Potential Mechanisms Underlying Adaptive Thermogenesis in Lean and Obesity-Prone Rats

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Degree of Doctor of Philosophy

by

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<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADRB2</td>
<td>Beta-2 adrenergic receptor</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti related peptide</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMR</td>
<td>Basal metabolic rate</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CR (only in figures)</td>
<td>Calorie restriction</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
<td>EAT</td>
<td>Exercise activity thermogenesis</td>
</tr>
<tr>
<td>EE</td>
<td>Energy expenditure</td>
</tr>
<tr>
<td>EEA</td>
<td>Energy expenditure of activity</td>
</tr>
<tr>
<td>EST</td>
<td>Eastern standard time</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GHSR</td>
<td>Growth hormone secretagogue receptor</td>
</tr>
<tr>
<td>HCR</td>
<td>High-capacity runners</td>
</tr>
<tr>
<td>KCNJ8 &amp;11</td>
<td>Potassium inwardly-rectifying channel, subfamily J, member 8 and 11</td>
</tr>
<tr>
<td>LCR</td>
<td>Low-capacity runners</td>
</tr>
<tr>
<td>LH</td>
<td>Lateral hypothalamus</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>MC3R</td>
<td>Melanocortin 3 receptor</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin 4 receptor</td>
</tr>
<tr>
<td>MC5R</td>
<td>Melanocortin 5 receptor</td>
</tr>
<tr>
<td>MCR</td>
<td>Melanocortin receptor</td>
</tr>
<tr>
<td>Med Gastroc</td>
<td>Medial gastrocnemius</td>
</tr>
<tr>
<td>MED1</td>
<td>Mediator of RNA polymerase II transcription subunit</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte stimulating hormone</td>
</tr>
</tbody>
</table>
NEAT  Non-exercise activity thermogenesis
NPY  Neuropeptide Y
NREE  Non-resting energy expenditure
PA  Physical activity
PC  Prohormone convertase
PeFLH  Perifornical region of lateral hypothalamus
POMC  Proopiomelanocortin
PVN  Paraventricular nucleus
qPCR  Quantitative PCR
Quads  Quadriceps
REE  Resting energy expenditure
RER  Respiratory exchange ratio
ROS  Reactive oxygen species
SERCA  Sarco/endoplasmic reticulum Ca\(^{2+}\) ATPases
SNS  Sympathetic nervous system
TDEE  Total daily energy expenditure
TEF  Thermic effect of food
UCP  Uncoupling protein
VCO\(_2\)  Volume of carbon dioxide release
VMH  Ventromedial hypothalamus
VO\(_2\)  Volume of O\(_2\) consumed
VO\(_{2\ max}\)  Maximal oxygen consumption
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Chapter 1: General Introduction

Obesity is a growing problem with more than one-third of US adults being obese and another one-third overweight [57, 134]. It is a major cause of many diseases including cardiovascular disease, type 2 diabetes mellitus, and some forms of cancer [6, 68, 72, 139]. Disease risk is ameliorated with weight loss, yet successful weight loss is notoriously hard to achieve and maintain. Although there are several genetic, environmental, physiological, and behavioral reasons influencing weight gain and weight loss, ultimately the main reason why a person loses weight is because they are taking in fewer calories than they are expending in the course of their daily activities, causing an energy deficit. Weight gain, on the other hand, occurs when the total daily energy intake exceeds the total energy expenditure (Figure 1.1). The loss of homeostatic balance results from an interaction between individual genetic predisposition and environmental factors. The major environmental factors leading to weight gain are a calorie-dense Western diet and lack of physical activity due to a modernized lifestyle, key components of an obesogenic environment [23, 68]. This obesogenic environment is supported by the luxuries of modern lifestyle where staircases have been replaced by elevators and a normal meal by an extra-large value sized meal [91]. Some people are more vulnerable to this obesogenic environment compared to the others.

In humans, body mass index (BMI) is used to detect normal, under/overweight and obese individuals (Table 1.1). It is derived by using the body mass (kg) and height (m²) of an
individual. In general the BMI correlates with body fat. However BMI does not always
directly reflect body fat, for example in the case of athletes who have greater body weight
due to their higher muscle mass (lean mass); BMI is an acceptable method to assess
obesity in population data and is commonly used in clinical studies as well.
1. CALORIES IN = CALORIES OUT $\rightarrow$ WEIGHT MAINTAINANCE

2. CALORIES IN $<$ CALORIES OUT $\rightarrow$ WEIGHT LOSS

3. CALORIES IN $>$ CALORIES OUT $\rightarrow$ WEIGHT GAIN

Calorie is the unit for energy defined by the energy required to raise the temperature of one gram of water by one degree Celsius.

Figure 1.1: Energetics of weight gain, loss, and maintenance.
Table 1.1: BMI and categorization of human obesity

<table>
<thead>
<tr>
<th>BODY MASS INDEX (BMI)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UNDER 18.5</td>
<td>UNDERWEIGHT</td>
</tr>
<tr>
<td>18.5-25</td>
<td>NORMAL</td>
</tr>
<tr>
<td>25-29.9</td>
<td>OVERWEIGHT</td>
</tr>
<tr>
<td>30 and UP</td>
<td>OBESE</td>
</tr>
</tbody>
</table>
1.1 COMPONENTS OF ENERGY EXPENDITURE (EE)

Total daily energy expenditure (TDEE) is subdivided into resting energy expenditure (resting EE, which generally corresponds to BMR, or basal metabolic rate), and non-resting energy expenditure (non-resting EE). Non-resting EE includes the thermic effect of food (TEF) and activity energy expenditure (activity EE). In humans, resting EE accounts for about 60% of TDEE in humans, TEF accounts for around 10%, and activity EE accounts for about 30% [45, 100, 101, 103] (Figure 1.2). Activity EE includes all of the calories burned in voluntary exercise plus the activity of daily living. Activity EE varies considerably between individuals [45].
Figure 1.2: Components of total daily energy expenditure in humans and rats.

1.2 NON-EXERCISE ACTIVITY THERMOGENESIS (NEAT)

The energy expenditure of daily activity is called non-exercise activity thermogenesis (NEAT) \([87, 98-101, 103]\), and includes activity other than voluntary exercise \([104]\).

NEAT can be further subdivided into posture which includes activities like standing, sitting, or lying down, and movement which includes activities like walking \([98, 101, 103, 104]\).

\[
\text{NEAT} = \text{Posture (e.g., standing)} + \text{Movement (e.g., walking)}
\]

There are individual differences in daily activity which are consistent over time \([104]\), and some individuals have a natural tendency to be more active than the others \([100, 101]\). Lean individuals tend to have higher daily physical activity and spend less time sitting \([77, 87, 103]\), and the ability of a person to resist weight gain has been related to a person’s NEAT \([102]\). Individuals with higher NEAT tend to be leaner than their low-NEAT counterparts \([87, 98, 101, 104]\) and tend to resist weight gain when overfed \([102]\).

There is evidence that in both humans and rats, many of these factors are biologically determined and individual differences in NEAT are not simply secondary to body weight \([63, 103, 159]\). Although NEAT plays a very important role in individual differences in energy expenditure, the underlying mechanisms are not clearly understood.
1.3 ADAPTIVE THERMOGENESIS

Energy expenditure depends on numerous factors including body weight and body composition, lean mass, fat mass, and age [13]. The energy expended increases with both higher body mass and higher lean mass. It follows, then, that weight loss leads to a decrease in a person’s EE. This phenomenon has been supported by numerous studies demonstrating that, with weight loss, energy expenditure also decreases [9-15]. Weight loss decreases the load carried, reducing the energy expenditure required for daily activities. With prolonged energy restriction this decrease in energy expenditure due to weight loss is beyond that what is predicted by the lost weight, a phenomenon called adaptive thermogenesis. Altogether, this counters the caloric deficit from the restricted diet, creating resistance to weight loss and increasing chances of fat accumulation. Adaptive thermogenesis varies between individuals. It varies with racial diversity, between lean and obese individuals, with climatic conditions, and with sex [111, 142, 151, 168, 182, 183]. The best way to figure out the underlying mechanism of this variation is to first identify the source of the variation—the component(s) of EE that is/are suppressed. If adaptive thermogenesis stems from suppression in EE beyond that predicted for a given weight and body composition, this can arise from any one or more of the components of TDEE outlined above—resting EE, TEF, and activity EE [180].

1.4 LEAN MASS AND ENERGY EXPENDITURE

The amount of energy an individual expends during the course of an activity depends largely on the body composition—the amount of fat mass and lean mass present [137]. A
person having greater lean mass will expend more energy compared to a person having greater fat mass even if the body weight is same [194]. Thus lean mass is a crucial component while considering energy expenditure and weight loss. Lean mass is responsible for the majority of resting EE [173] thus increased lean mass leads to an overall increase of EE. Lean mass also increases non-resting EE. It has been shown that changes in the energy use of muscle lead to an increase in NEAT [130]. Moreover, muscle undergoes structural and functional adaptations during the course of cycles of weight loss or gain [67, 96, 141, 142]. Thus even when changes in body weight have been taken into account there is a deficit of activity EE (non-resting EE) observed due to increased muscle efficiency after weight loss [11, 67, 141, 144], where a person can do the same amount of work while expending fewer calories. Thus adaptations in muscle play a very important role in NEAT and also in adaptive thermogenesis.

1.5 BRAIN MELANOCORTIN SYSTEM

Like appetite, the various components of EE are centrally modulated. There are multiple overlapping and redundant neural systems which modulates energy homeostasis. The melanocortin system seems to be an important system in integrating cues for food intake, physical activity, and EE. It also modulates autonomic outflow impacting metabolism and is the target for multiple mutations and variants in the genetics of human obesity.

Within a subset of cells in the arcuate nucleus of the hypothalamus (ARC), POMC (proopiomelanocortin) is cleaved post-translationally with the help of the enzymes
prohormone convertase 1 & 2 (PC1 & PC2) to form \( \alpha \)-MSH (melanocyte stimulating hormone), \( \beta \)-MSH, or \( \gamma \)-MSH [37, 51]. These are anorexigenic peptides controlling food intake and appetite [39]. The other hormones synthesized from POMC include adrenocorticotropic hormone (ACTH), \( \beta \)-endorphin, and \( \beta \)-lipotropin; the end product depends on the actions of PC1 or PC2, for example pro-ACTH is cleaved by PC1 to yield \( \alpha \)-MSH, whereas PC2 produces \( \beta \)-MSH (Figure 1.3). Altogether, melanocortin peptides exert their actions through activation of the melanocortin receptors (MCR) which include 5 subtypes, MCR1-5. MC1R is mainly present in peripheral tissue but is also found in periaqueductal gray area of midbrain [185]. The main function of MC1R is pigmentation. MC2R is present in the adrenal cortex and is the ACTH receptor [185] and thus plays a key role in triggering the release of glucocorticoids like cortisol and corticosterone due to the stimulation by ACTH. MC3R is found in the adult mammalian brain and some peripheral tissues, whereas MC4R is specific to the brain in adult mammals; both of these receptors play a prominent role in energy balance [30, 64, 185]. Deficiency or mutations in these receptors have been shown to be related to obesity in humans [37, 43, 95]. Lastly, MC5R, also linked to obesity [31], is found in the brain and also in several other tissues, and has close resemblance to MC3R and MC4R [31, 54]. More information of these receptors is given below. Within the hypothalamus, POMC, PC1, and PC2 are expressed in the ARC [136]; MC3R, MC4R, and MC5R are expressed in ARC, paraventricular nucleus (PVN), the perifornical orexin-containing region of the lateral hypothalamus (PeFLH), ventromedial nucleus (VMH), and dorsomedial nucleus (DMH), among other regions.
**Figure 1.3: Brain melanocortin system:** Cleavage of POMC by enzyme prohormone convertase (PC) 1 and 2 giving rise to various hormones and neuropeptides. Illustration from: Melanocortin Signaling Mechanisms (Paul C. Eves and John W. Haycock, 2010) with copyright permission from Springer.
1.6 HYPOTHALAMUS: THE SEAT OF OPPOSING POMC/CART AND NPY/AgRP NEURONS

Within the hypothalamus, the ARC is the seat of opposing POMC/CART neurons (proopiomelanocortin/cocaine- and amphetamine-regulated transcript) and NPY/AgRP neurons (neuropeptide Y and agouti related peptide) [32]. POMC/CART neurons are anorexigenic (i.e., inhibit food intake and increase physical activity) [37], while the NPY/AgRP neurons are orexigenic hence they increase food intake. The neurotransmitter GABA (γ-aminobutyric acid) released from the NPY/AgRP nerve endings inhibits the activity of POMC/CART cells [40, 190]. These two sets of neurons are controlled by several central and peripheral signals (Figure1.4). Hormones like insulin secreted from the beta cells of islets of Langerhans situated in the pancreas directly stimulate the activity of anorexigenic POMC/CART neurons. Leptin secreted from white adipose tissue acts on receptors on both the NPY/AgRP and POMC/CART neurons. Leptin stimulates the activity of the POMC/CART by directly activating these neurons and indirectly by inhibiting the release of GABA from the NPY/AgRP neurons. The “hunger hormone” ghrelin secreted from the stomach acts on its receptors (GHSR) on the NPY/AgRP neurons to activate these cells and inhibit POMC by releasing GABA [40, 119, 150, 170, 190]. These mechanisms allow ARC to monitor hormones and integrate the signals to adjust energy homeostasis.

It is evident that maintaining balance between these two opposing neurons is very important to maintain energy homeostasis. Thus calorie restriction will increase or
decrease the secretion of these and other hormones regulating these systems, causing a change in their activity and thus alter their downstream actions on their receptors, activating or inhibiting them to cause various outcomes. Since MC3R, MC4R, and MC5R are the receptors known to be present in the adult mammalian brain, any change in energy balance cues (e.g., hormones) in the periphery will affect their activation in the brain, and variation in these receptors and their actions will impact the response to calorie restriction.
Figure 1.4: Upstream and downstream effectors of POMC/CART and NPY/AgRP system in arcuate of hypothalamus: Illustration from: The genetics of human obesity (Christopher G. Bell, Andrew J. Walley & Philippe Froguel, March 2005) with copyright permission from Nature Publishing Group.
1.7 ROLE OF MELANOCORTIN RECEPTORS IN ENERGY BALANCE

1.7.1 Melanocortin receptor 3 (MC3R)

MC3R is centrally and peripherally located and has been found to be present in placenta, gut, heart, and brain [61]. In the brain it is found in the hypothalamus, specifically in the VMH and ARC, but also in the limbic system [35, 61, 105, 184] and in the septum, hippocampus, thalamus, and most of the regions of midbrain [74]. MC3R plays a very important role in controlling energy balance, and numerous studies have been performed to show the linkage of MC3R to obesity. MC3R gene deletion in mice increases fat mass, decreases lean mass, and increases food intake [26, 32]. Mice lacking MC3R have reduced physical activity on a running wheel, leading to a decrease in energy expenditure [26].

Mutations of the MC3R gene are associated with human obesity [95], and MC3R mutations might predispose humans to childhood obesity [94]. MC3R has been specifically implicated in energy balance and wakefulness around the feeding time, implicating an interaction with the circadian clock [15, 26, 163].
1.7.2 Melanocortin receptor 4 (MC4R)

MC4R is widespread in the brain, found in many regions including the cortex, thalamus, brainstem, and spinal cord [35, 81, 120]. Although it is brain-specific in humans, MC4R have been found to be present in the peripheral tissues in chickens [164].

In mice, MC4R knockouts are obese primarily due to hyperphagia [178]. Activation of MC4R reduces food intake and increases physical activity [12]. Melanocortin 4 receptor is extremely important in some cases of genetic obesity in humans [93], and deficiency of MC4R also leads to the inheritance of obesity due to hyperphagia [53]. Disruption of one or both alleles of the MC4R gene has been shown to be one cause of monogenic obesity. Mutations and variants in the MC4R gene in humans are associated with obesity and increased fat mass [71, 74, 148]. Due to the importance of MC4R in human obesity it has been the subject of intense focus in research, including pharmacological research, for example studies using MC4R agonists are being performed to attempt to identify a cure for the obesity epidemic [52, 53].

1.7.3 Melanocortin receptor 5 (MC5R)

MC5R is widely distributed throughout the body and is detected in adrenal glands, fat cells, kidneys, reproductive organs, pituitary, muscles (smooth and skeletal), stomach, and spleen, and brain [33, 108, 148]. It is a novel target in obesity study and in general has been known to be related to aggression and exocrine gland function [33, 116]. It has
close resemblance to MC3R and MC4R [31, 54], and may be relevant to the genetics of human obesity [31]. Studies from our laboratory have shown that lean and obesity-prone rats show different regional expression patterns in MC5R and differential response to MC5R agonists and antagonists [154].

1.8 ANIMAL MODEL OF LEANNESS AND OBESITY

To investigate the underlying mechanisms of adaptive thermogenesis and how it differentially affects weight loss between individuals, I used a contrasting rat model system of leanness and obesity developed through selective breeding for intrinsic aerobic capacity [82, 84]. Using heterogeneous N/NIH stock as founder population, two strains of rats were generated through artificial selection based on treadmill running capacity: high-capacity runners (HCR) and low-capacity runners (LCR). These rats are obtained from University of Michigan [84]. To maintain genetic variance, inbreeding was prevented with the help of rotational breeding scheme. After generations of artificial selection the two groups, HCR and LCR varied not only in their intrinsic aerobic capacity but had different body weights, body compositions, and health profiles [83, 103, 104](Table 1.2). The HCR were leaner and less prone to cardiovascular disease, visceral adiposity, impaired glucose tolerance, and dyslipidemia [83, 85, 125] compared to their obese counterparts. HCR were shown to have significantly higher energy expenditure and physical activity compared to the low-capacity rats [83, 85, 127, 129]. This higher physical activity in the lean rats is comparable to humans who have leaner body compositions and are generally more active than the obese individuals [103, 104]. In
humans, aerobic capacity is one of the best predictors of several diseases and mortality [36, 59, 177].

The HCR and LCR rat model was used to examine the differential suppression of EE during calorie restriction, their effects on skeletal muscle energetic and also the potential role of the brain melanocortin system in the energetic response to calorie restriction in these rats.
Table 1.2: The differences between HCR and LCR

<table>
<thead>
<tr>
<th>Key Trait: Aerobic capacity</th>
<th>High Capacity Runners (HCR)</th>
<th>Low Capacity Runners (LCR)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High intrinsic aerobic capacity</td>
<td>Low intrinsic aerobic capacity</td>
<td>[82, 83]</td>
</tr>
<tr>
<td>Physical Activity</td>
<td>Higher</td>
<td>Lower</td>
<td>[83]</td>
</tr>
<tr>
<td>Total Daily energy expenditure</td>
<td>High</td>
<td>Low</td>
<td>[83]</td>
</tr>
<tr>
<td>Economy of activity</td>
<td>Low</td>
<td>High</td>
<td>[83]</td>
</tr>
<tr>
<td>Overall Body composition</td>
<td>Lean</td>
<td>Fat</td>
<td>[83]</td>
</tr>
<tr>
<td>Metabolic Profile</td>
<td>Healthy metabolic profile</td>
<td>Prone to cardiovascular disease and metabolic syndrome</td>
<td>[83, 186]</td>
</tr>
</tbody>
</table>
1.9 SPECIFIC AIMS

To elucidate the relationship between calorie restriction and adaptive thermogenesis in HCR and LCR, I subdivided my entire study into the following three aims:

**Aim 1: Adaptive Thermogenesis and Energy Expenditure**

**Hypothesis:**

1. Differences in physical activity EE—part of non-resting EE—accounts for the individual differences in adaptive thermogenesis

**Aim 2: Effects of Calorie Restriction in HCR and LCR on Molecular Pathways Supporting Metabolism in Skeletal Muscle**

**Hypothesis:**

1. Expression of muscle molecular pathways important for thermogenesis decrease after calorie restriction.

2. Calorie restriction enhances expression of pathways important for energy conservation and lipid handling.

3. These muscle responses are dependent on aerobic capacity.
Aim 3: Identify Brain Mechanisms That Potentially Underlie Central Modulation of Adaptive Thermogenesis

Hypothesis:

1. Brain melanocortin signaling is altered in calorie restriction, and individual differences in melanocortin signaling may lead to variability in adaptive thermogenesis.
Chapter 2: Aerobic capacity modulates adaptive thermogenesis by impacting non-resting energy expenditure

2.1 INTRODUCTION

Weight loss requires the energy expenditure to exceed the energy intake. The energy expenditure of a person varies depending on several factors including the energy expended due to activity (non-resting EE, which includes voluntary activity levels and a person’s NEAT and TEF) and on their resting EE. The ability to lose weight or the tendency to gain weight varies between individuals. Some individuals are resistant to weight loss. This resistance could be attributed to greater adaptive thermogenesis, the suppression of EE beyond that which is predicted by the lost weight [137]. This suppression of EE hinders weight loss during calorie restriction and can prevent successful maintenance of lost weight.

Adaptive thermogenesis may differ due to race or ethnicity [58, 179], leanness vs. obesity [180], and sex. Though most investigations have focused on adaptive thermogenesis in resting EE or BMR, adaptive thermogenesis may also occur in NEAT. For example, some people might be more prone to decrease in their NEAT when on a diet compared to others, which would have the effect of countering weight-loss efforts. Human studies have revealed that during overfeeding some people gain fat, while others who do not gain as much fat as they increase their NEAT considerably during the process [102]. All components of EE may be suppressed during calorie restriction, but which component is
predominantly affected may vary over time and between individuals [96, 142]. The decrease in resting EE is similar among people. The role of suppression of resting EE during weight loss has been debated, but a decline in non-resting EE during dieting resulting in resistance to weight loss has been observed [142, 144]. The suppression of non-resting EE is higher during the loss of first 10% of the body weight [67, 96, 143, 144]. Weight loss induces a decrease in the total energy or kilocalories (Kcal) required to perform the daily activities of living. This is due in part to a phenomenon called skeletal muscle work efficiency [67] or locomotor efficiency (metabolic cost of movement), or increased economy of activity [129, 191]. The hypotheses of the mechanism underlying this could include altered sympathetic or thyroid hormone-related actions on muscle that may alter functioning of molecular pathways important in fuel uptake and use, as well as thermogenesis [9, 67, 143].

In summary, we know that adaptive thermogenesis varies between individuals and counteracts weight-loss efforts. Thus finding a way to counter adaptive thermogenesis could facilitate continued weight loss and prevent regain of the lost weight in humans [28, 46, 47, 66, 121, 182, 183]. The studies described here tested the hypothesis that differences in physical activity EE—non-resting EE—may account for the individual differences in adaptive thermogenesis and potentially impact weight loss on a diet.
2.2 ANIMAL MODEL USED AND ITS RELEVANCE

To investigate the underlying mechanisms of adaptive thermogenesis and how it differentially affects weight loss between individuals, I used a contrasting rat model system of leanness and obesity developed through selective breeding for intrinsic aerobic capacity [84]. These rat models were used to test out my hypothesis about how adaptive thermogenesis may oppose weight loss when on a diet (i.e., calorie restriction), and the importance of energy expenditure and NEAT in this process. HCR have elevated total daily energy expenditure due to higher physical activity combined with lower economy of physical activity [129, 130], and a difference in non-resting EE between groups [63]. Here, I examined which component of TDEE—resting EE or non-resting EE—changes before compared to after calorie restriction to determine which of these components is responsible for the differences in adaptive thermogenesis during calorie restriction.

We know that aerobic capacity is a very good predictor of long-term health in human beings [18, 92]. This rat model was developed to test the hypothesis about how aerobic capacity correlates with chronic diseases [21]. Some behavioral differences also accompany differences in aerobic capacity [25, 129]. People with higher NEAT gain less fat in a high-calorie diet, just like our high-NEAT HCR. Just as lean people generally tend to be more physically active, our lean HCR rats are also more active, and both have high intrinsic aerobic capacity. Similar to less active people, LCR rats are obesity prone and have a more difficult time shedding those extra grams on a “diet” (calorie restriction) [159]. Thus our rat model system is a good representation of humans in general.
2.2.1 Importance of using both males and females in EE studies

For this aim I have used both male and female rats. Female rats are generally more difficult to study because of the influence of the estrous cycle on energy balance [7, 22]. Others in our laboratory have found only minor variations in physical activity over the cycle, however [159]. Given access to running wheels, the sex differences observed in physical activity are much more pronounced [128]. In general, research on male subjects still predominates and females are studied less frequently, leading to a sex bias in neuroscience and behavioral research [14, 193]. Females can present advantages, particularly in energy-balance research as there is a smaller divergence of body weight and body composition between HCR and LCR, and females show a substantial overlap between groups (HCR and LCR) in both body weight and lean mass, facilitating direct comparison of EE [63]. In male HCR and LCR, on the other hand, differences in their body weight vary between approximately 100-200 grams. Since body weight is the primary determinant of energy expenditure, overlapping body weight helps factor out the error resulting from disparity in body weight between groups. Hence, using both males and females helps us to reach a more conclusive result regarding the effects of calorie restriction on energy expenditure in animals having a wide range of mass and body composition [63, 159].
2.3 MATERIALS AND METHODS

2.3.1 Food intake

Two separate studies were performed on individually housed rats, one on male rats [HCR (n=12); LCR (n=12)] and one on female rats [HCR (n=11); LCR (n=13)]. Male and female rats were measured and analyzed separately to examine individual differences in adaptive thermogenesis. A 12:12 hour dark and light cycle was maintained with the light cycle starting at 0700 EST. Rodent chow (5P00 MRH 3000, T.R. Last Co. Inc) and water were provided to each rat. In each case, daily food intake of each animal was calculated after measuring their baseline food intake for 8 days, feeding them at 1200 EST (±1 hr). Based on the average daily food intake of each rat and after the highest and the lowest values for each rat was discarded, the 50% food consumption of each rat was calculated individually. 50% calorie restriction was performed on each rat for 21 days. During calorie restriction, body weight was measured and animals were fed daily at 1200 (EST) with precision of ±1 hr.

2.3.2 Body composition analysis

Body composition measurement was done before 21 days of calorie restriction and again on the 21st day of calorie restriction using magnetic resonance spectroscopy with an EchoMRI-700 which uses nuclear magnetic resonance spectroscopy to assess masses of fat and muscle, as well as free water. The fat mass and lean mass data obtained (in grams) were used for further analysis.
2.3.3 Energy expenditure measurement using a calorimeter

Rats were acclimated for two days in the calorimetry room (isolation chamber) and cage prior to the energy expenditure measurement. I used an Oxymax FAST system (Columbus Instruments, Columbus, OH) to measure energy expenditure and physical activity for each animal with a temporal resolution of 30 seconds, which enabled detailed analysis of resting EE and non-resting EE. Rats were divided into cohorts of 4 to accommodate the 4-chamber calorimetry system. In each run, 2 HCR and 2 LCR rats were used; the measurement chamber was also randomly assigned to HCR and LCR so that each of type of rat had equal chance of being assigned to a particular chamber to prevent any group based chamber bias. The rats were monitored for ≥25 hours, and data from noon (EST) of day 1 to noon on day 2 were analyzed. The first 1-2 hours of data was not used for my analysis to prevent any error in the data due to the fact that the animals might have been agitated or more active immediately after they are placed in the chamber, and to allow the chamber air to settle. Energy expenditure was measured at 30-second intervals, and physical activity was measured at 10-sec intervals. Energy expenditure was separated into resting EE and non-resting EE using CLAX software [2, 63]; CLAX software was used to calculate the resting EE based on the lowest EE, after eliminating the 5 lowest episodes of EE using a previously validated method [63]. From there, non-resting EE was calculated using the formula: TDEE = resting EE + non-resting EE [63].
2.3.4 Maximal oxygen consumption (VO$_{2\text{max}}$)

VO$_{2\text{max}}$ was measured in male HCR and LCR before and after 21 days of 50% calorie restriction using a graded treadmill test (using protocol in Table 2.1). Each animal was first acclimated to a treadmill by allowing them to be on the treadmill for 5 minutes at 10m/min. The treadmills were randomly assigned to HCR and LCR to reduce experimental bias. After 2-3 days of acclimation each rat were run on a treadmill with variable speed and incline. The rats were allowed to run until a respiratory exchange ratio (RER) of 1 was reached and/or the animal was unable to run.
Table 2.1: Protocol used for graded treadmill test

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Speed (m/mins)</th>
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<tbody>
<tr>
<td>20</td>
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<td>37</td>
<td>25</td>
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</table>
2.3.5 Data Analysis

For both males and females, SPSS software was used to analyze the data. Analysis of covariance (ANCOVA) was used to compare groups that differ in body weight and composition, using body mass and lean mass as covariates in separate analyses for males and females. SPSS software was used to do post-hoc analysis using the least significant difference test (LSD) to probe interactions. A $p$-value of $\leq 0.05$ was considered as significant. Because normal SPSS analysis does not give the option of repeated-measures ANCOVA with different covariates for the same animals at different time points, I used an SPSS script (or syntax) to complete these analyses. Repeated-measures ANOVAs were used to compare change in EE and body composition over time between HCR and LCR. Figure 2.1 shows the symbols used to denote significance.
(*) Significant change ($p \leq 0.05$) over time compared to their respective baseline

# Significant interaction of HCR and LCR with CR.

* Significant change ($p \leq 0.05$) $HCR \neq LCR$ within the time

Significant change ($p \leq 0.05$) in main effect of calorie restriction

* beside top legend Significant change ($p \leq 0.05$) between the two compared variables

Figure 2.1: Symbols used to denote significance
2.4 RESULTS

2.4.1 Analysis of female HCR and LCR

a) Body composition

**Body weight:** Calorie restriction induced significant weight loss (in grams) \((p<0.001)\) but there was no significant interaction \((p=0.307)\) or difference of body weight between HCR and LCR \((p=0.082)\) observed. No significant differences in percentage of body weight loss \((p=0.198)\) between HCR and LCR were found.

**Fat mass:** Calorie restriction induced significant fat loss (in grams) \((p<0.001)\) and there was significant interaction \((p<0.001)\) but no main effect of group (i.e., fat mass did not significantly vary between HCR and LCR, collapsed across conditions) \((p=0.081)\). There was a significant difference in percentage of fat mass loss (compared to their baseline) \((p=0.02)\) where LCR lost a greater percentage of their fat mass compared to HCR.

**Lean Mass:** Calorie restriction induced significant lean mass loss (in grams) \((p<0.001)\) and lean mass was significantly different between HCR and LCR \((\text{LCR}>\text{HCR}, p<0.038)\). However no significant interaction \((p=0.566)\) or differences in percentage of lean mass loss \((p=0.396)\) between HCR and LCR were observed. Both HCR and LCR significantly loss a greater percent of fat mass compared to lean mass \((p<0.001)\), with the percentage taken with respect to each rat’s baseline levels.
Figure 2.2: Body composition loss analysis in female rats. Loss of body weight, lean mass, fat mass in female high- and low-capacity rats (HCR, LCR) before and after 21-day calorie restriction (CR). There was a significant overall loss (in grams) in body weight, fat mass, lean mass. There was a significant difference in HCR vs. LCR for lean mass loss (in grams LCR > HCR). Both HCR and LCR lost a greater percent of their fat mass lost compared to their lean mass. There was a significant difference in the percentage (%) of fat mass lost compared to baseline where LCR lost more % fat mass than the HCR. (± SEM: HCR; Fat mass: 3.3, lean mass: 2.3 and LCR; Fat mass: 2.3, lean mass: 1.6).
b) Respiratory exchange ratio (RER)

RER measured as a ratio of volume of carbon dioxide (VCO₂) released by volume of oxygen consumed (VO₂) (RER: VCO₂/VO₂). There was a significant effect of calorie restriction on RER was observed where it decreased compared to baseline ($p<0.001$). However there was no difference between HCR and LCR or differential effect on these groups due to calorie restriction (Figure 2.3).
Figure 2.3: Respiratory exchange ratio (RER) in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. For female high- and low-capacity rats (HCR, LCR), 21-day calorie restriction (CR) induced a significant overall decrease in respiratory exchange ratio (RER; VCO$_2$/VO$_2$) compared to baseline.
c) Physical Activity

Total horizontal activity was significantly suppressed by calorie restriction ($p<0.001$) and there was a significant main effect of line (HCR>LCR, $p<0.001$) (Figure 2.4). There was a significant interaction ($p<0.001$) where HCR showed a greater suppression in physical activity than LCR, though both HCR and LCR were less active after calorie restriction than before ($p<0.001$). Physical activity was greater in HCR before calorie restriction ($p<0.001$) but there was only a trend ($p=0.055$) towards higher physical activity in HCR after calorie restriction.
Figure 2.4: Physical activity in female high- and low-capacity rats (HCR, LCR) before and after calorie restriction. Calorie restriction (CR) induced a significant decrease in physical activity in both HCR and LCR, though the HCR changed significantly more than LCR. HCR were significantly more active than LCR before but not after CR.
d) Total daily energy expenditure (TDEE)

When either lean mass or body weight were taken into account in separate analyses, TDEE differed between HCR and LCR at baseline (HCR>LCR, \( p<0.001 \)) (Figure 2.5 A). No difference was observed in TDEE after 21-day calorie restriction (Figure 2.5 B). TDEE significantly covaried with both lean mass and body weight in HCR and LCR both before (\( p<0.001 \) for both lean mass and body weight) and after calorie restriction (\( p=0.002 \) for body weight and 0.043 for lean mass). Both HCR and LCR showed a suppression of lean mass-corrected TDEE after calorie restriction (\( p<0.001 \)) (Figure 2.6), whereas suppression of body weight-corrected TDEE was seen only in HCR (\( p=0.001 \)).
Figure 2.5: Total daily energy expenditure in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), total daily energy expenditure (TDEE) significantly differed in HCR vs. LCR before (A) but not after (B) calorie restriction (CR). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.6: Total daily energy expenditure change in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), total daily energy expenditure (TDEE) significantly differed before and after calorie restriction (CR) in both HCR (A) and LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
e) Resting EE

There was no significant difference in lean mass- or body weight-corrected resting EE between HCR and LCR before or after calorie restriction (Figure 2.7). HCR and LCR both showed significant suppression of their lean mass-corrected resting EE after calorie restriction (Figure 2.8), whereas suppression of body weight-corrected resting EE was seen only in HCR ($p<0.001$). Resting EE significantly covaried with body weight both before ($p<0.001$) and after ($p=0.026$) calorie restriction where lean mass significantly varied before ($p<0.001$) calorie restriction, but only a trend ($p=0.162$) was seen after calorie restriction. There was no significant difference in the percentage suppression of resting EE compared to baseline $\left[\frac{\text{baseline resting EE} - \text{post-calorie restriction resting EE}}{\text{baseline resting EE}}\right]$ between HCR and LCR. HCR suppressed their resting EE by 29.7% and LCR suppressed it by 30.2%.
Figure 2.7: Resting energy expenditure in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), resting energy expenditure (resting EE) did not differ between HCR and LCR before (A) or after (B) calorie restriction (CR). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.8: Resting energy expenditure change in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), resting energy expenditure (resting EE) significantly differed before vs. after calorie restriction (CR) both in HCR (A) and LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
f) Non-resting EE

When either lean mass or body weight was taken into account, non-resting EE differed between HCR and LCR before calorie restriction (HCR>LCR, \( p<0.001 \)) (Figure 2.9 A), but this difference was not observed after 21 days of calorie restriction (Figure 2.9 B). Non-resting EE significantly covaried with body weight both before \((p<0.011)\) and after \((p=0.016)\) calorie restriction, whereas non-resting EE significantly varied with lean mass before calorie restriction \((p<0.004)\) but a trend towards difference after \((p=0.069)\) calorie restriction with. Analysis within group (HCR alone, LCR alone) showed that HCR showed a significant suppression of both their body weight- and lean mass-corrected non-resting EE \((p=0.049\) and 0.001, respectively) (Figure 2.10 A), whereas the LCR did not show any significant suppression of their non-resting EE (Figure 2.10 B). There was a significant difference \((p=0.04)\) between the percent suppression of non-resting EE compared to the baseline between HCR and LCR. HCR suppressed their non-resting EE by 34.6\%, whereas LCR showed 23.5\% suppression.
Figure 2.9: Non-resting energy expenditure in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), non-resting energy expenditure (non-resting EE) significantly differed between HCR vs. LCR before (A) but not after (B) calorie restriction (CR). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.10: Non-resting energy expenditure change in female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In female high- and low-capacity rats (HCR, LCR), calorie restriction (CR) significantly suppressed non-resting energy expenditure (non-resting EE) in HCR (A) but not in LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
g) Repeated calorie restriction of the same females

After the regain of the original body weight, these females were used to perform a second round of 50% calorie restriction using the exact same protocol as the first round.

The results reflected similar difference as observed in the case of round one of calorie restriction. In short, a significant interaction ($p=0.013$) was observed where physical activity was suppressed only in HCR ($p=0.001$) (Figure 2.11). HCR had higher physical activity levels than LCR both before and after calorie restriction ($p=0.001$ and 0.03). Significant differences were seen in both lean mass and body weight-corrected TDEE before but not after calorie restriction between HCR and LCR. Lean mass- and body weight-corrected resting EE did not differ between HCR and LCR before or after restriction. Suppression was observed in both HCR and LCR in their lean mass- and body weight-corrected resting EE and TDEE. However non-resting EE was suppressed only in the HCR. Resting EE was suppressed by 39% in HCR and 34% in LCR compared to their respective baselines. A significant difference in percent suppression of non-resting EE observed between HCR (37%) and LCR (24%) compared to their respective baselines.
Figure 2.11: Physical activity in repeated calorie restricted female high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At both baseline and after 21-day calorie restriction (CR) in female high- and low-capacity rats (HCR, LCR) in which CR was repeated, physical activity was higher in HCR than LCR both before and after CR. In HCR, but not LCR, CR induced a significant decrease in physical activity.
2.4.2 Analysis of male HCR and LCR

Two sets of male data were analyzed where one set of rats were subjected to calorie restriction for the first time, and a second set were measured that had previously experienced calorie restriction.

a) Body Composition

**Body weight:** Calorie restriction induced a significant weight loss (in grams) \(p<0.001\), and a significant interaction \(p<0.001\) where body weight loss (in grams) significantly differed between HCR and LCR due to the LCRs’ larger starting body weight (LCR>HCR; \(p<0.001\)). However no significant differences were seen in percentage of body weight loss \(p=0.86\) between HCR and LCR.

**Fat mass:** Calorie restriction induced significant weight loss (in grams) \(p<0.001\) and a significant interaction \(p<0.005\) where LCR lost more fat mass than HCR \(p=0.001\). However no significant differences in fat loss as a percentage of baseline fat mass \(p=0.124\) between HCR and LCR were found.

**Lean mass:** Calorie restriction induced significant loss of lean mass (in grams) \(p<0.001\) and a significant interaction \(p<0.009\) were LCR lost more lean mass (in grams) than HCR \(p<0.001\). However HCR and LCR did not differ in the percent of their baseline lean mass that they lost \(p=0.586\). Both HCR and LCR lost a significantly greater
percent of their baseline fat mass compared to their baseline lean mass ($p<0.001$). HCR lost 12% of their lean mass and 47% of their fat mass. The LCRs lost 14% of their lean mass and 40% of their fat mass.
Figure 2.12: Body composition analysis in male high- and low-capacity rats (HCR and LCR). Body weight, lean mass, fat mass in male high- and low-capacity rats (HCR, LCR) before and after 21-day calorie restriction (CR). LCR> HCR in body weight, lean mass and fat mass (in grams both before and after CR). There was a significant overall suppression (in grams) in body weight, lean mass and fat mass and a significant interaction was observed in all the cases. Both HCR and LCR lost more % of fat mass compared to their lean mass % loss compared to their respective baselines. However there was no significant difference in the percentage of body weight, lean mass and fat mass lost compared to baseline between HCR and LCR. (±SEM: HCR; Lean mass: 2.8, fat mass: 1.5; LCR: lean mass 3.5, fat mass 3.1).
b) Respiratory exchange ratio (RER)

Calorie restriction significantly suppressed RER \((p<0.001)\) (Figure 2.13). However there was no significant interaction and no difference between HCR and LCR.
Figure 2.13: Respiratory exchange ratio (RER) in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At baseline for male high- and low-capacity rats (HCR, LCR), respiratory exchange ratio (RER; VCO$_2$/VO$_2$) was greater before calorie restriction (CR), where CR induced a significant overall decrease in RER similarly in HCR and LCR.
c) Total daily energy expenditure (TDEE)

There was no significant difference in lean mass or body weight-corrected TDEE found in HCR vs. LCR before or after calorie restriction (Figure 2.14). TDEE significantly covaried with body weight after \( p<0.001 \) but not before \( p=0.241 \) calorie restitution, and it significantly covaried with lean mass both before \( p=0.004 \) and after \( p<0.001 \) calorie. TDEE after calorie restriction was significantly suppressed in both HCR \( p<0.001 \) (Figure 2.15 A) and LCR \( p<0.001 \) (Figure 2.15 B).
Figure 2.14: Total daily energy expenditure in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), total daily energy expenditure (TDEE) did not significantly differ in HCR vs. LCR before (A) or after (B) calorie restriction (CR). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.15: Total daily energy expenditure change in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), total daily energy expenditure (TDEE) was significantly suppressed after calorie restriction (CR) compared to baseline in both HCR (A) and LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
d) Resting EE

No significant differences were seen in lean mass- or body weight-corrected resting EE in HCR vs. LCR before or after calorie restriction (Figure 2.16). In HCR vs. LCR, resting EE significantly covaried with body weight after \(p=0.001\) but not before \(p=0.212\) calorie restriction, and with lean mass both before \(p=0.006\) and after \(p=0.004\) calorie restriction. Both the groups significantly suppressed their resting EE after calorie restriction compared to their respective baselines \(p<0.001\) for both HCR and LCR (Figure 2.17). The HCR suppressed their resting EE by 35.8% and the LCR by 34.5%.
Figure 2.16: Resting energy expenditure in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), resting energy expenditure (resting EE) did not significantly differ in HCR vs. LCR before (A) or after (B) calorie restriction (CR). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.17: Resting energy expenditure change in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), resting energy expenditure (resting EE) was significantly suppressed by calorie restriction (CR) in both HCR (A) and LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
e) Non-resting EE

There was no significant difference in lean mass- or body weight-corrected non-resting EE in HCR vs. LCR before calorie restriction (Figure 2.18 A). However there was a significant difference in the lean mass- and body weight-corrected non-resting EE after calorie restriction where HCR>LCR (Figure 2.18 B). Non-resting EE significantly covaried with body weight after ($p=0.001$) but not before ($p=0.602$) calorie restriction, and with lean mass both before ($p=0.029$) and after ($p<0.001$) calorie restriction. When the lines were analyzed individually, compared to their respective baselines, only HCR ($p=0.002$) (Figure 2.19 A) showed significant suppression of their non-resting EE due to calorie restriction. HCR suppressed their activity EE by 21.7% whereas the LCR by 15.8% compared to their respective baselines.
Figure 2.18: Non-resting energy expenditure in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), non-resting energy expenditure (non-resting EE) did not significantly differ in HCR vs. LCR before (A) calorie restriction (CR). A significant difference was observed in HCR vs. LCR after CR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
Figure 2.19: Non-resting energy expenditure change in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. In male high- and low-capacity rats (HCR, LCR), non-resting energy expenditure (non-resting EE) was significantly suppressed by after calorie restriction (CR) in HCR (A) but not in LCR (B). Lean mass was taken into account using analysis of covariance (ANCOVA).
f) Physical activity

Calorie restriction significantly suppressed physical activity ($p<0.001$) (Figure 2.20). No interaction was observed but there was a main effect of line where HCR$>$LCR ($p<0.001$). The HCR were more active compared to LCR both before ($p=0.002$) and after ($p=0.007$) 21 days of 50% calorie restriction. There was a significant suppression of physical activity observed only in the HCR after calorie restriction ($p=0.01$).
Figure 2.20: Physical activity in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At both baseline and after 21-day calorie restriction (CR) in male high- and low-capacity rats (HCR, LCR), physical activity was higher in HCR both before and after CR. In HCR, CR induced a significant decrease in physical activity.
g) Repeated calorie restrictions in male HCR-LCR

Similar results were observed as in the male HCR/LCR experiment described above, except that in the rats undergoing their second round of calorie restriction there was no difference in any of the components of lean mass-corrected TDEE between HCR and LCR, either before or after calorie restriction. When HCR and LCR were analyzed separately, LCR showed suppression in their total and resting EE, but not in non-resting EE. HCR, on the other hand, showed a significant suppression of total daily EE, resting EE, and non-resting EE. This is consistent with what was seen in the previous results in rats experiencing their first calorie restriction.
2.4.3 Maximal oxygen consumption (VO$_2$\textsubscript{max})

This particular study was performed only in the repeated calorie restricted males.

a) Running performance

**Top interval:** There was no significant interaction in the top running interval reached (number of 10-sec intervals completed ($p=0.051$) between the HCR/LCR and calorie restriction (Figure 2.21 A). There was no significant main effect of calorie restriction on top interval ($p=0.376$). There was a significant main effect of line (HCR vs. LCR, where HCR$>$LCR, $p<0.001$) where HCR covered a significantly greater number of running intervals (number of 10-sec intervals in calorimetry after rest) compared to LCR both before ($p=0.001$) and after ($p<0.001$) calorie restriction.

**Top Speed:** The maximal running speed (Figure 2.21 B) achieved was not affected by calorie restriction ($p=0.068$). There was no significant interaction in top speed ($p=0.5$) and between HCR/LCR and calorie restriction suggesting that calorie restriction did not differentially affect HCR and LCR. There was a significant main effect of line (HCR vs. LCR, where HCR$>$LCR, $p<0.001$). HCR reached a greater treadmill speed compared to LCR both before ($p=0.001$) and after ($p<0.001$) calorie restriction.
Figure 2.21: Running performance (top interval and top speed) in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At both baseline and after 21-day calorie restriction (CR) in male high- and low-capacity rats (HCR, LCR), top interval (A) top speed (B) and achieved in a VO$_{2\text{max}}$ treadmill test were both significantly higher in HCR than LCR, both before and after CR. CR did not significantly suppress running performance (either top speed or total interval), however.
b) Respiratory exchange ratio (RER)

Food restriction did not significantly affect maximal RER during the VO$_{2\text{max}}$ test in either HCR or LCR ($p=0.259$) (Figure 2.22). There was no significant interaction ($p=0.864$) or main effect of line ($p=0.975$) observed.
Figure 2.22: Respiratory exchange ratio (RER) during VO$_2$$_{\text{max}}$ in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At both baseline and after 21-day calorie restriction (CR) in male high- and low-capacity rats (HCR, LCR), there was no significant suppression of, or group difference in, the maximal respiratory exchange ratio (RER; VCO$_2$/VO$_2$) reached during the VO$_2$$_{\text{max}}$ treadmill test.
c) Oxygen consumption during VO$_{2\text{max}}$ treadmill test

The body weight was factored in automatically (by the calorimetry computer program), yielding the data in ml/kg/hr. To more appropriately account for body mass, I first multiplied the VO$_{2\text{max}}$ (ml/kg/hr) by body weight in kilograms to yield ml/hr, and then subjected the data to ANCOVA with body weight factored out by using it as covariate. The maximal oxygen consumption reached (VO$_{2\text{max}}$, ml/hr) significantly decreased ($p=0.001$) after calorie restriction compared to baseline when either body weight or lean mass (Figure 2.23) were taken into account (using ANCOVA). Univariate analysis showed that HCR had higher VO$_{2\text{max}}$ both before ($p=0.032$) and after ($p=0.01$) calorie restriction (Figure 2.24). The significant reduction of VO$_{2\text{max}}$ suggests an increase in energy efficiency of running.
Figure 2.23: Maximal oxygen consumption (VO\(_{2\text{max}}\)) in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. At both baseline (A) and after (B) 21-day calorie restriction (CR) in male high- and low-capacity rats (HCR, LCR), maximal oxygen consumption (VO\(_{2\text{max}}\) in ml/hr) was significantly higher in HCR compared to LCR.
Figure 2.24: Maximal oxygen consumption ($\text{VO}_2\text{max}$) change in male high- and low-capacity rats (HCR and LCR) before and after calorie restriction. After 21-day calorie restriction (CR) in male high- and low-capacity rats (HCR, LCR), maximal oxygen consumption ($\text{VO}_2\text{max}$ in ml/hr) was significantly suppressed compared to baseline.
2.5 DISCUSSION

Weight loss on a diet and maintenance of this weight loss is a common struggle among many individuals. This is partly because of adaptive thermogenesis, which lowers the energy expenditure beyond a level predictable by the new, reduced body weight. Adaptive thermogenesis affects some people more than others [137]. This study focused on identifying the component of TDEE responsible for the differential suppression in EE by adaptive thermogenesis. This study was performed in both male and female rats to avoid overlooking potentially interesting results because of variances in body weight observed between male HCR and LCR.

In the females before calorie restriction there was no significant difference in the resting EE between HCR and LCR, but the HCR had higher non-resting EE. This is consistent with previous data from our lab [63]. This is also consistent with the idea that the non-resting EE is the factor differentiating the TDEE between HCR and LCR. After calorie restriction, no significant difference was observed between HCR and LCR in either component of EE, either resting or non-resting. This suggests that resting and non-resting EE showed differential suppression by calorie restriction in HCR and LCR. Indeed, resting EE was suppressed after calorie restriction, and the extent of this suppression relative to baseline resting EE was similar between phenotypes: 29.7% in HCR, and 30.2% in LCR. However the non-resting EE was suppressed by 34.6% in HCR, significantly higher than the 23.5% decrease seen in LCR. Therefore, non-resting energy expenditure is what distinguishes HCR and LCR energetic response to calorie restriction.
However an important point is that the physical activity of HCR was higher compared to LCR both before and after calorie restriction in most of cases. Thus the relatively greater suppression of the non-resting EE in HCR could be due to (a) lower physical activity, and/or (b) increased locomotor/energy efficiency.

These show that HCRs are adapting more to the calorie restriction compared to the LCR. They are expending less energy compared to their baseline levels. The LCR had a low baseline activity EE thus the only component of EE that suffered a significant change for them was the resting EE. Thus unlike the HCR, the LCR has their non resting EE pre-optimized to survive in conditions of lower food availability making them a thrifty phenotype to begin with [137]. At first glance, this does not necessarily correspond to human data where the weight-loss-resistant people showed more suppression of non-resting EE [137]. But in the context of the HCR-LCR rat model system, the suppression of non-resting EE in the more active HCR makes sense. The HCR show a suppression (or greater suppression) of physical activity compared to LCR, and in some cases LCR do not show a significant suppression in physical activity at all. HCR and LCR both showed plasticity in their resting EE, however only the HCR showed plasticity in their non-resting EE, similar to what is seen with the physical activity. Thus the adaptable nature of non-resting EE appears to be absent or at least minimized in the LCR. It could be hypothesized that the LCRs’ non-resting EE is suppressed all of the time, even without calorie restriction, giving them a better survival advantage during famine. The idea that LCR already have a “pre-suppressed” physical activity and activity EE during everyday baseline conditions is consistent with the idea that they have a thrifty phenotype better
suited to an environment that has less food, where energy restriction is an ever-present risk.

In male HCR and LCR, compared to the females, similar results were obtained where there was no difference between phenotypes in either TDEE or resting EE, either before or after calorie restriction. Non-resting energy expenditure differed between groups only after calorie restriction because HCR showed a greater suppression in the non-resting component of EE. Both HCR and LCR showed significant suppression in TDEE and in resting EE, but significant suppression in non-resting EE was observed only in HCR. While there were some differences between the males and females, overall both datasets highlight the relevance of non-resting EE and how it changes over the course of calorie restriction.

Again, in both males and females, calorie restriction significantly suppressed physical activity levels, and this was mostly seen in HCR, and rarely in LCR. HCR had higher physical activity levels compared to LCR throughout the course of calorie restriction. The only exception to this was observed in the first round of calorie restriction in female rats where both HCR and LCR suppressed their physical activity, though there was a significant interaction even in this case where HCR suppressed much more than LCR. This inconsistency could be because of relatively high variance in HCR and LCR physical activity (as physical activity is a notoriously labile behavioral variable). Still, activity levels in HCR were higher than LCR before but not after calorie restriction,
which was again consistent with the idea that LCR are already settled at a lower physical activity level under free-fed conditions and thus they did not have to undergo additional suppression to withstand the food restriction. On the other hand, HCR may have to suppress their physical activity to help survive the food restriction. This is consistent with previous data from our lab [159].

In lean and obese humans, the major reason for variation of their suppression of total daily EE during food restriction is the decline of non-resting EE (as TEF and resting EE are almost equivalently suppressed between phenotypes) [142]. A greater decline of non-resting is observed during the first 10% of body weight loss in a restricted diet between obese and non-obese individuals [67, 96, 144]. The 50% calorie restriction in the rats studied in these experiments induced a roughly equivalent decrease in resting EE in both the lean and obesity-prone HCR and LCR. Adding more time points would help discern differences in the speed of adaptation of calorie restriction, if there are any.

Calorie restriction induces increased skeletal muscle efficiency in humans [67] and changes in muscle energy use could impact both resting and non-resting EE. Locomotor efficiency is adaptable, for example it is altered with activation of brain melanocortin receptors also differs according to phenotype [63]. HCR and LCR were selected based on their aerobic capacity, but how their locomotor efficiency was impacted by calorie restriction was unknown. Therefore, I assessed VO$_{2\text{max}}$ using a graded treadmill exercise test, running the rats to exhaustion. As expected, the HCR ran significantly more in
regards to both time and distance compared to LCR, both before and after calorie restriction, having higher VO$_{2\text{max}}$. Interestingly, calorie restriction did not significantly compromise the maximal speed reached or distance run (intervals) in either HCR or LCR (Figure 2.20). Calorie restriction significantly impacted running efficiency, however, where the VO$_{2\text{max}}$ decreased after calorie restriction (Figure 2.24). Food restriction did not change the maximal respiratory exchange ratio (RER) during the VO$_{2\text{max}}$ test. Thus calorie restriction made HCR and LCR running more efficient without compromising their endurance. Again, HCRs’ running capacity and performance were significantly better than LCR both before and after calorie restriction, consistent with their phenotypes. Thus VO$_{2\text{max}}$ was also affected by adaptive thermogenesis by increasing running efficiency and conserving energy, making rats less susceptible to weight loss while protecting running performance.

Previous studies in my lab has showed that calorie restriction has resulted in a greater weight loss in HCR compared to LCR [159]. While this is consistent with the difference in non-resting EE seen here, in all my studies I observed a significant weight loss in HCR and LCR but there was no difference in weight lost between the HCR and LCR. This could be due to various reasons including differences in animal handling [160], temperature variations, cage size, or the severity of the calorie restriction inflicted. The difference in non-resting EE is consistent with previous data [63], and the HCR/LCR differences in muscle thermogenesis seen in previous studies done in the lab would be predicted to be eliminated after calorie restriction if muscle thermogenesis reflects non-resting EE or efficiency.
In summary, the HCR are leaner and more physically active and have higher aerobic capacity under normal baseline conditions compared to their obese counterparts, LCR. When HCR underwent food restriction, they adapted to the energy restriction by suppressing their spontaneous physical activity levels and non-resting EE, making their body equally energy efficient as the LCR. This phenomenon may protect them against continual weight loss when put through longer periods of food restriction, thus helping them survive. These results suggest that individual differences in calorie restriction-induced adaptive thermogenesis may be accounted for by variations in NEAT that are mechanistically linked to aerobic capacity. Specifically, it is likely activity EE, not resting or basal metabolism, accounts for individual variation in adaptive thermogenesis.
Chapter 3: Effects of calorie restriction in HCR and LCR on molecular pathways supporting metabolism in skeletal muscle

3.1 INTRODUCTION

Skeletal muscle plays a pivotal role in energy expenditure—the higher the lean mass, the greater the energy expenditure. Lean mass mainly affects resting EE, thereby with increase in lean mass there is an increase of total daily EE [112]. Alteration in the energy use of muscle has been implicated in increased NEAT [63]. Activity EE is influenced by variation in activity thermogenesis, which implicates adaptations in muscles. It is evident that sustained reduced body weight and/or negative energy balance impact skeletal muscle function [144]. Here, I proposed to examine how the changes in activity EE after 50% calorie restriction correspond to changes in the molecular pathways important for energy expenditure and conservation in tissues and organs important in metabolism, particularly skeletal muscle, at the level of both mRNA and protein. Since adaptive thermogenesis reduces the energy expenditure beyond predicted levels, I sought to determine how this relates to gene expression and protein levels of molecules important in cellular energy uptake, use, and conservation.
3.1.1 Molecular probes important in energy balance

a) Uncoupling proteins (UCP)

UCP is a mitochondrial inner-membrane protein that separates oxidative phosphorylation from ATP synthesis. In brown adipose tissue (BAT), UCP1 dissipates energy in the form of heat. It increases the permeability of the inner mitochondrial membrane, thereby facilitating the return of the protons already pumped in the intermembrane space back to the matrix [113, 145, 147]. The presence of uncoupling protein 1 (UCP1) is not seen in skeletal muscle, at least in myocytes, but is present in BAT [113]. The other forms of UCP, notably uncoupling proteins 2 and 3 (UCP2 and UCP3), have been identified in the skeletal muscle [149]. UCP2 helps in the control of mitochondrial derived reactive oxygen species. Another important role of muscle UCP is lipid handling [147]; accordingly UCP2 levels have been observed to be increased in starved rats [8, 27, 147]. Mutations in UCP3 have been seen in association with human obesity [8, 70]. Therefore, changes in muscle expression of UCP during calorie restriction may relate to both adaptive thermogenesis and metabolic fuel use.

b) Beta-2 adrenergic receptor (ADRB2)

ADRB2 is a G protein-coupled receptor. Binding of a ligand (epinephrine or norepinephrine) to this receptor mediates the response to sympathetic neural input. Action of the ligand on this receptor activates the protein kinase A (PKA) pathway.
activating adenyl cyclase, which in turn converts ATP to cAMP. Evidences points to the importance of ADRB2 signaling activation in the skeletal muscle in response to SNS input [152]. Though less recognized than its other functions, the SNS plays an important role in several aspects of muscle metabolism. One of the most important roles is preparing the muscle to deal with emergency situations. Catecholamines stimulate muscle glycogenolysis, decreasing the glucose uptake in the peripheral tissues while increasing glucose uptake in muscle, altering muscle fuel utilization [19]. The results of the study described in Chapter 1 point to a decrease in activity EE after calorie restriction, implicating altered muscle metabolism and suggesting a possible alteration in SNS signaling. This is supported by data from human clinical studies demonstrating that changes in SNS signaling directly relate to weight loss maintenance or weight regain following successful weight loss [9, 140, 141]. Decreased SNS tone has been related to weight regain [140].

c) Potassium inwardly-rectifying channel, subfamily J, member 8 and 11 (KCNJ8 & 11)

KCNJ8 and KCNJ11 are human genes encoding K_{ir}6.1 and K_{ir}6.2 proteins, respectively. These are the subunits of the ATP-gated K^{+} channel. These channels allow potassium to flow into a cell, and are constitutively closed in skeletal muscle. These channels are present in several places including pancreas, cardiac muscle, and skeletal muscle, and their function varies depending on the location. In skeletal muscle, these channels may modulate glucose uptake [56, 114], and also help in energy conservation [34]. The down-regulation of these channels can stimulate energy expenditure, thus countering body
weight gain [5]. Mice with deletion of sarcolemmal KCNJ11 gene are leaner, more thermogenic when at rest and expend more energy during activity, have higher endurance capacity, and are resistant to a high-fat diet [5]. Similarly disruption of these channels promotes increased thermogenesis in muscles [86]. Since manipulation of this gene in muscle has such a notable impact on muscle fuel economy, it may also differ in individuals based on the endogenous differences in fuel use of muscle. Thus, I investigated the difference in expression pattern of these channels across calorie restriction in the animal model which differs based on their intrinsic aerobic capacity.

d) Mediator of RNA polymerase II transcription subunit (MED1, also known as DRIP205 or Trap220)

In humans, this protein is encoded by the MED1 gene [55, 76]. MED1 interacts with numerous receptors and it has been found to play a very important role in adipogenesis [55]. In skeletal muscle, MED1 plays an important role in suppression of energy expenditure as MED1-deficient mice have been found to be glucose tolerant and resistant to obesity, even on a high-fat diet [34].

e) Sarco/endoplasmic reticulum Ca^{2+}- ATPases (SERCA 1 and 2a)

SERCA is present in the sarcoplasmic reticulum (SR) in the muscle cell. It aids in the Ca^{2+} transport from the cytoplasm to the lumen of SR leading to ATP hydrolysis, an energy-consuming process. Two different isoforms of SERCA (SERCA1 and SERCA
2a) are important in muscle work efficiency [11, 126, 158]. These are regulated by sarcolipin and phospholamban to mediate thermogenesis in skeletal muscle [10, 126, 158]. Human studies implicate increased muscle SERCA 2a in the enhanced skeletal muscle work efficiency seen in weight-reduced individuals [11], and uncoupling of SERCA by sarcolipin is thought to be one of the mechanisms underlying skeletal muscle thermogenesis due to the release of extra heat in the process of uncoupling [60]. In this study, I wanted to investigate potential changes in these channels associated with differential suppression of EE seen in association with obesity propensity in rats.

Here, I examined changes in the expression levels of both mRNA and protein of these molecules over calorie restriction and determined if these were impacted by aerobic capacity.

3.2 MATERIALS AND METHODS

3.2.1 Muscle groups used

To examine the molecular pathways affected by calorie restriction I have studied two sets of muscle: (1) quadricep femoris (quad), and (2) medial gastrocnemius (gastroc) (Figure 3.1). These muscle groups contain mixed muscle types, both slow twitch (type 1/oxidative) and fast twitch (type 2/glycolytic) muscle fibers (i.e., they have a mix of oxidative and glycolytic muscle fibers). However quad muscle is predominated by fast twitch muscle fibers and gastroc by slow twitch muscle fibers.
Figure 3.1: Muscle groups used for the study: medial gastrocnemius and quadriceps femoris of rat.
3.2.2 Animals

Male HCR (n=24) and LCR (n=24) rats were used for this study. Three different time points were considered for this experiment: baseline *ad libitum* feeding (HCR; n=8, LCR; n=8), 2-day 50%-calorie restricted rats (HCR; n=8, LCR; n=8), and 21-day 50%-calorie restricted rats (HCR; n=8, LCR; n=8). The two-day time point was added in this experiment to probe for differences between short-term (2-day) and long-term calorie restriction (21-day) in these rats. I selected the two-day time point based on a different study done in our lab which shows an increase in physical activity in 2-day calorie-restricted rats [159]. Initially when calorie restriction is started, rats are in negative energy balance but they do not yet show adaptive thermogenesis at this time, and they show increased rather than decreased physical activity relative to baseline levels [159]. Adding this 2-day time point in the study will show the impact of negative energy balance without adaptive thermogenesis. Rats were euthanized by rapid decapitation at these time points between noon and 1600 EST. Tissues were collected and rapidly frozen in liquid nitrogen, then stored at -80°C. These studies specifically focus on medial gastrocnemius (gastroc) and quadriceps femoris (quad) muscle groups.

3.2.3 RNA extraction and qPCR

Tissue of approximately 50 ug was collected from the frozen skeletal muscle. 1 ml of Tri reagent (Ambion Technologies) was used to homogenize the tissue. The total RNA was extracted using the Ambion ribopure kit. The purity and concentration of the collected RNA was measured using the Nanodrop (ND-1000; Nanodrop Technologies), with an
A260/280 ratio within the range of 1.8-2.1 considered to be acceptable. The RNA, diluted using elution buffer to reach a final concentration of 100 μg/μl (±10), was used to prepare cDNA using reverse transcription polymerase chain reaction (RT-PCR). Master mix was prepared using an Applied Biosystems kit, and PCR conditions corresponding to cycling at 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and holding at 4°C were maintained. The final cDNA concentration after dilution with nuclease-free water was 100μg/μl. This cDNA was used for quantitative PCR (qPCR) for the following molecules: UCP2, UCP3, ADRB2, KCNJ8 and 11, MED1, SERCA1 and SERCA 2a. All qPCR was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Probes were purchased from Taqman and ABI PRISM 7000 (Applied Biosystems). The relative expression was calculated using comparative Ct method (ΔCt) and data are expressed as a percent expression using the HCR baseline (ad libitum fed) as the reference value (defined as 100%).

### 3.2.4 Protein quantification and Western blot analysis

Proteins were extracted from tissue samples using a homogenization buffer (RIPA) containing protease inhibitors. Protein concentrations were measured using a Bradford assay (Biorad DC assay kit). Equal amount of protein was loaded on a SDS PAGE gel (gradient gel) and gel electrophoresis performed at 200 V for 30-40 min, and then the gel was transferred into a PVDF membrane. To prevent non-specific binding of the primary antibody, the membrane was blocked with 5% milk for 40 min-2 hrs at room temperature, and then the membrane was probed with the desired primary antibody and incubated
overnight at 4°C. The blots were washed 3-4 times for 5 min each and the secondary antibody was added the incubated at room temperature for 1-2 hours. After incubation with the secondary antibody, the blots were washed for 40-50 min with buffer (2X15-minutes washes followed by 3X5-minutes washes). Blots where then developed using a chemiluminiscence detector using an Amersham kit (GE Healthcare, UK; 2ml solution A, 50μl solution B). Total protein was used as a standard by staining the membranes with Coomasie Brilliant blue dye [4, 48, 49, 181] followed by a standard destaining procedure.

3.2.5 Statistical analysis

The expression levels were compared to HCR baseline which was considered as 100%. The expression levels of the animals which were above or below 3 standard errors was considered an outlier and not used for the analysis. Univariate analysis in SPSS statistical software was done to analyze the data using a 2X3 analysis of variance (ANOVA) with calorie restriction (3 time points: ad libitum, 2 day and 21 day-calorie restriction) and selected line (HCR, LCR) as the independent variables. Further post-hoc analysis was done using least significant difference test (LSD). A p-value of ≤ 0.05 was considered as significant. If a significant interaction was seen (i.e., if HCR and LCR reacted differently to calorie restriction), a 2-tailed t-test was done in Excel to observe additional differences. The symbols illustrated in Figure 3.2 have been used to show significant differences between HCR and LCR and at each of the time points studied.
| (*) | Significant difference ($p \leq 0.05$) over time compared to their respective baseline |
| #   | Significant difference ($p \leq 0.05$) in 21 days CR compared to their respective 2-day CR |
| *   | Significant difference ($p \leq 0.05$) HCR $\neq$ LCR within the time |
| ___ | Significant difference ($p \leq 0.05$) main effect of calorie restriction |
| ___*| Significant difference ($p \leq 0.05$) in post hoc analysis of main effect over time |
| * beside LCR | Significant difference ($p \leq 0.05$) in main effect of HCR vs LCR. |

**Figure 3.2:** Illustrations of the symbols used in the graphs.
3.3 RESULTS

UCP2 mRNA expression

Medial gastroc: No significant interaction or main effect of line (HCR/LCR) or calorie restriction was observed in the expression of UCP2 in the gastroc (Figure 3.3 A).

Quads: No significant interaction was observed suggesting that HCR and LCR were not differentially affected by calorie restriction. There was a main effect of calorie restriction ($p=0.05$) suggesting that 50% calorie restriction had a significant effect on the UCP2 expression in the quad which increased at 21-day calorie restriction compared to baseline ($p<0.018$) (Figure 3.3 B).
Figure 3.3: Quantitative PCR analysis of UCP2 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: No changes in gastroc (A) was seen. In quads (B), there was a significant main effect of calorie restriction (CR) in high capacity runners (HCR) and low capacity runners (LCR) where the overall expression of UCP2 increased after 21 days of calorie restriction compared to baseline.
UCP2 protein expression

**Medial gastroc:** Main effects of calorie restriction \( (p=0.035) \) and line (HCR vs. LCR, where HCR>LCR, \( p=0.009 \)) were observed (Figure 3.4 A). The overall expression of gastroc UCP2 increased after 2 days \( (p=0.018) \) compared to baseline and decreased after 21 days of calorie restriction compared to 2-day calorie restriction \( (p=0.015) \). There was a significant interaction between HCR and LCR \( (p=0.005) \). HCR significantly decreased its expression after 21-day calorie restriction compared to baseline \( (p=0.006) \), and HCR>LCR \( (p=0.005) \) at baseline but LCR significantly increased expression at 2-day calorie restriction \( (p=0.01) \) compared to its baseline, so at 2-day calorie restriction LCR>HCR \( (p=0.02) \).

**Quads:** There was a significant main effect of calorie restriction \( (p<0.001) \) (Figure 3.4 B). Post-hoc analysis showed that at 21-day calorie restriction, UCP2 protein expression was significantly higher than baseline expression \( (p<0.001) \) and 2-day calorie restriction \( (p<0.001) \). A significant interaction was observed, suggesting that the effect of calorie restriction was different between HCR and LCR \( (p=0.032) \). HCR increased quad UCP2 expression after 21 days of calorie restriction compared to baseline \( (p<0.001) \) and 2 days \( (p<0.001) \), whereas LCR increased quad UCP2 expression at both the 2-day \( (p=0.03) \) and 21-day \( (p=0.01) \) time points compared to baseline levels. LCR had higher expression levels compared to HCR at the 2-day time point \( (p=0.007) \).
Figure 3.4: Protein expression of UCP2 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: In medial gastroc (A) the expression at 2 days of calorie restriction was higher compared to baseline and 21 days of calorie restriction (CR) in high-capacity runners (HCR) and low-capacity runners (LCR), and there was a significant main effect of line and an interaction where HCR > LCR at baseline, but HCR < LCR at 2-day CR. HCR suppressed their expression significantly after 21 days of CR. In quads (B), there was a significant main effect of CR where the overall expression of UCP2 increased after 2 and 21 days of CR compared to baseline, and there was a significant interaction between HCR/LCR and CR.
**UCP3 mRNA expression**

**Medial gastroc:** There was a significant interaction \((p=0.027)\) observed between line (HCR/LCR) and calorie restriction, signifying that gastroc UCP3 reacted differently to the calorie restriction in HCR vs. LCR (Figure 3.5A). There was also a significant main effect of calorie restriction \((p<0.001)\). LSD analysis showed a significant increase in the expression levels of gastroc UCP3 between baseline and 2-day calorie restriction and decrease between 2-day and 21-day calorie restriction \((p<0.001\) for both). Further analysis showed that at baseline, HCR had significantly lower gastroc UCP3 expression than LCR \((p=0.03)\), but had a trend towards having higher expression compared to LCR after 2 days of calorie restriction \((p=0.06)\). HCR \((p=0.001)\) and LCR \((p<0.001)\) significantly increased their UCP3 expression after 2 days of calorie restriction compared to baseline, but both HCR \((p<0.001)\) and LCR \((p<0.001)\) showed suppression of UCP3 after 21 days of calorie restriction compared to 2 days of calorie restriction. Neither HCR nor LCR had any difference in gastroc UCP expression level between baseline and 21 days of calorie restriction.

**Quads:** A significant interaction was observed, suggesting that the effect of calorie restriction was different between HCR and LCR \((p=0.002)\) (Figure 3.5 B). There were significant main effects of both calorie restriction \((p=0.005)\) and line (HCR vs. LCR, where HCR>LCR, \(p=0.01)\), suggesting that calorie restriction affected quad UCP3, and that HCR and LCR significantly differed from one another. Post-hoc analysis showed a
significant increase in expression between baseline and 2-day calorie restriction
\((p=0.001)\), and a significant decrease between 2-day and 21-day calorie restriction
\((p=0.005)\). There were differences in the expression of quad UCP3 in LCR between the 3
time points. HCR showed a significant increase in UCP3 at 2-day calorie restriction
\((p<0.001)\) compared to baseline, but it was followed by a significant decrease in the
expression after 21 days compared to the 2-day expression \((p=0.02)\). At 2-day calorie
restriction, HCR showed higher expression of UCP3 compared to LCR \((p=0.015)\).
Figure 3.5: Quantitative PCR analysis of UCP3 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: There was a significant interaction and main effects of line—high-capacity runners (HCR) and low-capacity runners (LCR) due to calorie restriction (CR) was observed in both muscle types. The expression levels increased after 2 days and decreased after 21 days of CR in both HCR and LCR in gastroc (A). A main effect of line (HCR > LCR) was observed in quads (B), and a significant interaction where only HCR increased expression after 2-day CR and decreased after 21-day CR.
UCP3 mRNA expression

**Medial Gastroc:** A significant main effect of calorie restriction was observed (Figure 3.6 A). Post-hoc analysis showed an increase in gastroc UCP3 expression after 21 days of calorie restriction ($p=0.041$) compared to baseline. However no interaction was observed between HCR/LCR and calorie restriction.

**Quads:** I found no significant main effects of calorie restriction or line, and no interaction (Figure 3.6 B).
Figure 3.6: Protein expression of UCP3 in medial gastrocnemius (gastroc) and quadriiceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) In gastroc there was significant increase in expression after 21 days of calorie restriction (CR) compared to 2-day CR in high-capacity runners (HCR) and low-capacity runners (LCR). (B) No significant differences were seen in quads.
**KCNJ8 mRNA expression**

**Medial gastroc:** A significant interaction ($p=0.034$) was observed showing that gastroc KCNJ8 levels in HCR and LCR were affected differently by calorie restriction (Figure 3.7 A). There was a significant main effect of calorie restriction ($p=0.035$) but not line. Post-hoc analysis showed that there was a significant increase in the expression levels of KCNJ8 in gastroc between 2-day and 21-day calorie restriction ($p=0.035$) and a trend towards decrease in expression between baseline and 2-day calorie restriction ($p=0.052$).

At baseline, medial gastroc expression of KCNJ8 was significantly higher in HCR than in LCR ($p=0.04$). However there was no change in expression seen in the LCR over the course of calorie restriction. HCR showed a significant suppression in KCNJ8 expression after 2-day calorie restriction ($p=0.004$), and a trend toward a suppression after 21-day calorie restriction, compared to baseline ($p=0.052$).

**Quads:** A main effect of calorie restriction ($p=0.002$) was observed suggesting that calorie restriction affected the gene expression of KCNJ8 in quads. Post-hoc analysis showed a significant increase in quad KCNJ8 expression between baseline and 21-day calorie restriction ($p=0.010$), and also between 2-day and 21-day calorie restriction ($p=0.009$). HCR 21-day had lower animal number (n=2) (Figure 3.7 B).

Protein level of KCNJ8 was undetectable in both quad and medial gastroc.
Figure 3.7: Quantitative PCR analysis of KCNJ8 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: A significant interaction between high-capacity runners (HCR) and low-capacity runners (LCR) and main effect of calorie restriction (CR) were observed in gastroc (A). The expression levels increased at 21-day CR compared to 2-day CR. In Quads (B) significant main effect of CR was observed where expression was highest at 21 day CR compared to both baseline and 2 days of CR. HCR 21-day CR (n=2) was not included in the analysis.
KCNJ11 mRNA expression

**Medial gastroc:** No significant interaction was observed (Figure 3.8 A). There was a significant main effect of calorie restriction ($p<0.001$) where gastroc KCNJ11 was significantly lower at both 2-day calorie restriction ($p<0.001$) and at 21-day calorie restriction compared to baseline ($p<0.001$).

**Quads:** Similar to gastroc, no significant interaction was observed on the effects of calorie restriction and line on KCNJ11 expression in quad (Figure 3.8 B). There was a significant main effect of calorie restriction ($p<0.001$). Post-hoc analysis showed significantly greater expression at 21-day calorie restriction compared to baseline ($p<0.001$) and 2-day calorie restriction ($p<0.001$).
Figure 3.8: Quantitative PCR analysis of KCNJ11 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: A main effect of calorie restriction (CR) was observed in both muscle types. The expression levels increased compared to baseline after 21 days of CR in quads (A) but decreased in gastroc (B).
KCNJ11 protein expression

**Medial gastroc:** Significant main effect of calorie restriction ($p=0.044$) was observed where overall expression increased after 2 days of calorie restriction ($p=0.021$) compared to baseline (Figure 3.9 A). However no significant interaction or differences in HCR vs. LCR was observed.

**Quads:** Significant main effect of calorie restriction ($p=0.007$) was observed where overall expression was greatest at 21 day calorie restriction compared to baseline ($p=0.005$) and 2 days of calorie restriction ($p=0.006$) (Figure 3.9 B). However no significant interaction or differences in HCR vs. LCR was observed.
Figure 3.9: Protein expression of KCNJ11 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: A main effect of calorie restriction (CR) was observed in both muscle types. The expression levels increased compared to baseline after 2 days of CR in gastroc (A), and expression levels increased and were highest at 21-day CR in quads (B), compared to both baseline and 2-day CR.
**MED1 mRNA expression**

**Medial gastroc:** A significant interaction ($p=0.002$) was found, but no main effects of calorie restriction or line (HCR/LCR) were observed (Figure 3.10 A). Further analysis showed that the HCR had decreased expression of gastroc MED1 after 2 days of calorie restriction ($p=0.008$), however the expression significantly increased after 21 days of calorie restriction compared to the 2-day time point ($p=0.04$). LCR, on the other hand, significantly decreased their MED1 expression after 21 days compared to 2 days of calorie restriction ($p=0.001$). At the 2-day calorie restriction time point, HCR had significantly lower expression of MED1 than did LCR ($p=0.003$), however after 21 days of calorie restriction HCR showed a greater expression than LCR ($p=0.03$).

**Quads:** A significant interaction was observed ($p=0.002$) (Figure 3.10 B). There were also significant main effects of both calorie restriction ($p=0.004$) and line (HCR vs. LCR; where HCR<LCR, $p=0.024$). There was a significant increase in overall expression between baseline and 2-day calorie restriction ($p=0.003$), and significant decrease between 2-day and 21-day calorie restriction ($p=0.02$). There was no significant difference in the expression of quad MED1 in HCR between the 3 time points. LCR showed a significant increase after 2-day calorie restriction ($p=0.015$), but it was followed by a significant decrease in MED1 expression after 21 days compared to the 2-day expression ($p=0.03$). At the 2-day time point did LCR show greater MED1 expression compared to HCR.
Figure 3.10: Quantitative PCR analysis of MED1 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: Significant interactions between high-capacity runners (HCR) and low-capacity runners (LCR) were observed in both gastroc (A) and quads (B). Main effects of line (HCR vs. LCR) and calorie restriction (CR) were observed only in quads where overall expression increased after 2-day CR compared to baseline and decreased after 21-day compared to 2-day CR.
Expression of MED1 protein

No significant interactions or main effects of line or calorie restriction were observed for MED1 in medial gastroc or quads (Figure 3.11).
Figure 3.11: Protein expression of MED1 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: No significant differences were observed in either gastroc (A) or quads (B).
**SERCA1 mRNA expression**

**Medial gastroc:** No significant interaction or main effects of line or calorie restriction were observed for SERCA 1 in medial gastroc (Figure 3.12 A).

**Quads:** There was a significant main effect of calorie restriction ($p<0.001$). Post-hoc analysis showed a significant increase in the expression between baseline and 2-day calorie restriction ($p<0.001$), and decrease between 2-day and 21-day calorie restriction ($p<0.001$) (Figure 3.12 B). However, there was no significant interaction or main effect of line (HCR/LCR).
Figure 3.12: Quantitative PCR analysis of SERCA1 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) No change was observed in gastroc. (B) A main effect of calorie restriction (CR) was observed in high capacity runners (HCR) and low capacity runners (LCR) in quads. The overall expression levels were higher at 2-day compared to baseline and 21-day CR.
SERCA 1 protein expression

**Medial gastroc:** No significant interaction or main effects of line or calorie restriction were observed for protein expression of SERCA 1 in medial gastroc (Figure 3.13).

**Quads:** There were significant main effects of calorie restriction \((p<0.003)\) and line (HCR vs. LCR; where LCR>HCR, \(p=0.015\)) (Figure 3.13). Post-hoc analysis showed a significant increase in the expression at 2-day calorie restriction \((p<0.001)\) and 21-day calorie restriction \((p=0.001)\) compared to baseline. However, there was no significant interaction between HCR and LCR.
Figure 3.13: Protein expression of SERCA1 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) no significant effects of line or calorie restriction (CR) were seen in gastroc. In quads (B) there was significant effect of CR where there was an increase in overall expression at 2-day and 21-day CR compared to baseline in high-capacity runners (HCR) and low-capacity runners (LCR), and a significant main effect of and line (HCR vs. LCR).
**SERCA 2a mRNA expression**

**Medial gastroc:** There was a significant interaction observed between calorie restriction and HCR/LCR in the expression of SERCA 2a in gastroc ($p<0.001$) (Figure 3.14 A). This suggests that HCR and LCR reacted differently to the restricted diet. Significant main effects of calorie restriction ($p<0.001$) and line (HCR vs. LCR; where LCR>HCR, $p<0.001$) were also observed. Post-hoc analysis showed that gastroc SERCA 2a expression level was significantly higher at both 2 days ($p<0.001$) and 21 days of calorie restriction ($p<0.010$) compared to baseline, and lower at 21-day compared to 2-day CR ($p<0.011$). This effect was mostly due to the difference in gastroc SERCA 2a expression in LCR, which was highest at 2-day calorie restriction compared to both baseline ($p<0.001$) and 21-day calorie restriction ($p<0.001$) expression levels. LCR had significantly greater gastroc expression of SERCA 2a compared to the HCR at 2 days of calorie restriction ($p<0.001$). HCR did not show any significant difference in SERCA 2a expression throughout the course of calorie restriction.

**Quads:** No significant interaction or main effect of line or calorie restriction was observed for SERCA 2a in quads (Figure 3.14 B).
Figure 3.14: Quantitative PCR analysis of SERCA 2a in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) A significant interaction between high-capacity runners (HCR) and low-capacity runners (LCR) and main effects of calorie restriction (CR) and line (HCR vs. LCR) were observed only in gastroc. The expression levels increased at both 2 days and 21 days of CR compared to baseline, levels being highest at 2-day CR, with changes seen only in LCR. (B) No significant effects were seen in quads.
**SERCA 2a protein expression**

**Medial gastroc:** There was a significant interaction observed between calorie restriction and HCR/LCR in the expression of SERCA 2a in gastroc ($p<0.007$) (Figure 3.15 A). A significant main effects of calorie restriction ($p=0.020$) was observed. Gastroc SERCA 2a expression level was significantly higher at 2 days of calorie restriction compared to both baseline ($p=0.025$) and 21 days of calorie restriction ($p=0.029$). This effect was mostly due to the difference in gastroc SERCA 2a expression in LCR, which was highest at 2-day calorie restriction compared to both baseline ($p=0.017$) and 21-day calorie restriction ($p=0.021$) expression levels. LCR had significantly greater gastroc expression of SERCA 2a compared to the HCR at 2 days of calorie restriction ($p=0.04$). HCR did not show any significant differences in SERCA 2a expression throughout the course of calorie restriction.

**Quads:** No significant change in expression was observed (Figure 3.15 B).
Figure 3.15: Protein expression of SERCA 2a in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) A significant interaction between high-capacity runners (HCR) and low-capacity runners (LCR) and a main effect of calorie restriction (CR) were observed only in gastroc. The expression levels were highest at 2-day CR compared to 21-day CR and baseline, with changes seen only in LCR. (B) No significant differences were seen in quads.
ADRB2 mRNA expression

**Medial gastroc:** No significant differences were observed in ADRB2 in the medial gastroc (Figure 3.16 A).

**Quads:** There was a significant main effect of calorie restriction on quad ADBR2 expression ($p=0.018$) (Figure 3.16 B). Post-hoc analysis showed a significant increase in the expression of ABRB2 from baseline to 21-day calorie restriction ($p=0.005$). However, there was no significant interaction or main effect of line observed.
Figure 3.16: Quantitative PCR analysis of ADRB2 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) No significant differences were seen in gastroc. (B) A significant main effect of calorie restriction (CR) was observed in high-capacity runners (HCR) and low-capacity runners (LCR) in quads. The expression levels increased after 21 days of CR compared to baseline.
**ADRB2 protein expression**

**Medial gastroc:** No significant differences were observed in ADRB2 in the medial gastroc (Figure 3.17 A).

**Quads:** There was a significant main effect of calorie restriction on ADBR2 ($p=0.032$) (Figure 3.17 B). Post-hoc analysis showed a significant increase in the expression of ABRB2 from baseline to 2-day calorie restriction ($p=0.013$) and 21-day calorie restriction ($p=0.031$). However, there was no significant interaction or main effect of line observed. However due to various factor there was n=2 in HCR at 2-day calorie restriction.
Figure 3.17: Protein expression of ADRB2 in medial gastrocnemius (gastroc) and quadriceps femoris (quads): Expressed as percentage relative expression based on HCR baseline: (A) No significant differences were seen in gastroc. (B) A significant main effect of calorie restriction (CR) was observed in high-capacity runners (HCR) and low-capacity runners (LCR) only in quads (B). The overall expression levels increased after 2 and 21 days of CR compared to baseline. The HCR 2-day time point was n=2 for quads.
3.4 DISCUSSION

In the first study (Chapter 2), 50% calorie restriction for 21 days induced a greater suppression of non-resting EE in the HCR compared to the LCR. This suggests that HCR were undergoing adaptive thermogenesis to a greater extent than LCR, at least in the non-resting component of EE. Since muscle energetics play a very important role in activity EE, I sought to understand how the different molecules that were important for EE in muscles were affected by calorie restriction. I also wanted to determine if there was any difference in the expression of these molecules between short-term (2 days) and long-term (21 days) calorie restriction because of the difference between activity and non-resting EE under these conditions. This would reveal the most likely mechanisms underlying the differential adaptive thermogenesis in these rats.

UCPs are known to play an important role in lipid handling [27, 70, 147, 149] and gets activated by accumulation of reactive oxygen species (ROS) and its byproducts, thereby protecting against ROS accumulation, diminishing them by creating a proton leak leading to a negative feedback mechanism [29, 109, 110]. Its role in thermogenesis is suggested but is not universally accepted. If muscle UCPs are thermogenic then their expression levels should decrease over calorie restriction, but if lipid handling is their primary role then the expression of UCPs should increase during calorie restriction because of the reliance on fat oxidation under conditions of negative energy balance. Levels of UCP2 and 3 in skeletal muscle have been observed to increase due to starvation in rats [27, 70, 147]. In medial gastroc, although I found no difference in the mRNA expression levels of
UCP2, UCP3 showed significant change over calorie restriction. UCP3 mRNA expression increased significantly after short-term (2-day) calorie restriction in both HCR and LCR. Since the animals were still coping with the negative energy balance due to food restriction, there was an added amount of free fatty acids at 2 days of calorie restriction and thus the increase in UCP3 expression is consistent with its putative role in lipid handling. However, in quad for HCR and in gastroc for both HCR and LCR, UCP3 mRNA expression decreased after long-term (21 days) calorie restriction, suggesting an ongoing adaptation occurring within their systems. In medial gastroc, UCP3 protein expression increased at both 2- and 21-day calorie restriction compared to baseline, supporting its role in lipid handling. UCP2 overall protein expression in gastroc decreased after 21-day compared to 2-day calorie restriction, whereas in quads both UCP mRNA and protein expression increased after 21-day calorie restriction, and UCP protein also higher at 2-day calorie restriction compared to baseline, suggesting a differential adaptation in the muscle type to cope with energy requirements faced during calorie restriction. The differential effects of short-term vs. more severe energy restriction on muscle UCPs support potential roles of these proteins in both lipid handling and adaptation with calorie restriction, including thermogenic roles of UCPs in muscle. Moreover, there were some differences seen between HCR and LCR, specifically the greater suppression of UCP3 only in quads of HCR after 21 days of calorie restriction, suggesting that quads of HCR are adapting differently to the calorie restriction compared to LCR. This is consistent with the fact that HCR had a greater suppression of non-resting EE after 21 days of calorie restriction. Thus, HCRs are undergoing more adaptation in
their muscle UCP just as they had undergone more adaptation in their non-resting EE compared to the LCRs.

KCNJ8 and 11 promote energy conservation as down-regulation of KCNJ11 reduces body weight, increases EE, increases muscle thermogenesis at rest, and decreases muscle efficiency [5]. I saw an overall increase in quad KCNJ8 and KCNJ11 mRNA expression after 21 days of calorie restriction, and to some extent in gastroc KCNJ11, at least between 2-day and 21-day calorie restriction. Protein expression of KCNJ11 increased after 2 day in medial gastroc and both at 2 day and 21 days compared to baseline in quads. Thus the overall increase in expression of these channels with energy restriction supports their general role in energy conservation. This fits with the hypothesis that due to shortage of food supply the expression levels of these proteins were upregulated mainly in the quads of these animals to make muscle more efficient and conserve more energy. However, there might be a muscle fiber type difference in adaptation as I observed the opposite pattern in the medial gastroc, where KCNJ8 decreased after 2 days of calorie restriction (however the overall expression increased after 21 days compared to 2 days of calorie restriction) and overall gene expression of KCNJ11 decreased after 21 days of calorie restriction. The gene expression decrease was reversed at protein levels where there was an increase observed. This discrepancy in mRNA and protein levels could be because at the level of the protein, the energy conserving function becomes more potent where myocytes sense the decreased energy availability caused by food restriction and thus upregulate the translation process. Again, some differences were seen based on differential aerobic capacity. In HCR, gastroc KCNJ8 showed decreased gene
expression at both 2 and 21 days of calorie restriction compared to baseline. Since HCRs are generally more active—even after calorie restriction despite the significant calorie restriction-induced decrease (Chapter 2)—This higher physical activity level may have been influenced the muscle type specific (quads vs. gastroc) decrease in these channels seen in the HCR. Thus, overall this might suggest the potential role of these channels in suppression of activity EE leading to adaptive thermogenesis, however it is not consistent across both muscle types.

MED1 is also an energy conservation molecule and MED1 deficiency causes mice to resist fat gain even when on a high fat diet [34]. Accordingly, I predicted that the overall expression will increase after calorie restriction. I observed an increase in MED1 mRNA expression in quads after 2 days of food restriction, supporting the role of MED 1 in energy conservation. However, MED1 expression was significantly suppressed after 21 days compared to 2 days of calorie restriction, suggesting possible adaptation; this was specifically observed in LCR. In the gastroc, the HCR showed a decrease in MED1 expression at 2-day calorie restriction, however HCR increased and LCR decreased expression after 21 days compared to 2 days of calorie restriction. The increase in MED1 levels in HCR compared to the lower expression at 2-day calorie restriction supports the role of MED 1 in energy conservation and parallels the significant suppression of non-resting EE after 21 days of calorie restriction seen in HCR. This also implicates an increase in energy efficiency in the HCR. No change observed in protein levels of MED1 might be because of untranslated mRNA.
SERCA is thermogenic and therefore its expression would be predicted to decrease with calorie restriction, however the data did not support the hypothesis, as SERCA 1 (mRNA) in quad and SERCA 2a (both mRNA and protein) in the medial gastroc showed increased expression levels after 2 days of calorie restriction compared to the baseline. However there was a decrease in 21-day relative to 2-day SERCA 1 (quad) and SERCA 2a (gastroc) mRNA expression relative to baseline levels. The protein expression of SERCA1 in quads increased at both 2 days and 21 days compared to baseline. This increase is comparable to what is seen in humans, where in association with reduced weight, SERCA levels increase [11]. SERCA1 is found more in type 1 or oxidative muscle fiber and SERCA 2a in type 2 or glycolytic muscle fibers [11]. Thus focusing on each of these in varied muscle fiber type is important to get an overall idea about the effect of calorie restriction on thermogenic SERCA. Inhibitors like phospholamban and sarcolipin lower the ability of SERCA to bind to calcium, releasing more heat in the process [106]. However there are inadequate pharmacological and measurement tools to assess SERCA function in rats. Development of genetic tools will enable further investigation of the thermogenic roles SERCA and the more newly characterized modulators phospholamban and sarcolipin in the future.

Overall mRNA and protein expression of ADRB2 increased after 21-day calorie restriction in quads compared to baseline, as predicted. This is probably due to compensatory mechanisms occurring due to the decreased exposure of the muscle to catecholamines [75, 152, 171, 176].
Some of the expression changes were observed only in mRNA levels or protein levels, not both. Studies have implicated several factors in the variation that exists between mRNA and protein levels [172]. There are many stages between transcription and translation including post-transcriptional modifications (e.g., splicing, polyadenylation, etc.) which govern the stability and rate of translation. Moreover there is difference in rate of transcription and translation depending on the protein. The stability of mRNA and proteins also vary, where proteins are generally more stable than mRNA [172]. There could be post-transcriptional modifications like splicing which would create a different protein. The protein or its function could itself be impacted post-translational modifications. All these factors come to consideration when accounting for the variation between mRNA and protein levels and thus might not always be similarly expressed. However many of the mRNAs and their corresponding protein levels were consistent in a tissue-specific manner, including ADRB2, SERCA, UCPs and KCNJ.

Since rats are nocturnal animals, their normal feeding time is at night. I performed the feeding (both ad libitum and 50% calorie restriction) around noon, with lights-on at 700 EST. This might be expected to create a phase shift in peripheral tissues and change the expression of clock genes resulting in change of energy expenditure, physical activity, and adipose content [65, 138]. Dietary manipulations like a high-fat diet have a limited effect on clock genes in both muscle and liver, however muscle, unlike liver, does not show changes in metabolic genes with a high-fat diet [138]. Time-restricted feeding induces a phase shift in clock genes and metabolic genes in liver but not in the muscle [138]. Moreover, all HCR and LCR, including both control and calorie-restricted rats,
had similar daily stimulation like feeding, handling, and weighing that would be expected
to affect physical activity levels similarly across groups. If there is an effect of phase shift
in the genes due to feeding time, the changes should be similar at baseline and the
calorie-restriction time points. In our previous experience with food-restricted HCR and
LCR, they did not show overt food-anticipatory activity, though further analysis is
pending. Altogether, existing evidence does not support a role of daytime food access in
phase shifting the muscle molecular clock, so this was unlikely to contribute to the
differences I saw or obscure detection of existing changes.

In the gene expression of muscle, I observed a difference between short-term and long-
term energy restriction. Increase in levels of UCP3, KCNJ8 & 11, and MED1 in a
muscle-specific (quad vs. gastroc) fashion after 2 days of calorie restriction is consistent
with their individual functions. However, in many cases the increase observed after 2
days was reversed after the longer-term, 21-day calorie restriction. This is consistent with
the idea that duration or severity of the energetic challenge is important to the behavioral
and physiological adaptations that affect activity EE; with an extended but not short
period of food restriction, the body adapts to resist weight loss by suppressing EE and
increasing muscle efficiency. This phenomenon is also observed in animal behavior with
long-term calorie restriction where there is suppressed physical activity after 21-day
calorie restriction after having a sharp increase after 2-day calorie restriction [159]. The
persistent nature of the long-term effects on muscle might be one of the reasons why it is
difficult to keep losing weight or to sustain the lost weight.
Some of the changes in gene expression I demonstrate are muscle-type specific (i.e., seen in quads or gastroc, but not both), and some are observed only in HCR or LCR. HCR showed an increase in UCP3 (quads) and MED 1 (gastroc) expression followed by a decrease after 21 days, and a decrease in KCNJ 8 in medial gastroc at 2-day calorie restriction. Similarly, changes were observed only in the LCR for MED1 (quads), SERCA 2a (gastroc), where the expression increased after 2 days but decreased after 21 days of calorie restriction. However, with the limited exceptions of the above mentioned changes, there were few differences between HCR and LCR, no single protein or pathway that would clearly explain the large difference observed in non-resting EE adaptive thermogenesis (Chapter 2). However, some changes might have been obscured due to limited statistical power due to small sample size.

These data support the potential roles of UCPs, KCNJ, and MED1 in the differential adaptive thermogenesis observed between HCR and LCR. One or more of the changes observed in the HCR might be responsible for increasing muscle efficiency, thereby suppressing the non-resting EE a considerable level. Thus, from this study we can conclude that the effects of calorie restriction on muscle can be affected by aerobic capacity and especially the length or severity of food restriction, but no one particular protein stands out that may play a singular role in the adaptive thermogenesis induced by calorie restriction.
Chapter 4: Identify brain mechanisms that potentially underlie central modulation of adaptive thermogenesis.

4.1 INTRODUCTION

The hypothalamus plays an important role in control of energy balance homeostasis. Centrally, the hypothalamus modulates hunger, satiety, and physical activity to help maintain energy balance. Evidence also supports a role for the autonomic nervous system, especially the sympathetic nervous system (SNS) \cite{38, 41, 157, 187}, in the calorie restriction-induced suppression of activity EE \cite{165, 192}, and the hypothalamus is one controller of autonomic outflow and its modulation of metabolism \cite{78}. To determine how the changes in hypothalamic receptors and prohormones correspond to changes in EE during short- and long-term calorie restriction, I examined hypothalamic nuclei important in regulation of energy balance and autonomic outflow in rats with and without calorie restriction. Moreover, I compared how these changes differ between HCR and LCR. The nuclei under consideration were the ARC, PVN, LH--specifically the PeFLH which contains the brain’s orexin cell bodies--DMH, and VMH. Each of these nuclei modulates one or more process influencing energy balance, including appetite or satiety, physical activity and EE, or pre-autonomic modulation of peripheral systems.

Within the hypothalamus, as well as in extra-hypothalamic regions of the brain, the brain melanocortin system is particularly important in the control of food intake and maintaining homeostasis \cite{62, 187}. Activation of melanocortin receptors opposes positive
energy balance through inducing satiety, physical activity, and autonomic outflow, thus differential activation or suppression of this system during energy restriction could impact the magnitude of adaptive thermogenesis. Investigating the melanocortin system to find its possible role in adaptive thermogenesis will be useful in lending insight to the treatment of obesity, for example as a supplementary strategy during dieting [107, 108]. In this study I examined alterations in brain MCR and AgRP/NPY expression in hypothalamic regions across calorie restriction.

4.1.1 Importance of hypothalamic regions in energy balance

ARC: The arcuate nucleus is found at the base of the hypothalamus, and participates in several neuroendocrine and behavioral processes, including energy balance regulation (Figure 4.1). It is the seat of the POMC/CART and NPY/AgRP system described above [161]. Hence is an extremely important region as changes in its function during energy restriction will have an impact on the melanocortin systems thus affecting energy homeostasis. The arcuate sends projections to other regions of the hypothalamus including the PVN, VMH, DMH, and PeFLH, all of which are important in energy balance. Lesions of the ARC result in obesity [135].

PVN: The PVN receives projections from the ARC [20], and is a region important in satiety, physical activity, energy expenditure, and autonomic control (Figure 4.2). Lesions of this part of the brain cause hyperphagia [42, 97]. MCR antagonists in PVN stimulate food intake [79] whereas agonist microinjections into the PVN causes an
increase in physical activity leading to an increase in energy expenditure [153]. The PVN is complex in structure and function and has sub-regions containing characteristic neurotransmitters and peptides. The overall activity of the PVN and its peptides promotes negative energy balance through actions on feeding behavior (increase satiety), energy expenditure, and increased in physical activity and SNS outflow [1, 73].

**PeFLH:** The lateral hypothalamus (LH) receives projections from the ARC and contains melanocortin receptors [20] (Figure 4.4). The LH was once considered the “the feeding center,” one-half of the ”dual-center hypothesis [80].” This was due to the fact that lesions in LH lead to weight loss due to reduction in feeding [17, 118]. Today the view of the role of the LH in energy balance is more complex and is focused on the actions of specific neuropeptides. For example, the PeFLH, contains cell bodies that synthesize the neuropeptide orexin [117], which increases food intake, physical activity, and autonomic outflow [50, 124, 131, 133]. This region receives projections from the ARC, and ablation of orexin neurons leads to narcolepsy and obesity due to hyperphagia in mice [69]. Orexin has been found to decrease body weight by increasing spontaneous physical activity thereby increasing the overall EE [88, 117, 131, 133]. Other neuropeptides including neurotensin and melanin-concentrating hormone also plays roles in modulating energy balance [24].

**Ventromedial hypothalamus (VMH):** The VMH, also referred to as the VMN (ventromedial nucleus), is present on either side of median eminence close to arcuate, PVN, DMN, and PeFLH (Figure 4.3). It receives numerous projections from the ARC.
and is linked to the modulation of food intake. Numerous lesion studies of this region observed weight gain due to hyperphagia [3, 162], earning it the moniker “the satiety center” for some time during the age of the “dual-center hypothesis [80].” Nowadays, the VMH is recognized to be important in autonomic outflow and behaviors linked to energy balance. It is also critical to female reproductive behavior in rodents [146] and defensive behaviors in animals [90, 156, 175]. Data from our lab have implicated the VMH, specifically actions of MCR in the VMH, in the modulation of SNS outflow to skeletal muscle. This reinforces other evidence pointing to the importance of the VMH in the modulation of peripheral metabolism and fuel uptake and use [152, 166, 167].

**Dorsomedial hypothalamus (DMH):** The DMH, also referred to as dorsomedial nucleus (DMN), is an important hypothalamic region in the study of energy balance (Figure 4.5). It receives projections from the ARC [20] and has receptors for both melanocortins and NPY. Lesion studies have observed hypophagia along with stunted linear growth [16]. The DMH has been implicated in the control of autonomic outflow modulating BAT thermogenesis [90, 122]. Some thermogenic actions which are driven by leptin are also shown to be directly or indirectly modulated by DMH neurons [44].
Figure 4.1: Coronal section of rat brain showing the arcuate region of the hypothalamus (highlighted in red). Illustration from the *Rat Brain in Stereotaxic Coordinates* (Watson) with copyright permission from Elsevier Limited.
Figure 4.2: Coronal section of rat brain showing the paraventricular nucleus (PVN) (highlighted in red). Illustration from the *Rat Brain in Stereotaxic Coordinates* (Watson) with copyright permission from Elsevier Limited
Figure 4.3: Coronal section of rat brain showing the ventromedial nucleus (VMN)/ventromedial nucleus of hypothalamus (VMH) highlighted by red boundaries on either side of median eminence. Illustration from the *Rat Brain in Stereotaxic Coordinates* (Watson) with copyright permission from Elsevier Limited
Figure 4.4: Coronal section of rat brain showing the periforniocal region of lateral hypothalamus (PeFLH) highlighted by a red boundary on either side of median eminence. Illustration from the *Rat Brain in Stereotaxic Coordinates* (Watson) with copyright permission from Elsevier Limited
Figure 4.5: Coronal section of rat brain showing the dorsomedial nucleus (DMN)/dorsomedial nucleus of hypothalamus (DMH) highlighted by a red boundary on either side of median eminence. Illustration from the *Rat Brain in Stereotaxic Coordinates* (Watson) with copyright permission from Elsevier Limited
In this study I examined the mRNA expression of the peptides and receptors, focusing on the brain melanocortin system, in rats during short and long-term calorie restriction. The goal was to identify likely mediators the differential response in non-resting EE in HCR and LCR when subjected to calorie restriction.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male HCR (n=24) and LCR (n=24) rats were used for this study, divided into three different time points: baseline (HCR; n=8, LCR; n=8), 2-day 50%-calorie restricted rats (HCR; n=8, LCR; n=8), and 21-day 50%-calorie restricted rats (HCR; n=8, LCR; n=8). Rats were housed individually, maintained on a 12:12 hour dark and light cycle, with the light cycle starting at 700 EST. Rodent chow (5P00 MRH 3000, T.R. Last Co. Inc) and ad libitum water were provided to each rat. The ad libitum food intake was measured for each rat for 7 days, feeding them at noon each day (±1 hour). After 7 days, 50% food intake was calculated for each rat using their respective baseline food intake data. The 50% food was provided to each of them for the period of calorie restriction (2-day and 21-day). Rats were euthanized by rapid decapitation at these time points between 1200 and 1600 EST. Brains were collected rapidly and frozen with isopentane cooled in dry ice, then stored at -80°C. All studies were conducted according to the rules and approval of the Kent State University IACUC.
4.2.2 Cryostat & Brain Micropunches

Frozen brain tissue was sectioned using a cryostat (Leica Biosystem). The sectioning temperature was maintained at -20°C; 50μm sections were cut and mounted on plain glass slides. The slides were stored at -80°C for micropunching. The areas of interest were the ARC, PVN, PeFLH, VMH, and DMH; these were punched using micropunchers ranging between 0.5 mm to 2 mm. Alternate sections were used to collect tissues for RNA and proteins. The tissue was kept frozen on dry ice throughout the processes.

4.2.3 RNA extraction and qPCR

Tissues collected from micropunching were homogenized using TRI reagent (Ambion Technologies). An Ambion ribopure kit was used to extract the total RNA from the homogenized tissue. Nanodrop (ND-1000; Nanodrop Technologies) was used to measure the purity and concentration of the RNA. For purity, A260/280 ratio within the range of 1.8-2.1 is generally considered to be acceptable, however for this kit purity values different from the aforementioned range are also acceptable. This RNA was used to prepare cDNA. An Applied Biosystems kit was used to prepare the PCR master mixture, and PCR conditions corresponding to cycling at 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes, and holding at 4°C were used. The cDNA obtained was used for quantitative PCR (qPCR) for the molecules listed. All the qPCR were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control, using probes from Taqman and ABI PRISM 7000 (Applied Biosystems). The relative expression was
calculated using comparative Ct method (ΔCt) and data are expressed as a percent expression using the HCR baseline as the reference value (defined as 100%).

4.2.4 Data analysis

The expression levels were compared to HCR baseline which was considered as 100%. The expressions levels of the animals which were above or below 2 times standard error of the mean was considered an outlier and not used for the analysis. Univariate analysis in SPSS statistical software was done to analyze the data using a 2X3 ANOVA with calorie restriction (ad libitum, 2-day and 21-day calorie restriction) and selected line (HCR, LCR) as the independent variables. Further post-hoc analysis was done using the least significant difference (LSD) test. A p-value of ≤0.05 was considered as significant. If a significant interaction was seen (i.e., if HCR and LCR reacted differently to calorie restriction) a 2-tailed t-test was done to probe for additional differences. The symbols illustrated in Figure 4.6 have been used to show significant differences between HCR, LCR and at each of the time points studied.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>(*)</td>
<td>Significant difference ($p \leq 0.05$) over time compared to their respective baseline</td>
</tr>
<tr>
<td>#</td>
<td>Significant difference ($p \leq 0.05$) in 21 days CR compared to their respective 2day CR</td>
</tr>
<tr>
<td>*</td>
<td>Significant difference ($p \leq 0.05$) HCR ≠ LCR within the time</td>
</tr>
<tr>
<td></td>
<td>Significant difference ($p \leq 0.05$) main effect of calorie restriction</td>
</tr>
<tr>
<td></td>
<td>Significant difference ($p \leq 0.05$) in post hoc analysis of main effect over time</td>
</tr>
<tr>
<td>* beside LCR</td>
<td>Significant difference ($p \leq 0.05$) in main effect of HCR vs LCR</td>
</tr>
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**Figure 4.6: Illustrations of the symbols used in the graphs.**
4.3 RESULTS

Five hypothalamic regions (ARC, PVN, VMH, DMH, and PeFLH) were tested to determine the effect of calorie restriction on the POMC, MC3R, MC4R, MC5R, NPY, and AgRP in HCR and LCR.

**mRNA expression analysis**

**MC3R:** MC3R mRNA expression varied significantly in the PVN of the hypothalamus. Significant differences were observed in the interaction between line (HCR, LCR) and calorie restriction (baseline, 2-day, and 21-day calorie restriction; \( p=0.004 \)), indicating that calorie restriction affected the two groups differently (Figure 4.7). A significant main effect of calorie restriction \( (p=0.038) \) was also observed on MC3R in the PVN, but not a main effect of line (HCR/LCR). Post-hoc analysis in SPSS showed a significant difference between 2-day calorie restriction and 21-day calorie restriction \( (p=0.031) \). In HCR, I found a significant increase in MC3R expression between baseline and 2-day calorie restriction, and a significant decrease between 2-day and 21-day calorie restriction \( (p=0.03 \) and 0.0009, respectively). Moreover, in HCR there was a significant decrease \( (p=0.002) \) in MC3R expression after 21-day calorie restriction compared to baseline. PVN MC3R expression in the LCR did not change over time, and whereas HCR and LCR did not differ at baseline and 2-day calorie restriction, PVN MC3R expression in HCR was significantly lower than LCR at 21-day calorie restriction \( (p=0.0019) \). In short, HCR PVN MC3R changed over calorie restriction, increasing after 2 days and decreasing after 21 days, but the LCR showed no change.
No significant interactions or main effects were observed between HCR/LCR or over calorie restriction in MC3R expression in any other brain regions (Figure 4.8).
Figure 4.7: Quantitative PCR analysis of melanocortin receptor 3 (MC3R) in paraventricular nucleus (PVN): Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. A significant interaction was found signifying that calorie restriction (CR) affected high-capacity runners (HCR) and low-capacity runners (LCR) differently. HCR significantly decreased their expression after 21-day calorie restriction after an initial increase of expression after 2-day calorie restriction.
Figure 4.8: Quantitative PCR analysis of melanocortin receptor 3 (MC3R) in: (A) arcuate (ARC), (B) dorsomedial hypothalamus (DMH), (C) ventromedial hypothalamus (VMH), (D) perifornical region of lateral hypothalamus (PeFLF):
Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. No significant differences were observed between high-capacity runners (HCR) vs. low-capacity runners (LCR).
MC4R: Significant differences in MC4R expression over calorie restriction were observed only in the VMH (Figure 4.9). A significant interaction was observed between line (HCR/LCR) and calorie restriction ($p=0.015$) indicating that the impact of calorie restriction in MC4R expression in the VMH differed between HCR and LCR. A significant main effect of calorie restriction ($p=0.041$) was observed in the expression of MC4R in the VMH, and post-hoc analysis revealed a significant decrease in MC4R expression over calorie restriction after 2-day calorie restriction compared to baseline, and an increase in expression after 21-day calorie restriction compared to 2-day ($p=0.039$ and 0.042, respectively). HCR and LCR differed in expression at baseline ($p=0.01$) but not at any other time point. No significant interactions or differences were observed between HCR/LCR and calorie restriction in MC4R expression in any other brain regions (Figure 4.10). Besides the difference at baseline, the most salient effect is the increase in VMH MC4R between short-term and long-term calorie restricted rats.
Figure 4.9: Quantitative PCR analysis of melanocortin receptor 4 (MC3R) in ventromedial hypothalamus (VMH): Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain. The significant interaction suggests that high-capacity runners (HCR) and low-capacity runners (LCR) reacted differently to calorie restriction (CR). At baseline, a significant difference was observed between HCR and LCR. A significant decrease in expression was observed in LCR after 2-day calorie restriction compared to its baseline.
Figure 4.10: Quantitative PCR analysis of melanocortin receptor 4 (MC4R) in: (A) arcuate (ARC), (B) dorsomedial hypothalamus (DMH), (C) perifornical region of lateral hypothalamus (PeFLF), (D) paraventricular nucleus (PVN): Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. No significant differences were observed between high-capacity runners (HCR) vs. low-capacity runners (LCR) in any brain regions.
**MC5R:** A significant difference in MC5R expression was observed only in the ARC, where a trend towards significant interaction was observed \((p=0.051)\), however LCR baseline was \((n=2)\), and the ‘almost’ interaction was gone once it was taken out (Figure 4.11).
Figure 4.11: Quantitative PCR analysis of melanocortin receptor 5 (MC5R) in the arcuate nucleus (ARC): Expressed as percentage relative expression based on HCR baseline; in tissue obtained from micropunched brain samples, a trend towards significant interaction between high-capacity runners (HCR) vs. low-capacity runners (LCR) was observed. LCR baseline was n=2 and when this group was taken out of the analysis, the trend towards an interaction was no longer seen.
Figure 4.12: Quantitative PCR analysis of melanocortin receptor 5 (MC5R) in: (A) dorsomedial hypothalamus (DMH), (B) ventromedial hypothalamus (VMH), (C) perifornical region of lateral hypothalamus (PeFLH), (D) paraventricular nucleus (PVN). Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. No significant difference was observed between high-capacity runners (HCR) vs. low-capacity runners (LCR) in any brain regions.
**POMC in ARC:** There was a main effect of line on ARC POMC expression, where HCR > LCR ($p=0.005$) (Figure 4.13). No main effect of calorie restriction on POMC expression was found, and no interaction was observed. In short, HCR showed more POMC expression compared to LCR in the ARC over the course of calorie restriction.
Figure 4.13: Quantitative PCR analysis of Proopiomelanocortin (POMC) in the arcuate nucleus (ARC): Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. A significant main effect of line (high-capacity runners vs. low-capacity runners; HCR vs. LCR) was observed where HCR showed significantly higher POMC expression in the ARC, with no significant effect of calorie restriction (CR).
**NPY and AgRP in ARC**

The ARC is the major region for integration of central and peripheral signals related to energy balance within the hypothalamus. It contains receptors for hormones (e.g., leptin, ghrelin), and the actions of these hormones on ARC neurons impact the activation or inhibition of the orexigenic POMC/CART neurons and anorexigenic NPY/AgRP neurons. NPY and AgRP is orexigenic and promotes food intake and decreases energy expenditure. Therefore I examined how the expression pattern of these peptides changes with calorie restriction. I examined potential changes in these peptides in HCR and LCR over calorie restriction.

**NPY**: No significant interaction or main effects of calorie restriction or line (HCR vs. LCR) were observed in the expression of NPY in the ARC over the period of calorie restriction (Figure 4.14).

**AgRP**: AgRP expression showed significant main effects of line (HCR > LCR, \( p=0.041 \)) and calorie restriction (baseline, 2-day and 21-day calorie restriction; \( p=0.031 \)), where AgRP increased after 21-day calorie restriction compared to baseline (Figure 4.15). However, no difference in the response of HCR and LCR to calorie restriction was noted.
Figure 4.14: Quantitative PCR analysis of neuropeptide Y (NPY) in the arcuate (ARC). Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. No significant difference was observed between high-capacity runners (HCR) vs. low-capacity runners (LCR) during calorie restriction (CR).
Figure 4.15: Quantitative PCR analysis of agouti related peptide (AgRP) in the arcuate (ARC): Expressed as percentage relative expression based on HCR baseline; tissue obtained through micropunched brain samples. Significant main effects of line--between high-capacity runners (HCR) vs. low-capacity runners (LCR)--and calorie restriction (CR) were observed.
4.4 DISCUSSION

My previous studies (Chapter 2) indicated that calorie restriction induced adaptive thermogenesis and suppressed physical activity and activity EE, and that these responses varied depending on intrinsic aerobic capacity. There were also some changes observed in muscle molecular pathways important for EE and this also varied between long- and short-term calorie restriction, with some differential response seen between HCR and LCR. The behavioral and autonomic responses to changes in energetic demand are modulated by the hypothalamus. Therefore, in this study I examined changes in the brain melanocortin system with short- and long-term calorie restriction to determine likely substrates for the adaptive thermogenesis and suppressed physical activity seen during calorie restriction in HCR and LCR. I observed minimal changes in the brain melanocortin system during calorie restriction.

First, I observed that in HCR, the expression of MC3R in the PVN was elevated at 2-day calorie restriction; however HCR showed decreased PVN MC3R over 21 days of calorie restriction. Individually, both the PVN and MC3R have been shown to have a role in energy homeostasis including promoting energy expenditure and physical activity [26, 73, 153]. Microinjections of a mixed melanocortin receptor agonist in PVN has been shown to enhance spontaneous physical activity to a greater extent in HCR [153]. Thus, the change in PVN MC3R expression over calorie restriction in HCR parallels their suppression in physical activity after 21-day calorie restriction (Chapter 2, Figures 2.4, 2.11, 2.20). LCR showed no change in expression of MC3R in PVN, just as they showed
less suppression of physical activity and activity EE (non-resting EE) over the course of 21-day calorie restriction (Chapter 2). Thus the effect of calorie restriction on both the receptor expression and physical activity suggests a relationship between MC3R in the PVN and physical activity levels. For future investigations we could focus more on how the combination of MC3R and PVN is related to activity EE and adaptive thermogenesis.

Second, I also found a significant difference in the effect of calorie restriction on MC4R receptor in the VMH in HCR vs. LCR at baseline, where the most salient effects were a differential baseline expression, and the overall increase in VMH MC4R from 2 days to 21 days of calorie restriction. Both VMH and MC4R are linked to satiety and increased autonomic outflow. The changes in this receptor were mainly observed in LCR where the expression decreased at 2-day calorie restriction. Thus the changes I see could be linked to appetite or to autonomic outflow. For example, the decrease in MCH MC4R expression at 2-day calorie restriction parallels the increased appetite at this time. Since the LCR showed a short-term and not a long-term change in expression, VMH MC4R expression pattern parallels the pattern of adaptive thermogenesis, where LCR may not be undergoing adaptive thermogenesis to the same extent as HCR.

Third, I also observed a trend in changes of MC5R in the ARC, where the HCR and LCR showed a trend towards a difference in the effect of calorie restriction. Again, this is consistent with our previous findings on MC5R and calorie restriction, where MC5R
agonists promote physical activity and their antagonists suppress it [155], and physical activity is elevated at 2-day calorie restriction but gradually suppressed thereafter [159].

The melanocortin peptides that act as ligands of melanocortin receptors are cleaved form the POMC precursor. POMC null mice show increased obesity and does not increase their spontaneous physical activity when on a high fat diet [169]. In this study ARC POMC expression was significantly higher in HCR, but calorie restriction did not significantly affect this. Elevated POMC is consistent with the fact that HCR has higher physical activity and EE throughout the restricted feeding compared to the LCR. Melanocortin peptides derived from POMC are anorexigenic, thus the HCRs having more POMC is consistent with the phenotypic difference that exists between lean HCR and obese LCR.

While there was no significant change in NPY expression, expression of the appetite-related neuropeptide AgRP changed as predicted in the arcuate, with an overall increase after 21 days of calorie restriction and a trend toward a greater expression in HCR compared to LCR at 21 days. Since AgRP promotes appetite, it increases in response to cues that promote hunger [89]. In mice, activation of AgRP leads to food intake [89], and expression of AgRP increases during fasting, even independent of insulin or leptin levels [115]. Ablation or loss of signaling from AgRP leads to starvation [188, 189]. Given the prominent role of AgRP in appetite, EE, and physical activity [89], and the increase in AgRP during energy restriction [89, 115, 174], this peptide is likely to play an important
role in adaptive thermogenesis. AgRP acts as an inverse agonist on melanocortin receptors, increasing AgRP release during energy restriction [123, 185]. This increase could potentially be responsible for suppressed activity and activity EE seen during calorie restriction.

There was a marked difference observed in the expression pattern of some receptor in region-specific manner between short-term (2-day) and long-term (21-day) calorie restriction. Short-term fasting and calorie restriction induce a seemingly paradoxical increase in physical activity which has been attributed to a foraging response [132]. Furthermore, suppression of MC3R expression along with the reduction in non-resting EE seen in HCR suggest that in long-term calorie restriction the body becomes more efficient and expends less energy, lowering the EE thus hindering desired weight loss. This might help the HCR to cope with reduced food by lowering the physical activity helping them to survive during food shortage. Thus the changes observed in this study in the brain MC system may promote the adaptations observed in the HCR and LCR. It is crucial to differentiate between short-term and long-term changes in these molecules because of the fundamentally different functions that short- vs. long-term calorie restriction has on physical activity and on adaptive thermogenesis.

The statistical power of this study may have been diminished because of the low number of samples per group in some cases. For some of the animals, little tissue obtained and/or the quality of mRNA extracted was below the acceptable limit, hence those samples were
excluded from the analysis. The resulting lack of statistical power and elevated variance might have contributed to the lack of detection of some of the effects found by previous investigators of the lab and hindered the detection of potential effects of calorie restriction.

This study identified the potential mechanisms underlying suppressed physical activity observed during calorie restriction including the changes seen in MCR3, 4, 5 and AgRP in region-specific manner. AgRP appears more promising—if AgRP is increased during calorie restriction, it would be predicted to increase appetite and decrease physical activity and EE. Overall, these changes serve to counteract energy shortage by inducing adaptive thermogenesis and suppressing physical activity, promoting energy conservation during food shortages.
Chapter 5: General Discussion

Obesity, a growing problem in today’s society, is the result of a combination of numerous genetic, behavioral and environmental factors. A modernized Western lifestyle and diet makes weight maintenance a difficult task. Moreover there are individual differences in the ability of a person to lose or gain weight. When on a diet, some people are more resistant to weight loss than others, and some have a tough time maintaining the lost weight. This is partly because of adaptive thermogenesis which lowers the energy expenditure of the body beyond what could be predicted by the lost weight [142]. This makes it harder to create a negative energy balance which is essential for losing weight. NEAT plays a very important role in a person’s ability to lose or gain weight. A person with higher NEAT is more resistant to weight gain and they tend to lose weight easily than their low-NEAT counterparts [98, 99]. The energy expenditure though NEAT comprises non-resting energy expenditure which is about 80-85% of NEAT, with the remaining 10-15% EE due to the TEF (Chapter 1, Figure 1.2)

In my studies, I worked with the hypothesis that variations in adaptive thermogenesis are mainly due to differences in non-resting or activity EE, and that the differential suppression of non-resting EE is accompanied by some parallel changes in muscle energetic markers and some alterations in the molecular signaling important in energy homeostasis situated in the hypothalamus of the brain, focusing in the melanocortin system. I used the rat model system for leanness and obesity—HCR and the LCR—to
capture individual differences in EE that are intrinsic to the high- and low-capacity phenotypes.

I found that 21-day calorie restriction suppresses the resting EE to a similar extent in both HCR and LCR. However the major difference between phenotypes is observed in the non-resting EE, which is suppressed only in the HCR. Moreover physical activity is also generally higher in the HCR at both baseline and after 21-day calorie restriction, and in the majority of the cases only HCR suffered suppression in their physical activity. This is also observed in the human studies where the first 10% reduction of body weight in obese and lean subjects is accompanied by a equal suppression of resting but a greater suppression in non-resting EE resulting due to changes in muscle efficiency [11, 96, 143, 144].

In humans, obese individuals suffer a greater suppression in their non-resting EE with energy restriction, making them a thrifty phenotype, but show no differences at baseline [137]. In my study, the HCRs are generally leaner and more active than their obese counterparts, LCR. During food restriction, hypothetically to survive the shortage of food, HCR suppressed their non-resting EE to greater extent. In the human studies, those who decreased non-resting EE more were thriftier, but lean and obese people started at more or less the same baseline. But in my animals, the HCR and LCR had decidedly different baseline non-resting EE. HCR non-resting EE was elevated because of high physical activity and low economy of activity. But the adaptive thermogenesis brings
down HCRs’ non-resting EE, physical activity, and presumably locomotor efficiency to the level of LCR. From this we could further hypothesize that LCRs’ non-resting EE is essentially at a “pre-suppressed” level all the time—having a lower baseline physical activity and non-resting EE—which would make them better fit for a food-scarce environment, because they would not need time to adapt to low energy availability like the HCR do.

Non-resting EE differentially was suppressed during calorie restriction in HCR and LCR at least partly because of the differences in physical activity levels. Locomotor efficiency or skeletal muscle work efficiency at low or moderate levels of activity was not measured in this study. However, I ran a VO$_{2\text{max}}$ treadmill test, and running efficiency was assessed. In males, the VO$_{2\text{max}}$ was suppressed in the HCR and LCR without any differences in their capability to run—the total distance run or top speed reached. HCR always had a greater capacity to run (with respect to both attaining higher speed and distance covered) and had higher VO$_{2\text{max}}$ both before and after calorie restriction, consistent with their phenotype. In all rats, the suppression of VO$_{2\text{max}}$ with calorie restriction suggests an increase in locomotor efficiency during running, occurring without affecting their endurance. The efficiency changes because the skeletal muscle uses less energy to do the same amount of work (even after changes in body weight and composition were accounted for), that is to say there was a change in skeletal muscle work efficiency. This is also seen in humans, where their muscle efficiency increases after weight loss and decreases after gain weight [96, 144]. HCRs are making themselves more efficient.
causing a greater suppression of their non-resting EE and physical activity as a mode of survival during the food shortage.

There were some accompanying changes in the muscle molecular pathways, where the overall expression levels of some UCPs at the level of the protein (UCP2 and UCP3 in medial gastroc) and some at gene expression levels (UCP3 in both medial and quads) revealed an increase at 2-day calorie restriction and then a decrease after 21 days of calorie restriction, suggesting a difference in adaptation between short- and long-term calorie restriction. Protein and gene expression levels of UCP2 in quads increased after 21 days, consistent with its role in lipid handling. The increase in the levels of UCPs is observed in weight-reduced humans [147, 149]. This is mainly due to the primary function of the UCPs in muscle, which is lipid handling, and due to the presence of increased free fatty acid uptake from circulation during weight loss when on a restricted diet. The decrease after 21 days might be to reduce the energy cost involved in the production of these proteins. This may also suggest a possible dual function for this protein, depending on the metabolic demands placed on the myocytes. The increase at 2-day and decrease at 21-day calorie restriction seen in gene expression of UCP3 in the quads was observed only in the HCR. This suggests an additional muscle fiber specific adaptation going on in the HCR, which might be responsible for increased efficiency of their muscle by lowering the cost involved in production of the UCPs.
SERCA is a thermogenic molecule, however protein and gene expression levels of SERCA 2a in medial gastroc increased after 2-day calorie restriction, and the gene expression also increased at 21-day calorie restriction compared to baseline. However SERCA 2a decreased after 21-day compared to 2-day restriction where the expression level mainly decreased in the LCR. Gene expression level of SERCA 1 in quads was observed to increase, not decrease, after 2 days of calorie restriction, and then decrease after 21 days. Protein expression of SERCA 1 in quads, however, increased at both 2-day and 21-day calorie restriction. This increase after 2-day and 21-day calorie restriction parallels the increase seen in humans that could maintain lost weight [11]. In some of the cases there are changes occurring after 2 days which resulted in the observed reduction in the levels of SERCA after 21-day calorie restriction, where suppression is seen only in the LCR. Thus there is again a differential adaptation occurring between HCR vs. LCR muscle which might contribute to the variations in their adaptive thermogenesis. The changes seen in SERCA 1 levels were observed only in quad, and SERCA 2a only in the medial gastroc. Hence there are muscle-specific changes occurring for these molecules.

For $K_{\text{ATP}}$ channels, KCNJ11 (Kir6.2) gene expression levels increased in quad and decreased in medial gastroc after 21 days of calorie restriction, also showing a tissue-specific adaptation for the gene expression. However protein expression of KCNJ11 showed an increase in both muscle types. KCNJ8 (Kir6.1) expression increased after 21 days compared to baseline and 2-day calorie restriction in quad and compared to 2-day calorie restriction in medial gastroc. The differential increase in the two muscle types shows muscle-specific adaptations to calorie restriction. The increase in KCNJ8 and 11 due to calorie restriction in most of the cases is consistent with increased energy
conservation. Human data show that weight loss leads to an increase in skeletal muscle work efficiency [11, 67]. Thus the changes in the muscle molecular pathways with some differences seen in HCR vs. LCR along with some muscle type-specific changes might partly underlie for the differential adaptive thermogenesis observed in HCR and LCR. No single muscle molecule is responsible for modulating efficiency in these HCR and LCR rats.

The brain melanocortin system is important in modulating response to positive and negative energy balance. The autonomic outflow, including to skeletal muscle, helps modulate energy balance [166, 167]. In the brain melanocortin system I saw a suppression of MC3R gene expression in the PVN, but only in HCR. Microinjection of a mixed melanocortin receptor agonist in PVN has been shown to enhance spontaneous physical activity to a greater extent in HCR than in LCR [153, 155]. The change in PVN MC3R expression over calorie restriction in HCR seen here parallels the rats’ suppression in physical activity after 21-day calorie restriction (Chapter 2, Figures 2.4, 2.11, 2.20). LCR showed no change in expression of MC3R in PVN, just as they showed no or less suppression of physical activity and activity EE (non rests EE) over the course of 21-day calorie restriction (Chapter 2). Gene expression of MC4R receptor in the VMH showed a significant difference in HCR vs. LCR at baseline, and there was an overall increase in VMH MC4R from 2 days to 21 days of calorie restriction. Both VMH and MC4R are linked to satiety and increased autonomic outflow, and the changes in this receptor were mainly observed in LCR where the expression decreased at 2-day calorie restriction. The decrease in MC4R expression at 2-day calorie restriction parallels the
increased appetite at this time due to shortage of food. Gene expression of MC5R in the ARC for HCR showed increased expression after short-term calorie restriction with no changes seen in LCR. This is consistent with our previous findings on MC5R, where MC5R agonists promote physical activity and their antagonists suppress it [155], and physical activity is elevated at 2-day calorie restriction but gradually suppressed thereafter [159].

The gene expression of the anorexigenic peptide POMC in the ARC was significantly higher in HCR compared to LCR, consistent with their higher physical activity and phenotypic differences. POMC null mice show increased obesity and do not increase their spontaneous physical activity when on a high-fat diet [169]. Gene expression of the appetite-related neuropeptide AgRP changed as predicted in the arcuate, with an overall increase after 21 days of calorie restriction and a trend toward a greater expression in HCR compared to LCR at 21 days. Because of the prominent role of AgRP in appetite, EE, and physical activity [89], and the increase in AgRP during energy restriction [89, 115, 174], this peptide is likely to play an important role in adaptive thermogenesis. This increase could potentially be responsible for suppressed physical activity and activity EE seen during calorie restriction.

There was a marked difference observed in the expression pattern of some receptors in region-specific manner between short-term (2-day) and long-term (21-day) calorie restriction both in brain and skeletal muscle. Short-term fasting and calorie restriction
induces a seemingly paradoxical increase in physical activity which has been attributed to a foraging response [132], and melanocortins may play a role in this.

Along with the greater suppression of non-resting EE, there are many changes seen only in the HCR which point towards an increase in energy efficiency in these rats. Thus during calorie restriction they not only suppress their physical activity and non-resting energy expenditure, they exhibit some exclusive changes in their muscle molecular energetics and brain melanocortin system which parallel suppression in spontaneous physical activity and non-resting EE, potentially contributing to their differentia adaptation to food shortage.

Thus in conclusion these results suggest that differential adaptive thermogenesis depends more on activity (non-resting) energy expenditure and varies with the changes in molecular mechanisms in the skeletal muscle along with the with alteration of the signaling of brain melanocortin system during calorie restriction.
5.1 FUTURE DIRECTIONS

In this study I sought to determine the underlying mechanisms for adaptive thermogenesis in lean and obesity-prone rats. I determined that the primary source of differential adaptive thermogenesis was the suppression of non-resting EE and spontaneous physical activity along with some accompanying changes that occurred in skeletal muscle and brain pathways. I also found some difference that existed between short- and long-term calorie restriction suggesting adaptations leading to a greater struggle to lose weight.

The EE study was conducted in both males and females and very similar results were observed between sexes. However the studies examining skeletal muscle and brain melanocortin system were done only in males. If the same studies are also performed in the females it would tell us if the males and females skeletal system and brain were adapting similarly in the calorie restriction.

In the VO$_{2\text{max}}$ analysis it would be interesting to look at locomotor efficiency at lower running intensity because most of changes or differences are visible at low or moderate levels of activity. For example, in humans it has been observed that the suppression of non-resting energy expenditure due to increased skeletal muscle work efficiency is seen mainly during low-intensity exercise [142, 144]. This is also observed in enhanced muscle thermogenesis after intra-VMH MTII injections in rats, where the MTII induces changes in locomotor efficiency at low and moderate levels of activity (studies done in
my lab). Thus observing how calorie restriction affects HCR and LCR at low levels of exercise will give us more insight on the mechanism of suppressing non-resting EE.

Some of the changes in muscle molecular pathways varied between 2 days and 21 days of calorie restriction. Doing a study which has a greater number of time points will tell us when the switch between short- and long-term calorie restrictions happens and at which time point body starts to adapt more to hinder weight loss.

MC3R in PVN decreased in the HCR, we could do an agonist-antagonist study on HCR and LCR during calorie restriction and confirm how this decrease corresponds to the suppression in physical activity that I observed in the HCR. Further, the combination of MC3R in PVN could be used to find possible remedies to prevent suppression of physical activity, thereby preventing the decrease of non-resting EE. This would help us to prevent adaptive thermogenesis at least to a certain extent. While MC3R-specific agonists are available and have been used in HCR/LCR [154], there is no MC3R-specific antagonist currently available.

With respect to the brain melanocortin system, future hypotheses would also focus on the likely role of AgRP during calorie restriction, like if the increases in AgRP due to food restriction differ between HCR and LCR, would this be predicted to increase appetite and decrease physical activity and EE. Thus AgRP may differentially oppose negative
energy balance hindering weight loss by adding to the mechanisms leading to adaptive thermogenesis.

I also saw that, although SERCA is considered thermogenic, protein and gene expression levels actually increased during 2-day or short-term calorie restriction, and in quads SERCA 1 increased after 21 days of calorie restriction. This is similar to that is observed in weight-reduced humans, but does not explain the apparent inconsistency of having a thermogenic protein increase while thermogenesis or EE decreases. SERCA is regulated by sarcolipin and phospholamban. Uncoupling of SERCA by the binding of sarcolipin is thought to be one of the mechanisms underlying skeletal muscle thermogenesis due to the release of extra heat in the process of uncoupling Ca\(^{2+}\) transport from ATP use [60]. It is possible that the interactions actions between these proteins are changed with calorie restriction. Thus targeting SERCA and its regulators like sarcolipin and phospholamban in a muscle type-specific manner could be a good start to find their contribution to adaptive thermogenesis. For example experiments could be done comparing SERCA knockout, homozygous for SERCA (wild-type, or +/+ ) and heterozygous (+/-) animals and see how calorie restriction affect them in regards to adaptive thermogenesis. This would enable me to identify various measures to increase or maintain weight loss by mitigating adaptive thermogenesis.

If we concentrate on the mechanisms involved in the suppression of spontaneous physical activity and non-resting EE occurring during restricted feeding, we may find a way to
reverse these occurrences. If we can oppose adaptive thermogenesis to a certain extent, losing weight on a diet and maintaining lost weight would be far easier and accomplishable for many. Thus providing a way to fight the ever-growing problem of todays’ modern society, a disease named obesity.


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