenkel and Bakovic 2014). Less abundant phospholipid classes also found in both mitochondrial and plasma membranes include phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). In contrast, cardiolipin (CL) and phosphatidylglycerol (PG) are phospholipid classes unique to mitochondrial membranes and comprise approximately 15% of total mitochondrial phospholipids (Houtkooper et al. 2006; Osman et al. 2011). FAs most common within membrane phospholipids of mitochondria across tissues are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n9), linoleic acid (18:2n6),

and arachidonic acid (20:4n6), although several other FAs are often present in smaller, often undetectable amounts (Mulligan et al. 2014; see gas chromatography data in Chapter 2 Results).

Several studies have shown that alterations in dietary FA composition can influence remodeling of phospholipid acyl chains within the mitochondrial membrane as well as mitochondrial function, in both ectotherms and endotherms. While studies in ectotherms have focused mainly on muscle and liver mitochondrial capacity of various fish species, often in response to cold acclimation (Wodtke 1978; Guderley et al. 1997; Guderley et al. 2008), studies in endotherms have focused principally on liver and heart mitochondrial properties of rodents (Divakaran and Venkataraman 1977; Yamaoka et al. 1988; Monteiro et al. 2013). Mitochondrial alterations due to changes in dietary FA content may be tissue- and species-specific, and in general, changes to the FA makeup of the mitochondrial membrane can affect mitochondrial bioenergetics in a number of ways, from respiratory capacity and coupling to enzymatic activity. For example, trout fed a diet high in docosahexaenoic acid (DHA; 22:6n3) with increased DHA in their mitochondrial membranes had higher rates of OXPHOS than those fed a diet lower in DHA (Guderly et al. 2008), while liver mitochondria OXPHOS increased with glutamate and malate in rats fed a diet high in unsaturated fats versus saturated fats (Divakaran and Venkataraman 1977).

In addition to diet, Ta can also influence phase changes and physical properties of lipids within biological membranes, including modifications to mitochondrial membrane composition and metabolism. This process is generally known as homeoviscous adaptation, in which biological membrane lipids are able to maintain fluidity despite changing conditions (i.e. Ta; Hazel 1995). In ectotherms such as earthworms, acclimation to a lower Ta caused an increase in organization of lipid membranes in the body wall as well as in mitochondrial enzyme activity

(Dougherty and McNamer 2001); in carp, 10°C-acclimated individuals showed increased levels of unsaturated FAs in their mitochondria, which the authors suggest may be correlated to the simultaneous increase in ATP synthase activity (Itoi et al. 2003). Several studies regarding the effect of Ta on mitochondrial membrane composition and function have also been conducted in endotherms. Membrane-linked reactions within the mitochondria of rat liver were found to be affected by temperature changes (Lee and Gear 1974). More recently, the absence of cardiolipin in the mitochondrial membrane resulted in increased sensitivity in response to changing Ta within the mitochondria, as well as decreased respiratory capacity and increased occurrence of defects in mitochondrial DNA (Zhong et al. 2004). Much of this mitochondrial membrane research in mammals has focused on hibernating species, as these individuals experience seasonal temperature fluxes and undergo bouts of torpor to which their biological membranes must acclimate (Aloia and Raison 1989).

### I.E. Tissue-Specific Mitochondrial Respiration and Membrane Composition in Hibernating Mammals

This section focuses on tissue-specific differences in mitochondrial respiration and membrane composition as analyzed in Chapter 2. Unique physiological adaptations during hibernation are first discussed for each tissue, followed by a review of mitochondrial respiration studies as well as plasma and mitochondrial FA membrane composition studies. Although both plasma and FA mitochondrial membrane composition are discussed, it should be noted that no studies to my knowledge have reported on the interaction between the two in mammals that hibernate, nor if an interaction even exists. Therefore, for each tissue, measurements of both plasma and FA membrane composition are reviewed, but no correlations have been proposed.

Furthermore, it is important to note that many studies report fatty acid composition in terms of total phospholipids, which incorporates the mitochondrial membrane as well as membranes of the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). Major differences between existing fatty acid analyses in hibernating mammals, as well as my current data presented in Chapter 2, can be associated with the inconsistency of total versus membrane phospholipids extracted for gas chromatography.

### I.E.1. *Heart*

Despite enduring near freezing Ta, the hearts of hibernating mammals must maintain function, albeit drastically reduced, throughout torpor. The heart rates of hibernators can decrease to approximately 5 beats per minute (bpm) as compared to over 200 bpm at euthermic levels, generally paralleled by an increase in peripheral vascular resistance (Wang 1989). Although heart rate is reduced in these individuals, the force of myocardial contraction is actually increased during torpor (Fahlman et al. 2000), and unlike skeletal muscle, cardiac muscle tissue mass increased by approximately 21% in hibernating golden-mantled ground squirrels (Wickler et al. 1991). In regard to substrate utilization during hibernation when mammals are in a fasted, metabolically-suppressed state, the heart relies primarily on the beta-oxidation of FAs as the principal source of electrons within the cardiac mitochondria (Opie 2004).

Brustovetsky et al. (1992) measured an elevation in cardiac mitochondrial respiration during hibernation in torpid squirrels; this was paralleled by a 95% decrease in PDH activity, inhibiting carbohydrate oxidation in cardiac mitochondria from torpid GMGS (Brooks and Storey 1992). This inhibition of PDH activity, possibly resulting from an increase in pyruvate

dehydrogenase lipoamide kinase isozyme 4 (PDK4) levels (Andrews et al. 1998), has been linked to an absence of glycolytic intermediates entering the TCA cycle during hibernation (Tashima et al. 1970). Additionally, cardiac mitochondria in the Djungarian hamster (*Phodopus sungorus*) exhibit downregulated PDH activity during daily torpor (Heldmaier et al. 1999). ATP synthase transcripts encoded in the mitochondria increased in cardiac mitochondria from hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*; Hittel and Storey 2002).

Few studies exist which analyze the FA composition of the plasma membrane or mitochondrial membrane in heart tissue from torpid animals. Early studies found that in heart, there were no significant differences in whole-tissue phospholipid class composition between torpid and non-torpid European hamsters (*Cricetus cricetus*; Montaudon et al. 1983). Additionally, phospholipid fractions of the heart membrane in several torpid species, including the GMGS (Aloia and Pengelley 1979), European hamster (Montaudon et al. 1983), and thirteen-lined ground squirrel (Swan and Schatte 1977), contained fewer saturated and polyunsaturated FAs (SFAs and PUFAs, respectively) and more monounsaturated FAs (MUFAs) in torpid versus non-torpid individuals. More recently, an increase in dietary 18:2n6 was found to influence IMM phospholipid composition of cardiac mitochondria, subsequently affecting respiration and proton leak rates (Pehowich 1999).

### I.E.2. *Skeletal muscle*

Due to the inactivity associated with hibernation, skeletal muscles are often highly affected by disuse atrophy. In torpid ground squirrels and bats, skeletal muscle mass can decrease by 14-65% compared to euthermic levels, depending on muscle type and species (Steffen et al. 1991); often, this loss of skeletal muscle mass is correlated with a decrease in

OXPHOS capacity in the muscle mitochondria (Wickler et al. 1991). During disuse atrophy in the muscle, proteins are degraded and amino acids are subsequently released; these amino acids can then be taken up by the liver in order to generate both glucose and urea. Therefore, changes to skeletal muscle physiology during hibernation may also affect the physiology of other organs. More recent disuse studies in hibernators have focused on which, if any, mechanisms are responsible for increasing tolerance of skeletal muscle to disuse atrophy and overall dysfunction during the hibernation season (Carey et al. 2003; Rourke et al. 2004; Lee et al. 2008). Additionally, it has been suggested that as mammals prepare for hibernation, myoglobin protein levels in the skeletal muscle increase; perhaps this change allows the animal to prepare for IBAs during hibernation when disuse atrophy is most prevalent, facilitating shivering thermogenesis once the animal reaches approximately 15°C (Postnikova et al. 1999).

Muleme et al. (2006) reported no significant differences regarding any parameters measured in torpid versus active skeletal muscle mitochondria, including succinate oxidation, oxidation of different substrates such as pyruvate, glutamate, beta-hydroxybutyrate, and palmitoyl carnitine, and flux through ETS complexes. Studies in arctic ground squirrels (*Urocitellus parryii*) confirmed that respiration rates in skeletal muscle mitochondria do not vary over the hibernation season (Barger et al. 2003). No difference in expression of *nad4* mRNA, a subunit of Complex I in the ETS, was measured between skeletal muscle mitochondria of torpid versus active little brown bats (*Myotis lucifugus*; Eddy et al. 2006). Furthermore, regarding substrate utilization, the ability of skeletal muscle mitochondria to oxidize ketone bodies does not appear to differ between torpid and non-torpid ground squirrels, suggesting that *in vivo* control of ketone body utilization by skeletal muscle could be controlled by ketone bodies released into the blood via the liver when glucose is limited during hibernation, including

acetoacetate and 3-beta-hydroxybutyrate (McGarry and Foster 1980; Robinson and Williamson 1980).

Despite the lack of studies that have reported a difference in skeletal muscle mitochondrial properties between torpid and active mammals, respiration in skeletal muscle homogenate was inhibited in torpid arctic ground squirrels (Hannon et al. 1961), while Brustovetsky et al. (1992) reported that skeletal muscle mitochondrial respiration was enhanced in the same torpid species. These differences are almost certainly due to differences in homogenization and centrifugation techniques utilized in each study, as Hannon et al. (1961) used whole-tissue homogenates while Brustovetsky et al. (1992) actually isolated the mitochondria from the rest of the skeletal muscle tissue. Both Wickler et al. (1987) and Yacoe (1983) measured an increase in activity of beta-hydroxyacyl CoA dehydrogenase (HOAD; an enzyme of FA beta-oxidation which supplies acetyl CoA to the Krebs cycle) in skeletal muscle mitochondria isolated from torpid hamsters and bats, respectively. Boyer et al. (1998) noted that hibernation induced a large increase in UCP3 transcripts in the skeletal muscle mitochondria of arctic ground squirrels, while in hibernating jerboas (*Jaculus orientalis*), glyceraldehyde-3-phosphate dehydrogenase mRNA, protein, and activity are downregulated (Soukri et al. 1996). Additionally, citrate synthase reportedly increased in mitochondria isolated from the pectoralis muscle of bats (Brigham et al. 1990; Yacoe 1983) and leg muscles of GMGS and the golden hamster (*Mesocricetus auratus*; Wickler et al. 1987; Wickler et al. 1991) during torpor. Staples (2016) noted that in skeletal muscle mitochondria from hibernating mammals, ATP concentrations may decline by nearly 50%.

Very few studies on the FA composition of skeletal muscle mitochondria in hibernating mammals are available. As mentioned above, the majority of related studies focus on

mitochondrial respiration rather than mitochondrial membrane composition; even then, disuse atrophy appears to be the most prevalent topic in this field based on my literature review, and studies based on FA composition of plasma membrane phospholipids are also limited. An earlier study suggests that thermoregulatory uncoupling in skeletal muscle mitochondria may correlate with levels of free FAs in the whole-tissue and mitochondrial membranes (Brustovetsky et al. 1992). Pehowich (1994) reported that total levels of MUFA groups increased in PC, PE, and PI fractions of skeletal muscle sarcoplasmic reticulum in aroused Richardson's ground squirrels during hibernation, while DHA was lower in all phospholipids during arousal. A more recent study discusses the possible upregulation of heart-type FA binding protein (H-FABP), a FA importing isoform, in skeletal muscle mitochondria during hibernation; this would increase lipid delivery from exogenous sources during torpor, since skeletal muscle has few intracellular lipid depots from which to oxidize FAs (Hittel and Storey 2001). Based on my literature search, no data is available which measured specific FA components (i.e. 18:0, 20:4n6, etc.) of the skeletal muscle mitochondrial membrane in hibernating mammals. Therefore, despite the several respiratory studies that have been conducted in the muscle mitochondria of hibernating mammals, there is a lack of mitochondrial FA analyses across seasons, and these few FA analyses tended to be inconsistent in regard to methods used.

### I.E.3. *Liver*

While protein and lipid metabolism in the liver are often reduced during torpor (Burlington 1972), gluconeogenesis and carbohydrate metabolism can remain at levels similar to those of non-torpid individuals (Tashima et al. 1970). Since liver contributes 12-17% of whole-animal mass-specific oxygen consumption (Martin and Fuhrman 1955), suppression of liver

mitochondrial function during torpor could greatly increase metabolic efficiency throughout hibernation. As mentioned above, amino acids released from the skeletal muscle during disuse atrophy can be taken up by the liver to generate both glucose and urea during hibernation. In torpid animals, TCA cycle intermediates are diverted to gluconeogenesis, and fatty acids released from lipolysis in WAT are oxidized as fuel in the liver, producing acetyl-CoA. The build-up of acetyl-CoA leads to ketone body synthesis, and these ketone bodies can then be exported to the brain as a fuel source. Out of all tissues studied in hibernating mammals, the liver is by far the predominant experimental target for mitochondrial studies.

Liver mitochondria isolated from torpid thirteen-lined ground squirrels exhibited OXPHOS rates nearly 70% lower than those from non-torpid (or active) squirrels and hamsters, a readily reversible change often correlated with an inhibition of succinate dehydrogenase and overall suppression of the ETS at Complex II (Armstrong and Staples 2010; Muleme et al. 2006; Pehowich and Wang 1984; Brown and Staples 2010). An approximate 60-70% suppression in liver mitochondrial OXPHOS rate was also recorded in the Richardson's ground squirrel (*Urocitellus richardsonii*; Pehowich and Wang 1987) and the arctic ground squirrel (Barger et al. 2003; Fedotcheva et al. 1985), and a 30% suppression was measured in torpid Djungarian hamsters (Brown et al. 2007). Still, there was no significant difference between torpid and non-torpid (active) squirrels in terms of cardiolipin content of the liver mitochondrial membrane (Armstrong and Staples 2010), and Martin et al. (1999) actually recorded no evidence of OXPHOS suppression upon entrance into torpor.

Conflicting data exists for changes in flux through the ETS complexes in hibernating mammals; a 50-65% decrease in ETS flux at Complex IV was reported in torpid GMGS liver mitochondria (Shug et al. 1971), although in thirteen-lined ground squirrels, a 62-66% decrease

in ETS flux at Complex III was measured (Gehnrich and Aprille 1988). In terms of substrate preference, liver mitochondria from summer active ground squirrels oxidized both pyruvate and beta-hydroxybutyrate at significantly higher rates than torpid individuals (Muleme et al. 2006); it has been suggested that a downregulation of beta-hydroxybutyrate dehydrogenase and related enzymes may enhance the export of ketone bodies from the liver during hibernation for use by peripheral tissues (Muleme et al. 2006). Gerson et al. (2008) fed ground squirrels four different isocaloric diets containing 16, 22, 35, or 55 milligrams per gram of linoleic acid. In all but the 22 mg 18:2/gram diet groups, OXPHOS in liver mitochondria remained unsuppressed between torpid and active squirrels; however, proton leak was decreased in these groups, which the authors suggest may be a means of compensation by the liver mitochondria due to an inability to suppress respiration.

One of the first studies to measure FA composition of whole-tissue liver membranes between torpid and non-torpid individuals reported no significant differences in the thirteen-lined ground squirrel (Swan and Schatte 1977). However, in GMGS, a 16% increase in total liver membrane PE was measured in torpid versus non-torpid groups (Aloia 1981). Cremel et al. (1979) reported significant increases in 18:0 and 18:2n6 in the summer and winter, respectively, in liver mitochondrial membrane of the European hamster, as well as a general decrease in SFAs in torpid versus non-torpid liver mitochondria. Florant et al. (1998) also measured significant increases in 18:0 and 18:2n6 in liver plasma membrane in yellow-bellied marmots during hibernation versus summer. Platner et al. (1976) reported significant increases in 16:0 (palmitic acid) and 17:0 (heptadecanoic acid) and significant decreases in 18:0, 18:2n6, and 20:4n6 in liver mitochondrial membranes from hibernating thirteen-lined ground squirrels. However, the author notes that phospholipids were not separated from other lipids in this study, confounding analysis

of the data; this is further demonstrated by the fact that 17:0, generally used as an internal standard because it is not present in high amounts in biological membranes, was detected at high levels in torpid liver mitochondria.

More recently, mitochondrial membranes from non-torpid ground squirrel livers contained a higher proportion of 16:1 versus their torpid counterparts, which correlated highly to OXPHOS capacity (Armstrong et al. 2011). Gerson et al. (2008) reported significant increases in 18:0, 18:1n9, 18:2n6, 20:4n6, and 22:6n3 in liver mitochondrial membranes from hibernating ground squirrels; proton conductance was positively correlated with 18:2n6 and 20:4n6 levels in the mitochondrial membrane. Transient membrane remodeling only occurred in liver mitochondria isolated from ground squirrels transitioning from interbout euthermia to early entrance; 16:0 and 18:0 increased, while 18:2n6 and 20:4n6 decreased, although these changes in mitochondrial membrane composition did not correlate with mitochondrial respiration (Chung et al. 2011).

### I.E.4. *Brown adipose tissue*

Not only are hibernating mammals able to endure a Tb nearing zero degrees Celsius, but these individuals can also spontaneously arouse from torpor (i.e. interbout arousals) during which Tb reaches euthermic levels (~30°C) in just several hours. The energy required to warm the animal to near euthermic levels during this process is principally supplied via BAT, of which the mitochondria are almost completely uncoupled through the activity of UCPs. This collective process is known as non-shivering thermogenesis due to the animal's reliance on uncoupled heat production, in contrast to ATP-powered shivering thermogenesis occurring in the skeletal muscle (Klingenspor and Fromme 2012). The metabolic processes executed by BAT are fueled

primarily by glucose and FAs; sources of FAs include cellular uptake, *de novo* lipogenesis, and lipid droplets in brown adipocytes (Townsend and Tseng 2014).

Fewer BAT mitochondrial studies have been conducted in hibernating mammals compared to those conducted in the liver. Chaffee et al. (1966) measured an elevation in BAT mitochondrial respiration during hibernation in torpid squirrels, while Yacoe (1981) measured an increase in mitochondrial respiration and non-shivering thermogenesis of BAT upon exposing golden hamsters to cold. However, Staples and Brown (2008) note the conflicting hypotheses regarding whether BAT mitochondria are reversibly inhibited in hibernation; the absence of adrenergic stimulation in torpor may lead one to believe that BAT mitochondrial activity would be suppressed, while the thermogenic role of BAT during interbout arousals could suggest its mitochondrial capacities are increased throughout hibernation. Additionally, an increase in UCP1 mRNA as hibernation approaches has been reported in thirteen-lined ground squirrels (Nizielski et al. 1986), despite no difference in UCP1 mRNA levels between active and hibernating long-tailed ground squirrels (*Urocitellus undulatus*; Boyer et al. 1998). ATP synthase transcripts encoded in the mitochondria also increased in BAT mitochondria from hibernating thirteen-lined ground squirrels (Hittel and Storey 2002). BAT mitochondria in the Djungarian hamster (*Phodopus sungorus*) exhibited downregulated PDH activity during daily torpor (Heldmaier et al. 1999), while cytochrome *c* oxidase activity in BAT mitochondria of the little brown bat increased at least 3-fold during torpor (Eddy et al. 2006). Upregulation of *nad4* mRNA, unlike in skeletal muscle, was fourfold higher in BAT mitochondria of torpid versus active little brown bats (*Myotis lucifugus*; Eddy et al. 2006).

As in skeletal muscle, very few studies regarding the FA composition of BAT plasma membrane or mitochondria in hibernating mammals are available. The majority of related studies

focus on the mechanisms of non-shivering thermogenesis and how this could be transferred to human biomedical context rather than on mitochondrial membrane composition. Florant et al. (1998) measured significant decreases in myristic acid (14:0) and 18:0 and a significant increase in 18:1n9 in BAT plasma membrane in yellow-bellied marmots during hibernation versus summer. Carneheim et al. (1989) reported that at the start of hibernation in golden hamsters, the majority of FAs in BAT plasma membrane were considered "rare," including homo-gamma-linoleic (DGLA; 20:3n6), eicosadienoic (20:2n6), and lignoceric acids (24:0); the authors suggested that these three FAs were preferentially utilized during the early phase of hibernation.

Upregulation of muscle- and heart-type FA binding proteins (M-FABP and H-FABP, respectively) have been identified in BAT of hibernating thirteen-lined ground squirrels (Hittel and Storey 2001). Within the mitochondrial membranes of non-hibernating mammals, derivatives of 18:2n6 and 20:4n6 have been suggested to stimulate UCP1 activity in BAT (Brand et al. 2004). Furthermore, in non-hibernating mice, cold-induced alterations in the FA composition of BAT mitochondrial membranes, including a subsequent increase in 18:0, 18:2n6, and 20:4n6 and decrease in 16:0, 16:1, 18:1n7, and 22:6n3, were found to be independent of UCP1 (Ocloo et al. 2007). Based on my literature search, no data are available which measured specific FA components of the BAT mitochondrial membrane in hibernating mammals, similar to skeletal muscle.

### I.F. Experimental Questions and Hypotheses

Based on the important tissue-specific interactions between mitochondrial properties such as respiration and FA membrane composition in hibernating mammals discussed above, it seemed necessary to perform a comprehensive mitochondrial study across seasons in a

hibernator. We compared OXPHOS-linked respiratory capacities and coupling control with carbohydrate and FA substrates in mitochondria isolated from liver, heart, skeletal muscle, and BAT of GMGS in summer, fall (prehibernation), and winter (hibernation) seasons. To determine if changes in mitochondrial function paralleled changes in membrane composition, mitochondrial phospholipid FA composition was also evaluated in all samples.

We hypothesized that mitochondria respond to changing seasons in a tissue-specific manner based on the distinct metabolic demands and roles of the tissues they support, and that altered mitochondrial membrane composition may be partially responsible for these differences. Specifically, cardiac mitochondrial respiration would be upregulated across seasons due to the continuous and regulated contractile activity of the heart, even in hibernation (Carey et al. 2003). Cold-induced BAT mitochondrial respiration would be upregulated in prehibernation and hibernation due to the decreasing Ta associated with these seasons (Sundin et al. 1987; Milner et al. 1989; Staples and Brown 2008), while muscle mitochondrial function would be suppressed during these colder seasons due to progressive inactivity in the weeks preceding hibernation, which has been reported in a number of hibernating species (Musacchia et al. 1988; Hudson and Franklin 2002; Shavlakadze and Grounds 2006). Lastly, liver mitochondrial respiration would be suppressed in hibernation, reflecting an overall suppression of metabolic activity in the liver during this season, including decreased protein synthesis (Zhegunov et al. 1988) and translation (Knight et al. 2000; Van Breukelen and Martin 2001; Hittel and Storey 2002).

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# CHAPTER II: TISSUE-SPECIFIC SEASONAL CHANGES IN MITOCHONDRIAL RESPIRATORY FUNCTION AND MEMBRANE COMPOSITION IN THE GOLDEN-MANTLED GROUND SQUIRREL

## II.A. Introduction

Hibernating mammals such as the golden-mantled ground squirrel (*Callospermophilus lateralis*, GMGS) fast during the winter months when individuals enter extended periods of reduced metabolic rate known as torpor (Wang 1979). In preparation for this remarkable feat, animals increase food intake in the summer and fall while simultaneously decreasing energy demands (Pengelley and Fisher 1963; Muleme et al. 2006), leading to an approximate doubling of body mass primarily in the form of white adipose tissue (Florant et al. 2010). During hibernation, animals rely primarily on the oxidation of lipids from endogenous fat stores to fuel metabolic processes as their internal body temperature (Tb) decreases to near ambient temperatures (Ta) as low as 5˚C (Muleme et al. 2006). While most mammals increase metabolic rate in response to decreasing temperatures, hibernating mammals are unique in that they suppress metabolic processes as Ta drops (Geiser 2004; Heldmaier et al. 2004; Muleme et al. 2006).

Mitochondria, the cellular sites of oxidative metabolism, are known to respond to changes in both metabolic status and thermal stress in several tissues and species. However, whether mitochondria undergo metabolic and/or structural changes to facilitate or compliment seasonal variations in the organismal metabolism of hibernators is unclear. Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) exhibit 70% lower rates of succinate-supported oxidative phosphorylation (OXPHOS) in liver, but not muscle, mitochondria isolated from torpid

compared to summer active ground squirrels (Muleme et al. 2006). Similar suppression of liver mitochondrial respiration has also been reported in Arctic ground squirrels (*Spermophilus parryii*; Barger et al. 2003), long-tailed ground squirrels (*Spermophilus undulatus*; Brustovetsky et al. 1989), and GMGS (Martin et al. 1999) during hibernation. These decreases have been attributed to reduced capacities to oxidize substrates that supply reducing equivalents to Complexes I and II of the electron transport system (ETS) (i.e., carbohydrates such as pyruvate and succinate, respectively; Brustovetsky et al. 1989). However, effects on respiration supported by FA oxidation, the primary source of energy during winter months, were not investigated. Also unclear is the extent to which changes in liver mitochondria in torpor represent seasonal responses of mitochondria in other tissues critical for survival of hibernators, such as muscle, heart, and brown adipose tissue.

Changes in the FA composition of mitochondrial membranes have been reported in response to colder temperatures in both hibernating and non-hibernating mammals (Swan and Schatte 1977; Aloia and Pengelley 1979; Gerson et al. 2008), as well as functional adaptations in membrane-embedded enzymes and ion pumps (Yacoe 1983; Soukri et al. 1996; Lemieux et al. 2008). Whether altered membrane composition directly influences protein function and cellular adaptations to thermal and metabolic processes in hibernating mammals is not clear. Remodeling of mitochondrial membrane phospholipids occurs within several hours of entering torpor in the tissues of hibernating thirteen-lined ground squirrels (Chung et al. 2011), although the effect is only transient. Changes in mitochondrial membrane composition have been postulated to influence the activities of membrane-bound respiratory enzymes in response to cold (Hazel 1972). Gerson et al. (2008) described a positive correlation between linoleic acid (18:2n6) and arachidonic acid (20:4n6) with mitochondrial proton conductance in liver mitochondria of

hibernating thirteen-lined ground squirrels, suggesting a role for membrane polyunsaturated FAs (PUFAs) in regulating metabolic efficiency. Ruf and Arnold (2008) suggested that changes in levels of specific membrane polyunsaturated FAs (PUFAs) may be less important than shifts in the ratio of n6 to n3 PUFAs and relative proportions of PUFAs to monounsaturated FAs (MUFAs). However, few studies have investigated the interaction of mitochondrial membrane composition and respiratory function in endotherms, and none have examined both in multiple tissues across seasons in a hibernator.

In the present study, we compared OXPHOS-linked respiratory capacities and coupling control with carbohydrate and FA substrates in mitochondria isolated from liver, heart, skeletal muscle, and brown adipose tissue (BAT) of GMGS in summer, fall (prehibernation), and winter (hibernation) seasons. To determine if changes in mitochondrial function paralleled changes in membrane composition, mitochondrial phospholipid FA composition was also evaluated in all samples. We hypothesized that mitochondria respond to changing seasons in a tissue-specific manner based on the distinct metabolic demands and roles of the tissues they support, and that altered mitochondrial membrane composition may be partially responsible for these differences. Specifically, cardiac mitochondrial respiration would be upregulated across seasons due to the continuous and regulated contractile activity of the heart, even in hibernation (Carey et al. 2003). Cold-induced BAT mitochondrial respiration would be upregulated in prehibernation and hibernation due to the decreasing Ta associated with these seasons (Sundin et al. 1987; Milner et al. 1989; Staples and Brown 2008), while muscle mitochondrial function would be suppressed during these colder seasons due to progressive inactivity in the weeks preceding hibernation, which has been reported in a number of hibernating species (Musacchia et al. 1988; Hudson and Franklin 2002; Shavlakadze and Grounds 2006). Lastly, liver mitochondrial respiration would be

suppressed in hibernation, reflecting an overall suppression of metabolic activity in the liver during this season, including decreased protein synthesis (Zhegunov et al. 1988) and translation (Knight et al. 2000; Van Breukelen and Martin 2001; Hittel and Storey 2002).

## II.B. Methods

### II.B.1. *Ethical Approval*

The Colorado State University Institutional Animal Care and Use Committee approved all animal studies. All procedures were approved under IACUC Protocol #14-5137A.

### II.B.2. *Ground Squirrels*

Adult GMGS were trapped over two summers (June-August 2014 and 2015) in Larimer and Gunnison Counties, Colorado, under State of Colorado Department of Natural Resources Scientific Collection License #14TR099 (2014 season) and #15TR099 (2015 season). Male and female adult GMGS were transported live and housed in a coldroom animal facility at Colorado State University for both the 2014 season (n=15; males: n=8; females: n=7) and 2015 season (n=11; males: n=6; females: n=5). GMGS were housed in standard rodent cages in a temperature-controlled facility and provided standard rodent chow (Harlan 2918) and water *ad libitum* for the duration of the study. Food intake and body mass were measured daily until animals began torpor in mid-October, and Tb was estimated weekly by means of a handheld non-invasive infrared thermometer (Centech). GMGS were kept under a natural light:dark cycle for Colorado (Paragon Elec. Inc.) throughout the seasons.

GMGS were randomized into one of three seasonal groups: summer, prehibernating (fall), or hibernating (winter). In summer (June-August), GMGS were kept at a Ta of ~19-22°C in the housing facility for at least 6 weeks before sacrifice (n=9). During the prehibernation season (late September to mid-October), GMGS were kept at a Ta of ~12-15°C, maintained for at least 4 weeks at that Ta prior to sacrifice (n=9). During the hibernation season (mid-October to March or April), GMGS were kept in complete darkness to mimic the below-ground hibernacula where animals remain throughout the winter at a Tb of ~5-7°C. Hibernating GMGS (n=8) were defined by a Ta of ~4-11°C, a complete lack of food intake, a decrease in body mass, and an absence of any observable movement around the cage, and were not sacrificed during interbout arousals (IBAs) when their Tb was similar to summer GMGS (~31-37°C). Additionally, as monitored by the infrared thermometer, hibernating GMGS had been hibernating for at least four weeks prior to sacrifice. GMGS from summer and prehibernating groups were fasted overnight for ~16 hours prior to sacrifice.

### II.B.3. *Preparation of Isolated Mitochondria from Heart, Muscle, Liver, and BAT Tissue*

All GMGS were deeply anesthetized with isoflurane and sacrificed by decapitation and opening of the thoracic cavity. Generally, the heart was dissected first, followed by skeletal muscle, liver, and BAT tissues, which were all immediately transferred to ice cold buffers described below. Mitochondria were isolated from ~150mg of freshly extracted tissue from liver (left lateral lobe), BAT (left caudal subscapular depot), heart (left ventricle), and skeletal muscle (plantaris). Heart tissue was minced in ice-cold Chappell-Perry 1 (CP1) buffer containing (in mM) KCl (100), MOPS (50), EGTA (1), MgSO4•7H2O (5), and ATP (1), pH 7.4 with KOH. Skeletal muscle was minced in ice-cold CP0 buffer (CP1 without EGTA). Liver tissue was

minced in ice-cold IB buffer containing (in mM) sucrose (200), Tris-MOPS (10), and EGTA-Tris (1), pH 7.4 with KOH. BAT tissue was minced in ice-cold 250mM sucrose. It should be noted that WAT was not included in our mitochondrial respiration or fatty acid analyses due to its low level of metabolic activity and low density of mitochondria; additionally, in hibernating mammal studies, WAT has often been collected from inconsistent, and possibly incomparable, locations throughout the body (i.e. subcutaneous, gonadal, etc.). All tissues were washed 2-3 times in their respective buffers and kept on ice throughout the mitochondria isolation protocols until being used for respirometry experiments. Heart tissue was homogenized for 3-5 seconds using a polytron (Tissuemeiser, Fisher Scientific, medium speed) and transferred to a 10mL glass homogenizing tube. Heart homogenates and muscle tissue mince were trypsinized (10 mg/g tissue) for 7 minutes to dissociate mitochondria from myofibrils and disperse collagen matrices (in muscle tissue) to generate a mixed population of subsacrolemmal and intermyofibrilar mitochondia, after which CP2 (CP1 containing 0.2% fatty-acid free bovine serum albumin) was added to stop the trypsin reaction. The resulting slurries and minces of BAT and liver tissue were then homogenized 5-6 times via a Potter-Elvehjem Teflon/glass homogenizer (Fisher Scientific) and transferred to a centrifuge tube on ice. Muscle and BAT homogenates were pelleted at 9000 x g for 10 minutes, and resuspended in CP2 and sucrose, respectively. Mitochondria from all four tissues were then isolated using standard differential centrifugation methods consisting of low speed debris-pelleting spins (600 x g) and mitochondrial pelleting + 2 clarifying spins (7000 x g) as described in detail elsewhere (heart and muscle: Chicco et al. 2014; liver: Frezza et al. 2007; BAT: Cannon and Nedergaard, 2008). The resulting mitochondrial pellets were resuspended in 200 µL KME containing (in mM) KCL (100), MOPS (50), and ETGA (0.5), pH

7.4, and diluted in 25mM $KH_2PO_4$ at a ratio of 1:100 for protein assay (BCA assay, Pierce) prior to mitochondrial respiration experiments.

### II.B.4. *Mitochondrial Respiration*

Mitochondrial respiratory function of the isolated mitochondria from heart, muscle, liver, and BAT tissue was determined by high resolution respirometry via the Oxygraph-2K (O2K) high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). The specifications of the O2K are unique: the limit of detection of respiratory flux is 1 pmol·s -1 ·cm-3 (0.001 µM·s -1), and the limit of detection of oxygen concentration extends to 0.005 µM $O_2$ (Gnaiger 2008). A total of 75 µg of mitochondrial protein (as determined by BCA assay) were added to each chamber for muscle, liver, and BAT samples in order to produce a signal large enough to observe small changes on the O2K interface upon the addition of substrates. In contrast, a total of 50 µg mitochondrial protein were added for heart samples to prevent cardiac mitochondria from rapidly using up the oxygen contained within each chamber. Regardless, all four tissues were still normalized to milligrams per mitochondrial protein, so respiratory data remains comparable. The two chambers of the Oxygraph-2K to which the mitochondria were added each contained 2.3 mL of MiR05 respiration buffer containing (in mM): EGTA (0.5), $MgCl_2$ (3), K-lactobionate (6), taurine (20), $KH_2PO_4$ (10), HEPES (20), and sucrose (110), with 0.1% fatty-acid free bovine serum albumin (Fraction V).

Standardized instrumental and chemical calibrations were conducted to correct for background diffusion of oxygen into the chamber, oxygen consumption from MiR05, and oxygen consumption from the electrodes using Datlab software (Fasching and Gnaiger 2016; Oroboros Instruments, Innsbruck, Austria). Oxygen flux was monitored in real-time, which

resolved changes in the negative time derivative of the chamber oxygen concentration signal. Experiments were performed at 37°C beginning at room air oxygen saturation (~160 μmol $L^{-1}$). In isolated mitochondria, unlike in permeabilized muscle fibers, oxygen diffusion does not limit mitochondrial respiration, indicated by stable oxygen flux down to near 0 μmol $l^{-1}$. All respirometry data were collected at 37˚C with saturating amounts of each substrate established by titration experiments for each tissue mitochondria (unpublished data). To maintain consistency throughout the study, all FA-based respiration experiments (with palmitoyl carnitine) were carried out in chamber A, while all carbohydrate-based respiration experiments (with pyruvate) were carried out in chamber B. Detailed descriptions of the respiration protocols and associated respiratory states generated by the sequential titration of each substrate are provided in Table 2.1. Respirometry data are expressed per mg of total mitochondrial protein.

**Table 2.1. High resolution respirometry protocols and associated respiratory flux states assessed in mitochondrial respiration experiments.** Protocol constituents are listed in the order they are added in the respiration experiment, generating the cumulative respiratory states described in the right column. See text and ref. (Pesta and Gnaiger 2012) for additional explanation and interpretation of respiratory states. Abbreviations not listed below: ETF, electron transferring flavoprotein; OXPHOS, oxidative phosphorylation; CI/II, respiratory Complexes I/II; $\Delta\Psi_{mt}$; mitochondrial membrane potential.

| **Protocol constituents** (listed in order of titration) | **Abbreviation** | **Respiratory Flux State; Explanation** |
|---|---|---|
| ***Protocol 1: Fatty Acid + Carbohydrate Substrates*** | | |
| Malate + Palmitoylcarnitine (1 mM + 0.2 mM) | PalM | $FAO_L$; Non-phosphorylating LEAK respiration supported by fatty acids (without ADP) |
| ADP (4 mM) | ADP | $FAO_P$; Fatty acid OXPHOS capacity, limited by electron flux through ETF |
| Pyruvate (5 mM) | Pyr | $PM_P$; Fatty acid + pyruvate OXPHOS capacity; limited by PM oxidation capacity |
| Glutamate (10 mM) | Glut | $CI_P$; (also $PGM_P$) Complex I-supported OXPHOS capacity (+ fatty acids) |

| | | |
|---|---|---|
| Succinate (10 mM) | Succ | CI+II$_P$; Complex I+II-supported OXPHOS capacity (+ fatty acids); limited by ADP phosphorylation capacity |
| ***Protocol 2: Carbohydrate Substrates*** | | |
| Pyruvate + Malate (5 mM + 1 mM) | PM | PM$_L$; Non-phosphorylating LEAK respiration supported by pyruvate oxidation (without ADP) |
| ADP (4 mM) | ADP | PM$_P$; Pyruvate OXPHOS capacity, limited by pyruvate oxidation capacity |
| Glutamate (10 mM) | Glut | CI$_P$; (also PGM$_P$); Complex I-supported OXPHOS capacity (without fatty acids) |
| Succinate (10 mM) | Succ | CI+II$_P$; Complex I+II-supported OXPHOS capacity; limited by ADP phosphorylation capacity |

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### II.B.5. *Mitochondrial Phospholipid Extraction for Fatty Acid Analysis by Gas Chromatography*

Phospholipids were extracted from 20 µL of isolated mitochondria from each tissue stored at -80°C since completing the respiration experiments. 600 µL of methanol (precooled to 4°C) was added to the 20 µL of mitochondrial homogenate in an Eppendorf tube, as was 15 µL of a 10:1 (hexane:stock solution) dilution of heptadecaenoic acid (17:0) internal standard, and 10mM of butylated hydroxytoluene (BHT) to prevent peroxidation of the sample.
Tubes were vortexed for 30 seconds and then centrifuged at 900 x g (~3200 rpm) for 5 minutes to pellet the non-phospholipid fraction. The methanol supernatant was added to 25 µL of 25% sodium methoxide solution in a 13x100mm glass tube and incubated at room temperature for 3 minutes to allow the selective synthesis of methyl esters from glycerophospholipid FAs. The reaction was stopped after 3 minutes by adding 75 µL of methanolic HCl (3N) to each glass tube. FA methyl esters (FAMEs) were extracted by adding 700 µL of hexane to each tube and

vortexing the sample for ~20 seconds. Using a glass Pasteur pipet, the upper FAME-containing hexane layer was transferred to a 2 mL glass vial for each sample. This FA extraction was repeated with another 700 μL of hexane per glass tube, centrifuged for 1 minute at 2500 rpm, and the upper hexane layer was combined with the extracts already in the GC vial. FA extracts were dried down under nitrogen gas and resuspended in 50 μL of hexane for GC analyses.

### II.B.6. *Gas Chromatography*

GC analysis was performed as described by Mulligan et al. (2014) using an Agilent Technologies DB-225 30m x 0.250mm x 0.25μm column (model 122-2232, J&W Scientific) on an Agilent 6890 Series Gas Chromatograph with a flame ionization detector, with a flow rate of 1.7 ml/min and split ratio of 15:1 in 26 minutes. The initial temperature of the oven was 120°C with an initial ramp temperature of 10°C/min for 8 minutes, then 2.5°C/min for 6 minutes and held at 215°C for the remaining 6 minutes, for a total run time of 20 minutes. The front inlet split ratio was 15:1 with the column at constant flow and an initial flow, pressure, and velocity at 1.8 mL/min, 22.66 psi, and 42 cm/sec, respectively. Equilibration time per run was 1 minute. Samples were injected via auto-sampler, and the injector and column were rinsed with both acetone and hexane prior to each run. A standard solution of 1 mg/mL was injected into the GC every 5-6 runs in order to recalibrate the FA peaks on the chromatograph. GMGS phospholipid samples were analyzed for twelve FAs commonly found in mammalian mitochondrial membranes (Table 2.2); all other FAs were either not present in the membrane or were present but below detectable levels.

**Table 2.2. Mitochondrial membrane phospholipid fatty acids analyzed via gas chromatography and their nomenclatures.** Abbreviations not listed below: SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

| Palmitic | Palmitoleic | Stearic | Oleic | Vaccenic | Linoleic | alpha-Linolenic | Dihomo-gamma-linolenic | Arachidonic | Eicosa-pentaenoic | Docosa-pentaenoic | Docosa-hexaenoic |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Omega-7 | | Omega-9 | Omega-7 | Omega-6 | Omega-3 | Omega-6 | Omega-6 | Omega-3 | Omega-3 | Omega-3 |
| SFA | MUFA | SFA | MUFA | MUFA | PUFA | PUFA | PUFA | PUFA | PUFA | PUFA | PUFA |
| 16:0 | 16:1 | 18:0 | 18:1n9 | 18:1n7 | 18:2n6 | 18:3n3 | 20:3n6 | 20:4n6 | 20:5n3 | 22:5n3 | 22:6n3 |

### II.B.7. *Data Analysis*

All data were analyzed by one-way ANOVA with Tukey test post-hoc for pairwise comparisons (GraphPad Prism 6, San Diego, CA) when appropriate to test for significant differences across seasons for both mitochondrial respiration and membrane fatty acid composition data. A statistical significance of $P<0.05$ was set for all analyses. All data are presented as means of the experimental groups ± standard error of the mean (SEM).

## II.C. Results

### II.C.1. *Animal Characteristics*

Body weight, food intake, and Tb differed across the seasonal groups as expected and are presented in Table 2.3. Summer animals (n=9) exhibited a mean Tb from ~31-37°C and a steady increase in or maintenance of food intake and body mass. Prehibernating GMGS (n=9) had a mean Tb of ~25-30°C and increased food intake and body mass versus summer animals. Hibernating GMGS (n=8) were defined by a Tb of ~4-11°C, a complete lack of food intake, a decrease in body mass, and an absence of any observable movement around the cage for at least

four weeks prior to sacrifice. It should be noted that no trends existed for differences between males versus females in terms of both mitochondrial respiratory and FA membrane changes.

**Table 2.3**. **Mean GMGS body weight, food intake, and Tb.** Body weight and daily food intake values are means ± SEM representing 4 weeks prior to sacrifice for each seasonal group, with the exception of food intake during hibernation, since GMGS cease eating during this season. Tb, or internal body temperature, values are means ± SEM on the day of sacrifice. Within each row, different labels (letters) indicate a significant difference between seasonal groups at $P < 0.05$. For all measurements, Summer: N = 9; Prehibernation: N = 9; Hibernation: N = 8.

| | Summer | Prehibernation | Hibernation |
|---|---|---|---|
| **Body Weight (g)** | $243.3 \pm 5.6^{a}$ | $293.1 \pm 14.2^{b}$ | $234.7 \pm 24.1^{a}$ |
| **Daily Food Intake (g)** | $13.0 \pm 2.1^{a}$ | $20.4 \pm 2.2^{b}$ | $0.0 \pm 0.0^{a}$ |
| **Internal Body Temp, Tb ($^{o}$C)** | $34.0 \pm 3.0^{a}$ | $27.5 \pm 2.5^{b}$ | $7.5 \pm 3.5^{c}$ |

## II.C.2. *Heart Mitochondria*

### II.C.2.a. Mitochondrial Respiration

In cardiac mitochondria, OXPHOS-linked respiratory capacity was markedly elevated in the prehibernation season compared to summer and hibernation with Complex I and Complex I+II substrates (Pyr and Glut) in the presence of FAs (summer vs. prehibernation: p=0.015 and p=0.042, respectively; prehibernation vs. hibernation: p=0.047 and 0.027, respectively), with a similar but not significant trend measured after the addition of ADP and Succ. In prehibernation, mitochondrial respiration was approximately 35%, 55%, 59%, and 40% higher than hibernation rates after the addition of ADP, pyruvate, glutamate, and succinate, respectively, in the presence of FAs. (Fig. 2.1A). OXPHOS capacity was also significantly elevated in the prehibernation season compared to summer with Complex I and Complex I+II substrates (ADP, Glut, and Succ)

in the absence of FAs (summer vs. prehibernation: p=0.011, p=0.040, and p=0.025, respectively). Mitochondrial respiration in the absence of FAs during prehibernation was approximately 45%, 77%, 55%, and 44% higher than summer respiration rates after the addition of malate and pyruvate, ADP, glutamate, and succinate, respectively (Fig. 2.1B). No significant differences in coupling control factors were measured across seasons (p>0.05), indicating a similar control of FA ($FAO_{P-L/P}$; Fig. 2.1C) and pyruvate oxidation ($PM_{P-L/P}$; Fig. 2.1D) by ADP (the phosphorylation system) across seasons.

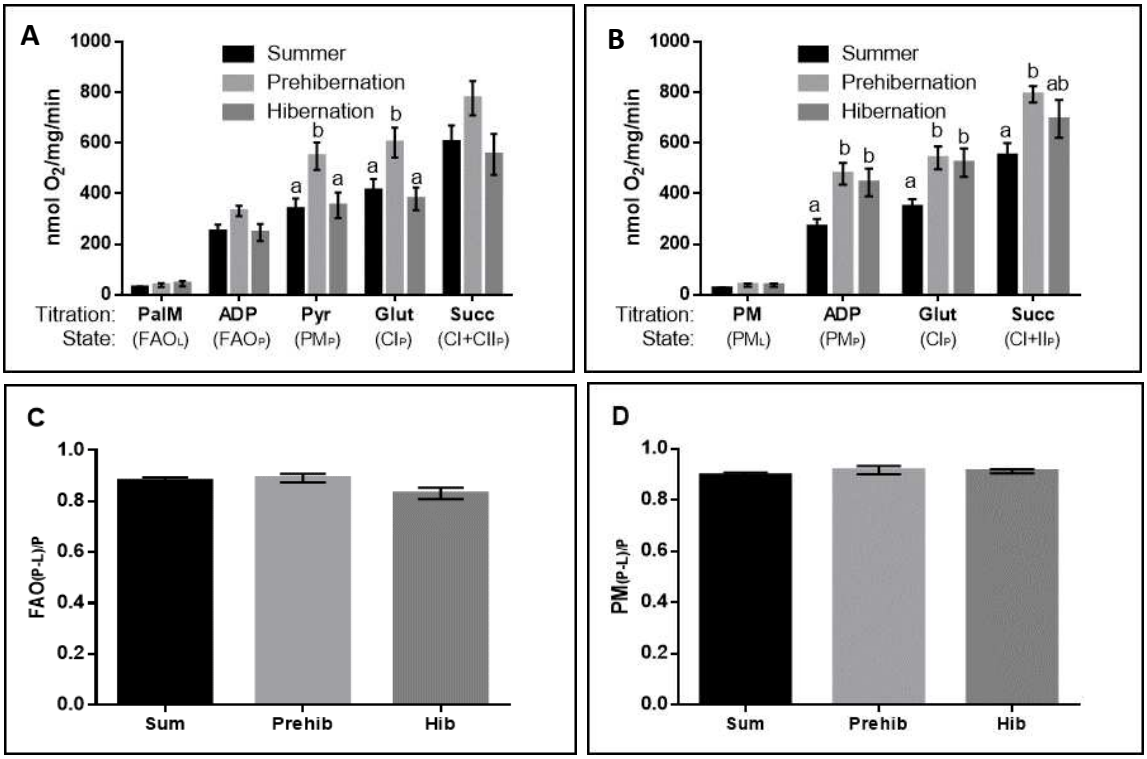


**Figure 2.1. Cardiac mitochondrial respiration.** (A) OXPHOS capacity was markedly elevated in the prehibernation season compared to summer and hibernation with Complex I and Complex I+II substrates (Pyr and Glut) in the presence of FAs (summer vs. prehibernation: p=0.015 and p=0.042, respectively; prehibernation vs. hibernation: p=0.047 and 0.027, respectively), with a similar but not significant trend measured after the addition of ADP and Succ. (B) OXPHOS capacity was markedly elevated in the prehibernation season compared to summer with Complex I and Complex I+II substrates (ADP, Glut, and Succ) in the absence of FAs (summer vs. prehibernation: p=0.011, p=0.040, and p=0.025, respectively). Note that across tissues, the range on the y-axis varies for A and B. (C) FA OXPHOS coupling control factor, calculated as (P-L)/P using the FAO OXPHOS capacity ($FAO_P$) and preceding LEAK respiration without ADP ($FAO_L$) in A. (D) OXPHOS coupling control factor for pyruvate oxidation, calculated as (P-L)/P using the pyruvate+malate OXPHOS capacity ($PM_P$) and preceding LEAK respiration without

ADP ($PM_L$) in B. Different labels (letters) within a substrate group indicate a significant difference between seasonal groups. Summer: N = 9; Prehibernation: N = 9; Hibernation: N = 8.

II.C.2.b. Phospholipid Fatty Acid Analysis

Significant differences across seasons were measured in three phospholipid FAs of the cardiac mitochondrial membrane (Fig. 2.2; Supplementary Table S.1). Percentages of 18:1n9 and 22:6n3 were significantly lower and higher in summer versus hibernation, respectively (p=0.025 and p=0.001, respectively) and lower in prehibernation versus hibernation for 18:1n9 (p=0.003). Percentage of 18:3n3 was only significantly lower in prehibernation vs. summer (p=0.0006). Although there was a trend of decreasing DHA measured in prehibernation versus summer, this was not statistically significant. The n3:n6 PUFA ratio, calculated as the sum of the proportion of n3 PUFA divided by the proportion of n6 PUFA, was significantly lower in hibernation compared to summer by 39% (p=0.048; Fig. 2.2; Supplementary Table S.1).

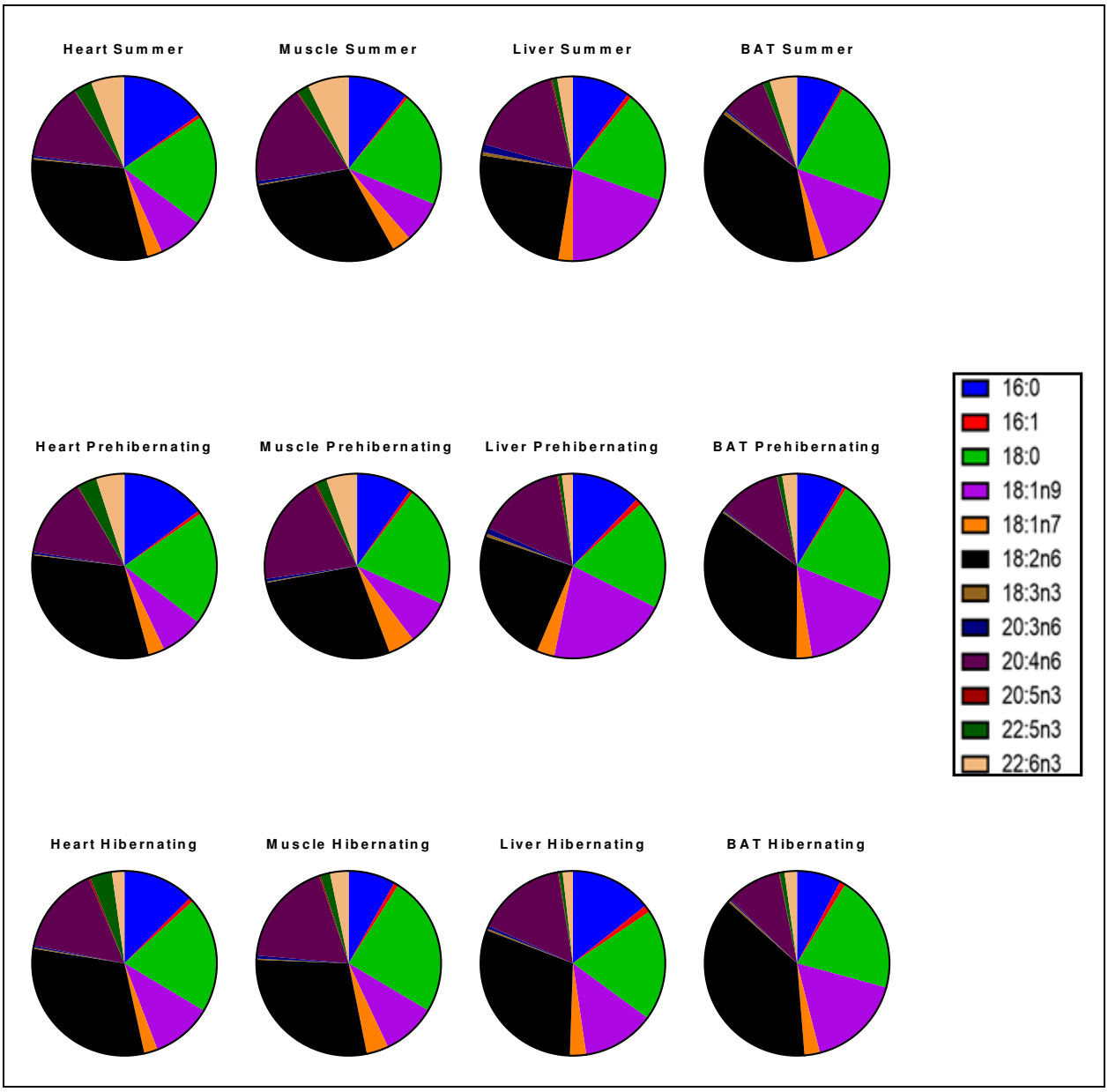


**Figure 2.2. Mitochondrial phospholipid fatty acid composition in GMGS across seasons, represented as mean percentages.** Each of the twelve phospholipid FAs analyzed in the mitochondrial membranes across tissues and seasons are represented as a percentage of the total FAs present. Differences within one tissue across seasons can be easily observed by comparing the three pie charts within each column. Mean percentage values and statistical differences across tissues and seasons for this figure are presented in Supplementary Table S.1. For heart, Summer: N = 8; Prehibernation: N = 9; Hibernation: N = 6. For muscle, liver, and BAT, Summer: N = 5; Prehibernation: N = 6; Hibernation: N = 3.

### II.C.3. *Muscle Mitochondria*

#### II.C.3.a. Mitochondrial Respiration

In skeletal muscle mitochondria, leak respiration supported by FAs (PalM; $FAO_L$) was significantly greater in hibernation than summer ($p=0.030$), while total (CI +CII) OXPHOS capacity in the presence of FAs was suppressed in hibernation ($CI+II_P$) compared to summer and prehibernation rates (summer vs. hibernation: $p=0.025$; prehibernation vs. hibernation: $p=0.049$; Fig. 2.3A). Similar trends were seen in the absence of FAs, but no significant differences in carbohydrate-supported leak or OXPHOS were seen across seasons in the absence of FAs ($p>0.05$; Fig. 2.3B). Consistent with higher leak and lower OXPHOS capacity in hibernation, a significantly lower index of FA OXPHOS coupling control was seen in hibernation versus other seasons (summer vs. hibernation: $p=0.040$; prehibernation vs. hibernation: $p=0.025$; $FAO_{P-L/P}$; Fig. 2.3C), indicating a greater control of FAO by ADP in summer and prehibernation seasons. Additionally, a significantly greater index of pyruvate + malate OXPHOS coupling control was seen in summer versus prehibernation ($p=0.007$; $PM_{P-L/P}$; Fig. 2.3D), consistent with greater muscle OXPHOS coupling efficiency in summer.

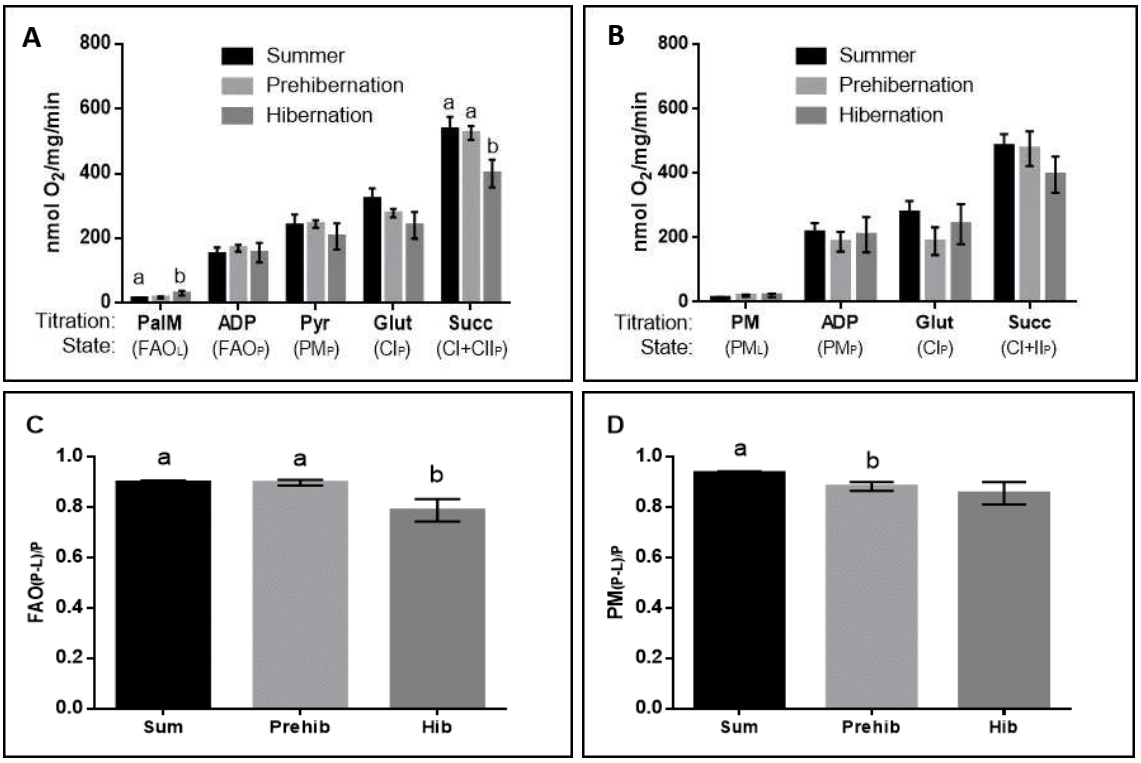


**Figure 2.3. Skeletal muscle mitochondrial respiration.** (A) LEAK respiration (PalM) differed significantly between summer and hibernation in the presence of FAs during OXPHOS (p=0.030). OXPHOS capacity in the presence of FAs was also markedly suppressed in hibernation (summer vs. hibernation: p=0.025; prehibernation vs. hibernation: p=0.049). (B) No significant differences in respiration were measured across seasons in the absence of FAs. Note that across tissues, the range on the y-axis varies for A and B. (C) FA OXPHOS coupling control factor, calculated as (P-L)/P using the FAO OXPHOS capacity ($FAO_P$) and preceding LEAK respiration without ADP ($FAO_L$) in A. (D) OXPHOS coupling control factor for pyruvate oxidation, calculated as (P-L)/P using the pyruvate+malate OXPHOS capacity ($PM_P$) and preceding LEAK respiration without ADP ($PM_L$) in B. Different labels (letters) within a substrate group indicate a significant difference between seasonal groups. Summer: N = 9; Prehibernation: N = 9; Hibernation: N = 8.

II.C.3.b. Phospholipid Fatty Acid Analysis

Percentages of 16:0 and 22:6n3 were significantly higher in summer versus hibernation (p=0.027 and p=0.005, respectively; Fig. 2.2; Supplementary Table S.1) and prehibernation versus hibernation (p=0.049 and p=0.010, respectively). Percentage of stearic acid (18:0) increased in hibernation versus summer by approximately 21% (p=0.002) and was also significantly different between summer and prehibernation (p=0.026) and prehibernation and

hibernation (p=0.049; Fig. 2.2; Supplementary Table S.1). Additionally, vaccenic acid (18:1n7) increased in prehibernation versus summer by approximately 38% (p=0.023). Similar to the trend measured in heart mitochondria, DHA (22:6n3) gradually decreased from summer to prehibernation to hibernation and was significantly different across all seasons. Percentage of DHA in summer was approximately 34% higher versus prehibernation and 123% higher versus hibernation, while percentage of DHA in prehibernation was approximately 67% higher versus hibernation (Fig. 2.2; Supplementary Table S.1). The unsaturation index (UI), calculated as the sum of the proportion of fatty acid multiplied by the total number of double bonds in the FA, was significantly lower in hibernation compared to summer and prehibernation (p=0.001 and p=0.017, respectively; Fig. 2.2; Supplementary Table S.1), by approximately 10%. A significant difference in the MUFA:PUFA ratio, calculated as the sum of the proportion of MUFAs divided by the proportion of PUFAs, was also measured in muscle mitochondria between summer versus prehibernation and hibernation (p=0.032 and 0.014, respectively; Fig. 2.2; Supplementary Table S.1); the ratio of MUFAs to PUFAs was approximately 38% higher in hibernation. In contrast, the n3:n6 PUFA ratio was significantly lower in hibernation compared to summer (p=0.003) and prehibernation (p=0.023), by approximately 44% and 31%, respectively (Fig. 2.2; Supplementary Table S.1).

## II.C.4. *Liver Mitochondria*

### II.C.4.a. Mitochondrial Respiration

In liver mitochondria, similar trends were seen in total OXPHOS capacity ($CI+II_P$) in the presence (Fig. 2.4A) and absence of FAs (Fig. 2.4B) across seasons, but significant differences were only detected as higher leak and Complex I-linked OXPHOS capacities ($PM_P$ and $CI_P$) in prehibernation (for PM, ADP, and Glut, summer vs. prehibernation: p=0.007, p=0.002, and p=0.023, respectively; prehibernation vs. hibernation: p=0.020, p=0.0002, and p=0.001, respectively) No significant differences (p>0.05) were measured across seasons regarding the coupling control factors (Fig 2.4C-D), though there was a trend for lower pyruvate OXPHOS coupling control in hibernation compared to summer ($FAO_{P-L/P}$; P = 0.184).

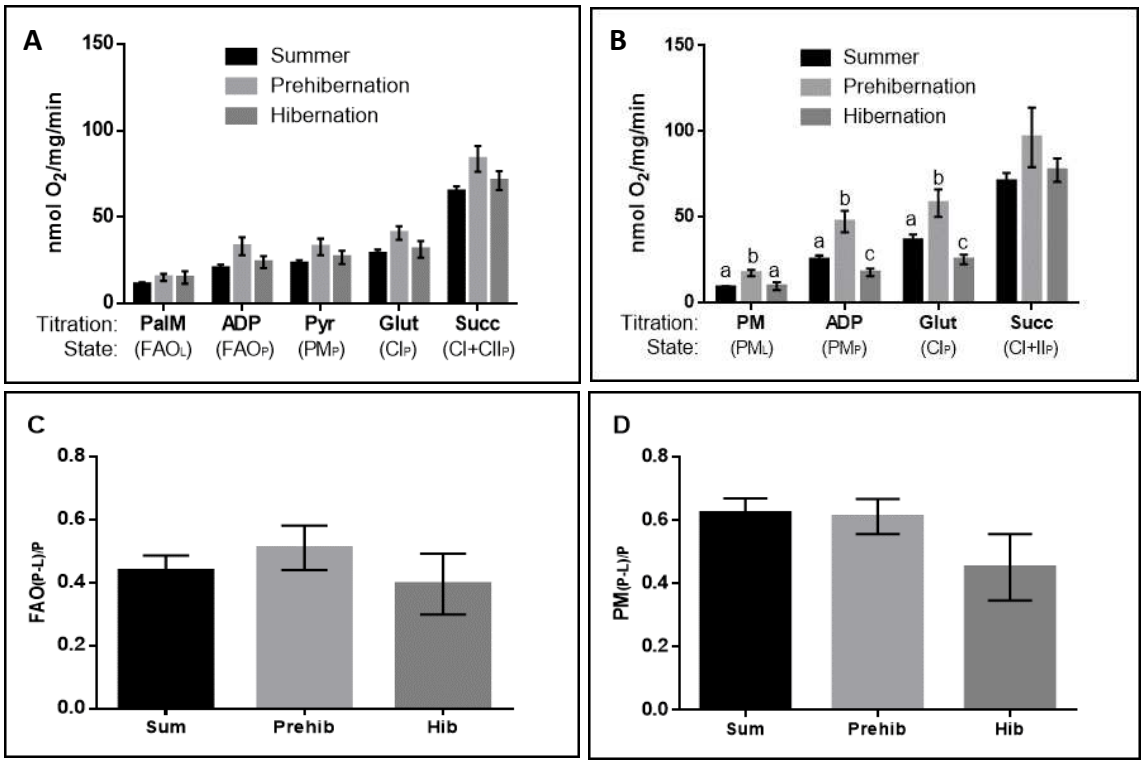


**Figure 2.4. Liver mitochondrial respiration.** (A) A trend of increased respiration in prehibernation was measured across substrates in the presence of FAs, but differences among seasons were not significant. (B) Pyruvate and Complex I-linked OXPHOS capacity (ADP and Glut) in the absence of FAs were elevated in prehibernation versus summer and hibernation.

LEAK respiration (PM) was elevated in prehibernation versus summer and hibernation (for PM, ADP, and Glut, summer vs. prehibernation: p=0.007, p=0.002, and p=0.023, respectively; prehibernation vs. hibernation: p=0.020, p=0.0002, and p=0.001, respectively). Note that across tissues, the range on the y-axis varies for A and B. (C) FA OXPHOS coupling control factor, calculated as (P-L)/P using the FAO OXPHOS capacity ($FAO_P$) and preceding LEAK respiration without ADP ($FAO_L$) in A. (D) OXPHOS coupling control factor for pyruvate oxidation, calculated as (P-L)/P using the pyruvate+malate OXPHOS capacity ($PM_P$) and preceding LEAK respiration without ADP ($PM_L$) in B. Different labels (letters) within a substrate group indicate a significant difference between seasonal groups. Summer: N = 9; Prehibernation: N = 9; Hibernation: N = 8.

II.C.4.b. Phospholipid Fatty Acid Analysis

Significant differences across seasons were measured in four phospholipid FAs of the liver mitochondrial membrane (Fig. 2.2; Supplementary Table S.1). Percentages of 16:0 significantly increased while percentages of 18:3n3 significantly decreased from summer to prehibernation (p=0.049 and p=0.047, respectively), summer to hibernation (p=0.002 and p=0.010, respectively), and prehibernation to hibernation (p=0.028 and p=0.038, respectively). Additionally, 18:2n6 significantly increased in hibernation versus summer and prehibernation by approximately 23-27% (p=0.017 and p=0.009, respectively), while 20:3n6 significantly decreased in hibernation versus summer and prehibernation (p=0.009 and p=0.001, respectively). A significant decrease in the MUFA:PUFA ratio was also measured in liver mitochondria from prehibernation to hibernation (p=0.018); the ratio of MUFAs to PUFAs was approximately 75% higher in prehibernation. Similarly, the n3:n6 ratio significantly decreased in hibernation versus summer by approximately 40% (p=0.031; Fig. 2.2; Supplementary Table S.1).

## II.C.5. *BAT Mitochondria*

### II.C.5.a. Mitochondrial Respiration

Maximal leak respiration, the primary role of BAT mitochondria, was approximately 95% higher in prehibernation versus summer (p=0.008) in the absence of FAs (Succ; Figure 2.5). In the presence of FAs (Pal), maximal leak respiration was approximately 106% and 73% higher in prehibernation and hibernation versus summer (p=0.011 and p=0.032, respectively), consistent with activation of BAT UCP1 and mitochondrial uncoupling by FAs. As expected, ADP suppressed respiration in BAT mitochondria (Cannon and Nedergaard, 2008), but was still 53% and 69% higher in prehibernation and hibernation versus summer, respectively, indicating an increase in total respiratory capacity of BAT mitochondria in colder seasons. No coupling control factors were calculated across seasons in BAT mitochondria due to the almost exclusively uncoupled respiration occurring in this tissue.

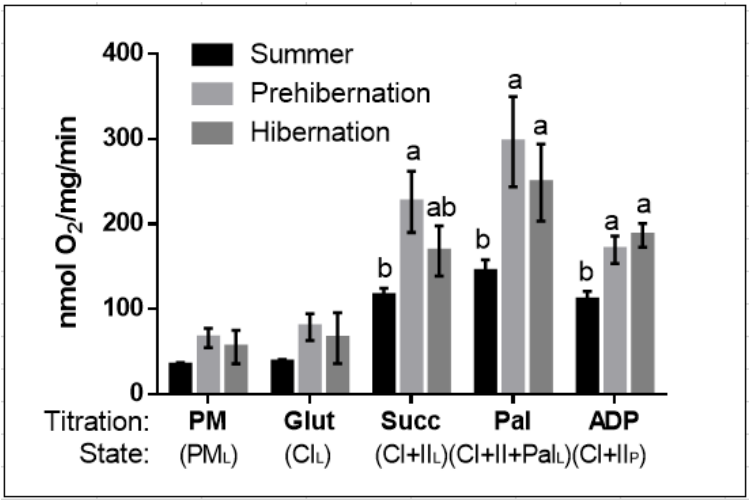


**Figure 2.5. BAT mitochondrial respiration.** Maximal leak respiration (without ADP) in the absence (Succ) and presence (Pal) of FAs, as well as maximal OXPHOS capacity (ADP), was highest in prehibernation and hibernation (for Succ, Pal, and ADP, summer vs. prehibernation: p=0.014, p=0.022, and p=0.017, respectively; for ADP only, summer vs. hibernation: p=0.005). However, this trend is not significant between summer and hibernation maximal LEAK rates in the absence and presence of FAs. Different labels (letters) within a substrate group indicate a

significant difference between seasonal groups. Summer: N = 9; Prehibernation: N = 9; Hibernation: N = 8.

II.C.5.b. Phospholipid Fatty Acid Analysis

Significant differences across seasons were measured in the three phospholipid FAs of the BAT mitochondrial membrane (Fig. 2.2; Supplementary Table S.1). The percentage of 16:0 was significantly higher in summer and prehibernation (p=0.031 and p=0.049, respectively) versus hibernation. Additionally, 18:3n3 and 20:3n6 significantly increased in summer versus prehibernation (p=0.004 and p=0.026, respectively) and hibernation (p=0.037 and p=0.015, respectively). Also, the n3:n6 ratio significantly decreased in hibernation versus summer by approximately 49% (p=0.044; Fig. 2.2; Supplementary Table S.1).

## II.D. Discussion

The present study is the first to examine tissue-specific responses of mitochondrial respiration and membrane composition to changing seasons in a hibernating mammal. While some consistent trends were found, functional responses of mitochondria appear to reflect the distinct physiological demands and plasticity of different GMGS tissues from the summer to winter hibernation seasons. Tissue-specific changes in mitochondrial membrane composition were also seen, but decreases in DHA and the n3:n6 ratio from summer to winter were among the most consistent changes across tissues. The uniformity of this trend suggests that changes in organismal PUFA metabolism occur from summer to hibernation, but argues against a major role for membrane FA remodeling as an explanation for seasonal changes in mitochondrial respiration.

### II.D.1. *Heart Mitochondrial Respiration*

Cardiac mitochondrial OXPHOS capacity increased from summer to the prehibernation season with all substrates, but tended to decline thereafter, particularly in the presence of FAs (Fig. 2.1A). This may reflect effects of cold exposure and/or excess nutrient supply on transcriptional regulators of mitochondrial protein expression in the heart previously reported by others (Klingenspor et al. 1996; Nisoli et al. 2003; Li et al. 2005; Wise et al. 2008; Gao et al. 2009). Wu et al. (1999) demonstrated that in mouse C2C12 heart and skeletal muscle cells, cold-exposure activates proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) to induce expression of uncoupling protein-2 (UCP2) and nuclear respiratory factors, which have been found to increase during cold exposure in rats and mice (Boss et al. 1997; Lelliott et al. 2006). PGC-1α is also recognized as a transcriptional regulator of adaptive thermogenesis by BAT in response to overfeeding (Rothwell and Stock, 1979). Consistent with these previous findings, increases in the respiratory capacity of cardiac mitochondria herein were not associated with improvements in coupling control, indicating a commensurate upregulation of both OXPHOS and leak respiratory capacities in the colder, hyperphagic weeks preceding hibernation.

Regarding substrate control of respiration, FAs appear able to suppress maximal rates of carbohydrate oxidation in hibernation (Fig. 2.1A versus Fig. 2.1B) compared to summer and prehibernation. This is consistent with a Randle-like inhibition of carbohydrate utilization by FAs in hibernation, when GMGS rely almost exclusively on lipolysis of adipose tissue FAs (South and House 1967). Additionally, previous studies have reported a decrease in pyruvate dehydrogenase (PDH) activity in the heart due to an upregulation of pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), which converts PDH to its inactive form via phosphorylation

(Andrews et al. 1998); this is consistent with the fact that glycolytic intermediates do not enter the tricarboxylic acid (TCA) cycle during hibernation (Tashima et al. 1970; Brooks and Storey 1992).

### II.D.2. *Muscle Mitochondrial Respiration*

OXPHOS capacities of skeletal muscle mitochondria were largely unaffected by seasons, but a greater inhibitory effect of FAs on Complex I+II OXPHOS capacity in hibernation months perhaps indicates a shift toward FA over carbohydrate oxidation during hibernation. As described for cardiac mitochondria, this is reasonable considering the whole-body shift to a greater reliance on lipids versus carbohydrates for energy during hibernation (South and House 1967; Carey et al. 2003). However, there was a trend for decreased OXPHOS capacity in hibernation across seasons (with the exception of increased leak respiration, $FAO_L$, with FAs present), with FAs both present and absent (Fig. 2.3A and 2.3B). This effect may reflect the progressive inactivity of GMGS in the weeks preceding hibernation, which has been reported in a number of hibernating species (Musacchia et al. 1988; Hudson and Franklin 2002; Shavlakadze and Grounds 2006). Moreover, James et al. (2013) reported decreased respiration, suppression of ATP production capacity, and reduced fatigue resistance in skeletal muscle mitochondria from hibernating thirteen-lined ground squirrels.

Notably, skeletal muscle was the only tissue to show significant differences between seasons regarding mitochondrial coupling control factors (Fig. 2.3C and 2.3D). We measured a significant decrease in FA OXPHOS coupling control and a trend for decreased pyruvate + malate OXPHOS coupling control in hibernation, caused primarily by an increase in leak respiration ($FAO_L$ and $PM_L$, respectively) during this season. Leak respiration is defined as

oxygen consumption that occurs in the absence of (i.e., is "uncoupled" from) ADP phosphorylation, reflecting a dissipation of mitochondrial membrane potential independent of proton flux through the ATP synthase (Chicco et al. 2014; van den Berg et al. 2011). This "uncoupled" respiration is classically associated with the thermogenic activity of BAT mitochondria facilitated by high levels uncoupling protein 1 (UCP1), but is hypothesized to occur to some degree in other tissues, including skeletal muscle (Rowland et al. 2015). UCP3 is found almost exclusively in the skeletal muscle of humans and rodents; however, unlike UCP1, UCP3 supposedly contributes more to the limitation of cellular free radical levels rather than to mitochondrial uncoupling and thermogenesis (Rousset et al. 2004). Harper et al. (2002) also suggested that in the skeletal muscle of rodents, the primary function of UCP3 is to export FA anions outside of the mitochondrial matrix when a large excess of fatty acids exists within the mitochondria. Perhaps the increase in fatty acid-driven leak respiration observed during hibernation in the muscle of GMGS could be due to a saturating amount of fatty acids and subsequent increase in UCP3 activity, rather than an increase in heat production for thermogenic purposes.

Additionally, overexpression of the cold-induced PGC-1α discussed above increases OXPHOS uncoupling in skeletal muscle mitochondria of mice (Miura et al. 2006), and Eddy et al. (2005) reported that in hibernating thirteen-lined ground squirrels, PGC-1α increased 1.8-fold in skeletal muscle. Brustovetsky et al. (1992) reported increased proton leak in skeletal muscle mitochondria from hibernating arctic ground squirrels, which may have been due to FA-induced uncoupling (Sultan and Sokolove 2001). Therefore, while intriguing, the mechanisms and physiological relevance of reduced OXPHOS coupling control of muscle mitochondria in the adaptive phenotype of hibernating mammals requires further investigation.

### II.D.3. *Liver Mitochondrial Respiration*

In liver mitochondria, CI and CI+II-linked OXPHOS capacities were greatest during the hyperphagic prehibernation period measured in the absence of FAs, suggestive of a greater hepatic carbohydrate oxidation capacity in this season. Suppression of this effect by the presence of FAs suggests a strong Randle-like inhibition of carbohydrate oxidation, perhaps favoring lipid and glycogen synthesis when FA fuel availability is high (Fig. 2.4A and 2.4B). When ATP demands are lower during prehibernation due to increased caloric intake and preparation for hibernation, GMGS liver may preferentially oxidize carbohydrates to increase the storage of lipids in the liver. As GMGS become progressively more inactive in the weeks preceding hibernation, the increase in the prevalence of skeletal muscle disuse atrophy causes amino acids to be released and subsequently taken up by the liver. The liver then utilizes these amino acids to generate glucose, which can contribute to the TCA cycle and carbohydrate oxidation within the mitochondria. This idea is reinforced by Brustovetsky et al. (1989), who reported that substances which feed reducing equivalents to Complexes I and II of the electron transport chain (ETC) (i.e. carbohydrates such as pyruvate and succinate, respectively) lead to significantly reduced respiration rates in liver mitochondria from hibernating ground squirrels, consistent with our data and several others demonstrating suppression of OXPHOS capacity during hibernation (Martin et al. 1999; Barger et al. 2003; Muleme et al. 2006), reflecting an overall suppression of metabolic activity in the liver during this season, including decreased protein synthesis (Zhegunov et al. 1988) and translation (Knight et al. 2000; Van Breukelen and Martin 2001; Hittel and Storey 2002). Additionally, the upregulation of liver mitochondrial OXPHOS respiration in the absence of fatty acids we measured in GMGS may not occur naturally *in vivo*, as our protocols use saturating amounts of each substrate and do not account for other factors

such as a high concentration of fatty acids in the bloodstream which could also influence the metabolism of the liver. Interestingly, while there is little in the hibernation literature regarding hepatic mitochondrial respiration in the prehibernation season, OXPHOS capacity of liver mitochondria in non-hibernating rodents is actually suppressed by hyperphagia and obesity (Chavin et al. 1999; Holmström et al. 2012). This suggests that the mechanisms underlying responses of hepatic fuel handling to hyperphagia in pre-hibernating mammals are distinct from classic "pathogenic" obesity in non-hibernators.

### II.D.4. *BAT Mitochondrial Respiration*

It is important to note that the order of titrated substrates added to BAT mitochondria was different compared to the other tissues included in our study. This is because in BAT, UCP1 dissipates much of the proton gradient generated by the electron transport system (ETS) as heat rather than harnessing it to generate ATP. This "uncoupled" respiration in BAT mitochondria is a primary source of non-shivering thermogenesis (NST) that occurs at a Tb generally below 15-16$^{o}$C when shivering thermogenesis in the skeletal muscle is nonfunctional (Hayward and Lyman, 1967; Hudson 1967). Therefore, our respirometry protocol prioritized assessment of leak respiration states ($PM_L$, $CI_L$, $CI+II_L$, and $CI+II+Pal_L$ in Fig. 2.5), and demonstrates that the addition of ADP actually leads to expected decreases in respiration in BAT mitochondria (Cannon and Nedergaard, 2008). These studies indicate that BAT mitochondrial respiratory capacity was highest during the prehibernation and hibernation seasons compared to summer, consistent with an increased reliance on NST during these periods. Consistent with this finding, cold exposure upregulated BAT oxidative capacity and UCP1 content in non-hibernating mammals such as the golden hamster (Sundin et al. 1987), which was further enhanced in cold-

exposed hibernators such as the Richardson's ground squirrel (Milner et al. 1989). However, it is worth noting that BAT activity may be reversibly inhibited in hibernation due to the absence of adrenergic stimulation in torpor (Staples and Brown 2008), with its thermogenic role being more important for interbout arousals and colder periods preceding hibernation. Thus, seasonal changes in the maximal respiratory flux of tissue mitochondria reflect adaptations of their capacities, but not necessarily their utilization in the complex metabolic maintenance of homeostasis in hibernating mammals throughout the year.

### II.D.5. *Mitochondrial Phospholipid Fatty Acid Composition*

While there were some tissue-specific differences in phospholipid FA composition of mitochondria that merit discussion, they did not appear to explain the distinct functional responses of respiration to changing seasons. Additionally, it is unclear based on previous studies whether fatty acid changes within mitochondrial membranes can cause long-term versus transient biological changes across seasons. DHA (22:6n3) significantly decreased from summer to hibernation in heart and skeletal muscle mitochondria, with a similar trend observed in liver and BAT (Fig. 2.2; Supplementary Table S.1). This is in contrast to increased proportions of DHA in mitochondrial membranes of cold-acclimated ectotherms such as fish (Hazel 1995), but consistent with the findings of Aloia and Pengelley (1979) in heart mitochondria of GMGS. Based on the existing literature, it is uncertain if products generated by DHA such as anti-inflammatory protectins and resolvins have any functional relevance during hibernation, particularly if DHA decreased over seasons. Ruf and Arnold (2008) noted a similar decrease in cardiac membranes that led to an overall reduction in the membrane unsaturation index and n3:n6 ratio as we observed in the present study. The decrease in the unsaturation index across

seasons in the GMGS is inconsistent with previous fatty acid analyses in cold-acclimated fish (Cossins et al. 1977; Guderley et al. 2004), non-hibernating mice and rats (Williams and Platner 1967; Hazel 1995; Jakobsson et al. 2006), and rodents that undergo hibernation or daily torpor (Geiser 1991; Geiser et al. 1992; Hiebert et al. 2000). However, most of these studies analyzed phospholipid fatty acid composition from either whole tissue samples or plasma membranes in contrast to the mitochondrial membranes we used for phospholipid extraction, which could explain the decrease in the unsaturation index we measured from summer to hibernation.

The decrease in the n3:n6 ratio is consistent with a conservation of the n6 PUFA linoleic acid during hibernation, and loss of n3 PUFA in hibernators upon entering a laboratory environment (Florant 1998). Generally, the percentage of 18:3n3 (a metabolic precursor of DHA) is highest in the summer, when GMGS consume large amounts of this essential n3 PUFA in the form of leafy vegetation in the wild. Once animals are transported to the lab and provided a rodent chow diet (relatively poor in 18:3n3), the percentage of 18:3n3 decreases in their membranes. This loss may be exacerbated by a preferential oxidation of 18:3n3 prior to hibernation (Frank and Storey 1995; Hill and Florant 1999). Thus, it is unclear whether these changes represent intrinsic effects of season on PUFA metabolism or confounding effects of the laboratory setting. A decrease in DHA in BAT mitochondrial membranes has been reported in laboratory rodents in response to cold exposure by Ohno et al. (1996) and Ocloo et al. (2007), but the functional relevance of this effect in BAT or other tissue mitochondria of hibernators will require additional studies specifically designed to investigate this phenomenon.

Another consistent finding among three of the four tissues was a significant decrease in palmitic acid (16:0) from summer to hibernation in skeletal muscle and BAT, which trended the same in heart, but opposite in liver mitochondria (Fig. 2.2; Supplementary Table S.1). A loss of

16:0 in BAT mitochondria is consistent with the findings of Ocloo et al. (2007) in cold-acclimated laboratory rodents, suggesting that at least in this tissue, a decrease in 16:0 may be cold-induced. The increased percentage of 16:0 in liver mitochondrial membranes has also been reported in hibernating thirteen-lined ground squirrels (Platner et al. 1976, Chung et al. 2011) compared to summer or periods of interbout euthermia. However, whether changes in mitochondrial membrane FAs reflect changes in other tissue FA depots or result from independent regulatory processes cannot be determined from this study.

### II.D.6. *Conclusions and Future Directions*

In summary, the present study demonstrates intrinsic adaptations of mitochondrial respiratory capacities, substrate utilization, and OXPHOS coupling control to changing seasons in the GMGS. Responses varied among the four tissues investigated, likely reflecting tissue-specific roles in metabolic substrate handling and energy supply/demand as animals progress from the active summer months to the colder, increasingly sedentary prehibernation and hibernation seasons. Dissecting out the relative contributions of seasonal temperature, diet, and activity to these responses merits further investigation, and may yield novel insights relevant to the study of hibernating mammal physiology and metabolic (dys)regulation in obesity. The incongruence of seasonal variations in tissue mitochondrial membrane composition with respiratory function, while potentially important, argues against a direct mechanistic link between these parameters in GMGS.

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# CHAPTER III: CONCLUSIONS AND FUTURE DIRECTIONS

## III.A. Conclusions

Mammals that hibernate, such as the golden-mantled ground squirrel (*Callospermophilus lateralis*; GMGS), cease to feed, reduce metabolic rate, and lower body temperature during the winter months, surviving almost exclusively on the oxidation of lipids from endogenous fat stores. Hibernating mammals exhibit remarkable metabolic plasticity that enables them to survive these seasonal changes in ambient temperature and food availability. However, whether mitochondria, the cellular sites of oxidative metabolism, undergo adaptive changes in response to hypometabolism and decreasing ambient temperature to facilitate this remarkable phenotype is unclear.

To my knowledge, my thesis project is the first study which comprehensively investigated mitochondrial respiratory function and membrane FA composition in heart, muscle, liver, and BAT from the warm summer months, to fall and winter in a hibernator. While some consistent trends were found, functional responses of mitochondria appeared to reflect the distinct physiological demands and plasticity of different GMGS tissues from the summer to winter hibernation seasons. This supports Staples' (2016) prediction that ATP supply and demand change synchronously in temporal heterotherms during the reversible metabolic suppression which characterizes torpor.

Cardiac mitochondrial OXPHOS capacity increased from summer to the prehibernation season with all substrates, but tended to decline thereafter, particularly in the presence of FAs; we associated this effect with cold exposure and/or excess nutrient supply on transcriptional regulators of mitochondrial protein expression in the heart as previously reported by others

(Klingenspor et al. 1996; Nisoli et al. 2003; Li et al. 2005; Wise et al. 2008; Gao et al. 2009). However, whereas mitochondrial respiration in thirteen-lined ground squirrels was suppressed by only 30% in hibernation (Brown et al. 2013) and was not at all suppressed in Djungarian hamsters (Kutschke et al. 2013), I would venture to say that out of the four tissues analyzed in our study, respiratory changes in GMGS cardiac mitochondria across seasons were most evident.

OXPHOS capacities of skeletal muscle mitochondria were largely unaffected by seasons, agreeing with what several other studies have reported in hibernating mammals (Barger et al. 2003; Muleme et al. 2006; Kutschke et al. 2013). However, a greater inhibitory effect of FAs on Complex I+II OXPHOS capacity during winter perhaps reflected a greater reliance on lipids versus carbohydrates for energy during hibernation in torpid GMGS (South and House 1967; Carey et al. 2003). Additionally, there was a trend for decreased OXPHOS capacity in hibernation across tissues in torpid GMGS, which we suggest may parallel the progressive muscle disuse that GMGS experience in the weeks preceding hibernation (Musacchia et al. 1988; Hudson and Franklin 2002; Shavlakadze and Grounds 2006).

In liver mitochondria, CI and CI+II-linked OXPHOS capacities were greatest during the hyperphagic prehibernation period measured in the absence of FAs, suggestive of a greater hepatic carbohydrate oxidation capacity in this season; this may allow prehibernating GMGS to preferentially oxidize carbohydrates to increase the storage of lipids in the liver. Similar to other studies, liver OXPHOS was also suppressed during hibernation in our GMGS, across most substrates (Martin et al. 1999; Barger et al. 2003; Muleme et al. 2006). In BAT mitochondria, respiratory capacity was highest during the prehibernation and hibernation seasons compared to summer, consistent with an increased reliance on cold-induced NST during these periods (Sundin et al. 1987; Milner et al. 1989; Ocloo et al. 2007). Our respiratory data in both liver and

BAT appears to be consistent with the published results from other related studies in the hibernation field.

Perhaps any differences between our mitochondrial respiration results and results from related studies in hibernators in heart, muscle, liver, and BAT are due to variations in methods, including respiratory equipment utilized and assay temperature of experiments. Although we exclusively analyzed our homogenized tissue samples at 37°C, Staples (2016) recommends a range of physiologically-relevant temperatures should be used to evaluate mitochondrial respiration in heterotherms, despite Brown et al. (2012) reporting 10°C is the minimum assay temperature for collecting reliable respiratory data. Differences in substrate protocols, or lack of physiologically-accurate amounts of substrates, may also contribute to inconsistencies in reported mitochondrial respiration data. For example, Staples (2016) notes that although adding succinate generally causes the greatest increase in OXPHOS during mitochondrial experiments, succinate is a less common substrate *in vivo* versus such substrates as pyruvate, glutamate, and palmitoyl carnitine. Any variations in our data across seasons (i.e. summer, prehibernation, and hibernation) versus previous studies could also in part be due to a lack of data measured over torpor bouts (i.e. entrance into torpor, early torpor, interbout arousals, late torpor, and cessation of torpor) in hibernation; Carey et al. (2003) describe these time points of torpor as physiological states distinct from the overarching state of hibernation. Furthermore, though daily torpor is also characterized by a suppression of metabolic processes as occurs in the aforementioned Djungarian hamster, comparisons between daily and obligate hibernators should be made cautiously, as metabolic rate is generally not suppressed below 26% of normal levels (Ruf and Geiser 2015). Animals which undergo daily torpor are also able to rely more on passive thermal effects, unlike obligate hibernators (Staples 2016).

Despite our original hypothesis suggesting that changes in the phospholipid FA composition of the mitochondrial membrane could influence the mitochondrial respiratory capacity, in part due to the dynamic remodeling of mitochondrial membranes reported in hibernating mammals both over torpor bouts (Armstrong et al. 2011; Chung et al. 2011) and hibernation (Arnold et al. 2011), our mitochondrial respiration and membrane composition results were not correlative. An increase in mitochondrial cristae abundance has also been reported during hibernation, and this increase has been correlated to a whole-body shift from carbohydrate to FA substrate utilization (Halestrap and Dunlop 1986; Halestrap 1987; Loncar et al. 1988; Morroni et al. 1995). Therefore, though our hypothesis was not supported, it seemed reasonable to postulate that changes in the FA composition of the mitochondrial membrane of different tissues could influence other mitochondrial properties.

Tissue-specific changes in mitochondrial membrane composition were seen, but significant decreases (and parallel trends) in DHA and the n3:n6 ratio from summer to winter were among the most consistent changes measured across all tissues. The uniformity of this trend suggests that changes in organismal PUFA metabolism occur from summer to hibernation, but argues against a major role for membrane FA remodeling as an explanation for seasonal changes in mitochondrial respiration. This modest lack of consistent, significant changes in phospholipid FAs across tissues and seasons could be partially due to the low sample size associated with our gas chromatography experiments (n=3) in hibernation, though our lack of significant differences in FAs of the mitochondrial membrane is supported by Chung et al. (2011), who reported that any transient changes in the mitochondrial membrane composition did not correlate with mitochondrial respiration.

### III.B. Future Directions

In summary, the present study demonstrated intrinsic adaptations of mitochondrial respiratory capacities, substrate utilization, and OXPHOS coupling control to changing seasons in the GMGS. Responses varied among the four tissues investigated, likely reflecting tissue-specific roles in metabolic substrate handling and energy supply/demand as animals progress from the active summer months to the colder, increasingly sedentary prehibernation and hibernation seasons. The incongruence of seasonal variations in tissue mitochondrial membrane composition with respiratory function, while potentially important, argues against a direct mechanistic link between these parameters in GMGS. Dissecting out the relative contributions of seasonal temperature, diet, and activity to these responses merits further investigation, and may yield novel insights relevant to the study of hibernating mammal physiology and metabolic (dys)regulation in obesity.

Further investigation of how mitochondrial respiration and phospholipid FA composition across seasons differ from the time points of a torpor bout would provide an interesting though time-consuming comparison across tissues. Additionally, measuring mitochondrial enzyme activity across tissues to compare with both mitochondrial OXPHOS capacity and membrane composition would be a logical next step in further clarifying dynamic mitochondrial properties of hibernators across seasons.

One of the principal questions that surfaced from our results was in relation to cold exposure in the cardiac mitochondria. OXPHOS was upregulated with both FAs present and absent in the cardiac mitochondria of prehibernating GMGS, but was this truly influenced by the effect of cold exposure? Prehibernation is the first season in which the GMGS experience a large drop in Ta, so it seemed reasonable that an increase in respiratory capacity of the mitochondria

could potentially be a cold-induced, cardioprotective adaptation of a hibernating mammal. In exposing a prehibernating group of GMGS to a Ta typical of the summer season (22°C) rather than the natural Ta of prehibernation (15°C), we measured OXPHOS with FAs both present and absent, body mass and body fat %, and glucose tolerance in heart, liver, and skeletal muscle (unpublished data). Based on our findings, we concluded that seasonal cold exposure modifies metabolic responses to obesity in "obese" pre-hibernating GMGS, leading to improvements in systemic glucose disposal, enhancement of cardiac mitochondrial OXPHOS capacity, and enhancement of lipid OXPHOS in liver. In a biological context, cold exposure appeared to facilitate tissue-specific alterations in mitochondrial metabolism that may be advantageous for successful hibernation; in a biomedical context, cold exposure appeared to modulate the metabolic effects of hyperphagia on glucose tolerance. Further investigation of this effect, including the occurrence of "beiging" in the WAT of hibernating mammals across seasons, may yield insights into the mechanisms that link obesity to diabetes in humans. Additionally, cold exposure has marked tissue-specific effects on mitochondrial metabolism that parallel improvements in glucose tolerance. Mechanistic links between these phenomena are unclear, but merit further investigation to better understand the role of mitochondria in thermal adaptation and diabetes.

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# APPENDIX: SUPPLEMENTARY MATERIALS

**Supplementary Table S.1. Mitochondrial phospholipid fatty acid composition in GMGS across seasons.** Values shown are mean percentages of total phospholipid FAs ± SEM. Within each tissue, different labels (letters) across a FA row indicate a significant difference between seasonal groups at $P < 0.05$. Abbreviations not listed above: DHGLA, dihomogammalinolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; UI, the unsaturation index; MUFA:PUFA, the ratio of monounsaturated FAs to polyunsaturated FAs; n3:n6, the ratio of n3 FAs to n6 FAs. For heart, Summer: N = 8; Prehibernation: N = 9; Hibernation: N = 6. For muscle, liver, and BAT, Summer: N = 5; Prehibernation: N = 6; Hibernation: N = 3.

| | Heart | | | Muscle | | |
|---|---|---|---|---|---|---|
| | **Summer** | **Prehibernation** | **Hibernation** | **Summer** | **Prehibernation** | **Hibernation** |
| **16:0, Palmitic** | 15.11 ± 0.51 | 14.81 ± 0.52 | 12.26 ± 1.06 | **10.58 ± 0.12[a]** | **9.73 ± 0.50[a]** | **8.19 ± 0.57[b]** |
| **16:1, Palmitoleic** | 0.60 ± 0.13 | 0.60 ± 0.06 | 0.72 ± 0.14 | 0.49 ± 0.10 | 0.60 ± 0.04 | 0.79 ± 0.14 |
| **18:0, Stearic** | 19.50 ± 0.49 | 19.98 ± 0.30 | 19.88 ± 0.97 | **20.20 ± 0.39[a]** | **21.39 ± 0.36[b]** | **24.44 ± 0.56[c]** |
| **18:1n9, Oleic** | **8.04 ± 0.89[a]** | **7.54 ± 0.44[a]** | **10.39 ± 0.80[b]** | 7.31 ± 0.31 | 7.92 ± 0.65 | 9.44 ± 0.47 |
| **18:1n7, Vaccenic** | 2.62 ± 0.20 | 2.81 ± 0.10 | 2.31 ± 0.24 | **3.35 ± 0.46[a]** | **4.62 ± 0.13[b]** | **3.82 ± 0.41** |
| **18:2n6, Linoleic** | 30.64 ± 0.89 | 31.15 ± 1.44 | 30.34 ± 2.47 | 30.17 ± 0.92 | 27.82 ± 1.28 | 28.68 ± 0.59 |
| **18:3n3, Linolenic** | **0.30 ± 0.02[a]** | **0.18 ± 0.02[b]** | **0.24 ± 0.04** | 0.22 ± 0.03 | 0.18 ± 0.01 | 0.21 ± 0.03 |
| **20:3n6, DHGLA** | 0.36 ± 0.07 | 0.37 ± 0.03 | 0.29 ± 0.09 | 0.56 ± 0.04 | 0.52 ± 0.05 | 0.54 ± 0.07 |
| **20:4n6, AA** | 13.74 ± 0.64 | 14.02 ± 0.67 | 15.21 ± 1.06 | 17.60 ± 0.38 | 19.57 ± 1.56 | 18.37 ± 0.20 |
| **20:5n3, EPA** | 0.09 ± 0.02 | 0.13 ± 0.02 | 0.34 ± 0.12 | 0.20 ± 0.01 | 0.31 ± 0.06 | 0.26 ± 0.10 |
| **22:5n3, DPA** | 3.15 ± 0.63 | 3.42 ± 0.79 | 3.75 ± 1.66 | 2.09 ± 0.22 | 1.90 ± 0.24 | 1.67 ± 0.26 |
| **22:6n3, DHA** | **5.85 ± 0.81[a]** | **4.99 ± 0.69[a]** | **2.15 ± 0.20[b]** | **7.26 ± 0.88[a]** | **5.43 ± 0.64[a]** | **3.26 ± 0.12[b]** |
| **UI** | 1.81 ± 0.05 | 1.79 ± 0.03 | 1.66 ± 0.11 | **1.99 ± 0.04[a]** | **1.93 ± 0.06[a]** | **1.76 ± 0.01[b]** |
| **MUFA:PUFA** | 0.21 ± 0.03 | 0.20 ± 0.01 | 0.27 ± 0.03 | **0.19 ± 0.02[a]** | **0.24 ± 0.01** | **0.27 ± 0.02[b]** |
| **n3:n6** | **0.21 ± 0.02[a]** | **0.20 ± 0.03** | **0.13 ± 0.04[b]** | **0.20 ± 0.02[a]** | **0.16 ± 0.02[a]** | **0.11 ± 0.01[b]** |

| | Liver | | | BAT | | |
|---|---|---|---|---|---|---|
| | **Summer** | **Prehibernation** | **Hibernation** | **Summer** | **Prehibernation** | **Hibernation** |
| **16:0, Palmitic** | **10.12 ± 0.77[a]** | **12.15 ± 0.78[b]** | **14.30 ± 0.51[c]** | **8.00 ± 0.59[a]** | **8.42 ± 0.95[a]** | **7.74 ± 0.15[b]** |
| **16:1, Palmitoleic** | 0.76 ± 0.16 | 1.07 ± 0.10 | 1.25 ± 0.35 | 0.33 ± 0.04 | 0.54 ± 0.14 | 1.00 ± 0.17 |
| **18:0, Stearic** | 19.68 ± 0.87 | 19.21 ± 1.22 | 19.51 ± 0.33 | 22.35 ± 0.59 | 22.20 ± 2.14 | 20.49 ± 0.80 |
| **18:1n9, Oleic** | 19.45 ± 2.61 | 20.87 ± 2.32 | 12.62 ± 0.52 | 13.89 ± 0.78 | 16.21 ± 1.68 | 16.81 ± 0.81 |
| **18:1n7, Vaccenic** | 2.55 ± 0.47 | 3.09 ± 0.25 | 2.84 ± 0.45 | 2.48 ± 0.40 | 2.73 ± 0.14 | 2.69 ± 0.26 |
| **18:2n6, Linoleic** | **24.75 ± 1.61[a]** | **23.87 ± 0.59[a]** | **30.32 ± 1.23[b]** | 38.11 ± 0.94 | 34.82 ± 0.98 | 37.89 ± 1.16 |
| **18:3n3, Linolenic** | **0.62 ± 0.05[a]** | **0.51 ± 0.03[b]** | **0.32 ± 0.06[c]** | **0.61 ± 0.06[a]** | **0.30 ± 0.07[b]** | **0.36 ± 0.09[b]** |
| **20:3n6, DHGLA** | 1.37 ± 0.24 | 0.99 ± 0.07 | 0.49 ± 0.06 | **0.34 ± 0.04[a]** | **0.22 ± 0.03[b]** | **0.21 ± 0.01[b]** |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **20:4n6, AA** | 16.81 | ± | 0.64 | 15.52 | ± | 1.21 | 15.76 | ± | 0.44 | 7.79 | ± | 0.66 | 10.99 | ± | 1.12 | 9.65 | ± | 0.92 |
| **20:5n3, EPA** | **0.25** | **±** | **0.03[a]** | **0.21** | **±** | **0.03[a]** | **0.14** | **±** | **0.02[b]** | 0.04 | ± | 0.03 | 0.06 | ± | 0.01 | 0.11 | ± | 0.03 |
| **22:5n3, DPA** | 0.90 | ± | 0.23 | 0.64 | ± | 0.14 | 0.66 | ± | 0.07 | 1.23 | ± | 0.24 | 0.88 | ± | 0.13 | 0.86 | ± | 0.18 |
| **22:6n3, DHA** | 2.75 | ± | 0.64 | 1.92 | ± | 0.43 | 1.79 | ± | 0.13 | 4.82 | ± | 1.16 | 2.63 | ± | 0.51 | 2.20 | ± | 0.58 |
| **UI** | 1.68 | ± | 0.07 | 1.55 | ± | 0.06 | 1.58 | ± | 0.01 | 1.62 | ± | 0.07 | 1.55 | ± | 0.04 | 1.55 | ± | 0.04 |
| **MUFA:PUFA** | **0.50** | **±** | **0.09** | **0.59** | **±** | **0.09[a]** | **0.34** | **±** | **0.03[b]** | 0.32 | ± | 0.02 | 0.39 | ± | 0.04 | 0.40 | ± | 0.03 |
| **n3:n6** | **0.10** | **±** | **0.02[a]** | **0.08** | **±** | **0.01** | **0.06** | **±** | **0.01[b]** | 0.15 | ± | 0.03 | 0.08 | ± | 0.01 | 0.07 | ± | 0.02 |

## LIST OF COMMON ABBREVIATIONS

**ADP** = adenosine diphosphate; **ATP** = adenosine triphosphate; **BAT** = brown adipose tissue; **CoA** = Coenzyme A; **CPT** = carnitine palmitoyltransferase; **DHA** = docosahexaenoic acid; **DHAP** = dihydroxyacetone phosphate; **ETF** = electron transferring protein; **ETS** = electron transfer system; **FA** = fatty acid; **FAD** = flavin adenine dinucleotide; **FAME** = fatty acid methyl ester; **GMGS** = golden-mantled ground squirrel; **GTP** = guanosine triphosphate; **G3P** = glucose-3-phosphate; **G6P** = glucose-6-phosphate; **IBA** = interbout arousal; **IMM** = inner mitochondrial membrane; **LDH** = lactate dehydrogenase; **MUFA** = monounsaturated acid; **NADH** = nicotinamide adenine dinucleotide; **NST** = non-shivering thermogenesis; **OXPHOS** = oxidative phosphorylation; **PDH** = pyruvate dehydrogenase; **PUFA** = polyunsaturated acid; **SFA** = saturated fatty acid; **Ta** = ambient temperature; **Tb** = internal body temperature; **TCA** = tricarboxylic acid; **UCP** = uncoupling protein; **WAT** = white adipose tissue.