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THE PHYSIOLOGICAL AND BIOCHEMICAL UNDERSTANDING OF 5'-AMP INDUCED DEEP HYPOMETABOLISM

William G. O'Brien

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**THE PHYSIOLOGICAL AND BIOCHEMICAL UNDERSTANDING OF 5'-AMP
INDUCED DEEP HYPOMETABOLISM**

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INDUCED DEEP HYPOMETABOLISM**

A
THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE

by

William Gerard O'Brien III, B.S.

Houston, Texas

December, 2009

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THE PHYSIOLOGICAL AND BIOCHEMICAL UNDERSTANDING OF 5'-AMP INDUCED DEEP HYPOMETABOLISM

Publication No. _____

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Supervisory Professor: Cheng Chi Lee, Ph.D.

Our laboratory's ability to induce deep hypometabolism (DH) via an injection of adenosine monophosphate (5'-AMP) followed by placement in an ambient temperature (T_a) around 15°C currently can last about 3-9 hours. While we have insight into how 5'-AMP induced hypometabolism is initiated, it remains unclear how arousal from hypometabolism is controlled. Other laboratory members have been unable to prolong this process safely and effectively with previous attempts of re-injecting a dose of 5'-AMP upon arousal or by decreasing the T_a . While these methods worked in suppressing arousal, the mortality rate is also increased. To gather a better understanding of the process, a metabolic panel from serum samples was run giving insight into possible biochemical events occurring from euthermia to DH to spontaneous arousal and back to euthermia. Analyzing this data revealed many changes in metabolites. A few metabolites that stood out such as glucose and pyruvate did not alter the outcome in arousal rate over saline injection. Additional metabolite analysis revealed that an amino acid, arginine, was significantly reduced during DH. This amino acid seems to be a key component in prolonging our process possibly due to its intricate role in the urea cycle. Supplemental arginine is clearly more beneficial both in suppressing arousal and increasing the survival rate after 12 hours in DH.

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LIST OF ABBREVIATIONS

5'-AMP	5'- Adenosine Monophosphate
AL	Argininosuccinate Lyase
AS	Argininosuccinate Synthetase
ATP	Adenosine Triphosphate
BUN	Blood Urea Nitrogen
CBT	Core Body Temperature
CPS1	Carbamylphosphate synthetase 1
DD	Dark/Dark
DH	Deep Hypometabolism
eNOS	Endothelial Nitric Oxide Synthase Isoform
ETC	Electron Transport Chain
iNOS	Inducible Nitric Oxide Synthase Isoform
LD	Light/Dark
mg/gw	mg / gram body weight
NAGS	N-acetylglutamate synthetase
nNOS	Neuronal Nitric Oxide Synthase Isoform
NO	Nitric Oxide
NST	Non-Shivering Thermogenesis
OAA	Oxaloacetate
OTC	Ornithine Transcarbamylase
PI	First Phase
PII	Second Phase
Pck1	Phosphoenolpyruvate carboxykinase
Pdk4	Pyruvate dehydrogenase kinase isoform 4

PEP	Phosphoenolpyruvate
RF	Reverse Flip
SA	Suspended Animation
T_a	Ambient Teperature
TCA	Tricarboxylic Acid
VCO_2	Carbon Dioxide Production
VO_2	Oxygen Consumption

CHAPTER ONE

Introduction

Animals are most commonly segregated into two categories: warm- and cold-blooded. Warm-blooded animals such as mammals and birds (also known as endotherms) maintain their core body temperature at an optimally efficient degree, and for humans this is 98.6°F or 37°C. In contrast, the core body temperature of cold-blooded animals such as reptiles and amphibians (also known as ectotherms) will fluctuate over a range, depending in part on their environmental temperature. For humans and other endotherms, this maintenance comes at an enormous energy cost compared to similarly sized ectotherms (**Heldmaier et al., 2004; Else and Hubert, 1981**). Mammals large and small alike go through daily sleep and awake cycles, during which the metabolic rate and body temperature drop slightly when resting (**Heldmaier et al., 2004**). However this drop is miniscule (less than 1°C) compared to the reduction that occurs in daily torpor or hibernation (**Lyman, 1982; Geiser and Ruf, 1995**). Animals that enter hibernation go through a prolonged hypometabolic state, which allows their body temperature to drop to around that of their environment (**Heldmaier et al., 2004**). For some hibernators, such as the arctic ground squirrel, the core body temperature at hibernation is just above freezing (**Barnes 1989**). Similarly, there are animals that undergo daily bouts of torpor, which is a less severe form of hypometabolic state that results in a lowering of the core body temperature (**Lyman, 1982**). Both hibernation and torpor involve the same 4 stages: entrance into hibernation/torpor, maintenance at the sub-basal metabolic rate, arousal from the deep hypometabolism, and a period at euthermia (**Heldmaier et al., 2004**). Another commonality between daily torpor and hibernation is that as these animals are ready to end their hypometabolic state, their bodies start the gradual rewarming process (arousal) that returns the body to euthermic temperature and normal metabolic activity (**Heldmaier et al., 2004**). The difference

between the hibernation and the torpor states lies in the second phase (maintenance) or the amount of time spent being metabolically depressed; hibernators can exist for days or even weeks at a time, whereas animals undergoing torpor fluctuate between normal function and the hypometabolic state daily (**Geiser and Ruf, 1995**). In other words, torpor is a daily, less severe form of hypometabolism. These physiological conditions have been observed in most of the mammalian orders in both small and large animals, from bats to lemurs to squirrels (**Heldmaier et al., 2004; Carroll, 1997**). The lemurs are old world primates and they are known to hibernate during periods of metabolic stress (**Kobbe et al., 2009**). Since humans are also primates it should be possible for humans to undergo either daily torpor and/or hibernation, but if/how this is possible is unknown (**Heldmaier et al., 2004; Carroll, 1997**). The reason we study these processes is to unlock the benefits of hypometabolism so that they can be applied to non-hibernating mammals like humans. The possibilities are vast; this research could potentially help with anything from increasing the survival rates in trauma patients by reducing their body's metabolic need, to reducing metabolic disorders such as obesity by changing the preferred fuel source from glucose in euthermia to fats in hibernation, to something as remote as revolutionizing space travel by allowing the transport of humans in the hypometabolic state (**Andrews, 2007**). Also, understanding and exploiting hibernation in humans could be the answer to that which people covet most: the fountain of youth (**Lyman et al., 1981**). It has been suggested that hibernation could have similar benefits to prolonging life as seen by the practice of caloric restriction (**Andrews, 2007**). Unfortunately, for the most part, the process of hibernation is not well understood and is still being extensively researched.

Our laboratory has discovered that a naturally produced metabolite, 5'-adenosine monophosphate (5'-AMP), can be used to induce a torpor-like state in mice without any apparent relation to either size or sex of the animal (**Zhang et al., 2006**). By injecting 0.5 mg/gram body weight (mg/gw) of 5'-AMP and placing the mice into an incubator with an ambient temperature (T_a) around 15°C, the mice will remain in this deep hypometabolic state anywhere between 3-9 hours. The reason for the range in the amount of time down is due to the fact that the arousal process from this hypometabolic state appears to be completely spontaneous. Attempts by other investigators in the laboratory to extend this hypometabolic process were largely unsuccessful due to high mortality rate. Approaches to extend the length of this hypometabolic process include increased dosage of 5'-AMP or additional injections of 5'-AMP when animals were about to arouse. Increasing the dose of 5'-AMP by several folds did not extend the length of this hypometabolic state. Giving additional 5'-AMP when an animal was about to arouse led to high mortality after the mouse had been in hypometabolism for 14-18 hours. However, animals that have returned to euthermia can undergo another episode of 5'-AMP induced hypometabolic state without the observed mortality outcome. Thus, my research aim was to investigate the physical, biochemical and physiological parameters that control the process of deep hypometabolism in mice. The hypothesis was that by understanding these parameters, the limitations of the non-hibernator's ability to maintain deep hypometabolism for prolonged periods could be expanded by administering key supplement(s). Through this expansion, we may allow non-hibernators to perform a deep hypometabolic process observed only in nature by hibernators. At the biochemical level, we began by obtaining a detailed metabolic analysis of serum metabolites of the animals at 4 distinct stages. These 4 stages, as defined by Heldmaier, are

entrance, maintenance, arousal and euthermia. However, since our animals are induced, the entrance samples were taken as euthermic samples prior to induction to establish a baseline to compare the later samples. At the physiological level, the core body temperature and the metabolic rate of the animals were examined via telemetry and oxygen consumption/carbon dioxide production during these 4 stages (**Figure 1**). The oxygen consumption (VO_2) graph revealed 2 distinct phases of deep hypometabolism present in every mouse that is injected with 5'-AMP. The first phase (PI) is the induction or entrance phase, portrayed by the steep and rapid drop in VO_2 from euthermic levels (~ 4000 ml /kg/h) to around 1500 ml/kg/h. The second phase (PII), characterized by the gradual decline from 1500 ml/kg/h VO_2 to about 300 ml /kg/h, is the stage in which deep hypometabolism is achieved, followed by the maintenance phase. The arousal phase is characterized when the VO_2 displays a steady increase from the basement level reached during the maintenance phase where VO_2 surpasses 2000 ml/kg/h. The recovery phase is started when the animal is back to euthermic temperature with VO_2 levels of ~ 4000 ml/kg/h. A telemetry device implanted in the mouse allowed us to simultaneously monitor the relation between the core body temperature and that animal's oxygen consumption (VO_2). At the physical level, the effect of environmental temperature on the physiological response of the mice during the hypometabolic state was investigated. My studies revealed several important conclusions: 1) The environmental temperature plays a major role in modulating the outcome of deep hypometabolism. 2) The dosage of 5'-AMP plays a key role only during the initial induction stage of hypometabolism, but not its prolonged maintenance. 3) Our biochemical analysis revealed that there are dramatic changes between the 4 behavioral stages in the flux of key metabolites which play a role in glycolysis/gluconeogenesis, transamination, purine

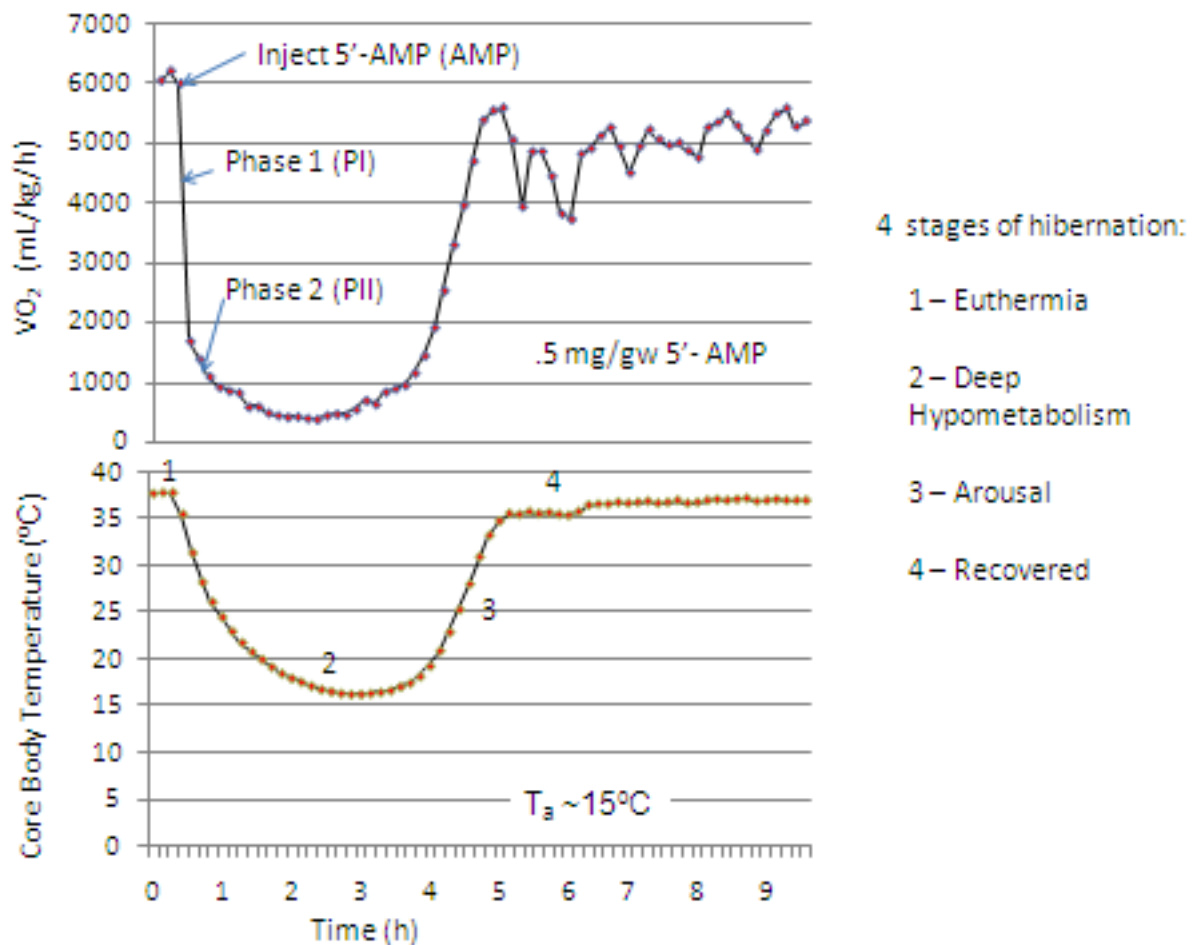


Figure 1. Normal mouse data of induced hypometabolism by 5'-AMP. The top graph represents oxygen consumption (VO_2) while the bottom is the correlated core body temperature (CBT) graph. After an injection of 5'-AMP, the mouse exhibits a quick and immediate drop in oxygen consumption (Phase 1) followed by a more gradual decrease (Phase 2). The 4 stages that the blood samples were collected for the Metabolon study that will be talked about later are labeled on the CBT graph.

catabolism and bile acids but fatty acid metabolism is less pronounced. 4) My studies investigate whether the supplementation of certain metabolites in combination with fluid could extend the length of hypometabolism in the mice.

A brief introduction to purine metabolism is provided to give some perspective to the fate of the 5'-AMP injected. The 5'-AMP that is used to induce hypometabolism is primarily metabolized to Inosine monophosphate (IMP) via AMP deaminase while there is a minor pathway that catabolizes 5'-AMP by dephosphorylation to adenosine using AMP nucleotidase. The 5'-AMP is also salvaged via the adenylate equilibrium to form ADP by utilizing ATP. Once 5'-AMP returned back to physiological levels, the ADP can then be reconverted back to ATP through the adenylate equilibrium. We have experimental evidence to indicate that the major pathway via AMP deaminase and the conversion to ADP are the main events in metabolizing the 5'-AMP injected to induce this DH state.

Glucose is usually broken down via glycolysis ultimately producing pyruvate and lactate in times of oxidative stress. The lactate can be recovered back to pyruvate by lactate dehydrogenase once it is transported to the liver. The pyruvate feeds into the TCA cycle after conversion to Acetyl-CoA. Throughout both of these processes, energy is stored as NADH, FADH₂ and ATP. However, in the red blood cells, glycolysis is the primary biochemical process to generate NADH and ATP. Oxidative phosphorylation in combination with glycolysis and the TCA cycle help make glucose a very efficient producer of energy; 1 glucose molecule is accepted to make around 38 ATP after it is completely metabolized. When the body is able, glucose is the preferred fuel source for its efficient ability to produce

energy. However, there are instances when glucose is limited and other fuel sources must be utilized. One of these alternative fuel sources comes from amino acid catabolism. Amino acids are different in their structure from glucose due to the amine group they possess. For the most part, both glucose and amino acids are hydrocarbons (consisting of hydrogen and carbon atoms) and therefore can both be readily used to produce energy. The difference is that the amine group on amino acids must be taken care of once it is free from its carbon chain. This is primarily done by removal from the body through the urea cycle since ammonia is highly toxic at high levels. Amino acids are described as either ketogenic, glucogenic or both based on where they are utilized in producing energy. Glucogenic amino acids can generate net glucose when it is metabolized. In contrast, ketogenic amino acids may not generate net glucose. Most amino acids are both ketogenic and glucogenic. All the amino acids except for leucine and lysine are glucogenic, which means the carbon skeleton of these amino acids can be used in the production of gluconeogenic precursors like pyruvate and TCA cycle intermediates like oxaloacetate and α -ketoglutarate. The two purely ketogenic amino acids above give rise to acetyl-CoA and acetoacetyl-CoA, which can produce energy via the TCA cycle; however, they cannot be used directly in gluconeogenesis. Once the amine group is removed, it must be discarded, which is done mainly by the urea cycle, which is responsible for taking ammonia out of the cells and removing it after being processed in the liver and filtered through the kidneys. The importance of these pathways will be examined further throughout this body of work to give a better idea of the metabolic changes occurring in the mice while in the DH state.

CHAPTER TWO

Methods

Female C57BL/6 mice from either Taconic Farms or Harlan were used in the current studies; however, the induction of DH has been shown to be just as effective in male and female mice. Fresh 9-12 week old mice were given a few weeks rest in our animal facilities with a 12h/12h Light Dark (LD) cycle to adjust to their new environment before being used for the proposed studies. In addition, mice that have undergone DH were rested for a few weeks before being reused in order to allow them to regain any lost body weight from the procedure.

The laboratory instrument used in the current studies is a reverse flow climate controlled metabolic chamber from Columbus Instruments, USA. It is a temperature-controlled incubator with 16 individually monitored cages with their own access to food and water. Each cage has its own monitoring device for oxygen consumption and CO₂ measurements. The information is then stored in an on-board computer that also controls the entire instrument.

First, an experimental regime was established to keep things consistent in order to define the effects of changing the desired parameters. From previous experimentations undertaken by the laboratory, it has been observed that a temperature at about 15°C was desirable. Thus, the thermostat was set at 14.5°C; in the metabolic chamber, the ambient temperature (T_a) at this setting is actually right around 15°C based on the readings from a thermometer placed in the chamber. Mice are nocturnal, therefore experiments were initiated at around 5 a.m. All mice were fasted for 12h but water is given ad libitum. For the fasting, the mice are removed from their normal cage and put into a fresh cage with fresh bedding and water. By putting them into a fresh cage, there is no possibility of food crumbs, droppings or deposits from prior feedings on the floor that could be a source of food. It is

not known if fasting plays an intricate role in either the suppression and/or the survival of the mice; it is done as a means to normalize the experiments and to eliminate unknown variables that could have an impact on the proposed study. Before the mice are placed in their respective cages in the metabolic chamber, they are weighed so the computational software can calculate the appropriate measurements (VO_2 , VCO_2 , etc.) during the experiment. Once the mice are in their respective cages, the experiment is set up by giving the experiment a label or file name, inputting animal tag numbers and their weights for the respective cages, and, when telemetry (Mini-Mitter Respironics, OR, USA) is used, the associated telemetry chip number with that mouse is also entered. The machine is then calibrated with a sample gas with predetermined concentrations of oxygen and carbon dioxide, and the experiment is ready to begin collecting data. After a few initial readings lasting for about 30 minutes to gather baselines of all the mice, hypometabolism is initiated with an intraperitoneal injection containing freshly prepared 5'-AMP (Sigma catalog # A1752-25G) [0.5 mg/g body weight] in 100uL saline. As will be demonstrated later, the 0.5 mg/gw is an efficient dose of initiating the deep hypometabolic process resulting from a 5'-AMP titration. The saline is made from OmniPur tablets (EMD chemicals product code 6501). 1 tablet is dissolved per 100uL water for a 1X solution and then pH adjusted to 7.2 and autoclaved to sterilize. Before injection, the saline is filter sterilized (.22 μm sterile syringe filters from Fisherbrand No. 09-719A). The first injection of tested metabolite was given 2 hours after the 5'-AMP injection chilled on a bucket of ice for 30 minutes prior. The injections between the initial 5'-AMP injection and the injections after the temperature is bumped up were all given chilled to administer a solution closer to that of their body temperature. The metabolites that will be tested in this study are glucose (Sigma D – (+) –

Glucose, G-8270), pyruvate (Sodium Pyruvate SigmaUltra P8574-100g), arginine (Acros Organics, L(+)- Arginine 104991000) and alanine (B-Alanine Eastman 4638). The injections were of a similar volume of saline as a control for each experiment. The concentration of metabolite, volume injected and frequency of metabolite given would vary depending on the hypothesis being tested. In addition, the T_a would be changed to test the effects of temperature on arousal and to establish a rewarming protocol that would allow for a safe return to euthermia after mice have been in DH for a prolonged period. Throughout the experiment, if mice aroused they would be taken out into normal room temperature and placed back into their original cages with food and water ad libitum. After the experiment was concluded, all remaining mice would be removed and put into their respective cages to be monitored for the next 1-2 days. If mice appeared to be in any discomfort, they were euthanized after symptoms were observed and noted. All experiments were carried out in accordance with protocols approved by the animal welfare committee at the UTHSC medical school (HSC-AWC-06-078 and HSC-AWC-08-015).

CHAPTER THREE

Results

Effect of 5'-AMP dosage on deep hypometabolism

We have been able to mimic the torpor process, in both metabolic rate depression and body temperature depreciation, in our laboratory with the administration of 5'-AMP. Previous studies by other members of the laboratory revealed that a deeper torpor state can be attained if the mice given 5'-AMP are cooled to a lower core body temperature. This deep torpor behavior, which we call deep hypometabolism (DH) or “suspended animation” (SA) has physiological parameters such as core body temperature and metabolic rate based on oxygen consumption that are much lower than natural torpor (**Heldmaier et al., 2004**). In general, it was observed that when a mouse's core body temperature (CBT) drops below 18°C, it lost the ability to right itself when laid on its side or back, thereby entering our DH or SA state. The length of time the mice are usually maintained in the DH state is currently about 3-9 hours on average.

To demonstrate that the 5'-AMP concentration is the initiator of the process and not the driver responsible for prolonging deep hypometabolism, mice were injected with a single dose of 5'-AMP ranging from 0mg to 1mg per gram body weight (mg/gw) and then kept in T_a of about 15°C. As seen in **Figure 2**, the length of time in the deep hypometabolic state, where core body temperature is below 17°C and VO_2 is below 1000 ml/kg/h, is independent of the amount of 5'-AMP used once an efficient dose (at least 0.5mg/gw) is reached that will ensure entrance into deep hypometabolism at 15°C T_a . As discussed previously, each mouse that is injected with 5'-AMP, regardless of the dose, exhibits the initial phase 1 drop in VO_2 but the phase 2 maintenance of DH is not apparent when the dosage of 5'-AMP is

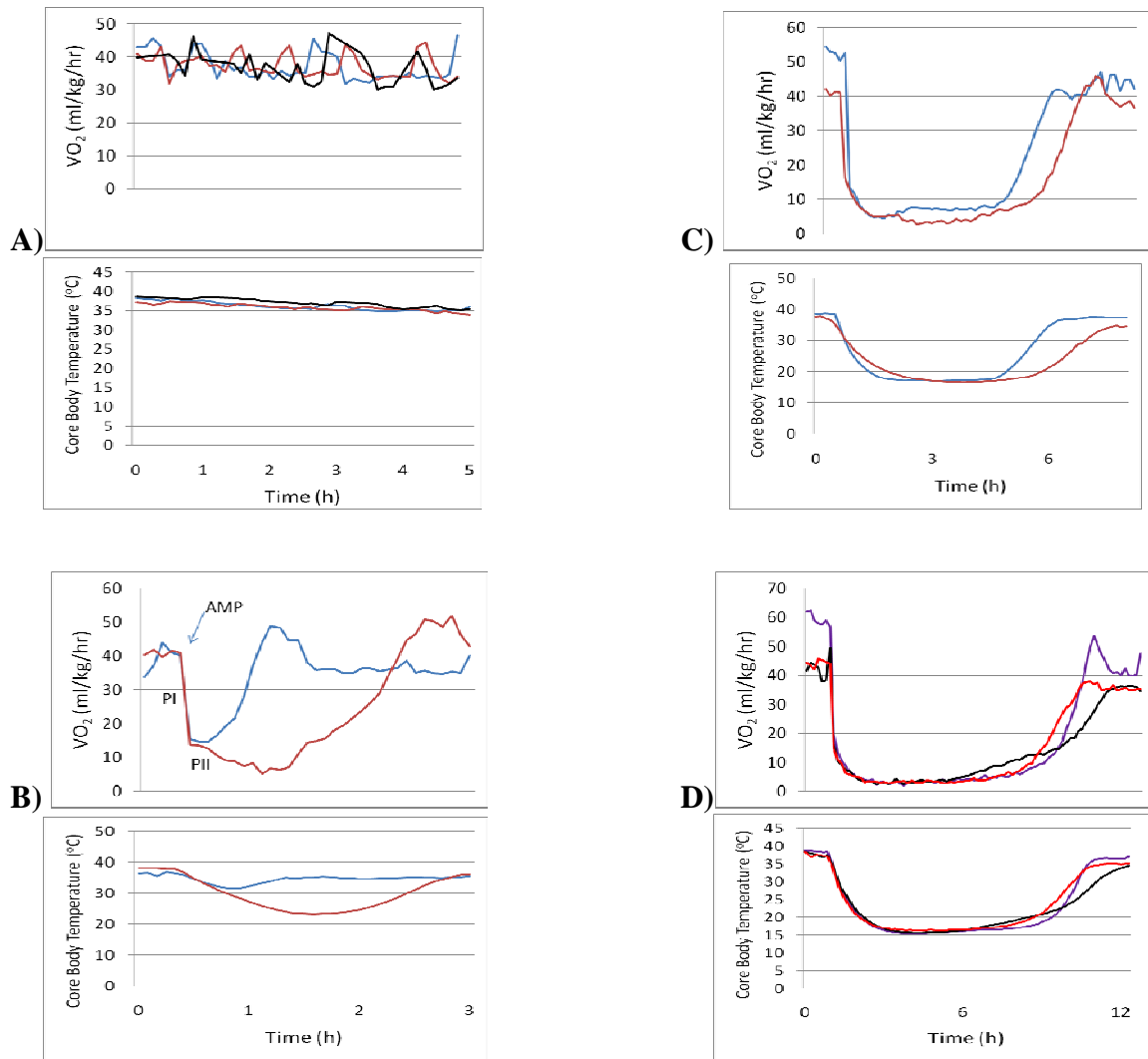


Figure 2. 5'-AMP titration. Mice were injected with A) Saline, B) .1mg / gram body weight (mg/gw), C) .5mg/gw and D) 1.0 mg/gw all at 15 $^{\circ}$ ambient temperature (T_a). B) Also shows the injection of 5'-AMP and the corresponding phase 1 and 2 (PI and PII) to show that even low concentrations of 5'-AMP respond similarly and therefore the concentration is only important to induce the process, not to prolong it. All VO_2 readings are in hundreds.

insufficient. This observation suggests that the concentration of 5'-AMP is necessary to cross the threshold and initiate the process. Once the mouse enters DH, the timing of arousal is not dependent on the dose of 5'-AMP used to induce DH. In addition, previous studies from other members of the laboratory have investigated whether arousal could be suppressed by re-injecting another dose of 5'-AMP. Logically, the process should be repeatable until the investigator decides to end the experiment and allow the mouse to arouse. However, what was observed was that suppression of arousal by additional injection of 5'-AMP led to increased mortality. It was observed that mice in DH after 14h have high mortality rate when arousal was suppressed by additional injections of 5'-AMP. The cause of death remains unclear, although dehydration is one possibility. Therefore, dehydration as a cause of death during prolonged DH was investigated.

Does giving saline during DH increase survival and help suppression of arousal?

Hibernators can spend weeks at a time in hibernation, which allows their bodies to go without food and, more importantly, without water for longer periods than is normally possible (**Heldmaier et al., 2004**). However, it has been observed that hibernators that undergo bouts of arousal during winter rise to drink but not to eat during their episodes of euthermia (**McManus 1974**). Since these mice are not natural hibernators, it was hypothesized that they may need certain supplements to maintain their deep hypometabolic state. The first supplement to test is the most important for sustaining life, water; but, in order to keep electrolyte levels at physiological norms, saline instead of water was used for these studies. The experiment was basic; one group of mice (n=8) received periodic injections of saline; another group of mice (n=8) received mock injections, during which they were taken out and handled, then subsequently returned to their cages. After about 18h

in DH, both groups of mice were aroused by raising the T_a to $\sim 16^\circ\text{C}$. One of the mice from the group not given fluid did rise before 12h and was excluded from the final analysis. The findings from this experiment are as follows: 5 of the 8 mice given saline survived, although 2 of these 5 mice had aroused spontaneously from DH at about 14 h. Only 1 of the 7 mock-injected mice survived (**Table 1**). After 12h, no mock-injected mice aroused and none survived. This experiment indicated that hydration for the mice in the DH state was beneficial. However, given that about half of the animals did not survive, it suggests that hydration alone is not enough. In addition, of the mice that died, some displayed apparent neurological defects, such as involuntary movements after they had re-warmed, and they were euthanized.

Fresh vs. reused mice

After performing multiple experiments, an apparent trend was observed between arousal rate and whether the mice were fresh (animals that had not been injected with 5'-AMP) or reused (mice that had been given 5'-AMP previously). Suppression, at the same temperature, seemed to favor mice that had never been administered 5'-AMP over those that had been used in an experiment regardless of whether this previous experiment was a week, a month or even months ago. When these data sets were examined, it proved that my initial observations were correct: the mice that were seeing 5'-AMP for the first time typically have a much lower rate (15%) of early arousal (arousal before 12h) when saline is provided after mice had entered DH. In contrast, mice that were being reused and had seen 5'-AMP before have a much higher rate of early arousal (51%) (**Table 2**). It was noticed that many of these early arousals appeared to be the heavier animals in the group. Therefore, the

Saline injections while in DH	No Saline injections
5/8 mice survived after 12h	1/7 mice survived after 12h

Table 1. Saline vs. Non-Saline Mice. Each group (n=8) were used to test the importance of saline. At ~15°C mice in the saline group were injected periodically while the non-saline mice were handled similarly to the saline treated mice without receiving an injection. 1 mouse from the non-saline group came out before 12 hours and therefore was not included in the final analysis.

Fresh mice arousal rates (in first 12 hours) Given saline	Reused mice arousal rates (in first 12 hours) Given saline
$3/20 = 15\%$	$20/39 = 51\%$

Table 2. Arousal rates between fresh and reused mice. Arousal rates in fresh and reused mice were examined to explore an observational trend. After examining the data, there seems to be a preference for the fresh mice to stay down in DH after 12 hours. Experiments were all run around 15°C with periodic injections of saline.

relationship between mouse weight and the time of spontaneous early arousal with the reused mice was examined. When the arousal time of the mice in DH given saline was plotted against their body weight, a trend was visible (**Figure 3**). Smaller mice tend to stay down longer than bigger mice. One possible explanation of this relationship could exist in the cooling efficiency between the smaller and larger animals. The larger animals have a smaller surface to volume ratio, which may not allow them to cool to a lower core body temperature as the smaller ones, and therefore they may not have fallen into as deep DH as the smaller mice. However, it is possible that other factors could be involved, since some of the early arousal animals were small. Nevertheless, the data would suggest that larger mice may need to be cooled in a slightly lower temperature than the smaller mice.

Does the circadian cycle have any influence on arousal?

The mice used in these experiments are typically housed in a 12h/12h light dark (LD) cycle in the animal facilities. However, some studies on natural hibernators conduct their research in 12h/12h Dark/Dark (DD) to coerce the animals into their hibernating bouts (**Heldmaier et al., 2004**). To address whether this change in environmental signal was important for preventing early arousal of mice in DH, a group of mice (n=16) with many habitual early arousals was put in a DD cycle for 3 days prior to DH induction. An earlier study with this group of mice when kept in an LD cycle showed that many were habitual early risers (**Figure 4**). As can be seen from the graph, about half of the 16 mice came out from DH during the first 12 hours. The same group of mice now maintained in a DD cycle did not show a significant reduction of early arousal. The LD and DD results with this group of mice revealed that the number of early arousals were similar at about 50% (**Figure 4**). Therefore, there is no apparent effect of the DD cycle on the suppression of early arousal.

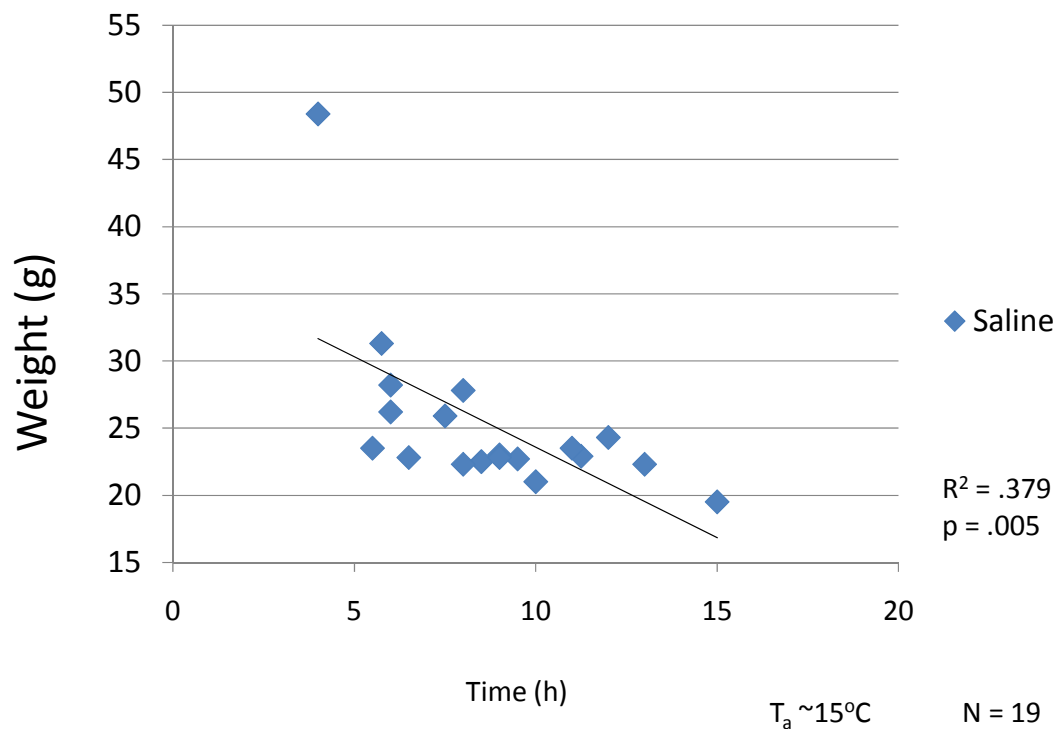


Figure 3. Examination of relationship between weight and arousal time. 19 reused saline mice were induced into DH and their arousal times were recorded and plotted against their weight to examine if there is a trend. Whereas a trend was apparent, showing that heavier mice tend to come out earlier than the lighter ones, some light ones also came out early suggesting there are other factors influencing arousal other than weight. Statistical analysis done by software from Wessa, 2008.

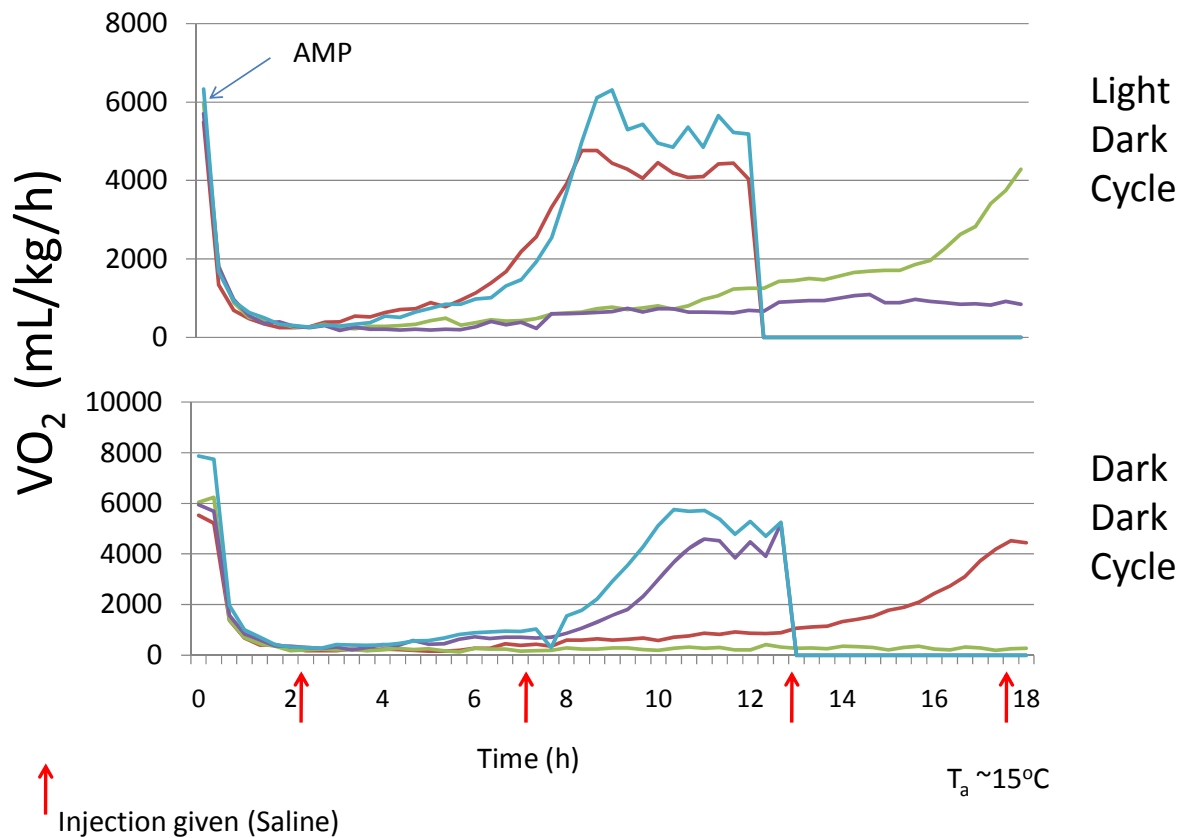


Figure 4. Role of Circadian Cycle in suppressing arousal. The same 4 saline mice were used once in a normal 12h:12h Light Dark (LD) cycle and then again in a 12h:12h Dark Dark (DD) cycle weeks later to test if there was a benefit to doing our studies in DD instead of LD. After examining the data, there did not seem to be any significant difference in suppression of arousal.

However, it was observed that in the few DD experiments that were run, the survival after 12 hours was impressive – 14 out of 16 mice that stayed beyond 12h survived. Many of these were in DH for 18h or longer. This is an interesting observation that will need to be explored further in the future.

Effects of environmental temperature on deep hypometabolism

The role played by environmental temperature in determining the length of the DH state was investigated. Previous studies have suggested that environmental temperature is a key exogenous factor in regulating hibernation (**Al-Badry and Taha, 1982**). Two groups of mice (n=4) were given the same dose of 5'-AMP (.5mg/gw) but were maintained at different ambient temperatures: 23°C and 15°C. **Figure 5** shows the effects of 5'-AMP induced hypometabolism at these two different ambient temperatures. The results show that the length of time the animal spends in deep hypometabolism is much longer when the ambient temperature is 15°C than at 23°C. Furthermore, the 15°C graph shows that the depth of DH attained and the severity of metabolic suppression were increased at this lower temperature when compared to the 23°C graph. Given that the same dose of 5'-AMP was used for both groups of mice, it can be concluded that at the lower temperature, the DH is prolonged and arousal further suppressed compared to the warmer T_a .

To further support these findings, an investigation was carried out to determine whether arousal from DH could be suppressed completely by further lowering the T_a . To exclude the possible contribution of individual mice variability, the same group of inbred mice (n=5) was used in sequential fashion for this study. When given 5'-AMP (0.5mg/gw), the same group of mice were maintained at ambient temperatures of 13.8°C, 13.6°C, 13.4°C

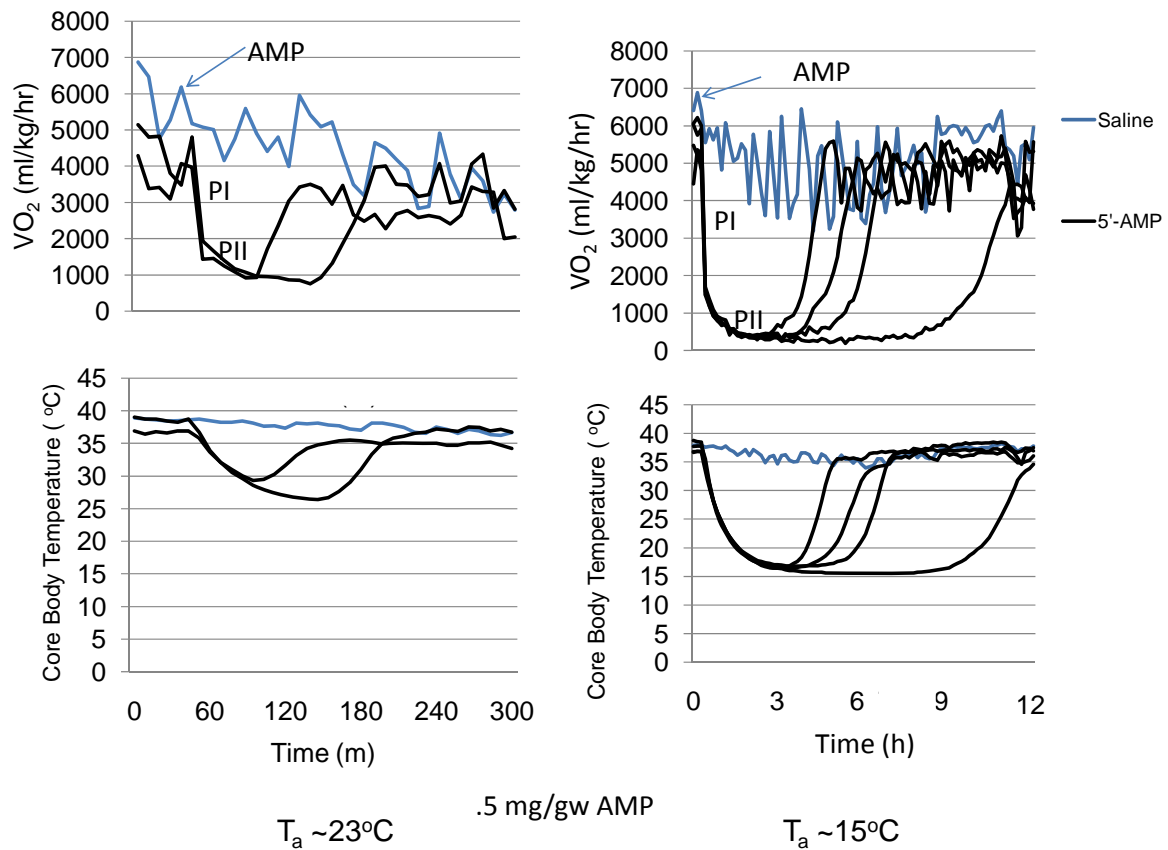


Figure 5. Effects of Environmental Temperature on DH. Mice were injected with .5 mg/gw 5'-AMP and placed at 2 different environmental temperatures, $\sim 23^{\circ}C$ and $\sim 15^{\circ}C$. Each group also has the phase 1 and phase 2 of induction labeled to point out the differences between the 2 temperatures. At $23^{\circ}C$, the phase 2 drop is short and approaches 1000 VO_2 before the mouse starts to come out from DH about 2 hours later. However at $15^{\circ}C$, the phase 2 drop is more pronounced and lower allowing the mice to stay in DH for up to ~10 hours. This experiment showed that the environmental temperature is the important factor in suppressing arousal for the DH state.

or 13.2°C. The VO₂ graphs in **Figure 6** show that there is a correlated linear relationship between the arousal rate of these mice and the T_a. The suppression of arousal is evident; not only do more mice stay down as the temperature is decreased, but the mice that come out do so significantly later than at the warmer temperatures.

From these results, subsequent experiments with fresh mice using a T_a of 13.2°C were undertaken. The suppression of arousal results were similar; however, the fresh mice were met with an astonishingly high mortality rate after 12h (>95%) even when saline was provided. Therefore, this strategy for preventing early arousal in mice needs to be investigated further.

In conclusion, the current investigation revealed that DH of mice can be induced by 5'-AMP. The amount of 5'-AMP only controls whether or not the mice will enter DH; the T_a is the key parameter regulating the DH state. If kept at about 15 °C, mice will safely enter DH and remain in such a state for about 3-9 hours following an injection of 5'-AMP of the appropriate dose.

One objective of this research is to extend the limit of DH in the mice. The above studies have shown that lowering the ambient temperature, giving injections of saline while in DH and administering an additional injection of 5'-AMP at the first sign of arousal all have significant drawbacks to their ability to extend the DH length. Primarily, it increases the mortality rate when T_a is too low after the mice have stayed in DH for more than 14 hours, even when saline was given. Additional injection of 5'-AMP prevented normal arousal but also enhanced mortality rate.

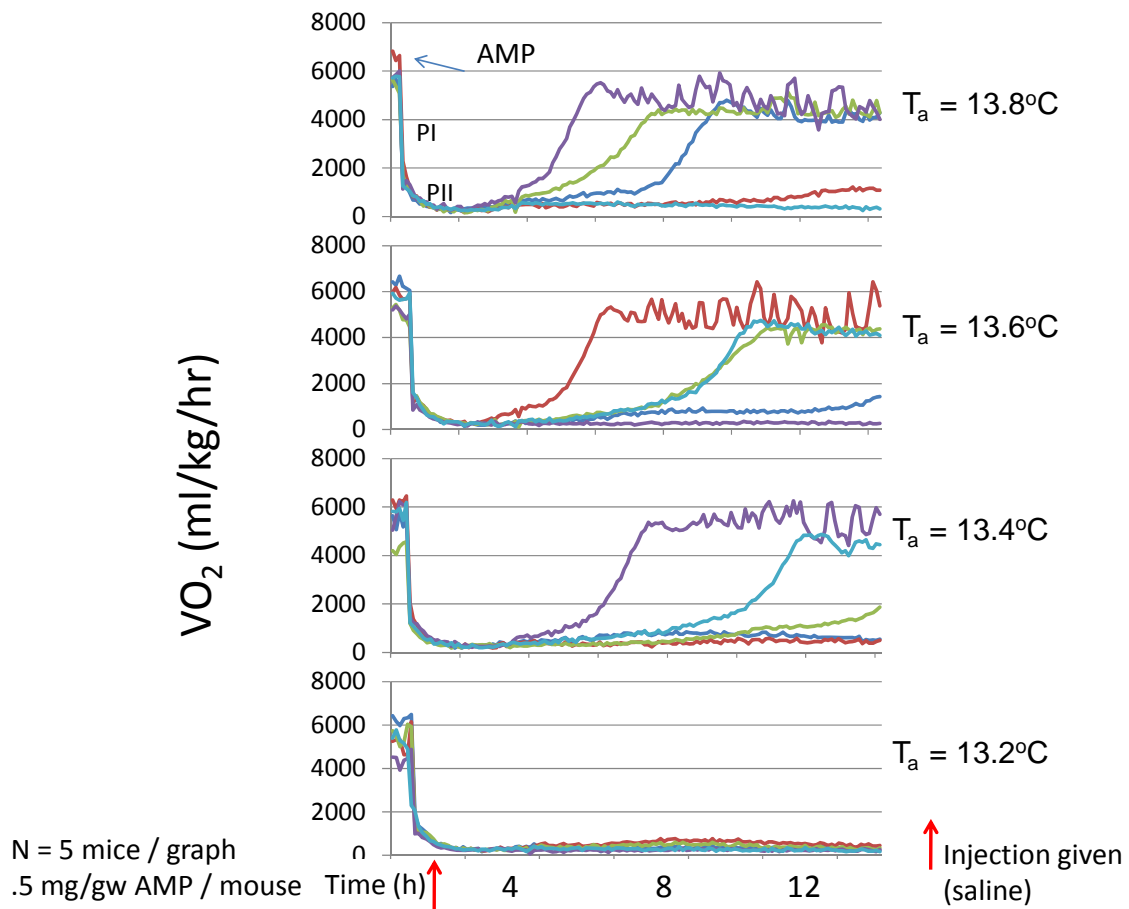


Figure 6. Temperature Titration. A group of 5 mice were used in subsequent experiments to establish a temperature that was beneficial to completely suppressing arousal. The temperatures ranged from 13.8 – 13.2 in .2°C increments. The experiment showed that as the temperature is decreased, arousal is delayed and in some cases inhibited altogether.

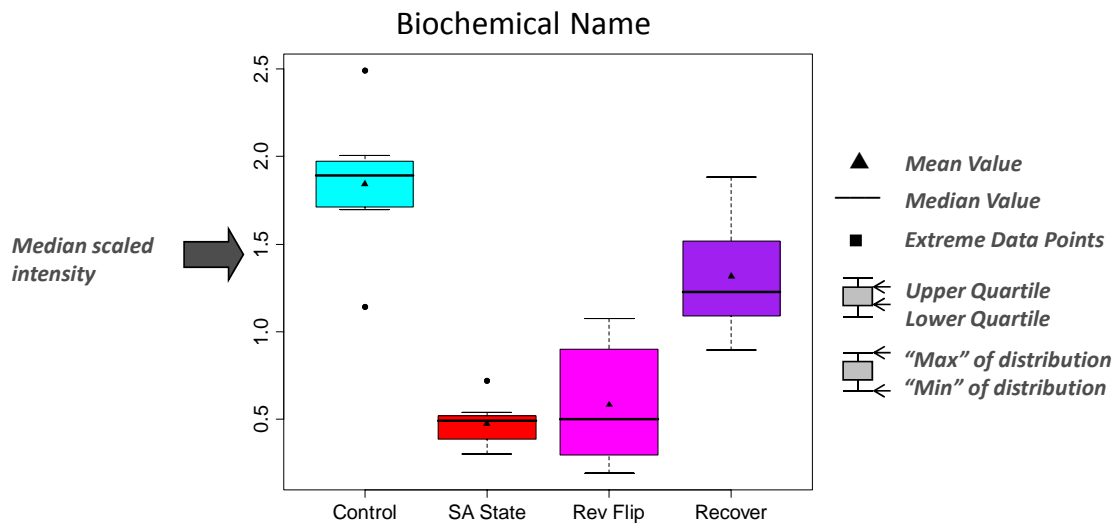
Changes in metabolites during various stages of 5'-AMP mediated hypometablism

To gather some insight into the changes of the metabolic state when mice undergo deep hypometabolism, serum was obtained from mice (each group n=8 mice) during the 4 stages; euthermia (control), deep hypometabolism (SA), arousal (reverse flip) and full recovery (recovery). These serum samples were examined at a commercial company specializing in metabolite analysis (METABOLOM). **Figure 7** illustrates the 4 different behavioral stages where the mice were sacrificed and their serum collected and how the metabolomic data was analyzed. For each group, the value of each metabolite is the average obtained from 8 mice. For each of the metabolites, the average value at euthermia is arbitrarily set at 1. The fold change in the DH, arousal and recovery is relative to the value obtained for euthermia. Therefore, a value of 1 indicates no change, a higher value indicates an increase and a value less than 1 indicates a decrease, all relative to euthermic levels.

Is glucose important in suppressing arousal?

The findings from the metabolomic analysis indicate that serum glucose was maintained at an elevated level for a prolonged period during DH (**Figure 8**). It has also been noted that a genetic shift from glycogen and fatty acid synthesis to gluconeogenesis is occurring in hibernating arctic ground squirrels, suggesting the animals are trying to provide themselves with increasing amounts of available glucose during their hibernation bouts (**Yan et al., 2008**). In addition, previous observations from the laboratory suggest that arousal coincides with decreased blood glucose and the activated expression of pro-colipase in the liver and other peripheral organs (**Zhang et al., 2006**). The metabolomic study also shows that glucose decreases between the DH state and arousal (reverse flip) state. It has

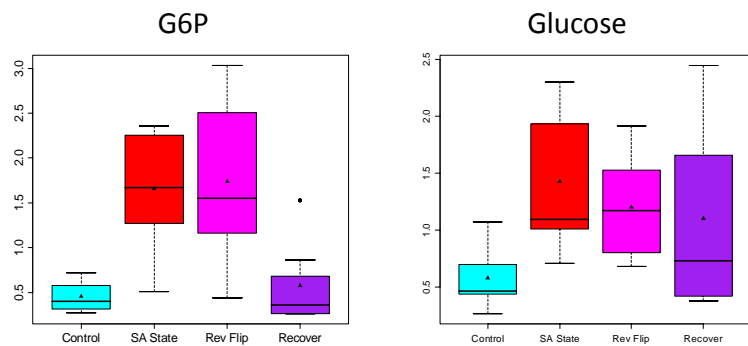
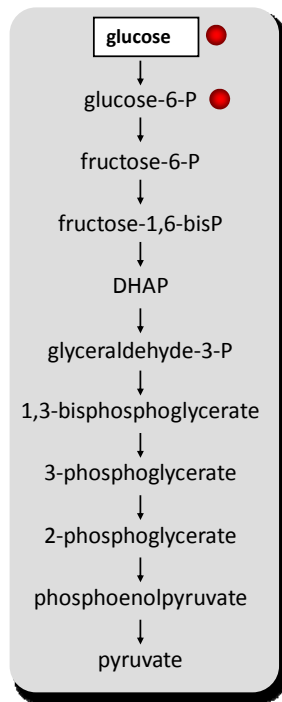
Example of Metabolon Data



Data from Metabolomic study done by Zhaoyang Zhao

Figure 7. Example of Metabolon Data. This is an example of how to read future figures that came from a Metabolomic Study done by Dr. Zhaoyang Zhao. Each group of mice (n=8) had blood collected at the 4 time points which were labeled in **Figure 1**. Control was prior to induction of DH, SA state is while in DH, Rev Flip is upon spontaneous arousal, and Recover is after the mice have returned to euthermia.

Glucose metabolism



- Glucose and glucose-6-phosphate elevated from control in SA and Flip states
- Glucose-6-phosphate restored to control levels in recovered group
- Glucose slightly reduced at Flip state from SA state.

Data from Metabolomic study done by Zhaoyang Zhao

Figure 8. Glucose Metabolon data. Shows the levels of Glucose and Glucose-6-Phosphate through the different stages of DH. As can be seen, both are highly elevated from control groups with a slight drop in glucose between DH (SA State) and spontaneous arousal (Rev Flip) which could signify an inability to produce large amounts of glucose anymore signaling arousal to the mouse.

further been suggested that hibernating ground squirrels use the increased availability of glucose to fuel their body's non-shivering thermogenesis (NST) maintaining their body at the right temperature while in hibernation (Yan et al., 2008). Together, this information led to a hypothesis that the mice may arouse when they can no longer produce the large amounts of glucose they need to sustain themselves in the DH state. Thus, whether injecting glucose could suppress arousal better than saline alone was investigated. Two groups of mice (n=3) were injected with similar amounts of saline at the same intervals; however, one group received glucose (20mg) at the 2-hour injection. After that injection, subsequent injections were solely saline for both groups. The experiments revealed that two mice from each group came out from DH at roughly 8-9 hours while the last of the saline group came out around 12-13 hours and the last of the glucose group around 20 hours after an increase in the temperature. Overall, the studies revealed that there was no major differential outcome in preventing early arousal whether the mice received saline or glucose (**Figure 9**). Additional experiments confirmed the conclusion that the early arousal rates continued to be about equal between glucose-injected mice and saline-injected mice. One possible reason for the elevated blood glucose is that the overall rate of glycolysis had been slowed at hexokinase and phosphofructose kinase, since there is also a 4-fold build-up of glucose 6-phosphate and fructose 6-phosphate according to the Metabolomic data.

Is pyruvate important for suppressing arousal?

Consistent with a reduced rate of glycolysis, the Metabolomic data indicated extremely low pyruvate and lactate levels in the DH state (**Figure 10**). Lactate is utilized by the liver to make pyruvate. Pyruvate is normally converted by pyruvate dehydrogenase to acetyl-CoA, a necessary input for the TCA cycle. However, ground squirrels undergoing

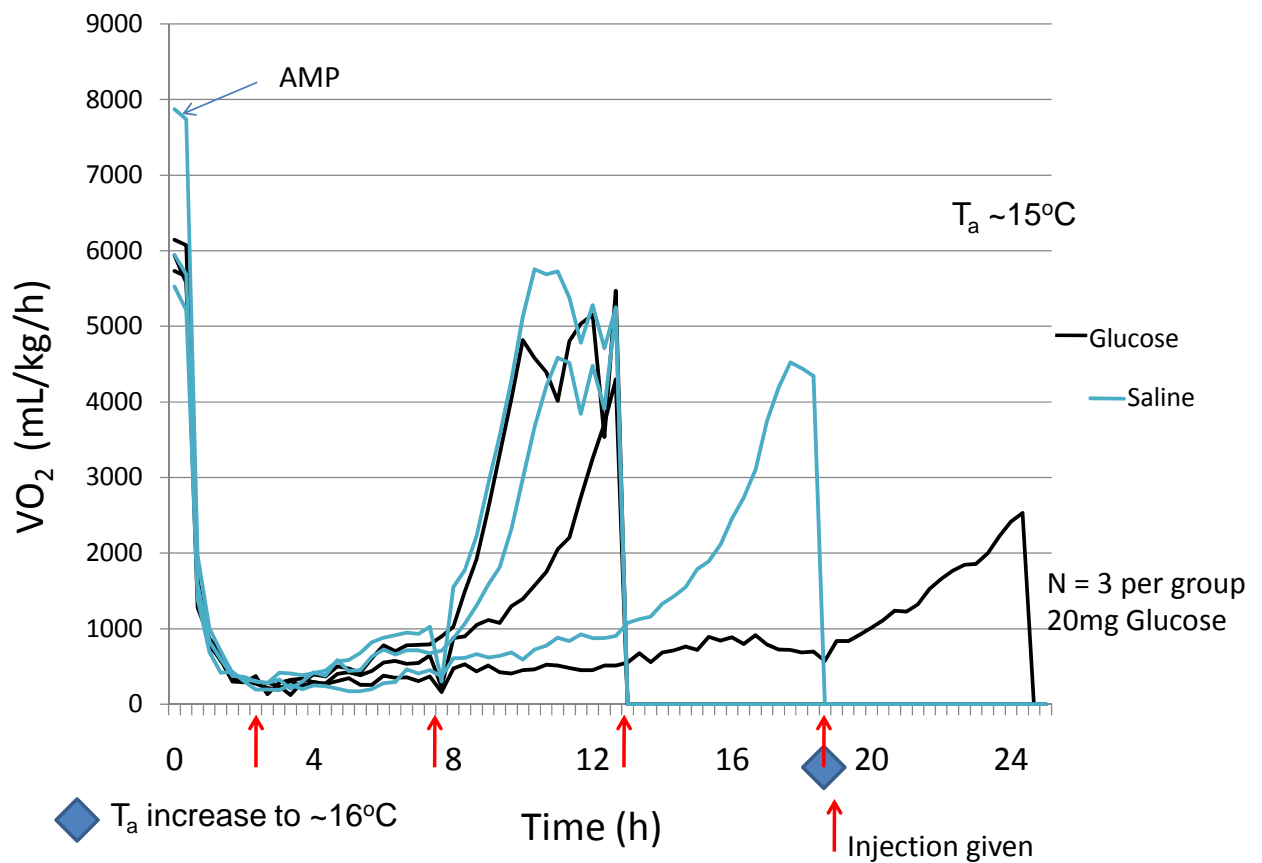
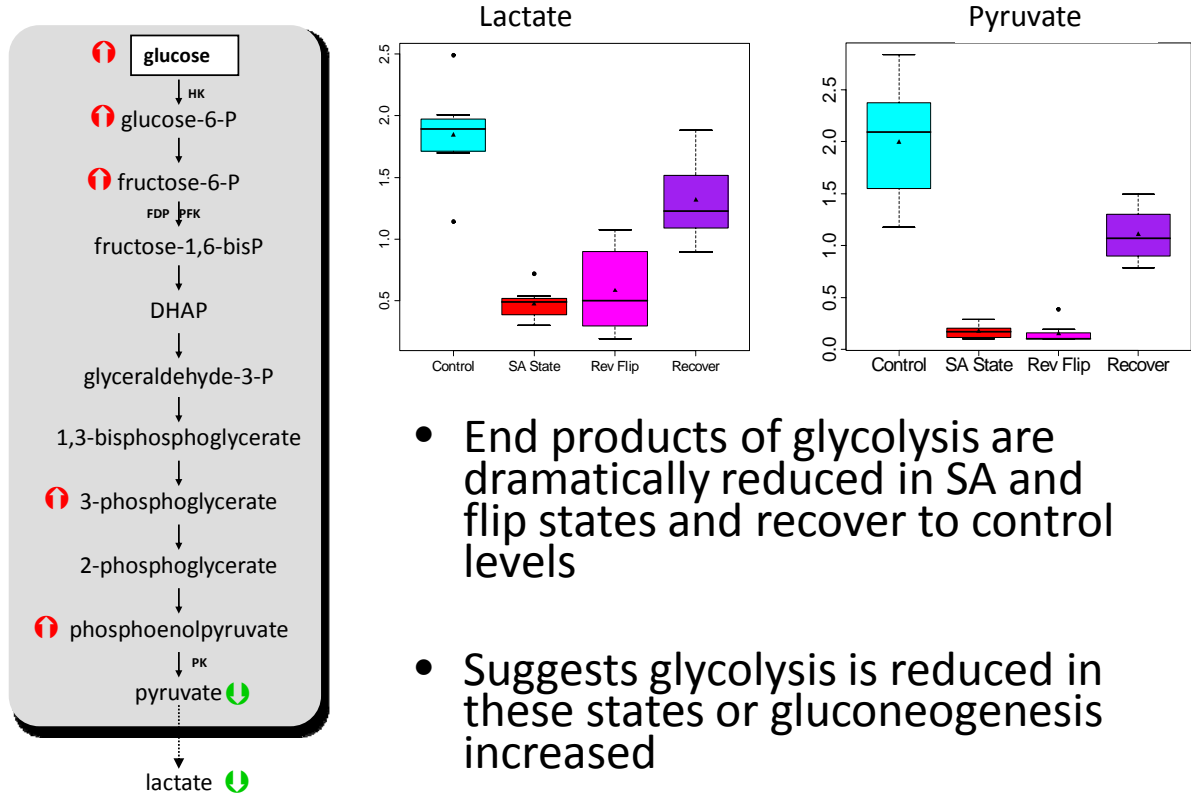


Figure 9. Glucose experiment. 2 groups of mice ($n=3$) were either given an injection of glucose or pure saline around 2 hours into the experiment followed by subsequent injections of saline. There appeared to be no benefit in suppressing arousal by injecting glucose over just saline.

Glycolytic products ↓ in SA & Flip states



- End products of glycolysis are dramatically reduced in SA and flip states and recover to control levels
- Suggests glycolysis is reduced in these states or gluconeogenesis increased

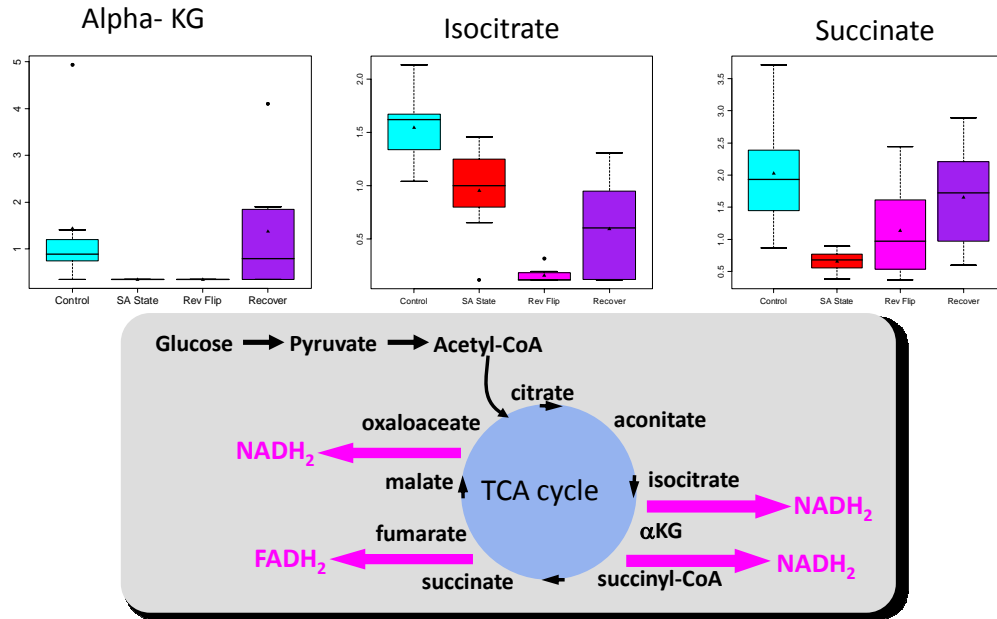
Data from Metabolomic study done by Zhaoyang Zhao

Figure 10. Glycolytic Products Reduced in DH. Metabolon data shows that there is a decrease in the glycolytic products of pyruvate and lactate while there is also an increase in glycolytic intermediates. This would point to either a reduction in glycolysis or an increase in gluconeogenesis.

torpor have shown an overexpression of Pdk4 (pyruvate dehydrogenase kinase isoform 4), an enzyme that deactivates pyruvate dehydrogenase, blocking the conversion of pyruvate to acetyl-CoA (Yan et al., 2008). In addition, in the liver, pyruvate can also be converted into oxaloacetate (OAA) then to malate which can exit the mitochondria and be used for gluconeogenesis by producing phosphoenolpyruvate. Interestingly, it has been recently shown that a key enzyme for the aforementioned process, Phosphoenolpyruvate carboxykinase (Pck1), is overexpressed in hibernating bears while some glycolytic enzymes are underexpressed, also hinting toward a state of gluconeogenesis during hibernation (Fedorov et al., 2009). It is also interesting that some TCA cycle intermediates, such as α -ketoglutarate, isocitrate and succinate, were also dramatically decreased (Figure 11). Slowing the TCA cycle causes the body to resort to a glycolytic state for ATP generation. Glycolysis is highly inefficient compared to oxidative phosphorylation where ATP is produced from the electron transport chain.

It is possible that the reduction in the TCA cycle intermediates could be a direct result of the decrease in pyruvate availability, since its absence could cause a slowdown in acetyl-CoA production that in turn would limit the amount available for the TCA cycle. Thus, supplemental pyruvate injected into the mice may restart the TCA cycle, which will in turn enhance the oxidative phosphorylation process allowing the mice to produce energy much more efficiently. Therefore, whether giving pyruvate to mice in DH might suppress arousal and prolong the amount of time down was investigated. Two groups of mice (n=3) were given either saline or pyruvate after they had entered DH after treatment with 5'-AMP. Similarly to the glucose experiment, the pyruvate group got the supplemental injection (20mg) at 2h and saline at subsequent injections. The study showed that there was no

TCA cycle reduced by SA and Flip states



- Consistent with reduced glycolytic flux and pyruvate production, several TCA cycle intermediates decline in the SA and flip states with levels nearing control in recovery group

Data from Metabolomic study done by Zhaoyang Zhao

Figure 11. TCA cycle intermediates reduced in DH. Metabolon data shows that there is a decrease in a few TCA cycle intermediates hinting at a slowing down of the TCA cycle in the DH state.

significant suppression of early arousal between the mice given saline and those given pyruvate (**Figure 12**). This result was fairly similar to that of the glucose experiment; however, the 3 mice from each group came out before or around 12 hours. The outcome of this experiment and several other subsequent larger studies indicated that pyruvate did not alter the level of early arousal, nor did it result in better survival compared with saline when animals stayed down for longer than 12 hours. Thus, it is concluded that a deficiency in pyruvate was not a major factor in regulating early arousal.

Are amino acids important to suppressing arousal?

Additional reevaluation of the Metabolomic data revealed that the mice had a very high level of glucose and glucose 6-phosphate while in DH. In the liver, glucose 6-phosphate is converted to glucose. However, in the muscle glucose and glucose 6-phosphate can be converted to glycogen for storage or used in glycolysis to generate ATP. Furthermore, during DH, other glycolytic intermediates like phosphoenolpyruvate (PEP) were increased (**Figure 13**). However, the products of glycolysis such as pyruvate and lactate were decreased. The increase in glycolytic intermediates, combined with the decrease in pyruvate and lactate, suggested that the mice were possibly undergoing gluconeogenesis or that glycolysis was highly reduced while in the DH state. Similarly, it has been shown recently that arctic ground squirrels have increased gene expression of gluconeogenic enzymes in their torpor state when compared to euthermic levels (**Yan et al., 2008**). Lactate is normally produced in the muscle when oxidative phosphorylation cannot sustain the demand for cellular ATP leading to anaerobic ATP generation via glycolysis. However, the mice displayed much lower levels of lactate, suggesting that there is actually very little demand for glycolysis. In addition, the extremely low levels of pyruvate also suggested that the level

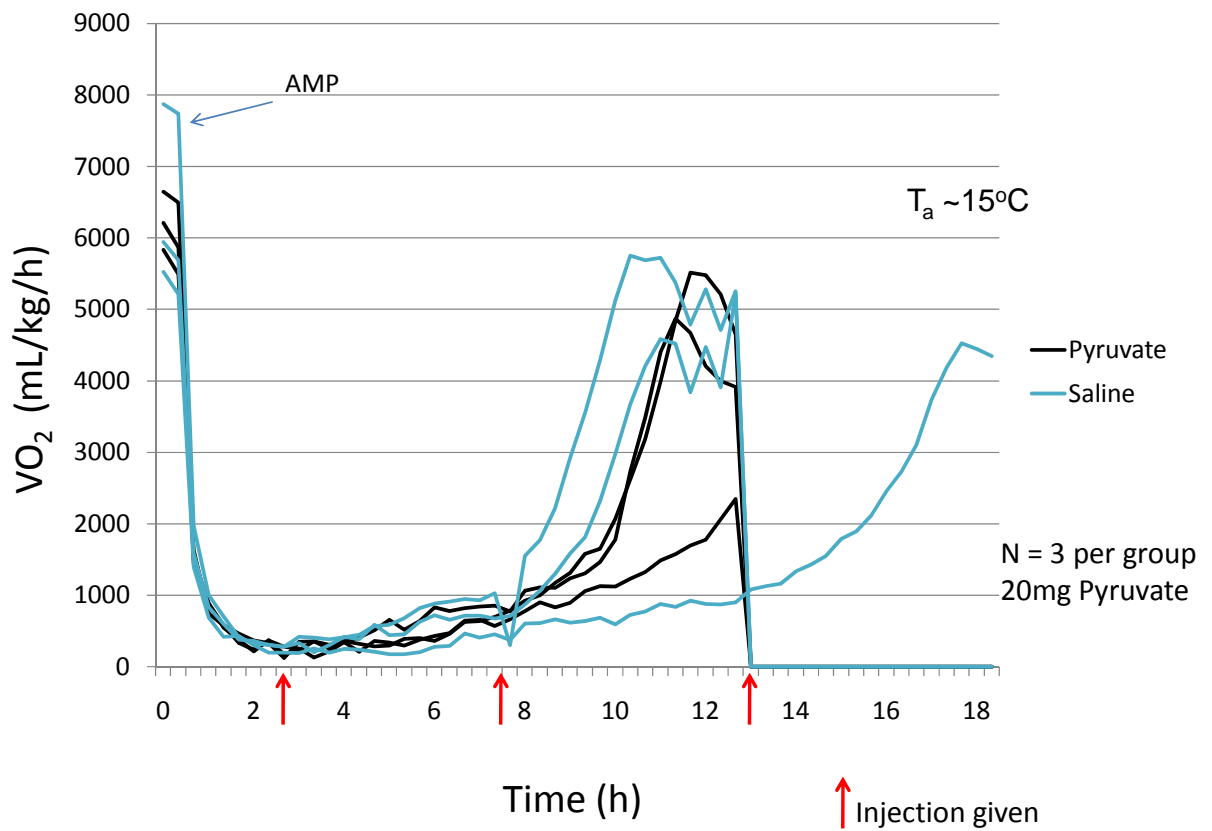
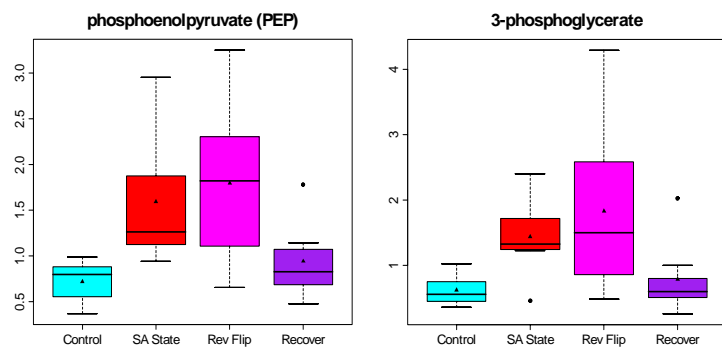
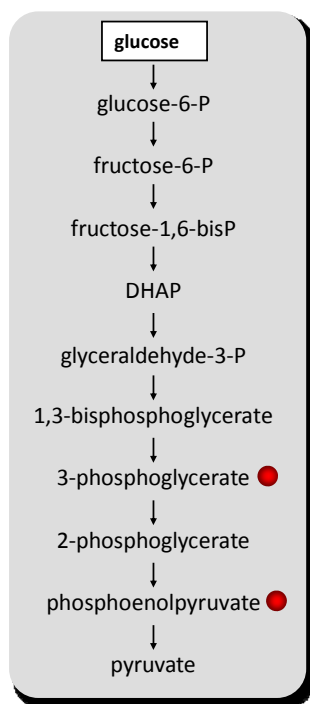


Figure 12. Pyruvate Experiment. 2 groups of mice (n=3) were either given pyruvate or saline 2 hours into the experiment followed by saline for all mice in following injections. This experiment shows, similarly to glucose, that there is no real difference in arousal rates between saline and pyruvate injected mice.

Glycolytic intermediates elevated



- Accumulation of some intermediates of glycolysis in the SA and flip states

Data from Metabolomic study done by Zhaoyang Zhao

Figure 13. Later glycolytic intermediates increased in DH. Metabolon data shows an increase in late stage glycolytic intermediates.

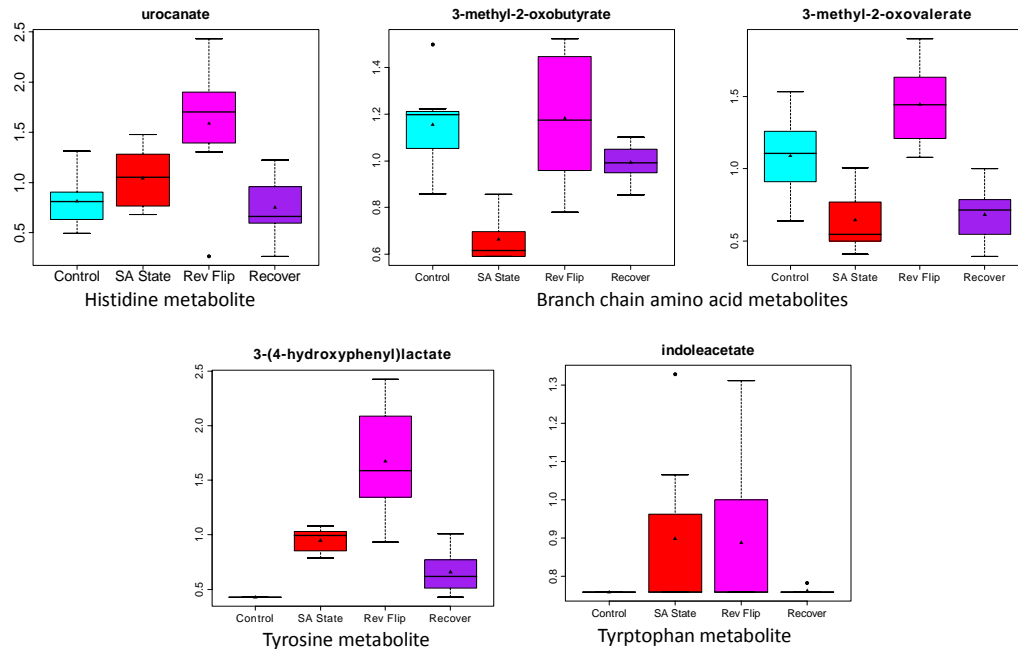
of glycolysis was low. This may explain why blood glucose and glucose 6-phosphate is significantly enhanced in the mice during DH.

If the hypothesis that the mice are in a gluconeogenic state is correct, then synthesis of glucose will require the availability of carbon skeletons, which are provided by amino acids, lactate or glycerol. The metabolomic data indicated that the catabolites of amino acids were significantly increased during DH suggesting that amino acids were indeed catabolized (**Figure 14**). Some genes in the liver, Glut1 and Got2, responsible for amino acid catabolism have also been shown to be overexpressed during torpor in ground squirrels (**Yang et al., 2008**). Therefore, whether giving amino acids to supplement the energy production would help suppress arousal was investigated.

In order to determine which of the amino acids to use, a serum panel was run of amino acid levels in normal mice (n=2), mice in DH (n=2) and mice at arousal (n=2) through a commercial service laboratory at Baylor College of Medicine. This study revealed that the mice in DH and at arousal were low in two amino acids, histidine and arginine, while the rest of the amino acids were greatly enhanced or not affected during DH and at arousal compared with euthermic control (**Table 3**). **Table 3** shows the complete panel with the two reduced amino acids indicated by the arrow. Each group of mice (euthermic, in DH and at arousal) had 2 sample points with the average value taken of those points and displayed in bold below the group. The most significant reduction is arginine. Why arginine?

To answer this question, the biological pathways that these amino acids are involved in were examined; importantly, arginine plays an intricate role in the urea cycle (**Figure 15**).

Amino acid metabolism altered in flip state



- Catabolites of amino acids also increase (for energy or overflow due to copious supply)

Data from Metabolomic study done by Zhaoyang Zhao

Figure 14. Amino Acid catabolites are increased in DH. Metabolon data shows an increase in amino acid catabolic products in DH.

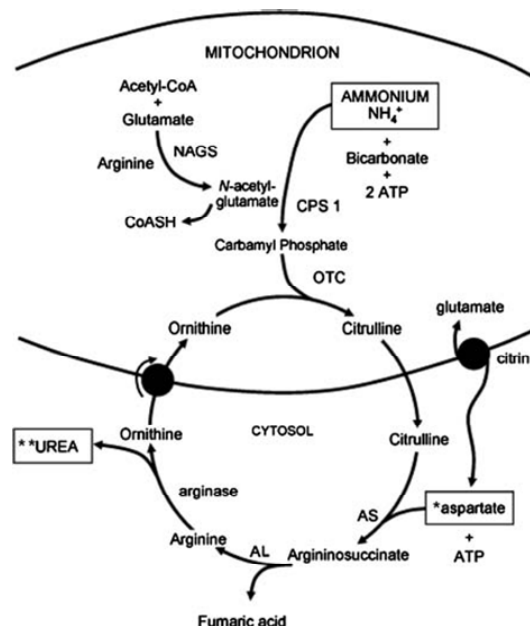
Amino Acid Assay

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Data from Zhaoyang Zhao

Table 3. Amino Acid assay. An amino acid assay from mouse serum samples (n=2) taken at 3 different stages throughout DH. First stage was euthermic (normal), second was while in DH (SA) and the third was at initial sign of spontaneous arousal. The 2 amino acids that are decreased are pointed out (histidiine and arginine).

Urea Cycle



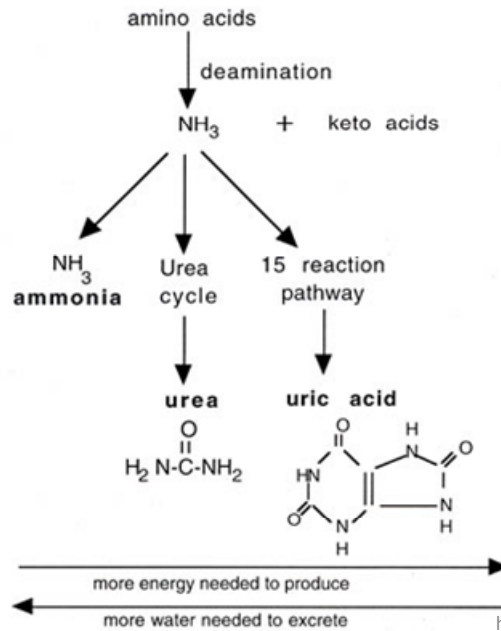
Walker 2009

Figure 15. Urea cycle. NAGS, N-acetylglutamate synthetase; CPS1, carbamylphosphate synthetase 1; OTC, ornithine transcarbamylase; AS, argininosuccinate synthetase; AL, argininosuccinate lyase; citrin, mitochondrial aspartate-glutamate carrier; ornithine transport system. The urea cycle displays the intricate role that arginine plays in the functionality of the entire cycle.

The reduction in the availability of arginine may compromise the urea cycle's ability to release urea and regenerate ornithine, which is essential to transport the NH_2^+ group on the

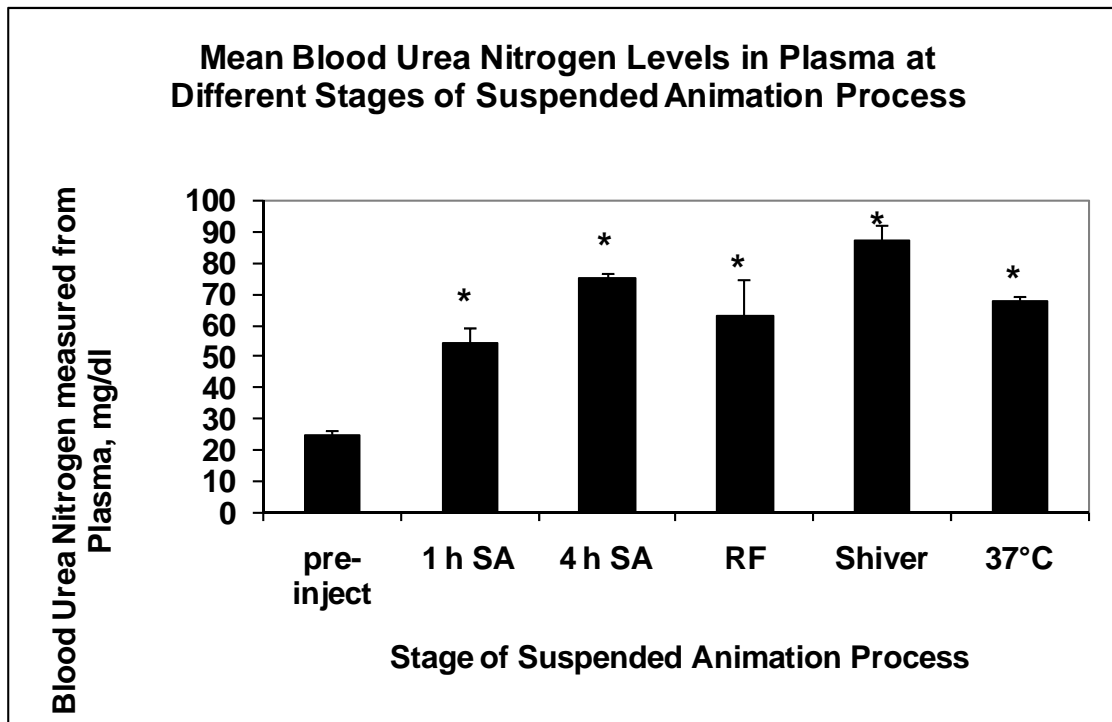
carbamoyl-phosphate out of the mitochondria by forming citrulline. Therefore, a shortage of arginine could significantly reduce the animal's ability to deal with an increased ammonia build up generated by transamination of amino acids (**Figure 16**). Since it requires more energy to produce uric acid than ammonia, the natural tendency would leave the ammonia groups as ammonia. Ammonia is highly reactive with lipids in plasma membranes, resulting in severe cellular damage; however, it is only toxic in the brain (**Walker, 2009**). Hibernating bears have means to recycle their nitrogenous waste, decreasing their urea cycle outputs (**Barboza et al., 1997**). The decrease in activity of the urea cycle noticed in the bears has also been shown in hibernating ground squirrels (**Williams et al., 2005**), which suggests all natural hibernators have an ability to suppress urea formation while in a hibernation-like state. Since these mice are not natural hibernators, they potentially do not possess these mechanisms to prevent the build-up of ammonia. Because of this possible inability to handle increases of ammonia while in DH, the mouse's ability to stay in DH for a prolonged time may be limited. It is possible that mice spontaneously arouse to handle the build-up of ammonia. To help gain insight into this theory, a colleague (Isadora Susan Daniels, Ph.D. student) in the lab ran a blood urea nitrogen assay (QuantiChrom™ Urea Assay Kit, DIUR-500 from Bioassay Systems) for mice throughout the DH process; the time points (n=20) were pre-injection, 1 hour into DH, 4h into DH, spontaneous arousal (RF), shivering, and return to euthermic temperature (**Figure 17**). The data shows a steady increase over the course of DH, with a decrease at the spontaneous arousal stage. This could suggest that the urea cycle is indeed shut down in the DH process, causing an accumulation of nitrogen in

Amino Acid Breakdown



<http://instruct1.cit.cornell.edu/courses/biog105/pages/demos/105/unit7/media/nitrogenouswastes.1.jpg>

Figure 16. Breakdown pathways of amino acids. Shows possible options in removing ammonia after the breakdown of amino acids. In the case of our mice, which it is hypothesized the urea cycle is compromised during DH, this figure suggests that the ammonia being produced would stay as free ammonia causing a build-up in vivo.



Unpublished data from Isadora Susan Daniels

Figure 17. Accumulation of blood urea nitrogen. Corroborating evidence from a member in the laboratory shows an increase of blood urea nitrogen (BUN) levels throughout the DH process with a slight decrease at the signal of spontaneous arousal (RF). Blood samples were taken from mice (n=20) in the different stages of DH (SA). This data lends some merit to our hypothesis of the urea cycle being compromised from the decrease in arginine throughout the DH process.

the blood. The spontaneous arousal could be a signal to the body that blood nitrogen levels are reaching a critical mass and the animal must come out to get rid of the waste. This result supported our theory and thus, it was investigated whether supplementing arginine could suppress early arousal. Two groups of mice (n=4) were used; one received an injection of arginine (2mg) at the 2h injection (same as pyruvate and glucose), and subsequent injections were pure saline for the rest of the experiment. **Figure 18** shows that the effect of arginine was striking. It was observed that 3 of the 4 mice that received arginine did not arouse early. None of the saline- injected mice (n = 4) displayed a similar ability to prolong DH. Not only was the early arousal suppressed, the survival after 12 hours was improved. The three arginine-injected mice that stayed down after 12h were able to stay in DH for up to 24 hours, and all survived without complications.

The hypothesis was made that the arginine had repaired the urea cycle; it has been shown previously that arginine can be used as an alternate pathway treatment to repair some urea cycle deficiencies in human patients (**Walker, 2009**). To investigate this hypothesis, another amino acid, alanine, was tested for a similar outcome. The transamination of alanine with α -ketoglutarate via alanine transaminase is directly converted into glutamate and pyruvate, one of the metabolites that is in very short supply during DH (**Strobel 2007**). If the mice given alanine showed similar results to the arginine mice, it could be inferred that it was not the urea cycle being repaired but simply that an amino acid fuel source was what the mice needed. A group of 7 mice (4 alanine, 3 saline) were given their respective injections 2h into the experiment. This experiment showed that giving alanine to mice only slightly increased the amount of time mice stayed in DH compared to saline-injected animals (**Figure 19**). However, the suppression was not nearly as dramatic as the arginine, and most

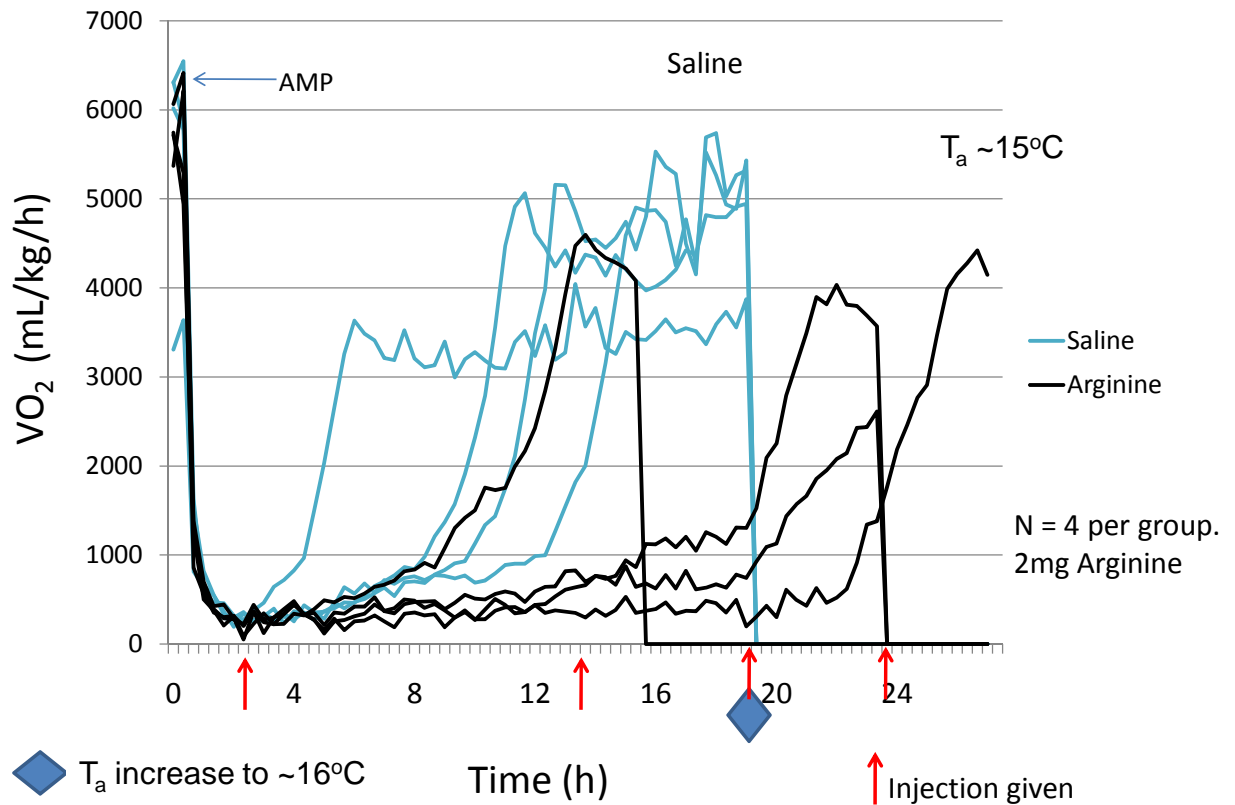


Figure 18. Arginine vs. saline experiment. 2 groups of mice (n=4) were given either saline or an injection of arginine 2 hours into the experiment and subsequent saline injections to both groups. This experiment suggested that arginine was beneficial in suppressing arousal and of those it suppressed the survival was better. The 4 saline mice all came out before or at 12 hours whereas only 1 arginine mouse came out before 12 hours and one lasted almost 24 hours in DH.

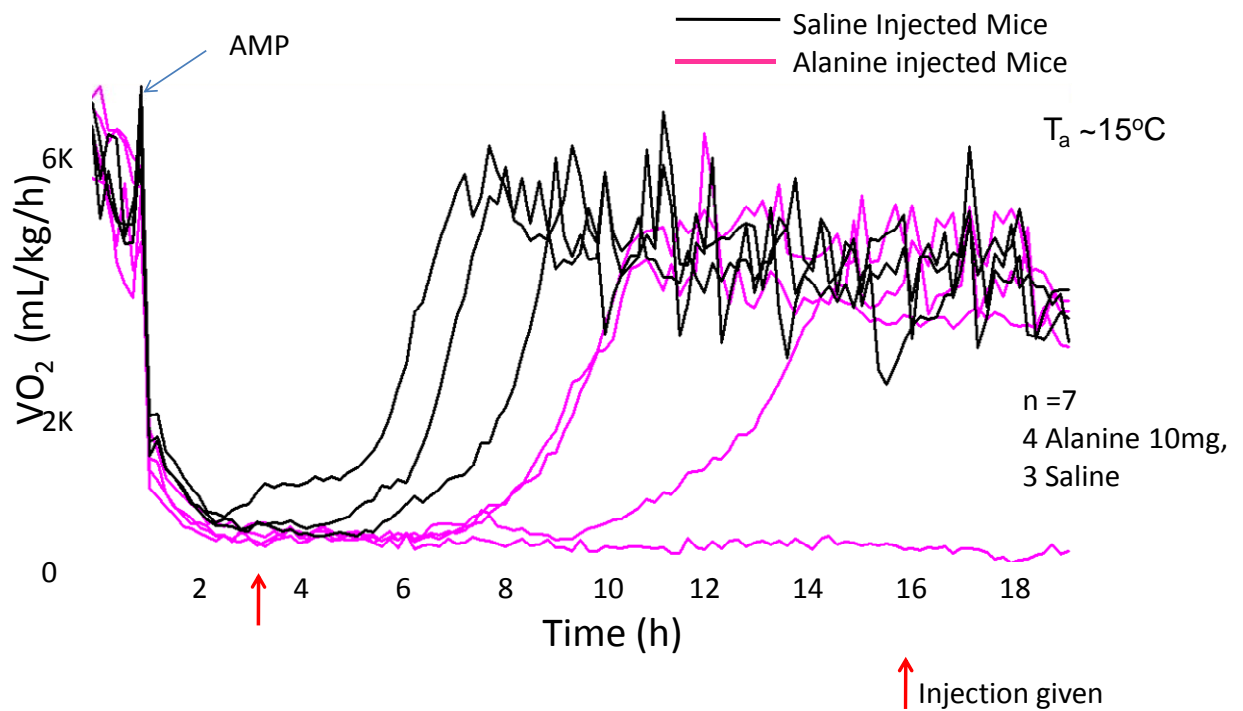


Figure 19. Alanine vs. saline experiment. 2 groups of mice ($n=4$ alanine and $n=3$ saline) were given either saline or an injection of alanine 2 hours into the experiment. The alanine injected animals lasted slightly longer in DH however most still aroused by 12 hours and the one that stayed down more than 15 hours wound up coming out with fatal side effects. Suggested that alanine could not be used in suppressing arousal.

of the mice still came out as early arousals, meaning arousal was not completely suppressed. Also, those that stayed down for more than 14-15 hours came out with unwanted side effects that affected their long term viability. These side effects include an inability to balance properly and constant shaking; these animals were euthanized. Descriptions of these side effects have been noted before in human cases dealing with arginase deficiencies (**Brusilow et al., 2001**), and it has also been noted that urea cycle deficiencies in general can lead to brain damage (**Walker, 2009**). These experiments suggested that the amino acids are important, however arginine's effect on the urea cycle appears to be crucial for both suppression and survival. Normally small hibernating rodents use their amino acids to produce energy through gluconeogenesis and the TCA cycle since these amino acids are not producing urea (**Yang et al., 2008**), however since these mice are being induced into this torpor-like state and do not prepare their bodies like typical hibernating animals (**Al-Badry and Taha, 1982**), the arginine given may aid in removing the nitrogenous waste that is being produced. In order to confirm arginine's importance, the successful experiment's parameters were repeated with all 16 mice given arginine (2mg). They all received an injection at 2h into DH and another arginine injection at the 28h mark for the mice still down. The intermediate injection was saline. **Figure 20** shows that the results from the initial experiment were highly replicable and further proof of the significance and effectiveness arginine has on the arousal rates and survival of the mice. All mice but 2 stayed down for the initial 12 hour period and upon an increase in temperature and removal, all mice came out and survived. One unfortunate fatality was due to human error a few days post experiment; however, I believe it could have been readily prevented if the right precautions had been taken. In addition, this study revealed that 2 mice were in DH for 30

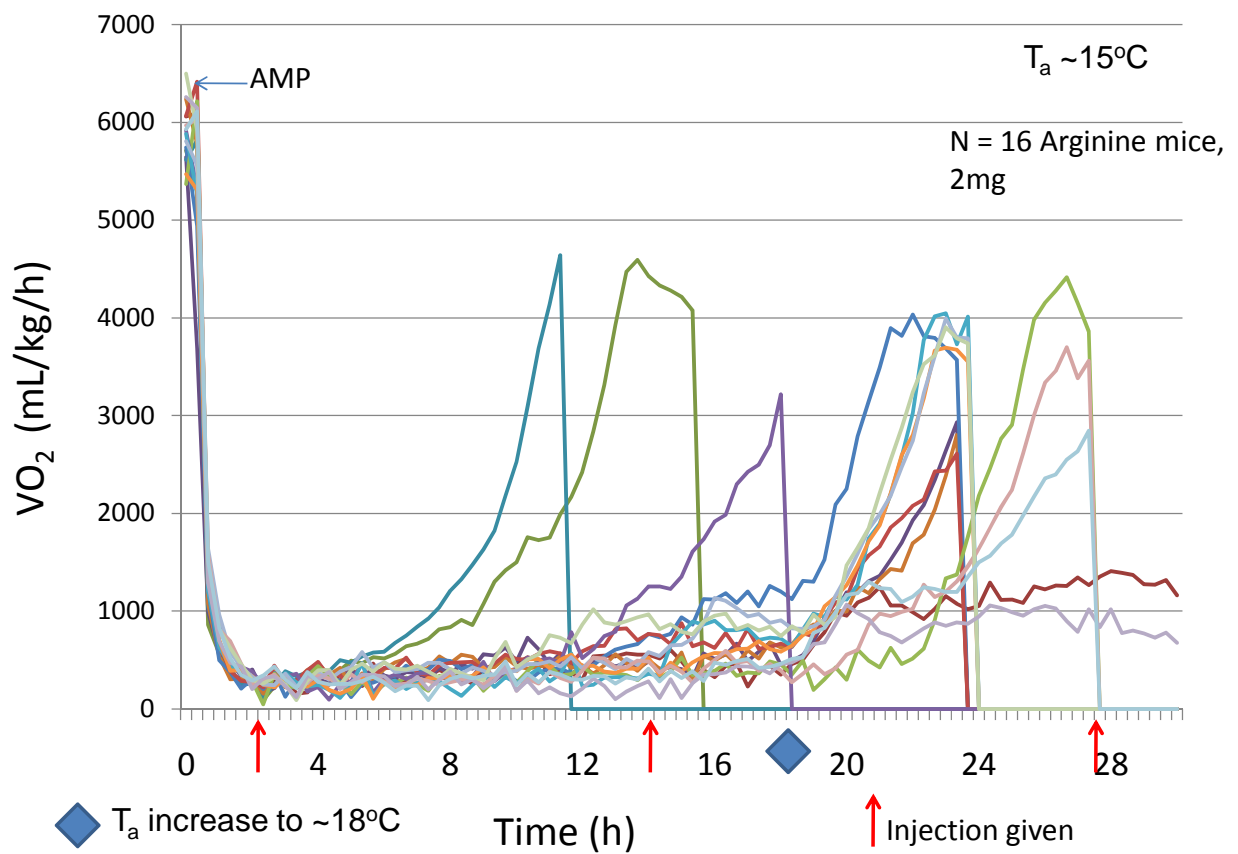


Figure 20. Arginine experiment. All mice (n=16) were given an injection of arginine 2 hours into the experiment. 2 mice came out in the first 12h however suppression was largely suppressed and there was 100% survival even for those mice that stayed down 28+ hours.

hours and when they were gradually rewarmed, they returned to euthermic state without obvious disabilities. Typically, from observations of many past experiments, if mice stay in DH after the temperature is bumped up they do not survive even for a day. These mice, however, were viable and appear normal even after 3 weeks of monitoring after undergoing this 30-hour DH. This experiment has since been repeated and similar results were obtained. Therefore, it has been concluded that at least part of the reason for arousal is tied to a build-up of ammonia in vivo. The ammonia build up is a consequence of the transamination of amino acids as the animal in DH is undergoing gluconeogenesis. A shortage of arginine compromised the animal's ability to safely remove the ammonia through the urea cycle. By arousing early, the gluconeogenic process would slow as the normal oxidative phosphorylation process to generate ATP returns and the production of ammonia is reduced.

Is any single metabolite better than the rest?

After a multitude of experiments using the metabolites above, all the data was pooled together and examined. From this data, 2 tables were created, providing an in-depth analysis. The first table shows the various metabolites and their benefit to suppress early arousal (**Table 4**). Even though some sample sizes were smaller than others, one metabolite stands out: arginine. Its 14% total arousal rate is half that of the next closest (pyruvate) and significantly less than saline alone (39%). The second table shows the overall survival after being in DH for 12 hours for the different groups of mice (**Table 5**). This is the most interesting data of all. While most of the metabolites are right around saline (58% saline, 64% glucose and 67% pyruvate), once again arginine is superior. The difference is phenomenal. Of the arginine mice that stayed down 12 hours or more, 94% survived. This

Metabolite	Arousal before 12h
Saline	23/59 = 39%
Glucose	5/16 = 31%
Pyruvate	25/89 = 28%
Arginine	6/42 = 14%

Table 4. Metabolite benefit for suppressing arousal. Table shows the metabolite and their corresponding percentage of mice that were early arousals (before 12h). Arginine had the best at 14% which was half of the next closest and almost a third of saline.

Metabolite	Overall Survival after 12h
Saline	21/36 = 58%
Glucose	7/11 = 64%
Pyruvate	43/64 = 67%
Arginine	34/36 = 94%

Table 5. Metabolite benefit for survival after 12h. Table shows the metabolite and their corresponding percentage of mice that survived after 12 hours in DH. Arginine is a clear favorite at 94%. The 2 deaths in the arginine group are believed to have been preventable since they were both due to human error during or after the experiment.

number would have been 100%; however; the two animals that did succumb died due to human error after having aroused from DH.

CHAPTER FOUR

Discussion

The fate of the 5'-AMP injected is the focal point around this study. Our studies revealed that 5'-AMP can be disposed of in 2 different ways; it can be catabolized to IMP and/or adenosine or it can be converted via the adenylate equilibrium to 2 ADP by combining with an ATP. The latter is the aspect believed to be important in the DH initiation process. Since ATP is essential for maintaining cellular activity levels at their optimum, by injecting AMP and removing ATP it would cause a slowdown in cellular functional levels throughout the body. Where this could possibly be of the utmost importance is in the red blood cells where their sole source of energy is the ATP dependant process of glycolysis. Our studies revealed that the red blood cells take up 5'-AMP readily. In turn, the increased intracellular 5'-AMP will force the adenylate equilibrium to form 2ADP utilizing the cellular ATP pool decreasing the level of intracellular ATP levels. Reducing ATP levels will affect glycolysis at two critical steps where ATP is required. The first is at hexokinase where glucose is converted to glucose 6 phosphate. The second is at phosphofructokinase which converts fructose 6 phosphate to fructose 1,6 biphosphate. The observed rise in glucose, glucose 6-phosphate and fructose 6-phosphate is consistent with a stalling of glycolysis at hexokiase and at phosphofructokinase. Metabolic modeling studies have shown that a decrease in ATP production in the RBC will lead to an increased production of 2,3 biphosphoglycerate causing an increase in oxygen's disassociation with hemoglobin. This is consistent with the phase I profile where a rapid decline in oxygen consumption was observed and is independent of the animal's core body temperature and the dosage of 5'-AMP given. In turn, the decrease in oxygen transport will slow down peripheral cell's metabolic needs by reducing oxygen availability. When the body cools, the demand for oxygen also decreases thereby creating and interdependent loop between metabolic need and

oxygen availability. This could explain the phase II profile where oxygen consumption was linked to the animal's body temperature.

These investigations of how the arousal process can be controlled have revealed several interesting findings. If no fluid is provided, very few mice will stay in the deep hypometabolic state beyond 12h under a permissive arousal temperature (T_a of 15°C). We classified arousal before 12h as early arousal for the remainder of this discussion. If arousal is suppressed by lowering the T_a to 13.2°C, then the mortality rate is increased if arousal from deep hypometabolism is blocked for more than 12h. When saline is provided during the DH state under T_a of 15°C, about 40% of the mice would be early arousals but, significantly, about 60% of the mice can remain in DH longer than 12h. It was observed that freshly bought mice that have not seen 5'-AMP previously have an early arousal rate of 15% compared with 50% for reused mice. Given that the reused mice were typically heavier, we investigated whether there was a relationship between the size of the mice and early arousal. Plotting the weight of the mice against its arousal time revealed a relationship between early arousal and the size of the animals. However, some of the smaller mice also displayed early arousal, suggesting that weight and size alone cannot explain early arousal and endogenous factor(s) are likely involved in the triggering of arousal.

While giving saline alone suppressed arousal for about half the mice in our study group (combining new and reused mice), mortality rate is still about 40% of those that stayed in DH longer than 12h. Whether optimizing the amount of fluid given over a 24h period could reduce this mortality rate is unclear. Rather, it was hypothesized that fluid alone is not enough to prevent death in mice that remain in DH longer than 12h. Through metabolic studies that have identified changes in metabolites, a series of experiments were

explored to test the usefulness of glucose (highly elevated in DH) and pyruvate (highly decreased in DH) in suppressing arousal. Giving glucose to mice (20mg/inj) had a 31% early arousal rate and about a 64% survival rate for the mice that stayed in DH longer than 12h. This was not much better than giving saline to the mice. It indicated that glucose is neither a major regulator of early arousal nor a metabolite that could improve survival when DH was prolonged.

Pyruvate is slightly better than glucose (28% versus 31% early arousal rate) at suppressing early arousal. About 67% of the mice given pyruvate that stayed in DH longer than 12h survived; this number too was right around that of glucose. The outcomes for pyruvate, glucose and saline were very similar in suppression of early arousal and overall survival rate after DH longer than 12 h.

To date, the most promising metabolite that showed a major differential outcome in suppression of early arousal and decreased mortality of mice in DH after 12h is the amino acid arginine. Mice given arginine have the lowest level of early arousal (14%) and mortality rate of mice in DH longer than 12h (6%). In fact, we have 3 mice that were in DH for 30h and they all survived without apparent neurological damage. We have attributed this success to arginine's role in the urea cycle; however, arginine is also a key component in creatine production and in nitric oxide production (**Walker, 2009**). The urea cycle and nitric oxide pathway actually have many other intermediates and enzymes in common other than just arginine (**M.A. Neill et al., 2009**). The urea cycle and NO pathway share arginine, citrulline, argininosuccinate, aspartate, argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) as well as a transporter, Citrin. Whereas the urea cycle has parts in both the cytoplasm and the mitochondria, NO production takes place solely in the

cytoplasm. From our current studies, it appears that the urea cycle is the key player in the survival and long term DH of our mice. However since the urea cycle and the NO pathway are intertwined and seem to regulate each other (**M.A. Neill et al., 2009**), we will have to examine both pathways to clarify their contribution to suppressing arousal. It has been hypothesized that short term regulation of the urea cycle takes place in the mitochondria at the enzyme CPS1 (Carbamoyl Phosphate Synthetase I), which is responsible for taking the ammonia groups out of the mitochondria and transferring them into the urea cycle when carbamoyl phosphate combines with ornithine (**Walker, 2009**). This enzyme has also been shown to be down regulated in hibernating bears (**Fedorov et al., 2009**). Since arginine plays a role not only directly in the urea cycle but also in the formation of a cofactor, NAGS, of carbamoyl phosphate production (**Walker, 2009**), if arginine is reduced as it is in our model, it would shut down both the shuttling out of ammonia from the mitochondria and the production of urea. In the future, there are proposed studies to investigate enzymatic genes of the urea cycle to determine which, if any, genes are being down regulated and/or up regulated throughout our DH process. There have been studies that show supplemental arginine, in instances of increased arginase activity that had led to human diseases such as asthma and pulmonary arterial hypertension, has been beneficial for treatment (**Morris et al., 2008**). There are other treatments besides arginine that have been effective in treating urea cycle deficiencies via alternate pathways (**Walker, 2009**), but it has been suggested that arginine may have advantages over other drugs (**Jobgen et al., 2006**). For future investigations, we plan to inject these drugs (Sodium Benzoate and/or Sodium Phenybutarate), or citruline (**Walker, 2009**) as it removes an ammonia group while also getting converted to arginine which could help replace the reduced amount of arginine in

vivo. Such studies, if successful in prolonging arousal, would give support to our current findings that the inability to regulate ammonia is the primary cause for early arousal and mortality if DH is prolonged.

After considering the urea cycle and its role, we must also examine the NO pathway for its redundancy in intermediates and enzymes with the urea cycle. The production of NO has numerous vital biological roles (**Nathan, 1992**) as it has 3 differentially expressed isoforms: the endothelial (eNOS) isoform primarily regulates vascular tone, the inducible (iNOS) isoform is mainly present in inflammatory and infectious conditions, and the neuronal (nNOS) isoform is mainly in neuronal tissue acting as a signaling molecule (**M.A. Neill et al., 2009**). It has also been suggested that NO plays a role in regulating nutrient metabolism in almost all types of mammalian cells and tissues (**Jobgen et al., 2006**) whether it is used in the cell that produces it or whether the NO gets taken up by the red blood cells and transported to distant target cells (**Stamler and Meissner, 2001**). The synthesis of NO requires more inputs than just arginine; it also requires tetrahydrobiopterin (BH₄), NADPH, Ca²⁺, calmodulin, FMN and FAD (**Wu and Morris, 1998**). NADPH and calmodulin are needed to give the NOS isoforms enzymatic activity while the Arginine, BH₄ and heme are all used as promoters and stabilizers (**Wu and Meininger, 2002**). Since NO is a highly reactive free radical species, it can have various effects depending on the concentration (**Jobgen et al., 2006**). At physiological levels, NO stimulates glucose uptake and oxidation, fatty acid oxidation, mitochondrial biogenesis, while enhancing lipolysis and ATP concentration and inhibiting synthesis of glucose, glycogen and fat (**Jobgen et al., 2006**). However if physiological levels are exceeded, it can disrupt the electron transport chain (ETC), resulting in reduced ATP from oxidative phosphorylation and causing an

increase in glycolysis to produce the required ATP. High levels of NO also decrease protein synthesis while increasing amino acid catabolism (**Jobgen et al., 2006**). Since the NO pathway and urea cycle are dependent upon each other, they must have mechanisms in place to regulate one another and provide feedback to make sure the NO pathway does not exceed the physiological levels and become a detriment to the cells. It has been suggested that NO may reduce expression of urea cycle enzymes and urea synthesis from ammonia while inhibiting amino acid oxidation in the liver and increasing the amount of dietary amino acids released into the circulation (**Jobgen et al., 2006**). In order to keep a balance on the NO production, the urea cycle can increase its rate in order to limit necessary substrates like arginine (**M.A. Neill et al., 2009**). These feedback mechanisms appear to be crucial in keeping each other in check. While it has been shown that arginine increases transcription of NOS and NO production (**Jobgen et al., 2006**), if our mice were running out of arginine, it was assumed these pathways would also be shut down in addition to the urea cycle. Supplying arginine has been shown to have numerous effects on both humans and test animals. In diabetic rats, it has been shown to reduce plasma levels of glucose, fatty acids and triglycerides (**Jobgen et al., 2006**) while also reducing fat mass and showing promise in uses for treating metabolic disorder (**Flynn et al., 2002**). In humans, it has been shown to increase insulin sensitivity (**Jobgen et al., 2006**). Therefore, it has been hypothesized that supplementing arginine, and maybe even citruline, might be able to help in treating metabolic disorder (**Jobgen et al., 2006**) as well as potentially saving our mice. These are testable hypothesis for future studies.

In summary, the following is what is believed to be occurring in this hypometabolic model. After induction by injection of 5'-AMP and placement at $\sim 15^{\circ}\text{C}$ T_a the urea cycle is

slowed and possibly halted due to a depletion of arginine levels. Therefore, the animals' ability to remove ammonia from the body is deficient, leading to a build-up of free ammonia in the blood. The spontaneous arousal that is seen is probably due to a critical limit in ammonia levels in the blood acting as a signal to the animal to arouse or die. Therefore, by supplementing the various other metabolites, we were not addressing the critical problem that existed while the animals were in DH and that is why the other metabolites did not work. Only once the urea cycle's importance and in vivo arginine levels were addressed did the mice start to prolong their time down in DH safely and consistently.

For future experiments, there are a few directions to test the various ideas to support our current data. The urea cycle enzymes should be compared between DH, euthermia and arousal to see if there are any changes. Citruline, Sodium Benzoate and/or Sodium Phenybutarate can be substituted for arginine to evaluate if it is in fact the urea cycle (**Walker, 2009**). If these treatments have similar effects to the arginine, that would point to the urea cycle as being deficient in our mouse model. We can also take blood ammonia levels during a time course throughout the DH process to see if there is an accumulation of free ammonia as time elapses. Unlike the previous data which measured blood nitrogen levels, this measurement would be solely for free ammonia and not total nitrogen levels. To test whether the NO pathway is the reason for the arousal and the lack of long-term survival, previous studies have used NO donors to treat NO deficiencies caused by an arginine shortage (**Morris et al., 2008**). By giving one or more of these donors to our mice, if the outcome is similar to the arginine experiments, we could suggest that the shortage of arginine and the consequences of our DH state were impacting the NOS pathways and not the urea cycle.

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