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Methionine restriction-induced metabolic changes in C57BL6J mice

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METHIONINE RESTRICTION-INDUCED METABOLIC CHANGES IN C57BL6J MICE

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ABSTRACT

Introduction: Eighty percent restriction of normal dietary methionine (MR) intake has been shown to increase energy expenditure and attenuate the rate of adiposity gain in rodents, despite a paradoxical increase in energy intake. Energy expenditure in rats was shown to increase, even though physical activity level stays the same. This observation suggests that metabolic mechanisms account for the majority of increased energy expenditure measured in methionine restricted animals.

Purpose: To observe and document the onset of physiological effects brought about and to determine the mechanistic role of the skeletal muscle on MR-induced metabolic changes in the C57BL6J mouse.

Methods: C57BL6J mice were fed a control (CON) or MR diet for eight weeks in which food consumption, effect on body composition, physical activity, and energy expenditure were documented. Expression of skeletal muscle genes known to change with increased fatty acid oxidation (*UCP3* and *CPT1b*) were measured post-mortem to determine any mechanistic changes of skeletal muscle fuel utilization.

Results: MR-fed C57BL6J mice gain less overall body mass than CON animals, which can be measured within the first few weeks of MR intervention. Additionally, differences in energy expenditure can be measured within the first 14 days. Despite alteration in energy expenditure, MR mice maintain similar levels of activity compared to control animals. Expression of *UCP3* and *CPT1b*, genes associated with increased fatty acid uptake and utilization in skeletal muscle do not change, suggesting increased metabolic affects of other tissues.

Conclusion: MR-fed mice exhibit a similar phenotype to that previously reported in rats on MR. Shortly after consumption of MR, C57BL6J mice exhibited an increase in energy expenditure. The increase in energy expenditure in these mice was not influenced by a change in physical activity, or genes associated with increased fatty acid utilization in the skeletal muscle tissue.

CHAPTER 1 INTRODUCTION

Obesity and the metabolic syndrome continue to plague developed countries around the world [1]. Despite advances in technology and nutritional research, rates of obesity continue to rise exponentially. It was recently reported that more than one third of U.S. adults are considered overweight and/or obese, a BMI of 25 to 30 $kg \cdot m^{-2}$ and $\geq$ 30 $kg \cdot m^{-2}$, respectively [2], which cost taxpayers more than $147 billion in 2008 [3]. Within the past year, the American Medical Association (AMA) recognized obesity as a disease for the first time in history [4]. The AMA, Nation Institute of Health, Centers for Disease Control and Prevention, and others have instituted a call-to-action in attempt to prevent obesity from escalating to epidemic proportions. The U.S. Department of Health and Human Services has assisted by recommending nutrition and physical activity standards for all Americans [5]. The actions of these organizations have failed to attenuate the increase in individuals who are overweight or obese.

While a number of voluntary factors have been associated with the development of adiposity accumulation, research has shown that advancing age may be the most significant risk factor in the development of obesity. This age-related adiposity gain is associated with a 7% per decade reduction in resting metabolic rate [6,7]. Physical activity and nutrition interventions have been shown to be marginally effective in reducing existing fat mass and at slowing the rate at which age-related adipose tissue is accumulated, even in healthy individuals [5]. Many of those suffering from increased adiposity are otherwise healthy individuals who cannot, or simply do not, follow the research-based nutritional and physical activity guidelines set forth by the government. Further, only half (48%) of Americans were able to meet the 2008 Physical Activity Guidelines for Americans [8] and it is reported that thirteen states in the US currently have obesity rates at or above 30% [9]. Despite the efforts of these organizations, obesity rates

continue to rise in the US. This has prompted researchers to explore a variety of other potential obesity treatments and preventative programs, including using dietary restriction models which have previously shown promise.

CHAPTER 2 REVIEW OF LITERATURE

Calorie Restriction

Calorie restriction (CR) is defined as a reduction in overall energy intake without inducing malnutrition [10]. In 1935, McCay *et al*. published a study which used CR by reducing the caloric intake of the treatment group by 54% *ad libitum* feeding of an otherwise complete diet without malnutrition. Results from the four-year experiment revealed that not only did CR slow maturation and weight gain in juvenile rats, but this dietary model also extended lifespan by 30-35% [11,12]. Observations were subsequently confirmed by several additional studies of CR in rats [13-15]. Calorie restriction also has the potential to prevent and treat cancer in animal models [13,16]. In humans, CR can decrease incidence of cardiovascular disease and improve memory function [17,18]. Despite the overwhelming health benefits associated with CR, implementing it in the human population has proven to be a truly difficult task [19], and other methods of dietary restriction have become the subject of current research.

Essential Amino Acid Restriction

Essential amino acid (EAA) restriction has emerged as a possible surrogate for CR [12]. For an amino acid (AA) to be considered essential, it must be extremely difficult, if not impossible, for the body to synthesize from other compounds and therefore must be consumed in the diet, whereas nonessential AAs are synthesized readily by the body [20]. Diets with restricted amounts of individual EAAs have caused rodents to exhibit a phenotype which very closely resembles that previously observed in CR studies [21].

Early work in the area of EAA restriction reported that diets low in tryptophan, an EAA, appeared to be a viable alternative to CR. Animals fed a diet restricted in tryptophan by 83% of normal feeding were found to live approximately 60 days longer and have lower organ and

whole body weights ($p<0.05$) when compared to control-fed animals [22]. Subsequent work found that rats consuming diets with a 60-70% restriction in tryptophan, compared to control, showed decreased mortality from aging and increased longevity. Unfortunately, this dietary restriction promoted higher rates of mortality in rats within the first few months after initiation of the diet, most likely as a result of the severe decrease in overall energy intake. Tryptophan-restricted diets had a tendency to induce food aversion in the test animals leading to reduced overall food consumption, and therefore restricting caloric intake of the test group. This decrease in energy intake made it difficult to determine if the reported changes in metabolism, body composition, and longevity were due to tryptophan restriction or to an overall reduction of caloric intake [23,24].

Methionine Restriction

Methionine restriction (MR) was also identified as an alternative CR mimetic years later. Methionine and cysteine are the only sulfur-containing amino acids [25]. Methionine is metabolized by the body through various steps which results in the formation of homocysteine. Homocysteine can be re-methylated to methionine, or condensed with serine to form cystathione, and ultimatley the nonessential amino acid cysteine (Figure 1) [25].

In 1993, Orentreich *et al.* discovered that by feeding Fischer 344 (F344) rats a diet void of cysteine and by reducing total methionine by 80% (total of 0.17% of normal methionine content per kg food weight) caused the rodents to experience a longevity phenotype similar to that of CR-fed rats [12]. A year later, researchers reported that initiating MR in juvenile F344 rats would extend mean lifespan by 42% and maximum lifespan by 44% [26], with similar results reported in the mature male BALB/cJ x C57BL/6J mouse [27]. Compared to control-fed rodents, rats on MR diet exhibited an overall 90% decrease in body weight gain over their

lifespan which coincided with a paradoxical increase in weight-adjusted food consumption [12,28-30]. On average, MR animals preserved a higher percentage of lean mass than control-fed animals and accumulated less overall adiposity over their lifetime [28,31]. Adult rats (e.g., 6 mo age) placed on the MR diet exhibited no additional gain in fat mass, where control animals experienced a 23% to 27% increase in adiposity deposition over six months [30]. Supplementing the MR diet with the nonessential AA cysteine caused the reduced adiposity phenotype to be muted in MR rats [32]. These findings suggest MR as a therapy to combat unhealthy weight gain in both juveniles as well as adults [27,31], and this overall decrease in age-related body weight gain is correlated with an increase in energy expenditure (EE) in MR animals when compared to control [30].

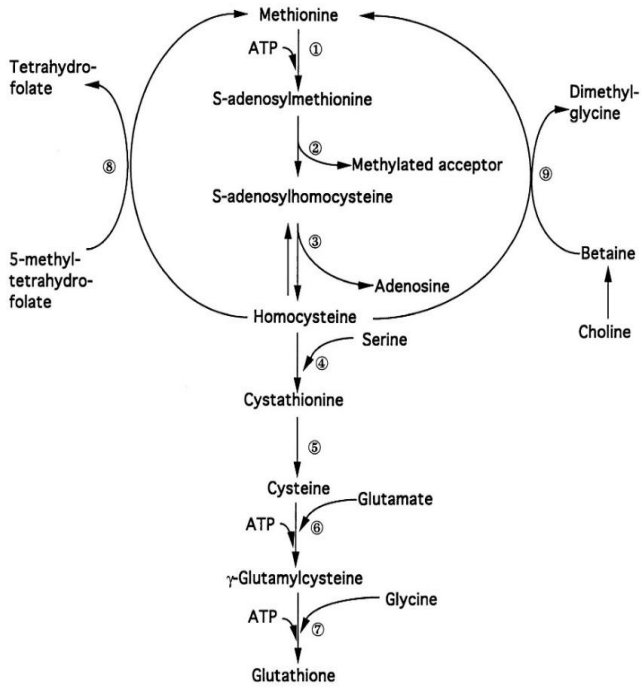

Figure 1. Methionine metabolism pathway.
Methionine is converted to cysteine via the transsulfuration pathway. Cysteine can then be utilized by the body or it can be converted to glutathione. Methionine can also be resynthesized from homocysteine if a deficiency is detected. Reprinted with the author's permission [27].

MR Increases Whole-body Energy Expenditure

The best method of measuring EE in rodents has been the source of much debate. Using indirect calorimetry, measures of resting and total daily EE can be calculated. To accurately assess EE in different animals, the calculated EE must be scaled to some function of body size. Scaling to total body weight assumes that every tissue has the same metabolic effect, whereas scaling to lean mass provides a more effective interpretation of EE [33-37]. More recently, the method of using an ANCOVA to determine the significance of many factors contributing to the overall increase in EE has been proposed and validated [35]. Although this is now the norm, one MR study using lean mass to normalize EE data reports that three months of dietary consumption of MR in rats lead to increased night-time weight-adjusted EE (kj/h/kg lean body mass) by 70%. This is the phase when rodents are typically active and are eating. EE was also 25% higher during the day-time phase, when the animals are typically sleeping (Figure 2) [30]. Preliminary data collected from mice has suggested an increase in energy expenditure using similar indirect calorimetry methods, but the use of the ANCOVA model has yet to be applied to the EE changes exhibited by mice on MR.

Substrate utilization, a measure of metabolic flexibility, has also been measured in rodents on MR using the respiratory quotient (RQ). The RQ provides information about what substrate is primarily being utilized at particular points throughout the metabolic cycle. RQ is calculated from the molar ratios of O_2 utilized and CO_2 produced during the utilization of glucose (1.00), lipid (0.70), and protein (0.80) [38]. The RQ will typically reside at 1 while utilizing glucose in the fed state, and will fall to 0.7 in the fasted state when the animal is

utilizing fat for fuel. Rats on MR have shown increased metabolic flexibility due to their dynamic range (measured by RQ) and ability to more efficiently switch substrates during times of feeding and times of fasting, typically avoiding the breakdown of proteins (Figure 2) [30].

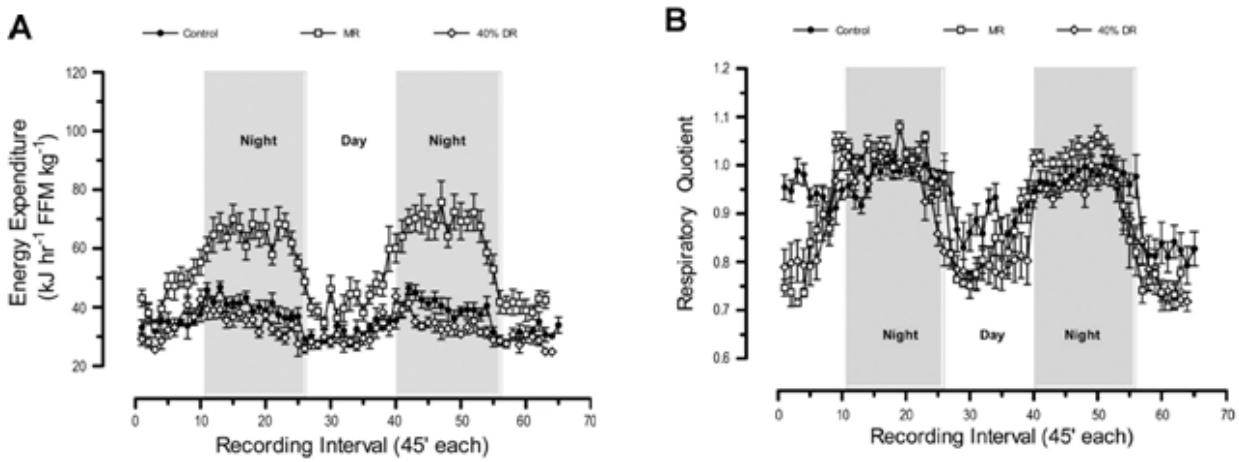

Figure 2. Energy expenditure and respiratory quotient analysis from F344 rat data. (A) Energy expenditure in F344 rats measured on the Comprehensive Lab Animal Monitoring System (CLAMS) show an increase in energy expenditure during both phases of the diurnal cycle in methionine restricted (MR) compared to control and dietary restricted rats. (B) Respiratory quotient (RQ) showing the dynamic range that MR animals exhibit while switching between fuel utilization [30].

MR and Physical Activity

In addition to resting metabolic rate and food-induced thermogenesis, physical activity is a large contributor to total EE [39]. Physical activity (PA) has been found to contribute to an overall increase in EE in MR rats, but it is not thought to be the main contributor to the increase in EE observed in response to MR. This is supported by ambulatory data of rats, expressed as beam-breaks, which showed a decrease in activity of MR animals during the day with no difference between groups at night [30]. This information coupled with the EE data suggests that there are other metabolic processes other than PA contributing to the EE increase previously measured in rats.

MR-induced Alterations in Gene Expression

Previous mechanisms such as increased uncoupling of the mitochondria, due to uncoupling protein 1 (*UCP1*), have been suggested as mechanistic contributors to the overall increased EE phenotype in MR rodents. Rats on MR exhibit increased gene expression of *UCP1* in brown and white adipose tissue, in addition to liver tissue [30]. Changes in gene expression of proteins involved in oxidative phosphorylation may be contributing to the increased EE in MR rodents. While much of the data published have favored liver and adipose tissue as main contributors to the increase in EE, these may not be the only tissues experiencing metabolic changes induced by MR. Skeletal muscle, a tissue which largely contributes to EE in mammals, has been previously overlooked in mice fed an MR diet.

In 2010, Perrone *et al.* reported changes in expression of genes related to fatty acid oxidation in skeletal muscle of MR rats [40]. A microarray of skeletal muscle genes revealed that genes associated with skeletal muscle fuel selection and EE were changed, while no change in mitochondria number was observed. Genes that were up-regulated include mitochondria transcription factor A (*TFAM*), pyruvate dehydrogenase kinase isozyme 4 (*PDK4*), and cytochrome C oxidase 4 isoform 1 (*Cox4i1*), which were up-regulated by 1.4, 3.0, and 1.3 fold respectively. These genes encode mitochondrial proteins involved in energy homeostasis. Two of the most promising increases in gene expression were Carnitine palmitoyltransferase 1 (*CPT1b*) and Uncoupling protein 3 (*UCP3*), genes associated with fatty acid oxidation in the skeletal muscle [40].

Uncoupling proteins (*UCP*s) are most often found in brown adipose tissue as the isoform *UCP1* [41], alternatively the isoforms *UCP2* and *UCP3* can be found in skeletal muscle [40]. The *UCP*s are best known for causing futile cycling of substrates which dissipates energy in the

form of heat [42]. The gene for *UCP1*, primarily found in brown adipose tissue has been shown to be up-regulated ~4 fold in white adipose tissue depots of MR F344 rats compared to control [30]. The uncoupling protein *UCP3* has been determined to be the skeletal muscle-specific *UCP* isoform [43]. Gene expression of UCP3, which serves the same purpose as the *UCP1* isoform, increases in conditions such as food deprivation and increased dietary fat consumption. Further, it has been suggested that *UCP3* is one of the major regulators of EE [44,45]. Both *UCP3* gene and protein expression are increased in rats by 1.8 and 1.9 fold, respectively in response to MR. Gene up-regulation of *UCP3* along with increased citrate synthase activity, an enzyme representative of mitochondrial aerobic capacity, suggests that the tissue favors increased fatty acid oxidation [40]. From careful review of the data, it is the belief of many researchers that mouse skeletal muscle fatty acid oxidation processes may significantly contribute to the overall increase in EE observed in rodents after dietary MR.

Carnitine palmitoyltransferase 1 (*CPT1*) is an enzyme which resides in the outer membrane of the mitochondria which functions to transfer long-chain fatty acids across the mitochondria membrane by binding them to carnitine. The protein *CPT1* is found in several tissues, including the liver and the skeletal muscle as isoforms *CPT1a* or *CPT1b* respectively [46]. The gene for the isoform *CPT1b* has been found to be up regulated in quadriceps tissue in MR rats by 1.5 fold [40]. Interestingly, significant increases in *CPT1b* gene expression have been observed in human skeletal muscle after chronic exercise [47,48]. This up-regulation is thought to be induced by the need of increased fat utilization by the muscle [49]. An increase in *CPT1b* gene expression in the muscle of F344 rats consuming MR may reflect the same increased demand to transport long-chain fatty acids to be utilized by the mitochondria.

Specific Aims

The purpose of this study is to determine the time course in which C57BL6J mice experience metabolic adaptations to MR diet. By exploring the effects of MR in a well characterized, diet-induced model of obesity, I will be able to more fully explore the changes in energy balance that were observed in F344 rats. Accordingly, the specific aims of the study are to determine the factors contributing to the increased energy expenditure induced by the MR diet by:

1. Determining the onset of increased food intake with dietary MR, and the changes in body composition over time. Hypothesis: Animals on MR diet will experience an almost immediate significant increase in food consumption, and a decrease in overall adiposity accumulation.
2. Determining whether changes in physical activity are related to overall energy expenditure. Hypothesis: Dietary MR induces an increase in energy expenditure in the absence of increased physical activity.
3. Evaluating *UCP3* and *CPT1b* gene expression in mouse skeletal muscle. Hypothesis: MR is associated with an increase in *UCP3* and *CPT1b* gene expression in mouse skeletal muscle.

CHAPTER 3 METHODS

Animals and Diets

C57BL/6J mice (n=28) were obtained from Jackson Laboratories (Bar Harbor, ME) at four weeks of age for use in two separate but structurally similar experiments of exposure to CON or MR diets. At five weeks of age, the animals were split into two cohorts: Cohort 1 (n=12) and Cohort 2 (n=16) and randomized to receive purified diets manufactured by Dyets Inc. (Bethlehem, PA) that contained 0.86% methionine (CON diet) or 0.17% methionine (MR diet) (Table 1). The animals were single-housed and received food *ad libitum* for the entirety of both experiments. All studies were reviewed by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. All of the National Institute of Health guidelines for the care and treatment of animals were observed.

At five weeks of age, Cohort 1 animals began CON diet and remained on the diet for one week. In Week 1, at 6 weeks of age, the animals were randomized into CON or MR, but remained on CON diet. Animals underwent nuclear magnetic resonance (NMR) to determine body composition before acclimating to the metabolic cages designed to measure body weight, food and water intake. In Week 2, after a seven-day acclimation period to the metabolic cages while on CON diet, six mice were switched to MR diet, while the other six continued consuming CON diet.

Food intake, water intake and body weight measurements were taken daily for two weeks. In Week 4, the animals returned to their home cages. In the home cages, weekly food intake, water intake, and body weight data were collected until Week 7. In Week 8, the animals returned to the metabolic cages, and the previously mentioned measurements were taken every

24 hours for two days before the mice were moved back to their home cages. In Week 9, mice were fasted for four hours before being euthanized by decapitation following CO_2-induced narcosis during the light part of the light/dark cycle.

Table 1. Composition of control (CON) and methionine restricted (MR) diets.
The composition of the control and methionine restricted diets used in MR experiments is identical, with the exception of L-Glutamic Acid which is increased to maintain the energy density of the MR diet.

	CON g/kg	MR g/kg
L-Arginine	11.2	11.2
L-Lysine HCl	18	18
L-Histidine	3.3	3.3
L-Isoleucine	8.2	8.8
L-Valine	8.2	8.8
DL-Methionine*	8.6	1.72
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Phenylalanine	11.6	11.6
Glycine	23.3	23.3
L-Leucine	11.1	11.1
L-Glutamic Acid*	27.1	33.88
Total Amino Acid	140.5	140.5
Dextrose	200	200
Dyetrose	50	50
Cornstarch	432.5	432.5
Corn Oil	80	80
Cellulose	50	50
Mineral Mix #200000	35	35
Vitamin Mix #300050	10	10
Choline Bitartrate	2	2
Total	1000	1000

Cohort 1 Timeline:

Week -1:

- Animals arrive at CBC (4 weeks of age)
- Animals into quarantine

Week 0:

- Animals acclimated to the CON diet in home cages
- Animals randomized into Cohort 1

- Cohort 1: Metabolic cages (n=12)

Week 1:

- Animals were randomized into CON or MR and remained on CON diet
- Animals underwent NMR
- Animals were transferred to metabolic cages to acclimatize

Weeks 2 & 3:

- Six pre-randomized animals began MR diet, while the other six animals remained on CON
- Food intake, water intake, and body weight measurements were taken every 24 hours

Week 4-7:

- Animals were moved back to home cages
- Weekly food intake, water intake, and body weight measurements were taken

Week 8:

- Animals were moved back to metabolic cages to acclimatize for two days
- Two days of food intake, water intake, and body weight measurements were taken
- Animals underwent NMR

Week 9:

- Animals were euthanized

At five weeks of age, Cohort 2 animals (n=16) began CON diet and remained on the diet for one week. In Week 1, at six weeks of age, animals were randomized into CON or MR groups, but remained on CON diet. Animals underwent NMR before being acclimated to the Comprehensive Lab Animal Monitoring System (CLAMS) device which is designed to calculate energy expenditure from indirect calorimetric methods on each animal every 18 minutes. The monitoring system also tracks ambulatory data in beam breaks, and food intake.

In Week 4, the animals were returned to their home cages. In home cages, weekly food and water intake, as well as body weight data were collected until Week 7. In Week 8, the animals returned to the CLAMS device, acclimated for 24hrs, and the previously described measurements were recorded for two days. At the completion of these two days, mice were

moved back to their home cages. In Week 9, mice were fasted for four hours, and then euthanized by decapitation following CO_2-induced narcosis during the light part of the light/dark cycle. The triceps surae muscle (gastrocnemius/soleus/plantaris) tissues were collected and snap frozen for future analysis.

Cohort 2 Timeline

Week -1:

- Animals arrived at CBC (4 weeks of age)
- Animal went into quarantine

Week 0:

- Animals were acclimated to the CON diet in home cages
    - Cohort 2: Indirect Calorimetry (n=16)

Week 1:

- Animals were randomized into CON or MR
    - Animals remained on CON diet
- Animals underwent NMR
- Animals were transferred to the CLAMS device to acclimatize
- Energy expenditure was measured every eighteen minutes while animals remained on CON diet

Weeks 2 & 3:

- Eight pre-randomized animals began MR diet, while the other eight remained on CON
- Food intake was recorded daily
- CLAMS device measured physical activity, as well as VO_2 and VCO_2 every eighteen minutes which was used to calculate energy expenditure

Weeks 4-7:

- Animals underwent NMR (week 4)
- Animals were moved back to home cages
- Weekly food intake, water intake, and body weight measurements were taken

Week 8:

- Animals underwent NMR
- Animals were moved back to CLAMS cages to acclimate for one day
- CLAMS device measured physical activity, as well as VO_2 and VCO_2 every eighteen minutes which was used to calculate energy expenditure

Week 9:

- Animals returned to home cages for three days
- Animals were euthanized
    - Skeletal muscle tissues were collected and flash frozen in liquid nitrogen

Body Composition

Prior to their individual interventions, mice from both cohorts were weighed, randomized, and body composition was determined by NMR using a Bruker Mouse Minispec (Bruker Optics, Billerica, MA). Body composition was measured prior to the initiation of any dietary changes, and was re-assessed at the completion of the eight-week studies. Daily or weekly body weight measurements were taken throughout the course of the study.

Indirect Calorimetry

The mice from Cohort 1 were single-housed in open-circuit oxymax chambers of the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). The mice were acclimated for 24 hours in the metabolic chambers after which, data was recorded for up to 72 hours before animals were removed for husbandry maintenance. Measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded electronically for each animal on an 18-minute cycle. Measurement of physical activity was accomplished by using the beam break method. Every time a beam was broken on the x-axis, a measurement was recorded. Using this method is a surrogate of physical activity for animals in each cage. These measures were taken for the entire span of time that the animals were in the CLAMS cages. Analysis was completed by looking at the differences in beam breaks between the groups during the light and dark phases. All mice were observed for three days while consuming the CON diet before any animals were switched mice to the MR diet. With the exception of husbandry maintenance, all mice remained in the indirect calorimeter for two weeks while information about energy expenditure was recorded by the CLAMS device. Energy expenditure was measured by VO_2 and RER (VO_2 * (3.815 + (1.23*RER)) * 40.1868) and expressed as kJ/hr.

Metabolic Cages

The animals from Cohort 2 were placed into individual metabolic cages to determine the energy input and output of the subjects. To measure food intake, a weighed amount of food was placed into the cage each day for a 24-hour period. Every 24 hours, the remaining amount of food was carefully collected and measured to determine the exact amount eaten. A screen placed under the wire-bottom cage allowed for collection of any wasted, powdered, diet. To measure water intake, the weight of the water bottle was measured at the beginning and the end of each 24-hour period.

Real Time PCR

Triceps surae muscle was quickly excised, snap frozen in liquid nitrogen, and then stored at -80°C until the tissue could be processed. Tissue was pulverized and homogenized in TRIzol (Invitrogen, Calsbad, CA) and mRNA was prepared following the kit manufacturer's instructions (QIAGEN, Valencia, CA). The following primers were used to amplify mouse UCP3 and CPT1b mRNA: mUCP3, forward: 5º-CGAATTGGCCTCTACGA-3º, reverse: 5º-TGTAGGCATCCATAGTCCC-3º; mCPT1b, forward: 5º-TCTAGGCAATGCCGTTCAC-3º, reverse: 5º-GAGCACATGGGCACCATAC-3º. Gene expression was normalized to the house-keeping gene, cyclophilin. qPCR was performed in duplicate or triplicate for each sample according to the manufacturer's protocol.

Statistical Analysis

Descriptive data (means, standard deviation, and standard error of the mean) were obtained for the following variables: food intake, water intake, body weight, body fat mass percent, body lean mass percent, respiratory quotient, energy expenditure, physical activity (expressed in beam breaks), and gene expression (measured in cycle counts). The data were

analyzed using GraphPad Prism (La Jolla, CA), and JMP Pro 10 (SAS Inc., Cary, NC) statistical analysis software and results reported as means ± standard error. A repeated measure analysis of variance (ANOVA) was used to determine differences in food intake, water intake, body weight, body fat percentage, lean body mass percentage, EE, RQ, physical activity and gene expression between MR and CON groups at time points relevant to each cohort. In order to detect the difference in EE while taking into account fixed variables (e.g. diet) and continuous variables (body composition, food intake, activity) an analysis of covariance (ANCOVA) was also performed. A P-value of <0.05 was be used to assess significance.

CHAPTER 4 RESULTS

Food Intake

A two-way repeated measures ANOVA was used to evaluate changes in food intake, and body composition measures. Food intake, expressed in grams of food per gram of body weight, was not different between CON and MR groups at baseline (p=0.97). Differences in food intake were observed within days of administration of the MR diet, but no statistical difference appeared between groups during the first two weeks (p=0.37) (Figure 3). However, a significant difference in food consumption (MR 0.16 ± 0.01, CON 0.12 ± 0.01) was observed at week 3 (p<0.0001), and this difference persisted until the end of the 8-week study (p=0.0006). At the end of 8 weeks, mean food intake of CON was 0.16 ± 0.02 g of food per g of body weight, 0.20 ± 0.02 g per g of body weight for MR. This resulted in MR consuming an average of 18% more food than CON at this time point.

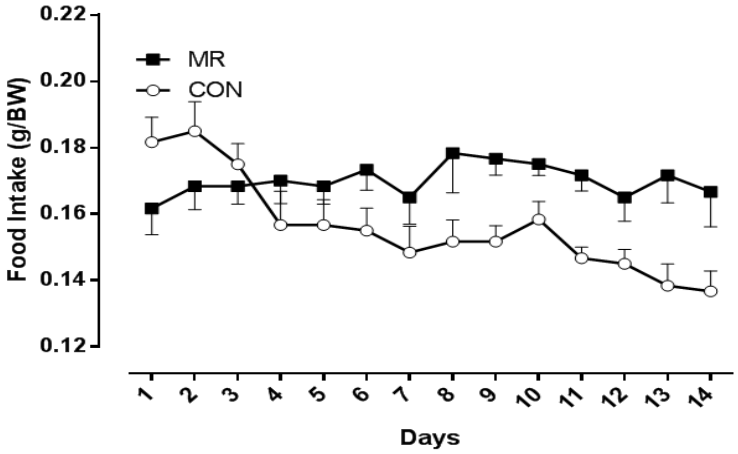

Figure 3. Two-week daily food intake measures of CON and MR mice.
C57BL6J mice were separated into CON or MR groups, and daily food intake was evaluated for 14 days. At baseline, there was no difference between groups (p=0.97).

Body Composition

In the first two weeks, neither CON nor MR gained significantly more weight when compared to baseline body weight measures (p>0.99 for both) (data not shown). Mean body

weights between CON and MR after two weeks of treatment were not significantly different (p=0.54). MR animals had significantly (10%) lower body weight when compared to CON after 3 weeks of dietary intervention (p=0.02) (Figure 4).

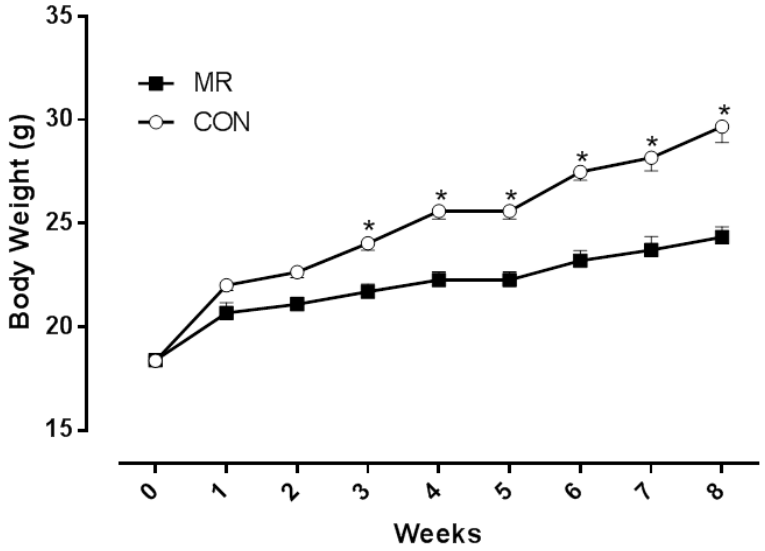

Figure 4. Body weight changes over eight weeks of MR intervention.
C57BL6J mice were placed on either a CON or MR diet, and weekly body weight measurements were taken for eight weeks. At baseline, both groups had similar mean body weights (p>0.99). At three weeks, mean body weights were statistically different from one another (p=0.02), and remained statistically different until the end of the study (p<0.0001).

Lean body mass, percent lean mass, body fat mass, and percent body fat mass were similar in both groups just before initiation of the MR diet. After two weeks of dietary intervention, there was no significant difference in mean lean body mass in CON and MR respectively (14.7 ± 0.52g vs. 13.9 ± 0.79g) (Figure 6). By the end of the 8 week intervention period, the mean lean body mass of CON increased 46% from baseline (p<0.0001), while MR experienced an increase of only 29% (p<0.0001). Mean lean body mass measurements between CON and MR were significantly different at the 8 week time point (CON =19.3 ± 0.70g; MR =17.4 ± 0.74g) (p<0.0001) (Figure 5).

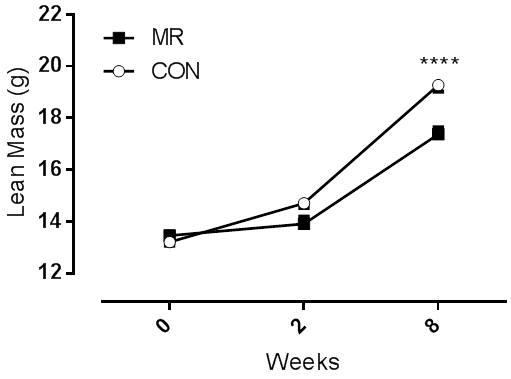

Figure 5. Change in lean body mass over eight weeks of MR intervention. C57BL6J mice were placed on either a CON or MR diet for eight weeks. Body composition was measured before initiation of the diets (Week 0), after 2 and 8 weeks on the diet. At baseline, lean mass measurements determined by NMR were not different between CON and MR groups. No significant change in lean mass between groups was observed until eight weeks, when the CON animals had gained significantly more lean mass than the MR animals ($p<0.0001$).

Percent lean mass measured at baseline was not different between CON and MR (72% and 73% respectively). At two weeks, CON had an average of 69% lean mass, and MR had an average of 67% lean mass, and there was not a significant difference between groups ($p=0.997$). CON maintained percent lean mass until week 8 of the study. At the end of the 8 weeks, CON had approximately 68% lean mass, only a 4% reduction from baseline. MR experienced an increase in percent lean mass from week 2 to week 8, with a final measurement of 73% lean mass. This discrepancy in percent lean mass lead to a significant difference between CON and MR at week 8 ($p=0.0015$) (Figure 6).

At baseline, mean fat mass was not statistically different between CON (2.6 ± 0.37g) and MR (2.5 ± 0.19g). At two weeks, mean fat mass of CON increased to 3.4 ± 0.44g, a 30% change in fat mass from baseline. MR had a mean fat mass of 3.1 ± 0.69g in week two; an increase of 24% from baseline. Changes in mean fat mass between CON and MR were not significantly different

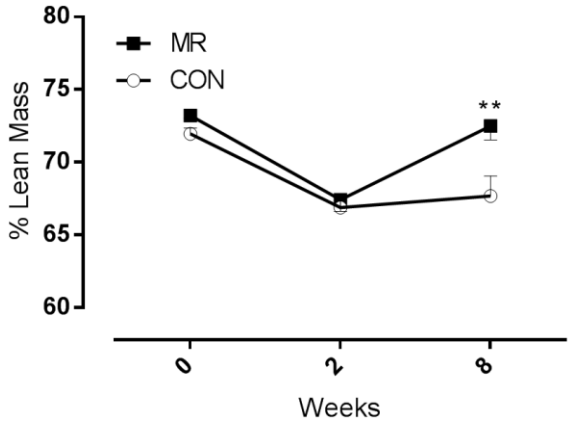

Figure 6. Change in percent lean body mass over eight weeks of MR intervention. C57BL6J mice were placed on either CON or MR diet for eight weeks. Body composition of the animals was measured at baseline (Week 0), 2, and 8 weeks. At baseline and at the 2 wk time point, percent lean mass was not significantly different between treatment groups. After eight weeks on the treatment diet, the percent lean mass of the MR animals significantly higher than the CON animals (p=0.0015).

after two weeks (p=0.97). At eight weeks, the mean fat mass of MR did not differ from the week two measurement, remaining at 3.1 ± 1.05g. On the contrary, CON animals continued to gain weight until 8 weeks, where fat mass was measured at 5.8 ± 1.66g, which was a 119% increase from baseline (p<0.0001). Fat mass measurements between CON and MR were significantly different at the 8 week time point (p<0.0001) (Figure 7).

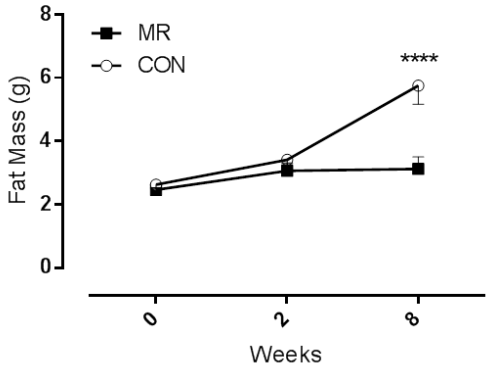

Figure 7. Change in body fat mass over eight weeks of MR intervention. C57BL6J mice were placed on either a CON or MR diet for eight weeks. Body composition measurements were taken at baseline (Week 0), 2, and 8 weeks. At baseline, mean fat mass was not different between the CON and MR groups. After eight weeks, the CON animals had significantly more mean fat mass than the MR group (p<0.0001).

Percent fat mass measured at baseline was not different between CON and MR (14% and 13% respectively). At two weeks, both CON and MR had gained approximately 2% fat mass (CON 16% fat mass; MR 15% fat mass) (p=0.994). At eight weeks, the percent fat mass in MR was 13%, and identical to baseline. CON gained approximately 6% fat mass over the eight week intervention period, resulting in 20% fat mass after 8 weeks of the intervention. Final percent fat mass measurements at 8 weeks between CON and MR were significantly different (p=0.0001) (Figure 8).

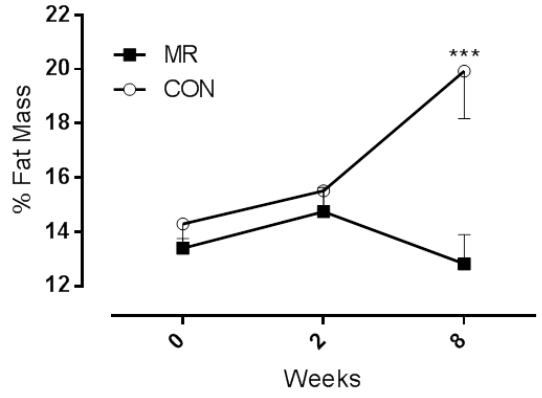

Figure 8. Change in percent body fat mass over eight weeks of MR intervention. C57BL6J mice were fed either a CON or MR diet for eight weeks. Body composition measures were taken at baseline (Week 0), 2, and 8 weeks. Baseline percent fat mass was not different between CON and MR groups. A difference in percent fat mass did not arise until eight weeks on the treatment diet, the percent fat mass of the MR group returned to baseline, while the percent fat mass of the CON animals increased. By eight weeks, this resulted in the MR animals having significantly less fat mass than the CON animals (p=0.0001).

Energy Expenditure

Despite the higher body weight of CON over time, MR exhibited 13% higher EE after only six days of dietary intervention, where the increase in EE was first measured in the night phase of the diurnal cycle (p=0.034), and 17% higher during the day phase of the diurnal cycle at day 11 (p=0.004) (Figure 9). EE of MR increased by 19% compared to CON within the first two

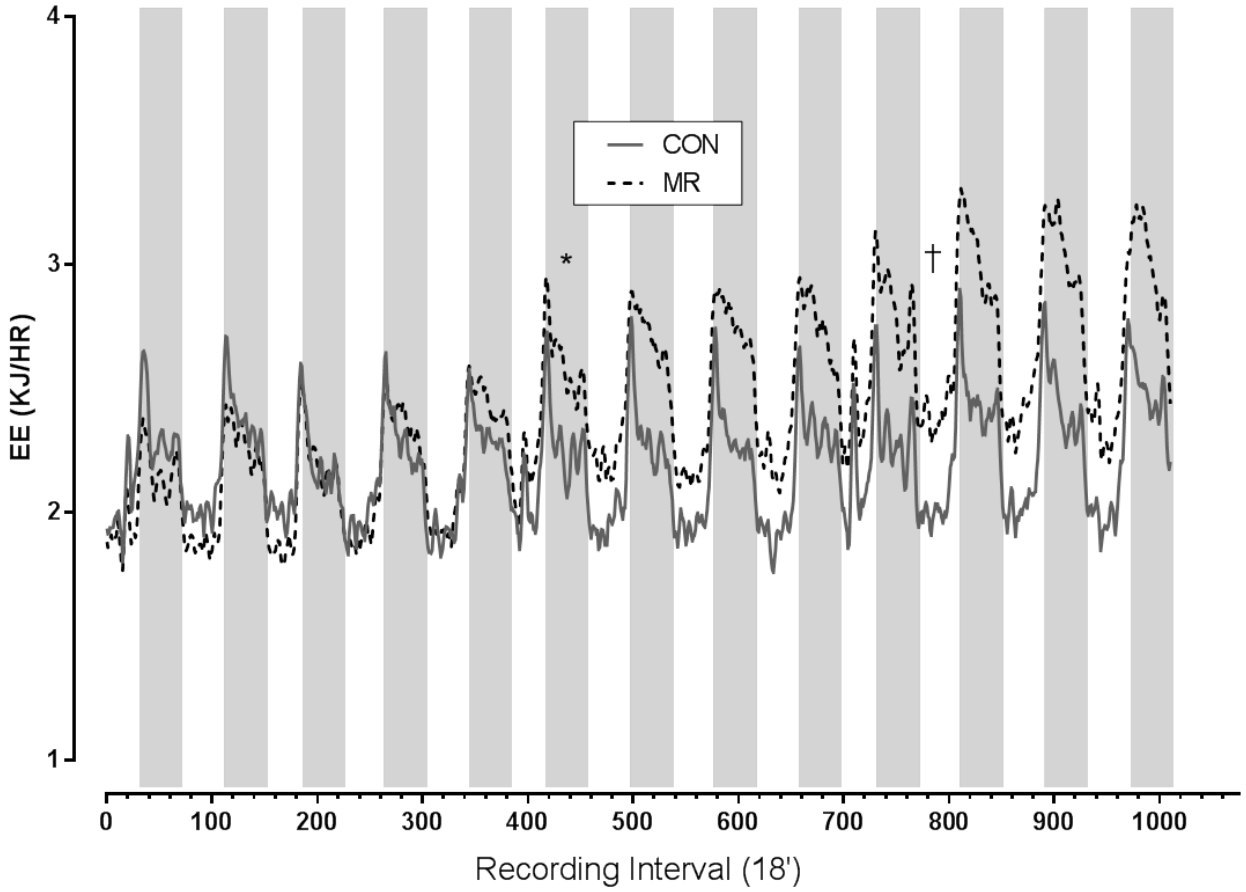

Figure 9. Energy expenditure data of C57BL6J mice on CON vs MR diet.
C57BL6J mice were put into the Comprehensive Lab Animal Monitoring System (CLAMS) device to acclimate for three days. At interval zero, half of the animals were switched to an MR diet, while the other half of the animals remained on the CON diet. Energy expenditure is expressed in $kJ \cdot hr^{-1}$. White bars signify the daytime phase of the diurnal cycle, while dark bars signify the night phase. Energy expenditure of MR increased above that of CON by the sixth night ($p=0.034$) and remained ~16-19% higher throughout the first two weeks.

weeks, and this increase was maintained with EE in MR 15% higher than CON at the end of the 8 week intervention ($p<0.0001$).

Over the course of the EE measurement period, both groups experienced a 55-69% increase in physical activity during the night phase compared to the light phase ($p<0.05$). There was no difference in physical activity between groups at any time point, with the exception of one; during the light cycle of day 10, the MR group was more physically active than the CON

group (p=0.046) (Figure 10). No difference was observed between groups at the 8-week time point as well (data not shown).

An ANCOVA, revealed that activity, measured in beam breaks, was a significant predictor of energy expenditure (p=0.003). However, when dietary treatment was assessed in the model, the relationship was not significant (p=0.079). Neither lean mass, nor food intake were significant predictors of energy expenditure in the animals in either group (p>0.05).

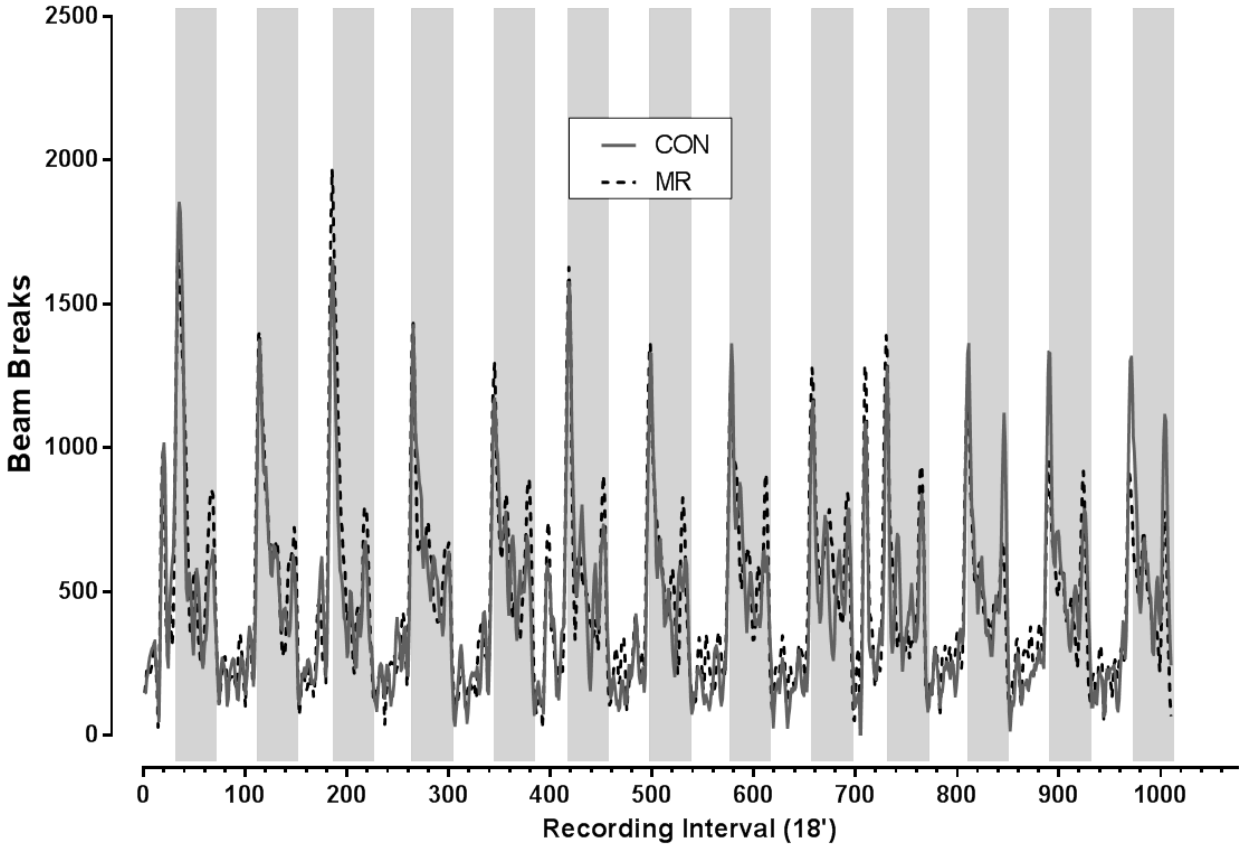

Figure 10. Physical activity data of C57BL6J mice on CON vs MR diet.
C57BL6J mice were placed into the Comprehensive Lab Animal Monitoring System (CLAMS) device and were allowed to acclimate for three days. At interval zero, half of the animals were switched to an MR diet, while half of the animals remained on CON diet. During this time, physical activity data was collected using beam breaks. Data are expressed in number of beam breaks on the y-axis, and intervals (each 18 minutes in length) on the x-axis. No difference in physical activity between groups was recorded with the exception of day 10 when MR mice exhibited a statistically higher number of beam breaks compared to CON.

Gene Expression in Mouse Skeletal Muscle

There was no difference in *CPT1b* (p=0.5237) or *UCP3* gene expression (p=0.844) in the triceps surae muscle after eight weeks of MR treatment (Figures 11 and 12).

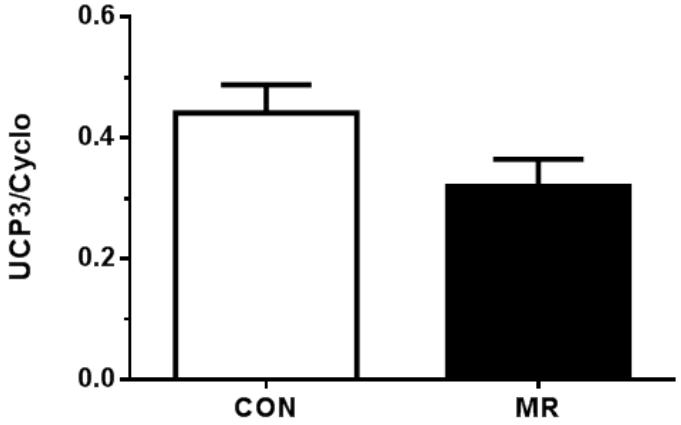

Figure 11. Gene expression of uncoupling protein 3 in mouse triceps surae muscle. Expression of the gene *UCP3* in skeletal muscle of CON and MR mice after eight weeks of dietary treatment. There were no significant differences between CON and MR groups.

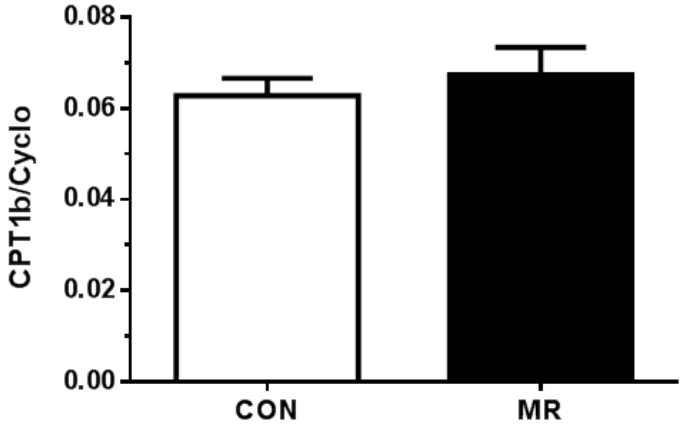

Figure 12. Gene expression of carnitine palmitoyltransferase 1 b in mouse triceps surae muscle. Expression of the gene *CPT1b* in skeletal muscle of CON and MR mice after eight weeks of dietary treatment. There were no significant differences between CON and MR groups.

CHAPTER 5 DISCUSSION

This study was designed to determine the time course in which C57BL6J mice fed a methionine restricted (MR) diet would exhibit the metabolic adaptations previously reported in rats. Food consumption and body composition, using nuclear magnetic resonance (NMR), were measured over the course of eight weeks while mice were being fed either a control (CON) or MR diet. Additionally, measurements of energy expenditure and physical activity level were obtained. Finally, transcriptional analysis was utilized to determine whether the MR diet induced alterations in gene expression of genes related to fatty acid oxidation.

In this study, C57BL6J mice fed MR exhibited a hyperphagic response after 3 weeks of feeding which continued until the cessation of the data collection period (8 weeks). This hyperphagic response resulted in 20% lower overall body weight of the MR (24.3 ± 1.39g) compared to the CON (29.7 ± 2.15g) after 8 weeks. Although the increase in food intake of MR rodents compared to CON has been previously established [12,28,31,50,51], the time course of this phenomenon is still unknown. Initially, food intake between groups was not different and when food intake was normalized to body weight, no difference between CON and MR mice existed ($p=0.97$). The MR animals exhibited a significant increase in food intake after three weeks on the diet when food intake was normalized to body weight. During the third week, MR mice were consuming 28% more food per gram of body weight than their CON counterparts ($p<0.0001$). MR mice ate an average of 24% more food per week than the CON animals for the remainder of the study ($p<0.05$). The measured increase in food intake normalized to body weight fits with what has been previously reported in rats (MR ate ~33% more than CON) [12,30] and mice (energy consumption of MR was 64% higher in MR than CON) [51]. This

significant increase in food intake by the MR animals within the first three weeks of feeding supports the hypothesis that the animals would become hyperphagic after the initiation of the MR diet.

Increased food intake with no change in energy expenditure is typically synonymous with increased adipose deposition in the C57BL6J mouse [52,53]. In rodents consuming MR, the increase in food intake does not lead to an increase in adipose deposition, even in the leptin-deficient *ob/ob* mouse model [54]. Despite being hyperphagic, MR gained less fat mass than CON over 8 weeks, while maintaining lean mass similar to baseline measures. Previous studies have determined that over 6 months of MR feeding, F344 rats did not have a significant change in adiposity from baseline measures, despite a 20% increase in weight-adjusted food consumption [30]. One study reported that rats consuming MR for 18 months exhibited a reduction of visceral fat mass, measured by weighing out the excised tissue, by an average of 52g compared to CON. However, this study failed to report weight-adjusted food intake [28]. Baseline body weight measurements for the first two weeks revealed that body weight MR was not statistically different than CON ($p>0.99$). Three weeks after baseline, MR weighed almost 9% less than CON ($p=0.02$). By the end of eight weeks of dietary intervention, MR weighed 20% less than CON ($p<0.0001$), similar to the results originally observed in rats [12,26]. MR also had 7% less body fat mass ($p=<0.0001$) than CON, yet maintained lean mass similar to baseline measures ($p=0.99$). Over the course of eight weeks, CON gained approximately 6% body fat mass ($p=0.004$). Body composition results are similar to those previously reported in which MR rats weighed approximately 66% less than CON rats at full maturity [28] and a 1.5-fold increase in epididymal white adipose tissue mass of CON rats compared to MR rats at 3

months [30]. These results support the hypothesis that MR would accumulate less adipose tissue than CON over the course of the study, despite an increase in weight-adjusted food intake.

The combination of increased food intake coupled with decreased adiposity accumulation suggests that MR has a tendency to induce a compensatory increase in overall EE in the mouse. A previous study reported an increased EE reported in kJ hr^{-1} FFM kg^{-1} in rats consuming an MR diet, compared to rats consuming CON at both 9 and 20 months after initiation. EE in the MR rats was 90% higher than CON during the day time of the diurnal cycle at 9 months and 25% higher than CON at 20 months [30]. EE in 63-week old MR rats was also 31% higher than CON when adjusting to total body weight [28]. As mentioned previously, each tissue contributes differently to basal metabolic rate depending on its fuel needs. Lean mass accounts for ~75% [19,55], and voluntary physical activity accounts for ~20% of the variation in total daily EE in humans [19,56] and presumably rodents. Rats on MR increase EE without an increase in voluntary activity [30], suggesting that other mechanisms may be responsible for the increase in EE reported. To determine how quickly a change in EE would occur after initiation of MR in the C57BL6J mouse, we measured VO_2 and VCO_2. An ANOVA of unadjusted EE, measured in kJ · hr^{-1}, revealed that despite an increase in body weight of CON, MR exhibited a 16% increase in EE by the sixth night initiation of MR ($p=0.034$). The increase in EE of MR was approximately 19% higher overall than CON after two weeks ($p<0.001$), and EE of MR remained ~15% higher than CON after 8 weeks ($p<0.0001$).

The main contributions to EE include basal metabolic rate, thermogenic effects of food intake, and physical activity [39]. Coupled with the increase in EE measure in MR, there was no statistical difference in physical activity, as measured by beam breaks, was apparent between groups during the first two weeks ($p=0.171$). Physical activity was found to be a direct

contributor to overall energy expenditure when both CON and MR were included in an ANCOVA analysis ($p<0.0001$). However, when dietary treatment was assessed in the model, the effect of physical activity on EE was no longer significant ($p=0.079$). Additionally, neither lean mass, nor food intake were significant predictors of energy expenditure in the animals ($p>0.05$). These findings suggest that although physical activity does play a role on overall energy expenditure, it is not a significant variable affecting the increased EE induced by the MR diet. These data suggest that the hypothesis was correct in that the MR animals experienced an increase in EE that was not affected by differences in physical activity.

In previous experiments using the F344 rats, expression of genes related to EE and fatty acid oxidation in skeletal muscle such as *UCP3* and *CPT1b* were found to be increased with MR [40]. The uncoupling proteins (*UCP3* in this case) are typically associated with the ability of a tissue to use futile cycling to burn energy and produce heat [42] and are speculated to be a key factor in the regulation of EE [44,45]. The CPT1 isoform CPT1b is a skeletal muscle specific isoform of *CPT1* which acts to translocate long-chain fatty acids across the mitochondria membrane [46]. Increases in gene expression of *CPT1b* have been measured in muscle tissue of individuals who require more fatty acid to be used as a substrate in ATP production, such as in humans after chronic aerobic training [47,48]. An increase in the expression of these genes typically leads to an increase in the translation of their corresponding proteins. Due to the increase percent lean mass, and decreased adiposity gain previously reported in MR rodents, we hypothesized that expression of genes associated with increased fatty acid oxidation (*UCP3* and *CPT1b*) will be increased in skeletal muscle of C57BL6J mice after eight weeks of MR consumption. To test this hypothesis, expression of the genes *UCP3* and *CPT1b* were measured in the triceps surae, a group of mixed muscle fibers, in all experimental mice. Neither the

expression of *UCP3* nor *CPT1b* genes was different between groups (p=0.844 and 0.524 respectively). These results do not support the hypothesis that fatty acid uptake and utilization in the triceps surae muscle of MR is different than CON. It is further possible that other tissues may be mediating this MR-induced increase EE. For instance, the previously mentioned increase in UCP1 in both white and brown adipose tissue published previously by our group [30].

CHAPTER 6 CONCLUSION

In summary, this study observed and documented the onset of physiological and metabolic effects brought about by MR in the C57BL6J mouse. Additionally, there is preliminary data which helps to describe the mechanistic role of the skeletal muscle on MR-induced metabolic changes in these mice. Although EE in MR mice increases within the first few days of dietary intervention, the effect is not mediated by changes in physical activity, and rather by metabolic adaptations. Consequently, these results suggest that MR may prove to be a viable intervention in humans who are disabled or suffering from obesity, and who are limited in their ability to perform physical activity. Although expression of skeletal muscle *UCP3* and *CPT1b* are unchanged between CON and MR mice, this does not completely rule out muscle tissue as an important contributor to the increase in EE observed in the MR animals.

Limitations

Measures of body composition in both groups were unchanged after the first two weeks, including overall body weight. One possible reason for this could be the stress that the animals incurred during the first two weeks. In both cohorts, the rodents were subjected to conditions that are not typical. The rodents could have experienced stress from the wire-bottom cages, or the environmental chambers used to measure metabolic activity. To resolve this, a third cohort of animals could have been placed in home cages with corncob bedding to assess the change in food intake on animals which were not being given additional stressors.

The OxyMax system was used to measure EE, and this system takes one measurement on each animal on an 18-minute cycle. Other researchers have made the claim that this 18-minute interval could possibly be too long of an interval to accurately determine if physical activity is a main contributor to EE.

In order to determine if there was a change in genes associated with fatty acid utilization in the skeletal muscle, we measured *UCP3* and *CPT1b*. These two genes were chosen because they were previously seen to have been up-regulated in the skeletal muscle of MR rats and are closely associated with fatty acid metabolism. To our surprise, the expression of these genes in the mouse skeletal muscle was unchanged. Our experimental design used a mixed muscle group to measure gene expression in order to obtain a more physiologically relevant answer. There remains a possibility that the mixed fiber types washed out any change in expression of genes which regulate fatty acid oxidation. In order to answer this question, the specific muscles would need to be split before mRNA is isolated, and the gene expression would need to be re-evaluated.

One issue brought to the researcher's attention is the increase in glutamic acid used to increase the energy content of the MR diet. Metabolic changes experienced by the C57BL6J mice in this experiment could have been exacerbated by the inclusion of more glutamate, rather than the exclusion of methionine. To gauge if this is a true concern, the diets used could be altered by increasing a different amino acid to equalizer the energy content of the diet. Other modifications include observing the differing effects brought about by adding glutamic acid in different proportions and studying different cohorts of mice.

Future Directions

Although regulation of the genes we chose to represent changes in fatty acid oxidation are not changed in the skeletal muscle of MR mice, other effects in the tissue may be taking place. For instance, in future research, mitochondria should be examined in mice after MR feeding to determine the diet's effects on mitochondrial biogenesis and efficiency in the skeletal muscle and other tissues. Mitochondria number in the tissues of MR mice, or that the metabolic processes of the citric acid cycle in the mitochondria are responsible for the increase in EE. A

citrate synthase activity assay could be performed on isolated mitochondria from specific tissues (white adipose, liver, etc.) of MR and CON mice to accomplish determine the specific contributions that these tissues have on overall EE.

Previously, it was established that other tissues, such as white and brown adipose, experience changes in gene and protein expression, and these tissues may be more important in defining the phenotype brought about by MR. For instance, the previously-mentioned increase in *UCP1* in the white adipose tissue suggests increased futile cycling in a tissue that is not typically known for energy dissipation. Additional effects of MR can be observed in the liver tissue where there is an increase in lipogenic gene expression which serves to help protect against hepatic steatosis [54].

More research in the promising area of dietary MR is required to determine the specific effects on the metabolism. The currently-observed effects on rodents appear to be promising, but many more questions remain to be answered before this intervention can be safely used in the human population. Whether the use of MR in humans is near, or is something that we will see in the near future, continues to remain unknown. However, the implications of utilizing such an intervention that allows a person to eat more and gain less weight without the need for increased physical activity is worth exploring further.

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VITA

Cory was born in March of 1989 in Metairie, Louisiana. He is the youngest of five children who were raised by his mother, Elizabeth, and the late Murphy Cortez. As a young man, he enjoyed participating in sports and activities, a passion that only grew as he aged. Cory graduated from St. Thomas Aquinas in Hammond, Louisiana in 2007 and attended Louisiana State University in Baton Rouge, majoring in kinesiology. During this time, he invested much of his time in promoting the university, and gaining research experience at Pennington Biomedical Research Center in the field of exercise science. Through his work experience he became interested in the effects on the body brought about by dietary nutritional interventions.

In anticipation of earning his undergraduate degree in the spring on 2011, Cory applied to the School of Kinesiology at Louisiana State University, under the mentorship of Dr. Laura Stewart. Shortly after admission to the university, he accepted a position as a research associate in the Nutrient Sensing and Adipocyte Signaling laboratory under Dr. Thomas Gettys. During his first year of graduate school, he completed many hours of laboratory bench work, cell culture studies, and animal husbandry tasks. Through his experience and the mentorship of Dr. Stewart and Dr. Thomas Gettys, he was able to develop and begin his thesis project involving aspects of both physiological and nutritional elements. Cory's immediate future plans are to graduate in May 2014 and continue his education at the doctorate level.