# The conflict of thermogenic responses in CD-1 mice to chronic hypoxic and cold environments

By

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TITLE: The conflict of thermogenic responses in CD-1 mice to chronic hypoxic and cold environments

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**ABSTRACT**

Surviving at high-altitudes requires organisms to endure the combined stresses of cold temperature and low atmospheric oxygen. Maintaining a stable body temperature ($T_b$) is a constant battle that endotherms must defend when they are subjected to temperatures beyond their thermal neutral zones (TNZ). Once outside their TNZ, they elicit mechanisms of adaptive thermogenesis which stimulate heat production via non-shivering (NST) and shivering thermogenesis (ST). These processes are carried out in brown adipose tissue (BAT) and skeletal muscle, respectively. Uncoupling proteins (UCP) carry out mechanisms of NST by uncoupling substrate oxidation from ATP synthesis to generate heat. Many studies have examined responses in endotherms to cold temperature acclimations as well as episodes of acute hypoxia. However very few studies have looked at the chronic effects of hypoxia and even less have investigated the combined effects of cold and hypoxia acclimations. Therefore, to address this issue we examined the chronic effect of four weeks of exposure to hypobaric hypoxia (H, 480mmHg), cold (C, 5°C) and the combination of the two stressors (HC) compared to normoxic thermoneutral controls (N, 28°C) in CD-1 mice. Overall we found that HC mice had significantly lower $T_b$ after periods of acclimation while still residing in hypoxia compared to all other treatment groups. However, experiments during acute temperature exposures in normoxia revealed that HC mice were able to increase $T_b$ as well as they demonstrated higher rates of thermal conductance at high ambient temperatures. Investigations into BAT and UCP regulation revealed that HC had a greater amount of BAT mass compared to

controls and lower UCP1 mRNA expression in BAT but no differences in UCP1 protein content. However, HC had lower mitochondrial density indicated by lower activity of citrate synthase in BAT as well as UCP3 mRNA expression. Together results from cold mice suggest that they exhibit an enhanced NST capacity through the up-regulation of BAT mass and UCP1 mRNA expression whereas hypoxia inhibits NST response by down-regulation of UCP1 and mitochondrial density. Moreover the acclimation to both hypoxia and cold may create barriers on an animal's ability to up-regulate NST in hypoxia by decreasing mitochondrial density, UCP1 and 3 mRNA expressions but still allows BAT mechanisms to generate heat through UCP1 protein that regulates $T_b$ in normoxia.

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## THESIS ORGANIZATION AND FORMAT

The format of this thesis is organized in a sandwich format approved by supervisory committee members and consists of four Chapters. Chapter one comprises an introduction and summary of the major findings in the literature and overall significance of the research conducted. Chapter two consists of a peer-reviewed manuscript. Finally, Chapter three concludes major findings of the study and relevance to recent studies in the literature and further suggests directions for future research.

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| **Chapter 4:** | | **References** |

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## ABBREVIATIONS

BAT, brown adipose tissue; C, cold; H, hypoxia; COX, cytochrome c oxidase; CS, citrate synthase; HC, hypoxic/cold; N, normoxic control; NE, norepinephrine; NST, non-shivering thermogenesis; PPAR gamma co-activator 1α; PGC-1α; RMR, resting metabolic rates; ST, shivering thermogenesis; $T_a$, ambient temperature; $T_b$, core body temperature; TNZ, thermal neutral zone; UCP1, uncoupling protein 1; $VO_2$, oxygen consumption; $VCO_2$, carbon dioxide production.

## Chapter 1

# INTRODUCTION

Maintaining physiological homeostasis within an environmental niche is a constant battle that an organism must endure. Very few environments challenge organisms with the combination of both low ambient temperature ($T_a$), that can be damaging to the tissues and low oxygen availability that places limits on oxidative metabolism. However, both of these environmental stressors exist at high altitudes (HA) which drive native organisms to adapt and evolve ultimately protective phenotypes to ensure survival.

The study of HA adaptations has been an ongoing research field for many years with various areas of interest using both humans and animals as experimental models. HA environments are extremely unique in that very few environments pose similar physiological challenges to organisms that combine both low ambient temperatures and low oxygen availability. The combination of these two stressors may greatly affect the way an organism controls and regulates thermogenic mechanisms. Cold exposure exerts pressure upon the individual to minimize heat loss and maximize heat production whereas hypoxia burdens the tissues with lower oxygen availability and demands efficient usage of oxygen. Therefore, this study's main objectives are focused on the combination of two extreme environmental stressors, hypoxia and cold and how acclimation may improve or limit an animal's ability to thermoregulate under these conditions.

## I. Thermoregulation

Trying to achieve an equilibrium between energy intake and energy output is a constant struggle that organisms combat to sustain total neutral energy balance.

**Total Energy Balance = Energy Intake - Energy Output + Energy Stored** (1)

According to equation 1 (Swinburn and Ravussin, 1993), energy taken in by the ingestion of food must be balanced by energy expended either by physical activity or thermogenesis to obtain total neutral energy balance. Otherwise an imbalance of this system can lead to negative or positive energy shifts that leave the body in a state of weight loss or gain, respectively. In endotherms, up to 95% of resting energy expenditures are spent on maintaining a balance between varying $T_a$ and a constant body temperature ($T_b$) (Hill et al, 2004). The thermal gradient between the organism's body and the surrounding environment increases the rate of heat loss to the environment as $T_a$ decrease below $T_b$ and vice versa if $T_b$ is lowered more than $T_a$. These energy costs are quite extreme compared to other organisms such as ectotherms or poikilotherms that rely mostly on behavioural tactics that significantly reduce thermoregulation costs. Furthermore, endotherms are usually found in smaller body sizes which greatly complicate the balance between thermal regulation and the surrounding environment. As the rate of heat loss is directly proportional to the total body surface area of an organism- high surface area to volume ratio found in smaller organisms greatly increases the

rate of heat lost to the surrounding atmosphere and creates immense energy costs in thermoregulation (Davenport, 1992). Advantageously compared to ectotherms and poikilotherms, endotherms are structurally designed to reduce thermal regulation challenges to combat unavoidable energy expenditures. Firstly, endotherms are able to maintain a more 'active' metabolism as they have larger sustainable organs that help to contribute to an increase in energy transfers per unit of time (Else and Hulbert, 1981). Secondly, they generate a large amount of inefficient metabolic heat by contraction of skeletal muscle that is used advantageously to offset the release of heat dissipated to the surrounding environment (Woledge, 1989). Conclusively, endotherms are highly unique in that they have adapted specific integrative thermoregulating techniques that help balance heat lost to the environment during cold $T_a$ that basal thermogenesis merely cannot match.

## II. Thermal Neutral Zone

Maintaining a normal $T_b$ set point is vital to all mammals in that it ensures proper enzyme functioning, diffusion abilities, and also avoids consequences of hypothermia (Chaffee and Roberts, 1971). Thermogenesis occurs in endotherms when $T_a$ decreases below an organism's thermal neutral zone (TNZ) (Figure 1.1). It is regulated by feed-forward afferent signals from cutaneous thermoreceptors that report to the preoptic area (POA) which is found in the rostral pole of the hypothalamus (Morrison et al, 2008). Interestingly, a drop in $T_a$ below skin

temperature signals thermoregulatory centers to reduce skin temperatures without affecting brain or heart tissue temperatures, preventing a fall in $T_b$ (Bratincsak and Palkovits, 2004). The range of temperatures in the TNZ is defined by a constant metabolic rate which is bordered by an upper and lower critical temperature (UCT or LCT) (Klaus et al, 1998). Therefore, as $T_a$ surpass the limits of the UCT or LCT organisms increase their metabolic rates to keep $T_b$ within a normal range.

## III. Obligatory Thermogenesis

Mechanisms of thermogenesis can be divided into two main categories of thermogenesis: simple obligatory (basal) and facultative thermogenic mechanisms. Obligatory thermogenesis maintains $T_b$ with minimal energetic costs and is the main source of heat production within the TNZ. This is achieved by passive mechanisms such as the development of a thick protective pelage, skin, and manipulation in postural positioning (Porter et al, 1994). Obligatory thermogenesis is controlled by thyroid hormones that stimulate the mobilization of necessary fuels from the breakdown of lipids, proteins and carbohydrates that contribute to metabolic heat (Silva, 1995). Although the exact role of the thyroid hormone in basal metabolism is controversial, it has been shown to affect other areas of thermogenesis especially in cold acclimation studies where it contributes to the up-regulation of muscle contractions and increases in $Na^+/K^+$-ATPase content which are not strictly related to obligatory thermogenic mehcnaims

(Simonides and Hardeveld, 1986). However, it is clear that these hormones substantially contribute to basal thermogenesis by providing essential substrates and initiating growth of pre-adipocytes necessary for the up-regulation of thermogenesis (Silva, 2006).

## IV. Facultative Thermogenesis

As $T_a$ drops below the LCT heat-conserving basal thermogenic mechanisms such as cutaneous vasoconstriction or piloerection, no longer is sufficient in regulating $T_b$ and endotherms are forced to become metabolically active (Hill et al, 2004) (Figure 1). This initiates the up-regulation of facultative or adaptive thermogenic mechanisms that serve to match the rate of heat loss to the environment and is reflected in an increase in oxygen consumption that happens almost instantaneously (Hill et al, 2004). As $T_a$ lowers, the most immediate response in facultative thermogenesis is the onset of shivering by the contraction of skeletal muscle where no useful work is produced (Himms-Hagen, 1985). Furthermore, longer periods of cold temperature exposure induces the up-regulation of adaptive thermogenic pathways referred to as non-shivering thermogenesis (NST) that are carried out by brown adipose tissue (BAT) found in rodents (Foster, 1984). The response of this thermogenic organ can be modulated by chronic exposure to cold environments which greatly enhances the capacity of NST and ultimately prolongs cold weather survival and thus is referred as an adaptive mechanism that helps to regulate $T_b$ (Lowell and Spiegelman, 2000).

## V. Shivering Thermogenesis

As previously mentioned, shivering is an instantaneous reaction in response to cutaneous cold afferent signals that stimulate $\alpha$-motor neuron receptors along skeletal muscles which initiate muscular contractions (Nakamura, 2008). Generally, shivering thermogenesis (ST) is termed as a source of metabolic heat that is generated inefficiently as no useful work is produced due to the substantial amount of ATP used to fuel cross-bridge cycling and calcium ion sequestration during muscular contractions (Jubrias et al, 2008 and Cannon and Nedergaard, 2004). Therefore, ATP supplies are short lived as the demand by muscles rapidly depletes sources and results only as an acute thermogenic response to cold temperatures. Most cold acclimation studies overlook the contribution of ST to facultative thermogenesis because previous reports have found little change in ST response after long term exposure to cold temperatures (Van Sant and Hammond, 2008). However, contrary to these recent studies it has previously been shown in the leaf-eared mouse (*Phyllotis xanthopygus* muridae) that ST can greatly increase its thermogenic response as much as 2-fold after cold acclimation exposure (Nespolo et al, 2001). This study by Nespolo et al (2001) is currently one of the only examples of a significant contribution of ST to increased thermogenic capacity and could be the result of an increase in skeletal muscle training. For example, as demonstrated in Davis et al (1960) skeletal muscle shivering continues until complete replacement by NST mechanisms. This continued muscle shivering over an extended period of time greatly improves

muscle shivering capacity as it is essentially a form of muscle exercise and stimulates endurance training. Over time with continuous stimulation, muscles become trained to withstand longer periods of contractions without fatigue (Cannon and Nedergaard, 2004). Although overall thermogenic capacities after acclimation or acclimatization may appear to be unaltered (Van Sant and Hammond, 2008), these studies may overlook important factors that are assumed to be constant in experimental conditions that could affect ST capacity. Only electromyography measurements can truly identify changes in ST capacity and therefore, it is still very important that studies consider all aspects of energy expenditures when observing changes in adaptive thermogenesis, especially those produced by shivering in skeletal muscle.

## VI. Non-shivering Thermogenesis

Interestingly, after a week exposure in cold temperatures animals completely abolish the use of ST and mostly rely on NST mechanisms to maintain body heat (Depocas et al, 1956, Davis et al, 1960). As $T_a$ drops below the range of the TNZ, shivering thermogenesis can no longer match the requirement for metabolic heat production. NST becomes a necessity to maintain $T_b$ and results in a gradual elevation in oxygen consumption as $T_a$ becomes increasingly lower (Sellers et al, 1954). NST can be defined as, any thermogenic process that does not occur through shivering, irrespective of whether it replaces shivering or not (Cannon and Nedergaard, 2004).

In this respect, NST is broadly described and can also be found during basal metabolism- as there is no muscle contraction during this type of thermogenesis. Therefore, to further clarify NST mechanisms this report will define NST as a mechanism of thermogenesis that does not require the contribution of shivering thermogenesis but exists only at $T_a$ outside the range of the TNZ.

NST capacity is highly sensitive to the length of cold acclimation periods which in turn is directly proportional to the growth in BAT mass (Smith and Hock, 1963). Therefore, BAT is known as the major site of adaptive NST capacity and has been found to contribute more than 60% to adaptive thermogenic mechanisms and greatly increases metabolic rate during cold temperature exposures (Foster, 1984).

**a. Brown Adipose Tissue (BAT)**

BAT is found in deposits around the body of rodents particularly in the intrascapular, subscapular, axillary, and intercostal regions where it lies along the major blood vessels of abdomen and thorax (Himms-Hagen, 1985). Besides BAT being visibly different from white adipose tissue (WAT), structurally it is unique in that it contains a vast amount of densely packed mitochondria and relatively little space is dedicated to the storage of fat(Sell and Richard, 2004). More importantly, cristae of BAT mitochondria are unique in that they contain a larger portion of uncoupling proteins (UCPs) than WAT (Nicholls and Locke, 1984)

which help to produce heat and classifies BAT as a main thermogenic organ in rodents (Smith and Hock, 1963).

Adaptive thermogenesis is hormonally regulated through the growth of BAT via the pre-synaptic sympathetic nerves that release norepinephrine (NE) and thyroid hormone upon activation. The release of NE in response to a decrease in $T_a$ stimulates the $\alpha$-$_1$-, $\alpha$-$_2$- and $\beta_3$-adrenergic receptors along the outer membrane in BAT (Figure 2). In mature BAT, only $\beta_3$-adrenergic receptors are most significantly expressed in rodents and increase thermogenic pathways, where the other $\beta$ receptors play a more important role in development of BAT (Bronnikov et al, 1999). As the $\beta_3$-adrenergic receptor is activated by NE, it generates a cascade of events through the modulation of a G-protein that leads to an increase in the activity of adenylate cyclase that turns on the production of cyclic AMP and further triggers protein kinase A (PKA) (Sell, 2004). Acute PKA stimulation allows for the activation of hormone-sensitive lipase (HSL) which helps to release free fatty acid (FFA) fuels stored as intercellular triglycerides (Sell, 2004). Transportation of FFA into the mitochondria occurs when lipids are transformed into acyl-CoAs and then transferred across the mitochondrial membrane as acyl-carnitine to be converted back to acyl-CoA in the matrix. Intracellular triglycerides stored inside the BAT are also liberated by the phosphorylation of perilipin by PKA that exposes FFAs to HSL and converts them to acyl-CoA which allows them to enter the interior of the mitochondria via carnitine transporter (Cannon and Nedergaard, 2004). Moreover, the release of

NE subsequently releases perilipins located on the surface of lipid droplets by HSL degradation (Belfrage et al 1982). Perilipins have been found on both WAT and BAT in mammals but their functioning in WAT is better known than in BAT. Perilipins contain six PKA receptor sites that help to maintain storage triacylglycerol (TAG) molecules during basal states. Souza et al (2007) recently looked at the role of phosphorylated PKA-dependent perilipins in BAT to observe and compare their functioning to that of WAT. Interestingly, perilipin knock-out mice were found to have a larger amount of TAG than the wild-types but when stimulated by NE there was no change in the release of FFA from BAT. In addition, Souza et al (2007) compared perilipin knock-out mice to genetically modified mice which did not contain active NE receptor sites in BAT TAG molecules that lead to no response in $T_b$ to NE injections. On the contrary, perilipin knock-outs and wild-type mice significantly increased their $T_b$ by ~3°C. Collectively these results suggest that adaptive thermogenesis is modulated through 'downstream' mechanisms of NE-induced thermogenesis where perilipin functioning helps to control proper thermal regulation.

Furthermore, after FFAs have been released from storage they undergo mechanisms of β-oxidation and the resulting acetyl-CoA is oxidized in the tricarboxylic acid (TCA) cycle. Here electrons are liberated and transferred to electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) that supply electrons to the electron transport chain (ETC). Each pair of electrons is transferred along a series of complexes (I, III and IV)

that pumps protons into the inner mitochondrial space. This generates a high electrochemical gradient in the inner mitochondrial space that favours the movement of protons to re-enter the matrix mainly through the ATPase synthase complex (ASC). ADP is phosphorylated to ATP by the release of energy as the proton transfers into the matrix that maintains the proton gradient between the inter membrane space and matrix (Lowell and Spiegelman, 2000) (Figure 1.3A).

However, in BAT a significant amount of protons are redirected away from the ASC and are 'leaked' back to the matrix through uncoupling proteins (UCPs) that are also situated along the inner mitochondrial membrane. This essentially uncouples the formation of ATP from substrate oxidation and as a result from the transfer of potential energy along the ETC complexes metabolic heat is generated as a by-product that stabilizes the proton gradient (Lowell and Spiegelman, 2000) (Figure 1.3B).

**b. Measurements of NST**

Many studies in the literature have already shown that BAT mass increases proportionally with cold acclimation (Biggers et al, 1957, Chappell, 1984, Rezende et al 2004, Chappell and Hammond, 2004, Rezende et al, 2004). In concert, NST capacity expands and allows cold acclimated animals to withstand longer periods in the cold (Jansky et al, 1967, Depocas et al, 1956). There are various methods to measure the capacity of NST although it is sometimes difficult to selectively measure NST without including contributions

of ST in metabolic rate. In theory, regulatory NST can be indirectly measured after muscular contractions have dissipated in response to the lowering of $T_a$. However, this is difficult to simulate as both mechanisms have been shown to coexist under extreme cold temperatures and teasing out the exact contributions of both ST and NST may present major problems (Jansky, 1973).

Moreover, understanding the theory behind the allometric relationship of metabolism in mammals provides greater insight into relative NST and ST contributions to maximal metabolism. Heldmaier (1972) demonstrated that maximal NST response to a minimal amount of NE is inversely proportional to body mass. For example when the basal metabolic rates (BMR) of mammals are plotted in terms of an allometric relationship a slope of 0.75 is calculated (Figure 1.4). Also, if $VO_{2max}$ (maximal amount of oxygen that can be consumed during exercise (Chappell, 1984)), in terms of increasing body mass is generated on the same graph two parallel lines with similar slopes of 0.75 (excluding additional factors that may heighten $VO_{2max}$ slope to 0.87) are formed that do not intersect. If an additional plot of NST vs. body size is added to the parallel plots its slope is calculated to be 0.5. Therefore, as body size along the x-axis increases then eventually both lines of BMR and NST will intersect (approximately at weights over 10kg). This intersection of NST at the BMR plot indicates the point of maximal contribution of NST towards BMR. Therefore, any additional contribution to basal thermogenesis must be contributed by ST as NST has established maximal capacity. According to this relationship it demonstrates that

a larger mammal (more than 10kg) will not use NST to sustain maximal metabolism but rather ST and a smaller mammal (less than 10kg) will greatly rely on NST mechanisms for thermoregulation (Figure 1.4). As NST is relatively more important in smaller sized mammals it suggests reasons why human neonates are born with approximately 1% of body weight in BAT which helps them to thermoregulate (Heaton, 1972). Heaton (1972) then describes that as humans develop into larger body masses they lose their ability to use NST mechanisms as BAT mass is slowly depleted and they are forced to rely on shivering to thermoregulate. However, recently BAT has been re-discovered in healthy human adult females at 7.5% of body weight and 3.1% in males around the cervical–supraclavicular, fascial plane in the ventral neck and superficial and lateral to the sternocleidomastoid muscles (Cypess et al, 2009). This re-discovery further complicates NST contributions to maximal metabolism in humans and especially relationships and exact mechanisms in small versus larger mammals remain to be elucidated.

Nevertheless, BAT is directly stimulated by NE in rodents and there is a non-linear saturation relationship between increasing concentrations of administered NE and oxygen consumption (Wunder and Gettinger, 1996). This technique allows the maximal stimulation of NST measured as the maximal oxygen consumption post-injection and a clear thermoregulatory role of BAT can be assessed in rodents. In addition, it is particularly important to measure the NST response while the animal is subjected to temperatures within their TNZ as this

will eliminate the effect of endogenous release of NE that cold temperatures may induce or enhance on the thermoregulating system and only allow for exogenous administered NE to stimulate BAT (Jansky, 1973).

### c. Uncoupling Proteins

Understanding NST mechanisms is a unique way to explore the complex inner workings of uncoupling oxidative phosphorylation in BAT mitochondria. UCPs which were first termed as thermogenin (Ricquier et al, 1976) are specifically up-regulated only in BAT in the response to chronic cold exposure. Today, UCP/thermogenin is simply referred to as UCP1 and is highly distinct from its other homologues UCP2 and UCP3 which are found in tissues such as the stomach and skeletal muscle, respectively (Klingenspor and Huang, 1999). UCP1 shares similarity between other members of its mitochondrial carrier proton family in that it takes on the tripartite structure with six transmembrane α-helices and acts under the form of a homodimer (Boss et al, 1998a). UCP1 expression in BAT is directly influenced by NE stimulation of β-and inhibition of $\alpha_2$-adrenergic receptors. In addition there have been previous studies that suggest a major role in up-regulation of the thyroid hormone that correlates with an increase in UCP1 expression (Rabelo et al, 1996). Thyroxine ($T_4$) secreted by the thyroid and tri-iodothyronine ($T_3$) which $T_4$ is eventually converted into are essential in the regulation of thermogenesis. They are found in increasing amounts in BAT and skeletal muscle that promotes higher amounts of $T_3$ that help to increase lipolysis

that up-regulates UCP1 expression (Bianco and Silva, 1988, Silva, 1988). Moreover, recent evidence strongly supports a more developmental role of the thyroid hormone that stimulates the up-regulation of UCP1 mRNA expression in BAT and allows newly born endotherms to enhance essential NST mechanisms (Cannon and Nedergaard, 2004).

In addition to the overwhelming amount of controversy in the literature to modulators of UCPs, fatty acids have been shown to influence the up-regulation of UCP1 expression and activity. As UCP1 along with UCP2 and 3 contain a unique GDP binding domain situated along the tripartite structures that inhibits proton conductance when occupied by purine nucleotides. It acts to assist fatty acid binding to UCP1 that enables it to act as an allosteric modulator, cofactor and transporter of protons that increases mitochondrial uncoupling (Cannon and Nedergaard, 2004). Although the fatty acid binding site on UCP1 has yet to be clarified, 'activated' fatty acids or acyl CoA compete for binding sites near the GDP domain which activates an increase in UCP1 expression and activity (Cannon et al, 1977). As well, fatty acids have been shown to relocate inside the structure of UCPs and help to guide protons down the electrochemical gradient and re-enter into the matrix (Klingenspor and Huang, 1999). Lastly, the exact transfer of protons back into the mitochondrial matrix may not be transported by the UCP1 themselves but rather by 'activated' fatty acids. For example, as suggested by Garlid et al (1996) fatty acids may act through a protonophore mechanism where a protonated fatty acid carries protons across the bilayer

membrane instead of UCP1. This mechanism is activated as high energy derived by fatty acids, which are situated next to UCP1 that attract protons to the carboxylic acid head located on the lipid bilayer membrane. The protonated fatty acid 'flip flops' the proton along the bilayer membrane so to face in the direction of the matrix. The proton is then released from the head of the fatty acid which leaves the electrochemical gradient neutral and the cycle continues. There is much evidence for this process happening and further suggests that the presence of fatty acids may play an essential role in uncoupling oxidative phosphorylation in BAT (Garlid et al, 2000). However, other studies suggest possible roles of other mitochondrial carrier proteins in BAT besides UCP1 such as ATP/ADP carrier proteins that have been found to transport protons in a similar matter (Andreyev et al, 1989) which further complicates that role of UCP1 as an uncoupler and raises more questions about its evolutionary significance.

Interestingly, the requirement of fatty acids for proper functioning of UCP1 helps to further define the roles of its two homologues UCP2 and 3. Klingenberg and Huang (1999) demonstrate that UCP1 loses its ability to transfer protons into the matrix when there is an alteration in two of its histidine residues that are proposed to help the binding of purine nucleotides. These authors predict that since UCP2 does not express either of these terminal residues then it should not support any function in the influx of protons across the mitochondrial membrane. Moreover, UCP3 contains one of the two histidine components and has been shown to support some proton flux across the mitochondrial membrane

(Klingenberg and Huang, 1999). However, most evidence in the literature reports that there are no differences in proton flux between both UCP2 and 3 compared to UCP1 so the exact roles of these proteins still remain highly debateable (Jaburek et al, 1999).

More recently, it appears that there is experimental evidence in the sensitivity of fatty acids to the up-regulation of UCPs that may further help to define the roles during energy metabolism. As Rial and Gonzalez-Barroso (2001) reports UCP1 significantly increases sensitivity in correlation to an increase in fatty acids while UCP2 and 3 remain relatively inactive. However, these observations remain highly controversial as recent evidence presented by Barger et al (2006) in arctic ground squirrels compares the roles of UCP1 and 3 in BAT and skeletal muscle, respectively. These results show that UCP3 mRNA may be more highly sensitive to fatty acid metabolism than what have been previously described. These authors found the expression of UCP1 mRNA to be elevated in squirrels that were active non-hibernators and lower in fasted hibernators upon cold exposure. Since the highest expression of UCP1 mRNA and protein was found in the hibernating squirrels, this supports a role of UCP1 in NST, which presumably helps contribute to the re-warming of the animals during periodic arousals. In contrast, the change in UCP3 did not parallel that of UCP1 and was found to be more essential in the metabolism of free fatty acids utilized by skeletal muscle for ATP synthesis. Barger et al (2006) found that fasting induced a 10-fold increase of UCP3 mRNA expression in active non-hibernators and

hibernators at both 5 and 10°C temperatures. Therefore, irrespective of the change in environmental temperature, UCP3 does not alter expression although the increase in UCP3 in unfed compared to fed squirrels supports the idea that UCP3 plays a larger role in fatty acid metabolism in skeletal muscle and UCP1 remains as the main uncoupler and regulator of adaptive thermogenesis in BAT. Therefore, UCP3 expression supports a bigger role in lipid metabolism where the increase in FFA concentrations in response to fasting, high-fat feeding, and exercise also induce an up-regulation in UCP3 (Schrauwen et al, 2002). However, the transport of FFA by UCP3 remains controversial and the exact mechanisms still need further investigation. Although it still remains unclear that all three of the above mentioned uncouplers increase proton conductance across the mitochondria membrane, UCP1 up-regulates adaptive thermogenic mechanisms unlike UCP 2 and 3 that are found to use other exogenous factors that stimulate thermogenic activity (Brand and Esteves, 2005).

It is clear that UCP1 expression in BAT is correlated with metabolic inefficiency in response to cold thermogenesis (Nespolo et al, 2001). The thermogenic roles of UCP2 and 3 have been suggested as being relatively minor compared to UCP1 but only recently UCP1 ablated mice conclusively support UCP1 as the only uncoupler that can mediate mechanisms of adaptive thermogenesis (Golozoubova et al, 2001). In other words, the ability to up-regulate NST in response to cold acclimation is only carried out by an increased expression and activity of UCP1 in BAT. This is demonstrated in UCP1 ablated

mice who do not possess the ability to metabolically respond after an injection of NE and remain cold-intolerant, allowing their body temperatures to fall beyond normal $T_b$ when exposed to cold or even a thermal neutral $T_a$ (Klaus et al, 1998 and Nedergaard et al, 2001). Enerback et al (1997) further demonstrated that UCP1 knockout mice are comparatively more sensitive to cold $T_a$ then wild-type mice as they exhibit an inability to maintain normal $T_b$ when exposed to $T_a$ of 5°C. Surprisingly, UCP1 ablated mice showed no evidence of obesity in this study and were able to maintain proper lean muscle as well normal rates of resting metabolism. Monemdjou et al (2000) reported that with the complete removal of UCP1 gene, that UCP2 mRNA expression in BAT increased suggesting compensation for the loss of UCP1 (Monemdjou et al, 2000). Together these results suggest that UCP1 is necessary for adaptive thermogenesis during cold $T_a$ but perhaps other UCP homologues are needed to support obligatory thermogenesis and control of lipid metabolism.

As the roles of UCP3 in lipid metabolism remain highly debateable, Costford et al (2008) sheds light on its various functional properties that may help to define its exact functioning mechanisms. Primarily, UCP3 may act as a bidirectional transporter of fatty acids that it increases fatty acid entry into the matrix to stabilize the proton electrochemical gradient and it also helps to shuttle fatty acids out of the cell to avoid lipotoxicity. Moreover, Costford et al (2008) examined the role of UCP3 in response to high fat feeding in UCP3 knock-out mice. From this investigation it appears that UCP3 plays a role in controlling

levels of body adiposity, as there were no differences between wild-type and knock-out mice in glucose tolerance and insulin sensitivity. Lastly, a study by Echtay et al (2003) proposes that fatty acids are used by UCP3 to successfully attenuate the accumulation of reactive oxidative species (ROS) in response to lipid peroxidation in cell. Moreover, UCP3 knock-out mice have been shown by Echtay et al (2003) to generate large amounts of ROS in response to normoxic conditions whereas wild-type mice produce lower levels of ROS and display a significant increase in UCP3 mRNA and protein. This result confirms a more prominent role of UCP3 in lipid metabolism that some authors may not support and further verifies the function of UCP1 in BAT to the up-regulation of adaptive thermogenesis.

## VII. Cold Acclimation

Many investigators have examined the response of animals exposed to the pressures of chronic cold environments (Biggers et al, 1957, Chappell, 1984, Rezende et al, 2004, Chappell and Hammond 2004). These studies show that endotherms increase their NST capacity to maintain normal $T_b$ outside their TNZ without the increased recruitment of ST (Deposcas et al, 1956). NST capacity is directly correlated with a change in BAT mass that increases along with a rise in UCP1 mRNA expression in response to chronic periods of cold $T_a$ (Watanabe et al, 2008). In addition to these changes in BAT composition and UCP expressions, animals subjected to cold temperatures have been shown to use other important heat conserving mechanisms. An increase in body mass is a common strategy that

cold acclimated animals use to prevent the increase in heat loss by decreasing body surface to volume ratios (Haim et al, 1993). Cold acclimated animals have increased organ sizes that are influenced by higher amounts of food consumed during cold exposure that also contribute to an increase in resting metabolic rate (RMR) (Hammond and Wunder, 1995, Rezende et al, 2009). However, some studies have reported no change in metabolic rate after cold acclimation and changes to RMR appear to be species specific. Van Sant and Hammond (2008) compared cold- to warm-acclimated deer mice and found no change in RMR while others found the complete opposite with an increase in RMR in *Spalacopus cyanus* (Rodentia) species (Nespolo et al, 2001) and Mongolian gerbils (*Meriones unguiculatus*) (Li and Wang, 2005). Although an increase in metabolism while at rest may be a strategy in some endotherms to increase heat production, it may not be the case for all species and especially in environments with multiple or variable stressors.

## VIII. Hypoxia Acclimation

Lower levels of ambient oxygen (hypoxia) activate hypoxic sensing mechanisms that turn down pathways that potentially increase oxidative demands and deplete valuable oxygen stores (Raguso et al, 2004). This adaptive strategy in hypoxic environments is extremely important for species such as turtles, carp and goldfish to survive periods of low levels of oxygen (Hochachka et al, 1996, Nilsson and Renshaw, 2004). In contrast, these strategies are not used by most mammalian species that experience HA environments. Rather pathways of higher

energy yields and enhanced delivery of oxygen to the tissues are emphasized in species experiencing low levels of oxygen (Hochachka et al, 1991). Efficiency of these pathways are increased in a variety of ways by 1) utilizing higher ATP yielding mechanisms such as aerobic vs. anaerobic pathways (Yoshino et al, 1990) 2) increasing oxidation of glycogen or glucose stores rather than lower ATP yielding fatty acid oxidation (Hutter et al, 1985, Hochachka, 1985) and 3) enhancing mechanical output to metabolic power input ratios thereby increasing overall work achieved by the organism (Hochachka et al, 1991).

Therefore, in states of hypoxia, especially in chronic situations, animals tend to use strategies to improve oxygen delivery and limit unnecessary use of valuable fuel stores. Typical exposure to hypoxia leaves the organism with a decrease in $T_b$ as blood flow is redirected away from the BAT to other tissues such as the heart and brain (Szelenyi and Donhoffer, 1968). In fact, when rats have been exposed to hypoxia they are shown to select $T_a$ that are cooler than their previously preferred TNZ which lowers the difference in the thermal gradient between $T_a$ and their lower $T_b$ set point (Dupre and Owen, 1992). This allows a decrease in the rate of heat transferred to the surrounding environment. In addition, similar to hypoxic animals, hibernating animals also readjust hypothalamic set-points that lower metabolic rates and reduce thermogenic costs. For example, in the golden-mantled ground squirrel metabolic rate was shown to decrease following a drop in $T_b$ triggered by hibernating conditions such as cold temperatures and hypoxia (Snapp and Heller, 1981). This demonstrates an

important strategy of hibernators that select lower $T_b$ to help reduce metabolism to decrease energy costs.

Although there are very few studies that have investigated the properties of BAT mitochondria under hypoxic conditions, it has been shown that hypoxia decreases BAT mass and UCP1 expression inhibiting the ability to up-regulate thermogenesis (Mortola and Naso, 1997). These studies were limited to relatively short hypoxic exposures of hours or days and perhaps longer acclimation periods may provide a more pervasive effect of hypoxia on integrative thermoregulating mechanisms.

Another typical gene response in low oxygen environments is the stabilization of hypoxia inducible factor 1 (HIF-1α) (Semenza, 2006). It is a dominant regulator in oxygen homeostasis as it is involved in the processes of angiogenesis (growth of new blood vessels), erythropoiesis (production of red blood cells) and metabolism (Raguso et al, 2004). Therefore, HIF-1α stabilization is a critical response to chronic hypoxia or oxidative stress that helps to maintain a steady internal milieu. It induces gene expression of glycolytic enzymes such as the up-regulation of phosphofructokinase, which is the rate limiting step during glycolysis (Raguso et al, 2004) (Figure 1.5). The oxygen level in the tissues greatly affects the actions of HIF-1 where in normoxia HIF-1 is hydroxylated and degraded but during bouts of hypoxia it is stabilized in the cytosol and translocates to the nucleus (Jaakkola et al, 2001). Interestingly, very few studies

have looked at the response of HIF-1α in BAT but more recently (Nikami et al, 2005) found that HIF-1α mRNA expression in BAT greatly increases in response to cold induced thermogenesis. In this study, cold exposure elicited an increased response in HIF-1α expression as well in UCP1 ablated mice where a simulated hypoxic environment showed no change in HIF-1α (Nikami et al, 2005). These findings suggest that the role of NE in the up-regulation of H1F-1α expression induced by lower $T_a$ may improve hypoxia tolerance for animals in the cold. This may suggest alternative strategies of animals at HA that help to combat extreme environmental stressors such as hypoxia and cold.

## IX. Hypoxia and Cold Acclimation

There are many studies that have demonstrated the effects of cold acclimation as well as acute hypoxic exposure on thermoregulation however there are few studies that try to define the thermogenic response of acclimation to both chronic hypoxia and cold environments. Previously, Gautier et al (1991) demonstrates that chronically cold acclimated rats exposed to short bouts of hypoxia rely more on ST rather than NST to compensate for lower $T_b$ found after exposure to hypoxia. Moreover, as this study tries to combine both stressors it is however limited by only demonstrating the effects of cold and intermittent hypoxia instead of allowing complete acclimation to occur under the combination of both environments. This is an extremely important component in HA studies as acclimation to both cold and hypoxia provides a better representation of the

natural environment and presents helpful insight into how animals may acclimatize to high elevations. Recently, Broekman et al (2006) reported lower LCT values in HA native mole rats that increased the range of the TNZ compared to other mole rats found at SL environments. In addition these animals had lower thermal conductance rates which indicated an enhanced use of insulation mechanisms that more than likely helping to reduce the rate of heat loss to the environment. Also, HA mole rats exhibited lower resting metabolic rates indicating a greater capacity to up-regulate NST mechanisms confirmed by an increased response to an injection of NE compared to sea-level (SL) animals. These results suggest that HA adaptations prominently enhance NST responses that may assist in effective and rapid heat production pathways necessary to battle the physiological conflicts at HA environments.

## X. Fuel Selection

It is important for organisms under hypoxic and cold stress to switch off energy costing pathways and turn on those that provide higher energy yields. Hypoxia and/or anoxia induce organisms to lower or completely shut down lipid utilizing pathways and up-regulate glycolytic mechanisms (Hochachka et al, 1991). Similarly, cold temperatures increase whole body glucose metabolism while in contrast BAT in endotherms are found to selectively use a higher amount of lipids as a fuel substrate (Cannon and Nedergaard, 2004).

Utilizing glucose or glycogen approximately yields 25-30% more ATP per mole of oxygen consumed than energy formed from lipolysis *in vivo* (in heart muscle) (Daut and Elzinga, 1989). Lipids inefficiently use more oxygen consuming pathways and essentially 'waste' valuable oxygen stores to produce energy as they require more oxygen per carbon unit than the oxidation of glucose (Holden et al, 1995). Therefore under extreme environmental stressors such as cold and hypoxia it is important to use fuel in an effective manner that uses a limited amount of oxygen per unit of fuel and also can sustain increased energy demands of heat production.

**a. Fuel selection under hypoxia**

Hypoxia/anoxia limits oxygen supply to the tissues and as a result promotes the use of glucose within the tissues as it provides higher energy yields than lipolytic pathways. However, animals exercising at moderate intensities under hypobaric hypoxia have been found to utilize lipids rather than carbohydrates (CHO). For example, Wistar rats acclimated for 10 weeks at ~4,300m above SL did not up-regulate glucose oxidation under sub-maximal exercise intensities at 60 or 80% of their $VO_{2max}$ (McClelland et al, 1998). The relative proportions of whole body CHO and plasma glucose oxidations remain unchanged in HA compared to control SL rats exercised at these moderate intensities. The authors point out that there may be a CHO sparing effect in HA animals that limits the use of precious CHO stores. This study highlights the effect of exercise intensity on patterns of fuel use. As exercise intensity increases so does the reliance on CHO

to power contraction. In fact previous studies on acclimation in humans that have suggested higher CHO use at HA may have been due to HA subject operating at a high intensity than SL controls (McClelland et al, 1998).

As demonstrated by many studies (McClelland et al, 1999, Jones et al, 1972, Young et al, 1982) plasma non-esterified fatty acid (NEFA) concentrations were found to be higher in HA compared to SL rats that indicates higher oxidation of free fatty acids under hypoxia. Moreover, despite no significant differences in lipid flux rates between HA and SL rats, HA rats had greater depletion of muscle triacylglyceride (TAG) stores from the soleus and red gastrocnemius muscle groups during exercise at 60% $VO_{2max}$ (Corbett, 2004). This suggests that higher circulating plasma NEFA concentrations found in HA rats may indicate that these animals rely more on intramuscular triglycerides (IMTG) to help fuel exercise than SL animals. Moreover, these changes in plasma NEFA concentrations in the HA rats could be explained by a variety of mechanisms. Hypobaric hypoxia could induce a greater uptake of NEFAs into the muscle from circulating plasma pools or NEFAs found within the muscle could be more selectively utilized. Furthermore, IMTG use could be triggered by hypoxia as fuel to spare use of muscle glycogen stores. In support of this, Ou and Leiter (2004) examined fatty acid metabolism in rats subjected to hypobaric hypoxia relative to 5,500m above SL and found that hypoxia enhanced liver lipid metabolism and increased greater uptake of palmitate concentrations into the skeletal muscle Furthermore, Galbes et al (2008) demonstrates that rats subjected to 5 weeks of hypobaric hypoxia

demonstrate a significant decrease in mCPT1 activity and mRNA expression in skeletal muscle where there were no changes in other mitochondrial marker enzymes such as citrate synthase and β-hydroxyacyl CoA dehydrogenase (HOAD) a fatty acid oxidation enzyme (Galbes et al, 2008). Conclusively these results suggest that under conditions of hypobaric hypoxia the up-regulation of fatty acid oxidation is more prominent than that of CHO oxidation and perhaps HA hypoxia induces a glucose sparing effect on tissues.

**b. Fuel selection in cold environments**

It has been well studied that cold temperatures increase metabolic rates in small mammals that up-regulate substrate turnover rates to increase fuel availability to the tissues (Depocas and Masironi, 1960). Glucose utilization has been shown to be a major contributor to fuel oxidation in cold acclimated rats which have been shown to have elevated glucose turnover, clearance and improvements to glucose tolerance (Vallerand et al, 1990).

During cold exposure animals can be found to increase their food intake by up to four times what they would normally ingest at room temperature (Cannon and Nedergaard, 2004). This extra intake of food provides more nourishment to vital organs and results in an increase in growth. More importantly, fuels such as triglycerides rather than CHOs fuel thermogenic organs such as BAT that increase mitochondrial uncoupling and are necessary for cold $T_a$ survival.

In addition, to an increase in lipid usage in BAT after cold exposure there is some recent evidence that glucose may also be up-regulated and utilized by BAT (Cannon and Nedergaard, 2004). NE is thought to help relocate glucose transporters to the outer membrane of BAT from cytosolic stores to assist in the uptake of glucose into the cell. GLUT1 has been found in BAT; however, it is mostly thought to contribute to basal glucose transport (Cannon and Nedergaard, 2004). Nevertheless, GLUT4 transporters that are expressed more intracellularly have been found to be up-regulated in response to cold temperatures (Shimizu et al, 1998). Although an injection of NE appears to have little affect on relocation of GLUT4 to the brown fat membrane. Instead some studies have found that it may be the activation and translocation of GLUT1 transporters that increases during NE stimulation (Shimizu et al, 1998). However, some studies claim that GLUT1 is only found on the plasma membrane and not manipulated by hormonal signals (Cannon and Nedergaard, 2004).

In addition, NE has been shown to induce mobilization of fatty acids stored in the blood as triglycerides through the activation of the enzyme lipoprotein lipase, which hydrolyzes the release of NEFA from storage and has also been found to stimulate UCP1 activity. Recently, Watanabe et al (2008) looked at global differences in genetic expressions of BAT using microarrays in cold-induced fed rats. These results suggest important roles of lipolytic mechanisms in BAT that indicates lipids as a main fuel substrate as there is an up-regulation of expression in lipoprotein lipase, fatty acid binding protein, long

chain acyl-CoA synthase, CPTI and II. Moreover, Watanabe et al (2008) concluded that very few studies have focused on the regeneration of fatty acids in BAT, where they show that BAT has an increased gene expression in glycolytic enzymes such as glycerokinase that help liberate glycerol-3-phosphate from glycerol that enhances re-esterification of triacylglycerol from glycerol-3-phosphate to regenerate fatty acids. As this mechanism is up-regulated in BAT it helps to mobilize fatty acids and enhances thermogenesis.

Previous studies have shown an increase in the oxidation of fatty acids such as arachidonic (20:4(*n*–6)) and linoleic (18:2(*n*–6)) acids after cold acclimation where their preferred use is in the phospholipid bilayer of the BAT membrane. Although the accumulation of fatty acids within the BAT membrane may help to stimulate gene expression of UCP1 they also may assist in maintaining regular fluidity of the BAT membrane. (Garlid et al, 2000). This suggests that changes in eating habits during cold exposure induce stimulation of lipoprotein lipases or other food degrading enzymes that could lead to necessary preventions against the loss of natural fluidity and immobilization of hydrophilic proteins that can produce disastrous effects with lower $T_a$. Therefore, taken together these results suggest that cold exposure induces a greater uptake of fatty acids in BAT that help to activate UCP1 and larger food consumption helps to up-regulate glycolytic pathways that circumvent membrane fluidity.

## XI. Mitochondrial Regulators

Citrate synthase (CS) plays an important role in the oxidative metabolism of all animals as it helps to catalyze the reaction converting oxaloacetate to form citrate in the Krebs cycle that yields electron donors such as NADH that are utilized in the electron transport chain (ETC) (Wiegand and Remington, 1986). Also, CS activity can be used as an estimate of mitochondrial density and functioning aerobic capacity of the cells in tissues (Reichmann et al, 1985). Stressors, such as exercise, help to stimulate muscle CS activity as a result of increased mitochondrial biogenesis as well as most mitochondrial enzymes (found in the matrix) (Houle-Leroy et al, 2000). Therefore, it is not surprising that studies that have examined the activity of CS in BAT and skeletal muscle have found a large increase in mitochondrial density after cold acclimation that helps to support high heat production (Wickler, 1981). Wickler (1981) specifically investigated the activity of mitochondrial marker enzymes and their responses during winter and summer acclimated environments in the white-footed mouse. CS activity in BAT and skeletal muscle increased after cold acclimation although there was a substantial difference in activity of both tissues. BAT and skeletal muscle showed a 200 and 80%, rise in CS activity, respectively, after winter acclimation compared to measurements taken after the summer months. As previously mentioned, BAT mass significantly increases after cold acclimation due to NE induced stimulation which greatly increases fatty acid mobilization which evidently leads to an increase in mitochondrial density. The smaller

increase in CS activity in skeletal muscle after acclimation provides evidence that these mice may be using more NST mechanisms rather than ST which could explain why mitochondrial density is higher in BAT rather than skeletal muscle. However, as this study did not test the response of BAT to NE these results may be misleading. As BAT tends to increase in size with cold exposure skeletal muscle remains relatively the same size after acclimation. This growth in BAT supports a greater number of mitochondria but in skeletal muscle space may be limited and may not be able to support growth for more organelles.

As mitochondrial density has been extensively researched after cold acclimation there is little data found in its response to hypoxia. In fact, Dill et al (2001) found no change in mitochondrial density in skeletal muscle in young and adult rats after exposure to hypobaric hypoxia. As there is little data in the literature that measures CS in BAT in response to chronic hypoxia, it is proposed here that there are a variety of factors that could contribute to the down-regulation in CS activity such as more reliance on anaerobic glycolysis instead of lipolytic mechanisms that have been shown to lower levels of peroxisome proliferators activated receptor (PPAR) that stimulate free fatty acids (Barbera et al, 2001).

Furthermore, cytochrome c oxidase (COX) activity reflects indirectly the level of overall functioning capacity of the ETC (Wickler, 1981). COX is composed of a 13 subunit complex, where subunits 1-3 are encoded within the mitochondrial genome while the remaining ten others are nuclear-coded and are expressed in

specific tissues (Ludwig et al, 2001). As it is the last complex (IV) in the ETC, it is considered the rate limiting step in mitochondrial respiration and is inhibited by a high ATP/ADP ratio (Ludwig et al, 2001). Similar to CS activity, COX has been shown to respond differently under the influence of hypoxia and cold acclimation. Cold induced thermogenesis has been shown to increase COX activity by 80 and 220% in skeletal muscle and BAT, respectively (Wickler, 1981). Again this can be explained by the up-regulation of thermogenic pathways via increasing fatty acid catabolism that ultimately increases the proton flux through the ETC. Although this study does not look at the individual subunits of COX but rather just the overall activity of COX, it does appear that different subunits have various functions. Cold-induced thermogenesis seems to up-regulate COX-II in rat BAT and skeletal muscles, but COX-I and III appear to show no changes which indicates a bigger role of nuclear-encoded mitochondrial proteins rather than transcriptional control of the mitochondrial genome after acute cold exposure (Martin et al, 1993). Moreover, COX-IV which is a nuclear encoded gene appears to be up-regulated in BAT of Djungarian hamster after cold acclimation that is suggested to lead to transcriptional control of early brown fat cells (Klingenspor, 2003). Although this work was examined only after 7 days of cold exposure there could be a more pronounced COX-IV expression after a longer acclimation period.

Further evidence for the role of COX during cold acclimation is found in mice that were deficient in up-regulating thermogenesis called uncoupling protein-

diphtheria toxin A-chain (UCP-DTA) transgenic mice (Klaus et al, 1998). These mice showed severe COX activity impairments and other forms of down-regulated BAT pathways. Therefore, measurements of mitochondrial marker enzymes such as CS and COX provide major insight into the functioning of BAT and skeletal mitochondria and relative contributions of NST and ST after cold and hypoxia acclimation.

Additionally, an important and major regulator of mitochondrial biogenesis is the peroxisome proliferators-activated receptor co-activator (PGC-1α) (Wu et al, 1999) that is up-regulated during environmental stressors such as skeletal muscle exercise (Holloszy and Coyle, 1984), cold acclimation (Nicholls and Locke, 1984) and in heart development (Attardi and Schatz, 1988). Also, this activator has recently been linked to HIF-1α signalling induced by an increase in oxygen consumption to skeletal muscle exercise that elevates mitochondrial biogenesis. PGC-1α in white adipocytes has been shown to up-regulate UCP1 expression along with other subunits of COX II and IV (Klingenspor, 2003). Cold-induced thermogenesis rapidly increases activity of PGC-1α in BAT and skeletal muscle that increases the binding of PPAR-γ and retinoid X receptor (RXR) to specific response elements that leads to the increase in UCP-1 gene expression (Puigserver et al, 1998, Boss et al, 1998) (Figure 1.2). PPAR-γ is only found in white and brown fat of animals and it is shown to increase transcription of target genes in response to an elevation in lipid metabolism (Tontonoz et al, 1994). The activation of PPARs is stimulated by the binding of fatty acid ligands

that increase their release from storage upon cold temperature stimulation by PGC-1 α (Barbera et al, 2001). Also, cold acclimation increases the response of PGC-1α that further increases the transcriptional expression of UCP1 in BAT. There are still many mechanisms involved in NE cold induced stimulated pathways that can alter PGC-1α functioning and further investigation into these enhancers or suppressors will certainly lead to more exact role of UCP1 mechanisms and other thermoregulating factors.

Moreover, the role of PGC-1α under hypoxia in skeletal muscle has been recently investigated where its regulation in BAT remains to be further clarified (Arany, 2008). As PGC-1α expression is strongly correlated with a decrease in ATP supply this may help predict its role in BAT after hypoxic exposure. Although the activation of HIF-1α in the presence of hypoxia has been shown to regulate COX and decreasing mitochondrial respiration this may indicate that mechanisms of PGC-1α may in turn be down-regulated in BAT. Therefore this present study examines the role of these above mentioned mitochondrial enzymes, regulators and genes after acclimation to hypoxia and cold in hopes to provide a more intricate understanding of BAT and skeletal muscle role in thermoregulation.

## XII. Objectives

Since HA environments experience severe weather conditions that are quite distinct to those found at sea level, survival in these conditions requires organisms to endure the combined stressors of cold temperature and lower partial pressure of oxygen. Although many studies have investigated thermogenic capacities of mice after cold acclimation little is known about the effects after chronic hypoxia exposure and even less is known about the combination of both environmental stressors, particularly using a chronic acclimation paradigm. Therefore, this investigation combines hypoxia and cold in addition to hypoxia and cold alone will contribute to a better understanding of thermogenic responses in endotherms and further clarify tissue and protein roles in BAT and skeletal muscle.

It is hypothesized that after cold acclimation NST capacities will increase by directly increasing BAT mass, mitochondrial density and UCP expression per unit mitochondria. On the contrary, it is proposed that the effect of hypoxia acclimation will lead to a reduction in NST mechanisms that will ultimately lower BAT mass and protein expressions that will lower $T_b$ set point. Finally the combination of cold and hypoxia acclimation will reverse negative effects of hypoxia and allows these mice to sustain minimal survival necessary under these stressful conditions. In this study the following four broad questions were addressed:

1. **Does acclimation to hypoxic and cold environments affect Tb set point of CD-1 mice?**

2. **Do metabolic responses change to acute temperature exposures after acclimation to hypoxia or cold?**

3. **Is the capacity for NST altered by chronic exposure to hypoxia or cold?**

4. **Does acclimation to hypoxia and cold show changes in BAT mass, mitochondrial marker enzymes and regulators or alter uncoupling protein content in BAT or skeletal muscle?**

This thesis will outline data collected from four different treatment groups of CD-1 strain mice that were acclimated to chronic cold, hypoxia, the combination of cold and hypoxia and a normoxic thermoneutral treatment group was used as a control group. Each of these treatment groups of mice were continuously monitored for core $T_b$ during and after acclimation periods using surgically implanted data loggers (iButtons). Various *in vivo* experiments using indirect calorimetry were carried out to measure $VO_2$ over varying $T_a$ and NST capacity by measuring oxygen consumption after an injection of NE. Further experiments carried out from tissue extractions in BAT and skeletal muscle investigated the effects of acclimation on UCP1 and 3 protein expressions and mRNA along with measurements of mitochondrial functioning enzymes and other factors contributing to mitochondria performance.

## XIII. Chapter Summary

HA environments pose a conflict between hypoxic and cold environments that may increase or decrease thermogenic pathways. This chapter has outlined major relevant findings in the literature and how they have formulated hypotheses, predictions and possible conclusions about the present study.

**Figure 1.1: Thermogenic mechanisms in mammals**

Figure1.1demonstrates that obligatory thermogenesis is used during the thermal neutral zone (TNZ) which is defined by a range of ambient temperatures where metabolic rate (MR) remains relatively constant (MR in TNZ indicated by ★ symbol). Facultative thermogenesis (FT) is activated as the lower critical temperature (LCT) of the TNZ is surpassed and metabolic rate gradually increases as ambient temperatures decrease (MR in the FT indicated by ■ symbol) (Image adapted from Silva, 2003).

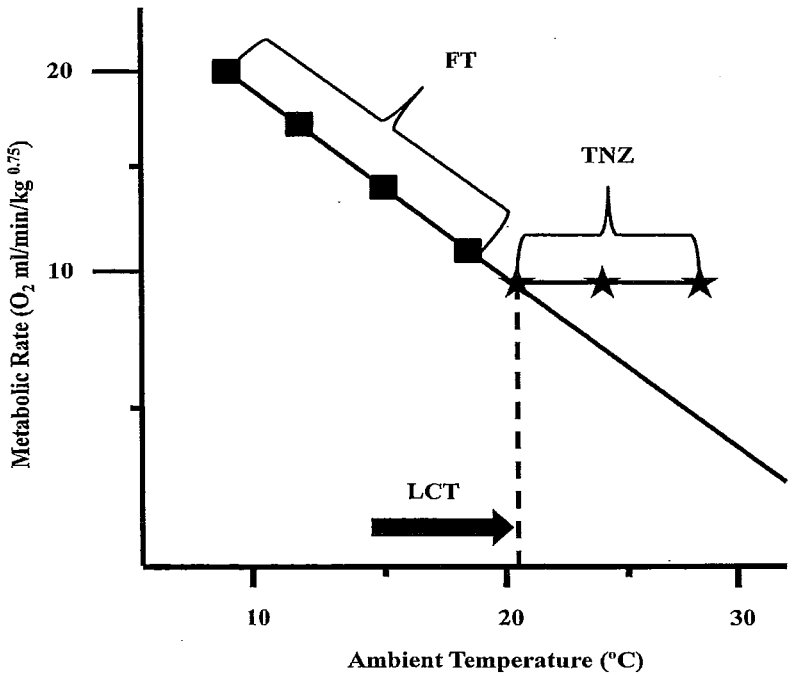

20
10
Metabolic Rate ($O_2$ ml/min/kg $^{0.75}$)
FT
TNZ
LCT
10
20
30
Ambient Temperature (°C)

**Figure 1.2: Mechanisms of non-shivering thermogenesis in brown adipose tissue**

In Figure1.2 cold temperatures activate the release of norepinephrine (NE) via the sympathetic nervous system (SNS). NE directly binds to $\beta_3$-adrenergic receptors that activate G-proteins linked to adenylyl cyclase (AC) that activates cAMP. Acute cold exposure initiates protein kinase A (PKA) to phosphorylate hormone sensitive lipase (HSL) that mobilizes free fatty acids (FFA) from storage and once converted to acyl-CoA it fuels substrate oxidation that increases uncoupling protein 1 (UCP1) activity. On the other hand longer term cold exposure increases PPAR$\gamma$ coactivator 1(PGC-1) by cAMP response element binding protein (CREB-P) that further binds PGC-1 to peroxisome proliferator-activated receptor-$\gamma$ (PPAR) and retinoid X receptor. This pathway increases the generation of mitochondria, hyperplasia of BAT as well as increased UCP-1 gene expression (Image adapted from Lowell and Spiegelman, 2000).

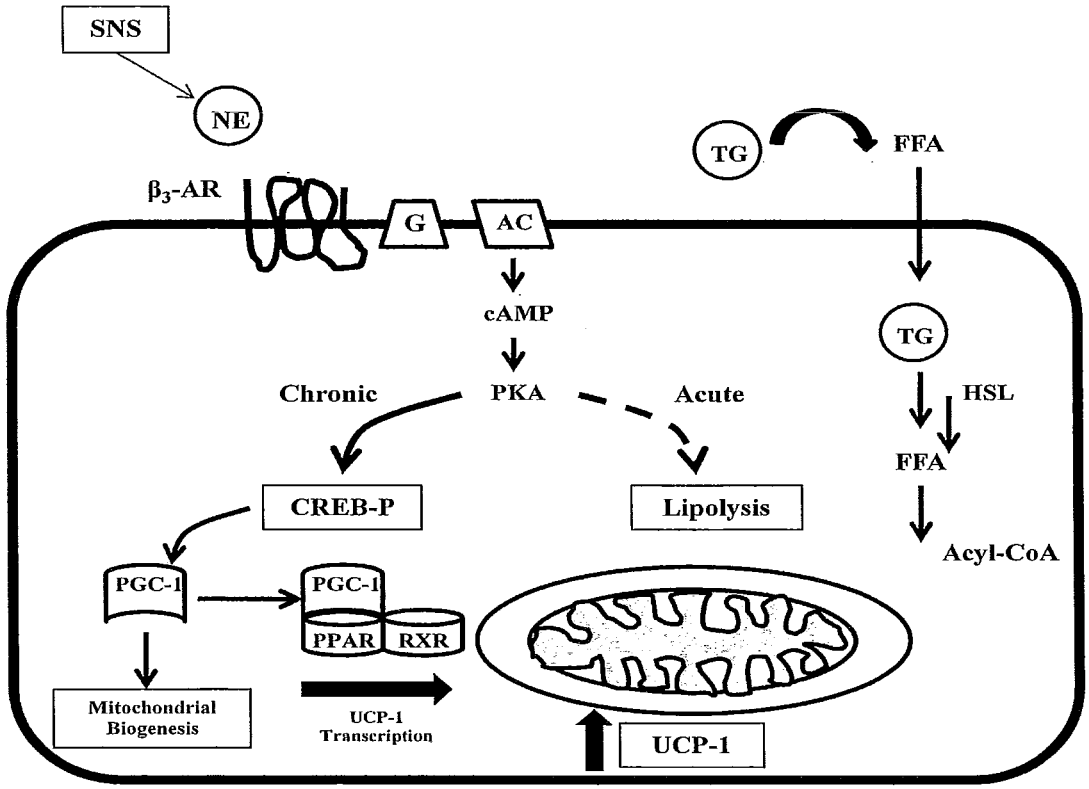
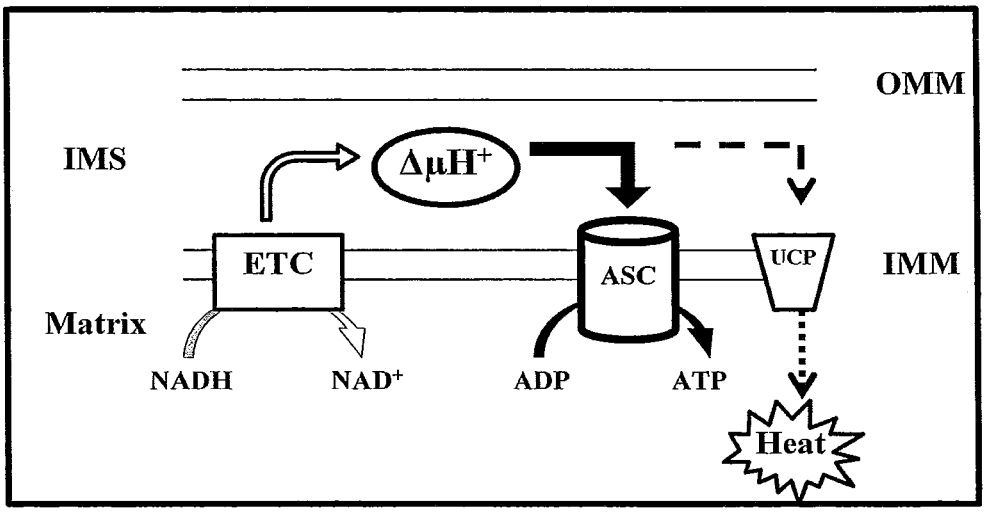

SNS
NE
TG
FFA
β3-AR
G
AC
cAMP
TG
Chronic
PKA
Acute
HSL
FFA
CREB-P
Lipolysis
Acyl-CoA
PGC-1
PGC-1
PPAR
RXR
Mitochondrial Biogenesis
UCP-1 Transcription
UCP-1

**Figure 1.3: Mechanisms of metabolic heat production**

Figure1.3**A** demonstrates protons pumped out into the intermembrane space (IMS) transferred downstream from the electron transport chain (ETC). A majority of protons are then leaked back through the ATP synthase complex (ASC) or $F_1/F_0$ ATPase to generate ATP while a smaller portion of protons are leaked thru uncoupling proteins (UCP) situated along the inner mitochondrial membrane (IMM). UCP essentially 'uncouples' substrate oxidation from the ETC by redirecting protons away from ASC which decreases the high proton gradient ($\Delta\mu H^+$) in the IMS. As a result metabolic heat is generated to balance the reduction in the gradient. Figure**1.3B** Displays mechanisms of substrate oxidation in brown adipose tissue (BAT) mitochondria where the majority of the protons bypass the ASC and leak back into the mitochondrial matrix via UCP and generate a significant amount of metabolic heat (Image adapted from Silva, 2003).

A.


OMM
IMS
ΔμH+
ETC
ASC
UCP
IMM
Matrix
NADH
NAD+
ADP
ATP
Heat

B.

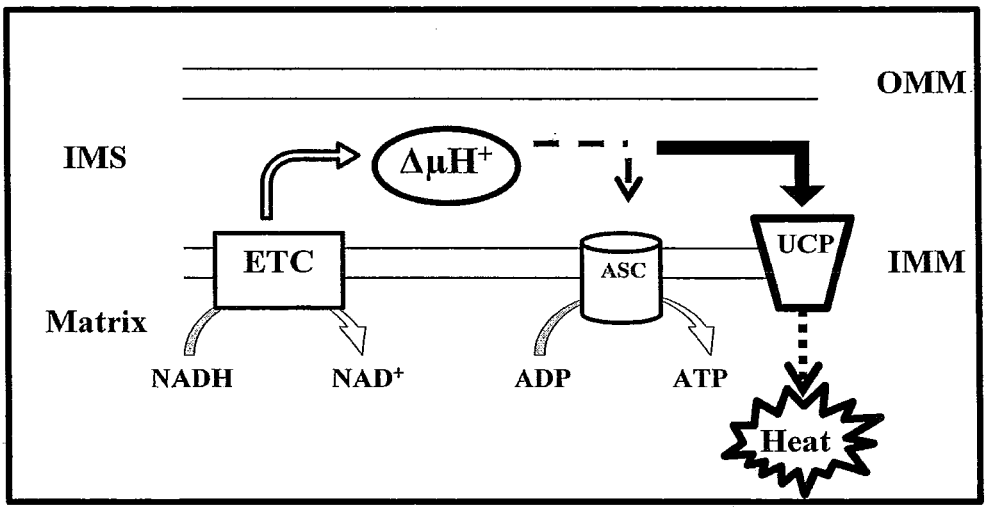

OMM
IMS
ΔμH+
ETC
ASC
UCP
IMM
Matrix
NADH
NAD+
ADP
ATP
Heat

**Figure 1.4: Allometric relationship between whole body metabolism, maximal metabolism and NST**

Figure1.4 shows the allometric relationship in basal metabolic rate (BMR), maximal metabolism ($VO_{2max}$) and non-shivering thermogenesis (Image adopted from Wunder and Gettinger, 1996). These data points were collected from animals acclimated to 5°C.

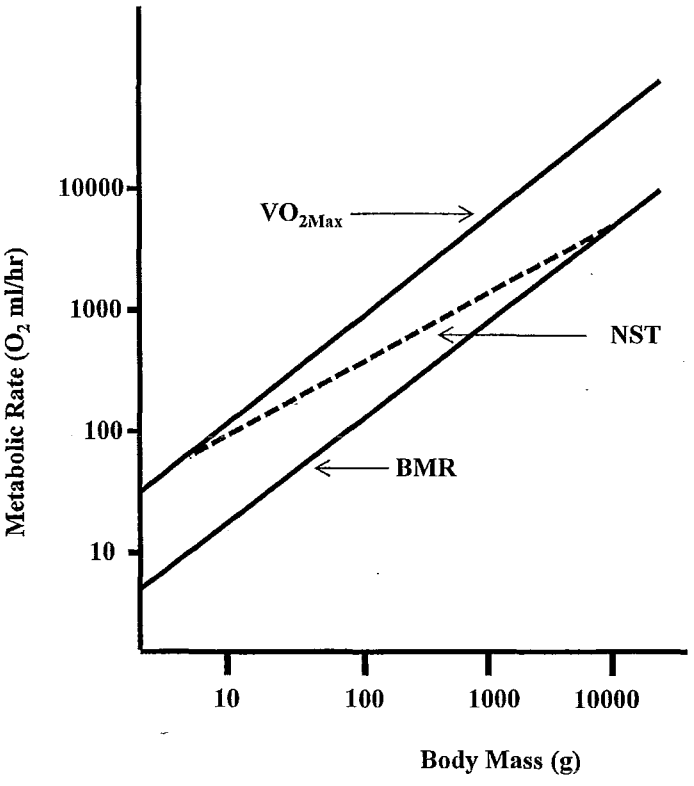
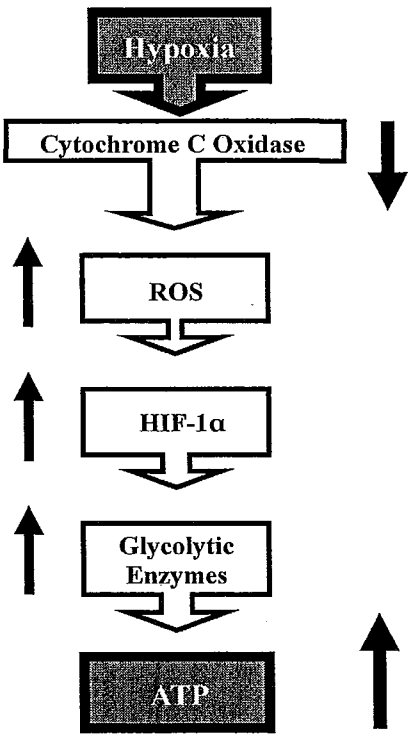

Metabolic Rate ($O_2$ ml/hr)
10000
1000
100
10
$VO_{2Max}$
NST
BMR
10
100
1000
10000
Body Mass (g)

**Figure 1.5: Down-regulation of metabolic pathways induced by hypoxia**

Figure1.5 shows the response of cytochrome C oxidase decreases expression in response to hypoxia that increases the generation of reactive oxygen species (ROS) that promotes the activation of hypoxia-inducible factor-1 (HIF-1) which in turn increases the activity of glycolytic enzymes that increases adenosine triphosphate (ATP). (Image adopted from Raguso et al, 2004).


Hypoxia
Cytochrome C Oxidase
ROS
HIF-1α
Glycolytic Enzymes
ATP

## Chapter 2

### MANUSCRIPT

### Abstract

Maintaining a constant body temperature ($T_b$) independent of varying ambient temperatures ($T_a$) is critical for survival in endotherms. In most small mammals thermoregulation may occur through shivering and / or non-shivering thermogenesis (NST). Brown adipose tissue (BAT) carries out NST through mitochondrial uncoupling proteins (UCPs) that aid in uncoupling substrate oxidation from ATP synthesis to generate heat. An up-regulation of this mechanism would be advantageous in a cold environment but not in conditions of low oxygen. High altitude, which exhibits both hypoxia and cold, may have conflicting influences on an animal's thermogenic capacity. To address this issue we examined the chronic effect of four weeks of exposure to hypobaric hypoxia (H, 480mmHg), cold (C, 5°C) and the combination of the two stressors (HC) compared to normoxic thermoneutral controls (N, 28°C) in CD-1 mice. We found HC had significantly lowered $T_b$ by 3.5±0.4°C compared to controls. Oxygen consumption ($VO_2$) over decreasing acute changes in $T_a$ from 32 to 4°C in normoxia was significantly higher in all treatment groups compared to N, within individual thermal neutral zones. Norepinephrine (NE) stimulated $VO_2$ was greater in C (5.06±0.18 $ml/min^{-1}$) and decreased in H (2.63±0.16$ml/min^{-1}$) compared to N (3.64±0.17$ml/min^{-1}$ ($p<0.001$)). Furthermore, HC showed increased BAT mass (0.33±0.01gm) versus N at (0.25±0.02gm) ($p<0.05$) but

showed no change in UCP1 protein content per mg mitochondria (0.66±0.14 vs. 0.99±0.09 in N). However HC had lower mitochondrial density indicated by lower activity of citrate synthase in BAT (9.08±0.6504 $\mu mol/min^{-1}/g^{-1}$ and 18.55±1.04$\mu mol/min^{-1}/g^{-1}$ ($p<0.05$), respectively). Together these results suggest that cold enhances NST capacity through the up-regulation of BAT mass and UCP1 mRNA expression whereas hypoxia inhibits NST response and may induce greater use of other thermogenic pathways such as shivering thermogenesis. Moreover the acclimation to both hypoxia and cold places limits on an animal's ability to up-regulate NST through impairments in BAT mechanisms that resets $T_b$ at a lower temperature to help reduce costs of thermoregulation.

## Introduction

Endotherms maintain a constant body temperature ($T_b$) by a combination of passive mechanisms within a narrow range of ambient temperatures ($T_a$) referred to as the thermal neutral zone (TNZ; Romanovsky *et al*, 2002). At $T_a$ below this zone most small mammals engage in facultative thermogenic mechanisms through non-shivering thermogenesis (NST) and shivering thermogenesis (ST) to elevate metabolic heat production (Foster, 1984, Himms-Hagen, 1985). Chronic cold exposure is known to enhance the capacity for NST in rodents (Biggers et al, 1957, Chappell, 1984, Rezende et al, 2004, Chappell and Hammond, 2004) whereas acute hypoxia can have negative effects on the ability to up-regulate thermogenesis (Mortola and Naso, 1998) and generate unnecessary energetic costs. Since high-altitude (HA) environments typically combine chronic hypoxia and cold, it poses a particular challenge to an animal's ability to thermoregulate. For example, lower $T_a$ requires an individual to minimize heat loss and maximize heat production, while hypoxia requires the most efficient use of oxygen that is available. These challenges are accentuated in small mammals owing to their high mass specific metabolic rates and high surface area to volume ratios.

Many studies have examined endotherms' thermogenic response to lower $T_a$ but most have neglected to investigate the combined effects of cold and hypoxia that are found at HA. This elicits facultative thermogenic mechanisms

which stimulate heat production via NST and ST carried out in brown adipose tissue (BAT) and skeletal muscle, respectively.

As colder temperatures are sensed by the brain, norepinephrine (NE) is released via the sympathetic nervous system and directly stimulates $\beta_3$-adrenergic receptors on BAT (reviewed in Cannon and Nedergaard, 2004). Unlike other tissues BAT expresses a large amount of uncoupling protein 1 (UCP1) along the inner mitochondrial membrane which redirect protons into the mitochondrial matrix thus uncoupling substrate oxidation from ATP production. Heat is generated as a by-product of increased cellular metabolism as energy transferred down the electron transport chain helps to stabilize the high proton gradient of the intermembrane space (Lowell and Spiegelman, 2000). Previous cold acclimation studies at $T_a$ lower than 5°C and for more than 4 weeks have shown that endotherms gradually decrease skeletal muscle shivering while increasing NST (Depocas et al, 1956). It is thought that the continuous NE release and subsequent BAT stimulation in response to cold temperatures increases BAT mass, mitochondrial biogenesis and UCP1 mRNA expression (Klaus et al, 1998). In addition, maximal *in vitro* activities ($V_{max}$) of mitochondrial marker enzymes citrate synthase (CS) and cytochrome c oxidase (COX), have been shown to increase their activity in BAT in response to cold acclimation (Wickler, 1981) and (Barnd et al, 1970) which indicates an enhanced capacity to generate heat in this tissue and contribute to elevated NST mechanism.

On the other hand, there are relatively few studies that have investigated the properties of BAT mitochondria under hypoxic conditions or after chronic hypoxia acclimation. Results presented from Mortola and Naso (1998) show that after chronic hypoxia exposure there is a significant decrease in BAT mass and UCP1 protein content. In addition, after acclimation periods animals were found to have lower $T_b$ than controls suggesting lower NST capacities. Although these studies provided some insight into the effects of chronic hypoxia only exposures of hours or a few day were used. Longer exposures may result in a greater acclimation response and a more comprehensive insight into how chronic hypoxia affects thermoregulation. In addition, there are very few studies that try to define the effects of the combination of both hypoxic and cold exposures on thermoregulation (Gautier et al, 1991). Previously, Gautier et al (1991) demonstrated that chronically cold acclimated rats exposed to short bouts of hypoxia rely more on ST to compensate for lower $T_b$ rather than utilizing NST mechanisms. Chronic acclimation to both cold and hypoxia would provide a better representation of the natural environment and provide helpful insight into how animals might adapt to HA.

Therefore, this study's main objectives were to examine the consequences of chronic hypoxia and cold on an animal's ability to regulate thermogenesis. We hypothesize that cold will increase NST capacities by up-regulating BAT mechanics. Whereas hypoxia will inhibit NST activation as a result of lower BAT mass and additional impairments to the functioning capacity in BAT. Finally,

acclimation under both chronic hypoxia and cold will allow animals to sustain NST functioning by counteracting the effects of hypoxia stress through the effects of cold acclimation.

## Methods

*Animals*

The present study was approved by the McMaster University Animal Research Ethics Board according to the guidelines of the Canadian Council for Animal Care (CCAC). CD-1 strain male out-bred mice were purchased (Charles River Laboratories Incorporated, Wilmington, Massachuetts, USA) at 13 weeks of age and weighed over 38 grams at the time of experiments. Animals were housed at 25±1°C for one week under 12:12-h light-dark cycle photoperiod and fed laboratory chow ad libitum before treatments.

*Body Temperature*

After one week in normoxic conditions an electronic data logger, (iButton, DS1922L, Embedded Data Systems, Lawrenceburg, Kentucky, USA) was surgically implanted into the abdominal cavity. Each iButton weighed ~3gm before and 3.94-3.99gm after being coated in melted paraffin elvax (Mini Mitter, Bend, Oregon, USA). The iButtons were then sized matched to the mice so that the iButton weight was no more than 10% of the mouse's body weight.

*Surgical Procedure*

Before any surgical procedures were performed the mice were initially anaesthetized with 4% iso-flurane and remained at 1% until surgery was complete. iButtons were implanted according to previous published protocols in Gusztak et al (2005). A small incision was made about 2-2.5cm along the midline which was followed by an opening through the body wall along the linea alba. The wax coated iButton was dipped in diluted disinfectant (Germex, Vetoquinol, Cambridge, Ontario, Canada), then rinsed in sterile water and inserted into the abdominal cavity. The incision along the body wall and skin was sutured with a 4-0 silk using a simple interrupted suture pattern. Tissue glue (Vetbond) was used over top of the sutured wound to help adhere the skin and eliminate the mice from chewing out the threads and exposing the lesion. Mice were monitored post-surgery for a decline in weight and euthanized if dropped more than 10% of original body weight.

*Acclimation and experimental design*

At least 4 days of recovery from surgery the mice were separated into groups of 5-7 and placed into acclimation environments; hypoxia (H,4wks at 480mmHg), cold (C,4wks at 5°C) and the combination of the two (HC, 4 weeks at 480mmHg and 5°C) and normoxic thermoneutral controls (N, 4wks at 28°C). Cold and hypoxic/cold mice were transferred into a cold room initially kept at 12°C±1 and either placed into a hypoxic hypobaric chamber at -0.1Barrs or under

normoxic conditions. The room temperature was progressively decreased every 3 days by 3-4°C until 5±1 °C. In addition, for the HC and H group mice, pressure was lowered every 3 days to a final pressure of -0.4Barrs (480mmHg). This pressure is equivalent to approximately 4300m altitude (McClelland et al, 1998). The mice were then left for an additional 4 weeks under these conditions while their food, water, and cages were changed once a week. Therefore the mice in the hypobaric chamber were brought back to normoxia every 6-7 days for about an hour each time.

*Indirect calorimetry*

After the acclimation period the metabolic response to acute temperature changes were assessed using the Peltier effect temperature controller and insulated cabinet (Sable Systems, Henderson, Nevada, USA). A flow-through respiratory system was used to measure oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$). Briefly $CO_2$- and $H_2O$-free air was pushed through a cylindrical Plexiglas chamber (~500ml in volume) at ~250-280ml/min$^{-1}$ (STPD) by a mass flow controller (Sable Systems, Henderson, Nevada, USA). A sub sample of excurrent air was passed through an $O_2$ and $CO_2$ analyzer (Withers, 1977). Temperature in the cabinet was kept at 32°C for at least 30 minutes and then lowered by 2 °C every 25 minutes until a final temperature of 4±1°C. The $VO_2$ and $VCO_2$ measurements were then used to calculate the resting metabolic

rates (RMR) during each individual temperature. Each experiment was carried out between the daytime hours of 10am-8pm.

Mice were familiarized to temperature controlled chamber about 1-2 days prior to temperature-dependant measurements and kept at 28°C for ~1hour. Each mouse was individually fasted under normoxia for 3 hours prior to each experiment while remaining in either their respective cold or warm environments. Mice in the hypobaric chamber were removed, and fasted 3 hours prior to each individual experiment. The mice left in the chamber were then resealed in the hypobaric chamber and trial mice were returned to the chamber the following day after remaining in normoxia warm or cold overnight.

During temperature-dependent experiments the system was calibrated and a baseline measurement was recorded without the mouse in the chamber for ~10 minutes, the mouse was added to the chamber at 32°C and allowed to settle for a minimum of 30 minutes. Once the mouse was calm, ambient temperature was lowered by 2°C every 25 minutes until 4°C±1 which took up to a total ~7hours or more.

Data analysis of measured $VO_2$ during various temperature intervals were issued individually by written scripts into an interpreter program (Perl Express version 2.5). $VO_2$ measured data was averaged at each $T_a$ from the 3 lowest periods of 2 minutes of activity. This was then normalized to (body weight) $^{\wedge 0.75}$.

Thermal conductance rates were calculated according to the equation $C=VO_2/(Tb-Ta)$ ($mlO_2/g/h/°C$) (Bradley and Deavers, 1980).

*Norepinephrine injections*

Each animal was placed in random order into the temperature-controlled chamber (Sable Systems, Henderson, Nevada, USA) at 28°C and metabolic rate was recorded for at least 30 minutes. Animals were removed from the chamber and 500µl of saline was injected into the intrapreoneal cavity and returned to the chamber for at least 30 minutes of data collection. Animals were removed once more from the chamber and injected with Norepinephrine (NE) at a dose according to the equation; NE dose mg/kg=2.53x $M^{-.04}$, M=mass in grams found in Wunder and Gettinger (1996). The NE was diluted at room temperature saline for a total of 500µl which was injected into the intrapreoneal cavity and the animal was returned to the chamber. Metabolic measurements were continued up to 2 hours or until $VO_2$ returned to control values. Peak oxygen consumption was defined as the highest oxygen consumption recorded over a 5minute period after post NE injection.

*Tissue Analysis*

Animals were euthanized by cervical dislocation at least 24 hours after NE injection. BAT, left and right gastrocnemius muscles were weighed and then either freeze clamped with liquid nitrogen-cooled aluminium tongs and stored at -80 °C or placed into ice cold buffer for mitochondrial isolation. Hematocrit levels

were measured using capillary tubes of sampled blood from cheek vein that was centrifuged and spun down to separate whole blood from plasma to quantify amount of read blood cells present in the whole blood and presented as a percentage.

Mitochondrial isolation for BAT was dissected from the intrascapular area and placed in 10 ml of ice cold buffer containing 250 mM sucrose, 1 mM HEPES, and 0.2 mM EDTA (pH 7.2 with KOH) and was minced with pre-cooled scissors and then homogenized using a Teflon on glass Potter-Elvehjem homogenizer (Monemdjou et al, 1999). Fractionation of homogenate was carried out by spinning at 1,500x *g* at 4°C for 10 min. The supernatant was then poured into a another cooled centrifuge tube and centrifuged at 12,000x *g* for 10 min at 4°C to obtain a mitochondrial pellet. The pellet was resuspended (on ice) in 175 µl of a suspension medium containing 120 mM KCl, 20 mM sucrose, 3 mM HEPES, 2 mM $MgCl_2$, 2 mM EGTA, and 0.5% BSA (pH 7.2 with KOH). Isolated fractions were than frozen at -20°C until immunoblotting.

*Western blots*

Isolated BAT mitochondrial protein was quantified using a Bradford Assay (Bradford, 1976) to ensure equal amounts of protein were loaded on gels. 30µg BAT were separated on a 12% SDS-Page gels (Bio-Rad) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for ~2 hours. The membranes were then washed in methanol for 10 seconds and blocked in 10% powdered milk. The membranes were then washed twice for 5 minutes

and once for 15 minutes in 10 mM phosphate buffer, 0.09% NaCl, and 0.05% Tween 20, pH 7.5 (PBST) and then BAT membranes were probed with 1:7500 diluted primary antibody UCP-1 (UCP11-A, Alpha Diagnostics) and then washed in PBST twice for 5 minutes and then once for 15 minutes before being probed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Perkin Elmer Life Science, Boston MA, USA, NEF812) at a dilution of 1:20,000. Membranes were washed once more in PBST twice for 5 minutes and then once for 15 minutes. Then blots were developed using an ECL detection system (Perkin and Elmer), and immunoreactions were visualized by exposure to BioMax XAR film (Kodak, Carestream Health France, Paris, France). Bands were quantified by scanning photodensitometry using Chemilmager Software 5.5 (Alpha Innotech Corporation, San Leandro, CA, USA).

*mRNA expression*

Total BAT and skeletal muscle RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified immediately by ultraviolet spectrophotometry at 260 nm, and RNA purity was verified by the 260- to 280-nm ratio (Nanodrop ND-1000; Fisher Scientific, Wilmington, DE). First-strand cDNA was synthesized from 1 μg of total RNA treated with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H-RT (Invitrogen). 5μl of mRNA was quantified in duplicate on a Stratagene MX3000P real-time PCR machine using SYBR green

with ROX as reference dye (Bio-Rad, Mississauga, ON). Each reaction contained 12.5 µl SYBR green mix, 1 µl of each forward and reverse primer (5 µM), and 5.5 µl RNase-free $H_2O$. The PCR protocol conditions consisted of an initial denaturation for 3 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, annealing at 60°C for 45 seconds, and extending at 72°C for 30 seconds. This was followed by a melting curve analysis to verify the specificity of the PCR products within and between tissues. Standard curves were constructed for each target gene using serial dilutions of cDNA from a combination of all treatment groups for BAT and skeletal muscles. To account for differences in cDNA and loading differences, all samples were normalized to the expression level of the housekeeping gene beta actin, which did not change significantly over the course of the experimental treatments. Accession numbers and primers can be found in Table 2.1.

*Enzyme Activities*

BAT and skeletal tissues were powdered using a liquid $N_2$-cooled mortar and pestle. Then ~20mg of each tissue were weighed into 2ml cryovial containers (Nalgene) and homogenized on ice using a glass on glass homogenizer for 1min in a 20 x dilution of extraction buffer consisting of 20mM of Hepes (pH 7.0) and 1mM of EDTA. All enzymes were measured at 37°C on a Spectromax Plus 384, microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assays were performed in triplicate and control rates without substrate were determined for each assay. Enzyme activity of COX was determined, soon after homogenization

and activity of CS was measured after a two freeze and warm flash cycles. Assays condition were, COX: 20mM Tris-HCL (pH 7.2), 50mM reduced cytochrome c; CS: 50mM oxaloacetate (omitted in control), 30mM acetyl-CoA, and 10mM dithiobisnitrobenzoic acid (DTNB) in 20 mmol $l^{-1}$ Tris (pH 8.0).

*Statistically analysis*

Data was analyzed using one-way or two-way ANOVAs. Multiple comparisons were made by using the Holm-Sidak method. Also significant differences were expressed with p values of <0.05 and <0.001.

## Results

*Body composition*

Table 1.2 displays body mass (gm), BAT weight (gm), percent of BAT weight per gm of body weight and percent of red blood cell mass (hematocrit) in mice after 4 weeks of acclimation. Hypoxic mice had significantly lower body weights than those of control and cold groups (38.1±1.1gm vs. 43.0±1.03gm and 45.5±.09gm; p<0.001) and hypoxic/cold mice were significantly lower than cold treatment group (40.0±0.9gm vs. 45.5±.09gm; p<0.001).

After mice were acclimated their BAT weights were weighed and scaled per gm of body weight. Cold mice displayed a significant increase compared to controls in raw BAT weight as well as when whole animal weight was considered (0.18±0.02 vs. 0.11±0.01gm; p<0.05 and 0.40±0.05 vs. 0.25±0.02gm; p<0.05). Hypoxic/cold mice displayed a significant increase in the amount of BAT

expressed per gram tissue of body weight compared to controls (0.33±0.01 vs. 0.25±0.02mg/g; $p<0.05$). Hypoxic mice compared to cold had significantly lower percentages of BAT interscapullary (0.25±0.02 vs. 0.40±0.05mg/g; $p<0.05$). The percentage of red blood cells was found to be significantly higher in hypoxic/cold compared to controls (68.5±6.6 vs. 48.4±1.5; $p<0.05$) and no other differences between treatment groups were found.

*Body Temperature*

Figure 2.1A displays $T_b$ recorded by surgically implanted data loggers (iButtons) over a 48 hour time period after 4 weeks of acclimation. $T_b$ were recorded every 5minutes or every 15 minutes and were averaged over an hour time period. $T_b$ in hypoxic/cold mice were significantly lower at an average of 33.1°C compared to controls who maintained $T_b$ around 36.9°C. As well $T_b$ at 12am and 8am were significantly different between controls compared to cold mice ($p<0.05$).

*Indirect calorimetry*

Measurements of oxygen consumption ($VO_2$) were taken from each individual mouse at ambient temperatures starting at 32°C and slowly decreased by 2°C every 25minutes until the chamber temperature reached ±4°C. Figure 2.2A shows that controls had significantly lower rates of $VO_2$ compared to all other treatment groups from temperatures of 32-22°C; $p<0.05$. $T_a$ below 22°C to 4°C had no effect on the varying acclimated mouse groups.

Figure 2.2B relates $T_b$ of acclimated mice while indirect calorimetric measurements were being performed in the temperature controlled chamber. At 4°C hypoxic mice had a significantly lower $T_b$ than all other treatment groups ($p<0.05$). Over the varying ambient temperatures in the temperature controlled chamber hypoxic/cold mice showed a significantly elevated $T_b$ than those of the all other treatment groups.

*Thermal conductance*

Figure 2.2C displays average thermal conductance (C) rates of each treatment group according to the equation $C=VO_2/ (Tb-Ta)$ ($mlO_2/g/h/°C$) (Bradley and Deavers, 1980). This equation helps to calculate how easily heat can be transferred from the core of an organism's body to the surrounding environment. Therefore if an organism has a higher rate of C compared to another organism that has a lower C than this indicates that heat can move more readily from the organism with the higher C and indicates a greater resistance for insulation (Hill et al., 2004). Figure 2.2C indicates that hypoxic/cold mice have a higher C than controls at $T_a$ of 30°C (0.16±0.01 vs 0.12±0.01) and 32°C (0.15±0.01 vs 0.1±0.003). Therefore hypoxic/cold mice have a greater rate of heat being transferred out to the environment than control mice and as a result require higher rates of metabolism to maintain $T_b$s that are evidently shown in Figure 2.2C.

*Non-shivering Thermogenesis*

Each mouse was placed in the temperature controlled chamber at their previously determined TNZ 28°C. Oxygen consumptions after NE injections significantly increased in all treatment groups compared to resting oxygen consumption which were measured over a 2hr period following NE injection ($p<0.001$). Cold mice experienced a significant increase by almost 30% in oxygen consumption after NE injections compared to controls (5.06±0.18ml/min vs. 3.64±0.17ml/min; $p<0.001$) and hypoxic mice significantly decreased oxygen consumption after NE injection by almost 30% (2.63±0.16ml/min vs. 3.64±0.17ml/min). There were no differences found between controls and hypoxic/cold (Figure 2.3).

*UCP1, UCP3 and PGC1-α mRNA expression and protein content*

UCP1 protein abundance was quantified in isolated BAT mitochondria using western blotting and normalizing samples to a pooled sample of all treatment tissues. No differences were found between controls and hypoxic/cold, cold and hypoxic groups (0.66±0.14 vs. 0.99±0.09, 0.67±0.13 and 0.50±0.08). However, there is 49% difference between hypoxic compared to hypoxic/cold UCP1 content (Figure 2.4).

Relative UCP1, 3 and PGC1-α mRNA expression was normalized to β-Actin mRNA expression in whole BAT and skeletal tissues of all treatment groups using Real-Time PCR. There was no significant difference in UCP1 mRNA expression found between hypoxic/cold, cold and hypoxic compared to

controls (0.49±0.18, 1.59±0.46, and 0.5±0.19 vs. 1.0±0.37; p=0.09). There was a significant increase in UCP1 mRNA between cold compared to hypoxic/cold and hypoxic group (1.58±0.5 vs. 0.49±0.2 and 0.49±0.2; $p<0.05$) (Figure 2.7).

Figure 2.8 shows PGC-1α relative mRNA expression in BAT where there was no statistical significance found between controls and treatment groups although it may appear that cold group has a larger expression than controls (N=1.0±0.2 vs. HC=1.2±0.3, C=2.1±0.7 and H=0.8±0.2; p=0.496). Relative mRNA expression in skeletal muscle to β-Actin shows a significant difference between control, cold and hypoxic treatment groups (1.0±0.2 vs. 2.5±0.5 and 3.9±0.6; p=0.008). There was no significant difference found between hypoxic/cold and control group (2.3±0.5 vs. 1.0±0.2; p=0.101) (Figure 2.8).

There was a significant increase in UCP3 mRNA between cold and hypoxic compared to control group (9.4±1.6, 8.9±2.1 vs. 1.0±0.2; $p<0.05$). But no differences found between hypoxic/cold and controls (2.5±1.1 vs. 1.0±0.24; p=0.212) (Figure 2.9).

*Citrate Synthase and Cyctochrome c activities*

Mitochondrial density and electron transport chain capacity were measured by citrate synthase (CS) and cyctochrome c (COX) activities in whole tissue homogenates. Increases in CS were found in cold mice compared to control mice (25.04±0.6 vs. 18.6± 1.04μmol/min/g; $p<0.05$) as well a significant decrease of CS in hypoxic/cold and no differences in hypoxic compared to controls

(9.1±0.7, 21.3±1.2 vs. 18.6±1.04μmol/min/g; $p<0.05$) (Figure 2.5). CS activity in skeletal muscle did not change among acclimated groups compared to controls (12.8±0.7, 14.05±0.4, 13.04±0.6 vs. 13.4±0.4μmol/min/g).

COX activity in BAT significantly increased compared to controls values in hypoxic/cold and hypoxic groups (4.2±0.4 vs. 8.3±0.5 and 8.3±0.7μmol/min/g; $p<0.05$) and no differences were seen in cold compared to controls (5.03±0.3 vs. 4.2±0.4μmol/min/g). No changes in COX activity were found in treatment groups compared to controls (5.5±0.6, 6.1±0.9, 5.9±0.4 vs. 6.5±0.2 μmol/min/g) (Figure 2.6).

## Table 2.1: Primers and accession numbers

Table2.1 shows a list of all primers and accession numbers used for Real-Time PCR.

| Gene | Primer | Accession Number |
|---|---|---|
| Beta-Actin | F- 5'-GGCTCCTAGCACCATGAA-3'<br>R- 5'-CTGGAAGGTGGACAGTGA-3' | X03672 |
| UCP-1 | F- 5' –AGAAACTGCCTCTCTCG- 3'<br>R- 5' –TCTGACCTTCAGACCTCTG- 3' | U63418 |
| UCP-3 | F- 5'-GGATGCCTACAGAACCATCG-3'<br>R- 5'-GGTCACCATCTCAGCACAGTT-3' | AB010742 |
| PGC1-α | F-5'-CATGGATGGCCTATTTGATGAC-3'<br>R-5'-CACGGAGAGTTAAAGGAAGAGC-3' | NM133249 |

**Table 2.2: Whole body mass, BAT mass, BAT mass per gm of body weight and hematocrit percentage in whole blood extractions**

Table2.2 shows whole body weights, BAT and percentage of hematocrit in whole blood after acclimation was completed in CD-1 mice held under hypoxia, cold and hypoxia/cold in comparison to controls. Samples sizes were as follows: N n=16, H n=14, C n=7 and HC n=9. The * indicates significant difference from N and ** indicates significant difference from C where $p<0.05$ using a one-way ANOVA. All values are means ± SEM.

| | Weight (gm) | BAT Weight (gm) | Relative BAT Weight (mg per gm body weight) | Hematocrit |
|---|---|---|---|---|
| N | 43.0± 1.0 | 0.11± 0.01 | 0.25± 0.02 | 48.4± 1.5 |
| H | 38.1± 1.1* | 0.09± 0.01 | 0.25± 0.02** | 56.7± 4.2 |
| C | 45.5± 0.9 | 0.18± 0.02* | 0.40± 0.05* | 52.4± 3.5 |
| HC | 40.0± 0.9** | 0.13± 0.01 | 0.33± 0.01* | 68.5± 6.6* |

**Figure 2.1: Average hourly body temperature over 48 hours**

Figure2.1 shows body temperatures measured every hour for 48hours after acclimation period in all 4 treatment groups. N=normoxic thermoneutral control represented in closed circle, C=cold by closed triangle, H=hypoxia open triangle and HC=hypoxic/cold in open circle. Samples sizes were as follows: N and H n=4, C n=6 and HC n=5. The *indicates significant differences between HC group and all other treatment groups using a two-way ANOVA ($p<0.05$). Values are means ± SEM.

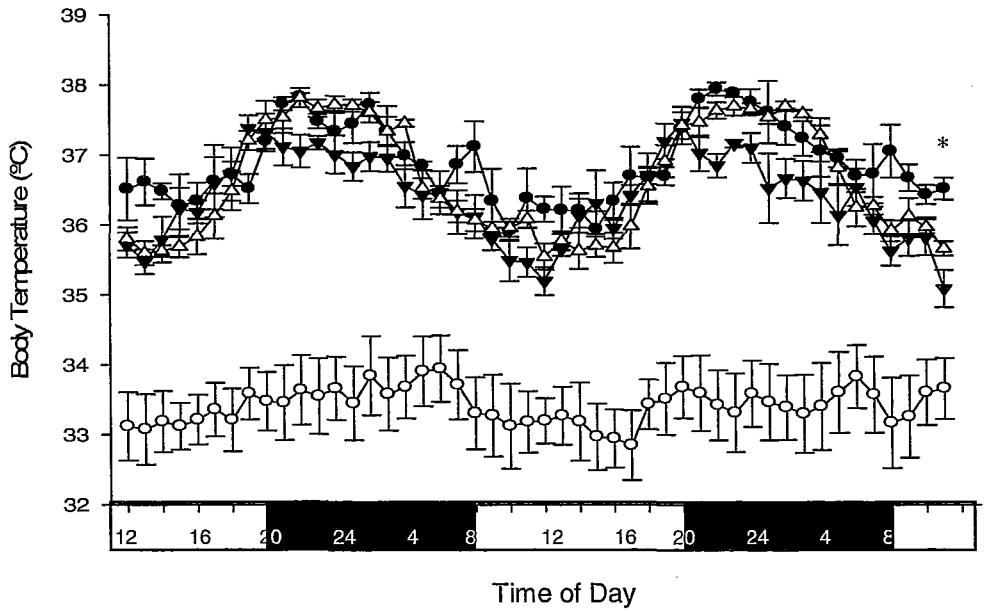

Body Temperature (ºC)
39
38
37
36
35
34
33
32
*
12
16
20
24
4
8
12
16
20
24
4
8
Time of Day

**Figure 2.2: Metabolic rate, body temperatures and calculated thermal conductance rate during acute temperature exposures (4-32°C)**

Figure**2.2A** shows average oxygen consumption ($VO_2$) measured over ambient temperatures ($T_a$) 4-32°C. The * indicates values of significance ($p<0.05$) from N group where HC across all varying ambient temperatures is found to be statistically significant using a two-way ANOVA. Figure**2.2B** shows average body temperature at ambient temperatures between 4-32°C. The # indicates a significant difference between H and all other treatment groups at ambient temperature of 5°C ($p<0.05$) from HC using a two-way ANOVA. Samples sizes were as follows: N n=6, H, C, HC n=4. Figure**2.2C** shows calculated thermal conductance rates at ambient temperatures between 4-32°C. Samples sizes are all n=5. N=normoxic thermoneutral control represented in closed circle, C=cold by closed triangle, H=hypoxia open triangle and HC=hypoxic/cold in open circle. All values are ±SEM.

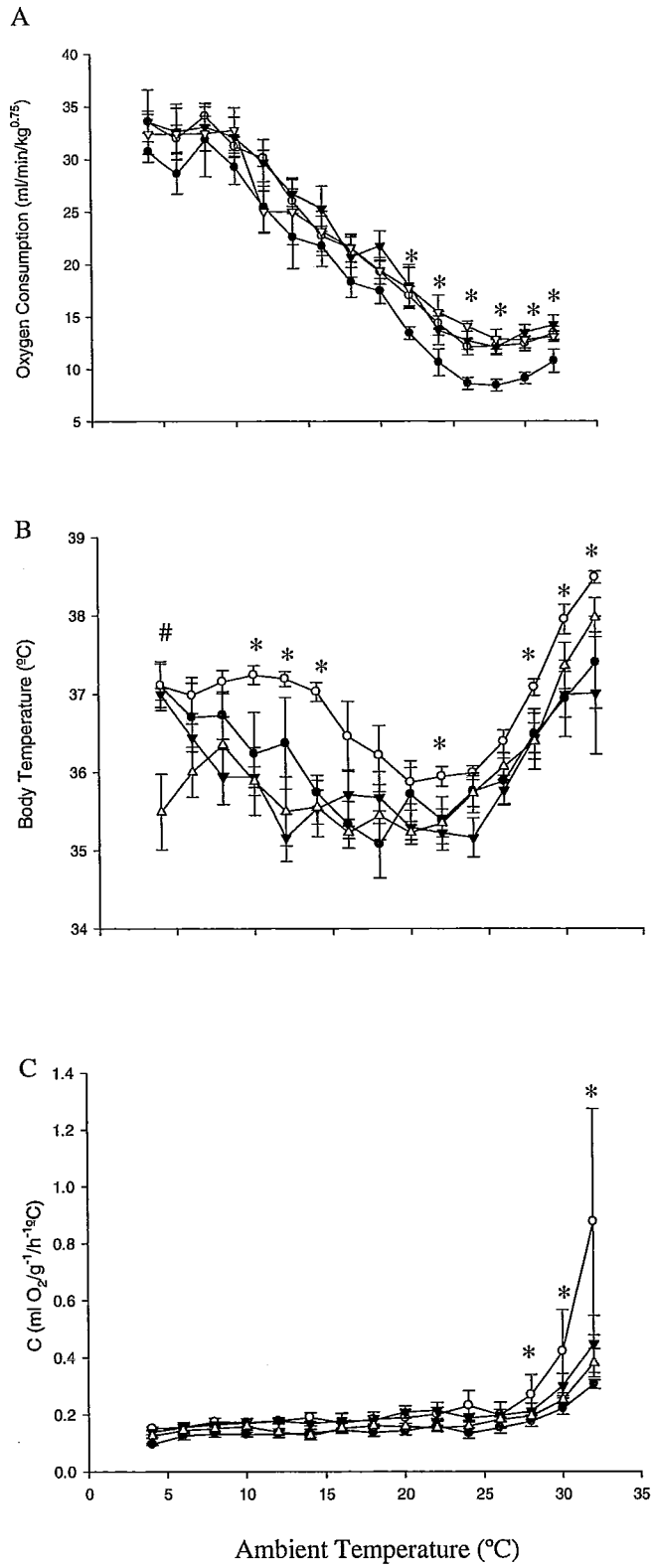

A
Oxygen Consumption (ml/min/kg^0.75)
40
35
30
25
20
15
10
5
B
Body Temperature (°C)
39
38
37
36
35
34
#
C
C (ml O2/g-1/h-1°C)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Ambient Temperature (°C)

**Figure 2.3: Metabolic rate in response to an injection of saline and NE**

Figure2.1 shows maximum resting oxygen consumption after an injection of saline and then again after an injection of NE. Different letters denote statistical significance between treatment groups after saline injection ($p<0.001$) using a two-way ANOVA. No statistically differences were found between treatment groups after Saline injections. Sample sizes were as follows: N n=7, H n=8, C n=6 and HC n=9. Values are ±SEM.

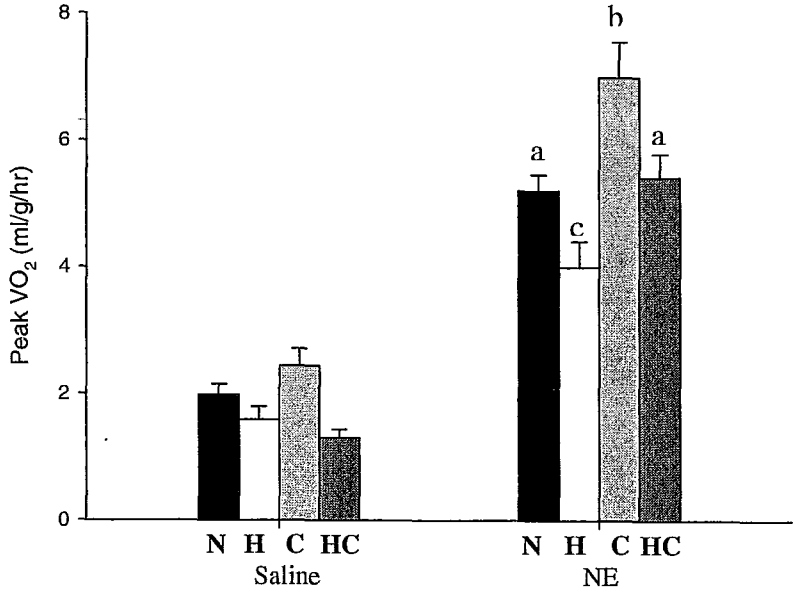
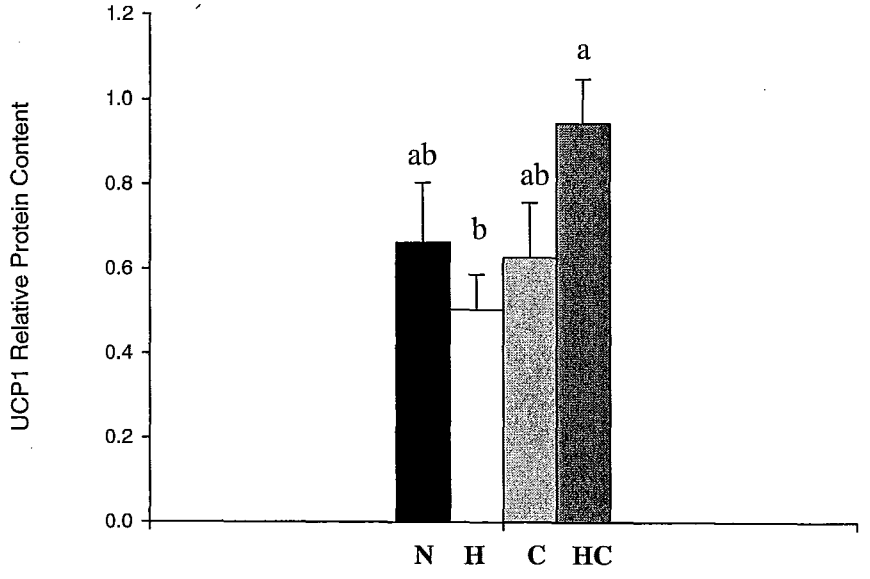

Peak $VO_2$ (ml/g/hr)
8
6
4
2
0
a
c
b
a
N H C HC
Saline
N H C HC
NE

**Figure 2.4: UCP1 protein content per unit mg of mitochondria in BAT**

Figure2.4 shows average UCP1 protein content in isolated BAT mitochondria relative to pooled sample of all treatment samples. Different letters indicate statistical significance between treatments ($p=<0.05$) using a one-way ANOVA. Sample sizes were as follows: N and HC n=7, H n=10 and C n=6. Values are ±SEM.


UCP1 Relative Protein Content
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ab
b
ab
a
N
H
C
HC

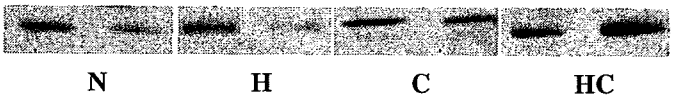

N
H
C
HC

**Figure 2.5: Citrate synthase activity in BAT and skeletal muscle**

Figure2.5 shows average citrate synthase (CS) activities in whole BAT tissues and gastrocnemius muscle. Different letters indicate statistical significance between treatments ($p<0.001$) using a one-way ANOVA. There was no statistical significance in skeletal muscle between treatments. Sample sizes are as follows: N and C n=7, H and HC n=6 and skeletal muscle N, HC and C n=8 and H n=8. All values are ±SEM

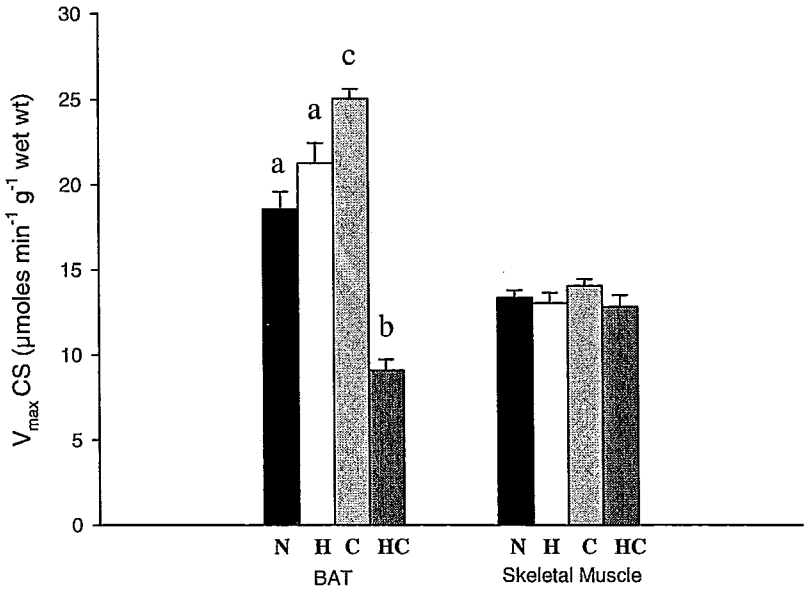

30
25
20
15
10
5
0
$V_{max}$ CS (µmoles $min^{-1}$ $g^{-1}$ wet wt)
a
a
c
b
N H C HC
BAT
N H C HC
Skeletal Muscle

**Figure 2.6: Cytochrome c oxidize (COX) activity in BAT and skeletal muscle**

Figure2.6 shows average cytochrome c oxidize (COX) activities in whole BAT tissues and gastrocnemius muscle. Different letters indicate statistical significance between treatments ($p=<0.001$) using a one-way ANOVA. There was no statistical significance in skeletal muscle between treatments. Sample sizes were as follows in BAT: N and C n=7, H and HC n=6. Sample sizes were as follows in skeletal muscle: N and H n=8, HC n=9 and C n=7. All values are ±SEM

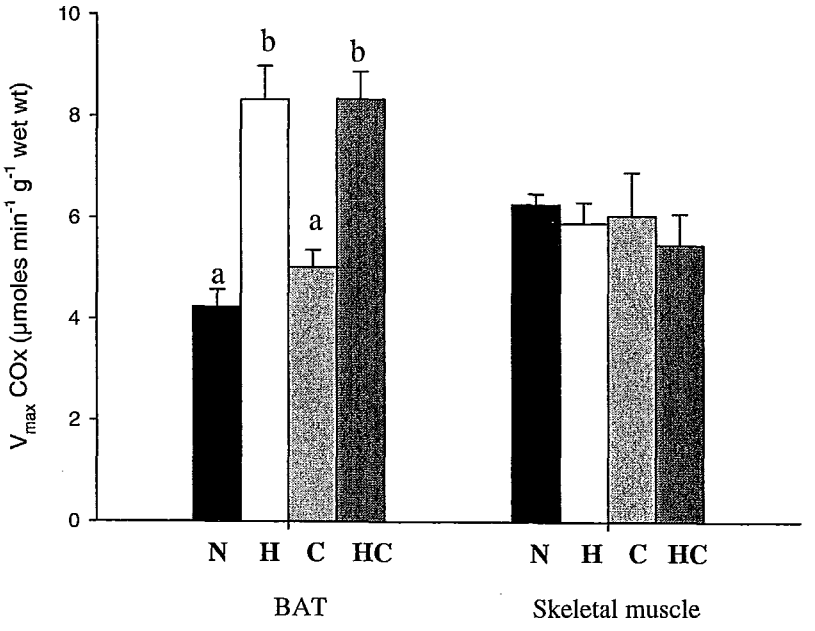

10
8
6
4
2
0
$V_{max}$ COx (µmoles $min^{-1}$ $g^{-1}$ wet wt)
a
b
a
b
N H C HC
BAT
N H C HC
Skeletal muscle

**Figure 2.7: UCP1 mRNA expression in whole BAT**

Figure2.7 shows UCP1 mRNA expressions in BAT relative to controls. All treatments were normalized expression in β-Actin. Different letters indicate statistical significance between treatments ($p<0.05$) using a one-way ANOVA. Sample sizes were as follows: N and H n=7, C and HC n=6. All values are ±SEM

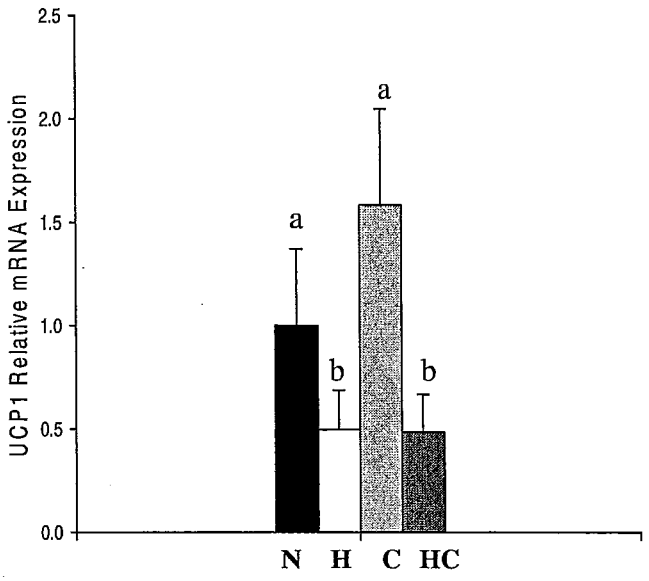

UCP1 Relative mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a
b
N
H
C
HC

**Figure 2.8: PGC-1α mRNA expression in BAT and skeletal muscle**

Figure2.8 shows PGC-1α mRNA expression in BAT and gastrocnemius muscle relative to control treatment. All samples were normalized to expression in β-Actin. Different letters indicate statistical significance between treatments ($p<0.05$) using a one-way ANOVA. Samples sizes were n=7 for all treatments. All values are ±SEM.

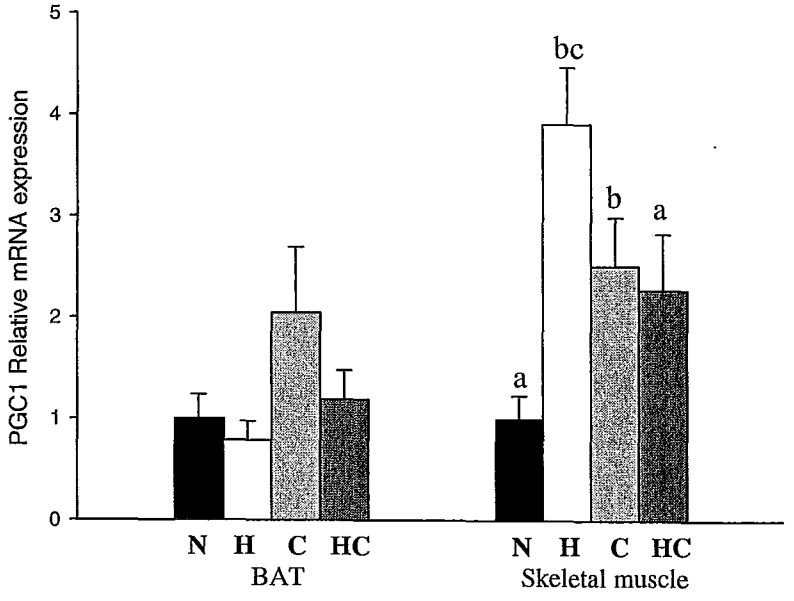
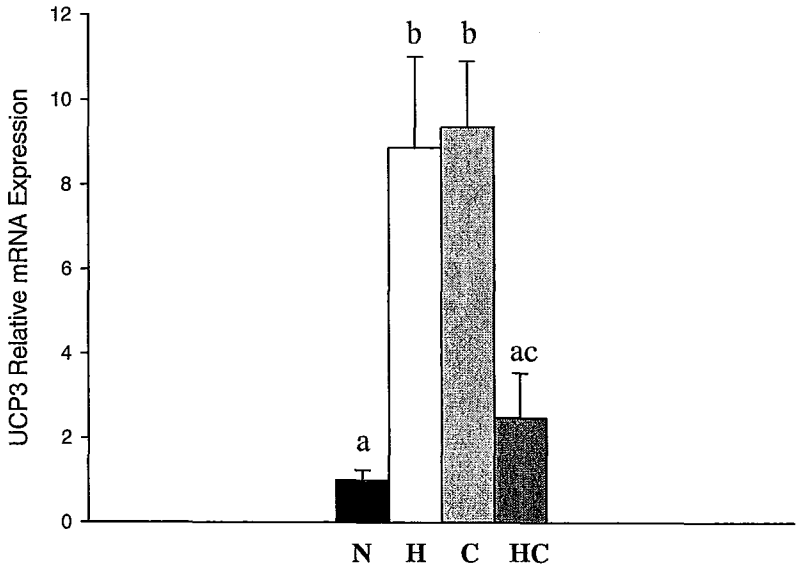

PGC1 Relative mRNA expression
5
4
3
2
1
0
bc
b
a
a
N H C HC
BAT
N H C HC
Skeletal muscle

**Figure 2.9: UCP3 mRNA expression in skeletal muscle**

Figure2.9 shows UCP3 mRNA expressions in skeletal muscle relative to controls. All treatments were normalized to expression in β-Actin. Different letters indicate statistical significance between treatments ($p<0.05$) using a one-way ANOVA. Samples sizes were as follows: N, HC, C n=5 and H n=6. All values are ±SEM.


UCP3 Relative mRNA Expression
12
10
8
6
4
2
0
a
b
b
ac
N
H
C
HC

## Discussion

Physiological responses of mice after exposure to cold $T_a$ and low atmospheric oxygen have been studied extensively (Biggers et al, 1957, Chappell, 1984, Rezende et al, 2004, Chappell and Hammond, 2004). However, there are a few studies that have examined these two environmental stressors together, especially chronically exposed animals (Gautier et al, 1991). Due to the relevance of these combined stressors to high-altitude (HA) environments we examined the metabolic response of laboratory CD-1 mice exposed to environmentally realistic levels of cold and hypoxia for more than a four week period. For many of the variables measured the combination of cold and hypoxia had distinct effects from either cold or hypoxia alone. In general, hypoxia on its own had a negative effect of NST where these mice exhibited lower $T_b$ during certain times of the day than controls (Figure 2.1). Cold acclimation enhanced NST capacity through up-regulation of BAT heat generating machinery. Hypoxic/cold mice were not able to maintain normal $T_b$ and lost diurnal $T_b$ rhythms after acclimation (Figure 2.1). Although this group maintained a lower $T_b$ they were able to engage in sufficient thermoregulation when presented with an acute temperature challenge in normoxia (Figure 2.2). This may be the result of a slightly increased BAT mass, increased UCP1 protein abundance per mg of mitochondria but a blunted response in oxygen consumption after an injection of NE. Moreover, significantly lower amounts in UCP1 mRNA expression may result from lower mitochondrial densities of BAT with the combination of cold and hypoxic acclimation. The

reason for this strategy remains unclear but may be a mechanism for these animals to conserve energy in an oxygen poor environment.

**Brown adipose tissue**

BAT is extremely crucial for heat production in small mammals and serves to protect an organism from becoming hypothermic with cold exposure (Himms-Hagen, 1985). Therefore, as a result of acclimation to cold environments endotherms increase body weight as well as BAT mass that greatly increases capacity for NST (Reviewed in Cannon and Nedergaard, 2004). However, in the very few studies that have investigated the properties of BAT mitochondria with a four day hypoxic exposure suggest a decrease in the capacity of BAT for thermogenesis (Mortola and Naso, 1998). Comparable to these previous studies, our hypoxic mice show significantly lower BAT mass compared to controls when normalized to body mass. As well, cold mice exhibited an increase in body mass after acclimation (Table 2.2). Interestingly, hypoxic/cold mice have significantly higher amounts of BAT compared to controls but have lower BAT weight compared to cold mice. This may suggest that cold exposure improves NST capacities, in part by slightly increasing BAT weight. However, in combination with hypoxia, BAT mass remains up-regulated as is found in cold acclimated mice but perhaps may not elicit the same capacity to carry out NST mechanisms (See below).

**Body temperature set points**

Continuous monitoring of $T_b$ via iButtons data loggers show that hypoxic/cold mice maintain a significantly lower $T_b$ than all other treatment groups (last 48 hours of acclimation shown in Figure 2.1). It is well known that $T_b$ set point in mammals is regulated through the hypothalamus which helps to control and maintain $T_b$ independently from the environment (Heller et al, 1977). Chronically lower $T_b$ found in hypoxic/cold mice is likely due to a re-setting of the hypothalamic $T_b$ set point. This suggests an adaptive strategy to combined cold and hypoxic conditions to decrease energy expenditure involved in thermogenesis, presumably to cope with lower amounts of available oxygen. Moreover, this response in hypoxic/cold mice may be due to an inability to shiver in the hypobaric chamber. Again this may help to lower energetic costs of thermoregulation in hypoxia. Interestingly, hypoxia has been shown to inhibit ST in ground squirrels (Barros et al, 2001) which could suggest that despite moderate changes to NST in hypoxic/cold mice, ST could also be vastly down-regulated in hypoxia and lead to an inability to maintain $T_b$ relative to controls.

Hypoxia on its own generally has been found to decrease $T_b$ as blood flow is redirected away from the BAT to other more oxygen sensitive tissues such as the heart and brain (Szelenyi and Donhoffer, 1968). This redistributes nutrients required for vital tissue functioning but also results in greater heat loss. Indeed, in this present study hypoxic mice display lower $T_b$ during later evening hours but

not at other times of day (Figure 2.1). Perhaps since hypoxic mice were housed within their TNZ, only moderate effects of hypoxia on $T_b$ were seen which help reduce the already low metabolic costs of thermoregulation. Moreover, cold mice showed no changes in $T_b$ after acclimation compared to controls indicating an ability to adjust to colder temperatures and the effective use of heat generating pathways such as NST. Taken together these results suggest that although BAT mass is greater in hypoxic/cold mice it may be limited in its ability to up-regulate NST with cold exposure and these mice may be forced to rely on other less effective heat generating mechanisms such as shivering which may be impaired under a state of hypoxia (Barros et al, 2001).

In addition it is interesting to note that all groups except hypoxic/cold display a clear diurnal rhythm in $T_b$ (Figure 2.1). Classically $T_b$ should rise during night time hours where nocturnal animals are most active and fall during daytime hours where sleeping and other less taxing activities are most likely to occur (Mousel et al, 2001). However, hypoxic/cold mice seem to display a relatively stable $T_b$ ~33°C and no evidence of fluctuations between night time and daytime temperatures. Thus the environmental stress of hypoxia/cold lowers $T_b$ and blunts regular diurnal $T_b$ oscillations. Regular daily observations of mouse behaviour confirmed that hypoxic/cold mice remained relatively immobile and in a curled up position while all other treatment groups kept normal behavioural patterns.

**Metabolic rates**

Adaptive thermogenesis is an overall increase in heat production by the up-regulation of NST which represents an overall increase in $VO_2$ over any given $T_a$ range. In Figure 2.2A, all experimental groups, including hypoxic/cold mice, showed significantly higher oxygen consumption rates compared to controls within their respective TNZ (~22-32°C). This may be the result of larger organs such as BAT in the hypoxic/cold and cold acclimated groups. Recently a study by Rezende et al (2009) has shown that higher metabolic rates are associated with an increase in size of the heart, liver and digestive organs after chronic (7 weeks) cold exposure in deer mice. There were no specific reports of BAT mass by Rezende et al (2009) but cold acclimated male and female mice that possessed higher metabolic rates were correlated with having lower amounts of absolute fat and more lean muscle. Therefore higher metabolic rates within TNZ in hypoxic/cold and cold mice may be a consequence of larger organs and evidently by higher amounts of BAT shown in Table 2.1 that increase thermoregulatory efficiency. Moreover, during exposure to the acute temperature challenge in normoxia, Figure 2.2B demonstrates higher $T_b$ ~38 °C in hypoxic/cold mice. This suggests that these mice elevate $T_b$ higher than $T_b$ in the hypobaric chamber to perhaps in response to eliminate excessive heat gain from higher $T_a$. This is further demonstrated in Figure 2.2C where the rate of thermal conductance in hypoxic/cold mice is extremely elevated at $T_a$ above 30°C compared to controls. This indicates that these mice are losing heat more readily to the environment

possibly in an attempt to return $T_b$ to normal. This may help to explain the increased rates of metabolism that these mice have demonstrated within TNZ (Figure 2.2A) as possible mechanisms to eliminate excessive heat gain. Also, hypoxic/cold mice may selectively raise their $T_b$ in colder acute temperature exposures as they may elicit an increase in shivering or NST that were shut down during the acclimation periods in an attempt to eliminate excessive heat gain.

**Non-shivering thermogenesis induced by norepinephrine**

Norepinephrine (NE) directly stimulates $\beta_3$-adrenergic receptors on BAT and effectively elevates oxygen consumption due to the increase in metabolic heat production. Therefore, direct stimulation of this mechanism can be used to quantify the capacity for adaptive NST (Jansky, 1973, Wunder and Gettinger, 1996). In response to NE injection hypoxic/cold mice showed little difference in NST capacity compared to controls. However, cold and hypoxic mice display higher and lower oxygen consumption rates respectively, relative to controls (Figure 2.3). The capacity for NST can result from changes in BAT mass, mitochondria density of BAT and/ or UCP1 density per unit mitochondria. BAT weight was slightly increased in hypoxic/cold and cold mice but lower in hypoxic mice, compared to controls. Therefore, hypoxia may limit NST capacity by reducing BAT. In support of this idea cold mice greatly increase oxygen consumption immediately after NE injection compared to controls which indicates rapid up-regulation and greater capacity for NST. In contrast, although there is some activation of thermogenesis via BAT stimulation in hypoxic mice,

oxygen consumption did not reach levels as high as those of the controls. Therefore, results seen in hypoxic/cold mice are not surprising in that they resemble similar oxygen consumption levels as controls. Acclimation to hypoxia/cold may inhibit NST capacity because of overriding effects of hypoxia. For this reason they do not resemble cold acclimated mice, possibly due to a hypoxia-induced shutdown of thermogenesis that hinders NST pathways in hypoxic/cold mice. Along with BAT mass, BAT mitochondrial density and UCP1 content can also affect NST. These qualitative features of BAT have been commonly found in cold exposed endotherms (Jakus et al, 2002) and are suggested to greatly facilitate animals that live in environments with variable seasonal temperatures (Haim et al, 1993, Himms-Hagen, 1985).

**UCP1**

Long-term cold exposure is known to increase whole tissue UCP1 mRNA expression in BAT (Watanabe et al, 2008, Nedergaard et al, 2001, Golozoubova et al 2001 and Dulloo et al, 2001). Surprisingly, we found that after more than four weeks of cold exposure cold mice did not up-regulate UCP1 protein content per unit mitochondria (Figure 2.4). Moreover, hypoxia on its own decreased whole cell UCP1 mRNA and protein but this effect in UCP1 protein was reversed when combined with cold acclimation (Figure 2.4). These results suggest mitochondrial UCP1 protein may be differentially regulated compared to UCP1 mRNA expression under various environmental stressors. Previous studies have

shown that full recruitment of UCP1 at the mRNA level is established ~4 hrs after exposure to the cold whereas UCP1 protein requires ~3 wks to complete its full stabilization (Jacobsson et al, 1994). This delay between UCP1 mRNA and UCP1 protein abundance is related to the half-life of UCP1 mRNA that is directly proportional to the rate of synthesis of UCP1 protein compared to controls (Nedergaard et al, 2001). Perhaps although cold mice do demonstrate an increase in UCP1 mRNA expression and no changes in UCP1 protein content (Figure 2.4) they do have higher mitochondrial densities that lead to an overall increase in NST ability. Where hypoxia has been shown to decrease thermogenic machinery such as BAT mass and UCP1 expression (Mortola and Naso, 1998) and further reports of blunted NE response may indicate impairments in UCP1 functioning (Nikami et al, 2005). In this study and in concert with previous findings, UCP1 protein expression along with mRNA is lower in hypoxic mice compared to controls. However, exposure to hypoxia/cold may up-regulate UCP1 protein expression in mitochondria above those observed in hypoxia alone. Higher amounts of UCP1 protein may help these mice to stimulate NST mechanisms under normoxic conditions which are demonstrated by an increase in $T_b$ during acute temperature exposures (Figure 2.2) and when ST may not be inhibited.

CS is an enzyme that plays an important central role in metabolism of all animals maintaining flux through the Kreb's cycle. It can also be used as an indicator of tissue oxidative capacity (Wiegand and Remington, 1986) and is more commonly used to estimate the amount of mitochondrial density (Janssens

et al, 2000). COX activity reflects the functioning capacity of the electron transport chain and gives estimates of mitochondrial respiratory capacity. Figure 2.5 shows a lower mitochondrial density in hypoxic/cold mice compared to all treatment groups. Furthermore, increases in COX activity in both hypoxic/cold and hypoxic treatment groups suggest increased mitochondrial cristae surface area perhaps to accommodate higher amounts of UCP1 protein content found in hypoxic/cold mice (Figure 2.4).

In contrast, cold acclimated mice demonstrate higher levels of mitochondrial density as indexed by an increase in BAT CS activity (Figure 2.5). Combined with a greater BAT mass, cold acclimated mice also demonstrate a greater capacity in NST (Figure 2.3) in agreement with previous studies that, in addition also show high expression levels of UCP1 mRNA (Watanabe et al, 2008). In contrast to previous studies, lower UCP1 protein levels in cold mice found in this study were surprising but may indicate differences in the regulation of mRNA and mitochondrial proteins in chronic cold exposures. Moreover, lower COX activity per gm tissue found in cold mice compared to hypoxic and hypoxic/cold mice may contribute to lower oxidative capacity and amounts of UCP1 protein found in cold acclimated mice (Figure 2.6).

Furthermore, lower CS activity and UCP1 mRNA expression seen in hypoxic/cold treatment group may indicate lower mitochondrial densities and thermogenic capacity (Figure 2.5). Therefore, to confirm these results we measured the expression levels of a key player in mitochondrial biogenesis,

PPAR gamma co-activator (PGC-1α) (Figure 2.8), which more importantly has been shown to directly control UCP1expression in BAT (Janssens et al, 2000). However, none of the experimental treatments significantly induced PGC-1α expression but there was a trend in the down-regulation of PGC-1α in BAT after hypoxic exposure and an increase in PGC-1α mRNA in cold acclimated mice (Figure 2.8). Moreover, a rise in BAT PGC-1α mRNA after cold exposures may be responsible for higher expressions of UCP1 mRNA and more importantly increases in mitochondrial density. Therefore, it can be concluded that cold acclimated mice increase NST capacity through increased mitochondrial biogenesis leading to increased mitochondrial density, concurrent with increased UCP1 mRNA expression. However, acclimation to hypoxia and/cold combined decreases mitochondrial density and UCP1 mRNA but enhances NST in normoxia through the up-regulation of UCP1 protein that maintain $T_b$ during acute temperature exposures but lower $T_b$ in hypoxia.

**UCP3**

The exact role of UCP3 in thermogenesis remains a highly controversial topic in recent literature (Brand and Esteves, 2005) but current evidence suggests that it plays a more significant role in the metabolism of lipids (Barger et al, 2006). In this present study higher UCP3 mRNA expression in skeletal muscle were found in both cold and hypoxic treatment groups that may perhaps aid in ST mechanisms (Figure 2.9). ST may be a more prominent form of thermogenesis in

normoxia after hypoxia exposure as the hypoxic treatment group displays a higher expression of UCP3 in skeletal muscle. Additionally, UCP3 expression has been shown to be up-regulated in skeletal muscle upon exposure to hypoxia to help reduce the generation of reactive oxidative species (Zhou et al, 2000). Previous studies have shown there are effects after an acute hypoxic exposure and perhaps UCP3 expression continues to remain elevated after chronic exposure as it is evident in this present study. Moreover, acute cold exposure studies have also shown an up-regulation of UCP3 in skeletal muscle but further evidence in UCP1 ablated mice have shown that UCP3 plays little role in adaptive thermogenesis (Cline, 2006). Therefore elevated levels of UCP3 found in this present study may suggest that after chronic cold exposure there may be an up-regulation of ST in addition to NST that may improve these animal's abilities to withstand longer periods of cold exposures by continuous muscle contractions (Cannon and Nedergaard, 2004).

In contrast, there were no significant differences found in CS or COX activity in skeletal muscle amongst any of the treatments, however, PGC-1α mRNA expression was found to increase in hypoxic and cold acclimated mice skeletal muscle. Previous reports have found a significant increase in PGC-1α expression in skeletal muscle in response to bouts of hypoxia induced by exercise or lower amounts of ambient oxygen (Arany, 2008) as well as increased expression with chronic cold (Wu et al, 1999). Therefore although no differences in mitochondrial density or respiration were obvious in skeletal muscles of the

treatment groups there may be an up-regulation of PGC-1α mRNA expression that may in turn increase UCP3 mRNA in hypoxic and cold acclimated groups.

**Conclusions**

Taken together results suggest that the combination of hypoxic and cold exposure limits an animal's thermogenic responses. Whereas cold acclimation alone develops an enhanced adaptive thermogenic response, hypoxia acclimation may inhibit some NST pathways while the combination of these two stimuli has a distinct effect on NST capacity. The hypoxic/cold treatment group may impose a limited thermogenic response under hypoxia that reduces $T_b$. Also, PGC-1α expression down-regulates mitochondrial density and UCP1 mRNA expression in BAT. Moreover, BAT mass is up-regulated in hypoxic/cold mice to help these animals maintain some NST capacity with acute temperature exposures in normoxia that could be sustained through high UCP1 protein pools that help to maintain $T_b$.

Further studies should be carried out to examine species-specific responses to cold and hypoxia which could provide a more in-depth analysis on the response to hypoxic/cold seen in other mouse species or if this is a unique response to the CD-1 male mouse strain. In addition, further examinations of specific neural control in hypothalamic set points should be carried out in mice that are acclimated from sea-level to natural HA environments as well as mice acclimated to laboratory hypoxic/cold simulated settings.

## Chapter 3

# GENERAL SUMMARY and CONCLUSIONS

Managing a neutral energy balance drives survival under various environmental stimuli and influences a bodily system either negatively or positively. HA environments can place extreme stress on an organism's metabolic reserves and greatly amplify thermogenic challenges that exist within an organism especially those that require the maintenance of a constant $T_b$. Endotherms residing at HA must defend mechanisms of thermoregulation while battling the consequences that hypoxia may pose on the entire energy balance of their system. Many researchers have previously investigated the acute and chronic effects of cold acclimation on endothermic thermoregulating systems as well as the response to acute hypoxia, but rarely have they examined the response of chronic exposure to both these extreme stressors. Therefore, this study's objectives were designed to allow a unique examination of how these environments affect a regular thermoregulating system using chronically exposed endotherms to both hypoxia and cold. The subsequent paragraphs include a discussion on additional data that was collected from the same acclimated treatment groups as presented in Chapter 2. This is followed by a brief overall conclusion of all results, future directions for this area of research and a detailed methods section for this additional data.

## I. Tissue composition

Generally, small endotherms have very high surface area to volume ratios, which allows body heat to move more readily into the surrounding environment. Therefore, endotherms are forced to dedicate a large portion of their acquired energy towards generating metabolic heat necessary to maintain a constant $T_b$ (Davenport, 1992).

Cold acclimated animals typically increase their food consumption that enlarges body mass and organ size. This leads to an increase in metabolic rate of these functioning organs and further reduces surface area to volume ratios so heat can remain inside the body (Haim et al, 1993). In this thesis, body mass in cold mice were slightly higher compared to controls although they were not statistically significant (Table 3.1). However, hypoxic mice had lower body mass compared to controls and the hypoxic/cold were slightly smaller than cold mice but were found to be larger than controls. Typically a drop in body weight is a general consequence of hypoxia as blood flow becomes redirected towards more tissues such as the brain and heart, while non-vital organs such as the skeletal muscle and fat deposits tend to reduce in size which leads to an overall loss in body weight (Szelenyi and Donhoffer, 1968).

Hypoxic/cold acclimated mice significantly show lower body weights compared to cold mice (Table 3.1) which could be due to the overriding effects of hypoxia on tissue development. Furthermore, hypoxic/cold mice appeared to have

larger hearts, which is probably due to larger left and right ventricles compared to controls, whereas hypoxic mice tend to have smaller heart sizes and much larger skeletal muscles compared to hypoxic/cold mice. Larger heart muscles were expected in hypoxic/cold mice as blood and nutrients are typically directed towards these vital organs and away from other less critical organs such as skeletal muscles. Nevertheless, it was surprising to find that hypoxic mice had significantly larger skeletal muscles than hypoxic/cold mice but did indicate a response of increased muscle shivering possibly due to a reduction in BAT mass that these mice may enlist instead of up-regulating NST pathways.

## II. Body Temperature

In response to an injection of NE, normoxic thermoneutral controls appear to express a trend towards an increased $T_b$ until 30 minutes post-injection and returned to baseline after 120minutes (Figure 3.1). In contrast, the hypoxic/cold mice had a more exaggerated response which resulted in a rapid increase in $T_b$ which remained elevated for 30minutes until returning to baseline after 120minutes. Although only core $T_b$ were measured in this present study it has been previously shown by Souza et al (2007) that core $T_b$ along with BAT temperature rapidly increased by ~1 and ~3°C and remained elevated for at least 30 minutes post-injections of NE in cold acclimated mice. The NE response shown in the hypoxic/cold mice $T_b$ is much greater than those of the controls and perhaps was related to the larger amount of BAT mass found in hypoxic/cold

mice (Table2.1) than controls; however, there were no differences in oxygen consumption post-injection (Figure 1.3) between hypoxic/cold and controls. Thus, these mice may have trouble dissipating heat generated by NST. Moreover, as hypoxic/cold mice have a large amount of BAT mass they also have more UCP1 per mg of mitochondria (Figure 1.4), but lower mitochondrial densities (Figure 1.5) and UCP1 mRNA expression (Figure 1.7). Therefore, it may be plausible that these mice do not employ NST through increasing mitochondrial density or up-regulation of UCP1 transcription but rather under normoxic conditions up-regulate BAT mechanisms through an increase in mitochondria surface area present in an increase in COX activity in BAT (Figure 1.6). Therefore, an increase in BAT mass and UCP1 abundance enables a response to NE injection by a significant rise in $T_b$ but does not affect the rate of oxygen consumption. This may help counteract the negative effects that hypoxia alone has on NST capacity.

### III. Thermal Neutral Zone

Lower critical temperature (LCT) of each treatment group represents the final $T_a$ of the TNZ beyond which lower $T_a$ requires the use of facultative thermogenesis which can be indirectly measured as an increase $VO_2$. Hypoxic mice show an increase in metabolic rate at a relative high $T_a$ of 27.3°C compared to normoxic thermoneutral controls, which significantly increase oxygen consumption at 26.9°C. Previous reports on animals exposed to hypoxia have shown that in general, the animals prefer lower $T_a$ as a response to the resetting of

lower thermoregulatory set points (Ramirez et al, 2007). As hypoxic mice were shown to display lower $T_b$ (Figure 1.1) during certain daily hours this may explain reasoning behind lower LCT of these animals. However, it may be also be a reflection of their lower thermogenic ability to maintain $T_b$ during acute temperature exposures below 27°C. These mice are demonstrating that they require higher metabolic demands at higher $T_a$ than other treatment groups and as a result need to expend more energy at a higher $T_a$ to maintain $T_b$. In addition, as previously demonstrated these mice show smaller BAT mass and a lower metabolic response in BAT after an injection of NE (Figure 1.3) similar to BAT ablated mice (Klaus et al, 1998). Therefore, a narrow TNZ range may indicate behavioural differences at lower $T_a$ in hypoxic mice. These mice may change postural positioning or develop thicker fur coats as ways to conserve heat; however, there were no differences found in fur densities (Figure 3.2) of hypoxic mice compared to controls and further, no differences in the rates of thermal conductance (Figure 1.2C) in these mice. Therefore, as these mice demonstrated a lower NST capacity and BAT mass it is more than likely they are relying heavily on other heat generating mechanisms such as shivering to maintain $T_b$ and not necessarily NST.

In addition, hypoxic/cold were found to have a broader TNZ range than controls where LCT were calculated to be 26.77 compared to 26.9°C, respectively. Evidently, this suggests that hypoxic/cold mice may be less sensitive to cooler $T_a$ than controls and thus able to remain thermal neutral during cooler

temperatures. As hypoxic/cold mice were shown to have no differences in NST capacity compared to controls but had larger amounts of BAT and overall body weights it is possible that these mice may decrease body surface area to volume ratios and fat composition to maintain $T_b$ instead of directly increasing NST capacity. Also, similar to hypoxia acclimated mice they may increase their abilities to elicit mechanisms of shivering under normoxic conditions and are able to broaden their TNZ with the addition of larger amounts of BAT that set them apart from the other treatment groups.

**IV. Fur Density**

Although there is no significant differences amongst treatment groups in fur density there appears to be a trend that hypoxic/cold and chronic hypoxic mice have a slightly higher and lesser amount of fur than controls, respectively (Figure 3.2). Fur growth is vastly dependent on seasonal conditions that influence an organism to accommodate physiologically as well as behaviourally. For example, during winter months where some animals tend to hibernate to avoid cold temperatures, fur growth it induces changes in fur pelages to become finer and denser to enhance insulation. Nonetheless, summer months encourage weight loss and fur growth changes to become coarser and more sporadic to increase ventilation and circulation through to the skin (Johnson, 1977). In this present study, similar changes in fur growth were expected to be seen in treatment groups exposed to colder $T_a$; however, only the hypoxic/cold treatment group appeared to show some increase in density compared to controls. Moreover, it is difficult

determine whether these changes in fur growth are due to changes in fur characteristics (i.e. fur length, spacing, or thickness of hair follicles) or hair-type changes because the data measures only the fur density. An increase in density may suggest a change in one of these fur characteristics in response to avoid body heat escaping and increase mechanisms of insulation. Furthermore, it appears that chronic hypoxic mice may have a lower amount of fur growth in what appears to be smaller measurements in fur density. These mice were housed in $T_a$ within their TNZ (~28°C), which may inhibit stimulation of facultative thermogenic mechanisms and force them to rely on mechanisms of basic thermogenesis which may be less effective in regulating $T_b$. Therefore, it is not surprising that this treatment group does not require extra insulator properties to prevent heat loss but rather a pelage that increases air flow and ventilation the only requirement to properly thermal regulate.

## V. Activity

As demonstrated by Davis et al (1960) ST is progressively reduced with longer periods of acclimations to the cold and NST starts to become an increasingly more significant source of metabolic heat. Consequently, it was hypothesized that cold acclimated mice in this present study would likely acquire an up-regulation of NST (demonstrated in Figure 1.3) complemented by a down-regulation in ST. Additionally, the opposite was predicted in hypoxic mice that were thought to exhibit inhibited NST mechanisms that would be balanced by an increase ST or other passive means of thermogenesis. This indicates that, an

increase in ST would likely be shown in lower bouts of activity seen in the chamber as $T_a$ progressively decreased; however, there were no significant differences in activity between cold and hypoxia groups compared to controls during experiments of acute temperature exposures (Figure 3.3). Likewise, both treatment groups increased their activity levels similar to controls during $T_a$ at around 10°C where it is more than likely that these mice are starting to increase movement to increase $T_b$ before the onset of shivering takes place, presumably at a lower $T_a$. Hypoxic/cold activity levels start to decline after $T_a$ 12°C when compared with controls as these mice may start to huddle or shiver, an activity that cannot easily be distinguished by the motion detector plate and therefore recorded simply as lower levels of activity. These mice were observed during acclimation periods as being generally less active than other acclimating groups which suggests that limited activity in these mice are still present during lower $T_a$ and could represent the onset of shivering in hypoxic/cold mice.

## VI. UCP3 protein

UCP3 is expressed in BAT and cardiac muscle of humans and rodents but is more abundantly found in skeletal muscle (Vidal-Puig et al, 2000). This protein has been linked to various functions such as fatty acid metabolism, prevention against reactive oxidative species (ROS) generation and helps protect against the accumulation of fatty acids inside skeletal muscle and therefore is strongly related to the development of Type II diabetes mellitus (Nabben and Hoeks, 2008). As UCP1 functions in BAT to increase mitochondrial uncoupling and promote

thermogenesis, UCP3 has been shown to function in a similar way. In addition, it has been shown to be up-regulated in response to other promoters of thermogenesis such as increases in thyroid hormone, $\beta_3$-adrenergic agonists and leptin (Gong et al, 1997). More specifically, starvation induces an immediate increase in skeletal muscle UCP3 mRNA and decrease in BAT UCP3 where re-feeding has been found to reverse these effects. This further suggests a more prominent function of UCP3 during starvation conditions that sets it apart from UCP1 and 2.

Although there are many studies that have examined the effects of cold acclimation on the expression of UCP1 mRNA and protein and claim it has a distinct role in adaptive thermogenesis (Matthias et al, 2000) UCP3 exact role in rodent muscle remains debateable. As UCP3 has been shown to be directly regulated hormonally and nutritionally there are many reasons to suggest that it plays a role in the up-regulation of NST during cold exposure. However, some studies state that its roles are only minor in adaptive thermogenesis (Boss et al, 1998) and others claim there is no evidence in controlling uncoupling during cold exposure and there is a complete shut down of this mechanism (Larkin et al, 1997). Previously, Lin et al (1998) has shown that UCP3 mRNA and protein are up-regulated during the first 3 hours of cold exposure and return to basal levels only 6 days after cold exposure along with the down-regulation of GLUT4 in the skeletal muscle of rats. This study has lead to subsequent examinations of UCP3 after chronic cold and starvation exposures where UCP3 appears to play a larger

part in the control of thermogenesis during unfed states rather than after cold exposure in the hibernating ground squirrel (Barger et al, 2006). Recent studies on UCP1 ablated mice (Golozoubova et al, 2001) further support the role of UCP3 in energy starvation states where UCP1 ablated mice had normal rates of basal metabolism but could not be stimulated adrenergically despite high levels of UCP3 mRNA expression. Therefore, UCP1 ablated mice confirm that the role in up-regulating NST is strictly conserved through UCP1 in BAT and although UCP3 may assist during acute cold exposure, it is relatively unimportant in the enhancement of adaptive thermogenesis.

In this present study UCP3 mRNA expression in skeletal muscle (Figure 1.9) appear to be up-regulated in hypoxic and cold treatment group compared to controls. This may suggest that an increase in UCP3 occurs after a chronic cold exposure extending more than 4 weeks whereas, previous studies have shown UCP3 is up-regulated during acute cold exposure and down-regulated during longer periods of cold. Nevertheless, the up-regulation of UCP3 may result as a compensatory method that these mice utilize as a way to enhance thermogenesis through ST along with thermogenic routes through UCP1 to maintain $T_b$. Therefore, the role of UCP3 may be more closely related to the increase in shivering in the skeletal muscle rather than NST that is manipulated through UCP1 activation. Although UCP3 protein expression data in this present study (Figure 3.4) does not support this conclusion and in fact UCP3 has less protein content in cold acclimated mice compared to controls. In light of this, UCP3

transcriptional response to cold is significantly increased compared to controls (Figure 1.9) and may be a result of increased food consumption usually found in cold acclimated animals (Hammond and Wunder, 1995). However as UCP3 protein content was found to remain low in cold mice in this present study, it may further suggest that UCP3 limits its protein expression similarly to UCP1 protein content in BAT (Figure 1.4) and instead up-regulates UCP1 and 3 mRNA expression (Figure 1.7 and 1.9).

Previous studies on acute hypoxia in the skeletal muscle of mice have shown that UCP3 mRNA and protein greatly increase with exposure as well up-regulate after stimulation to exercise and an increase in AMP-activated protein kinase (AMPK) (Zhou et al, 2000). Also it has been shown to attenuate the generation of ROS that follows the induction and chronic hypoxic exposure in the mitochondrial matrix (Talbot and Brand, 2005). As UCP3 knock-out mice have been shown to generate large amounts of ROS under hypoxic and normoxic conditions, UCP3 mRNA and protein increases in wild-type mice measurements of ROS are shown to be low (Lu and Sack, 2008). In this present study (shown in Figure 1.9), UCP3 mRNA expression is greatly increased in hypoxic mice compared to controls. However, UCP3 protein content is not significantly different compared to controls but is slightly elevated compared to cold and hypoxic/cold treatment groups (Figure 3.4). Therefore, it can be concluded that in concert with acute hypoxic studies that episodes of chronic hypoxia may continue to stimulate the up-regulation of UCP3 transcription and content in skeletal

muscle. Although ROS generation was not measured in this study, it may be plausible that UCP3 is continually up-regulated to attenuate and control high amounts of ROS that are generated during long periods of hypoxia. Alternatively, hypoxic mice may have higher levels of circulating plasma free fatty acids as this is a general preference in fuel use under hypoxia (McClelland et al, 1999, Jones et al, 1972, Young et al, 1982) that could stimulate higher levels of UCP3 mRNA and protein.

Hypoxic/cold mice did not display any significant differences in UCP3 mRNA (Figure 1.9) data compared to controls but although not significant showed lower levels of UCP3 protein content (Figure 3.4) compared to cold and hypoxic treatment groups. This data is harder to justify as it does not follow previous predictions however hypoxic/cold mice may down regulate UCP3 expression in concert with UCP1 mRNA found in BAT (Figure 1.7). Since these mice are able to maintain a normal $T_b$ during acute temperature exposures in normoxia, this could suggest that they are able to utilize NST mechanisms thru UCP1 protein content rather than UCP1 mRNA expression seen in cold acclimated mice. Although UCP1 and UCP3 have been shown to play opposing roles during adaptive thermogenesis the present data may suggest that while residing in chronic environments of hypoxia/cold, UCP1 and UCP3 expression may be limited and the content that greatly contributed to lower $T_b$ which were displayed in hypoxic/cold mice. Lastly, the change to a normoxic environment

from a hypoxic/cold condition may stimulate thermogenic mechanisms that were dormant while residing in hypoxia.

## VII. Conclusions

The final results presented from hypoxic/cold mice demonstrate clear consequences of the combination of hypoxia and cold that greatly affect the ability of these animals to maintain a $T_b$ similar to control mice. The hypoxic/cold treatment group have a clear down-regulation in $T_b$ set point after four weeks of chronic exposure (Figure 1.9). $T_b$ was found to sequentially decrease ~7 days after introduction to the hypobaric chambers and fell far below $T_b$ of controls and other treatment groups until a constant $T_b$ ~33°C was maintained for the reminder of the acclimation period (Figure 3.5A). Interestingly and unexpected results occurred when the hypoxic/cold mice were exposed to acute temperature challenges in normoxia starting at a $T_a$ of 32°C, their $T_b$ dramatically increased by ~5°C and remained significantly higher than $T_b$ of controls for the remainder of the exposures (Figure 1.2B). Therefore, although it may appear that hypoxic/cold mice have an inability to thermoregulate under hypoxic conditions they still retain the capacity to up-regulate these mechanisms during acute episodes in normoxia. Differences between normoxic and hypoxic effects on $T_b$ regulation may indicate important strategies that these endotherms utilize to possibly conserve energy reserves and maintain energy balance under stressful environments. Previous acute and chronic hypoxic experiments indicate lower levels of oxygen consumption as an attempt to lower energy demands in a low oxygen environment

(Dupre and Owen, 1992). Therefore, the lowering of $T_b$ in hypoxic/cold mice seems inevitable as this helps to decrease thermal gradients between $T_a$ and $T_b$ and helps to preserve energy stores during hypoxia. Also, it is quite possible that the detection of more available oxygen in a normoxic environment may stimulate thermogenic pathways and up-regulate $T_b$. This indicates that although thermogenic pathways appear to be impaired while animals are residing in hypoxia they may still retain the ability to activate these mechanisms under the normoxic conditions. In support of this argument, hypoxic/cold mice were found to have larger BAT mass and UCP1 protein expressions compared to controls. Although these mice expressed no differences in the capacity of NST compared to control mice, they were still able to significantly increase oxygen consumption after injections of NE compared to after saline injections indicating the capacity for NST. In addition, it may be possible that hypoxic/cold mice may up-regulate UCP1 mRNA earlier on in acclimation periods that leads to high pools of UCP1 protein that are later utilized with the onset of normoxia.

The results of this present thesis propose different strategies that each group of acclimated mice may employ to correctly balance energy stores and maintain $T_b$. Cold acclimated mice were found to enhance NST mechanisms by up-regulating BAT mass, mitochondrial density and UCP1 mRNA expression and a trend appears to up-regulate PGC-1α. Hypoxic acclimated mice were shown to down-regulate NST mechanisms in normoxia and decreasing Tb during certain hours of a 24-hour cycle after acclimation. The mice also displayed lower

mitochondrial densities and UCP1and PGC-1α mRNA in BAT while keeping high levels of UCP1 protein and UCP3 mRNA. Together these results suggest that the different environmental stimuli elicit various thermogenic strategies and may provide further insight into the exact roles of these mechanisms. Thus, it may be possible that cold acclimated mice do not up-regulate UCP3 despite, these mechanisms possibly being conserved for other possible roles in skeletal muscle such as increasing lipid metabolism or may be used as a way to attenuate further ROS generation in the case of hypoxic mice. Ultimately, the results from all treatment groups confirm that roles of thermogenesis are greatly enhanced upon exposure to cold environments alone and are limited under hypoxia. Acclimation to both hypoxia and cold limits thermogenesis while organisms reside in hypoxia but allow partial up-regulation of NST mechanisms with the onset of normoxia. This may be a conserved strategy of endotherms to turn down thermogenesis during episodes of hypoxia such as hibernation or burrowing but still allows these mechanisms to remain dormant until they are required i.e. arousal during warmer months or more simply during times of foraging.

## VIII. Future directions

These experiments present very interesting results; however, a complete understanding of thermogenesis under hypoxia and cold require more intricate investigations to define the exact mechanisms under these stressful conditions.

Many studies have investigated the role of BAT in small mammals and it remains clear that its functional importance is to help maintain $T_b$ regardless of

changing $T_a$. Nevertheless, its significance in humans remains to be elucidated, as it only has recently been rediscovered in adult humans. Currently investigations into BAT and UCPs in humans have been linked to disease models such as obesity and Type II diabetes mellitus where further research into regulatory pathways and mechanisms will provide helpful therapies and alleviations to improve human life.

Moreover, although this present study investigates the response of UCP1 and 3 to various environmental stimuli in BAT and skeletal muscle respectively, future studies should try to examine the role of UCP2 and 3 in BAT, skeletal muscle as well as other organs such as the liver and stomach. Understanding triggers and inhibitors of these UCPs in various tissues may further define or eradicate roles in thermogenesis and possibly provide insight into other uses in energy expenditure or storage dynamics.

Furthermore, as the hypoxic/cold treatment group may pose a limited thermogenic response in hypoxia, it still remains unclear whether CD-1 mice may be responding with an increased sensitivity to chronic hypobaric hypoxia. As previous acute hypoxic studies have shown CD-1 mice counteract hypoxia exposure by lowering overall metabolic rate that could explain reasons for lower $T_b$ set-points (Zwemer et al, 2007). Future investigations should focus more closely on the specific hypothalamic control that these mice may increase or decrease in response to these environmental stimuli as well as the response of other mouse species. A strong comparison between species as well as HA native

species will provide an improved insight into how endotherms enhance or limit thermogenic mechanisms in simulated or real-life HA conditions.

## IX. Chapter Summary

This present thesis uniquely examined endotherms that were chronically acclimated to the combination of both hypoxia and cold. Four main objectives that were outlined in Chapter One were explored throughout the course of this Master's thesis and overall conclusions of the work were provided in the present chapter. In conclusion, hypoxic/cold mice were found to utilize mechanisms of thermogenesis during acute temperature exposures in normoxia but were unable to maintain normal $T_b$ during acclimation periods in hypoxia. Cold acclimation enhances the capacity of NST by increasing BAT mass, mitochondrial density and UCP1 mRNA expression. Hypoxic acclimation lowers the ability to up-regulate NST by decreasing BAT growth, mitochondrial density and UCP1 mRNA expression. Stimulation of NST mechanisms in normoxia in hypoxic/cold mice imply that these mechanisms are highly sensitive to exposures of hypoxia and in combination with cold further down-regulate these pathways that severely limit a normal $T_b$ set point.

## Table 3.1: Tissue weight of whole heart, left and right ventricle, gastrocnemius and liver

Table3.1 shows specific tissue weights of whole heart, left and right ventricle, gastrocnemius and liver expressed as a percentage of whole body weight (gm). The * indicates significant difference from N, ** indicates significant difference from C and *** indicates significant difference from HC where $p<0.05$ using a one-way ANOVA. Sample sizes were as follows: N and C n=7, HC n=9 and H n=8. All values are means ± SEM.

| | Body Weight (gm) | Whole Heart | L. Vent. | R. Vent. | Gastroc | Liver |
|---|---|---|---|---|---|---|
| N | 43.0± 1.0 | 0.38± 0.38 | 0.23± 0.23 | 0.10± 0.11 | 0.4± 0.4 | 3.97± 3.97 |
| H | 38.1± 1.1* | 0.37± 0.12*** | 0.23± 0.01*** | 0.11± 0.01*** | 0.43± 0.03*** | 4.67± 0.13 |
| C | 45.5± 0.9 | 0.48± 0.03 | 0.30± 0.02 | 0.12± 0.01 | 0.43± 0.02 | 4.03± 02 |
| HC | 40.0± 0.9** | 0.59± 0.12* | 0.34± 0.01* | 0.19± 0.01* | 0.28± 0.04 | 3.57± 0.47 |

**Figure 3.1: Body temperature after injections of saline and norepinephrine**

Figure3.1 shows body temperatures in hypoxic/cold and control mice while living in the hypobaric chamber, at rest in temperature controlled chamber, after injection of saline and after an injection of NE every 15minutes for a total of 120minutes. The * indicates significant difference from control group. Hypoxic/cold group represented by open white circles and normoxic thermoneutral control group represented by closed black circles. All values are ±SEM and sample sizes were n=4 for both treatment groups. Data was analyzed using a Two-way ANOVA statistical test.

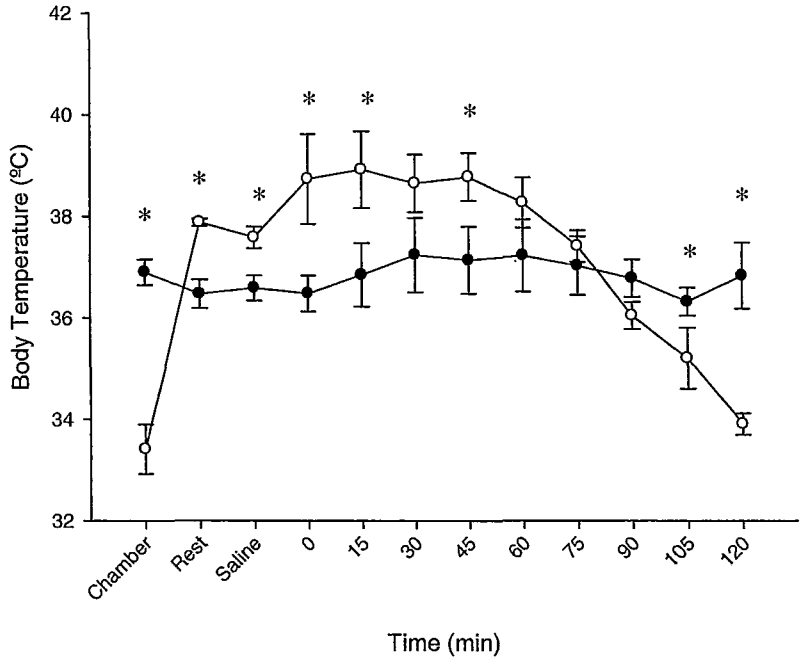

42
40
38
36
34
32
Body Temperature (ºC)
Chamber
Rest
Saline
0
15
30
45
60
75
90
105
120
Time (min)

**Figure 3.2 Fur density**

Figure3.2 shows measured fur density before acclimation, after and the difference between the two measurements. Sample sizes were as follows: N, H n=4, C n=3and HC n=5. All values are ±SEM.

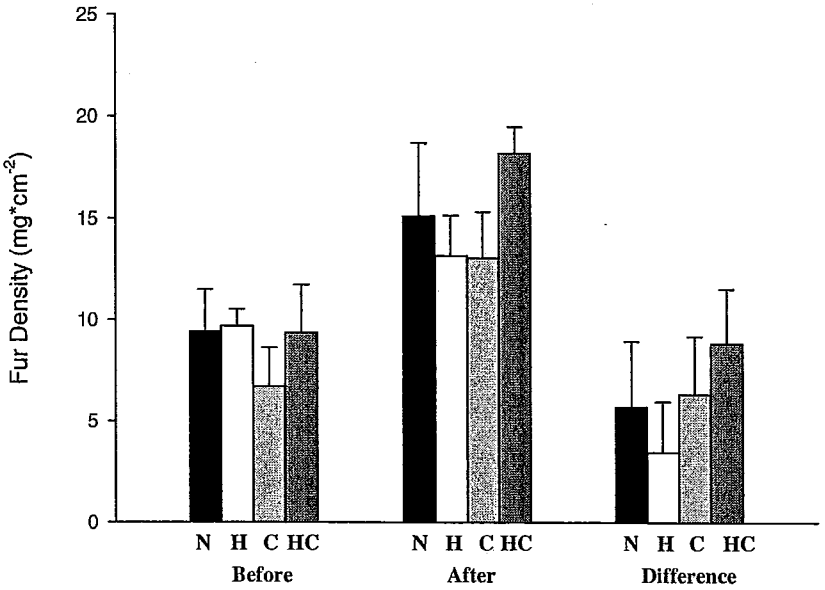

25
20
15
10
5
0
Fur Density (mg*cm-2)
N H C HC
Before
N H C HC
After
N H C HC
Difference

**Figure 3.3: Activity during to the acute lowering of ambient temperature**

Figure3.3 shows the percentage of activity monitored at each ambient temperature from 4-32°C. All measurements of activity were normalized as a percentage of activity at 32°C. N=normoxic thermoneutral control represented by solid black bars. HC=hypoxic/cold by solid light grey bars. C=cold by solid darker grey bars and H= hypoxia by open white bars. The * indicates a significant difference between N and HC treatment groups during Ta of 6-12 °C using a one-way ANOVA. All values are ±SEM and sample size for all treatment groups is n=5.

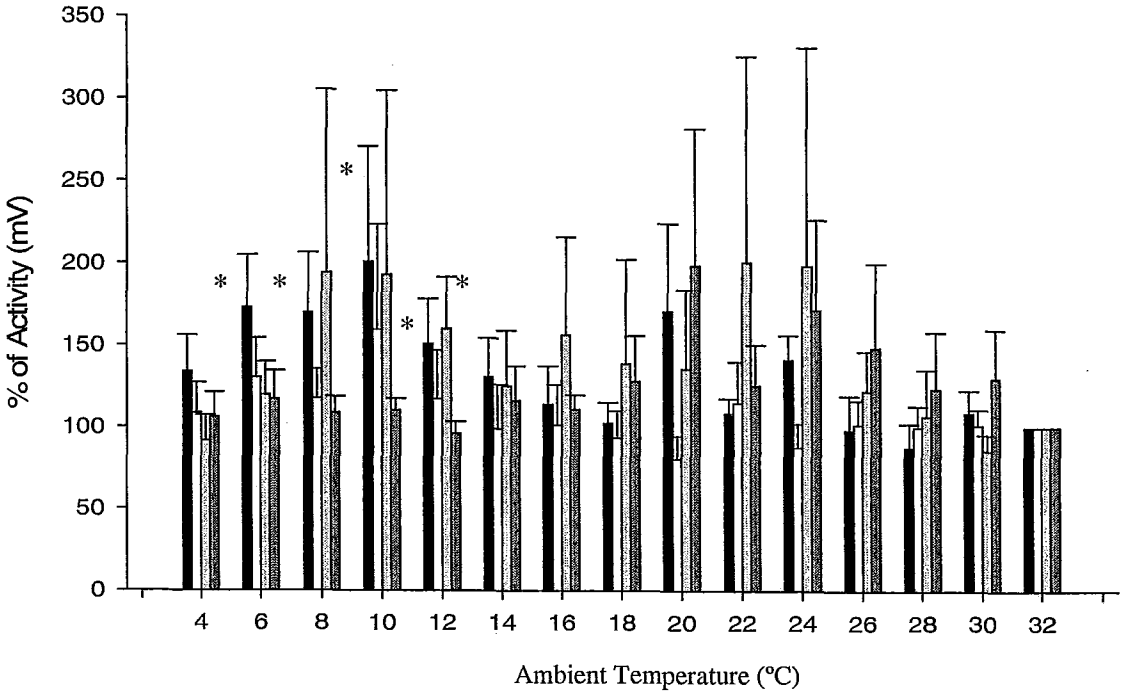

350
300
250
200
150
100
50
0
% of Activity (mV)
*
*
*
*
*
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Ambient Temperature (°C)

**Figure 3.4 UCP3 protein expression in BAT per mg of mitochondria**

Figure3.4 shows relative UCP3 protein content in isolated BAT mitochondria to a pooled sample of all treatment samples. Different letters indicate statistical significance between treatments ($p<0.05$) using a one-way ANOVA. All values are ±SEM and sample sizes for all treatment groups were n=4.

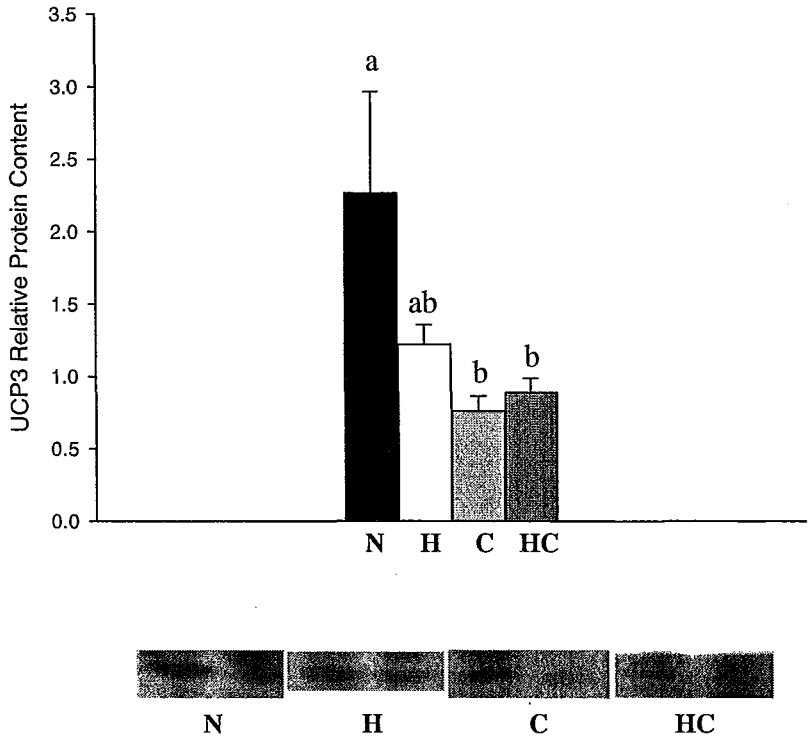
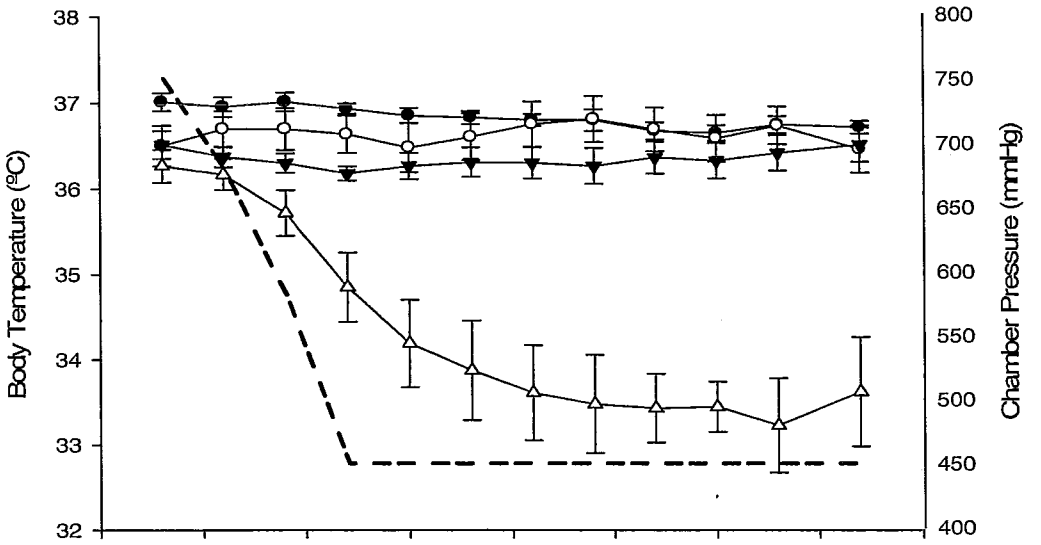

UCP3 Relative Protein Content
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
N
H
C
HC
N
H
C
HC

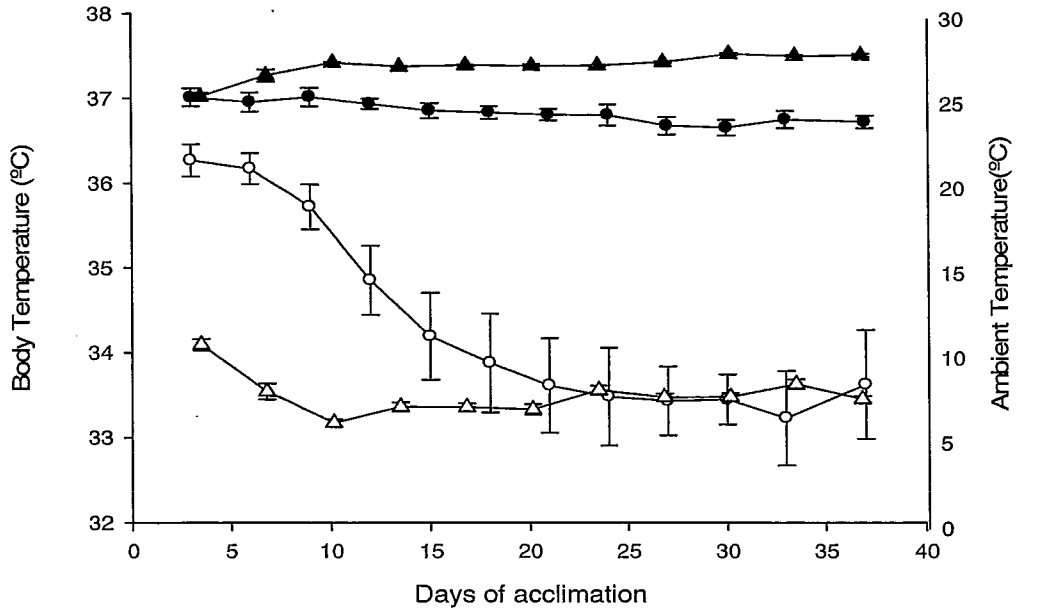
**Figure 3.5: Body and ambient temperatures every 3 days during 4 weeks of acclimation**

Figure**3.5A** shows pressure changes every 3 days for 4 weeks of acclimation on the right hand y axis plotted against body temperatures of each of the treatment groups on the left hand y axis. Dashed line represents barometric pressure in hypobaric chamber (mmHg). Normoxic thermoneutral group is represented by closed circles, hypoxic group is represented by open circles, Cold group represented by closed triangles and hypoxic/cold group is represented by open triangles. All values are ±SEM. Figure**3.5B** shows body temperature of normoxic and hypoxic/cold treatment group plotted on left hand y axis plotted against ambient temperature in the respective chambers on the right hand y axis. Normoxic thermoneutral group body temperatures are represented by closed circles and ambient temperature in normoxic thermoneutral chamber is represented by closed triangles. Hypoxic/cold group body temperatures are represented by open circles and ambient temperature in hypoxic/cold chamber are represented by open triangles. All values are ±SEM.

A


Body Temperature (ºC)
38
37
36
35
34
33
32
800
750
700
650
600
550
500
450
400
Chamber Pressure (mmHg)

B


Body Temperature (ºC)
38
37
36
35
34
33
32
30
25
20
15
10
5
0
Ambient Temperature(ºC)
0
5
10
15
20
25
30
35
40
Days of acclimation

## METHODS

The following is a detailed section of methods relevant to chapter 3 discussion and data.

*Animals*

The present study was approved by the McMaster University Animal Research Ethics Board according to the guidelines of the Canadian Council for Animal Care (CCAC). CD-1 strain male out-bred mice were purchased (Charles River Laboratories Incorporated, Wilmington, Massachuetts, USA) at 13 weeks of age and weighed over 38 grams at the beginning of the experiments. Animals were housed at 25±1°C for one week under 12:12-h light-dark cycle photoperiod and fed laboratory chow ad libitum.

*Body temperature*

After one week in normoxic conditions an electronic data logger, (iButton, DS1922L, Embedded Data Systems, Lawrenceburg, Kentucky, USA) was surgically implanted into the abdominal cavity of the mice. However approximately two mice out of each treatment group did were treated as a sham surgery and did not receive an iButton. This was carried out to ensure surgery or iButtons did not affect the mouse behaviourally or metabolically. Each iButton weighed 2.94-2.99gm before surgery and was coated in melted paraffin elvax (Mini Mitter, Bend, Oregon, USA) then weighing approximately 3.94-3.99gm.

The iButtons were then sized matched to the mice so that the iButton weight was no more than 10% of the mouse's body weight.

*Surgical Procedure*

Prior to surgery, mice were administered 0.1ml/30grams of body weight of a painkiller, Buprenorphine, this dose was advised from the Central Animal Care Facility (CAF) at McMaster University. Before any surgical procedures were performed the mice were initially anaesthetized with 4% iso-flurane. A patch of fur no more than 2cm x 2cm on the dorsal and ventral side of the animal was shaved and the hairs were collected and weighed while the animal was maintained at 1-2% iso-flurane. These hair patches were then later used as a way to determine any changes in fur growth and density. Before an incision was made the mouse was placed in a supine position under a heating pad and sterile cloths. Lacrilube was placed over the eyes and the shaved patch of fur over its stomach was cleaned with Iodine and a solution of Final Prep (diluted iodine in alcohol). The surgical implantation of the iButton was followed by similar protocol in Gusztak et al., 2004). A small incision was made about 2-2.5cm along the midline which was followed by an opening through the body wall along the Linea Alba. The wax coated iButton was dipped in diluted disinfectant (Germex, Vetoquinol, Cambridge, Ontario, Canada) and rinsed in sterile water was inserted into the abdominal cavity. The incision along the body wall and skin was sutured with a 4-0 silk using a simple interrupted suture pattern. Tissue glue (Vetbond) was used

over top of the sutured wound to help adhere the skin and eliminate the mice from chewing out the threads and exposing the lesion. Iso-flurane was then decreased to less than 1% and the mice were transferred to sterile cages and allowed to recover for 4 days while monitoring weight and body condition. After surgical procedures were carried out the mice were administered 0.1ml of Paediatrics Tylenol (80mg/ml) and then 24-hours later received another dose at 0.1ml/30grams of body weight of Burprenorphine. If the surgical wounds were re-opened and body weight had dropped to less than 10% of the original body weight than the mouse was euthanized. Following the next two days after surgery mice were administered 1 drop of the antibiotic, Cepricol in their water bottles to help prevent infection or other possible harmful growths.

*Acclimation and Experimental Design*

At least 4 days of recovery from surgery the mice were separated into groups of 5-7 and then acclimated to hypoxia (H=4wks at 480mmHg), cold (C=4wks at 5°C) and the combination of the two (HC, 4 weeks at 480mmHg and 5°C) and normoxic thermoneutral controls and 28°C (N). Cold and hypoxic/cold mice were transferred into a cold room initially kept at 12°C±1 and either placed into a hypoxic hypobaric chamber at -0.1Barrs or under normoxic conditions. The room temperature was progressively decreased every 3 days by 3-4°C until 5±1 °C. In addition, for the HC and H group mice, pressure was lowered every 3 days to a final pressure of -0.4Barrs (480mmHg). This pressure is equivalent to

approximately 4300m altitude (McClelland et al., 1998). A dehumidifier was placed in the room to help eliminate any excess moisture in the air and maintained around 30-50% relative humidity. The mice were housed in chambers for a total of 4 weeks while food, water, and cages were changed once a week. Therefore the mice in the hypobaric chamber were brought back to normoxia every 6-7 days for about an hour each time.

*Body Temperature Analysis*

Surgically implanted iButtons recorded body temperatures ($T_b$) throughout entire acclimation periods, temperature controlled experiments and during NE injection experiments. Data analysis of recorded body temperatures were interpreted by a One Wire Viewer Net program (Maxim Integrated Products, Sunnyvale, California, USA), reader cable and adaptor (DS1402D-DR8- Blue Dot Receptor iButton Reader Cable and DS9490R-USB to 1-Wire / iButton Adaptor from Embedded Data Systems, Lawrenceburg, Kentucky, USA) and then transferred into a Microsoft Excel spreadsheet (Version 2007). Each data set from each mouse was individually analyzed by written scripts into an interpreter program (Perl Express version 2.5) which took $T_b$ recordings every 5 or 15 minutes and averaged over a 1hr time period. These recordings were then averaged every 3 hours over the entire acclimation period or extracted during various ambient temperatures. Data is represented as ±SEM in all $T_b$ figures.

*Indirect calorimetry*

A flow-through respiratory system (Sable Systems, Henderson, Nevada, USA) (Figure 2) was used to measure mass-specific oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) during decrementing ambient temperatures of 32°C to 4°C in a temperature controlled chamber (Sable Cooler, Sable Systems, Henderson, Nevada, USA). A previous study was conducted where mice were first introduced into temperature controlled chamber at 4°C and then increased until 32°C. No significant differences were found in oxygen consumption measurements between mice started at an ambient temperature of 4 or 32°C. Furthermore, ambient temperatures commenced at 32°C to enable the capture of the onset of shivering thermogenesis with decreasing ambient temperatures.

Calibration of the flow-through chamber was conducted in the absence of the animals by sub-sampling atmospheric air through the chamber (~500ml) at a rate of ~250-80ml/min through a Sable System FC-1B oxygen analyzer and a CA-2A carbon dioxide analyzer (Sable Systems, Henderson, Nevada, USA). Air was essentially scrubbed clean through a network of plastic tubing consisting of a series of Drierite and soda lime to ensure air was $CO_2$-and $H_2O$-free air and continued to flow into a Plexiglas cylindrical chamber housed inside the Sable Cooler at a flow rate ~500ml/min (STPD) controlled by a mass-flow controller (Sable Systems, Henderson, Nevada, USA). Appropriate stoichiometric $VO_2$

consumption and $VCO_2$ production ratios of 0.667±2% were obtained after measuring the combustion of methanol in the Plexiglas cylindrical chamber which confirmed the accuracy of the flow-through system (as previously described McClelland et al., 1998).

Prior to temperature dependent oxygen consumption measurements were recorded mice were introduced into cylindrical Plexiglas chamber inside the Sable Cooler at an ambient temperature of 28°C and allowed to become familiarized in their new surroundings for ~1hour (Figure 3). After at least 1 day of recovery from introduction into the chamber each mouse was individually fasted for a minimum of 3 hours prior to each experiment while remaining in their respective treatment environments. Mice that were used from the hypobaric chamber were depressurized and fasted prior to each individual experiment while mice not used from the chamber were immediately resealed and returned to prior pressure levels. Trial mice were returned to the chamber the following day after remaining in normoxia warm or cold overnight.

ExpeData (1.0.15) computer programming recorder was used to generate a baseline measurement for at least 10minutes in the absence of the mouse in the chamber. The mouse was added to the chamber at an ambient temperature of 32°C and allowed to settle for a minimum of 30 minutes. $VO_2$, $VCO_2$ and motion activity measurements were then recorded every 100msec while ambient temperatures decreased every 2°C after being held at each temperature for

25minutes until an ambient temperature of 4°C±1 was reached. Data analysis of measured $VO_2$ during various temperature intervals were issued individually by written scripts into an interpreter program (Perl Express version 2.5). $VO_2$ measured data was averaged at each ambient temperature from the 3 lowest periods of 2 minutes of activity. This was then normalized to (body weight) ^0.75 although the weights were not significantly different between each of the treatment groups ($P<0.05$).

*Activity Analysis*

Activity recorded in millivolts by a motion detection plate for each $T_a$ was measured every 100msec for 25minutes and interpreted into an Expedata program file. Absolute values of the recorded moments of movement were taken directly from Expedata and placed into a Microsoft Excel (Version 2007) sheet where activity was then averaged every 10msec for the entire section of selected data. These averaged values were than calculated to the find the mode of activity which is the most frequent set of data recorded during that $T_a$. This was done to help limit ambiguous and misleading recorded data that could skew results. For example, if abnormally large spikes were recorded it could indicate a period of intense activity by the animal that may be unrelated to changes in $T_a$ and therefore results could be overestimated and wrongly interpreted as high levels of activity. Furthermore, once modes for each $T_a$ were found then these were scaled as a

percentage based on recording found at 32°C for each mouse. % of activity = (100*(Voltage value/ (Voltage value at 32°C)).

*Lower Critical Temperature*

Lower critical temperatures (LCT) for each individual treatment group were calculated using a 2-phrase straight-line regression model that is previously described in Campbell and Hochachka, 2000 originally adopted from Nickerson et al (1989). This model calculated the outer limit of the TNZ by evaluating the relationship between $VO_2$ of the animal and Ta of the chamber during acute temperature exposure. These calculations were performed using a regression and solver functions of Microsoft Excel (2007 version) where the point of inflection was used to determine the LCT for each treatment group of mice.

*Non-shivering Thermogenesis response*

After temperature controlled experiments were completed animals were allowed to rest for at least one day prior to NST experiments. Each animal was placed into the temperature controlled chamber at 28°C and metabolic rate was recorded for at least 30 minutes. Animals were quickly removed from the chamber and 500µl of saline was injected into the intrapreoneal cavity and returned to the chamber. Measurements were continued for at least 30 minutes or until oxygen consumption levels resembled those at rest. Animals were removed once more from the chamber and injected with Norepinephrine (NE) at a dose of ($2.53x\ M^{-.04}$, Wunder and Gettinger, 1996) which measured approximately 20-

26µl dose of NE for each animal. NE was diluted in room temperature saline for a total of 500µl and quickly injected into the intrapreoneal cavity and returned to the Sable Cooler chamber. Metabolic measurements were continued up to 2 hours or until the measurements resembled recordings prior to injections.

*Brown Adipose Tissue and Skeletal muscle collection*

Animals were euthanized by cervical dislocation at least 24 hours after NE injection. Blood was extracted from the facial artery and samples were spun in a centrifuge at 10,000 rpm for 10minutes. Hematocrit levels were measured using capillary tubes of sampled blood from cheek vein that was centrifuged and spun down to separate whole blood from plasma to quantify amount of read blood cells present in the whole blood and presented as a percentage. The remaining blood sample was then transferred into a -20°C freezer until later used for NEFA quantifications. Fur patches of no more than 2cm X 2cm of surface area were removed from the dorsal surface side of the animal and weighed using a scale accurate to 0.0001 decimal places, similar protocol was performed in Paul et al (2007).

Whole hearts were removed and weighed and left and right ventricles were divided and weighed separately. BAT and left and right gastrocnemius, tibialis anterior, soleus and thigh muscles were weighed and then either freezed clamped and stored at -80 °C or put into ice cold buffer for mitochondrial isolation.

Mitochondrial isolation for skeletal muscle was performed according to Vidal-Puig et al., 2000, respectively. Gastrocnemius, tibialis anterior, soleus and thigh muscles was dissected from the hindlimb area and placed in 10 ml of ice cold buffer (containing 250 mM sucrose, 10 mM HEPES, and 0.5 mM EDTA , pH 7.2 with KOH, 0.1% bovine serum albumin) and was homogenized with cooled scissors and then using a glass-Teflon Potter-Elvehjem tissue grinder. Fractionation of homogenate was carried out by spinning at 3,500x *g* at 4°C for 10 min. The supernatant was then poured into a another cooled centrifuge tube and respun at 12,000x *g* for 10 min at 4°C to obtain a mitochondrial pellet. The pellet was resuspended (on ice) in 175 µl of a suspension medium (containing 120 mM KCl, 20 mM sucrose, 3 mM HEPES, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.2 with KOH, 0.5% BSA). Isolated fractions were than frozen at -20°C until they were used for immunoblotting.

*Western blots*

Gastrocnemius muscle proteins were quantified using a Bradford Assay (Bradford, 1976) to ensure equal amounts of protein were loaded on the gels. 100 µg of skeletal muscle proteins, were separated on a 12% SDS-Page gels (Bio-Rad) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) for ~2 hours. The membranes were then washed twice for 5minutes and once for 15minutes in 10 mM phosphate buffer, 0.09% NaCl, and 0.05% Tween 20, pH 7.5 (PBST). The membranes were then washed in methanol for 10seconds and were blocked in 2.5 % BSA/PBST overnight. The membranes

were washed twice for 5minutes and once for 15minutes in PBST and membrane was probed with 1:2500 of UCP-3 (UCP32-A, Alpha Diagnostics). Membranes were washed again in PBST twice for 5minutes and once for 15minutes before being probed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Perkin Elmer Life Science, Boston MA, USA, cat no. NEF8129) at a dilution of 1:20,000. Membranes were washed once more in PBST twice for 5minutes and once again for 15minutes. Blots were developed using an ECL detection system (Perkin and Elmer), and immunoreactions were visualized by exposure to BioMax XAR film (Kodak, Carestream Health France, Paris, France). Bands were quantified by scanning photo densitometry using Chemilmager Software 5.5 (Alpha Innotech Corporation, San Leandro, CA, USA).

*Statistically analysis*- Data was analyzed using one and two-way ANOVAs. Multiple comparisons were made by using the Holm-Sidak method. Also significant differences were expressed with p values of $<0.05$ and $<0.001$.

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