THESIS

FUNCTIONAL RESPONSES OF CARDIAC AND SKELETAL MUSCLE MITOCHONDRIA TO SHORT-TERM OBESITY: ARE ALL OBESITIES THE SAME?

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ABSTRACT

FUNCTIONAL RESPONSES OF CARDIAC AND SKELETAL MUSCLE MITOCHONDRIA TO SHORT-TERM OBESITY: ARE ALL OBESITIES THE SAME?

Obesity is associated with metabolic alterations, specifically in the mitochondria, that may play a role in the development of insulin resistance and type 2 diabetes. In nature, there are circumstances where obesity is normal, even favored as seen in mammals that hibernate (hibernators). Understanding the consequences of obesity in 'hibernators' versus 'disease' models might yield novel insights on the effect of short-term obesity on mitochondrial function. The aim of this study is to compare mitochondrial function in a hibernator, the golden-mantled ground squirrel (*Callospermophilus lateralis*) and Ob/Ob mice following 4-6 weeks of hyperphagia versus their respective lean controls. Glucose tolerance tests were administered in lean, summer active squirrels (summer); hyperphagic obese, pre-hibernation period squirrels (prehib); and 5-week-old leptin-deficient (*ob*) and control (WT) C57-BL6J mice prior to sacrifice. High-resolution respirometry was used to examine mitochondrial function in permeabilized solei muscle fibers and isolated cardiac and skeletal muscle mitochondria obtained from all study groups. Body mass of obese animals was 30 and 50% greater than respective lean controls in squirrels and mice, respectively. Glucose intolerance developed with obesity in both species. Skeletal muscle mitochondria exhibited a greater capacity to oxidize pyruvate in the presence of lipids in squirrels, but not in mice. Cardiac oxidative phosphorylation (OXPHOS) capacity significantly increased only in squirrels, and exhibited a significantly greater capacity to

oxidize pyruvate in the presence of lipids. No significant change was observed in mice cardiac mitochondria. Mitochondrial responses to obesity differed between models despite nearly identical effects of weight gain and glucose intolerance. Therefore, the observed differences between squirrel and mice likely reflect responses to different environmental cues or genetic background independent of 'classic' effects of obesity. The observed mitochondrial responses are not simply responses of obesity and glucose intolerance.

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INTRODUCTION

Obesity continues to plague modern society and is associated with metabolic alterations that contribute to the pathogenesis of type 2 diabetes mellitus (DM) and cardiovascular disease (CVD). Diet induced obesity occurs when caloric intake exceeds energy expenditure, and typically is associated with sedentary behavior. Chronic nutrient excess is related to the development of insulin resistance, hyperglycemia, and hyperlipidemia, which are the well-established hallmark characteristics of DM (Guo, 2014). Obesity and DM are independent risk factors for the development of heart failure (HF) (Bertoni, Tsai, Kasper, & Brancati, 2003; Kenchaiah et al., 2002). In fact, HF is the principle cause of morbidity and mortality in diabetic individuals (CDC, 2013; Candido, Srivastava, Cooper, & Burrell, 2003). Both clinical and epidemiological studies have indicated that cardiomyopathies associated with obesity and DM can be unrelated to microvascular and macrovascular complications, hypertension and other comorbidities classically associated with HF (Bell, 1995; Boudina & Abel, 2007; Wong & Marwick, 2007). Although the exact mechanisms leading to the pathogenesis of obesity and DM cardiomyopathy are undefined, symptoms are associated with hyperglycemia and hyperlipidemia, indicating that myocardial metabolism may play a major role in the development (Bajraktari et al., 2006; Belke, Larsen, Gibbs, & Severson, 2000).

Skeletal muscle is proposed to play a large role in the development of DM and insulin resistance. During the postprandial phase, skeletal muscle is the major site of glucose uptake. Reduced insulin-simulated glucose uptake into the skeletal muscle is suggested by many to play an important role in the pathogenesis of insulin resistance and DM (Shrauwen-Hinderling et al.,

2015). At the molecular level, reduced overall mitochondrial oxidative capacity of the skeletal muscle has been postulated to underlie the development of insulin resistance and DM (Lowell & Shulman, 2005). A decline in mitochondrial function has also been reported to occur in the heart of type 2 diabetics, underlying certain forms of CVD and HF (Boudina & Abel, 2006). Therefore, it is important to study both heart and skeletal mitochondrial respiratory function in states of obesity to help determine what role they might play in the development of obesity related diseases.

Mitochondrial processes are tightly regulated and have the ability to adapt to different metabolic conditions (Balaban, 2010). Therefore, states of chronic nutrient excess, as seen in obesity, may have an impact on mitochondrial function. In fact, studies on obese humans and rodent models have demonstrated alterations in energy substrate utilization and mitochondrial function in association with obesity-related disorders (Anderson et al., 2009; Belke, Larsen, Gibbs, & Severson, 2000; Boudina et al., 2005, 2007; Boushel et al., 2007; Mazumder et al., 2004; Peterson et al., 2004). Mitochondria have tissue-dependent functions and have the ability to adapt to the energetic and metabolic demands of the given tissue. Studies in rodent tissues indicate that mitochondrial mass, DNA copy number, and cytocrome c oxidase activity is different between tissues, including skeletal and cardiac muscle, and even varies within a given tissue under different metabolic conditions (Balaban, 2010). Therefore, it is reasonable to hypothesize that because mitochondria are finely tuned to respond to metabolic stress, obesity may be accompanied by tissue-specific changes in mitochondrial function. In particular, alterations in skeletal and cardiac muscle mitochondria have each independently been implicated in the development of obesity related diseases, and therefore will be discussed separately.

Most studies analyzing cardiac energy metabolism in obese humans and rodents indicate the development of "metabolic inflexibility", characterized by a greater reliance on oxidation of fatty acids (FA) and a decreased reliance on glucose oxidation (Belke et al., 2000; Mazumder et al., 2004; Peterson et al., 2004). Alterations in mitochondrial function have been proposed to play a key role in the development of metabolic inflexibility. Most animal studies have reported a decrease in cardiac mitochondrial respiratory capacity in obesity (Boudina et al., 2005, 2007; Kuo, Giacomelli, & Wiener, 1985; Kuo, Moore, Giacomelli, & Wiener, 1983), whereas results from human studies have been equivocal. A recent study by Montaigne et al. (2014) found that cardiac mitochondrial respiratory function is impaired in DM, but not obesity (Montaigne et al., 2014). The recent study by Montaigne highlights the importance of differentiating between obesity and DM when studying the role of mitochondria in obesity-related diseases.

Most studies do not study obesity independently; instead, researchers typically study mitochondrial respiratory function in the presence of insulin resistance and DM, making it difficult to distinguish the effects of established DM from obesity per se. Studies that investigated obesity independent of diagnosed DM have found decreased mitochondrial function in the skeletal muscle of obese subjects compared to lean controls (Karakelides et al., 2010; Vijgen et al., 2013). However, Karakelides et al., 2010 did find that although not diabetic per se, the obese participants had significantly reduced insulin sensitivity compared to lean participants (Karakelides et al., 2010).

Although several lines of evidence suggest that alterations in skeletal muscle mitochondrial function are associated with obesity and insulin resistance in humans and animal models, the role of skeletal muscle mitochondria in the pathogeneses of diabetes has been controversial. Estimates of skeletal muscle mitochondrial content have been consistently

reported to be 14-38% lower in obesity and DM (Holmström, Iglesias-Gutierrez, Zierath, & Garcia-Roves, 2012; Kelley, He, Menshikova, & Ritov, 2002; Ritov et al., 2005). Decreased mitochondrial function has been observed in some studies of obese humans and animals respiration (Mogensen et al., 2007; Phielix et al., 2008), but not others (Anderson et al., 2009; Boushel et al., 2007; Holmström et al., 2012; Karakelides, Irving, Short, O'Brien, & Nair, 2010; Kelley et al., 2002; Lefort et al., 2010). Similar to the heart, most studies involve severely obese, insulin resistant mice, or humans with DM, making it difficult to understand what role mitochondrial function plays in the pathogenesis of DM.

Most of the discrepancies seen in the literature can be attributed to the lack of consistency in methods. Differences in experimental design, diet, age, genetic background, and sex of the individuals/ animals under investigation may independently influence mitochondrial function. For example, Boushel et al., 2007 found that obese patients with DM have normal mitochondrial function in skeletal muscle compared to healthy overweight individuals but only when data was normalized the number of copies of mtDNA per μg of tissue ×10,000 (Boushel et al., 2007). However most studies do not normalize mitochondrial respiratory data to content markers. Other discrepancies in the literature can be attributed to differences in experimental protocol. For example, one study found that obese insulin-resistant adults had normal mitochondrial respiration of the skeletal muscle compared to lean adults when using a mitochondrial respiration protocol that included the presence of FA (Palmatile Carnitine (PC)) (Fisher-Wellman et al., 2014). However, another study found that adults with DM had normal skeletal muscle respiration compared to healthy obese results when the mitochondrial respiration protocol included FA, but had decreased mitochondrial respiration when in the presence of a carbohydrate (pyruvate) and no FA (PC) (Mogensen et al., 2007). These and other discrepancies in the literature make it

difficult to determine whether changes in mitochondrial function contribute to or result from the development of obesity related diseases. In addition, most rodent studies use severely obese genetically altered models or animals on a high-fat diet that might not be relevant to the human population. Therefore, to elucidate whether changes in mitochondrial respiratory function are a cause or consequence of obesity related disorders, it will be beneficial to study short-term obesity and its effects on mitochondrial function and glucose tolerance before the onset of overt disease.

As previously stated, the majority of studies analyzing mitochondrial respiratory function and obesity use genetically modified rodent models. The leptin-deficient *ob* mouse is common model for investigating how obesity leads to cardiometabolic disorders and DM. Leptin deficiency causes hyperphagic obesity and glucose intolerance on a standard chow diet (Buchanan et al., 2005). Obesity also occurs seasonally in nature in hibernating animals such as the golden-mantled ground squirrels (Callospermophilus lateralis) (GMGS). Hibernators must gain a substantial amount of weight in the summer and fall in order to successfully undergo a heterothermic period of hibernation in the winter (Van Breukelen & Martin, 2002). Hibernators have been shown to develop glucose intolerance in the fall that resolves after the winter hibernation period (Buck, Squire, & Andrews, 2002; Florant, Lawrence, Williams, & Bauman, 1985). The development of obesity in both the ob mouse and hibernating ground squirrels has been shown to be attenuated by exogenous leptin administration, suggesting some physiological similarities between the two rodent species (Ormseth, Nicolson, Pelleymounter, & Boyer, 1996; Weigle et al., 1995) However, it remains unclear whether the cell/molecular aspects of the obesity phenotype and associated cardiometabolic disorders are shared between these species.

The leptin-deficient *ob* mouse has been used in obesity and diabetes research since it was first discovered by the Jackson Laboratory in 1949 (Ingalls, Dickie, Coleman, 1950). In humans with leptin deficiency, treatment with exogenous leptin administration has been shown to have the same profound effects on food intake and body weight as seen in *ob* mice. However, most obese humans do not have leptin deficiencies (Nilsson, Raun, Yan, Larsen, & Tang-Christensen, 2012). In fact, they typically suffer from hyperleptinemia and leptin resistance and therefore typically do not respond to exogenous leptin treatment. Although the *ob* mouse model is a valuable and relevant model for the study of obesity, its monogenic cause of obesity does not completely reflect the background of obesity in humans. Therefore, we thought it might be fascinating to also study obesity in a natural model that does not lack leptin (GMGS). In fact, leptin levels have been shown to increase with increasing fat mass in hibernators, suggesting that during autumn/winter hibernators may switch from a lipgenic to a lipolytic state, which may even include leptin resistance as seen in obese humans (Florant et al., 2004).

The primary aim of this study is to investigate whether alterations in cardiac and skeletal muscle respiratory function occur during the early stages of obesity in prehibernating GMGSs and the hyperphagic ob mouse model. We chose to study two models independent models of obesity - one natural through seasonal weight gain in preparation for hibernation and one through leptin deficiency. We found that given similar extents of short-term (4-6 weeks) obesity on the same diet, ob mice exhibited similar degrees of glucose intolerance as pre-hibernating GMGS. We hypothesized that because cardiac and skeletal muscle mitochondrial respiratory function reflect overall metabolic status, similar extents of obesity and glucose intolerance will result in changes in mitochondrial function in both species, as suggested by the literature.

LITERATURE REVIEW

Basic Physiology

When analyzing the impact of obesity on cardiac and skeletal muscle mitochondria, it is important to understand basic metabolic and mitochondrial function under normal conditions. This section will focus on basic metabolism of the heart and skeletal muscle, specifically the utilization of FA and carbohydrates (CHO) in energy metabolism, before briefly reviewing aspects of mitochondrial function.

Metabolism in the Normal Heart

The heart has a high-energy demand that requires a constant supply of adenosine triphosphate (ATP) to sustain its function. Under normal conditions, 95% of ATP generated in the heart is derived from oxidative phosphorylation (OXPHOS) in the mitochondria. Glycolysis and the tricarboxylic acid cycle (TCA) contribute the remaining 5% (Opie, 2004). The majority of heart-generated ATP is dedicated to contraction with the remaining used to maintain various ion pumps. In the fasted state, fatty acid oxidation is the main substrate of the heart as it fuels 70% to 90% of the cardiac ATP. The remaining is supplied from the oxidation of CHO, as well as minimal amounts of ketone bodies (Opie, 2004). In the postprandial state, plasma glucose and insulin rise and the contribution of CHO as a cardiac fuel increases. Therefore, the normal heart is metabolically flexible and is able to switch from FA to CHO oxidation and vise versa depending on their availability and ATP demand.

Metabolism in Normal Skeletal Muscle

Skeletal muscle metabolism during exercise or at rest depends on a balance between CHO and FA oxidation. The contribution of ATP generated to maintain function by the muscle depends on the activity state of the muscle and muscle fiber type. Type I oxidative skeletal muscle fibers rely heavily on OXPHOS from the mitochondria, whereas type II glycolytic fibers depend more on glycolysis and carbohydrate metabolism. In healthy states, skeletal muscle is also metabolically flexible, able to use diversity variety of substrate as fuel to match ATP demand.

Fatty Acid Oxidation FA Use

There are three major steps of FA use: uptake into cytosol (facilitated by transport proteins such as FA translocase, the homolog of CD36 (FAT/CD36)), transportation across the mitochondrial membrane, and oxidation inside the mitochondria (Abumrad, el-Maghrabi, Amri, Lopez, & Grimaldi, 1993; Holloway et al., 2006). Once FAs are transported into the cytosol, free FAs are esterified to fatty acyl-coenzyme A (CoA). Fatty acyl-CoA is then converted to fatty aclycarnitine by carnitine palmitoyltransferase (CPT) I, located in the outer mitochondrial membrane. Acylcarnitine is subsequently transported into the mitochondrial matrix where CPT II converts it back to fatty acyl-CoA, which enters the β-oxidation pathway, producing acetyl-CoA, NADH and $FADH_2$ (van der Vusse, van Bilsen, & Glatz, 2000). Importantly, the reactions catalyzed by CPT I are an important regulator of FA oxidation in vivo, and can be inhibited by malonyl-CoA (Zammit, Fraser, & Orstorphine, 1997), generated by the carboxylation of cytosolic acetyl-CoA.

Glucose Use

Glucose as a substrate either comes from the uptake of exogenous glucose or from stored glycogen in the muscle and liver. Glucose can also be synthesized by non-carbohydrate precursors, such as glycerol from the lipolysis of triglycerides, through a process called gluconeogenesis. Glucose is transported into the cytosol by glucose transporters (GLUTs), specifically GLUT4 in adults (Abel, 2004; Richter & Hargreaves, 2013). Post transport, free glucose is phosphorylated into glucose-6-phosphate (G6P), which can enter many metabolic pathways, mainly glycolysis. Glycolysis generates pyruvate, NADH, and a small amount of ATP. Pyruvate can either be converted to lactate in the cytosol or transported to the mitochondrial matrix where it is oxidized and decarboxylated into acetyl-CoA. Pyruvate oxidation to acetyl-CoA is catalyzed by pyruvate dehydrogenase (PDH). The activity of PDH is regulated by the activity of pyruvate dehydrogenase kinase (PDK), which inhibits PDH in response to high levels of ATP, NADH and/or acetyl-CoA.

The TCA Cycle, Electron Transfer System, and Mitochondrial Respiration

Mitochondrial ATP production relies on a continuous flow of substrates across the inner mitochondrial membrane into the mitochondrial matrix, which are oxidized in the beta-oxidation cycle (FAs) and/or tricarboxylic acid (TCA) cycle (pyruvate, amino acids), generating reducing equivalents (NADH and $FADH_2$) that power the electron transfer system (ETS). The TCA cycle is series of chemical reactions that oxidizes acetyl-CoA to generate GTP (an ATP equivalent), CO_2, NADH and $FADH_2$.Substrates for the TCA cycle enter the mitochondrial matrix through various carrier proteins, which are ultimately oxidize to feed electrons to specific ETS complexes. For example, oxidation of malate, pyruvate and glutamate reduce NAD+ to NADH, feeding electrons to Complex I of the ETS. Succinate, in contrast, provides electrons to FAD+,

generating $FADH_2$ that enter the ETS at Complex II. Fatty acyl-CoAs oxidized in the β-oxidation cycle provide electrons as NADH (for Complex 1) and $FADH_2$ to the electron transferring flavoprotein (ETF), in addition to acetyl-CoA that can be oxidized in the TCA cycle. Finally, electrons can also be donated ultimately as $FADH_2$ derived from glycerol 3-phosphate (from triacylglycerols or glycolysis) by mitochondrial glycerol 3-phosphate dehydrogenase (G3PDH) on the outer face of the inner mitochondrial membrane.

Thus, electrons enter the ETS at four separate convergent sites that eventuate in the reduction of coenzyme Q (ubiquinone). Once the mobile electron carrier ubiquinone is reduced to ubiquinol, is directs its electrons to complex III (ubiquinol-cytochrome *c* oxidoreductase) where it is oxidized (Scheffler, 2007) and its electrons are transferred to cytochome *c*, another mobile electron carrier. Cytochrome c directs flow to Complex IV (cytochome *c* oxidase). Complex IV catalyzes the final reaction of the ETS, the reduction of oxygen, the terminal electron acceptor, generating water. Electron flow through complexes I, III, and IV facilitate transport ("pumping") of protons into the inter mitochondrial membrane space, generating a proton gradient. In perfectly "coupled" mitochondria, the entire proton gradient would be used to generate ATP through Complex V (ATPase), which harnesses the proton gradient to power phosphorylation of ADP to ATP. However, some of the protons pumped by the ETS (or the membrane potential it generates) are dissipated through the activity of uncoupling proteins (UCPs).

When studying mitochondrial respiration *in vitro*, the aforementioned substrates can be used to evaluate the capacities of specific pathways generating reducing equivalents to the ETS, as well as rates of electron flow through the Complexes. Respiratory states were classically defined by Chance and Willams in 1955, who were the first to separate Complex I or II specific

substrates for a functional diagnosis of OXPHOS (Chance & Williams, 1955). In this study, we used certain substrate combinations to evaluate various aspects of respiratory function *in vitro* defined in Table 1 (see methods).

Mitochondria and Obesity

This section will focus on the relation of mitochondrial function and obesity. The heart and skeletal muscle will be discussed in separate sections. Although alterations in mitochondrial number and morphology, biogenesis, dynamics, and reactive oxygen species (ROS) production have all been postulated to be associated with obesity and DM, this section will mainly focus on mitochondrial respiratory function and substrate utilization as that is the primary focus of the study.

Cardiac Mitochondrial Adaptations to Obesity and DM

Cardiovascular disease is the primary cause of mortality among diabetic patients (Candido, Srivastava, Cooper, & Burrell, 2003). Studies of obesity and DM have identified metabolic inflexibility (an inability to readily switch from CHO to FA substrates) and reductions in mitochondrial OXPHOS capacity in mitochondrial function to be the major characteristic of the diabetic heart (Scheuermann-Freestone et al., 2003). Obese and diabetic individuals have been shown to have high circulating levels of glucose and FAs (Cascio, Schiera, & Di Liegro, 2012). In the early stages of obesity, excess FA delivery is associated with insulin resistance that switches cardiac metabolism to favor FAs. In accordance with this shift, there is an increase in the expression of the nuclear receptor transcription factor peroxisome proliferator activated receptor alpha (PPARα), which regulates genes involved with FA uptake and oxidation (Finck et al., 2002). However, one study employing PET metabolic imaging demonstrated higher rates of

FA oxidation in the heart of obese diabetics compared to obese non-diabetics, indicating that the development of DM (rather than obesity *per se*) may play a key role in the increased rate of myocardial FA oxidation (Peterson et al., 2012).

Early upregulation of FA oxidation pathways may be an advantageous adaptation to manage excess circulating FA; however, sustained activation can be detrimental. A chronic high level of FAs and associated PPARα activity increases in delivery of FA substrates to the mitochondria for β-oxidation, but excessive delivery can have negative consequences. If delivery of FAs and subsequent production of reducing equivalents outpace the energetic needs of the cell, continued flux through the ETS can increase mitochondrial membrane potential, and increase generation of reactive oxygen species (ROS). The abundance of FA and ROS can activate uncoupling proteins, which may partially mitigate mitochondrial ROS production and FA accumulation, but also reduce the efficiency of OXPHOS, leading to inefficient myocardial ATP production (Boudina et al., 2007). In addition, acetyl-CoA levels may increase due to excessive β-oxidation, which inhibits the pyruvate dehydrogenase complex and limits glucose oxidation, causing metabolic inflexibility (Batenburg & Olson, 1976). Finally, the resulting accumulation of FA intermediates resulting from incomplete catabolism may lead to a state of cardiac "lipotoxicity" leading to cardiomyocyte dysfunction and death by multiple mechanisms (Muoio et al., 2012).

Studies specifically examining cardiac mitochondrial respiratory function in humans and rodent models mostly support decreased metabolic flexibility, reduced OXPHOS capacity, and increased uncoupling. Boudina et al., 2005 found decreased OXPHOS capacity and increased mitochondrial uncoupling concomitant with impaired cardiac function in obese *ob* mice (Boudina et al., 2005). Two-years later, the same group did a comprehensive study on obese

db/db mice that demonstrated reduced OXPHOS capacity in the presence of FA and CHO as well as increased "Leak" (Boudina et al., 2007).

Studies in humans are more difficult to conduct, as researchers are only able to extract atrial tissue from patients receiving a heart transplant. Nevertheless, researchers such as Montaigne and Anderson obtained right atrial myocardium from patients receiving open-heart surgery that presented no signs of cardiomyopathy that enabled them to conduct human studies on cardiac mitochondrial function in obesity and DM (Anderson et al., 2009; Montaigne et al., 2014). Anderson et al., 2009 found that OXPHOS capacity is decreased in DM versus obese controls (Anderson et al., 2009). In addition, Montaigne et al., 2014 found decreased mitochondrial OXPHOS capacity in in DM; however, obese, non-diabetic individuals did not show any alterations in mitochondrial OSPHOS capacity compared to lean controls (Montaigne et al., 2014). These recent studies by Montaigne and Anderson highlight the importance of differentiating between obesity and DM when studying the role of mitochondrial in obesity-related diseases.

Skeletal Muscle Mitochondrial Responses to Obesity and DM

Impairments of both CHO and FA metabolism have been reported in skeletal muscle of obese and diabetic individuals (D. E. Kelley et al., 1996; D. E. Kelley & Simoneau, 1994). In particular, reductions in FA oxidative capacity have been associated with lipid accumulation and insulin resistance in skeletal muscle of obese and diabetic individuals, which may contribute to impairments in whole-body glucose metabolism (D. E. Kelley & Mandarino, 2000). While the exact mechanisms of impaired FA oxidation in skeletal muscle of obese and diabetic individuals is unknown, it is hypothesized that there is a) reduced tissue mitochondrial content, b) reduced mitochondrial OXPHOS capacity, or c) a combination of both. Several studies in humans in the

early 2000s provided evidence of lower oxidative enzyme activities and decreased lipid metabolism in the skeletal muscle of obese and diabetic individuals compared to lean controls (David E. Kelley, He, Menshikova, & Ritov, 2002b; D. E. Kelley, Goodpaster, Wing, & Simoneau, 1999; Kim, Hickner, Cortright, Dohm, & Houmard, 2000). Additionally, Kelley et al., 2002 demonstrated decreased mitochondrial content in combination with reduced NADH:O2 oxidoreductase activity (Complex I-linked respiration) in the skeletal muscle of obese, insulin-resistant subjects versus lean controls (David E. Kelley et al., 2002b). Another study by Ritov et al., 2005 concluded that impairments in mitochondrial ETS activity exceeded decreases in tissue mtDNA content, indicating intrinsic loss of mitochondrial respiratory capacity in the skeletal muscle of obese and DM individuals (Ritov et al., 2005). These studies were some of the first to indicate decreased skeletal muscle mitochondrial content and function in obese individuals. Since then, several other studies have employed more comprehensive assessments of mitochondrial function, such as high-resolution respirometry, which have demonstrated conflicting results.

To determine whether mitochondrial function and/or content are impaired in obesity, Holloway et al., 2006 measured palmitoylcarnitine (FA) oxidation in isolated mitochondria and premeabilized fibers in obese when compared to lean individuals (Holloway et al., 2006). Obesity did not alter FA oxidation measured in isolated mitochondria, but whole muscle FA oxidative capacity was decreased by 28%, suggesting a decrease in muscle mitochondrial content rather than decreased mitochondrial function (Holloway et al., 2006). In contrast, a study analyzing permeabilized fibers in young, obese individuals found no significant differences in mitochondrial oxidative capacity in the presence or absence of FA, suggesting that neither function nor content were impaired (Fisher-Wellman et al., 2014). While both of these studies

tested obese, insulin sensitive subjects, another study by Mogensen et al., 2007 examined mitochondrial respiration in obese, insulin sensitive subjects versus individuals with DM. They reported decreased pyruvate oxidative capacity in isolated mitochondria in diabetics, but no change in FA oxidation compared to obese controls (Mogensen et al., 2007). Other studies analyzing both obesity and DM in humans and rodent models have reported decreased mitochondrial oxidative function (Iossa et al., 2003; Phielix et al., 2008), or no change in either population (Boushel et al., 2007; Trenell, Hollingsworth, Lim, & Taylor, 2008; van Tienen et al., 2012). Finally, a study by Karakelides et al., 2010 found that decreases in mitochondrial function are independent of adiposity and insulin sensitivity, but indicated effect of age (Karakelides, Irving, Short, O'Brien, & Nair, 2010). Therefore, defining the role of mitochondrial function in the pathogenesis of skeletal muscle insulin resistance remains challenging. Collectively, it appears that alterations in mitochondrial content and function are not a requisite for the development of insulin resistance in all circumstances. Differences in results may be attributed to the extent of obesity studied, the population examined (humans versus rodents; age; gender), and the methodological approach.

Obesity and Metabolism in Hibernating Mammals

Hibernation is an adaptive strategy used by mammals to save energy in winter, typically when environmental temperatures drop below freezing with lack of access to food. There are two main phases for hibernators: a homothermic phase of reproduction, growth and fattening in the summer through early fall, and a heterothermic phase of hibernation in winter (Van Breukelen & Martin, 2002). During hibernation, core body temperatures drop as low as 0°C in GMGS, heart rate slows to ~5bpm, breathing rates are infrequent, and metabolic rates are ~5% of what they are during euthermia (Carey, Andrews, & Martin, 2003; Lyman & Chatfield, 1955). After the

energy demands of growth and reproduction are met in late summer, animals gain massive amounts of weight, mainly attributable to large stores of triglycerides in their adipose tissue. This fat mass is consumed when hibernators forgo food and water for many consecutive months in winter (Serkova, Rose, Epperson, Carey, & Martin, 2007). Hibernators have been shown to develop glucose intolerance and insulin resistance in the fall that is rescued after the winter hibernation period concomitant with reduction in adipose tissue (Buck, Squire, & Andrews, 2002; Florant, Lawrence, Williams, & Bauman, 1985).

Studies on mitochondrial function in the heart and skeletal muscle of hibernators have only compared the differences in summer active and hibernating animals. For example, Brown et al., 2013 found pronounced seasonal (summer versus winter (interbout) euthermia) changes in respiration rates in isolated heart mitochondria concomitant with a shift in fuel use away from CHO and towards FA in thirteen-lined ground squirrels (Brown, & Staples, 2013). In contrast, these seasonal findings were not observed in isolated hind-limb skeletal muscle mitochondria. Thus, our analysis of mitochondrial function of heart and skeletal muscle in summer and prehibernating animals will provide novel insight to the field.

METHODS

To test the hypothesis that similar extents of obesity and glucose intolerance will result in similar changes in mitochondrial function in both species independently, we used the following methods:

Ethical Approval

The Institutional Animal Care and Use Committee approved all animal studies.

Ground Squirrels

Adult golden-mantled ground squirrels (GMGS) were trapped throughout the months of June and July in Larimer and Gunnison Counties, Colorado under State of Colorado Department of Natural Resources Scientific Collection License #14TR099. Eight males were transported live and housed in an animal facility at Colorado State University. Animals were separated into two study groups (n= 4 males/ group). The summer group was sacrificed in the summer active period (early August) and the prehib group was sacrificed after obesity was clearly evident toward the end of fall prehibernation period, before any torpor bouts were observed (late September- early October).

Animals were housed in standard rodent cages and restricted to sedentary cage activity, artificial for these animals, in a temperature-controlled room under a 12 hour light/ 12 hour dark cycle. The ambient temperature where the animals were housed was decreased from 18°C to 15°C in late August to mimic natural conditions found in their natural habitat. Standard chow (Harlan Teklod 8604) and water were accessible *ad libitum* and food intake and body mass were measured daily.

Mice

Five-week-old male genetically hyperphagic *ob* mice on the C57BL/6J background and their lean controls (WT) were obtained from Jackson Laboratory in late winter (Febuary – March) and used in this study. All mice were housed in standard rodent cages and restricted to sedentary cage activity and provided standard Purina 2819 chow *ad libitum*. Animals were sacrificed for mitochondrial experiments 4-6 weeks after arrival to Colorado State (~9-10 weeks of age). Although these animals were very young, it was important to study the animals in a state of short-term obesity.

Glucose Tolerance Tests

About four days before sacrifice, animals were fasted overnight for 12-16 hours to prepare for an interperitoneal (IP) glucose tolerance test. Animals were weighed prior to the test. A small tail incision was made for blood collection. After baseline blood glucose was recorded, a 1g/kg bolus of glucose was injected IP. Blood glucose concentrations were measured from tail incisions at baseline, 30, 60, 90, and 120 minutes post bolus glucose injection using a small rodent glucometer (AlphaTrak, Abbott Laboratories, Abbott Park, IL).

Preparation of Isolated Skeletal and Cardiac Muscle Mitochondria

Following confirmation of deep anesthesia with isoflorane, animals were sacrificed by thoracotomy and removal of the heart. Mitochondria were isolated from ~100mg freshly extracted heart or hindlimb muscle tissue (plantaris and gastrocnemius together) in ice cold Chappel-Perry (CP) buffer consisting of (in mM) KCl (100), MOPS (50), $MgSO_4 \cdot 7H_2O$ (5), and ATP (1), pH 7.4 (with KOH) for muscle, and CP + 1 mM EGTA for heart, using standard differential centrifugation methods (7000xg pelleting spin) as previously described with minor modifications (Sparagna et al., 2007). To avoid extracellular matrix contamination from muscle

tissue and obtain a mix of both subsarcolemmal and intermyofibrillar mitochondria from both tissues, trypsin was included in the initial homogenization step for 7 minutes at 5 and 1 mg/g tissue for muscle and heart, respectively, then quenched by the addition of 0.2% bovine serum albumin. Isolated mitochondria were suspended in buffer containing 100mM KCl, 50 mM MOPS and 0.5 mM EGTA on ice, and added to the Oxygraph chamber containing MIR05 respiration buffer consisting of (in mmol l^{-1}) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES and 110 sucrose, with 1 g l^{-1} fatty-acid free BSA at 37°C for 5-10 minutes of stabilization prior to respiratory experiments. A total 75 μg mitochondrial protein (determined by the BCA assay) were added to each chamber. Respirometry data are expressed per mg of total mitochondrial protein.

Saponin Permeabilized Soleus Muscle

Once sacrificed, intact soleus muscle was immediately extracted from animals and placed in ice-cold biopsy preservation medium (BIOPS) consisting of (in mmol l^{-1}) 10 Ca^{2+} -EGTA, 20 imidazole, 50 potassium-4-morpholinoethanesulfonic acid, 0.5 dithiothreitol, 6.56 $MgCL_2$, 5.77 ATP and 15 phosphocreatine at pH 7.1. Muscle samples were then mechanically separated, removing any visible connective and adipose tissue using fine-tipped forceps and scissors under a dissecting microscope. Once a high degree of fiber separation was visually verified, teased fiber bundles (~4-6 mg each) were chemically permeabilized by incubation in ice-cold BIOPS with saponin (50 μg ml^{-1}) for 20 minutes, followed by 2x15 minute washes in ice-cold MIR05 respiration buffer consisting of (in mmol l^{-1}) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES and 110 sucrose, with 1 g l^{-1} fatty-acid free BSA. Permeabilized fibers were quickly and carefully blotted on clean Whatman filter paper for ~5 seconds to remove excess buffer, weighed (~2.0 g) and immediately placed in the Oxygraph chamber containing

MiR05 at 37°C for 5-10 minutes of stabilization prior to respiratory experiments. Respirometry data were normalized to fiber weight.

Mitochondrial Respiration

Mitochondrial respiratory function was determined in isolated cardiac and skeletal muscle mitochondria as well as saponin permeabilized solei from each study group by high-resolution respirometry using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). Standardized instrumental and chemical calibrations were conducted to correct for back diffusion of oxygen into the chamber, oxygen consumption from MiR05, and oxygen consumption from the electrodes using Datlab software (Oroboros Instruments). Oxygen flux was monitored in real-time by software that resolved changes in the negative time derivative of the chamber oxygen concentration signal (oxygen flux). A detailed description of the respiration protocols used in this study and their associated respiratory states generated by the sequential titration of each substrate are demonstrated in Table 1.

All respirometry experiments from the permeabilized muscle fibers were carried out in a hyperoxegenated environment (250-400 μmol l^{-1}) to prevent any potential oxygen diffusion limitation (Gnaiger, 2009). In isolated mitochondria, oxygen diffusion does not limit mitochondrial respiration (indicated by stable O2 flux down to 0 μmol l^{-1}; unpublished observations), therefore experiments were calibrated at ambient temperature (160 μmol l^{-1}) and were be carried out until oxygen reached near 0 μmol l^{-1}Biological duplicates were performed on some, but not all of the samples to ensure data were repeatable.

Respiratory Titration Protocol

Two titration protocols were used in this experiment specifically chosen to examine individual aspects of respiratory control through a series of substrate states induced via separate titrations in the presence or absence of fatty acid (; FA). Palmitoylcarnitine,a 16 carbonsaturated FA bound to carnitine, was used as the fatty acid substrate, which enters the mitochondrial matrix independent of the carnitine palmitoyltransferase (CPT1) enzyme.

Table 1: High-resolution respirometry protocols and associated respiratory flux states assessed in mitochondrial respiration experiments

Protocol constituents (listed in order of titration)	**Abbreviation state**	**Respiratory flux**
PROTOCOL 1		
Palmitoylcarnitine + malate (0.2 mmol l^{-1} + 1 mmol l^{-1})	FA Leak	Leak respiration in the presence of a FA with no ADP present
ADP (5 mmol l^{-1})	FA OXPHOS	FA OXPHOS capacity limited by electron flux through ETF
Pyruvate (5 mmol l^{-1})	FA + CHO OXPHOS	FA + CHO OXPHOS capacity limited by pyruvate + malate oxidation capacity
Glutamate + Succinate (10 mmol l^{-1} + 10 mmol l^{-1})	FA TOTAL OXPHOS	Complex I+II supported OXPHOS capacity in the presence of FA, limited by ADP phosphorylation capacity and mitochondrial membrane potential
PROTOCOL 2		
Pyruvate + malate (5 mmol l^{-1} + 1 mmol l^{-1})	CHO Leak	Leak respiration in the presence of pyruvate with no ADP present
ADP (5 mmol l^{-1})	CHO OXPHOS	CHO OXPHOS capacity limited by pyruvate oxidation capacity
Glutamate + Succinate (10 mmol l^{-1} + 10 mmol l^{-1})	CHO TOTOAL OXPHOS	Complex I+II supported OXPHOS, limited by ADP phosphorylation capacity and mitochondrial membrane potential

Protocol constituents are listed in the order that they were added in the respiration experiments.

Data Analysis

For all statistical evaluations, a statistical significance was set at $P<0.05$. All data were presented as group means ± s.e.m., with the number of samples per group stated in the results section. Duplicates were run on some, but not all samples. Area under the curve (AUC) was

calculated using trapezoidal integration. Data from respiration experiments comparing species-specific lean control and obese were compared by independent-sample *t*-tests.

RESULTS

Obesity and Glucose Intolerance in Prehibernating GMGSs and *Ob* Mice

A significantly greater body mass was observed in prehibernating GMGS ($P < 0.05$) and ob ($P < 0.01$) mice versus their respective lean controls (Figure 1). Prehib GMGS increased body mass by 67% from August to time of sacrifice in early October. Fasting blood glucose concentrations of *ob* mice were significantly higher than WT (190.0 ± 22.2 and 133.6 ± 26.7 mg/dl, respectively, $P = 0.02$). In contrast, fasting blood glucose concentrations were no different between summer and prehib GMGS (193.3 ± 7.2 and 168.2 ± 7.6 mg/dl, respectively, $P = 0.45$). However, glucose tolerance was significantly impaired in both *ob* mice and prehib GMGS versus their respective lean controls, indicated by a greater peak blood glucose response and area under the curve following the 1 g/kg i.p. glucose injection (Figure 1). Peak blood glucose was 461.4 ± 12.0 mg/dl in *ob* mice versus and 253.1 ± 35.6 mg/dl in WT lean controls (($P = 0.0002$), and 404.0 ± 38.4 in prehib versus 242.3 ± 11.6 in summer GMGS (and mg/dl, ($P = 0.0006$), indicating similar degrees of glucose intolerance in the obese animals of both species.

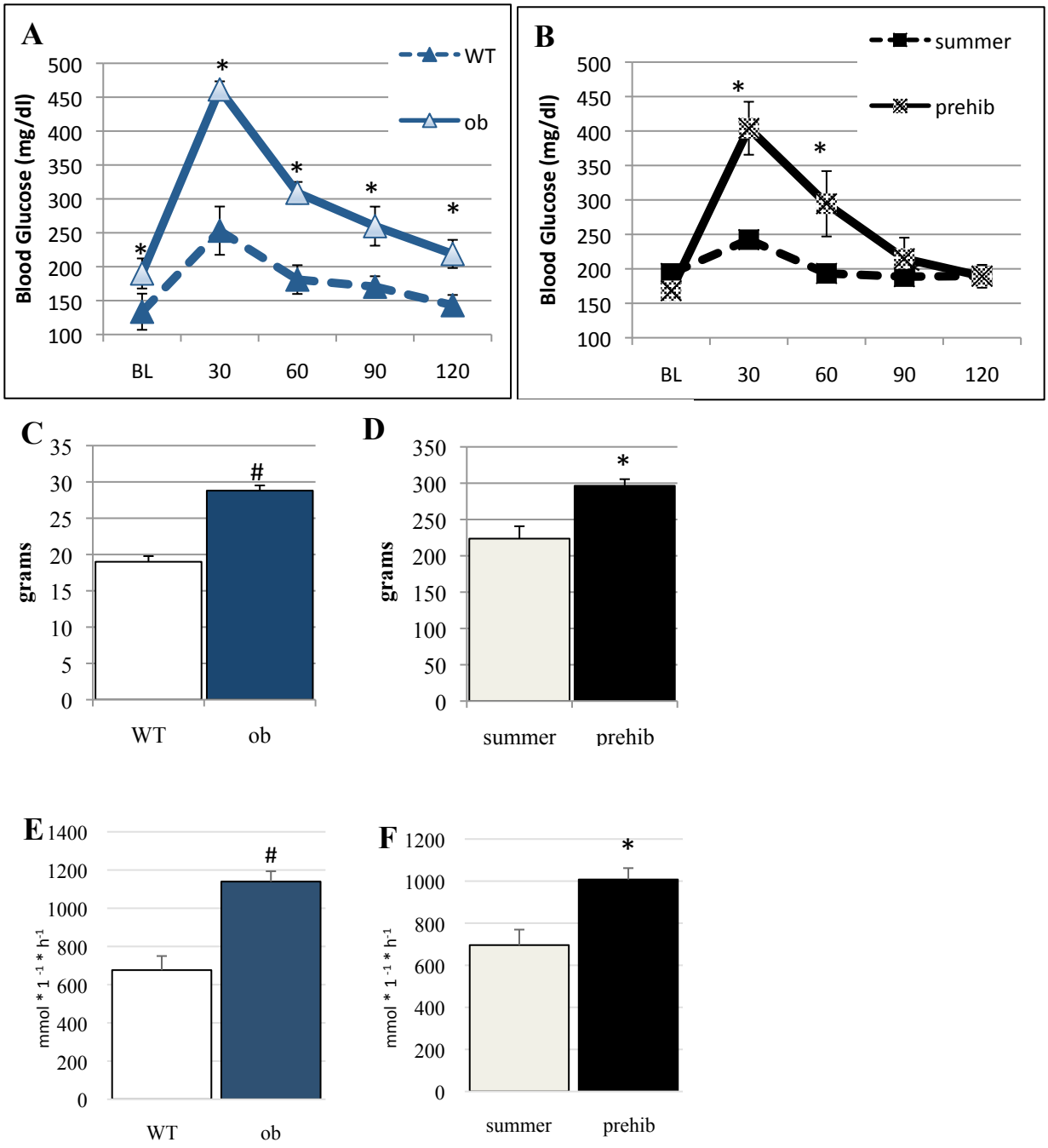

Figure 1. IP glucose tolerance test and body weight in *ob* and WT mice and summer and prehib GMGS. (A) IP glucose tolerance test for *ob* versus WT mice. Baseline (BL) fasting glucose and peak glucose post injection was significantly higher in *ob* than WT. (B) IP glucose tolerance test for prehib versus summer GMGS. Peak glucose post injection was significantly higher in prehib versus summer GMGS. (C) Body mass was significantly higher in *ob* versus WT mice. (D) Body mass was significantly higher in prehib versus summer GMGS. (E) Area under the curve from IP glucose tolerance test in mice. (F) Area under the curve from IP glucose tolerance test in GMGS. *P<0.05 prehib versus summer and *ob* versus WT.

Mitochondrial Respiration: Cardiac Mitochondria

To determine whether aspects of mitochondrial respiratory capacities were impaired after a period of short-term obesity in GMGS and mice, isolated cardaic mitochondria were studied using high-resolution respirometry in the presence or absence of FA (palmitoylcarnitine). Mitochondrial OXPHOS capacity (oxygen flux achieved after addition of substrates in the presence of ADP) was analyzed under various substrate conditions to delineate substrate specific alterations in OXPHOS capacity (Figure 2). FA-supported OXHPOS capacity was 31% greater in prehib versus summer GMGS ($P < 0.05$), while pyruvate-supported OXPHOS was 67% greater ($P < 0.05$) indicating greater mitochondrial flexibility in prehib versus summer. OXPHOS supported by a combination of FA and CHO was 62% greater in prehib versus summer ($P < 0.01$)(Figure 2A, B, &C.). Total OXPHOS capacity (CHO, FA and succinate) was 24% greater in prehib versus summer GMGSs ($P < 0.01$). No significant differences in OXPHOS capacities were observed between *ob* and WT mice.

To examine the capacity of cardiac mitochondria to oxidize pyruvate in the presence of saturating levels of FA substrate and ADP, pyruvate (CHO) was added to the respiratory chamber after stable FA-supported OXPHOS flux was established. The additive effect of pyruvate on respiratory flux was calculated as a percent increase from the previous FA-supported OXPHOS state ((FA+ CHO) – FA)/ FA), and compared between summer versus prehib GMGS and *ob* versus WT mice. The additive effect of pyruvate on FA-supported OXPHOS increased by 69% in prehib versus summer ($P < 0.01$) but decreased by 18% in *ob* mice versus WT ($P < 0.05$) (Figure 2 D), indicating a much greater responsiveness to pyruvate in prehib versus summer GMGS, and decreased responsiveness in the ob versus WT mice

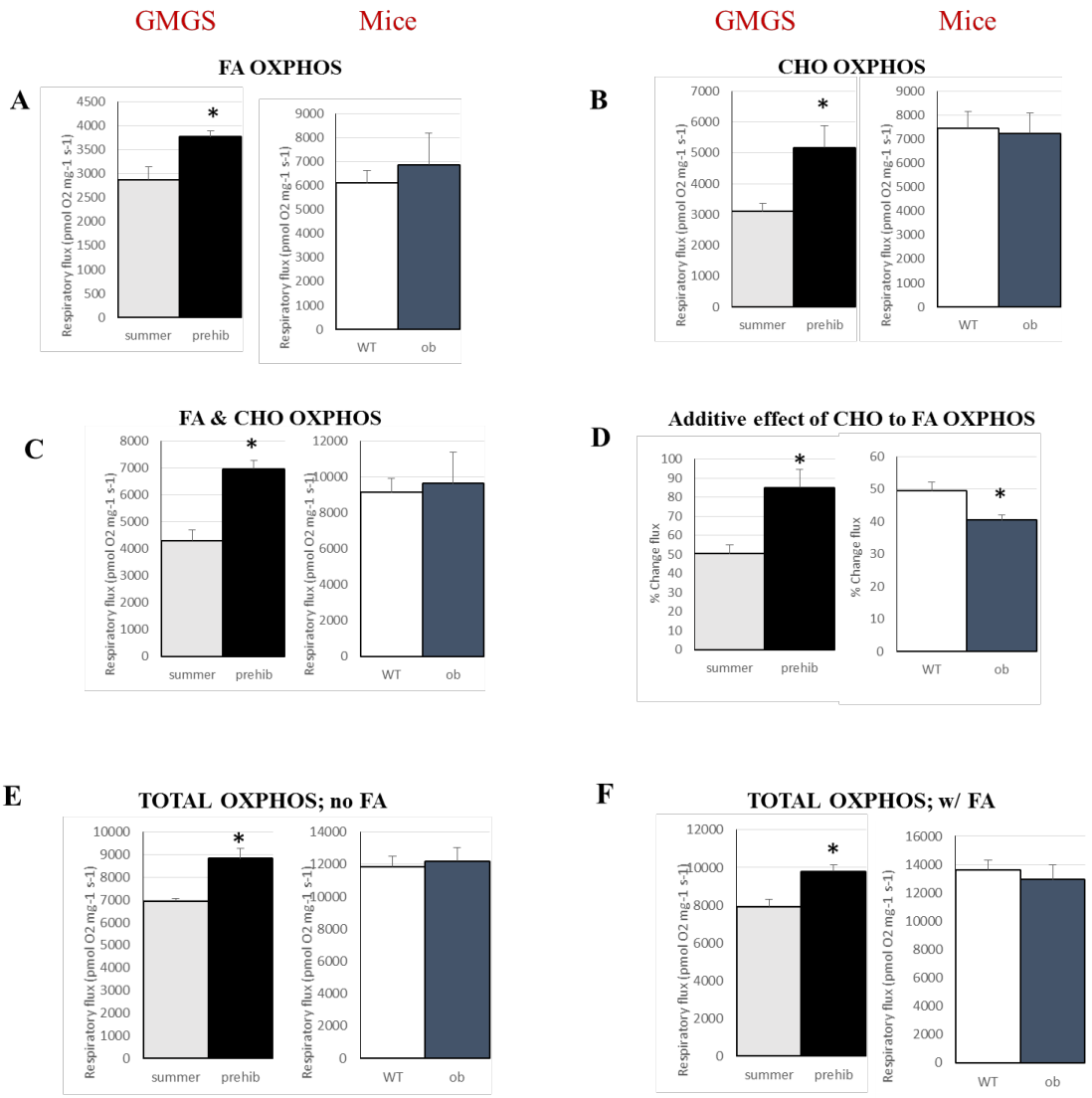

Figure 2. The effect of short-term obesity on isolated cardiac mitochondrial respiratory function in GMGS and mice;. (A) Fatty acid (FA) supported oxidative phosphorylation (OXPHOS);. (B) Pyruvate (CHO) supported OXPHOS; (C) Both FA and CHO supported OXPHOS; (D) Additive effect of CHO on FA OXPHOS ((FA + CHO OXPHOS – FA OXPHOS)/ FA OXPHOS); (E) Total OXPHOS with no FA; (F) Total OXPHOS in the presence of FA.

Mitochondrial "Leak" Respiration and Coupling Control: Cardiac Mitochondria

To determine whether there were alterations in mitochondrial "leak" (non- coupled respiration) after a period of short-term obesity in GMGS and mice, isolated cardiac mitochondria were studied using high-resolution respirometry in the presence of pyruvate or FA (+ malate), but in the absence of ADP. Without ADP present, Complex V cannot harness the proton motive force to generate ATP; therefore, respiration proceed only to the extent that the protons "leak" down their concentration gradient, or mitochondrial membrane potential is dissipated by other mechanisms (e.g., uncoupling protein). Thus, in the leak state, ETS flux is "uncoupled" from ADP phosphorylation. In GMGS, FA- and CHO-supported leak were 23% and 21% greater in prehib versus summer ($P < 0.05$), whereas no significant differences were observed in mice (Figure 3, A & C). OXPHOS coupling control can be calculated by evaluating the additive effect of ADP on leak respiration ((OXPHOS-LEAK)/OXPHOS)) in the presence of FA or CHO substrates. Interestingly, cardiac mitochondria from both GMGS and mice were highly (~90-95%) coupled with both FA and CHO substrates (Figure 3, B & D), with no difference between lean and obese cohorts in either species (prehib versus summer, $P > 0.1$; *ob* versus WT, $P > 0.6$).

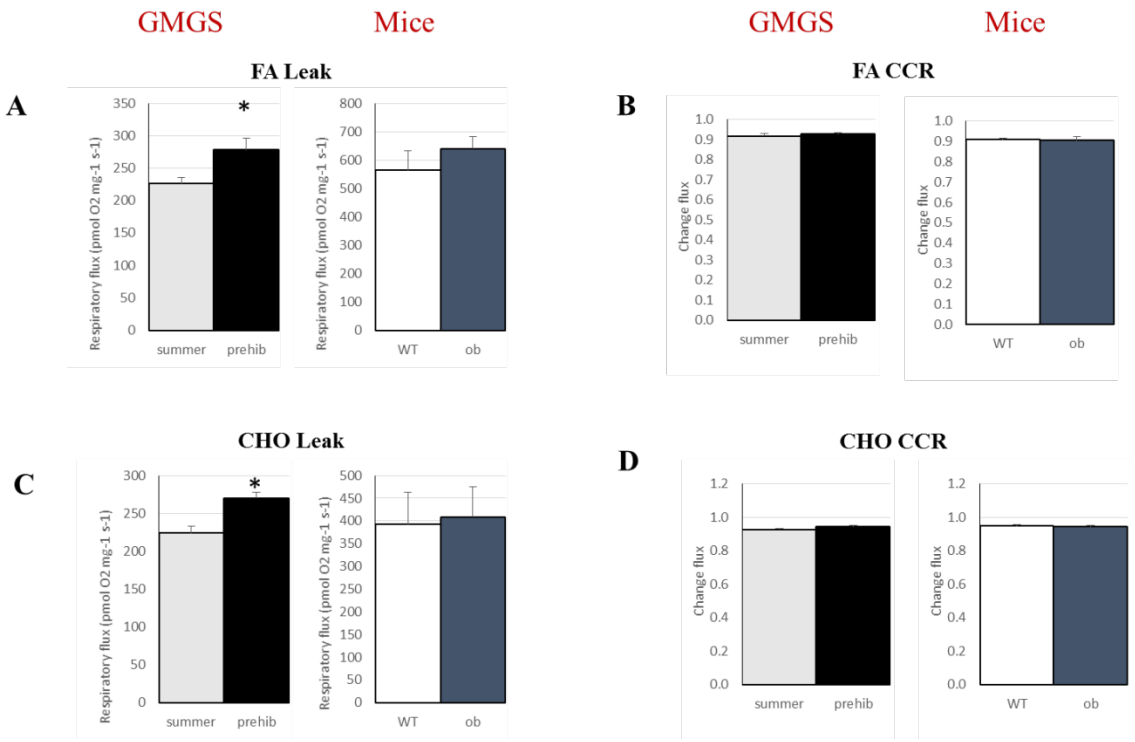

Figure 3. The effect of short-term obesity on isolated cardiac mitochondrial respiratory function in GMGS and mice. (A) Fatty acid (FA) supported "Leak". (B) FA oxidative phosphylation (OXHPOS) coupling control factor, calculated as (FA OXPHOS – FA LEAK/ FA OXPHOS) (C) Pyruvate (CHO) supported "Leak" D) CHO OXHPOS coupling control factor, calculated as (CHO OXPHOS – CHO LEAK/ CHO OXPHOS)

Mitochondrial Respiration: Skeletal Muscle Mitochondria

To determine whether mitochondrial OXPHOS capacities were impaired after a period of short-term obesity in GMGS and mice, isolated skeletal muscle mitochondria were studied using high-resolution respirometry in the presence or absence of FA. FA-supported OXHPOS capacity was 44% greater in prehib versus summer GMGS ($P < 0.05$). However, CHO supported OXPHOS capacity was 37% lesser in prehib versus summer GMGS ($P < 0.05$). OXPHOS supported by a combination of FA and CHO was 44% greater in prehib versus summer GMGS ($P < 0.05$) (Figure 4 A, B, &C.). FA-supported OXHPOS capacity was 39% greater in *ob* versus WT mice ($P < 0.05$). OXPHOS supported by saturating levels of FA and CHO was 23% greater in *ob* versus WT mice ($P < 0.05$). No significant differences were observed in CHO OXPHOS capacity in *ob* versus WT mice (Figure 4 A, B, &C.). No significant differences in total OXPHOS capacity (CHO, FA and succinate) were observed in summer versus prehib GMGS or *ob* versus WT mice (Figure 4 F)

The additive effect of pyruvate on FA-supported OXPHOS decreased by 21% indicating decreased responsiveness to CHO in *ob* mice versus lean controls ($P < 0.05$) (Figure 4 D). There was no significant additive effect of pyruvate on FA-supported OXPHOS in summer versus prehib GMGS.

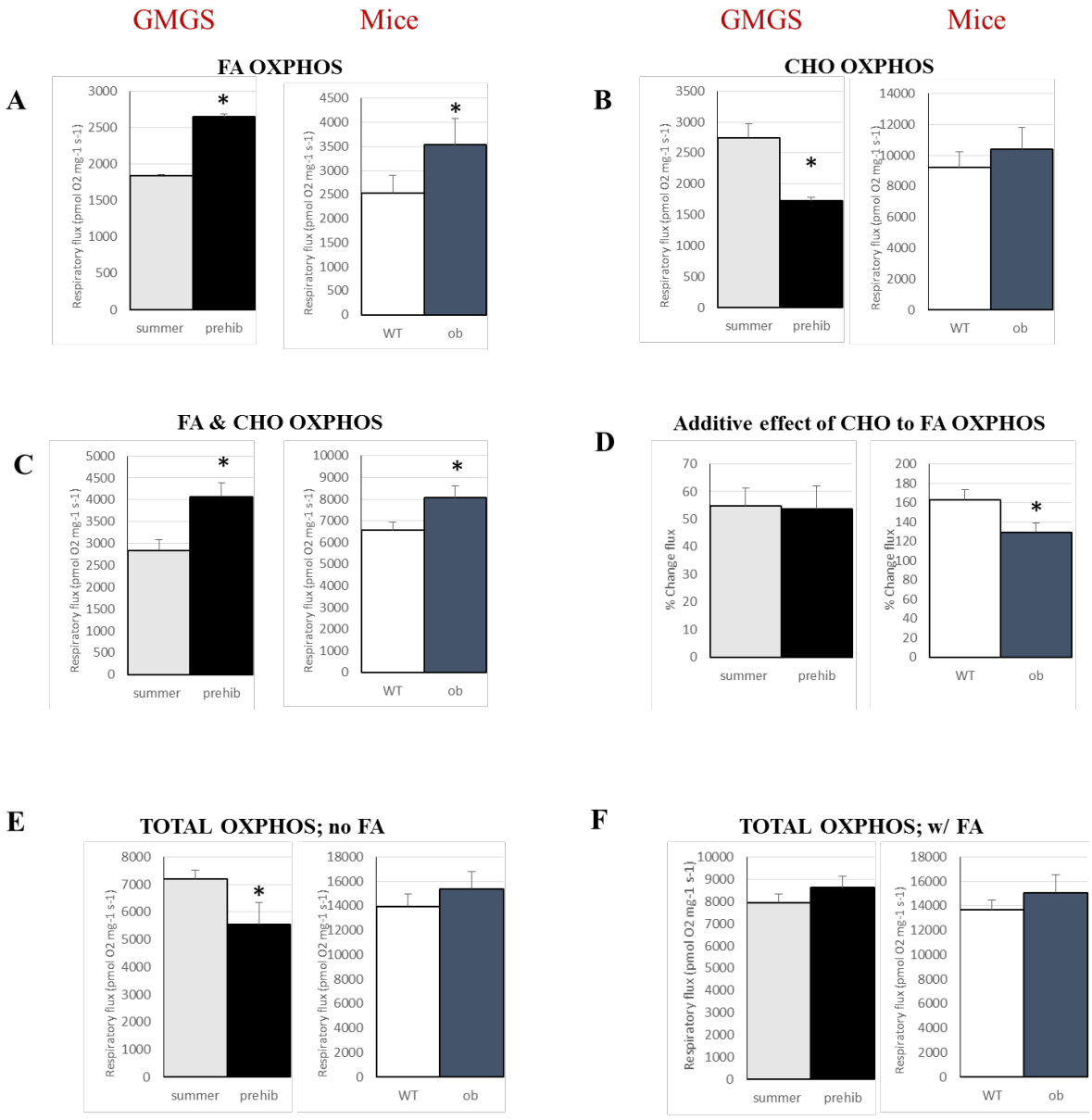

Figure 4. The effect of short-term obesity on isolated skeletal muscle mitochondrial respiratory function in GMGS and mice. (A) Fatty acid (FA) supported oxidative phosphorylation (OXPHOS). (B) Pyruvate (CHO) supported OXPHOS (C) Both FA and CHO supported OXPHOS D) Additive effect of CHO to FA OXPHOS ((FA + CHO OXPHOS – FA OXPHOS)/ FA OXPHOS) (E) Total OXPHOS with no FA (F) Total OXPHOS in the presence of FA.

Mitochondrial "Leak" Respiration and Coupling Control: Skeletal Muscle Mitochondria

To determine whether there were alterations in mitochondrial leak after a period of short-term obesity in GMGS and mice, isolated skeletal muscle mitochondria were studied using high-resolution respirometry in the presence or absence of FA. In GMGS, FA- supported leak was 32% greater and CHO-supported leak was 39% greater in prehib versus summer ($P < 0.05$) (Figure 5, A & C). In mice, FA- supported leak was 49% greater in *ob* versus WT ($P < 0.05$); however, no significant differences were observed in CHO-supported leak in *ob* versus WT (Figure 5, A & C)

Isolated skeletal muscle mitochondria from GMGS and mice were highly (~85-90%) coupled with FA (Figure 3, B & D), with no difference between lean and obese cohorts in either species (prehib versus summer, $P > 0.3$; *ob* versus WT, $P > 0.2$). Isolated skeletal muscle in the presence of CHO were highly coupled (~95%) in mice with no difference between *ob* and lean mice ($P > 0.3$) (Figure 5, D). In contrast, isolated skeletal muscle in GMGS in the presence of CHO, skeletal muscle were significantly less coupled by 9% in prehib versus summer GMGS ($P < 0.01$) (Figure 5, D).

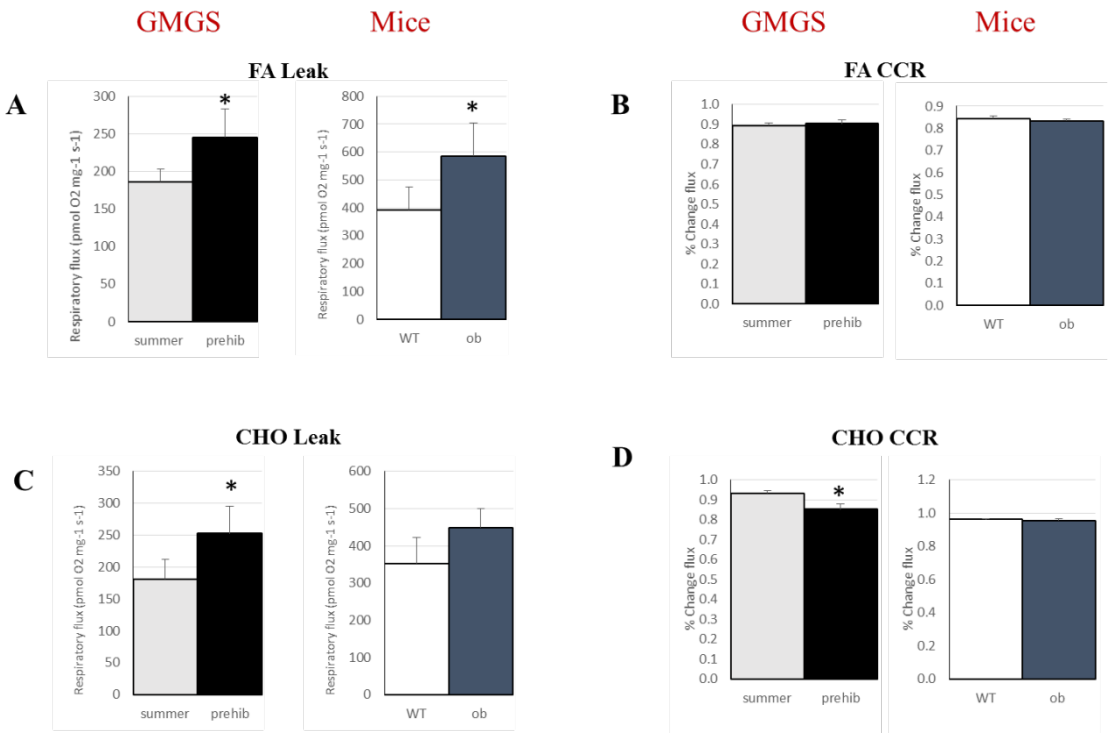

Figure 5. The effect of short-term obesity on isolated skeletal mitochondrial respiratory function in GMGS and mice. (A) Fatty acid (FA) supported "Leak". (B) FA oxidative phosphylation (OXHPOS) coupling control factor, calculated as (FA OXPHOS – FA LEAK/ FA OXPHOS) (C) Pyruvate (CHO) supported "Leak" D) CHO OXHPOS coupling control factor, calculated as (CHO OXPHOS – CHO LEAK/ CHO OXPHOS)

Mitochondrial Respiration: Permeabilized Muscle Fibers

Analyzing permeabilized fibers can give insight on *in situ* mitochondrial function. The use of permeabilized muscle fibers allows a detailed analysis of functional mitochondrial in their normal intracellular position and assembly, preserving the essential interactions with other organelles in the tissue (Kuznetsov et al., 2008). Mitochondrial FA-supported OXHPOS capacity was 110% greater in prehib versus summer GMGS, but CHO-supported OXPHOS capacity was not significantly different. However, combined FA+CHO-supported OXPHOS was 20% greater on prehib versus summer GMGS ($P < 0.05$) (Figure 6 A, B, &C.). No significant differences were observed in OXPHOS capacities in mice; however, there was a trend towards increased FA OXPHOS capacity ($P = 0.08$) in *ob* versus WT (Figure 6 A, B, &C.). Total OXPHOS (with the addition of glutamate and succinate) in the presence of FA was 20% greater in prehib versus summer GMGS ($P < 0.05$). No significant differences in total OXPHOS capacity (CHO, FA and succinate) were observed in *ob* versus WT mice (Figure 6 F).

There was no additive effect of pyruvate on FA-supported OXPHOS in *ob* mice or prehib GMGS compared to their respective lean controls (Figure 6 D).

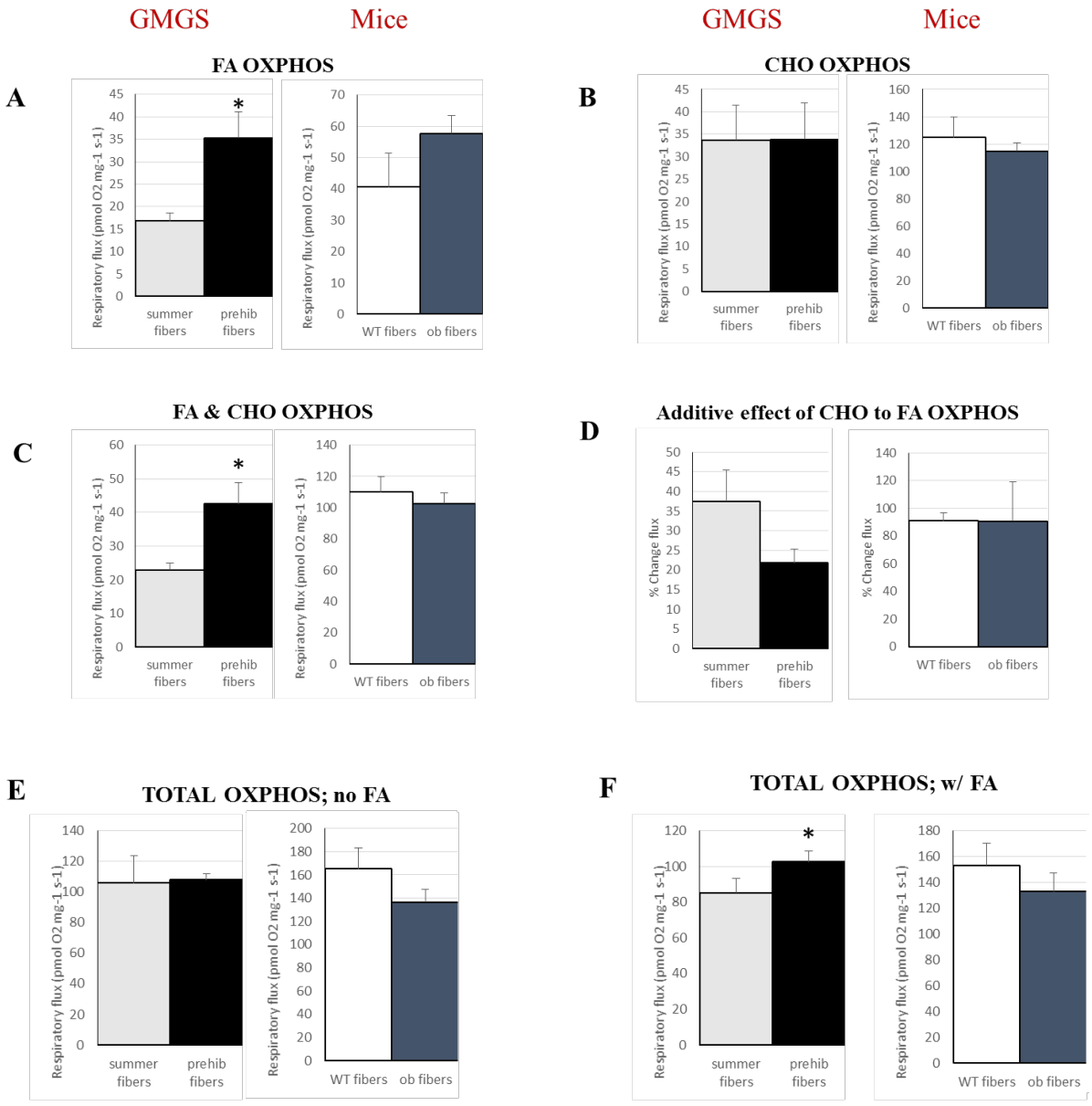

Figure 6. The effect of short-term obesity on permeabilized skeletal muscle fiber mitochondrial respiratory function in GMGS and mice. (A) Fatty acid (FA) supported oxidative phosphorylation (OXPHOS). (B) Pyruvate (CHO) supported OXPHOS (C) Both FA and CHO supported OXPHOS D) Additive effect of CHO to FA OXPHOS ((FA + CHO OXPHOS – FA OXPHOS)/ FA OXPHOS) (E) Total OXPHOS with no FA (F) Total OXPHOS in the presence of FA.

Mitochondrial "Leak" Respiration and Coupling Control: Permeabilized Skeletal Muscle Fibers

To determine whether there were alterations in mitochondrial leak after a period of short-term obesity in GMGS and mice, permeabilized skeletal muscle fibers were studied using high-resolution respirometry in the presence or absence of FA. In GMGS, FA- supported leak was 110% greater and CHO-supported leak was 84% greater in prehib versus summer GMGS ($P < 0.05$) (Figure 7, A & C). In mice, FA-supported leak was 59% greater in *ob* versus WT mice ($P < 0.05$). Non-significantly, CHO-supported leak was 43% greater in *ob* versus WT mice ($P= 0.068$) (Figure 7, A & C)

In GMGS and mice permeabilized skeletal muscle fibers in the presence of FA, there was no significant difference in coupling control between lean and obese cohorts in either species. (Figure 7, B & D) In contrast, in GMGS and mice permeabilized skeletal muscle fibers in the presence of CHO, coupling control decreased by 22% in prehib versus summer GMGS ($P < 0.05$) and 14% in *ob* versus WT mice ($P < 0.01$) (Figure 7, B & D).

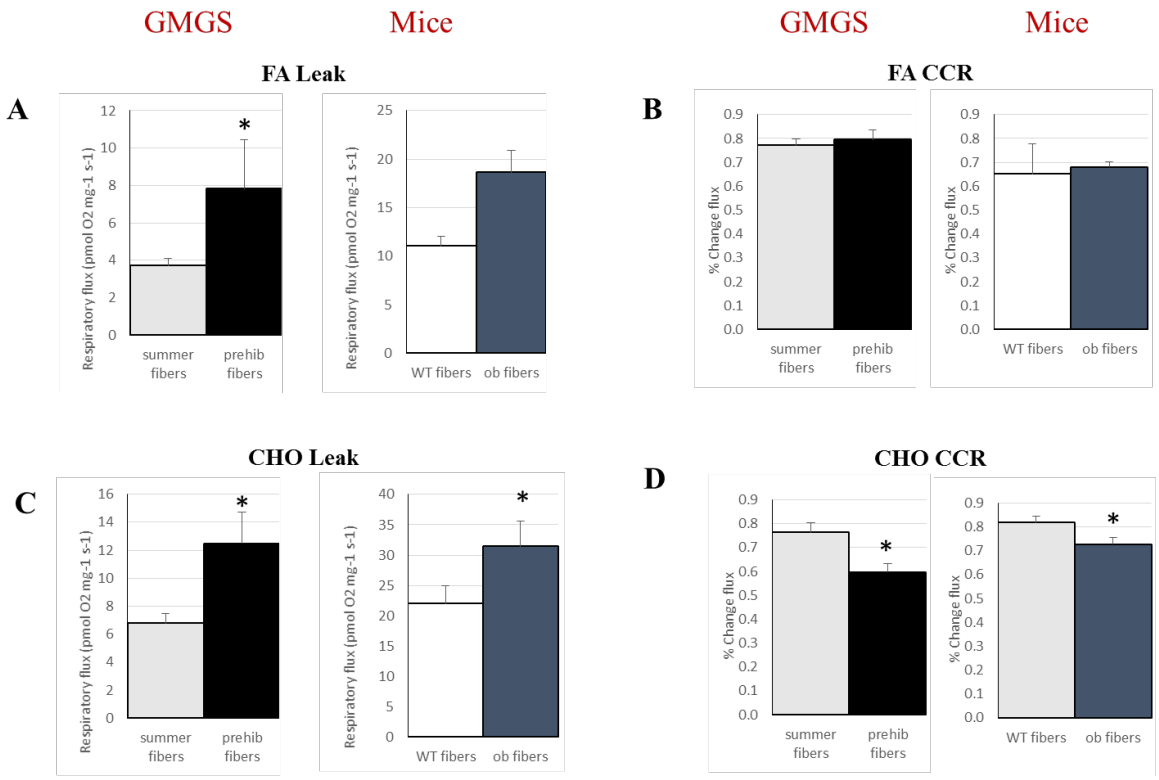

Figure 7. The effect of short-term obesity on permeabilized skeletal muscle fiber mitochondrial respiratory function in GMGS and mice. (A) Fatty acid (FA) supported "Leak". (B) FA oxidative phosphylation (OXHPOS) coupling control factor, calculated as (FA OXPHOS – FA LEAK/ FA OXPHOS) (C) Pyruvate (CHO) supported "Leak" D) CHO OXHPOS coupling control factor, calculated as (CHO OXPHOS – CHO LEAK/ CHO OXPHOS)

DISCUSSION

Obesity induced alterations in mitochondrial function has long been proposed as one of the mechanisms contributing to the development of insulin resistance and DM. Transcriptional and posttranslational events that occur in metabolic states of nutrient overload finely adjust the mitochondria to meet the metabolic and energy demands of the tissue (Balaban, 2010). Most previous studies assessing mitochondrial respiratory function in the heart and skeletal muscle have studied chronically obese humans and rodents, making it difficult to elucidate if changes in mitochondrial respiratory function are the cause or consequence of obesity-related disorders. To our knowledge, this study is the first to assess the effects of short-term obesity on cardiac and skeletal muscle mitochondrial function in natural hibernating animal and a genetic obesity model independently. We have several main findings: (1) both species showed significant weight gain and glucose intolerance versus their respective lean controls; (2) isolated cardiac mitochondrial OXPHOS capacities were significantly greater in prehib versus summer GMGS, whereas no significant differences in OXPHOS capacities were observed between *ob* and WT mice; (3) there were substrate specific differences in isolated skeletal muscle mitochondrial OXHPOS capacities when in the presence of CHO in prehib versus summer GMGS as well as *ob* versus WT mice characteristic of "metabolic inflexibility" seen in diabetics; (4) In the presence of FA in isolated skeletal muscle, neither species exhibited the reduced OXPHOS capacity or uncoupling reported in obesity/ diabetes in the literature. In fact, FA OXPHOS was greater in prehib versus summer GMGS as well as *ob* versus WT mice; (5) mitochondrial respiratory responses to short term obesity were not consistent between species, indicating species-specific responses that appear to be independent of weight gain and glucose intolerance *per se*.

Obesity and DM are associated with reduced cardiac mitochondrial oxidative capacity (Boudina et al., 2005; Montaigne et al., 2014). In our study, isolated cardiac mitochondrial OXPHOS capacities were significantly greater in prehib versus summer GMGS, whereas no significant differences in OXPHOS capacities were observed between ob and WT mice. Neither species exhibited reduced cardiac mitochondrial respiratory capacity, nor uncoupling reported with obesity or DM in the literature, suggesting these are later-stage effects requiring longer duration and/ or more severe obesity. It is curious that cardiac mitochondrial OXPHOS capacity are greater with obesity in GMGS. To our knowledge, this is the first study to assess cardiac mitochondrial function in summer versus prehib hibernators and therefore difficult to compare findings with other studies. It must be reminded that the ambient temperature of the animal holding room was decreased from ~18°C to 15°C in September, ~5 weeks before the prehib animals were sacrificed to mimic seasonal changes experienced by this species. Interestingly, a recent unpublished study on 13 lined ground squirrels by a group from the University of Duluth reported greater brain mitochondrial function during hibernation compared to summer active controls (Ballinger, 2015). Perhaps such adaptations must occur for the myocardium and brain to remain healthy for prolonged periods of low temperatures and hypometabolism during the winter hibernation period. During hibernation, GMGS left ventricular mass increases by ~30% from the summer active period (Nelson & Rourke, 2013). Maybe the drop in temperature initiated a biologically adaptive response to increase left ventricular heart mass, mitochondrial content, and potentially, intrinsic mitochondrial OXPHOS capacity. Fascinatingly, marsupials that were exposed to chronic cold exposure (~12°C) increased heart mass and whole body mitochondrial volume compared to thermoneutral controls, indicating a physiological response to cold exposure (Schaeffer, Villarin, & Lindstedt, 2003). Although we do not have direct metabolic or body

temperature data on the GMGS in this study, a study by Sheriff et al., 2012 discovered that thermoregulatory adjustments occur up to 45 days prior to the onset of hibernation in arctic ground squirrels when average ambient temperature is ~15°C, suggesting that even gradual reductions in ambient temperature can have an impact in metabolic and physiological adjustments in hibernating ground squirrels (Sheriff et al., 2012).

It is unclear what could cause the increase in cardiac mitochondrial OXPHOS capacity mammals; however, a study on GMGS found PPARgamma co-acitivator (PGC-1alpha), a key regulator of energy metabolism and mitochondrial biogenesis, demonstrated at a greater level in the heart during hibernation compared to summer controls (Eddy et al., 2005) and heart mitochondrial OXHPOS capacity was significantly greater in hibernating painted turtles versus normothermic controls (Birkedal et al., 2004). However, in thirteen-lined ground squirrels, cardiac mitochondrial OXPHOS capacity was lower during hibernation torpor and interbout euthermia when compared to summer euthermia, even when data was normalized to citrate synthase activity (Brown & Staples, 2014). Further mechanistic studies need to be conducted on hibernators to elucidate what may cause the increase in cardiac mitochondrial OXHPOS capacity in prehibernation animals as observed in this study.

There was a greater additive effect of pyruvate on isolated cardiac respiratory capacity in the presence of saturating levels of FA in prehib versus summer GMGS, but a lesser additive effect in *ob* versus WT mice. The trend shown in mice is characteristic of "metabolic inflexibility" seen in states of nutrient excess (S. Lee, Rivera-Vega, Alsayed, Boesch, & Libman, 2015). The greater additive effect in prehib GMGS compared to summer controls could be attributed to the cardiac remodeling discussed earlier. Short-term obesity appears to induce

species-specific responses to cardiac mitochondrial function that are independent of weight gain and glucose intolerance.

Several lines of evidence link decreased mitochondrial function in skeletal muscle with the emergence of insulin resistance and the switch between normal glucose tolerance and DM; however, the evidence remains inconclusive (Holmström, Iglesias-Gutierrez, Zierath, & Garcia-Roves, 2012; David E. Kelley, He, Menshikova, & Ritov, 2002a; D. E. Kelley & Mandarino, 2000; Ritov et al., 2010). In vivo data either supports (Ritov et al., 2010), or challenges (Holmström et al., 2012) the notion that mitochondrial function plays a role in the development of insulin resistance and DM. In our study we provide evidence for greater OXPHOS capacity in the presence of FA in both species versus their respective lean controls. During states of nutrient overload, there is an increase in triglycerides and free FAs that have been shown to activate PPARα, which is a major regulator of many genes involve with lipid metabolism (Kersten, 2014). The activation of PPARα has been shown to increase rates of FA oxidation (J.-Y. Lee et al., 2011). Therefore, the increase in isolated skeletal muscle OXPHOS capacity in the presence of FA supports what has been seen in the literature.

Conversely, in the presence of CHO and no FA, OXPHOS capacity is lower in prehib versus summer GMGS whereas no significant difference was observed in mice. Unlike laboratory animals that are born and raised in laboratory cages, we trapped the GMGS in the wild where they were extremely active in search of food, mates, etc. The decreased OXPHOS capacity in the presence of CHO in prehib versus summer GMGS could be attributed to a reduction in activity level once animals were restricted to sedentary cage activity. Training induces skeletal muscle mitochondrial metabolic alterations that increase flexibility and glycogen storage and usage (Hoppeler & Fluck, 2003). The length of time removed from the active

environment could have a large impact on the reduction in CHO OXPHOS capacity in prehib versus summer GMGS.

On the other hand, in permeabilized soleus fibers, no decrease in CHO OXPHOS capacity is observed in prehib versus summer GMGS. The difference between findings could be attributed to differences in skeletal muscle used in the isolated skeletal versus permeabilized fiber experiments. Skeletal muscle differs greatly due to different locomotor, structural and metabolic demands (Rabøl et al., 2010). To isolate skeletal muscle, we used the entire hind limb, which includes a combination of differing fiber types and characteristics, whereas the soleus tissue used for permeabilized fiber experiments is primarily oxidative. A study by Holmström et al., 2012 found greater OXPHOS capacity in the presence of pyruvate but not FA in glycolytic skeletal muscle of obese *db* mice compared to lean WT, but a slight decrease in isolated oxidative skeletal muscle OXPHOS capacity in the same experimental animals suggesting differences in mitochondrial oxidative capacity between skeletal muscle types (Holmström et al., 2012). Another study by Jacobs et al., 2013 found substrate specific significant differences in mitochondrial function in mouse soleus, gastrocnemius, and quadriceps in healthy mice, further elucidating differences in respiratory capacity between skeletal muscles (Jacobs, Díaz, Meinild, Gassmann, & Lundby, 2013).

A major limitation to our study involves a lack of complimentary mechanistic data that would help elucidate causality of our seen changes in mitochondrial function. It is well established in the literature that ROS may play an essential role in the development of reduced mitochondrial oxidative capacity in obese and diabetic individuals (Lefort et al., 2010; Montaigne et al., 2014). Our lab is perfecting the measurement of real-time hydrogen peroxide (H2O2) using florometry during mitochondrial respiratory experiments that will help determine

differences in H2O2 production after periods of short-term obesity. It would also have been helpful to gather systemic data from our subjects, including circulating hormones and FFAs, which would help further elucidate the observed changes in mitochondrial function and glucose intolerance. Another limitation is the decrease in ambient temperature in GMGS animal holding room ~5 weeks prior to the sacrifice of prehib GMGS. As discussed earlier, cold exposure can cause cardiac and mitochondrial remodeling in mammals and may have contributed to the species-specific differences seen in GMGS. A follow up study is currently being conducted by our laboratory to examine the effects of cold exposure in *ob* and WT mice and the absence of cold exposure in prehibernating GMGSs, to determine if the differences between species are attributable to cold-exposure.

In summary, our study indicates that both *ob* mice and prehib GMGS showed significant weight gain and glucose intolerance versus their respective lean controls. However, changes in cardiac and skeletal muscle respiratory function after a period of short-term obesity was unique to each species, indicating species-specific responses independent of weight gain and glucose intolerance. Neither species exhibited reduced OXPHOS capacities in isolated cardiac mitochondrial nor uncoupling reported with obesity/ DM, suggesting these are later-stage effects requiring longer duration and/ or more severe obesity in the heart. Isolated skeletal muscle respiratory function demonstrated lesser CHO OXPHOS capacity and coupling control in the prehib versus summer GMGS, but no effect of obesity was seen in mice, which may be attributed to the changes in activity levels in the species once restricted to sedentary cage activity. The greater FA OXPHOS capacity seen in both species versus their respective lean controls may indicate an increase in genes involved with lipid metabolism. Mitochondrial responses to obesity differed between models despite nearly identical effects of weight gain and glucose intolerance.

Therefore, the observed differences between squirrel and mice likely reflect responses to different environmental cues or genetic background independent of 'classic' effects of obesity. The observed mitochondrial responses are not simply responses of obesity and glucose intolerance.

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