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Mice Deficient in Sympathetic Activation of Brown Fat Gain Weight When Housed at Thermoneutrality (30°C)

By

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A thesis submitted in partial fulfillment

of the requirements for the

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In Biology

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Table of Contents

Abstract

When exposed to dietary excess, mice release norepinephrine (NE) onto brown fat, activating uncoupling protein-1, and dissipating caloric excess as heat. Mice lacking dopamine beta-hydroxylase(DBH) cannot produce NE, making them cold-sensitive and unable to activate a sympathetic response to thermal stress or dietary excess. However, DBH-/- mice fed a high fat diet (HFD) and housed at 22°C gain weight normally due to compensatory thermogenic mechanisms which raise metabolic rate. The hypothesis that NE is essential for diet-induced thermogenesis was tested by housing DBH-/- mice at thermoneutrality on a HFD or low fat diet (LFD) for 8 weeks. The thermoneutral zone and basal metabolic rate (BMR) of female DBH-/- mice and littermate controls were calculated. There was no difference in the mass specific BMR between DBH-/- mice and control mice (24.3 ± 0.6 vs. 24.6 ± 0.9 ml O_2/min/g). A trend of a reduced lower critical temperature was observed in the DBH-/- mice in comparison to controls (28.4 ± 0.1 vs. 28.6 ± 0.1 °C). DBH-/- mice gained weight on either a HFD or a LFD (52.2 $\pm$ 7.0% and 19.2 $\pm$ 3.2% of body weight, respectively.) Control mice on either a HFD or LFD at thermoneutrality gained significantly less weight than the DBH-/- mice (11.5 $\pm$ 3.3% and 16.3 $\pm$ 2.6% of body weight, respectively). These results demonstrate that while DBH-/- mice have a normal BMR, they are more susceptible to weight gain at thermoneutrality than control mice.

Introduction

Homeostasis is an organism's ability to maintain a constant physiological state despite changes in internal functioning or the external environment. Any change within an organism is immediately met with a series of mechanistic adjustments working together to re-establish steady-state conditions (Cannon, 1932). Energy balance and thermoregulation, two important homeostatic concepts, are necessary for the well-being and survival of mammals.

Every organism must produce or obtain usable energy to maintain homeostasis. The majority of that energy comes in the form of the chemical, adenosine triphosphate(ATP). Hydrolysis of ATP to form adenosine diphosphate(ADP) is a highly exothermic reaction, releasing large amounts of energy that can be coupled to other biosynthetic and biochemical reactions within a cell. Production of ATP occurs in the mitochondria of cells. The rate at which ATP and other energy-rich molecules are produced is known as energy metabolism. Energy metabolism per unit time is defined as metabolic rate. (Nielsen-Schmidt, 1997) As the majority of ATP is produced using pathways that require oxygen, and the amount of energy produced per liter of oxygen consumed is the same whether fats, carbohydrates, or protein are used as the energy source, metabolic rate can be calculated by measuring the oxygen consumed per unit time (Nielsen-Schmidt, 1997). Many factors influence the metabolic rate of an organism, including food intake and availability, ambient temperature, and activity level.

Thermoneutrality

The majority of mammals and birds are homoeothermic endotherms, generating internal heat to maintain an elevated and constant internal body temperature (T_B). Hydrolysis of ATP to ADP, which occurs in most metabolic processes that require energy, releases heat. To ensure a constant body temperature, heat loss from an organism must equal heat gain produced by hydrolysis of ATP in metabolic processes (Moyes and Schulte, 2006). For every species, a temperature zone exists, known as the thermalneutral zone, in which energy expenditure needed to maintain body temperature is at its lowest (Lodhi and Semenkovich, 2009). **Figure 1.** represents the correlation between T_A and metabolic rate.

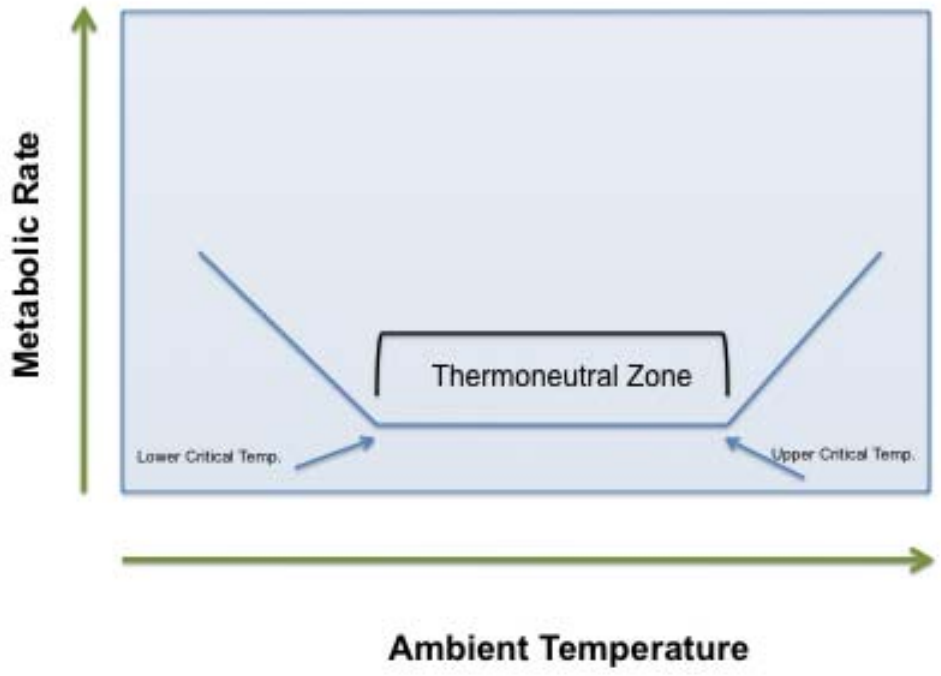

Figure 1. Metabolic Rate as a Function of Ambient Temperature.

When an animal stays within the ambient temperature that represents its thermoneutral zone, metabolic rate remains constant and at a minimum level. If the animal is at rest and fasted, this minimum metabolic rate within the thermoneutral zone represents the basal metabolic rate, or the minimum level of energy production needed to sustain vital organs only (Gordon, 1993). When ambient temperature reaches an upper limit, known as the upper critical temperature (UCT), the animal must induce cooling mechanisms that require an increase in energy expenditure and subsequent rise in metabolic rate. When ambient temperature reaches a lower limit, known as the lower critical temperature (LCT), the animal must increase heat production, raising the animal's energy needs and metabolic rate. The greater the deviation in T_A from an animal's thermoneutral zone, the greater the rise in metabolic rate (Moyes and Schulte, 2006). The ease of heat loss from an animal, known as thermal conductance, is measured in watts per unit body mass per degree Celsius and can be calculated using **Equation 1.,** where M represents the rate of heat loss and T_B-T_A represents the difference between the body temperature of the animal and the ambient temperature (Nielsen-Schmidt, 1997).

$$\mathbf{C=M/(T_B-T_A)} \qquad \mathbf{(1)}$$

Equation 1. Thermal Conductance of a Homeothermic Animal

As heat loss must equal heat production, than the variable **M,** representing rate of heat loss, is equal to rate of heat production(metabolic rate), and so thermal conductance of an animal can be calculated by using the metabolic rate of the animal, as measured in ml O_2 consumed per gram body weight per unit time. Conductance(**C**), therefore, represents the slope of the T_A/Metabolic Rate regression line below the LCT (Gordon, 1993).

The thermoneutral zone of a naked human is 28-30°C while the thermoneutral zone of a clothed human drops to 22-25°C which represents conventional "room temperature" (Lodhi and Semenkovich, 2009). While mice have a thermoneutral zone similar to humans at around 28-32°C, in laboratory

conditions they are often housed at room temperature, 20-22°C. Mice living in cold laboratory conditions are constantly exposed to thermal stress and must increase their metabolic rate by 50-60%. Food intake increases by about 50% as well (Golozoubova, 2004). Mice who were exposed to thermoneutral conditions (T_A=30°C) after living at room temperature(T_A=23°C) exhibited a decrease in mean arterial pressure and heart rate (Williams et al., 2002) indicating that exposure to thermal stress has important cardiovascular implications as well.

Thermoregulation

In order to maintain steady T_B despite fluctuations in ambient temperature (T_A), mammals have developed a series of thermoregulatory mechanisms. As T_A rises, mammals initiate cooling mechanisms to increase heat loss and lower the rising T_B. After sensing a rise in ambient temperature, vasodilation occurs. Arterioles dilate, increasing blood flow to the skin, where heat is exchanged with the skin, increasing the temperature of the skin and resulting in increased heat loss through the skin. Larger mammals utilize sweat to increase cooling, through evaporative heat loss.

Smaller mammals alter their ventilation rate and pattern to increase heat loss in rising T_A. Rapid, shallow respiration increases air-flow across the moist, vascularized respiratory surfaces, resulting in loss of heat through convection and increased evaporation of water from these surfaces (Moyes and Schulte, 2006).

When T_A drops, an organism can either decrease their heat loss or raise their rate of heat production. Vasoconstriction reduces blood flow to the extremities of an organism, lowering the skin temperature and reducing heat loss. In piloerection, erector muscles contract, pulling body hair into a perpendicular position to increase insulation and reduce heat loss (Moyes and Schulte, 2006).

Thermogenesis, the production of heat through metabolic pathways, is essential for maintaining an elevated $T_{B.}$ in endothermic animals. Hydrolysis of ATP to ADP, which occurs in most metabolic processes that require energy, releases heat. Any activity that increases the energy needs of an organism increases ATP hydrolysis and energy output of that output, resulting in increased heat production. An animal that is faced with a cold environment may voluntarily increase its activity level, causing an increase in energy utilization in skeletal muscle and generating heat. Involuntary muscle contractions, known as shivering, may also occur in an animal placed in a low T_A, with the same, thermogenic result (Moyes and Schulte, 2006). A second type of thermogenesis, non-shivering thermogenesis, is prevalent in small mammals. One example is the uncoupling of the proton gradient in brown adipose tissue (BAT).

Non-Shivering Thermogenesis

ATP is produced in the mitochondria of cells. Transport of electrons through a series of proteins that make up the electron transport chain (ETC) located on the inner mitochondrial membrane provide energy to pump protons from the mitochondrial matrix into the inner membrane space. This pumping of protons produces an electrochemical gradient that is known as the proton motive force. The potential energy stored in this proton gradient is used in the synthesis of ATP. The protons flow down their gradient through ATP synthase and back into the mitochondrial matrix. Their movement through ATP synthase drives the phosphorylation of ADP to form ATP, which can then be used as an energy source for other cellular processes (Moyes and Schulte, 2006).

The mitochondria of the thermogenic organ known as brown adipose tissue (BAT) contain uncoupling protein one (UCP1). This protein, located in the inner mitochondrial membrane, takes the protons that have been transported into the inner membrane space to form a proton gradient and allows them to move down their gradient back into the mitochondrial matrix. However, the energy

released from the proton movement is not coupled to production of ATP or any other high-energy molecule. It is instead released as heat energy. (**Figure 2.)** Thus, stimulation of BAT and UCP1 upon exposure to a cold environment would lead to increased heat production and increased metabolic rate via uncoupling of the proton gradient.

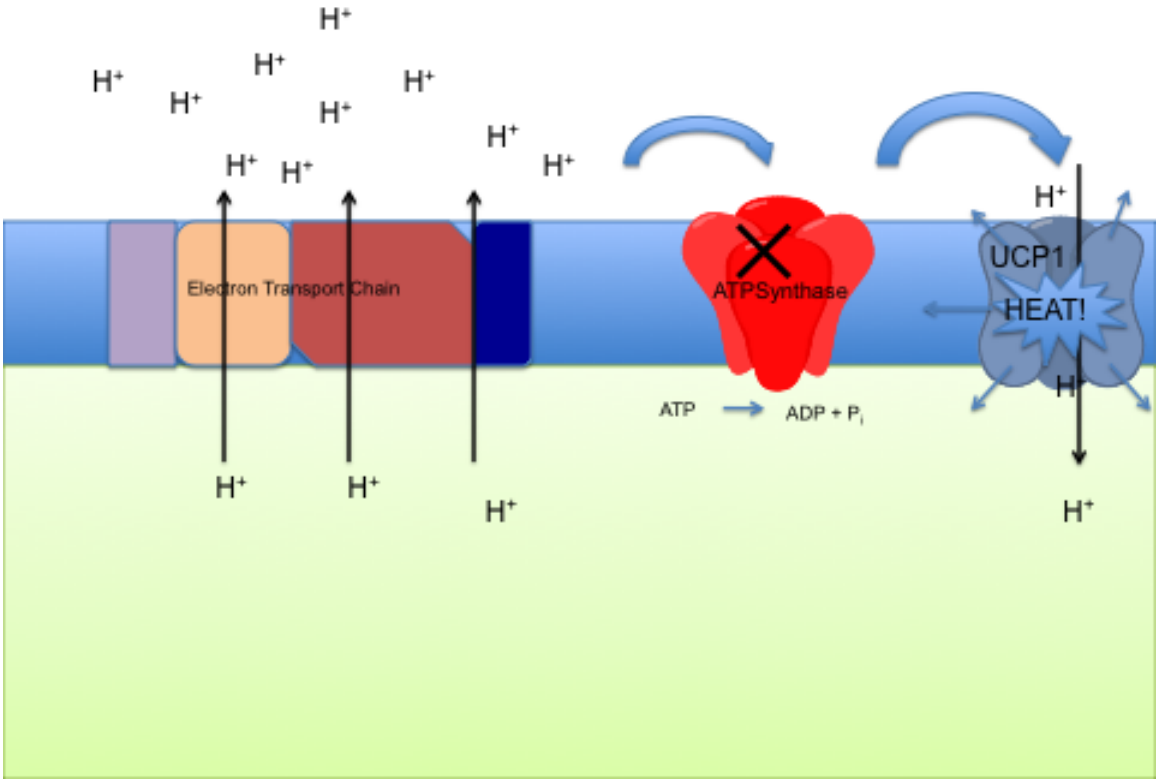

Figure 2. UCP1 Mediated Thermogenesis. Protons are transported across the inner mitpochondrial membrane to form the proton gradient. In addition to cycling through ATP synthase and driving heat, the protons move through UCP1 and the energy stored in the proton gradient is dissipated as heat.

Proper UCP1 function is dependent on the presence of fatty acids (Klingenberg and Huang, 1998). Fatty acids are thought to interact with UCP1 as allosteric activators of proton transport. However, evidence also exists for a mechanism in which fatty acids, which are anions, become transported out of the mitochondrial matrix through UCP1. In the intermembrane space they become

protonated, allowing them to pass through the membrane back into the mitochondrial matrix, thereby helping to dissociate the proton gradient (Susulic and Lowell, 1995). Fatty acids also act as the main source of oxidative phosphorylation in mitochondria BAT, providing the biochemical energy to drive non-shivering thermogenesis (Palou et al., 1997).

UCP1 is located in BAT, which differs morphologically from white adipose tissue in that it contains multiple droplets per adipocyte, exhibits a higher capillary density representing an increased demand for oxygen, is highly innervated by sympathetic nerves, and contains an abundance of iron containing mitochondria, giving it a brownish color. BAT is found primarily in small hibernating mammals and newborn infants (Cannon and Nedergaard, 2004); however, recent studies also provide evidence for the presence of functional BAT in adult humans (Nedergaard et al., 2007). After labeling with FDG-PET, increased tracer uptake was seen in the upper body of adult human males corresponding to pockets of BAT (Nedergard et al., 2007). In a different study, adult males were kept in either a thermoneutral (22°C) or mild cold environment (16°C) and then injected with the FDG-PET tracer. BAT was observed in 96% of the cold exposed subjects but was not present in those subjects residing at thermoneutrality, suggesting that BAT in humans is activated by a cold environment (Lichtenbelt et al., 2009). However, the idea of functional brown adipose tissue and its exact physiological role in adult humans remains a controversial subject.

UCP1 mRNA levels are increased in BAT after a 48hr cold exposure in rats (Daikoku et al., 2000), indicating that upon activation of BAT in a cold environment, levels of UCP1 rise in order to mediate the increased need for thermogenesis (Ricquier et al., 1986). Similarly, hyperplasia, hypertrophy, and increased blood flow is seen in BAT of cold acclimated animals.

Small mammals produce heat to maintain body temperature when exposed to a cold ambient temperature. The method of heat production depends on length of exposure to the cold. Upon initial or

acute exposure to cold, animals initiate a shivering response to generate heat. However, after 48hrs of cold exposure, levels of shivering thermogenesis decrease and the main mechanism of heat production switches to non-shivering thermogenesis. Non-shivering thermogenesis mediated through BAT and UCP1 is therefore a plastic component of thermogenesis and plays the greatest role in heat generation during chronic cold exposure once the animal has acclimated to the cold.

Additional homologs of UCP1, named UCP2 and UCP3, have been recently discovered. UCP2 mRNA is expressed in low levels throughout the body while UCP3 mRNA is mainly expressed in muscle tissue and BAT, although the actual UCP3 protein has only been identified in muscle tissue (Nedergaard and Cannon, 2003). The exact mechanism and function of these proteins remains unknown with some evidence suggesting a possible role for UCP2 and UCP3 in thermogenic processes. However, evidence supports UCP1 as the main protein involved in proton gradient dissipation in DIT and NST, while UCP2/3 provide minimal, if any, contribution at all (Nedergaard et al., 2001).

Sympathetic Control of Thermogenesis

To maintain homeostasis, most physiological functions must be under constant regulation and control. The autonomic nervous system, under the control of a central regulatory center in the brain, works to maintain homeostasis in organisms. The autonomic nervous system consists of the sympathetic and parasympathetic nervous system. Most organs are innervated by both sympathetic and parasympathetic neurons and the two systems are antagonistic to each other. For example, parasympathetic stimulation of the heart results in a reduced heart rate while sympathetic stimulation results in an increased heart rate. The two pathways work together to maintain homeostasis, inhibiting or provoking a certain organ depending on the current environment and an organism's need. While the parasympathetic nervous system is most active when an animal is at rest, the sympathetic nervous system (SNS) takes over in response to stressors and physical activity (Moyes and Schulte, 2006).

The hypothalamus plays an integral role in CNS control of autonomic regulation. In response to signals from sensory neurons that relay information about the state of the organism, the hypothalamus alters its activity to increase or reduce autonomic nervous activity. Body temperature, food intake, and water balance are all regulated by the hypothalamus (Moyes and Schulte, 2006).

Upon activation, sympathetic neurons release the catecholamines epinephrine(E) and norepinephrine(NE) onto adrenergic receptors located in the CNS, peripheral nervous system(PNS) as well as cardiac and smooth muscle. NE and E are synthesized and released from the adrenal medulla glands as well as post-ganglionic neurons in the SNS. NE binds to and acts upon the adrenergic receptors including the alpha 1, 2 and beta 1,2,3 receptors.

Any significant decrease in body temperature is sensed by the hypothalamus as an environmental stressor and the SNS is activated. NE is released from post-ganglionic sympathetic neurons and increases heart rate, initiates vasoconstriction in the extremities, contracts the smooth muscles controlling hair movement resulting in piloerection, and activates NST in brown fat. Studies have shown that cold exposure results in a sharp increase in blood plasma levels of NE in mice (Ibraimova 1997). Extensive innervation of BAT by sympathetic neurons has been observed and levels of both BAT innervation and synaptic NE turnover increase in cold acclimated rats (Cottle and Cottle, 1969 and Young et. al. 1982). These results support the role of the sympathetic nervous system in cold initiated BAT mediated thermogenesis.

NE released from adrenergic neurons synapsing on BAT binds to beta-adrenergic receptors on the cell surface of brown adipose cells, and activates UCP1 protein, uncoupling the proton gradient and generating heat. While UCP1 mediated NST plays an integral in the thermoregulation of mammals in a cold environment, this pathway is also involved in body weight regulation and energy balance.

Diet- Induced Thermogenesis

Dietary excess can lead to weight gain. In order to maintain a stable body weight, an animal must increase energy expenditure to offset this excess or decrease food intake to prevent continued consumption of unnecessary calories. Regulation of food intake is controlled by a complex group of hormones and neurotransmitters that monitor the metabolic status, environmental conditions, and bodily energy stores and adjust hunger and satiety signals accordingly. Factors such as ghrelin, the orexins, and neuropeptide Y act as powerful appetite stimulants. Insulin, released from the pancreas in response to high levels of circulating glucose, binds to receptors in the hypothalamus, inhibiting food intake (Hellstrom et al. 2004). The hormone leptin, secreted from adipose tissue, also acts as an anorexigenic compound by decreasing appetite and reducing food intake. Both leptin and insulin circulate in levels directly proportional to body fat. Ingestion of caloric excess leading to an increase in adiposity will also lead to increased leptin levels, signaling the brain to decrease appetite and food intake (Houseknect et al. 1998).

In addition to regulating food intake, an animal can also increase its energy expenditure in response to caloric excess. An increase in energy expenditure results from an increase in metabolic rate, indicating that many of the mechanisms used in thermoregulation could also work to maintain energy balance by producing an increase in metabolic rate through heat production. Fasting inhibits the sympathetic nervous system while overfeeding increases its activity as measured by NE turnover rates (Landsberg 2006). Increased sympathetic activity results in increased activation of BAT, leading to mitochondrial uncoupling, a reduction in metabolic efficiency and rise in metabolic rate than can offset the caloric excess. The ability of animals to increase the capacity for energy expenditure in response to certain foods and diets is known as diet-induced thermogenesis(DIT) and follows the same pathway used in NST. (**Figure 3.)**

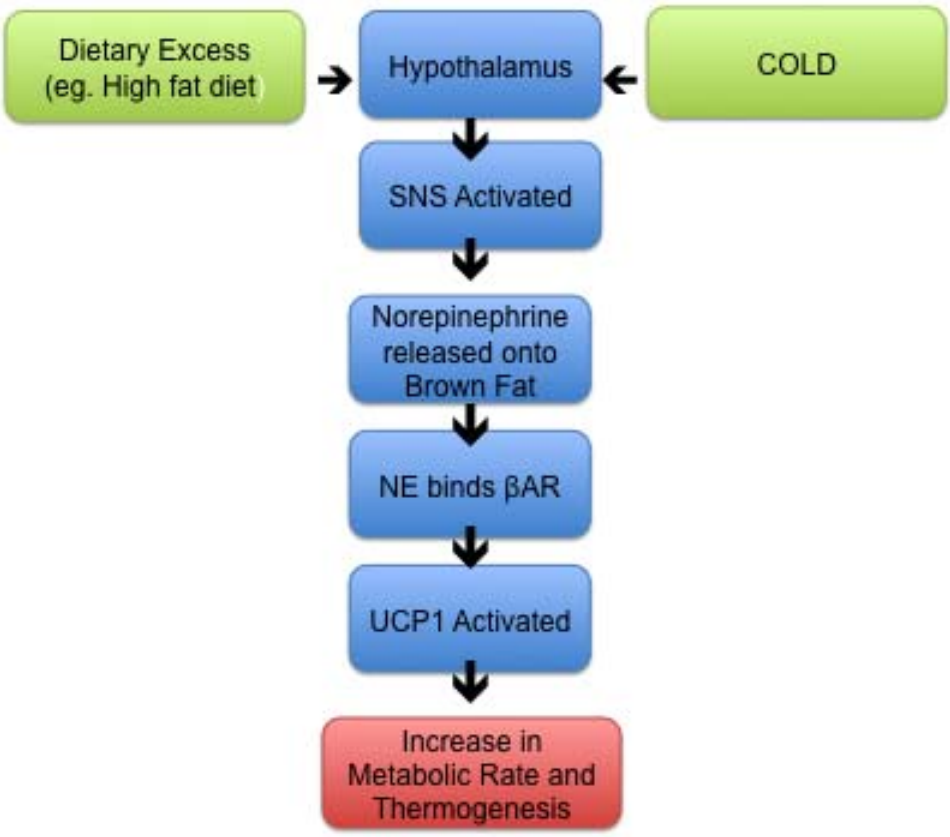

Figure 3. Pathway of Activation in Non-Shivering Thermogenesis and Diet Induced Thermogenesis

Obesity

However, appetite regulation and DIT can fail to compensate for caloric excess, leading to storage of extra energy in white adipose tissue (WAT). Over time, as triglycerides continue to accumulate in WAT, obesity develops. Obesity is a major health problem facing the United States and other developed countries. As of 2008 estimates, 36 states had a prevalence of obesity greater than 25%, with six states of those states having a prevalence greater than 30%, accounting for nearly 1/3 of the population.(CDC) Increased body mass can lead to health problems such as diabetes, hypertension, heart disease, stroke, and premature death.

The prominence and severity of the obesity epidemic has led to research into the causes of obesity and possible therapeutic agents to combat the disease. In humans, BAT levels have been inversely correlated with body mass index (Cypess et al. 2009), leading to a focus on UCP1 mediated thermogenesis as a potential target for obesity. Mice that contain genetically altered white fat in which the UCP1 protein is overexpressed do not become obese in response to a high fat diet (Kopecky et al. 1996), further supporting a role for UCP1 and brown fat manipulation in obesity treatment. The magnitude of diet-induced thermogenesis can be measured by the ability of an animal to increase metabolic rate in response to injection of NE (Cannon and Nedergaard, 2004).

Obesity can be modeled in a laboratory rodent model in various ways, including overfeeding, feeding on a high fat diet, and use of genetically altered mice. The *ob/ob* mouse does not produce the hormone leptin and displays uncontrolled food intake resulting in an obese phenotype. Even when caloric intake is severely limited in these animals, weight gain continues (Coleman 1982). These mice display high levels of insulin and circulating fatty acids, glucose intolerance, insulin resistance and diabetes (Cullen et al. 1995).

Obesity can also be developed by feeding an animal a high fat diet. Mice fed a high fat diet display a significant increase in body weight compared to controls. After 8 weeks of high fat feeding, mice were not responsive to leptin injections, indicating that leptin resistance had developed in these animals (Thomas et al., 2000). Chronically high circulating levels of leptin result in the inability of the body to respond to leptin signals in the brain, resulting in decreased satiety signaling.

Activation of Diet-Induced Thermogenesis

As a variety of genetic and environmental factors are responsible for obesity in humans (Das and Rao, 2007), it makes sense to study the DIT pathway using a model that takes into account both genetics

and environmental influences such as diet content. As metabolic inefficiency is the direct result of proton uncoupling in the UCP1 protein, genetic ablation of this protein affects the ability of an organism to dissipate excess energy. Original studies of UCP1 knockout mice (UCP1-/-) indicate that UCP1-/- mice kept at room temperature display cold sensitivity but do not become obese in response to a high fat diet (Enerback et al. 1997). However, UCP1 ablation in mice housed at thermoneutrality prevents diet-induced thermogenesis and induces obesity in mice fed both a control and a high fat diet. Control animals fed a high fat diet display a robust metabolic response (as measured by oxygen consumption) to NE injection in comparison to controls fed a regular diet while UCP1-/- animals fed a HFD showed a response to NE injection similar to UCP1-/- animals fed a normal diet. (**Figure 4.)** These results indicate that the UCP1-/- animals could not activate diet-induced thermogenesis in response to a high fat diet (Feldmann et. al. 2009). Housing at thermoneutrality is necessary for appearance of an obese phenotype as UCP1-/- animals placed at room temperature must initiate other compensatory thermogenesis mechanisms to maintain a constant body temperature in the cold environment, which increases metabolic rate and offsets the dietary excess of the high fat diet.

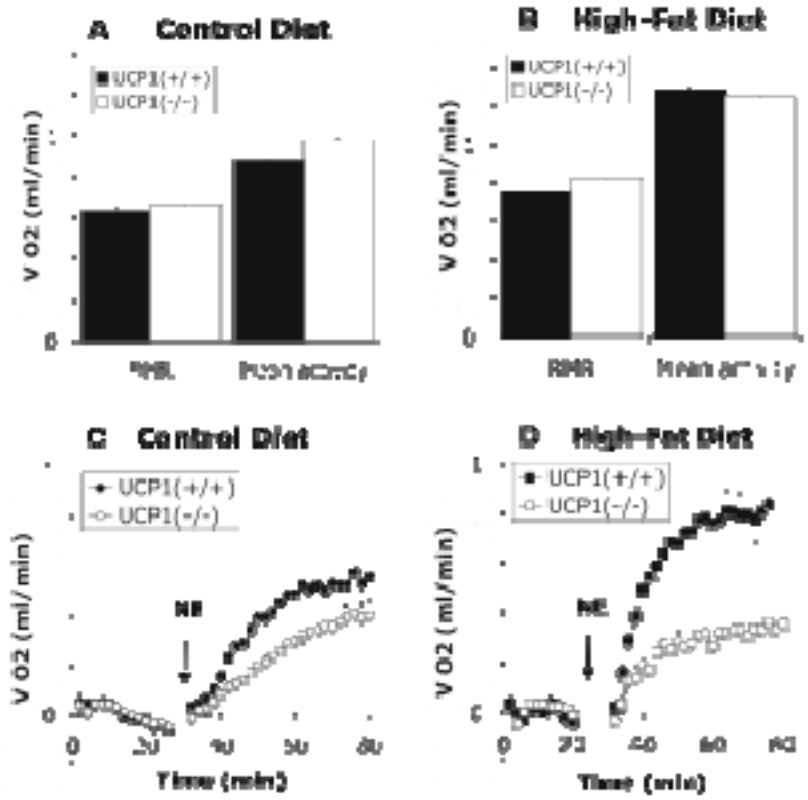

Figure 4. Feldman et al., 2009 Diet Induced Thermogensis in UCP1-/- Mice fed a High Fat Diet as measured by metabolic response to Injection of Norepinephrine.

Activation of UCP1 protein is dependent on NE binding to beta adrenergic receptors (βARs). Mice in which all three βARs have been genetically ablated are mildly obese on a regular diet in comparison to controls. These mice also display a decreased metabolic rate. When housed at 22°C,

mice lacking β-adrenergic receptors displayed enlarged and pale BAT that contained detectable levels of leptin and lower than expected levels of UCP1 mRNA. When placed on a high fat diet at thermoneutrality, mice lacking β-adrenergic receptors became very obese despite comparable levels of food intake compared to WT controls and did not display the increase in metabolic rate seen in controls fed a HFD (Bachman et al., 2002). These results support the role of BAT in diet-induced thermogenesis and indicate that βARs are necessary for normal BAT morphology. Ablation of βARs in the presence of dietary excess leads to an inability to dissipate extra calories, resulting in development of obesity in these mutant mice.

Dbh-/- Mice

Going one additional step back in the pathway and removing the β-adrenergic receptor ligand NE, one would expect to see the same disruption of DIT and NST seen in the UCP1 or β-adrenergic receptor deficient animals. Norepinephrine(NE) is synthesized from dopamine(DA) by the enzyme dopamine beta-hydroxylase(DBH). A rare human genetic disorder exists in which a failure to produce the DBH enzyme responsible for synthesizing NE and E causes hypoglycemia, hypotension, and hypothermia in affected patients(Robertson et al., 2006). Disruption of the *Dbh* gene in laboratory mice results in NE and epinephrine (E) deficient animals with a distinct mutant phenotype. *Dbh*-/- mice are 80-90% of normal weight and display a 25% elevated resting metabolic rate (RMR) even at thermoneutral temperatures (Thomas and Palmiter, 1998). To offset this increase in RMR and maintain a normal energy balance, *Dbh-/-* animals display elevated food and water intake. The increase in RMR cannot be attributed to hyperthyroidism, increased shivering, or compensation by UCP2 (Thomas and Palmiter, 1997). As NE mediates vasoconstriction, NST, and piloerection, *Dbh*-/- animals are cold-sensitive, surviving only 1-2 hrs at 4°C in comparison to controls, which survive indefinitely. Even at room temperature (22°C), a less extreme cold environment, *Dbh*-/- mice have a lower body temperature than

do controls. Upon exposure to 4°C, both the vasoconstriction and piloerection response to cold was reduced in *Dbh*-/- mice in comparison to controls. Double the normal amount of BAT is observed in these animals. The BAT in *Dbh*-/- mice is paler than normal, corresponding to an increased fat content of the tissue. At room temperature (22°C), UCP1 mRNA levels were only 10% of normal, further confirming the inability of *Dbh*-/- mice to activate brown fat, NST and DIT (Thomas and Palmiter 1998).

Additionally, *Dbh*-/- mice have blood glucose levels 25% lower than controls and a 4-6 fold higher insulin concentration than controls (Marie and Palmiter 2004). Low glucose levels correspond with increased appetite while high insulin levels correspond with increased rates of energy storage, both of which can lead to weight gain and obesity. Leptin increases UCP1 mRNA levels in BAT and this effect is abolished in *ob/ob* mice, supporting a role for leptin in initiating DIT (Commins et al. 1999). Leptin injections in *Dbh*-/- mice produced no effect on UCP1 mRNA expression in BAT and this effect was reversed upon co-administration of CL-316,243, a B_3AR agonist, with leptin, indicating that NE is required for leptin induced increased mRNA expression in BAT (Commins et al. 1999).

A normal phenotype can be restored in *Dbh*-/- mice by administration of L-threo-3, 4-dihydroxyphenylserine (DOPS), a synthetic amino acid that is converted to NE in adrenergic cells. DOPS crosses the blood brain barrier and therefore can act in both peripheral and central adrenergic systems. 5hr after administration of a single dose of DOPS, NE was restored to normal levels in the heart, brown fat, liver, muscle, spleen, seminal vesicle, and olfactory bulb; there was higher than normal levels of NE in the lung, kidney, intestine, and pancreas, and lower than normal levels in the frontal cortex, cerebellum, and brainstem. No restoration of NE was observed in the adrenal gland (Thomas et al., 1998) Administration of DOPS also lowered RMR and food intake to normal levels in comparison to controls (Thomas and Palmiter, 1998). These studies suggest that the cold-sensitivity and energy expenditure deficiency in *Dbh*-/- animals can be reversed by treatment with DOPS, indicating that the observed

mutant phenotypes are caused by a physiological NE deficiency at the time of testing versus a developmental NE deficiency earlier in life.

The *Dbh*-/- mouse therefore provides a valuable model for studying the effects of NE and E deficiency on thermoregulation and energy balance systems mediated through the sympathetic nervous system. One would expect that NE and E deficient mice fed a high fat diet (HFD) would be unable to activate compensatory sympathetic mediated DIT to burn excess energy and would become obese in comparison to normal mice fed the same HFD. However, *Dbh*-/- mice housed at room temperature and fed a HFD diet for 6 weeks gained weight normally in comparison to controls fed the same diet. (**Figure 5.)** On a normal diet, both *Dbh*-/- and *Dbh*+/- mice displayed an average 20% increase in body weight while both genotypes fed the HFD displayed a 54% increase in body weight. There was no significant difference in body weight between *Dbh-/-* and *Dbh+/?* mice fed the HFD (Marie et al., 2005).

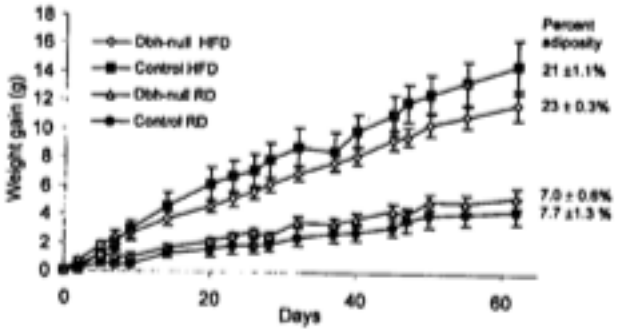

Figure 1: Dbh-null mice gain weight normally and have normal adiposity when fed an HFD. Weight gain vs. time is plotted because *Dbh*-null mice are smaller than controls; starting weights of *Dbh*-null mice and the controls were 22.98 ± 0.77 and 27.13 ± 0.91 grams, respectively. *P* = not significant.

Figure 5. Marie et al., 2005. Results of HFD on Body Weight in DBH-/- Mice

As the mice were kept at a temperature (20-22°C) below their thermoneutral zone, metabolic rate in both *Dbh*-/- and control mice would have been elevated in order to maintain a stable body

temperature. The elevation in metabolic rate could have been sufficient to offset the caloric excess ingested in those mice fed a HFD, resulting in normal weight gain of the *Dbh*-/- mice.

Hypothesis

I hypothesize that *Dbh*-/- mice, when fed a HFD and housed in their thermoneutral zone (28-30°C), will become obese in comparison to control mice also fed a HFD. No thermoregulatory mechanisms will need to be activated to reduce heat loss and initiate heat production, thereby minimizing metabolic rate and eliminating any mechanism for dissipation of caloric excess in the *Dbh*-/- mice, resulting in the development of obesity. And so, disruption of the pathway involved in UCP1 mediated thermogenesis results in an inability to activate the BAT in DIT upon ingestion of dietary excess, causing weight gain and obesity in a rodent model.

Materials and Methods

Animals:

20 nine month old female *Dbh-/-* and *Dbh+/?* mice were obtained from the Weinshenker Laboratory at the Emory School of Medicine in Atlanta, Georgia. Mice were housed individually on a 12:12 hour light cycle and were allowed *ad libitum* access to food and water. Mice were housed at 28-30°C unless otherwise indicated. Feeding and handling of mice occurred in the last hour of the light cycle and any telemetry or metabolism data obtained during this period was omitted from analysis.

Metabolic Testing:

All metabolic testing and energy expenditure calculations were obtained using indirect calorimetry and measurement of the respiratory gasses CO_2 and O_2. **Figure 6.** depicts the metabolic set-up used.

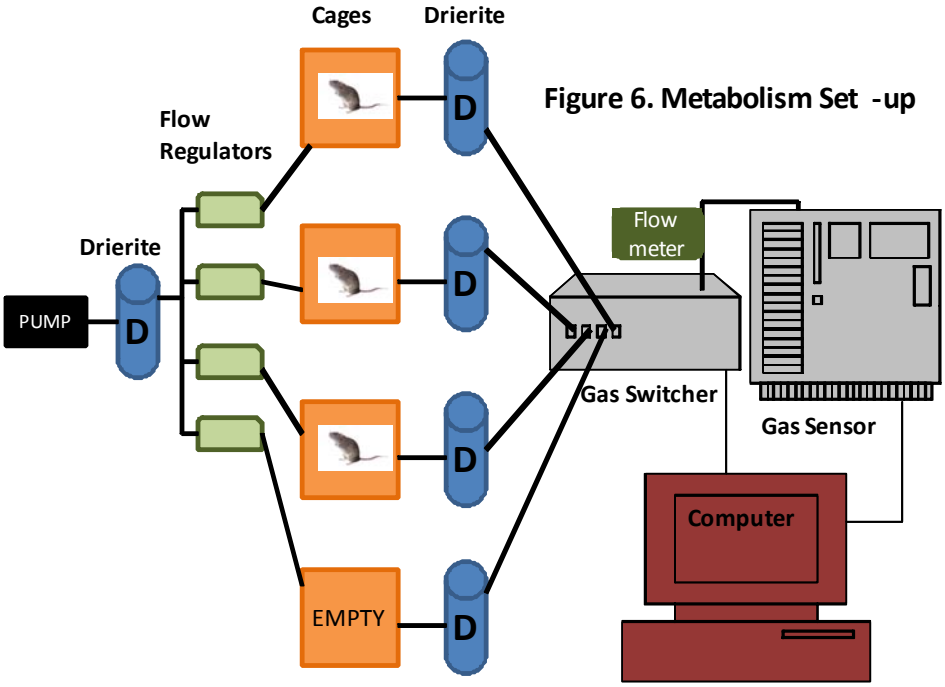

Figure 6. Metabolism Set -up

Animals were placed in an airtight cage and air was pumped through the cage. Flow rate was controlled using a flow meter and water vapor was removed from the air before and after passage through the animal cage using Drierite columns. Air from the cage was passed through a flow meter to measure flow rate and then passed through an O_2 analyzer and then a CO_2 analyzer. One cage always acted as a control to monitor the ambient percent concentrations of O_2 and CO_2. A gas switcher connected to a computer controlled the rate and order in which the gas sample from each cage was tested. This program also recorded the flow rate, $\%O_2$ and $\%CO_2$ of each sample. Metabolic rate and the respiratory quotient could be calculated from these values.

Telemetry:

All animals were implanted with Data Science International (DSI) PhysioTel® radio telemeters that measured body temperature, activity levels, and in some cases, heart rate. Telemeters were turned on with a magnet only during periods of data collection. Animals' cages were placed on DSI PhysioTel receivers that were connected to a DSI Data Exchange Matrix. The DSI ART 4.0 software was used to control and record sampling duration and frequency of the various receivers. An ambient pressure and room temperature receiver were also connected to the matrix to record ambient conditions. A light sensor was connected and used to verify beginning and end of the light and dark phases.

Temperature Control:

For the major duration of the study, all mice were housed at 28-30°C. Room temperature was raised with the use of 2-3 space heaters placed around the room, making sure the hot air was not being blown directly at any animals. When data collection required the need of higher or lower temperatures, cages were placed in Styrofoam boxes with Plexiglas tops that were fitted with copper piping connected to plastic tubing. This tubing allowed the flow of ethylene glycol from a water bath that had heated or

cooled the ethylene glycol to the appropriate temperature through the copper tubing in the boxes, thereby heating or cooling the air in the boxes. Box temperature was monitored manually using small glass thermometers placed in each cage and electronically by a temperature sensor placed in one of the cages and connected to the DSI matrix.

Diet:

During the first two months of thermoneutral zone testing and prior to implantation of telemeters, all mice were fed Harlan Teklad® mouse/rat laboratory diet. After a week of recovery after surgery, each mouse was placed on either a high or low fat diet mixed in lab. Due to staggering of surgeries, the mice implanted with ECG telemeters were started on their respective diets 3 weeks after those implanted with temperature telemeters had started. **Table 3.** summarizes the ingredients and nutritional content of each diet.

Table 1. Ingredients and Nutritional Information in High and Low Fat Diets (Ingredient weights are per every kg produced)

Ingredient	Amount Low Fat Diet(g/kg)	Amount High Fat Diet (g/kg)
Corn Starch	**600 g**	**300 g**
Casein	**207 g**	**261 g**
DL-methionine	**3 g**	**4 g**
Celufil	**79.96 g**	**78.96 g**
Rogers-Harper Mineral Mix	**50 g**	**50 g**
Teklad Vitamin Mix	**10 g**	**10 g**
Zinc Carbonate	**0.04 g**	**0.04 g**
Lard	**15.8 g**	**296 g**
Corn Oil	**34.2 g**	**0 g**
Nutritional Information	**High Fat Diet**	**Low fat Diet**
Kilocalories/ gram	**4.8 kcal/g**	**3.45 kcal/g**
% Calories from Fat	**55.4%**	**13.0%**
% Calories from Protein	**22.1%**	**24.3%**
% Calories from Carbohydrates	**23.5%**	**62.7 %**

The diets were made in lab on an as needed basis and stored in the refrigerator. To create similar consistencies between the two diets, the low fat diet was stored as a powder and 35 g of water for every 65 g of powdered food was mixed together prior to feeding. The high fat diet was of a solid but malleable consistency and needed no water addition prior to feeding. Mice were fed daily for the first 12 weeks in the hour before onset of dark cycle in rectanglular plastic containers placed inside the cages. All food was broken up into chunks about the size of a 1 inch cube. After the first 12 weeks, mice were fed once every three days, with increased food given to compensate for the greater time in between feedings.

Thermoneutral Zone Testing:

To calculate the thermoneutral zone of the *Dbh-/-* and *Dbh*+/? animals, each animal was placed in a metabolic cage within the temperature control boxes without access to food. The temperature was set to 26°C and animals were allowed to equilibrate to the new environment for 2 hours before the temperature was raised one degree every hour. When the temperature in the cages reached 32°C, the animals were allowed to remain at this temperature for 2 hours before the temperature was lowered back to 26°C, two degrees every hour. The air from each cage was sampled for 5 seconds every four minutes over the course of testing.

Implantation of Radio Telemeters

All 20 mice were implanted 2 months after delivery with DSI PhysioTel® radio telemeters. 5 *Dbh-/-* and 5 *Dbh+/?* mice were implanted with a TA-F20 (3.8g) telemeter that measured body temperature and activity level. 5 *Dbh-/-* and 5 *Dbh+/?* mice were implanted with a ETA-F20 (3.9g) telemeter that measured heart rate, body temperature and activity level. Mice were anesthetized with the inhaled anesthetic isoflurane, mixed with oxygen. Mice were first exposed to 5% isoflurane in an airtight chamber until mice became unconscious and unresponsive at which point they were moved to the surgical table. Nose cones supplying 1-2% isoflurane allowed maintenance of anesthesia during surgery. Mice were placed ventral side up on a sterile surgical pad placed over a heating pad and secured. Lower abdominal hair was removed using NAIR® Hair Removal Lotion and the surgical site was cleaned with saline and iodine. A 2-3cm vertical incision through the skin and body wall was made using scissors on the midline between the lower two quadrants of the abdomen.

The TA-F20 telemeters were placed free-floating in the body cavity and the body wall was sutured shut. The skin incision was stapled closed. The ETA-F20 telemeters were placed in the body cavity and sewn into the body wall using two sutures. A 20G needle was inserted on either side of the

wound and the ECG leads were threaded through the body wall. The body wall was fully sutured shut. By separating the skin from the body wall, a tunnel was created through which the ECG leads were placed in proper positions above and below the heart to obtain a signal. The leads were sutured to the body wall and the skin was stapled shut.

Antibacterial ointment was placed on the wound and the animal was allowed to regain mobility before being transferred to the recovery room and placed on a heating pad. Animals undergoing TA-F20 implantation stayed for 1 day in recovery while those undergoing ETA-F20 implantation stayed for two days after surgery. All animals received a low dose of acetaminophen in their drinking water for two days following surgery to minimize discomfort. Staples were removed seven days after surgery. Although all efforts were made to minimize time under anesthesia, two of the mice implanted with ECG telemeters died from complications during the surgery.

Data Collection during High or Low Fat Feeding:

All animals were placed on a low fat diet for three days to allow adaptation to eating food placed in a dish in the cage. Half of the animals were then given a high fat diet. High and low fat diet groups were adjusted to ensure that an equal number of knockout and control mice were placed on each diet and that an equal number of mice with ECG and temperature telemeters were placed on each diet. **Table 4.** represents the sample sizes for each experimental group.

Table 4. Sample Sizes for Each Experimental Group

High Fat *DBH-/-*	Low Fat *DBH-/-*	High Fat *DBH+/?*	Low Fat *DBH+/?*
Temp. Telem. (3) **ECG Telem. (2)** **Total: 5**	**Temp. Telem. (2)** **ECG Telem. (2)** **Total: 4**	**Temp. Telem. (2)** **ECG Telem. (2)** **Total: 4**	**Temp. Telem. (3)** **ECG Telem. (2)** **Total: 5**

Body temperature, heart rate (in the ECG mice), and activity were monitored continuously for the three days preceding and the three days after commencement on the high or low fat diets. Subsequently, body temperature, activity and heart rate (in mice implanted with ECG telemeters) were monitored for a 22 hour period every week for 12 weeks. Animals were sampled for 1 second every minute starting at the beginning of the dark cycle and ending two hours before the end of the light cycle. Body weights were taken daily for 16 weeks.

At the end of 12 weeks, mice were placed in the metabolic cages at 30°C without access to food and metabolic rate was monitored over 22 hours. Each animal was sampled for 5 seconds once every four minutes. The same experiment was performed allowing access to food. After sixteen weeks, mice were placed in the metabolic cages at 20°C with access to food, and metabolic rate was monitored over 22 hours, with each animal being sampled for 5 seconds every four minutes. All animals were initially placed in the metabolic cages in the hour before the onset of the dark cycle and sampling began at the start of the dark cycle.

Shivering:

Shivering analysis was performed on the animals implanted with ECG telemeters. Telemeters were turned on and animals were placed in the Styrofoam boxes equilibrated to 20°C. Each animal was sampled for 120 seconds every 10 minutes. Raw data waveforms were exported into DSI Ponemah

Physiology Platform®. ECG recordings were generated as graphs that allowed visualization of the tracings of each heartbeat. The noise in the tracing, which represents electrical signals from involuntary muscle activity involved in shivering, was quantified. This number was used as a measure of the levels of shivering in each animal.

Statistical Analysis of Data:

All data was managed and all graphs were generated in Microsoft Excel. Values represent means ± standard error. Students T-tests were performed in Excel. One-way ANOVAs' were performed in SPSS. Diagrams and schematics were generated in Microsoft Powerpoint.

Results

Thermoneutral Zone Testing

To establish the temperature range over which metabolic rate is at a minimum, metabolic rate of *Dbh-/-* and control animals was measured as ambient temperature was raised from 26°C to 32°C, and then lowered back to 26°C. When faced with a changing ambient temperature, *Dbh-/-* and *Dbh +/?* mice displayed similar metabolic profiles. Typical metabolic rates as a function of ambient temperature for a *Dbh-/-* mouse and littermate control are plotted in **Figure 7**.

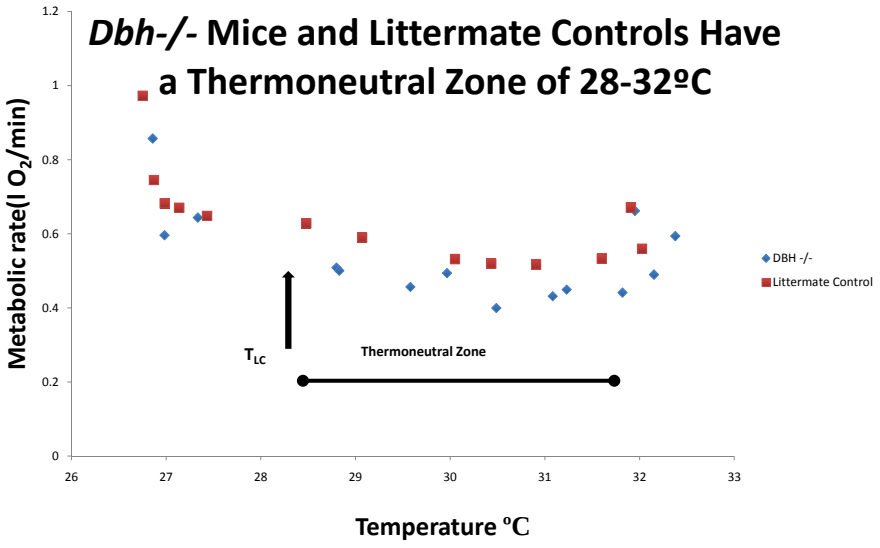

Figure 7. Thermoneutral zone and lower critical temperature of *Dbh-/-* mice and littermate controls. *Dbh-/-* and controls display a similar thermoneutral zone profile. Both *Dbh-/-* and littermate controls displayed a thermoneutral zone(black line with rounded ends) in the range of 28-30ºC . The lower critical temperature (T_{LC}), black arrow falls around 28.5ºC. The two data sets represent typical *Dbh-/-* and control animals.

Both groups of mice displayed an elevated metabolic rate between 26-27°C and as ambient temperature increased, metabolic rate decreased. When the temperature reached 28-29°C, metabolic rate stopped decreasing and remained steady despite subsequent rises in ambient temperature. At around 32°C, metabolic rate starts to rise again. From data like those in **Figure 7**, I was able to calculate basal metabolic rate and lower critical temperature.

There was no difference in the specific basal metabolic rate of *Dbh-/-* and *Dbh+/?* mice. (**Figure 8.)**

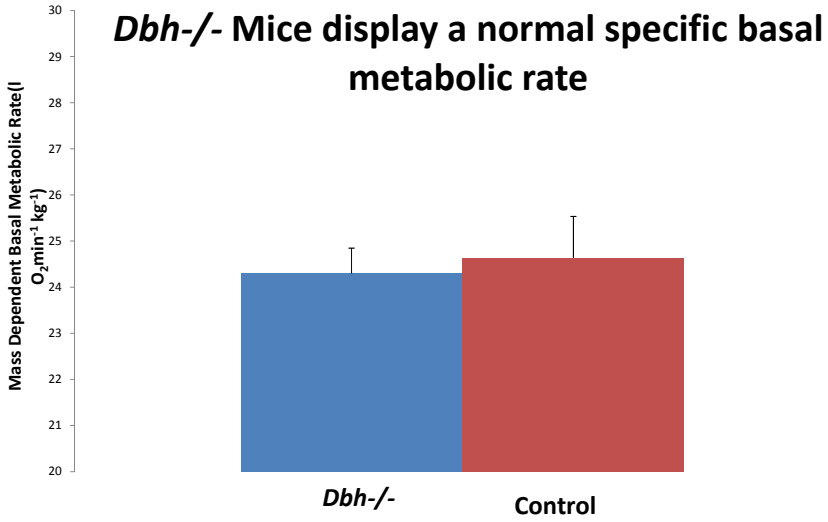

Figure 8. *Dbh-/-* mice do not display an elevated basal metabolic rate (BMR). The oxygen consumption of *Dbh-/-* mice and littermate controls was measured over the span of their thermoneutral zone (28-30°C). (See Figure 7.) BMR is expressed as ml O_2 /kg min. There was no difference in between the BMR of *Dbh-/-* and control animals. (24.3±0.6 versus 24.6±0.9).

Dbh-/- mice had a specific basal metabolic rate of 24.3±0.6 ml O_2/ kg min (n= 10), and the control mice had a mean basal metabolic rate of 24.6±0.9 ml O_2/ kg min (n=11). Basal metabolic rate was calculated as the average metabolic rate over the thermoneutral zone (28-30°C) of each mouse. At the time of thermoneutral zone testing, *Dbh-/-* mice were smaller in size than the littermate controls. (22.8±0.6 versus 23.9±0.3, $p<0.07$) To account for this discrepancy in weight, metabolic rates (ml O_2/ min) were expressed relative to body weight.

Although no difference was detected in specific basal metabolic rate, *Dbh-/-* animals exhibited a trend for a cooler lower critical temperature than controls (28.3±0.1 versus 28.6±0.1). (**Figure 9.)**

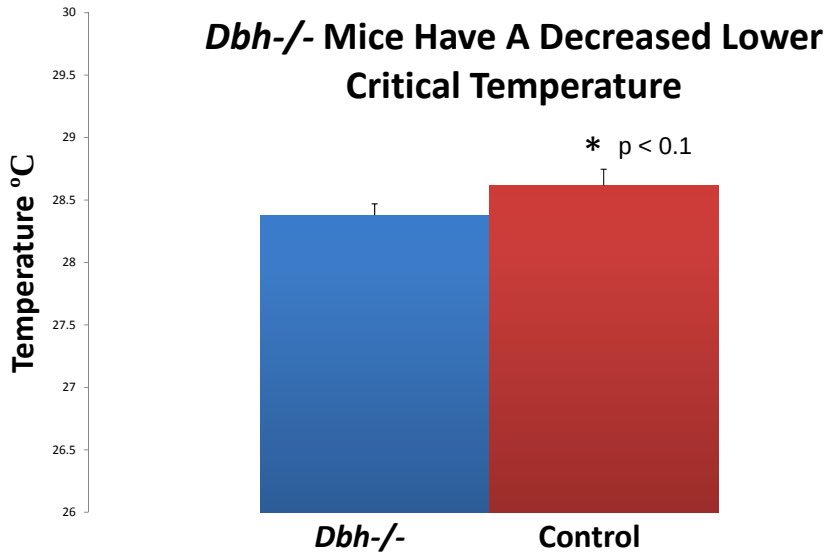

Figure 9. *Dbh-/-* mice have a decreased lower critical temperature in comparison to littermate controls(28.3± 0.1 versus 28.6± 0.1 °C). T_{LC} was calculated as the temperature at which the metabolic rate began to rise in response to decreasing temperature. <*p=0.08>

These results indicate that *D*bh-/- and control mice have very similar metabolic parameters and did not display gross differences in metabolic susceptibility to temperature change within an ambient temperature range of 26-32°C.

High or Low Fat Feeding

As depicted in **Figure 10,** prior to beginning of high or low fat feeding, while all on identical diets and housed at 24-25°C, *DBH-/-* mice were significantly smaller in size than controls (24.8±0.6 versus 28.4±0.3, p<0.001)

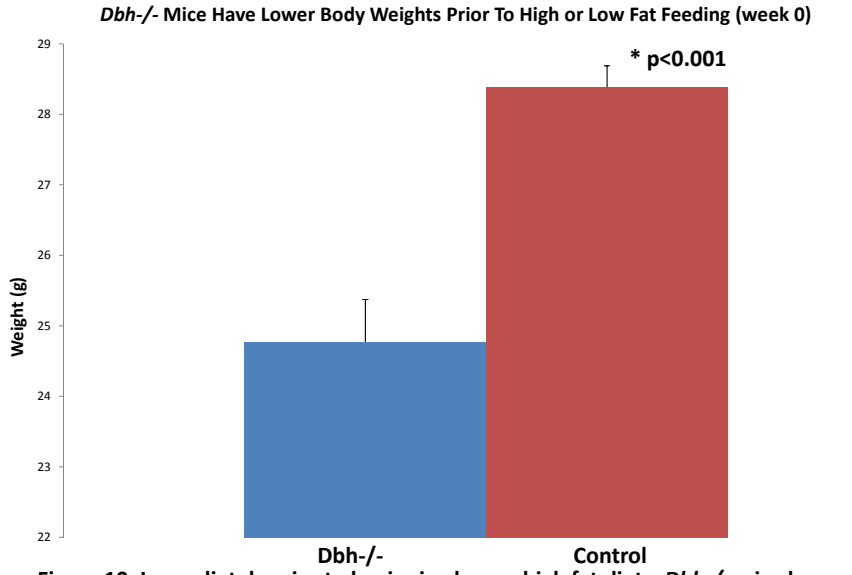

Figure 10. Immediately prior to beginning low or high fat diets, *Dbh-/-* mice have significantly lower body weights than control animals. (p<0.001)

Dbh-/- mice had a lower heart rate at week 0, prior to high or low fat feeding. (**Figure 11.)** *Dbh-/-* mice demonstrated a lower heart rate during both the light and dark cycles and when calculated as a 22 hour average.

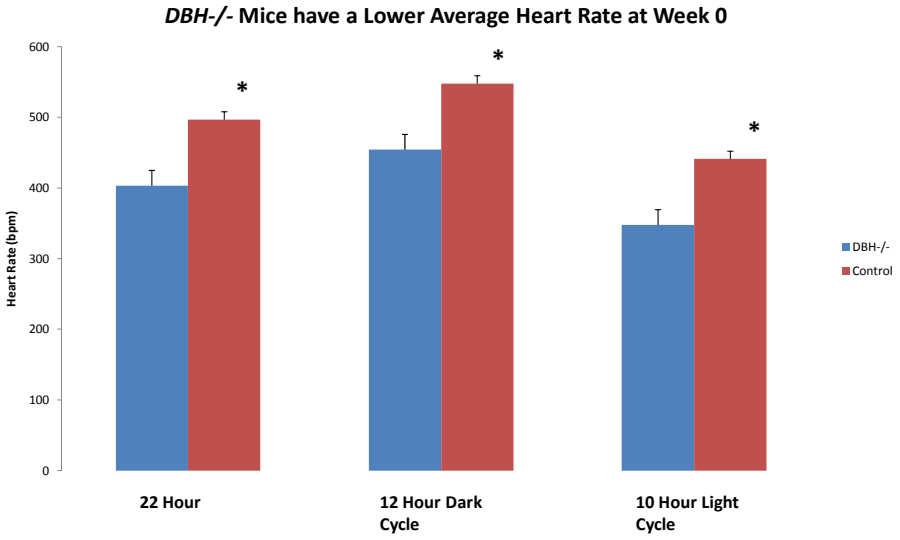

Figure 11. Before the start of high or low fat feeding, *DBH-/-* animals had a lower average heart rate(Bpm). Average heart rate was calculated for the entire 22 hour testing period, for the 12 hour dark cycle, and for the 10 hour testing period during the light cycle. * P< 0.001.

*D*bh-/- mice also demonstrated lower activity levels as compared to control animals during the dark cycle at week 0 prior to high or low fat feeding. (5.94 ± 1.2 versus 9.08 ± 1.4, **Figure 12.)** *DBH-/-* mice were significantly less active than controls ($P < 0.05$) when average activity levels of the 12 hour dark cycle (5.9 ± 1.2 versus 9.1 ± 1.4) and the entire 22 hour sampling period (4.3 ± 0.8 versus 6.2 ± 0.8) were calculated. However, *DBH-/-* mice displayed normal activity levels during the 10 hour light cycle sampling period (2.5 ± 0.7 versus 3.1 ± 0.5).

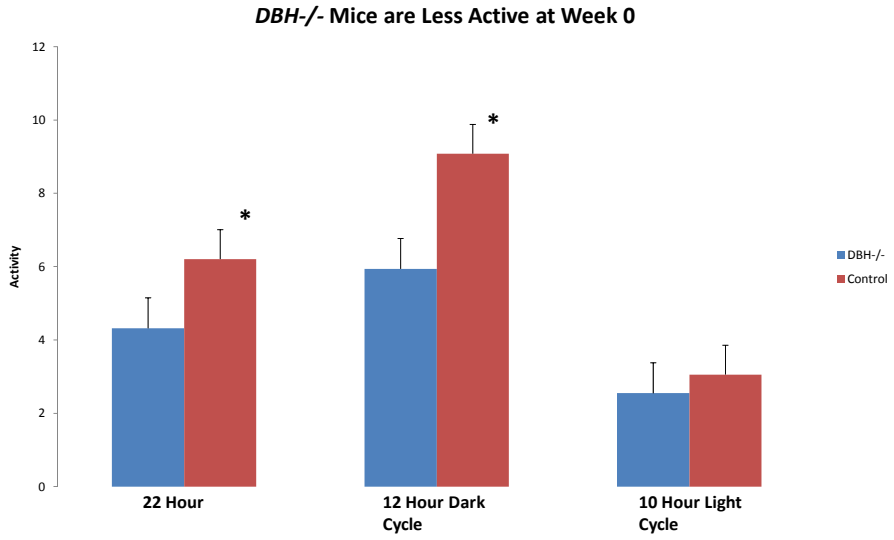

Figure 12. Before the start of high or low fat feeding, *DBH-/-* animals had a lower average activity level. Average activity level was calculated for the entire 22 hour testing period, for the 12 hour dark cycle, and for the 10 hour testing period during the light cycle. * P< 0.05.

DBH-/- and control mice had similar body temperatures at week 0 prior to high or low fat feeding over the 22 hour sampling period ((37.1 ± 0.1 °C versus 37.4 ± 0.9 °C.). (**Figure 12.)**

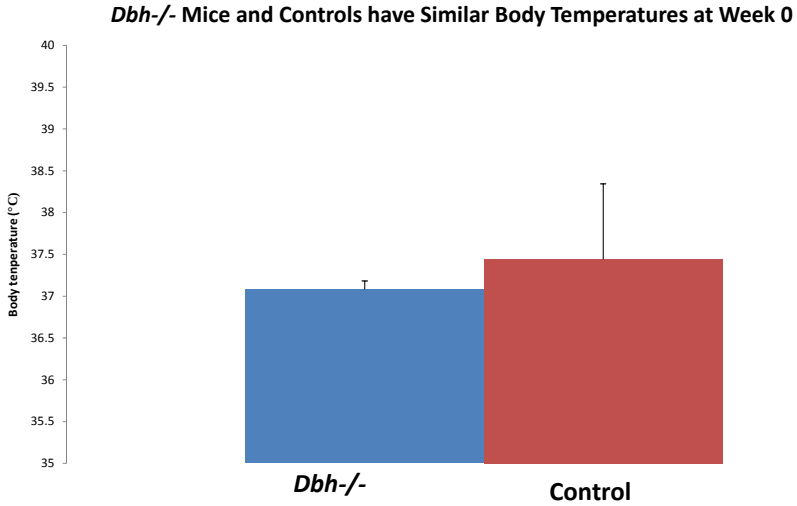

Figure 12. Before the start of high or low fat feeding, *Dbh-/-* animals had equal body temperatures. Average body temperatures were calculated over the entire 22 hour testing period.

Dbh-/- and control mice consumed equal number of calories on a low fat diet over a 24 hour period at week 0 (0.65 ± 0.06 kcal/g versus 0.75 ± 0.09 kcal/g, **Figure 13.)**.

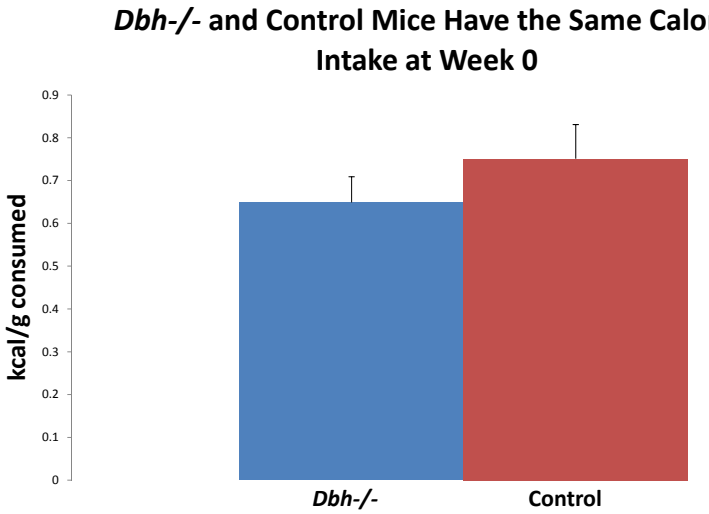

Figure 13. *Dbh-/-* and control mice consumed equal number of calories on a low fat diet over a 24 hour period at week 0. Values represent kcal/g consumed over the 24 hour period.

Mice were placed on a high or low fat diet and body weights were monitored over the course of 16 weeks. (**Figure 14.)**

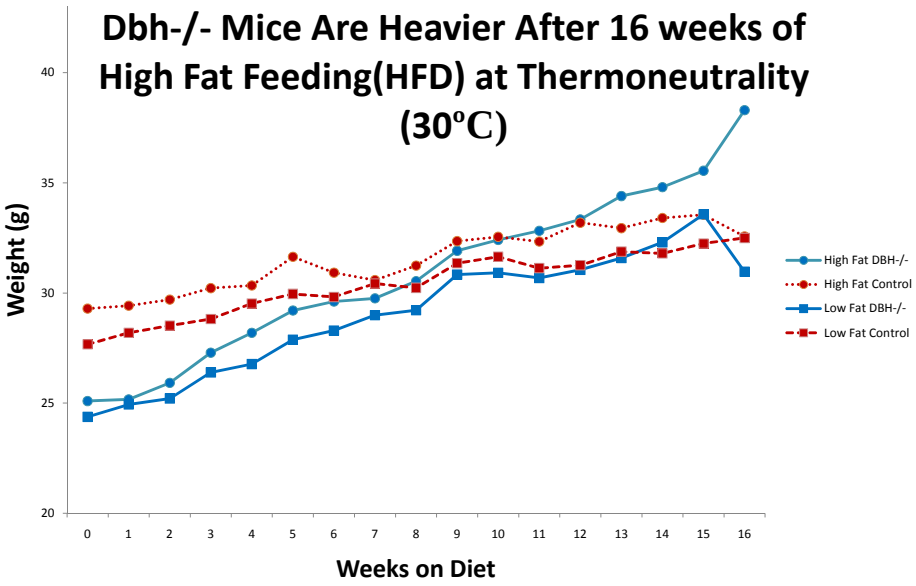

Figure 14. Response of *Dbh-/-* mice to a high fat diet over 16 weeks. *Dbh-/-* mice (blue line) and littermate controls(red line) were fed either a low fat diet(squares)or a high fat diet (circles).For the final four weeks of high or low fat feeding(after week 12) the *Dbh-/-* mice on the HFD exhibited higher body weights.

After 15 weeks of high or low fat feeding, diet did not have a significant effect on body weight in control or *Dbh-/-* mice. **(Figure 15.)**

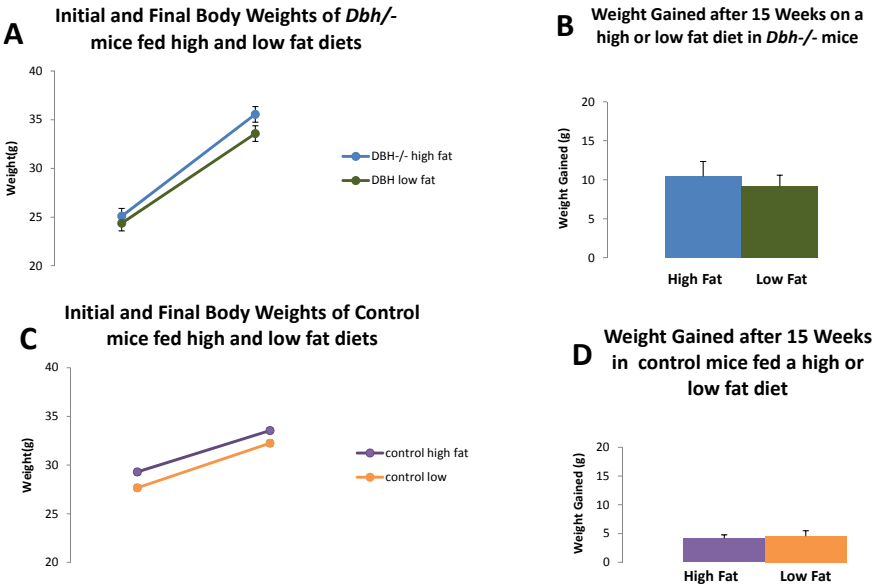

Figure 15. Initial and final body weight of *Dbh-/-*(A) and control mice (C) after 15 weeks of high or low fat feeding and the total change in body weight over 15 weeks of feeding in *Dbh-/-*(B) and control (D) mice. No difference in body weight or weight gained was seen in between the mice fed high and low fat diets in each group.

Dbh-/- animals fed both a high and a low fat diet displayed similar profiles of weight gain. Body weights at week fifteen were not significantly different between *Dbh-/-* mice fed a high fat diet and a low fat diet. (35.6±2.4 versus 33.6±1.3, respectively). *Dbh-/-* mice fed a high fat diet gained an average of 10.5 ± 1.9 g while knockout mice fed a low fat diet gained an average of 9.2 ± 1.4 g. Control animals fed a high fat diet showed an average final body weight at week fifteen of 33.6 ± 1.0 g and gained an average of 4.2 ± 0.6 g. Control animals fed a low fat diet had an average final body weight at week fifteen of 32.3 ± 1.0 g and gained an average of 4.6 ± 0.9 g during the fifteen weeks on the diet. Diet did not have a significant effect on body weight in control mice over the 15 week feeding period.

However, the *Dbh-/-* mice gained significantly more weight over the 15 weeks than control animals, regardless of diet. (**Figure 16.)**

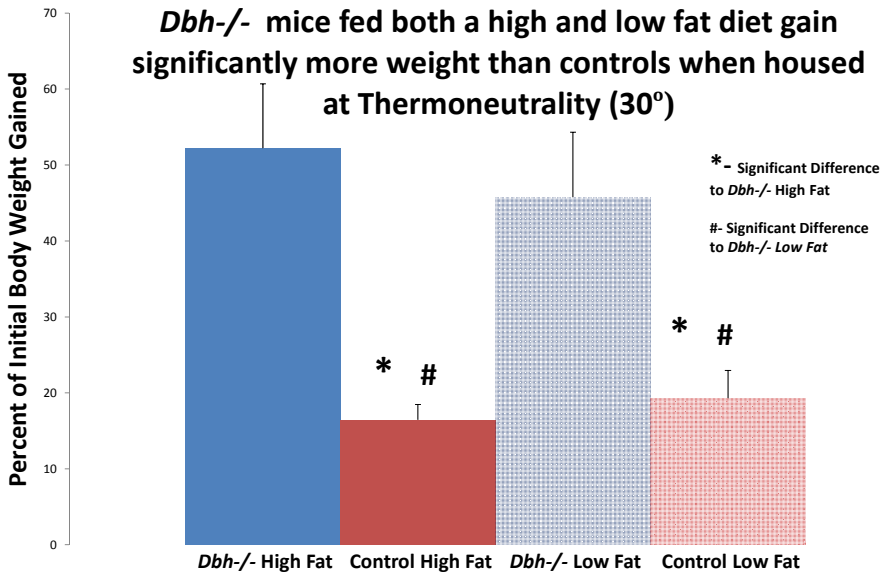

Figure 16. Percentage of initial body weight gained in *Dbh-/-* mice and littermate controls fed a high or low fat diet. Initial measurements of body weight were taken on the day before high or low fat feeding began and final measurements were taken at the end of week 15 of low or high fat feeding. (*p-values *Dbh-/-*high fat > 0.01, # p-values *Dbh-/-* low fat >0.05)

The *Dbh-/-* mice fed a high fat diet gained significantly more weight than controls fed either a high or low fat diet(52.2 ± 8.4 % versus 16.4 ± 2.0% and 19.3 ± 3.7 % of initial body weight, p<0.01.) The

Dbh-/- mice fed a low fat diet gained significantly more weight than control animals fed either a high or low fat diet. (45.8 ± 8.5 % versus 16.4 ± 2.0% and 19.3 ± 3.7 % of initial body weight, $p<0.05$.)

Diet had no effect on specific basal metabolic rate after 12 weeks of high or low fat feeding in both *Dbh-/-* and control mice. (**Figure 17.)**

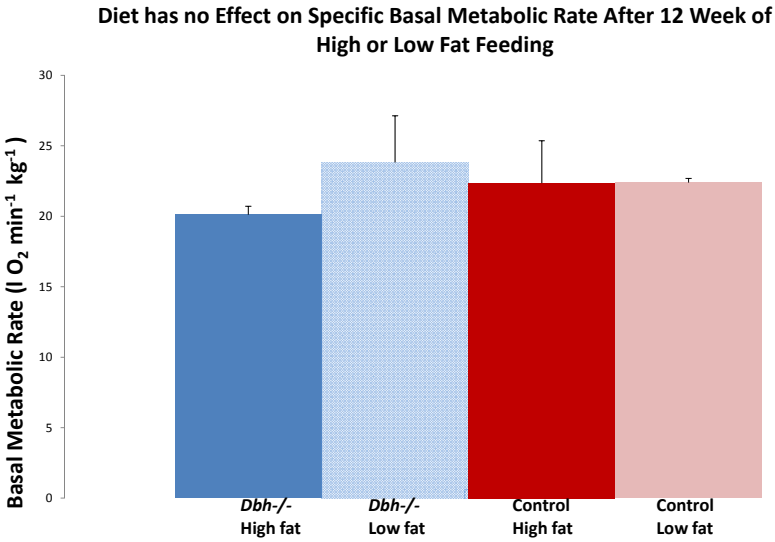

Figure 17. After 12 weeks of feeding, *Dbh-/-* and control animals fed a low or high fat diet show no difference in mass dependent basal metabolic rate. Values represent average metabolic rate recorded over 24 hours.

Table 1. summarizes the average body temperature, activity levels, and heart rate in mice after 11 weeks of high or low fat feeding.

Table 1. Body Temperature, Activity Level, and Heart Rate in *DBH-/-* and Control Mice fed a High or Low Fat Diet for 11 weeks.

Experimental Group	***DBH-/-* High Fat**	***DBH-/-* Low Fat**	**Control High Fat**	**Control Low Fat**
Body Temperature (°C)(22 hours)	37.4 ± 0.1°C	37.5 ± 0.1°C	37.0 ± 0.3°C	37.2 ± 0.1°C
Activity Levels (Dark Cycle)	7.85 ± 0.8	12.83 ± 3.4	10.55 ± 1.8	10.35 ± 1.4
Heart Rate (bpm) 22 Hours	472.6 bpm	481.6 ± 20.9 bpm	536.7 ± 59.9 bpm	465.7 ± 17. 2 bpm

No significant difference in the above parameters was observed between the various experimental groups with the exception of activity levels in *Dbh-/-* mice. *Dbh-/-* mice fed a low fat diet were more active than *DBH-/-I* animals fed a low fat diet during the 12 hour dark cycle (12.83 ± 0.97 versus 7.85 ± 3.4, **Figure 18.)**

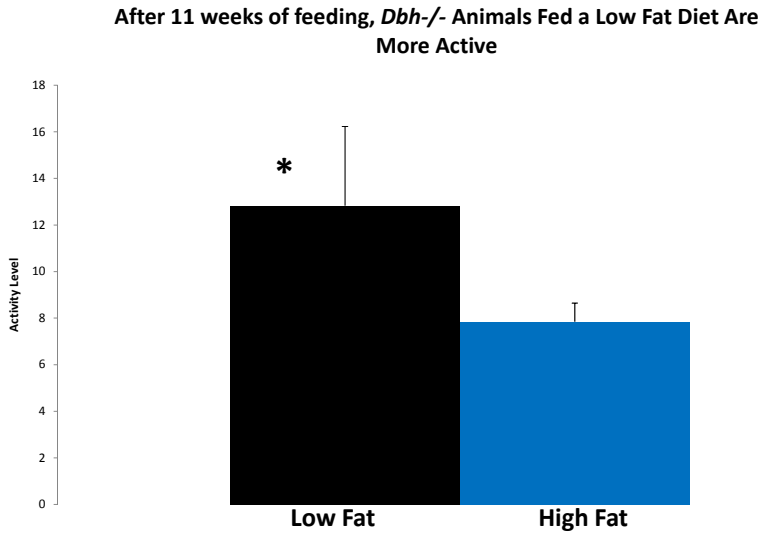

Figure 18. After 11 weeks of feeding, *Dbh-/-* animals fed a low fat diet are more active than *Dbh-/-* animals fed a high fat diet. Values represent average activity levels recorded over the 12 hour dark cycle. * P < 0.05

Caloric intake remained the same between *Dbh-/-* and control mice at week 4 (0.25 ± 0.03 kcal/g versus 0.35 ± 0.08 kcal/g) as well as at week 11 (0.45 ± 0.07 kcal/g versus 0.43 ± 0.06 kcal/g). **Figure 19** displays caloric intake of mice fed a high or low fat diet at week 4 and week 11. At week 4, mice fed a high and low fat diet had equal caloric intakes. (0.26 ± 0.01 kcal/ g versus 0.34 ± 0.09 kcal/g). However, at week 11, mice fed a low fat diet consumed significantly more calories than mice fed a high fat diet (0.28 ± 0.03 kcal/g versus 0.58 ± 0.04 kcal/g, $p < 0.01$).

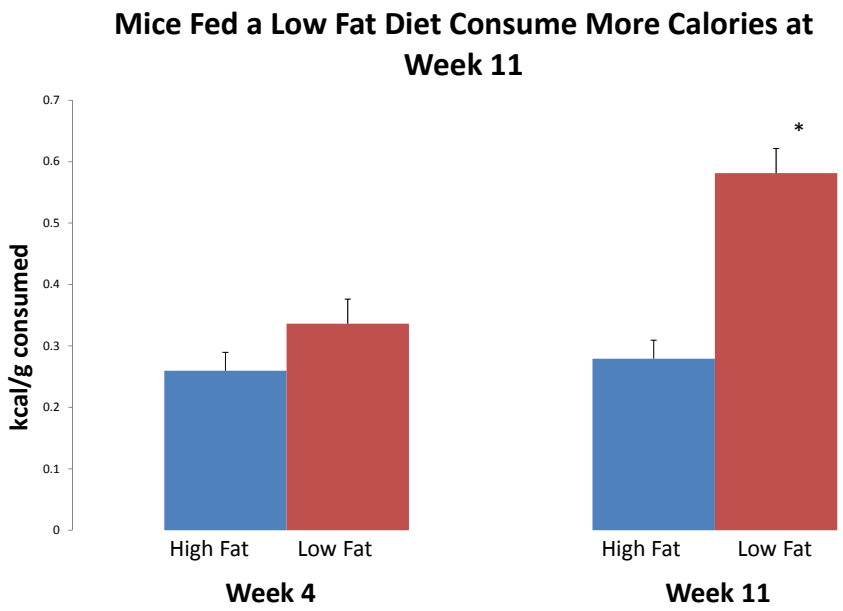

Figure 19. Mice fed a high or low fat diet consume equal calories at week 4 but mice fed a low fat diet consume more calories at week 11 ($p < 0.01$) Values represent kcal/g consumed over a 24 hour period.

Shivering and Resting Metabolic Rate at 20°C

To further characterize the metabolic profile of the experimental groups, resting metabolic rate was measured at 20°C. The resting metabolic rate of *Dbh-/-* was not significantly different from control animals. (**Figure 20.)**

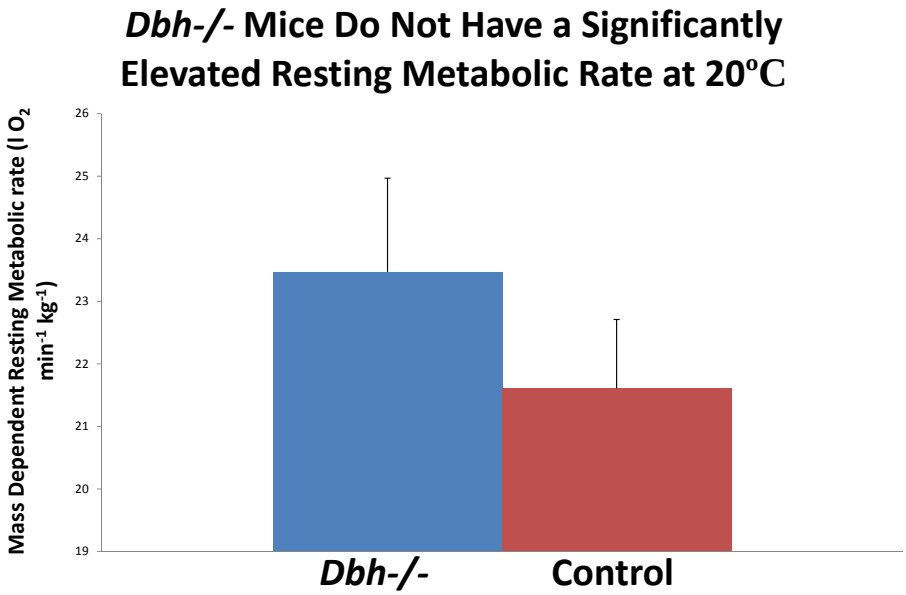

Figure 20. *Dbh-/-* mice placed at 20ºC have a slightly elevated metabolic rate. Measurements represent average oxygen consumption and were taken over a 24 hour period. Only those values collected during the light cycle were used in the calculation.

Dbh-/- mice had an average resting metabolic rate of 23.5 ± 1.5 l O_2/ kg min while control animals had an average resting metabolic rate of 21.6 ± 1.1 l O_2/ kg min ($p<0.17$). While this result was not significant, *Dbh-/-* trended towards having an elevated resting metabolic rate and this result may become significant if a larger sample size were available.

Shivering was quantified at 20°C. **Figure 21.** represents a typical ECG tracing of a *DBH-/-* animal. The noise highlighted on the graph represents the electrical signals generated by the involuntary muscle contractions in a shivering animal.

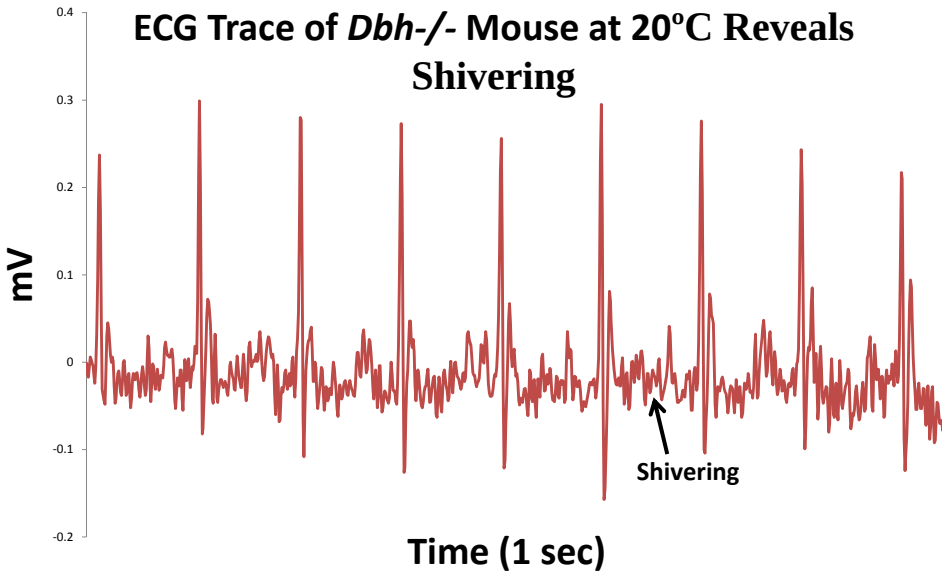

Figure 21. ECG trace of typical *Dbh-/-* mouse over 1 sec time interval. Noise in between QRS waves represent EMG signal of muscle contraction in shivering.

Dbh-/- do not shiver more at 20° in comparison to control animals (13.1 ± 2.7 versus 9.1 ± 2.8, p<1.8). (**Figure 22.)**

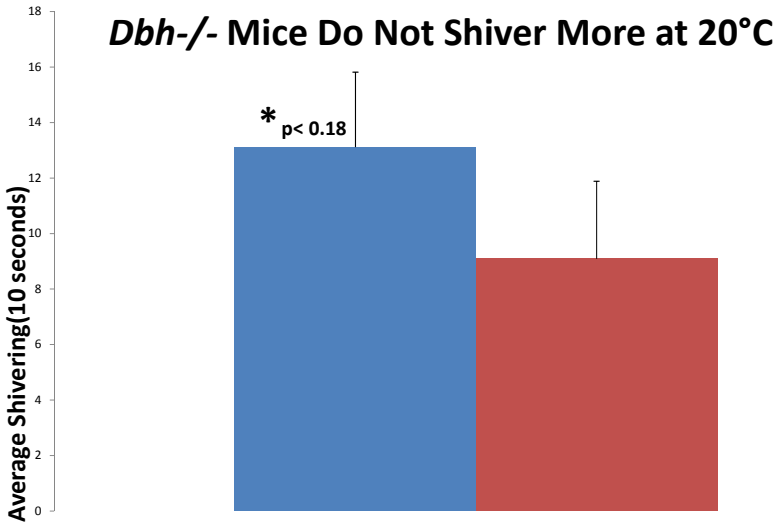

Figure 22. *Dbh-/-* mice shiver more than controls at 20°C. Shivering values represent average shivering rates during 10 second runs over 24 hours.

While this difference does not approach a significant value, small sample size (*DBH-/-* n=3 and control n=4) limited the extent of the shivering analysis. This result may become significant should a larger sample size be used.

Discussion

Dbh-/- mice cannot synthesize norepinephrine and should not be able to activate brown fat through the sympathetic pathway. Without a mechanism to dissipate caloric excess, I hypothesize that these mice will become obese when placed on a high fat at thermoneutrality (30°C).

Most experimental studies looking at the brown fat pathway in mice have been performed at room temperature (20-22°C), which represents thermal stress for these animals. Mice under thermal stress elevate metabolic rate and increase energy expenditure. Animals exposed to concurrent dietary excess and low ambient temperature could hypothetically dissipate the extra calories through the increased metabolic rate caused by thermal stress. Before placement of *Dbh-/-* mice on a high fat diet, the thermoneutral zone and metabolic parameters must be established to ensure the animals are housed at a temperature that allows animals to maintain metabolic rate at a minimum.

Dbh-/- and control mice have similar metabolic profiles with comparable basal metabolic rates. *Dbh-/-* and control animals have a thermoneutral zone of 28-30^{o}C. Animals housed at this temperature do not increase metabolic rate and energy expenditure to maintain body temperature. Previous studies have shown that *Dbh-/-* mice are smaller in size and have an elevated metabolic rate. While the *Dbh-/-* mice in this study were smaller in size at the beginning of the study, they did not show an elevated basal metabolic rate, as BMR values were taken as a function of size, thereby eliminating the discrepancy in metabolism possibly caused by the difference in size. However, *DBH-/-* mice did have a decreased lower critical temperature in comparison to controls. Mice deficient in sympathetic activation of thermoregulation are cold sensitive. As ambient temperature drops, normal thermoregulatory mechanisms cannot be activated and *Dbh-/-* mice must rely on other methods of thermogenesis to defend body temperature. This limited ability to initiate thermogenesis allows *Dbh-/-* mice to

experience lower ambient temperatures without activating the thermogenic mechanisms that increase metabolic rate.

Various physiological parameters in the *Dbh-/-* and control mice were monitored at week 0 before placement of mice at high or low fat diets to establish baseline levels that might affect weight gain later in the experiment.

At week 0, *Dbh-/-* mice were less active and had a lower heart rate than control mice. *Dbh-/-* mice exhibit a low heart rate and hypotension in comparison to controls during baseline conditions and do not raise heart rate and blood pressure in response to acute stress (Swoap et al., 2004). The low heart rate observed in the *Dbh-/-* mice tested in this study is in agreement with previous findings. Additionally, the sympathetic nervous system is involved in an acute stress response. Although entry into the room where mice were housed was kept to a minimum during sampling, it is possible that outside noises or slight changes in room conditions would elicit a temporary stress response in the mice. A sudden stressor would elicit increased movement and a rise in heart rate in control mice but not in the *Dbh-/-* mice. A rise in activity would cause a subsequent rise in heart rate to account for the increased locomotor action. Decreased baseline activity in *DBH-/-* mice, in combination with the hypothesized deficiency in brown fat activation, would only increase the likelihood of weight gain when exposed to caloric excess. Body temperature was comparable in both groups of animals at week 0. Metabolic rate is correlated with body temperature and as specific basal metabolic rate is equal between *Dbh-/-* and control animals, it is expected that body temperature would be similar as well.

At week 0, caloric intake in *Dbh-/-* and control mice is equal. Previous studies have shown that *Dbh-/-* animals display elevated food and water intake. (Thomas and Palmiter, 1998) However, no difference in calories consumed, (expressed in kcal/g) was observed between *Dbh-/-* and control mice.

DBH-/- mice fed a high fat diet and housed at thermoneutrality did not gain significantly more weight than *DBH-/-* mice fed a low fat diet. However, *DBH-/-* animals gained significantly more weight than control animals, regardless of diet.

Body temperature did not differ between groups at the end of 11 weeks of high or low fat feeding. However, DBH-/- mice fed a low fat diet were significantly more active than *DBH-/-* mice fed a high fat diet. The weight gain in *DBH-/-* animals fed a low fat diet can therefore not be explained by decreased locomotor activity. Diet did not have an effect on locomotor activity in control animals.

Caloric intake did not differ between *Dbh-/-* and control animals at week 4 or at week 11. Caloric intake in animals fed a low fat diet, while higher, was not significantly different from the caloric intake of animals fed a high fat diet at week 4. However, at week 11, caloric intake of animals fed a low fat diet was significantly higher than the caloric intake of animals fed a high fat diet. Rats fed a high fat diet become obese, eating larger portions less frequently, than mice fed a control diet (Furnes et al., 2009). However, low-carbohydrate diets have been associated with a reduction in circulating insulin, preventing the development of insulin resistance that occurs with high carbohydrate diets (Perrot et al., 2006). As insulin affects satiety signaling and food intake patterns, the difference in food intake between mice fed a low and high fat diet could be explained by the marked difference in carbohydrate consumption between the high and low fat diets (23.5% kcal from carbohydrates versus 62.7 % kcal from carbohydrates.) Additionally, the timing and calorie consumption of each meal could differ between mice fed a high or low fat diet, also affecting satiety and total calorie consumption over a 24 hour period. However, the decreased food intake is not in agreement with previous studies that show mice fed a high fat diet develop leptin resistance (Thomas et al., 2000), which would result in decreased satiety and increased food intake.

In previous studies, mice deficient in the UCP1 protein and mice deficient in β-3 agonists gained significant weight over controls when fed a high fat diet and housed at thermoneutrality (Bachman et al., 2002. Feldman et al., 2009.). One would expect that mice deficient in NE, the signaling ligand in the brown fat pathway, (**Figure 3)** that were fed a high fat diet at thermoneutrality would gain significant weight over controls. However, this result was not observed. These unexpected results could be explained by one or more of many possibilities.

1. Animals fed a low fat diet consumed more calories per gram body weight by the end of the study. Therefore, the dietary excess induced by the high fat diet would be matched by the caloric excess in mice fed a low fat diet. This increased caloric intake in mice fed a low fat diet would induce weight gain in the *Dbh-/-* mice which theoretically have limited capability to disspate caloric excess through brown fat activation. Both groups of *Dbh-/-* would gain weight. Both groups of control animals would resist excessive weight gain by activation of brown fat and diet-induced thermogenesis. As control and *Dbh-/-* animals demonstrated comparable caloric intakes within each of the two diets, it could be concluded that caloric excess could not be attenuated in the *Dbh-/-* animals and that NE is essential for dissipation of caloric excess.
2. As the *Dbh-/-* mice fed both a low or high fat diet gained large amounts of weight throughout the study, it is possible that movement to a warmer environment caused this weight gain. *Dbh-/-* animals must work harder to defend body temperature at a lower ambient temperature as they are deficient in vasoconstriction and piloerection. Brown fat mediated non-shivering thermogenesis is impaired as well. These animals must rely on shivering to generate heat at a low ambient temperature, resulting in elevated metabolic rates. Upon long-term exposure to an ambient temperature within their thermoneutral zone, they are no longer under thermal stress. A large decrease in energy expenditure would be observed, resulting in significant weight gain regardless of diet. The *Dbh-/-* animals fed a high fat diet could very well be deficient in activation

of brown fat and susceptible to weight gain on a high fat diet; however, it is possible that the weight gain induced by movement to a warmer ambient temperature masks the weight gain induced by the high fat diet. This would explain why 1) the *Dbh-/-* mice fed a high fat diet did not gain more weight than knockout animals fed a low fat diet and 2) why both groups of *Dbh-/-* mice gained significantly more weight than controls, for whom the thermal stress was less than at the 23-24^{o}C in which they were originally housed. Animal number was limited, so it was not possible to form control groups of animals housed at 22°C and fed low or high fat diets to see if similar patterns of weight gain occurred at a low ambient temperature. However, if *Dbh-/-* mice fed a high fat diet and housed at 22°C gained more weight than those fed a low fat diet at 22°C, one could conclude that movement to thermoneutrality is correlated with increased body weight in *Dbh-/-* mice.

Significant weight gain in mice deficient in β3-receptors or UCP1 protein fed a high fat but not a low fat diet at thermoneutrality could be explained by a decreased cold-sensitivity in animals that produce NE. Although these animals cannot activate NST, they can still mediate vasoconstriction and piloerection to defend body temperature. They would not need to elevate metabolic rate as high at a cool ambient temperature, making the decrease in energy expenditure upon movement to a thermoneutral temperature less severe. The change in temperature would have a smaller effect on the energy balance of mice lacking β-adrenergic receptors and UCP1-/- mice than *Dbh-/-* mice.

3. Finally, these results could indicate that these *Dbh-/-* mice employ other unknown mechanisms for dissipation of caloric excess and/or that norepinephrine is not essential for brown fat activation. Some other ligand or signal could be activating uncoupling protein-1 in brown fat. Very little is known about other members of the uncoupling protein family (uncoupling proteins 2 and 3), found mainly in heart and muscle tissue. While these proteins are thought to decrease

reactive oxygen species and minimize cell oxidative damage, an increase in activation of these proteins via a signal other than norepinephrine could theoretically speed up energy metabolism and work to dissipate caloric excess.

Figure 23. represents possible mechanisms for dissipation of caloric excess in mice unable to activate brown fat via norepinephrine.

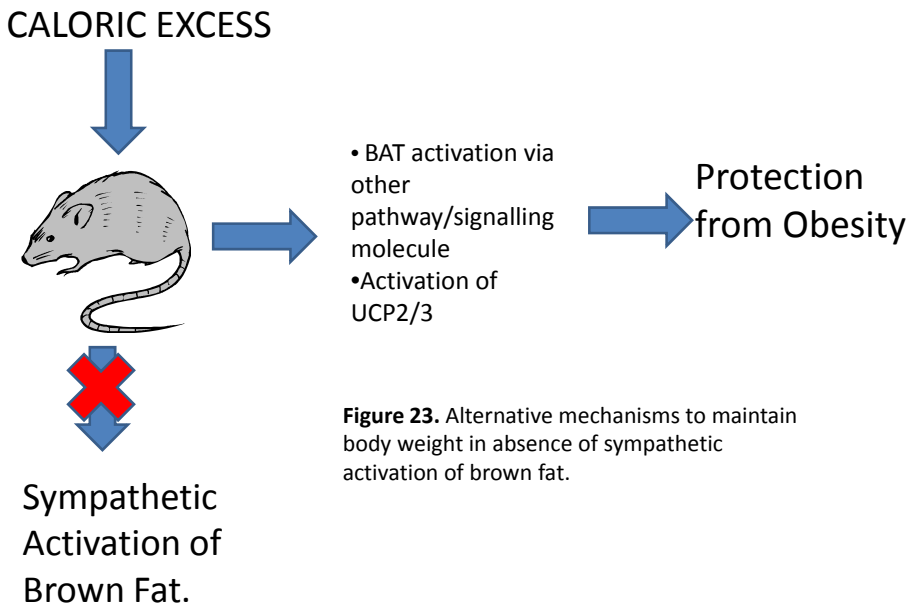

Figure 23. Alternative mechanisms to maintain body weight in absence of sympathetic activation of brown fat.

Although not statistically significant, resting metabolic rate was slightly elevated in the *Dbh-/-* mice at 20°C. This elevation was most likely due to an increased amount of shivering as shivering levels were elevated by a similar magnitude in the *Dbh-/-* animals. *Dbh-/-* animals cannot induce non-shivering thermogenesis nor vasoconstriction to defend body temperature at lower ambient temperatures and so must rely heavily on shivering mechanisms to generate heat. This large amount of shivering would raise resting metabolic rate at a low ambient temperature. Although results were not significant, increasing the sample size would most likely induce a significant difference between *Dbh-/-*

mice and controls in both shivering levels and resting metabolic rate at 20°C. Significant increases in resting metabolic rate and shivering at 20°C in *Dbh-/-* mice would support the above theory that movement of mice to 30°C would diminish shivering levels, lower energy expenditure, and induce possible weight gain.

As the study progressed, animal deaths caused an imbalance in subjects in each of the experimental groups leading to data with large variances and standard error, affecting the significance of results. Repetition of this experiment using larger sample sizes might induce different results. With a larger sample size, one could sacrifice mice throughout the duration of the high or low fat feeding and examine BAT tissue levels and morphology. *Dbh-/-* mice display hypertrophic BAT that contains enlarged lipid vacuoles, suggesting decreased tissue activity (Thomas and Palmiter, 1997). The appearance of hypertrophy in BAT tissue in *Dbh-/-* mice fed a high fat diet at thermoneutrality would suggest that the tissue was inactive and not involved in DIT or dissipation of caloric excess. Additionally, it would be important to house the animals at thermoneutrality from birth as the switch from a cool ambient temperature to 30°C at the beginning of the experiment could affect body weight and metabolism, interfering with the effects of a high fat diet.

Overall, the results of this study were inconclusive as to the role and necessity of norepinephrine in brown fat activation in *Dbh-/-* animals fed a high fat diet and housed at thermoneutrality. Further work using larger sample sizes is needed before concrete conclusions can be obtained.

References

Adam-Perrot A, Clifton P, Brouns F. (2006) *Low-carbohydrate diets: nutritional and physiological aspects*. Obesity Review. 7(1):49-58.

Bachman, ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Koblika BK, and Lowell BB. (2002) *BAR Signalling Required for Diet-Induced Thermogenesis and Obesity Resistance.* Science, 297, 843-845.

Cannon, Walter B. (1932) *The Wisdom of the Body*. (W.W. Norton and Company) New York, NY.

Coleman DL. (1982) *Thermogenesis in Diabetes-Obesity Syndromes in Mutant Mice.* Diabetologia. 22, 205-211.

Commins SP, Marsh DJ, Thomas SA, Watson PM, Padgett MA, Palmiter R, Gettys TW. (1999) *Norepinephrine Is Required for Leptin Effects on Gene Expression in Brown and White Adipose Tissue.* Endocrinology. 140(10), 4772-4778.

Commins SP, Watson PM, Padgett MA, Dudley A, Argyropoulos G, Gettys TW. (1999) *Induction of uncoupling protein expression in brown and white adipose tissue by leptin.* Endocrinology, 140(1), 292-300

Cottle MKW and Cottle WH. (1970) *Adrenergic fibers in brown fat of cold-acclimated rats.* J Histochem Cytochem. 18(2), 116-119

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR. (2009) *Identification and importance of brown adipose tissue in adult humans.* N Engl J Med., 360(15), 1509-17.

Daikoku, T., Shinohara, Y., Shima, A, Yamazaki, N., and Terada, H. (2000) *Specific elevation of transcript levels of particular protein subtypes induced in brown adipose tissue by cold exposure.* Biochimica et Biophysica Acta (BBA), 1457(3), 263-272.

Das UN, Rao AA. (2007) *Gene expression profile in obesity and type 2 diabetes mellitus.* Lipids Health Dis., 6, 35.

Dulloo, A.G.(2002) *A Sympathetic Defense Against Obesity.* Science, 297, 780-1.

Enerbäck S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP. (1997) *Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese*. Nature., 387(6628), 90-4.

Feldman HM, Golozoubova V, Cannon B, Nedergaard J. (2009) *UCP1 Ablation Induces Obesity and Abolishes Diet-Induced Thermogenesis in Mice Exempt from Thermal Stress by Living at Thermoneutrality.* Cell Metabolism. 9, 203-209.

Furnes MW, Zhao CM, Chen D. (2009) *Development of obesity is associated with increased calories per meal rather than per day. A study of high-fat diet-induced obesity in young rats*. Obesity Surgery. 19(10):1430-8

Golozoubova, V, Gullberg, H, Matthias, A, Cannon, B, Vennström, B, and Nedergaard, J. (*Depressed Thermogenesis but Competent Brown Adipose Tissue Recruitment in Mice Devoid of All Hormone-Binding Thyroid Hormone Receptors*. Molecular Endincronology. 18(2), 384-401.

Gordon, C.J. (1993) *Temperature Regulation in Laboratory Rodents*. (Cambridge University Press.) New York, NY.

Granneman, J.G., Burnazi, M., Zhu, Z., and Schwamb, L.A.(2003) *White adipose tissue contributes to UCP1-independent thermogenesis.* Am J Physiol Endocrinol Metab., 285, 1230-1236.

Houseknecht, K.L., Baile, C.A., Matteri, R.L., and Spurlock, M.E. (1998) *The biology of leptin: a review.* J Anim Sci, 76, 1405-1420.

Ibraimova GI. (1997) *Effects of hypoxic and cold stimuli on heat production and catecholamine content in mice.* Aviakosm Ekolog Med., 31(6), 59-63.

Inokuma K, Okamatsu-Ogura Y, Omachi A, Matsushita Y, Kimura K, Yamashita H, Saito M. (2006) *Indispensable role of mitochondrial UCP1 for antiobsity effect of B_3-adrenergic stimulation.* Am J Physiol Endocrinol Metab 290, E1014-E1021.

Kopecký J, Rossmeisl M, Hodný Z, Syrový I, Horáková M, Kolárová P. (1996) *Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology***.** Am J Physiol. 270(5 Pt 1), 776-786

Landsberg, L. (2006) *Feast or Famine: The Sympathetic Nervous System Response to Nutrient Intake.* Cellular and Molecular Neurobiology, 26, 495-506.

Lichtenbelt, W.V.D., Vanhommerig,J. W., Smulders, N.M., Drossaerts, J., Kemerink, G.J., Bouvy, N.D., Schrauwen, P., and Jaap Teule, G.J.(2009) *Cold-Activated Brown Adipose Tissue in Healthy Men.* N. Engl. J. Medicine. 360(18), 1500-1508.

Lin S, Thomas TC, Storlien LH, Huang XF. (2000) *Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice.* Int J Obesity, 24, 639-646.

Lodhi IJ, Semenkovich CF. (2009) *Why we should put clothes on mice.* Cell Metabolism. 9(2), 111-112.

Moyes, CD and Schulte, P.M. (2006) *Principles of Animal Physiology.* (Pearson Benjamin Cummings) San Francisco, CA.

Nedergaard, J., Bengtsson, T., Cannon, B.(2007) *Unexpected evidence for active brown adipose tissue in adult humans* Am J Physiol Endocrinol Metab, 293.

Nedergaard, J. and Cannon, B. (2003) *The 'novel' 'uncoupling' proteinsUCP2 and UCP3: what do they really do? Pros and cons for suggested functions.* Experimental Physiology, 88(1), 65-84.

Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. (2003) *UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency*. Biochim Biophys Acta., 1504(1), 82-106.

Overton T. D. Williams, J. B. Chambers, R. P. Henderson, M. E. Rashotte and J. M. (2002)

Cardiovascular responses to caloric restriction and thermoneutrality in C57BL/6J mice. Am J Physiol Regulatory Integrative Comp Physiol, 282, 1459-1467.

Palou, A, Picó, C, Bonet, M.L., and Oliver, P. *The uncoupling protein, thermogenin.* (1997) Int J. Biochem.and Cell Biology, 30(1), 7-11.

Pelleymounter, MA, Cullen, MJ, Baker, MB, Hecht, R, Winters, D, Boone, T, Collins, F.(1995) *Effects of obese gene product on body weight regulation in ob/ob mice.* Science, 269, 540-543.

Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J., and Pénicaud, L. (1986) *Expression of Uncoupling Protein mRNA in Thermogenic or Weakly Thermogenic Brown Adipose Tissue.* J Biol. Chem., 261, 1390-13910.

Robertson D, Haile V, Perry SE, Robertson RM, Phillips JA. (1991) *Dopamine B-hydroxylase deficiency: A genetic disorder of cardiovascular regulation.* Hypertension. 18, 1-8.

Schmidt-Nielsen, K. (1997) *Animal Physiology; Adaption and Environment*. (5th Ed. Press Syndicate University of Cambridge) Cambridge, UK.

Ste. Marie L, Palmiter RD. (2003) *Norepinephrine and epinephrine-deficient mice are hypoinsulinemic and have lower blood glucose.* Endocrinology. 144(10) 4427-4432.

Ste Marie L, Luquet S, Curtis W, Palmiter RD. (2005) *Norepinephrine- and epinephrine-deficient mice gain weight normally on a high fat diet.* Obesity Research. 13, 1518-1522.

Swoap SJ, Weinshenker D, Palmiter RD, Garber G. (2004) *Dbh(-/-) mice are hypotensive, have altered circadian rhythms, and have abnormal responses to dieting and stress.* Am J Physiol Regul Integr Comp Physiol. 286(1):R108-13

Thomas SA, Marck BT, Palmiter RD, Mastumoto AM. (1998) *Restoration of norepinephrine and*

reversal of phenotypes in mice lacking dopamine B-hydroxylase. J Neurochemistry. 70(6), 2468-2476.

Thomas SA and Palmiter RD. (1998) *Examining adrenergic roles in development, physiology, and behavior through targeted disruption of the Mouse Dopamine B-Hydroxlase Gene.* Advances in Pharmacology. 42, 57-60.

Thomas SA, Palmiter RD.*Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline.* Nature. 387(6628), 94-7

Ukropec J, Anunciado RVP, Ravussin Y, and Kozak LP. (2006) *Leptin is Required for Uncoupling Protein-1-Independent Thermogenesis during Cold Stress.* Endocrinology, 147(5), 2468-2480.

U.S. Obesity Trends. http://www.cdc.gov/obesity/data/trends.html#County

Young, J.B., Saville, E., RothwellL,N.J., Stock, M.J., and LANDSBERg, L. (1982*) Effect of Diet and Cold Exposure on Norepinephrine Turnover in Brown Adipose Tissue of the Rat.* J Clin Invest, 69(5), 1061-1071.

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