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## Physiological implications of natural versus induced arousal from torpor

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PHYSIOLOGICAL IMPLICATIONS OF NATURAL VERSUS INDUCED  
AROUSAL FROM TORPOR

by

Jenifer C. Utz

Bachelor of Science  
University of Nevada, Las Vegas  
2004

A dissertation submitted in partial fulfillment of  
the requirements for the

**Doctor of Philosophy in Biological Sciences  
School of Life Sciences  
College of Science**

**Graduate College  
University of Nevada, Las Vegas  
December 2010**

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## THE GRADUATE COLLEGE

We recommend the dissertation prepared under our supervision by

**Jenifer C. Utz**

entitled

### **Physiological Implications of Natural Versus Induced Arousal from Torpor**

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### **Doctor of Philosophy in Biological Sciences**

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**December 2010**

## ABSTRACT

### **Physiological Implications of Natural Versus Induced Arousal from Torpor**

by

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During the hibernation season, animals oscillate between periods of torpor and periods of interbout arousal (IBA). During torpor, body temperature is often near 0° C and metabolism is severely depressed. Oxygen consumption, a proxy for aerobic metabolism, may fall to 1% of active values. Many physiological processes including cardiovascular, respiratory, and cellular functions nearly cease. During the IBA, euthermic body temperature is restored and most systemic and cellular processes function at fully active levels. The transition period between these two physiologically dissimilar states is called arousal.

The rate of rewarming (RRW) during arousal was previously expected to progressively increase until a euthermic set point was approached. However, my data contradict this expectation. I monitored the body temperature ( $T_b$ ) of golden-mantled ground squirrels (*Spermophilus lateralis*) housed at 4, 8, 12, and 16° C during natural arousals. The various housing temperatures facilitate manipulation of torpid  $T_b$ , since torpid  $T_b$  is usually within 1° C of ambient temperature ( $T_a$ ). The maximum RRW, the time required to reach a maximum RRW, and the relative time index all demonstrated negative relationships with  $T_a$ . The  $T_b$  corresponding to maximal RRW demonstrated a positive relationship with  $T_a$ .

One parameter was independent of ambient temperature. Squirrels reached maximal RRW when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$ . These data suggest that arousal is more constrained than expected and that both time and temperature influence the RRW.

Much hibernation research involving arousal has been conducted on animals that were induced to arouse prematurely. Natural arousal from torpor occurs spontaneously with highly predictable timing. However, animals can also be induced to arouse prematurely in response to various disturbances. While many investigations have used natural and induced arousals synonymously, direct comparisons of these two types of arousal have been lacking. I addressed the question of whether natural and prematurely induced arousals are the same. I compared the effects of ambient temperature on the dynamics of natural versus induced arousals. Arousal duration, maximum rewarming rate, and the variance associated with increases in body temperature increased during induced arousals. Prematurely inducing arousal also decreased the duration of the interbout aroused (IBA) period, and the responsiveness to the induced arousal stimulus was influenced by torpor duration.

The metabolic cost of natural versus induced arousal from torpor was also investigated. Metabolic activity was estimated through indirect calorimetry and assays of metabolites in blood plasma. Although initial rates of oxygen consumption were comparable for natural and induced arousal, initial rates of carbon dioxide production and respiratory quotient values were significantly reduced during induced arousal. Plasma lactate levels were significantly elevated

during induced arousal whereas glucose and free fatty acids levels were similar. Previous work has indicated oxidative stress and elevated antioxidant defenses during hibernation. However, the effectiveness of these defenses has not been as well characterized. Indicators of oxidative damage to lipids and proteins in heart, liver, kidney, and brain tissues were measured to investigate potential oxidative damage to cellular macromolecules. Lipid peroxidation products and protein carbonyl levels were low in all tissues for both types of arousal. Thus, hibernators appear to be well protected against oxidative damage.

Arousal was once regarded as a sole effort to rewarm as quickly as possible, thereby facilitating conservation of energy stores. However, data indicate that arousal is a more constrained process where animals regularly experience less than maximal rewarming rates. Further, prematurely inducing arousal alters key aspects of the rewarming process as well as metabolic activity. Considering these differences, I recommend that careful consideration be given to experimental design and data interpretation when arousing animals are utilized.

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## CHAPTER 1

### INTRODUCTION

#### Hibernation Overview

Metabolic depressions like hibernation are a common physiological response to challenging environmental conditions such as cold temperatures and limited food supplies (Carey et al., 2003; Guppy and Withers, 1999; Storey and Storey, 1990). Hibernation is a dynamic physiological process wherein animals cycle between periods of torpor, characterized by low body temperature ( $T_b$ ) and severely depressed metabolic rates and periods of interbout arousal, characterized by euthermic  $T_b$  and high metabolic rates (Figure 1; from Utz et al., 2007).

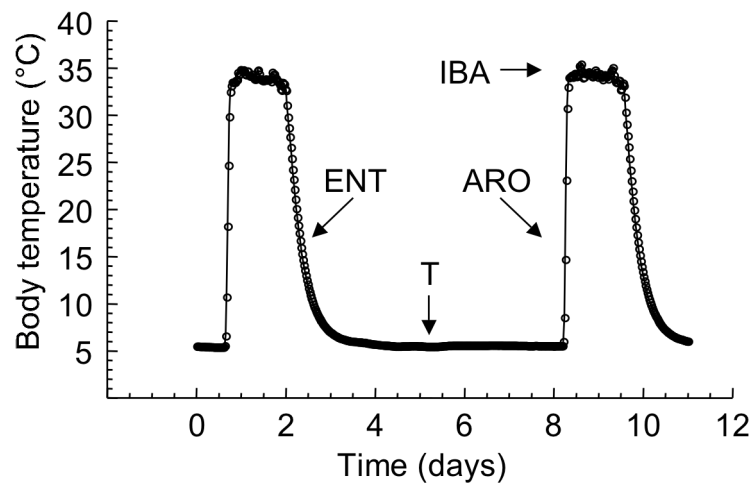


Figure 1. Body temperature ( $T_b$ ) in a hibernating ground squirrel.  $T_b$  was measured every 30 minutes for an animal housed at 4°C. During entrance (ENT) into torpor,  $T_b$  slowly decreases from euthermic (~36°C) to near ambient temperature ( $T_a$ ). Torpor (T) lasts approximately 1 week, and  $T_b$  is maintained within 1°C of  $T_a$ . Animals rapidly rewarm to euthermic  $T_b$  during arousal (ARO). Euthermic  $T_b$  is maintained during the interbout arousal (IBA).

Ground squirrels are exceptional hibernators, spending 6 to 9 months in hibernation each year (Frank et al., 2008). During the hibernation season, an animal typically experiences 15 bouts of torpor. The length of each bout may range from 1 to 3 weeks and is influenced by species, ambient temperature, and time of year (Buck and Barnes, 2000; French, 1982; Wang, 1979; Geiser and Baudinette, 1990; Twente et al., 1977).

During torpor, ground squirrels can experience oxygen consumption rates as low as 1% of active rates, and body temperatures as low as  $-2.9^{\circ}\text{C}$  have been recorded (reviewed in Wang and Lee, 2000; Barnes, 1989). The effects of this drastically reduced energy supply can be seen in the depression of many physiologically significant processes. Transcription, translation, mitosis, and mitochondrial respiration are all depressed during torpor (Carey et al., 2003; van Breukelen and Martin, 2001; 2002a and b). In addition to these cellular processes, the activity of entire organ systems such as the cardiovascular, renal, and immune systems are also depressed during torpor (Zatzman, 1984; McArthur and Milsom, 1991; Prendergast et al., 2002).

Periodically, animals spontaneously exit torpor and rewarm to euthermic  $T_b$ ; this rewarming process is called arousal. During the euthermic period of interbout arousal, the processes of transcription, translation, mitosis, and mitochondrial respiration are restored; organ system functions that are depressed during torpor also resume (reviewed in Carey et al., 2003). Animals typically spend 12 to 24 hours in the interbout aroused state before spontaneously entering another bout of torpor.

It seems likely that a hibernator experiences many physiological stressors during the transitions between the torpid and interbout aroused states. Several mismatches occur during these transition periods i.e. not all processes are depressed or reengaged concurrently. For example, during entrance into torpor heart rates drop prior to any substantial decrease in  $T_b$  (Milsom et al., 1999). This mismatch between supply mechanisms and tissue demand may result in reduced blood supply to tissues that are still metabolically active. However, some of the physiological insults associated with entrance into torpor may be mitigated due to the fact that squirrels gradually enter torpor over a period of approximately 24 hours.

In contrast, arousal from torpor is an extremely rapid event. Hibernators rewarm from near freezing temperatures to euthermia in a mere 2 to 3 hours. Maximum rewarming rates for individual ground squirrels can approach  $1.5^{\circ}\text{C}$  per minute (Utz et al., 2007). The process of arousal likely involves numerous physiological mismatches. The most noticeable incongruence is between increases in oxygen consumption,  $T_b$ , and the restoration of blood flow. For example in arousing thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), blood flow posterior to the diaphragm is restricted until the thoracic temperature is  $\sim 25^{\circ}\text{C}$  (Bullard and Funkhouser, 1962). In arousing arctic ground squirrels (*Spermophilus parryi*), oxygen consumption peaks when abdominal  $T_b$  is  $\sim 5^{\circ}\text{C}$  (Tøien et al., 2001). During arousal, animals must reconcile the mismatches between  $\text{O}_2$  consumption, blood flow, and changing body temperature in the midst of rapidly changing metabolic demand as

processes like transcription, protein metabolism, mitochondrial respiration, and mitosis are restored. Instances of mortality hint at the cost of repeatedly experiencing such physiological hardships. As many as 70% of juvenile Belding's ground squirrels (*Spermophilus beldingi*) die during their first hibernation season in the wild, and adult mortality ranges from 36 to 39% for a given year (Sherman and Morton, 1984).

Although much hibernation research has focused upon understanding the metabolic depression of torpor, I contend that a better understanding of arousal is required.

### Experimental System

Although the hibernation phenotype is widely distributed across the mammalian phylogeny, much experimental research has been conducted in sciurid rodents (Carey et al., 2003). Ground squirrels are exceptional hibernators; during torpor they can experience body temperatures below zero and function at 1% of active oxygen consumption rates (Barnes, 1989; Wang, 1979; Wang and Lee, 2000). Additionally, populations are widely distributed. There is a solid collection of hibernation literature for ground squirrels, and they experience predictable torpor bouts when housed in environmental chambers.

All experiments were conducted with golden-mantled ground squirrels (*Spermophilus lateralis*) at the University of Nevada, Las Vegas. Animals were live trapped from local populations in the Southern Nevada, Southern California, or Southern Utah. Prior to the beginning of the hibernation season, temperature

sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) were surgically implanted into the abdominal cavity. These radiotelemeters allow for precise measurement of  $T_b$  throughout the hibernation season. Unless otherwise noted, animals were housed in an environmental chamber at an ambient temperature of 4°C during the hibernation season; 8 days is a typical torpor bout length under these conditions (personal observation). The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures related to the experiments discussed in this dissertation.

### Historical Perspectives and Rationale for this Work

Although hibernation physiology has been scrutinized for over a century, we are just beginning to appreciate that we must consider more than a bipartite physiology. Hibernating animals were initially regarded as being in one of two dissimilar states, torpid or euthermic. In recent years, we have begun to understand that not all torpid animals are the same. The internal workings of an animal in the first days of torpor are different than for an animal approaching the end of a torpor bout. For example, the distribution of the ribosome pool and the types of transcripts associated with ribosomes differ markedly between early and late torpor (van Breukelen and Martin, 2001; Pan and van Breukelen, unpublished data). In addition to changes in cellular physiology, important differences are also evident at the level of systemic function. Cardiovascular and respiratory system functions vary across a torpor bout (Steffen and Riedesel, 1982; Milsom et al., 1999; 2001). Changes in heart rate during entrance and



early torpor are mediated via the parasympathetic nervous system, whereas arousal from torpor is mediated via sympathetic tone (Milsom et al., 1999).

Increased awareness of the spectrum of physiological activity represented throughout a torpor bout necessitates a more careful consideration of the hibernation literature. In particular, greater consideration must be given to arousal, the transition from torpor to the interbout aroused state. At a fundamental level, heat is generated as a byproduct of biochemical reactions. As temperature increases, the rate of most biochemical reactions also increases (Withers, 1992). Therefore, the rate at which animals rewarm has been expected to progressively increase as body temperature increases until a euthermic set point is approached. Further, models have been presented for animals to rewarm as rapidly as possible (Stone and Purvis, 1992). Rewarming quickly minimizes heat loss to the environment and conserves an animal's fuel stores. Figure 2 presents an idealized arousal according to the aforementioned models. Although these models are sensible, this depiction of arousal is inaccurate. Animals do not rewarm as quickly as possible (Figure 11), and the rate of rewarming decreases long before a euthermic set point is approached (Figures 3,5,6, and 7).

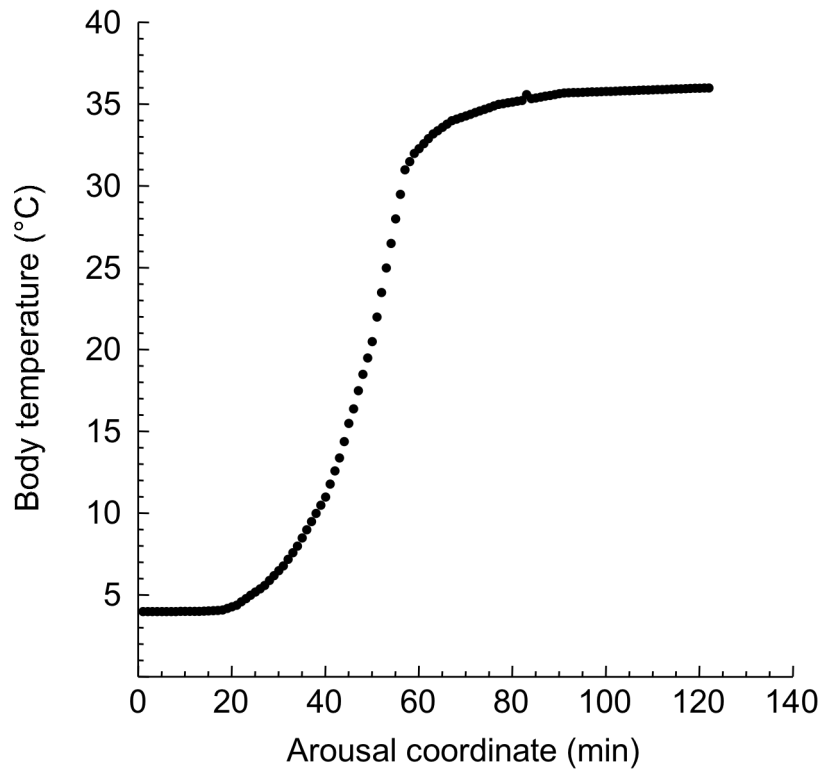


Figure 2. Previous model of rewarming. An idealized depiction of progressive increases in the rate of rewarming until a euthermic set point is approached.

Recognizing arousal as a multifaceted, regulated process necessitates consideration of how an animal arouses from torpor. Although the occurrence of a natural arousal from torpor can be reliably predicted, most hibernation research has utilized animals that were induced to arouse prematurely (Table 1; Twente and Twente, 1965a and b; Twente et al., 1977). Premature arousals occur in response to rapid changes in body temperature, loud noises, or physical disturbances (Twente and Twente, 1965a; 1968; Pengelley and Fisher, 1968). Table 1 presents a random sample of 50 publications investigating arousal; 9 records utilized natural arousals, 35 records utilized induced arousals, and the nature of the arousal was unclear for the remaining 6 records. Of the 35 studies

utilizing induced arousals, only 8 represent intentionally induced arousals i.e. prematurely inducing arousal was part of the experimental design. Experimental procedures, lack of knowledge regarding arousal, or convenience for the investigator are the primary sources of the remaining prematurely induced arousals.

To my knowledge, only one study has directly compared natural and induced arousals (Tähti and Soivio, 1977; 1978). Tähti and Soivio found that increases in oxygen consumption, heart rate, and blood pressure occur more rapidly during induced arousal (1978). Further, the relative timing of changes in ventilation, heart rate, and blood pressure were altered during induced arousal (1977). These two articles by Tähti and Soivio have been referenced in the literature only 5 times (personal observation). Studies utilizing aroused animals have primarily centered upon understanding the energetics of hibernation, cardiovascular, ventilatory and circulatory changes, metabolism and metabolic fuels, or stress (Table 1). Although many investigations have utilized aroused animals, the actual process of arousal has not been well described (Table 1).

My work provides a more thorough characterization of arousal itself. I examined temporal and temperature effects on natural arousal to better understand putative regulatory constraints affecting this complex physiological process. The rate of rewarming (RRW) during arousal was previously expected to simply increase as a function of time until a euthermic set point was approached (Figure 2).

Table 1. Categorization of 50 Publications Utilizing Aroused Hibernators

Item	Year	Arousal Type	Title
1	1950	induced	Circulatory changes during the process of arousal in the hibernating hamster
2	1959	induced	Notes on hibernation and awakening in arctic ground squirrels
3	1962	induced	Estimated regional blood flow by rubidium 86 distribution during arousal from hibernation
4	1965	unclear	Oxidation of glucose-U-C <sup>14</sup> and palmitate-1-C <sup>14</sup> by liver, kidney, and diaphragm from hamsters in cold exposure and hibernation
5	1965	natural	Effects of core temperature upon duration of hibernation of <i>Citellus lateralis</i>
6	1968	induced	Estimated heat contribution of brown fat in arousing ground squirrels ( <i>Citellus lateralis</i> )
7	1968	induced	Carbon dioxide fixation during hibernation and arousal from hibernation
8	1968	induced	Plasma free amino acids in hibernation and arousal
9	1968	intentionally induced	Ability of the ground squirrel, <i>Citellus lateralis</i> , to be habituated to stimuli while in hibernation
10	1968	natural	Simultaneous recording of core temperature and energy expenditure during the hibernation cycle of <i>Mesocricetus auratus</i>
11	1968	intentionally induced	Progressive irritability of hibernating <i>Citellus lateralis</i>
12	1969	intentionally induced	Alteration of activity rhythm after induced arousal from hibernation
13	1970	intentionally induced	Sliding set points for body weight in ground squirrels during the hibernation season
14	1970	induced	Regional distribution of blood flow in the bat ( <i>Myotis lucifugus</i> ) during arousal from hibernation
15	1971	unclear	Circulatory patterns of hibernators
16	1974	induced	Temporal changes in AA catabolism during arousal from hibernation in the golden-mantled ground squirrel
17	1975	induced	Comparison of regional blood distribution in big brown bat during torpor (summer), hibernation (winter), and arousal
18	1977	intentionally induced	Respiratory and circulatory differences between induced and spontaneous arousals in hibernating hedgehogs
19	1977	natural	Regulation of arousal from hibernation by temperature in three species of <i>Citellus</i>

20	1978	intentionally induced	Comparison of induced and spontaneous arousals in hibernating hedgehogs
21	1979	induced	Effect of cerebral injection of biogenic amines during arousal from hibernation
22	1982	induced	Concentrations of lactate and pyruvate and temperature effects on lactate dehydrogenase in the tissues of the big brown bat during arousal from hibernation
23	1982	intentionally induced	Effect of temperature on the duration of arousal episodes during hibernation
24	1982	natural	Urine concentration by an undisturbed, naturally arousing hibernator ( <i>S. lateralis</i> ): water balance implications
25	1982	natural	Pulmonary ventilation and cardiac activity in hibernating and arousing golden-mantled ground squirrels
26	1983	unclear	Body temperature, heart rate and oxygen consumption of normothermic and heterothermic western jumping mice ( <i>Zapus princeps</i> )
27	1987	unclear	Glucose oxidation by adipose tissue of the edible dormouse during hibernation and arousal: effect of insulin
28	1988	induced	Cardiac arrhythmias during arousal from hibernation in 3 species of rodents
29	1988	induced	The influence of ambient temperature on the rate of arousal and behavioral changes during arousal from hibernation in the 13-lined ground squirrel
30	1988	induced	Time-course of blood acid-base state during arousal from hibernation in the European hamster
31	1993	intentionally induced	Induction of arousal in hibernating European hamsters by vasopressin infusion on the lateral septum
32	1997	induced	Rates of rewarming, heart, and respiratory rates and their significance for oxygen transport during arousal from torpor in the smallest mammal, the Etruscan shrew ( <i>Suncus etruscus</i> )
33	1999	induced	Arousal from torpor in the Chilean mouse-opossum ( <i>Thylamys legans</i> ): does non-shivering thermogenesis play a role?
34	1999	induced	Reversible depression of oxygen consumption in isolated liver mitochondria during hibernation
35	2000	unclear	Hibernation induces oxidative stress and activation of NF-KB in ground squirrel intestine
36	2001	induced	Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels

37	2002	induced	Activation of stress signaling molecules in bat brain during arousal from hibernation
38	2002	unclear	Ubiquitin conjugate dynamics in the gut and liver of hibernating ground squirrels
39	2003	induced	Brown fat and nonshivering thermogenesis in the gray mouse lemur ( <i>Microcebus murinus</i> )
40	2003	induced	State-dependent regulation of cortical blood flow and respiration in hamsters: response to hypercapnia during arousal from hibernation
41	2004	induced	Comparison of surface temperature in 13-lined ground squirrel and yellow-bellied marmot during arousal from hibernation
42	2004	natural	Increased heat loss affects hibernation in golden-mantled ground squirrels
43	2004	natural	Patterns of body temperature variation and torpor in the numbat, <i>Myrmecobius fasciatus</i> (Marsupialia: Myrmecobiidae)
44	2005	induced	Sympathetic alpha-adrenergic regulation of blood flow and volume in hamsters arousing from hibernation
45	2005	induced	Arousal from hibernation alters contextual learning and memory
46	2007	natural	Temporal and temperature effects on the maximum rate of rewarming from hibernation
47	2008	induced	Simultaneous measurement of brain tissue oxygen partial pressure, temperature, and global oxygen consumption during hibernation, arousal and euthermia in non-sedated non-anesthetized Arctic ground squirrels
48	2008	natural	Rewarming rates and thermogenesis in hibernating echidnas
49	2009	induced	Energetics of arousal episodes in hibernating Arctic ground squirrels
50	2009	induced	Physiological oxidative stress after arousal from hibernation in Arctic ground squirrels

Table 1 references correspond to the item number. (1) Chatfield and Lyman (2) Muscaccia and Hamilton (3) Bullard and Funkhouser (4) Baumber and Denyes (5) Twente and Twente (6) Horwitz et al. (7) Klain and Whitten (8) Klain and Whitten (9) Pengelley and Fisher (10) Robertson et al. (11) Twente and Twente (12) Popovic and Kent (13) Mrosovsky and Fisher (14) Rauch and Hayward (15) Wells (16) Whitten et al. (17) Rauch and Beatty (18) Tähti and Soivio (19) Twente et al. (20) Tähti and Soivio (21) Glass and Wang (22) Cuddihoe and Fonda (23) French (24) Muchlinski and Carlisle (25) Steffen and Riedesel (26) Cranford (27) Castex et al. (28) Eagles et al. (29) Hintz and Hall (30) Malan et al. (31) Hermes et al. (32) Fons et al. (33) Opazo et al. (34) Martin et al. (35) Carey et al. (36) Tøien et al. (37) Lee et al. (38) van Breukelen and Carey (39) Genin et al. (40) Osborne and Hashimoto (41) Phillips and Heath (42) Kauffman et al. (43) Cooper and Withers (44) Osborne et al. (45) Weltzin et al. (46) Utz et al. (47) Ma and Wu (48) Nicol and Andersen (49) Karpovich et al. (50) Orr et al.

However, my data contradict this expectation. I monitored the body temperature ( $T_b$ ) of golden-mantled ground squirrels (*Spermophilus lateralis*) housed at 4, 8, 12, and 16° C during natural arousals. The various housing temperatures facilitate manipulation of torpid  $T_b$ , since torpid  $T_b$  is usually within 1° C of ambient temperature ( $T_a$ ). The maximum RRW, the time required to reach a maximum RRW, and the relative time index all demonstrated negative relationships with  $T_a$ . The  $T_b$  corresponding to maximal RRW demonstrated a positive relationship with  $T_a$ . One parameter was independent of ambient temperature. Squirrels reached maximal RRW when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$ . These data suggest that arousal is more constrained than expected and that both time and temperature influence the RRW.

Further, I examined a long ignored question: Are natural and induced arousals the same? My characterization of natural arousal facilitates a direct comparison with prematurely induced arousal. I compared the effects of ambient temperature on the dynamics of natural versus induced arousals. Arousal duration, maximum rewarming rate, and the variance associated with increases in body temperature increased during induced arousals. Prematurely inducing arousal also decreased the duration of the interbout aroused (IBA) period, and the responsiveness to the induced arousal stimulus was influenced by torpor duration.

The metabolic cost of natural versus induced arousal from torpor was also investigated. Metabolic activity was estimated through indirect calorimetry and

assays of metabolites in blood plasma. Although initial rates of oxygen consumption were comparable for natural and induced arousal, initial rates of carbon dioxide production and respiratory quotient values were significantly reduced during induced arousal. Plasma lactate levels were significantly elevated during induced arousal whereas glucose and free fatty acids levels were similar. Previous work has indicated oxidative stress and elevated antioxidant defenses during hibernation. However the effectiveness of these defenses has not been as well characterized. Indicators of oxidative damage to lipids and proteins in heart, liver, kidney, and brain tissues were measured to investigate potential oxidative damage to cellular macromolecules. Lipid peroxidation products and protein carbonyl levels were low in all tissues for both types of arousal. Thus, hibernators appear to be well protected against oxidative damage.

Arousal was once regarded as a sole effort to rewarm as quickly as possible, thereby facilitating conservation of energy stores. However, my data indicate that arousal is a more constrained process where animals regularly experience less than maximal rewarming rates. Both time and body temperature influence the rate of rewarming during natural arousal. Further, prematurely inducing arousal alters key aspects of the rewarming process including maximum rewarming rates and arousal duration. Initial metabolic activity is also altered during induced arousal; rates of carbon dioxide production are significantly less than those associated with natural arousal. Considering these differences, I recommend that careful consideration be given to experimental design and data interpretation when arousing animals are utilized.



## CHAPTER 2

### CHARACTERIZATION OF NATURAL AROUSAL

#### Abstract

During hibernation animals oscillate from near ambient ( $T_a$ ) to euthermic body temperatures ( $T_b$ ). As animals arouse, the rate of rewarming (RRW) might be expected to simply increase as a function of time. We monitored the  $T_b$  of golden-mantled ground squirrels (*Spermophilus lateralis*) housed at 4, 8, 12, and 16° C during natural arousals. The maximum RRW, the time required to reach a maximum RRW, and the relative time index all demonstrated negative relationships with  $T_a$ . The  $T_b$  corresponding to maximal RRW demonstrated a positive relationship with  $T_a$ . Squirrels reached maximal RRW when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$ . These data suggest that arousal is more constrained than expected and that both time and temperature influence the RRW.

#### Statement of Previously Published Work

The contents of this chapter were previously published in the Journal of Thermal Biology (Utz et al., 2007). The copyright release is included as Appendix 1. Jenifer Utz is the first author for the publication; contributing authors include Vanja Velickovska, Anastacia Shmereva, and Frank van Breukelen. All work was conducted in and financially supported by the van Breukelen laboratory. Jenifer Utz solely performed the data collection and the initial writing of the manuscript. Jenifer Utz and Frank van Breukelen jointly conducted the statistical analysis and

edited the manuscript. Jenifer Utz and Vanja Velickovska participated in animal care responsibilities. Anastacia Shmereva was included in recognition of time spent on other projects in the van Breukelen laboratory.

## Introduction

For many mammals, the onset of winter presents formidable challenges in the form of cold temperatures and scarce food supply. Hibernation provides a means of evading many of these challenges and is a strategy employed throughout the mammalian class (see reviews by van Breukelen and Martin, 2002a; Carey et al., 2003). Hibernators experience tremendous energetic savings as a result of severely reduced metabolic rates. Torpid metabolic rates may be as low as ~ 1% of euthermic rates in ground squirrels (reviewed in Wang and Lee, 2000). The effects of this drastically reduced energy supply can be seen in the depression of many physiologically significant processes during torpor. Transcription, translation, mitosis, and mitochondrial respiration are all depressed during torpor (Carey et al., 2003; van Breukelen and Martin, 2001; 2002a and b and references therein).

However, hibernators are not always torpid. Across a hibernation season there are dynamic fluctuations in body temperature ( $T_b$ ) and physiological activity as they oscillate from near ambient ( $T_a$ ) to euthermic body temperature (Figure 1). The duration of torpor phases can change with species,  $T_a$ , and progression through the hibernation season (Buck & Barnes, 2000; French, 1982; Carey et al., 2003). As ground squirrels enter into torpor,  $T_b$  falls slowly and normally

stabilizes within  $\sim 1^\circ \text{C}$  of  $T_a$ . When challenged with temperatures below freezing, animals actively thermoregulate to maintain  $T_b$  near  $0^\circ \text{C}$  (Buck and Barnes, 2000). The length of a typical torpor bout for golden-mantled ground squirrels (*Spermophilus lateralis*) housed at  $4^\circ \text{C}$  is  $\sim 8$  days (personal observation). Torpor bouts are regularly interrupted by arousals wherein animals rapidly rewarm to euthermic levels ( $T_b$  is  $\sim 36^\circ \text{C}$ ). Upon completion of the interbout arousal (IBA), animals spontaneously reenter another torpor bout.

During arousal, animals may increase  $T_b$  by  $> 30^\circ \text{C}$  within a period of 2 to 3 hours, which represents a substantial quantity of heat generation. Given the general relationship between biological reactions and temperature, a simple expectation might be that as animals arouse from torpor and rewarm to euthermic  $T_b$ , an increasing number of biochemical processes will be restored. Biological reactions release heat, and their rates are usually temperature dependant (Withers, 1992). Thus, the rate of rewarming (RRW) might be expected to simply increase as a function of time during the arousal process. To better understand heat gains during arousal, golden-mantled ground squirrels were implanted with temperature sensitive radiotelemeters and housed at 4, 8, 12, and  $16^\circ \text{C}$ . Since torpid  $T_b$  is generally within  $1^\circ \text{C}$  of  $T_a$ , the various housing temperatures allow for modulation of torpid  $T_b$ . All squirrels were allowed to proceed through natural undisturbed arousals.  $T_b$  was recorded; relationships between  $T_a$ , RRW, time to reach maximal RRW, and  $T_b$  at maximal RRW were investigated.

## Materials and Methods

### Animals

Golden-mantled ground squirrels (*Spermophilus lateralis*) were live-trapped from the Southern California/Southern Nevada area. Prior to the beginning of the hibernation season, temperature sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) were surgically implanted in the abdominal cavity. Animals were housed in an environmental chamber at 4, 8, 12, and 16° C. Body temperature was recorded every minute. The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures.

### A Relative Definition of Arousal

It has been noted that the time required to complete an arousal changes with  $T_a$  (i.e. it takes longer to rewarm from 4° C to ~ 36° C than from 16° C to ~ 36° C; French, 1982). In order to compare arousal dynamics across varying  $T_a$ , the initiation and termination of an arousal must be clearly defined.  $T_b$  was recorded every minute. The first derivative (FD) and its associated standard deviation (SD) for temperature as a function of time were calculated. The onset of arousal was defined as the point where the instantaneous rate of rewarming (RRW) exceeded the  $FD \pm 3 SD$  (threshold rate; TR) for a 10-minute period of consistent  $T_b$ . The first  $T_b$  measurement with a  $FD > TR$  was defined as the onset of arousal. The onset of arousal was typically associated with a rate of rewarming of  $0.02^\circ \text{C} \cdot \text{min}^{-1}$ . The termination of arousal was defined as the first point at which the instantaneous rate was  $< TR$  for the following 5 min.

### Calculations and Statistical Analyses

For each  $T_a$ , arousals from 5 animals were examined. A mean value was used in analysis of rewarming rates. Mean maximum rate of rewarming was calculated for each animal from the 5 highest RRW values for a given arousal. The period of time between the onset of arousal and the single highest RRW value was used for analysis of the time required to attain maximum rewarming rate. The relative time index is the time to reach maximum RRW divided by the arousal duration. The  $T_b$  at which maximum RRW occurs was investigated; the 5 highest RRW values within a single arousal were identified, and the 5 corresponding  $T_b$  values were used to calculate a mean  $T_b$ . Mean  $T_b$  was also used to calculate the temperature gain index wherein it was divided by the difference in  $T_b$  between the initiation and termination of arousal. When appropriate, data were subjected to regression analyses. Statistical significance was indicated when  $p < 0.05$ .

## Results

### The Dynamics of a Typical Torpor Bout

The body temperature during a typical torpor bout for golden-mantled ground squirrels is depicted in Figure 1. Changes in body temperature during arousal from torpor for an animal housed at 4, 8, 12, and 16° C were analyzed (Figure 3a). A pattern of a relatively slow increase in  $T_b$ , followed by a faster increase in  $T_b$ , and finally an additional period characterized by a slow increase in  $T_b$  was noted for all animals at all ambient temperatures. The onset of arousal was

typically marked by instantaneous rates of rewarming of  $0.02^{\circ}\text{C} \cdot \text{min}^{-1}$  (Figure 3b). During early arousal, the RRW progressively increases and may be as high as  $1.4^{\circ}\text{C} \cdot \text{min}^{-1}$ . Interestingly, the RRW begins to decrease during mid arousal despite as much as an additional  $25^{\circ}\text{C}$  yet to be gained in  $T_b$ .

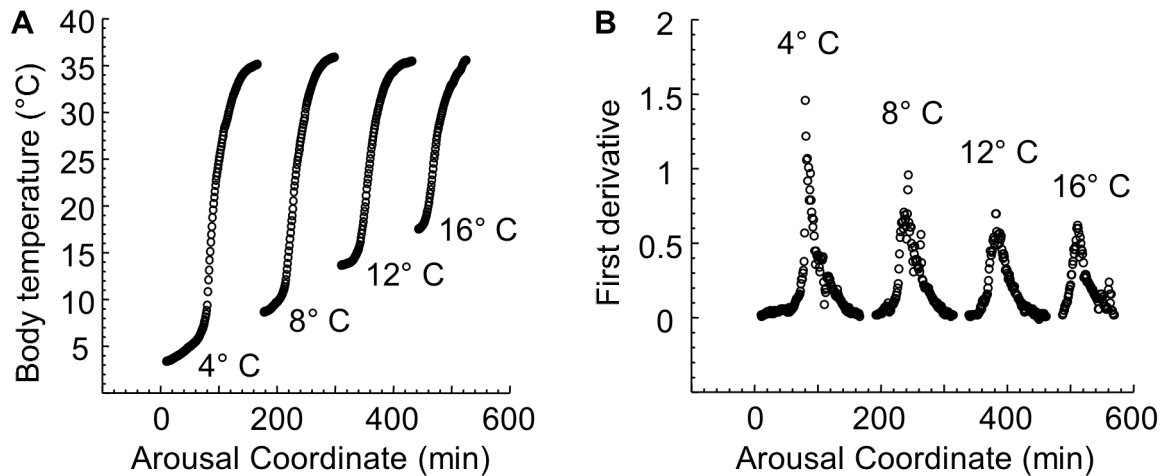


Figure 3. Body temperature as a function of time during arousals from one individual. Panel A) Body temperature was measured every minute for a squirrel housed at 4, 8, 12, and  $16^{\circ}\text{C}$ . Panel B) Instantaneous rate changes as demonstrated by plotting the first derivative as a function of time across the same range of ambient temperatures.

### Maximum Rewarming Rate

The maximum rate of rewarming was determined. A negative relationship between mean maximum RRW and  $T_a$  was found (Figure 4). Mean maximum RRW decreased  $\sim 35\%$  from  $0.86 \pm 0.09^{\circ}\text{C} \cdot \text{min}^{-1}$  for squirrels housed at  $4^{\circ}\text{C}$  to  $0.55 \pm 0.08^{\circ}\text{C} \cdot \text{min}^{-1}$  for squirrels maintained at  $16^{\circ}\text{C}$ .

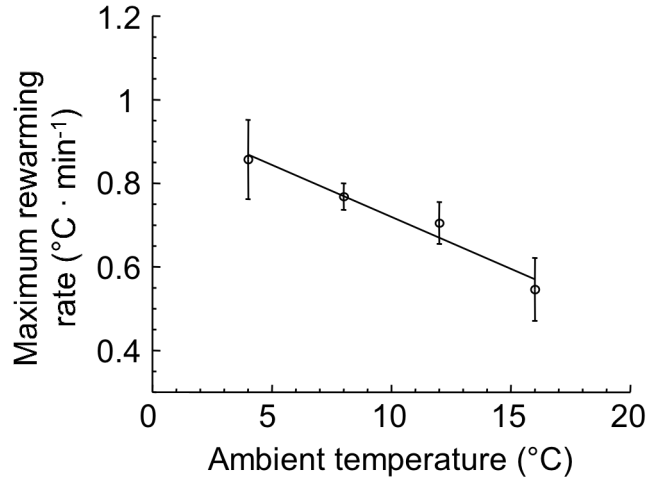


Figure 4. Effect of ambient temperature on maximum rewarming rate. Symbols represent means  $\pm$  SE,  $n = 5$ . There is a significant effect of  $T_a$  on maximum RRW,  $p < 0.05$ ,  $r^2 = 0.96$ .

#### Time to Attain Maximum Rewarming Rate

Data were analyzed to determine whether there is a defined period of time required to reach maximum RRW. In other words, is the RRW a simple function of time with no effect of  $T_a$ ? There is a negative relationship between the time required to attain maximum RRW and  $T_a$  (Figure 5). Squirrels housed at 4° C took  $\sim 3.5$  fold more time to reach a maximum RRW.

#### Relative Time Index

Hibernators arouse more rapidly when housed at elevated ambient temperatures (French, 1982). Thus, the waxing and waning pattern observed for RRW may be dependant on a relative rather than absolute amount of time. For example, it is plausible that maximum RRW is reached when 40% of the time required to complete arousal has passed. To investigate this possibility, the relative time index was examined. Even when accounting for differences in

arousal duration, animals housed at 16° C reached maximal rates approximately twice as fast as those housed at 4° C (Figure 6).

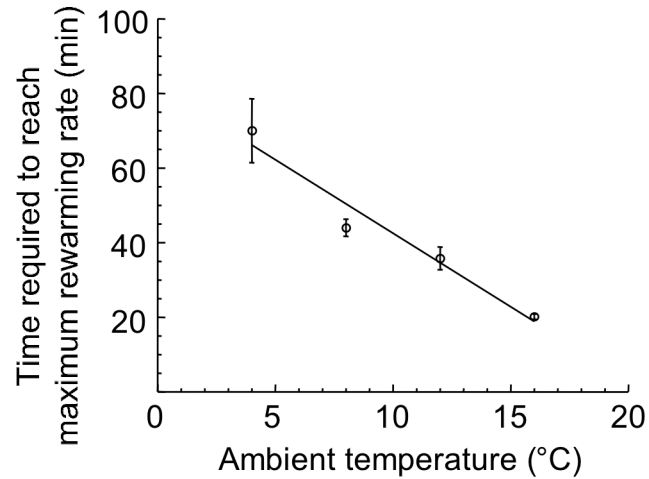


Figure 5. Effect of ambient temperature on time to reach maximum rewarming rate. Symbols represent means  $\pm$  SE,  $n = 5$ . There is a significant effect of  $T_a$  on time to attain maximum RRW,  $p < 0.05$ ,  $r^2 = 0.96$ .

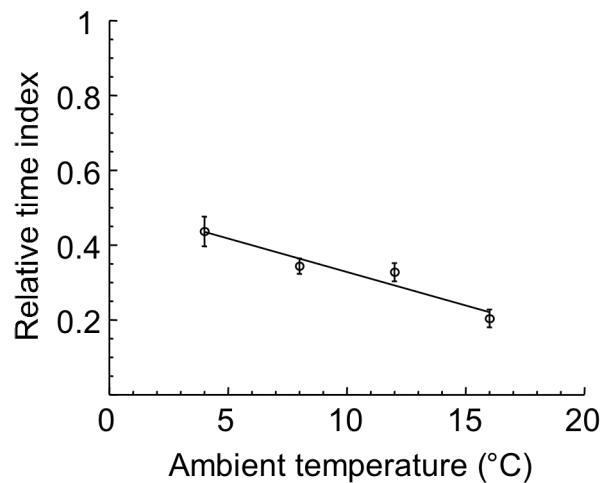


Figure 6. Effect of ambient temperature on relative time index. Relative time index was calculated as the fraction of elapsed arousal duration required to reach maximum RRW; i.e. 40 min to maximum RRW/100 min total arousal duration. Symbols represent means  $\pm$  SE,  $n = 5$ . There is a significant effect of  $T_a$  on relative time index,  $p < 0.05$ ,  $r^2 = 0.93$ .



### Body Temperature Associated with Maximum Rewarming Rate

Since we found that the RRW is not a simple function of time (Figures 5 and 6), we investigated the role of  $T_b$  in determining RRW. Does maximum RRW occur at a specific  $T_b$ ? There is no specific temperature for maximum RRW (Figure 7). Rather, the mean  $T_b$  for the occurrence of maximal RRW at  $T_a$  of 16° C is ~ 6.5° C higher than for  $T_a$  of 4° C.

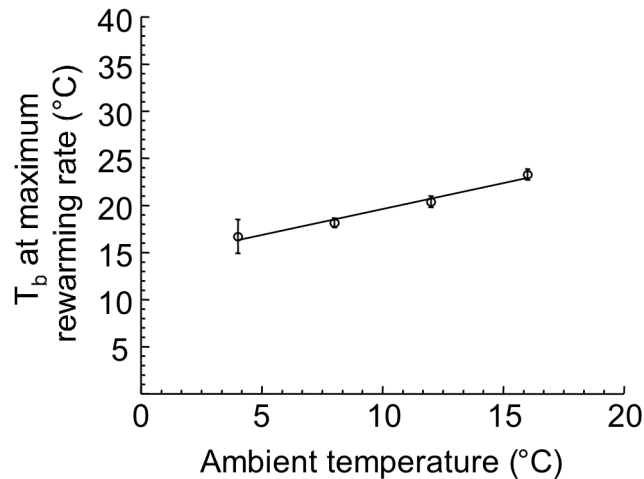


Figure 7. Effect of ambient temperature on the body temperature at which the maximum rewarming rate occurred. Symbols represent means  $\pm$  SE,  $n = 5$ . There is a significant effect of  $T_a$  on the  $T_b$  at which the maximum RRW occurred,  $p < 0.05$ ,  $r^2 = 0.98$ .

### Temperature Gain Index

The heat required to transition from a torpid  $T_b$  of 16° C to ~ 36° C is much less than from 4° C to ~ 36° C. It is plausible that maximum rewarming rates are reached when an animal has generated enough heat to reach some fraction of the required temperature gain. For all ambient temperatures, squirrels reached

maximum rewarming rates when they had generated 30 to 40% of the heat required to reach euthermic body temperatures (Figure 8).

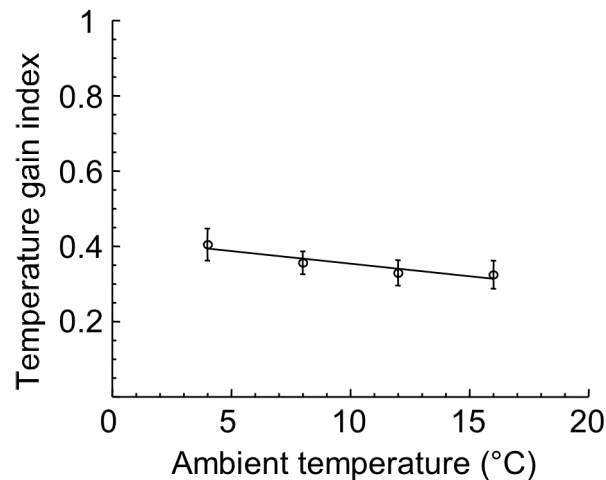


Figure 8. Effect of ambient temperature on the temperature gain index. Temperature gain index is defined as the fraction of temperature gain achieved when animals reach maximal rewarming rates; i.e. an animal housed at 4° C must gain ~ 32° C to reach euthermic  $T_b$ . If a maximum RRW occurs at a  $T_b$  of 14° C, the animal has gained 10° C thus the temperature gain index is 0.31 (10° C/32° C). Symbols represent means  $\pm$  SE,  $n = 5$ .  $T_a$  has no effect on the temperature gain index,  $p > 0.05$ ,  $r^2 = 0.89$ .

## Discussion

The maximum RRW, the time required to reach a maximum RRW, and the relative time index all demonstrated negative relationships with ambient temperature ( $T_a$ ). As torpid body temperature ( $T_b$ ) increases, animals reach their maximal rewarming rates in less time e.g.  $20.2 \pm 0.73$  min for animals housed at 16° C compared to  $70.0 \pm 8.5$  min for animals housed at 4° C (Figure 5). When changes in arousal duration are considered, animals at 16° C still reach a maximum RRW ~ 2 fold faster than those at 4° C (Figure 6). Although animals

housed at warmer temperatures reach a maximum RRW faster, the rates themselves are reduced when compared to animals housed at cooler temperatures e.g. maximum RRW was  $0.546 \pm 0.075^{\circ} \text{C} \cdot \text{min}^{-1}$  for  $16^{\circ} \text{C}$  and  $0.857 \pm 0.095^{\circ} \text{C} \cdot \text{min}^{-1}$  for  $4^{\circ} \text{C}$  (Figure 4). The  $T_b$  corresponding to maximal rewarming rates demonstrated a positive relationship with  $T_a$  (Figure 7). Squirrels housed at  $16^{\circ} \text{C}$  reached maximal rewarming rates at a  $T_b$  of  $23.3 \pm 0.58^{\circ} \text{C}$  while those housed at  $4^{\circ} \text{C}$  were at a  $T_b$  of  $16.7 \pm 1.8^{\circ} \text{C}$ . Interestingly, one parameter was unchanged by  $T_a$ , the temperature gain index. For all ambient temperatures, squirrels reached maximal rewarming rates when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$  (Figure 8).

A simplistic explanation of heat production at the subcellular level is based on the release of heat from inefficient biological reactions (Withers, 1992). When one couples this inefficiency with the notion that most enzymatic rates are temperature dependent, a simple expectation might be that as  $T_b$  increases and biochemical processes are restored during arousal from torpor the RRW would increase as a function of time. In a review of standard metabolic rate, Rolfe and Brown (1997) compiled data for major oxygen consuming reactions in six rat tissues. Protein synthesis generally accounts for 20 to 30% but may account for as much as 74% of total oxygen consumption depending on tissue type. Initiation of translation is acutely depressed at  $T_b \leq 18^{\circ} \text{C}$  during entrance into hibernation and is fully recoupled with elongation as animals arouse at  $T_b \geq 18^{\circ} \text{C}$  (van Breukelen and Martin, 2001). Thus, it appears that there is a critical temperature for the bulk of initiation at  $18^{\circ} \text{C}$ . Given the role of protein synthesis in generating

metabolic heat, one might assume that at  $T_b \geq 18^\circ \text{C}$ , there would be a significant input of heat as initiation of translation was restored. The data do not support a significant heat input at  $18^\circ \text{C}$  (Figure 7). One explanation may be that this heat input is obfuscated by heat generation at other sites.

A consideration of major sites of heat production in the whole organism reveals localized thermogenesis. During arousal from hibernation, the primary sources of heat generation are skeletal muscle (SM) and brown adipose tissue (BAT; Carey et al., 2003; Fons et al., 1997; Genin et al. 2003 and references therein). For a typical arousal, initial increases in  $T_b$  appear to be due to heat production in BAT with SM beginning to contribute heat midway through the arousal process (Fons et al., 1997). One plausible explanation for our data may be that the relative contributions or timing of SM and BAT thermogenesis is shifted depending on  $T_a$ . The relative contribution of SM activity during arousal following transfer from  $4^\circ \text{C}$  to  $22^\circ \text{C}$  in Etruscan shrews was examined; only after  $T_b > 17^\circ \text{C}$  did SM activity occur (Fons et al., 1997). In two species of ground squirrels, the onset of SM thermogenesis may vary from when  $T_b > 12^\circ \text{C}$  (housed at  $5^\circ \text{C}$ ) to  $T_b > 15^\circ \text{C}$  (housed at  $2^\circ \text{C}$ ; taken from Tøien et al., 2001; Phillips and Heath, 2004). We found that the body temperature at which maximal RRW was achieved was correlated with  $T_a$  (Figure 7). Future efforts will directly ascertain the effects of  $T_a$  on the onset of shivering thermogenesis in hibernators.

Body temperature is not just a function of heat gain but may also be influenced by heat loss. Models for animals to rewarm as fast as possible have been addressed in the literature; one of the consequences of rewarming slowly is

increased heat loss to the environment and thus a greater overall cost of rewarming to the animal (c.f. Stone and Purvis, 1992). Changes in regional blood flow dramatically influence both heat gain and loss. Regional blood flow is regulated during arousal from torpor. Blood flow to the hind foot is markedly reduced during early arousal in hibernators (Osborne et al., 2005). Blood flow posterior to the diaphragm is restricted in arousing thirteen-lined ground squirrels until the thoracic temperature is  $\sim 25^{\circ}\text{C}$  (Bullard and Funkhouser, 1962). These data are consistent with data from the big brown bat where anterior organs receive greater fractions of cardiac output during early arousal (Rauch and Beatty, 1975). The restoration of blood flow to the periphery and skin may also have large effects on the level of heat loss to the environment. In larger mammals like marmots, redistribution of blood flow to peripheral circulation has been shown to slow rates of rewarming (Phillips and Heath, 2004). We are not aware of any studies to date that have examined the effects of  $T_a$  on regional blood flow regulation. If the regulation of regional blood flow were dependent on  $T_a$ , temporal and temperature effects consistent with our findings might be expected.

The sigmoidal nature of the  $T_b$  increase during arousal has been noted before (Stone and Purvis, 1992). This sigmoidal pattern is characteristic of issues with substrate delivery and utilization. Animals that are arousing from warmer ambient temperatures have lower maximal RRW (Figure 3B, Figure 4). One explanation may be that tissue metabolism cannot be supported by available supply mechanisms e.g. since much of thermogenesis occurs in SM and BAT, perhaps

there is insufficient blood flow to these regions to support additional thermogenesis at warmer  $T_b$ . Interestingly although maximum RRW is much higher for squirrels housed at lower ambient temperatures, the time required for an animal to rewarm when  $T_a$  is 16° C is equal to the amount of time required for an animal housed at 4° C to complete the final 20° C (i.e. from 16° C to 36° C;  $p > 0.05$ ; data not shown). Thus, it appears there are constraints on the maximum RRW achievable at warmer  $T_b$ .

Biological reaction rates are temperature dependent and many reactions release heat. Therefore as  $T_b$  increases, an increasing number of biochemical processes should contribute additional heat. Using this model, RRW might be expected to simply increase as a function of time until a euthermic set point is approached. While this assertion is sensible, the data support a more complex regulation. A more likely model for heat production during arousal integrates systemic physiology with the subcellular biochemical processes that underlie thermogenesis. Contributions of regional blood flow, substrate delivery and utilization, and unequal contributions of heat from different tissues must be reconciled in the context of both heat gain and heat loss. Our data indicate the dynamics of rewarming are affected by both time and temperature. Maximum RRW and the time to reach maximum RRW decrease with increasing  $T_a$ . The  $T_b$  associated with maximum RRW increases with increasing  $T_a$ . Thus, there does not appear to be a simple relationship for heat gain during arousal. Instead, it appears that the process of arousal is more constrained than we initially expected. All animals reached their maximum rewarming rates when they had

generated 30 to 40% of the heat required to reach a euthermic  $T_b$ . Future efforts will be directed towards further elucidating the interactions among the systemic and subcellular events that modulate thermogenesis.

## CHAPTER 3

### COMPARISON OF NATURAL AND INDUCED AROUSAL DYNAMICS

#### Abstract

The regulation of arousal has garnered attention since the inception of hibernation research. Natural arousal from torpor occurs spontaneously with highly predictable timing. However, animals can also be induced to arouse prematurely in response to various disturbances. While many investigations have used natural and induced arousals synonymously, direct comparisons of these two types of arousal have been lacking. I address the question of whether natural and prematurely induced arousals are the same. I compare the effects of ambient temperature on the dynamics of natural versus induced arousals. Arousal duration, maximum rewarming rate, and the variance associated with increases in body temperature differed between natural and induced arousals. Prematurely inducing arousal also alters the duration of the interbout aroused (IBA) period, and the responsiveness to the induced arousal stimulus is influenced by torpor duration. I recommend that careful consideration be given to experimental design and data interpretation related to the arousal phase of a torpor bout.

#### Introduction

Metabolic depressions like hibernation are a common physiological response to challenging environmental conditions such as cold temperatures and limited food supplies (Carey et al., 2003; Guppy and Withers, 1999; Storey and Storey,



1990). Hibernation is a dynamic physiological process wherein animals cycle between periods of torpor and periods of interbout arousal. Torpor is characterized by body temperatures ( $T_b$ ) as low as  $-2.9^{\circ}\text{C}$  and severely depressed metabolic rates; interbout arousal is characterized by euthermic  $T_b$  and high metabolic rates (Barnes, 1989; Carey et al., 2003). Ground squirrels are exceptional hibernators, spending 6 to 9 months in hibernation each year (Frank et al., 2008). During the hibernation season, an animal typically experiences 15 to 20 bouts of torpor. The length of each bout may range from 1 to 3 weeks and is influenced by species, ambient temperature, and time of year (Buck and Barnes, 2000; French, 1982; Wang, 1979; Geiser and Baudinette, 1990; Twente et al., 1977).

The process of rewarming from torpor is called arousal. Normally animals spontaneously arouse from torpor, presumably in response to endogenous cues. However, animals can also be induced to arouse prematurely in response to external disturbances such as handling, loud noises, or rapid temperature shifts (Pengelley and Fisher, 1968; Twente and Twente, 1965a; 1968). Arousal from torpor is a rapid event; squirrels can rewarm from near freezing temperatures to euthermia in a mere 2 to 3 hours (Figure 9). Maximum rewarming rates for individual ground squirrels can approach  $1.5^{\circ}\text{C}$  per minute (Utz et al., 2007).

The process of arousal involves incongruent changes in various physiological systems i.e. not all processes are reengaged concurrently. For example in arousing thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), blood flow posterior to the diaphragm is restricted until the thoracic temperature is ~

25° C (Bullard and Funkhouser, 1962). In arousing arctic ground squirrels (*Spermophilus parryi*), oxygen consumption peaks when abdominal  $T_b$  is ~ 5° C (Tøien et al., 2001). During arousal, animals must reconcile the incongruence in oxygen consumption, blood flow, and changing body temperature in the midst of rapidly changing metabolic demand as processes like transcription, protein metabolism, mitochondrial respiration, and mitosis are restored (van Breukelen and Martin, 2001, 2002a, 2002b; van Breukelen et al., 2004; Velickovska et al., 2005; Velickovska and van Breukelen, 2007; Martin et al., 1999; Kruman et al., 1988).

Data from a previous study investigating the influence of ambient temperature on the process of natural arousal indicate that both time and body temperature affect rewarming dynamics (Utz et al., 2007). One parameter was found to be independent of ambient temperature; all animals reached their maximum rewarming rates when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$  (Utz et al., 2007). These data support a multifaceted regulation of the arousal process.

Natural and prematurely induced arousals have traditionally been regarded as synonymous events, though this assertion has not been well investigated. Table 1 presents a random sample of 50 publications investigating arousal; 9 records utilized natural arousals, 35 records utilized induced arousals, and the nature of the arousal was unclear for the remaining 6 records. Of the 35 studies utilizing induced arousals, only 8 represent intentionally induced arousals i.e. prematurely inducing arousal was part of the experimental design. To my knowledge, only

one study has directly compared natural and induced arousals (Tähti and Soivio, 1977; 1978). Tähti and Soivio found that increases in oxygen consumption, heart rate, and blood pressure occur more rapidly during induced arousal (1978). Further, the relative timing of changes in ventilation, heart rate, and blood pressure were altered during induced arousal (1977). Indeed, these alterations in cardiovascular and respiratory system function may be physiologically meaningful.

I question whether natural and prematurely induced arousals are physiologically synonymous events. Premature arousal from torpor might be difficult for an animal to regulate due to mismatches between external stimuli and endogenous timing cues. It seems reasonable to expect that rewarming dynamics may differ between natural and induced arousals. Here, I investigate the relationship between ambient temperature and induced arousal dynamics. I also examine the effect of induced arousal on the duration of the interbout aroused (IBA) period. Further, I investigate the effect of torpor duration on responsiveness to the induced arousal stimulus.

## Materials and Methods

### Animals

Golden-mantled ground squirrels (*Spermophilus lateralis*) were live trapped from local populations in Southern Nevada, Southern California, or Southern Utah. Prior to the beginning of the hibernation season, temperature sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) were surgically

implanted into the abdominal cavity. These radiotelemeters allow for precise measurement of  $T_b$  throughout the hibernation season. Animals were housed in an environmental chamber at ambient temperatures of 4, 8, 12, and 16°C during the hibernation season. The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures.

Body temperature was recorded every minute. The initiation and termination of an arousal were determined based on the rate of change in body temperature; initiation of arousal typically occurred when  $T_b$  increased at a rate of 0.02° C per minute (Utz et al., 2007). For each temperature, 5 animals were allowed to proceed through a natural arousal. Subsequently, these same animals were induced to arouse prematurely by mild shaking for 30 seconds (Figure 9). Rewarming dynamics were compared for natural and induced arousals from the same animal.

### Calculations

Body temperature ( $T_b$ ) was recorded every minute to the hundredth of a degree. The first derivative (FD) and its associated standard deviation (SD) for temperature as a function of time were calculated. The initiation of arousal was defined as the point where the instantaneous rate of rewarming (RRW) exceeded the FD + 3 SD (threshold rate; TR) for a 10-minute period of consistent  $T_b$ . The first  $T_b$  measurement with a FD > TR was defined as the onset of arousal. The termination of arousal was defined as the first point at which the instantaneous rewarming rate was  $\leq$  TR for the following 5 min.

Maximum rate of rewarming is a mean value of the 5 highest values for a given arousal. The time required to achieve maximum RRW is defined as the period of time spanning the initiation of arousal to the single highest RRW value. Arousal duration is the period of time spanning from the initiation of arousal to the termination of arousal. The relative time index is a ratio of the time required to reach a maximum RRW divided by the arousal duration. The  $T_b$  associated with maximum RRW is a mean of 5 values corresponding to the 5 highest instantaneous rewarming rates. The temperature gain index is a ratio of the gain in  $T_b$  achieved at the maximum RRW divided by the temperature gain achieved at the termination of arousal.

Interbout arousal duration is defined as the period of time spanning the termination of arousal to the initiation of entrance. Initiation of entrance is defined as the first point after which there are only decreases in body temperature. Time torpid is defined as the period of time spanning the start of torpor to the application of the arousal stimulus. Start of torpor is the first point following entrance where there is a 5 minute period with no decrease in body temperature. Lag time is defined as the period of time spanning the application of the arousal stimulus to the initiation of arousal.

### Statistical Analyses

A two-sample test for variance was performed on rewarming data from all animals at all temperatures. This test analyzed the rate of change in  $T_b$  between the natural and induced arousal for each individual. The F-statistic was calculated as a ratio of the variances of the two samples, and an F-test was

performed to determine whether the variance for induced arousal was greater than the variance for natural arousal (OriginPro 8). In order to examine the effect of ambient temperature and type of arousal, rewarming dynamics data were subjected to ANCOVA (Statview 4.1). Records from 10 animals housed at 4°C were subjected to a paired t-test to determine the effect of arousal type on the duration of the interbout arousal (IBA; Statview 4.1). A simple regression analysis was performed to determine whether the amount of time spent in torpor affected responsiveness to the arousal stimulus (Statview 4.1). A p-value < 0.05 was considered significant for all analyses.

## Results

### The Dynamics of Natural and Induced Arousal from Torpor at 4, 8, 12, and 16° C

The experimental design is depicted in Figure 9. Following a natural spontaneous arousal from torpor, each animal was subsequently induced to arouse from torpor prematurely. The induced arousal stimulus was 30 seconds of mild shaking.

Increases in body temperature during both natural and induced arousal from torpor were measured for animals housed at 4, 8, 12, and 16° C (Figure 10). Previously, a rewarming pattern for natural arousals was observed where initial increases in  $T_b$  were relatively slow, followed by faster increases, with the final period also characterized by slow increases in  $T_b$  (Utz et al., 2007). This pattern of rewarming was maintained for induced arousals at all ambient temperatures

(Figure 10). Interestingly, there was greater scatter in the rate of change in  $T_b$  during induced arousals (Figure 10B).

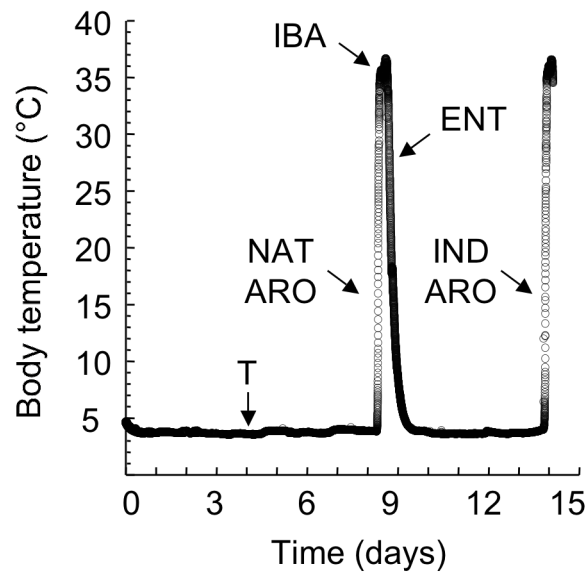


Figure 9. Occurrence of natural and induced arousals. Body temperature ( $T_b$ ) was measured every minute for an animal housed at 4° C. During the ~1 week long period of torpor (T),  $T_b$  is maintained within 1° C of ambient temperature ( $T_a$ ). During natural arousal (NAT ARO), animals spontaneously rewarm to euthermic  $T_b$  (~36° C). Euthermic  $T_b$  is maintained during the interbout arousal (IBA). During entrance (ENT) into torpor,  $T_b$  gradually falls from euthermic (~36° C) to near  $T_a$  (~4° C). Following an undisturbed torpor bout, animals were induced to arouse (IND ARO) prematurely in response to 30 seconds of mild shaking.

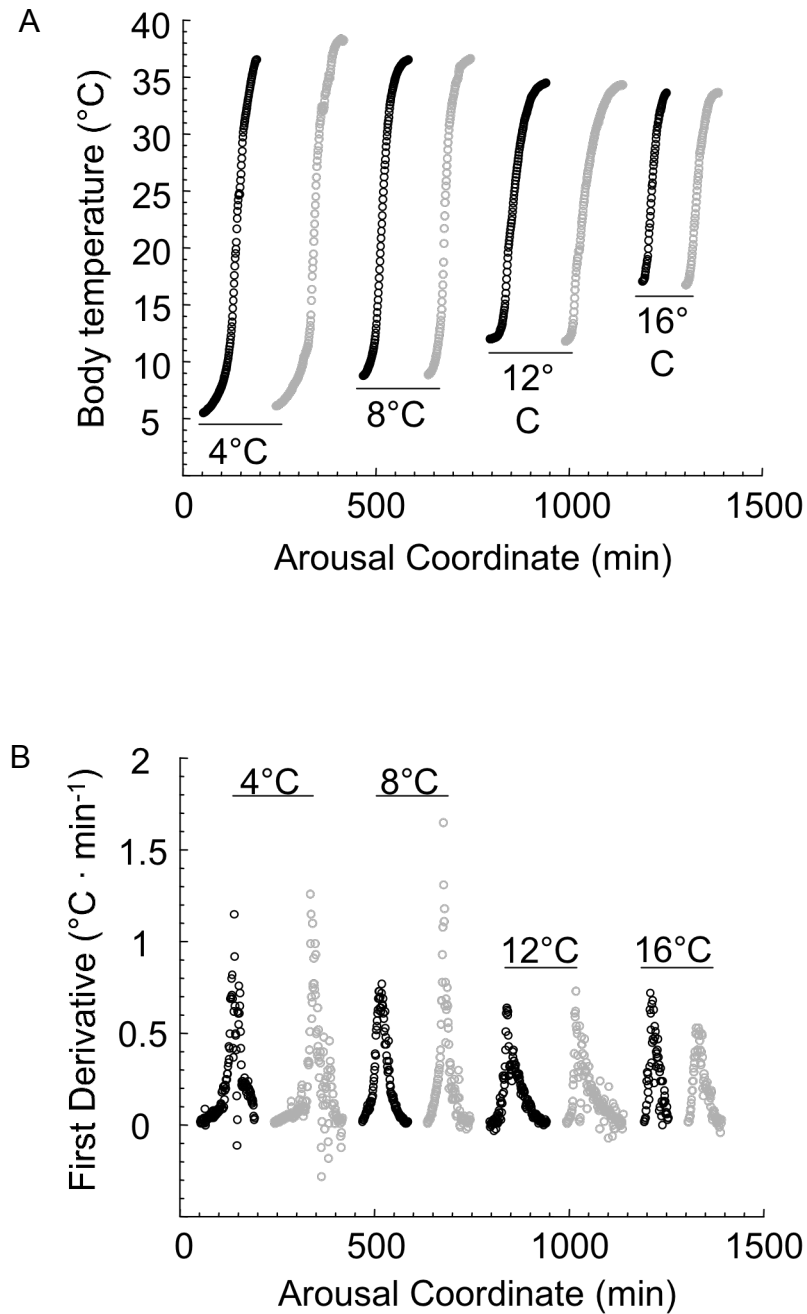


Figure 10. Body temperature as a function of time during arousals from four individuals for natural (black symbols) and induced (gray symbols) arousals. (A) Body temperature was measured every minute for squirrels housed at 4, 8, 12, and 16°C. (B) Instantaneous rate changes as demonstrated by plotting the first derivative as a function of time across the same range of ambient temperatures.



### Variability of Increases in Body Temperature

Increases in  $T_b$  during induced arousal may be more erratic than during natural arousal. A two-sample test for variance was performed to determine whether the variance of the rate of change in  $T_b$  for induced arousal was greater than the variance for the paired natural arousal (Table 2). For 80% of the pairs analyzed, induced arousals had greater variance than natural arousals. Induced arousals had significantly greater variance than natural arousals for ambient temperatures of 4 and 8° C (Table 2). At higher ambient temperatures, the variance for natural and induced arousals was comparable.

Table 2. The Effect of Ambient Temperature on Body Temperature

#### Variance during Arousal

	4°C	8°C	12°C	16°C
Natural Arousal	0.0374 ± 0.006	0.0524 ± 0.005	0.0278 ± 0.003	0.0474 ± 0.012
Induced Arousal	0.0510 ± 0.006*	0.0800 ± 0.010*	0.0416 ± 0.013	0.0482 ± 0.012

The variance of the rate of change in body temperature was calculated for both natural and induced arousal; n = 5 animals for each ambient temperature. \*At ambient temperatures of 4 and 8° C, the variance associated with induced arousal is significantly greater than the variance associated with natural arousal ( $p < 0.05$ ).

### Maximum Rewarming Rate

The maximum rate of rewarming was determined for both natural and induced arousal from torpor (Figure 11). There was a significant effect of  $T_a$  on the maximum RRW for both natural and induced arousal ( $p < 0.05$ ). The type of arousal also had a significant effect on the maximum RRW ( $p < 0.05$ ). For natural

arousals, the maximum RRW decreased  $\sim 32\%$  from  $0.87 \pm 0.06^\circ \text{C} \cdot \text{min}^{-1}$  for animals housed at  $4^\circ \text{C}$  to  $0.59 \pm 0.06^\circ \text{C} \cdot \text{min}^{-1}$  for animals housed at  $16^\circ \text{C}$ . For induced arousals, the maximum RRW decreased  $\sim 42\%$  from  $1.13 \pm 0.09^\circ \text{C} \cdot \text{min}^{-1}$  for animals housed at  $4^\circ \text{C}$  to  $0.65 \pm 0.05^\circ \text{C} \cdot \text{min}^{-1}$  for animals housed at  $16^\circ \text{C}$ . The maximum rate of rewarming for induced arousals was  $\sim 28\%$  greater than for natural arousals at  $4$  and  $8^\circ \text{C}$ .

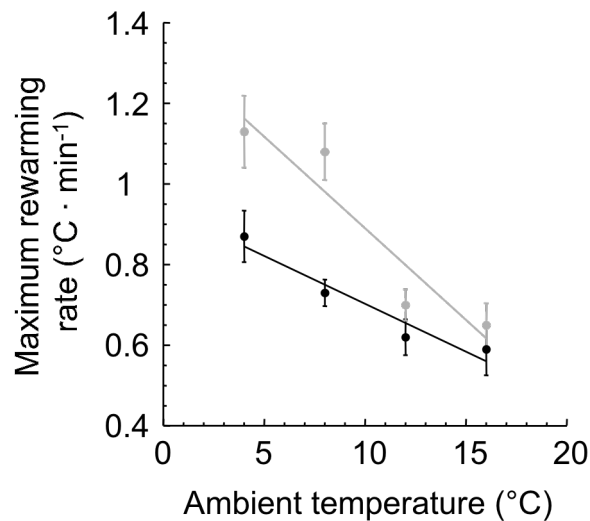


Figure 11. Effect of ambient temperature on maximum rate of rewarming for natural and induced arousal from torpor. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . There is a significant effect of  $T_a$  on the maximum rate of rewarming for both natural and induced arousals,  $p < 0.05$ ,  $r^2 = 0.93$ ,  $r^2 = 0.88$  respectively. There is a significant effect of arousal type on the maximum rate of rewarming,  $p < 0.05$ .

### Time to Attain Maximum Rewarming Rate

For both natural and induced arousals, the time required to attain maximum rewarming rate was determined (Figure 12). There was a significant effect of  $T_a$  on the time required to achieve maximum RRW for both natural and induced

arousal ( $p < 0.05$ ). For natural arousals at 4° C, maximum rewarming rates were achieved after  $73.8 \pm 8.2$  min; at 16° C the time required was  $22.6 \pm 4.6$  min. For induced arousals at 4° C, the time required to achieve maximum rewarming rates was  $77.0 \pm 7.4$  min; at 16° C the time required was  $22.0 \pm 1.5$  min. Squirrels housed at 16° C reached their maximum rewarming rate  $\sim 3.5$  times faster than those housed at 4° C for both natural and induced arousal from torpor. The type of arousal did not have a significant effect on the time required to achieve maximum RRW ( $p > 0.05$ ).

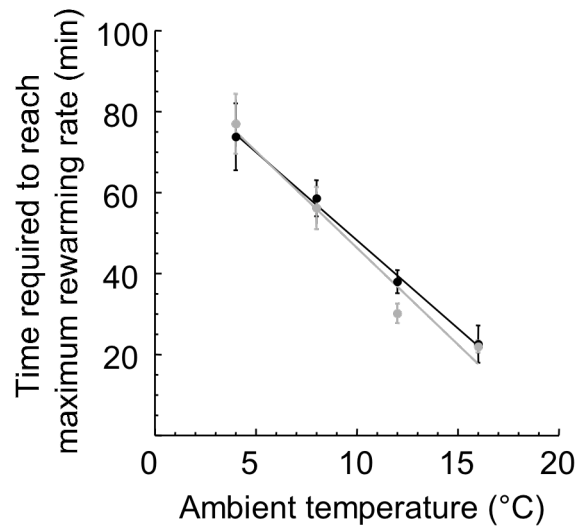


Figure 12. Effect of ambient temperature on time required to achieve maximum rewarming rate for natural and induced arousal from torpor. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . There is a significant effect of  $T_a$  on the time required to achieve maximum RRW for both natural and induced arousal,  $p < 0.05$ ,  $r^2 = 0.99$ ,  $r^2 = 0.97$  respectively. Type of arousal did not affect the time required to achieve maximum rewarming rate,  $p > 0.05$ .

### Arousal Duration

Data were analyzed to determine if there are differences between natural and induced arousal for the amount of time required to complete arousal (Figure 13). There was a significant effect of  $T_a$  on arousal duration for both natural and induced arousal ( $p < 0.05$ ). Arousal duration at 4° C for natural and induced arousals, respectively, was  $144.6 \pm 8.6$  min and  $170.6 \pm 8.2$  min. At 16° C duration for natural and induced arousals, respectively, was  $66.25 \pm 8.1$  min and  $77.3 \pm 9.9$  min. The type of arousal had a significant affect on arousal duration ( $p < 0.05$ ).

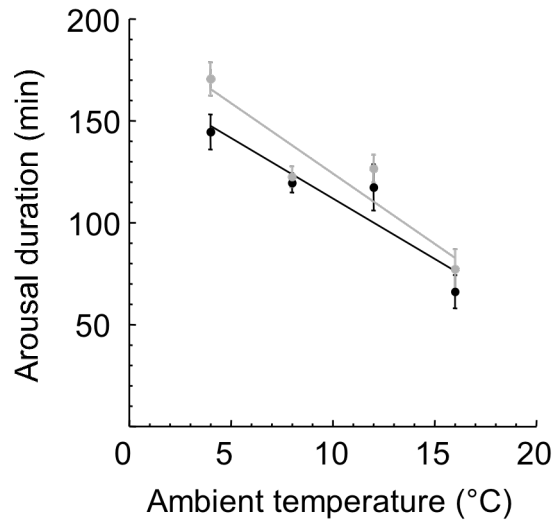


Figure 13. Effect of ambient temperature on arousal duration for natural and induced arousal from torpor. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . There is a significant effect of  $T_a$  on arousal duration for both natural and induced arousal,  $p < 0.05$ ,  $r^2 = 0.87$ ,  $r^2 = 0.87$  respectively. There is a significant effect of arousal type on duration,  $p < 0.05$ .

### Relative Time Index

The amount of time required to complete arousal changes with housing temperature (Figure 13). Thus, it seems appropriate to evaluate the time required to reach maximum rewarming rates in relative as well as absolute terms (Figure 14). The relative time index is the ratio of time required to reach maximum RRW/ arousal duration. There was a significant effect of  $T_a$  on relative time index for both natural and induced arousal ( $p < 0.05$ ). At  $4^\circ\text{C}$ , animals representing both types of arousal reached their maximum RRW when the arousal was  $\sim 50\%$  complete. At  $16^\circ\text{C}$ , animals reached their maximum RRW when the arousal was  $\sim 30\%$  complete. The type of arousal did not have a significant affect on the relative time index ( $p > 0.05$ ).

### Body Temperature Associated with Maximum Rewarming Rate

The data were analyzed to determine whether the type of arousal affects the relationship between ambient temperature and the body temperature associated with maximum rewarming rate (Figure 15). There was a significant effect of  $T_a$  on the  $T_b$  at which the maximum RRW occurred for both natural and induced arousal ( $p < 0.05$ ). For both types of arousal,  $T_b$  increased  $\sim 6^\circ\text{C}$  for arousals at  $16^\circ\text{C}$  as compared to arousals at  $4^\circ\text{C}$ . The type of arousal did not have a significant affect on the body temperature associated with maximum rewarming rates ( $p > 0.05$ ).

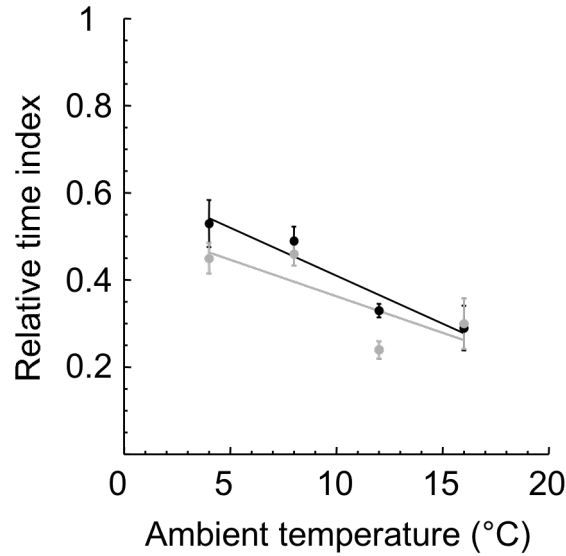


Figure 14. Effect of ambient temperature on relative time index for natural and induced arousal from torpor. Relative time index is the ratio of time required to reach maximum RRW divided by arousal duration. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . There is a significant effect of  $T_a$  on relative time index for both natural and induced arousal,  $p < 0.05$ ,  $r^2 = 0.93$ ,  $r^2 = 0.62$  respectively. Type of arousal did not affect the relative time index,  $p > 0.05$ .

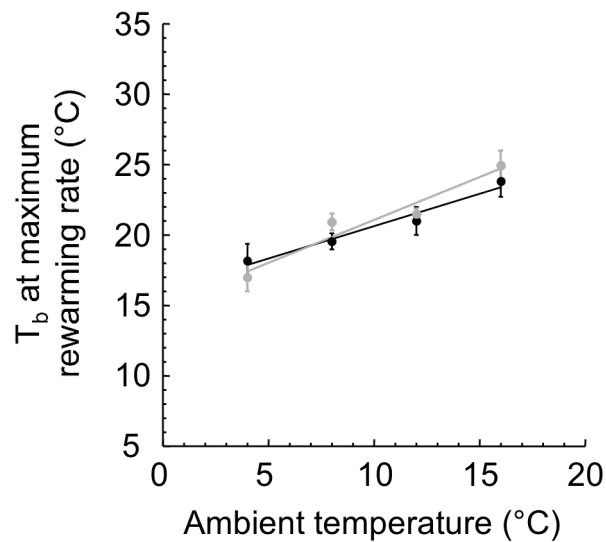


Figure 15. Effect of ambient temperature on the body temperature at which the maximum rate of rewarming occurred for natural and induced arousal from torpor. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . There is a significant effect of  $T_a$  on the  $T_b$  at which the maximum RRW occurred for both natural and induced arousal,  $p < 0.05$ ,  $r^2 = 0.97$ ,  $r^2 = 0.94$  respectively. Type of arousal did not affect the  $T_b$  at which the maximum RRW occurred,  $p > 0.05$ .

### Temperature Gain Index

As ambient temperature is increased, the body temperature associated with the maximum RRW also increases (Figure 15). The temperature gain index reflects the relative heat gains associated with the occurrence of the maximum RRW. The temperature gain index is the ratio of heat gains required to reach the body temperature associated with maximum RRW divided by heat gains required to reach euthermy. Concordant with a previous study, ambient temperature did not have a significant effect on the temperature gain index (Utz et al., 2007;  $p > 0.05$ ). For all ambient temperatures, animals reached their maximum rewarming rates when they had generated ~ 40% of the heat required to reach a euthermic body temperature (Figure 16). The type of arousal did not have a significant effect on the temperature gain index ( $p > 0.05$ ).

### IBA Duration

Records from 10 animals housed at 4° C were analyzed to determine the effect of induced arousal on the duration of the interbout arousal (IBA). Interbout arousals following an induced arousal were ~ 25% shorter than the corresponding IBA following a natural arousal (Figure 17). The average IBA duration following natural arousal was ~ 12.5 hours whereas it was ~ 9 hours following induced arousal. This reduction in IBA duration was significant (paired t-test,  $p < 0.05$ ).

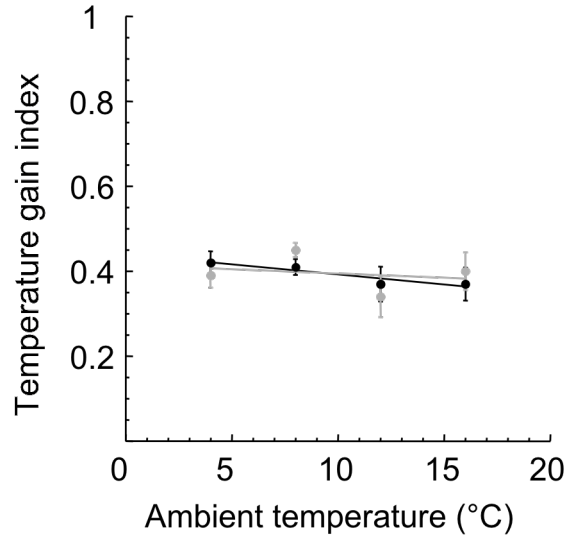


Figure 16. Effect of ambient temperature on temperature gain index for natural and induced arousal from torpor. Temperature gain index is defined as the fraction of temperature gain achieved when animals reach maximal rewarming rates. Symbols represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . Ambient temperature has no effect on temperature gain index for natural or induced arousal,  $p > 0.05$ ,  $r^2 = 0.87$ ,  $r^2 = 0.20$  respectively. Type of arousal did not affect the temperature gain index,  $p > 0.05$ .

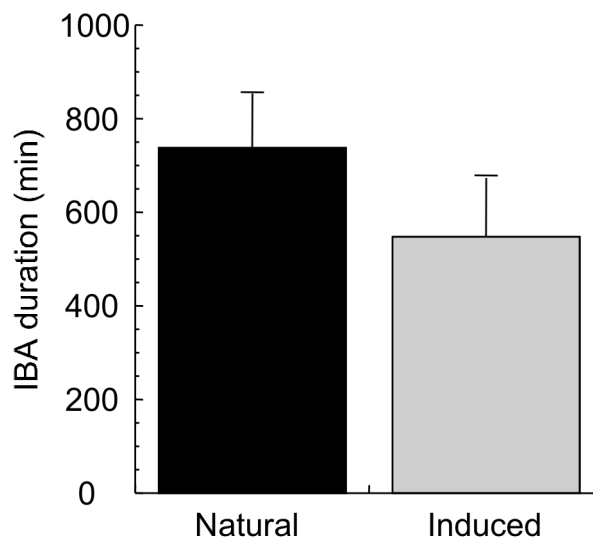


Figure 17. Effect of induced arousal on IBA duration. Bars represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 10$ . All animals were housed at  $4^\circ\text{C}$ . Type of arousal has a significant affect on IBA duration, paired  $t$ -test  $p < 0.05$ .



### Torpor Duration Influences Responsiveness to Induced Arousal Stimulus

The period of time spanning the application of the induced arousal stimulus to the initiation of arousal was measured (Figure 18). This “lag time” was influenced by the amount of time the animal had spent in torpor. Lag times were relatively shorter when an animal was induced to arouse early in the torpor bout. As animals progressed throughout the torpor bout, the lag time increased. The amount of time spent in torpor had a significant affect on the lag time ( $p < 0.05$ ).

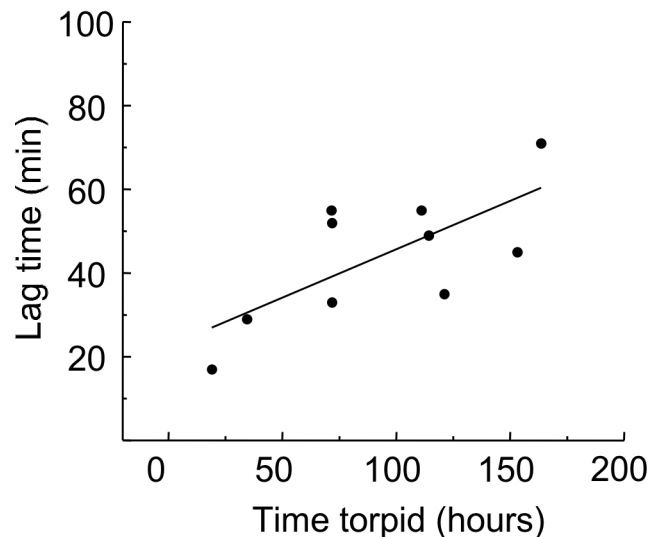


Figure 18. Effect of torpor duration on responsiveness to the arousal stimulus. 10 animals housed at 4° C were subjected to 30 seconds of mild shaking, following differing amounts of time in torpor. The period of time between the application of the arousal stimulus and the initiation of arousal was measured and is referred to as the lag time. Time torpid had a significant effect on lag time,  $p < 0.05$ ;  $r^2 = 0.49$ .

### Discussion

The process of arousal exemplifies the dynamic nature of the torpor bouts that constitute a hibernation season. Arousal is an intricate and rapid process.

Animals may increase their body temperature ( $T_b$ ) by more than 35° C in a mere 2 to 3 hours. During arousal, animals must reconcile differing rates of oxygen consumption, blood flow, and changing  $T_b$  in the midst of rapidly changing metabolic demand as processes like transcription, protein metabolism, mitochondrial respiration, and mitosis are restored (van Breukelen and Martin, 2001, 2002a, 2002b; van Breukelen et al., 2004; Velickovska et al., 2005; Velickovska and van Breukelen, 2007; Martin et al., 1999; Kruman et al., 1988).

Models for animals to rewarm as fast as possible have been addressed in the literature; rewarming at slower rates represents increased energy expenditure, as more heat is lost to the environment (Stone and Purvis, 1992). I previously investigated the affect of ambient temperature on the process of natural arousal. Ambient temperature was found to impact the rate of rewarming (RRW), the time required to achieve maximum RRW, and the body temperature associated with maximum RRW (Utz et al., 2007). One parameter was found to be independent of ambient temperature; all squirrels reached their maximum RRW when they had generated 30 to 40% of the heat required to reach a euthermic body temperature (Utz et al., 2007). These data suggest that arousal is more regulated than previously expected. Rather than simply allowing temperature to dictate metabolic reactions and thus rewarming rates, the data indicate complex regulation of heat gain and heat loss that is influenced both by temperature and time.

Natural arousals occur spontaneously with highly predictable timing. The duration of consecutive torpor bouts during mid-winter often varies by less than

10% (Twente and Twente, 1965a, 1965b; Twente et al., 1977). In addition to natural arousal, animals can be induced to arouse prematurely in response to various disturbances (Pengelley and Fisher, 1968; Twente and Twente, 1965a). Although there are data that indicate important differences, natural and induced arousals have historically been utilized rather interchangeably (Tähti and Soivio, 1977; 1978; Table 1).

Here, I compare the affects of ambient temperature on the dynamics of natural versus induced arousals. Natural and induced arousals differed considerably for the maximum rate of rewarming and the variance associated with increases in body temperature (Figure 11; Table 2). For 80% of the arousal pairs analyzed, increases in body temperature were more variable during induced arousal (Table 2). The maximum rate of rewarming is ~ 30% greater for induced arousals at 4 and 8° C (Figure 11). Thus it seems animals may not necessarily rewarm as fast as possible during natural arousal. Such a finding reinforces the idea that natural arousals are a regulated processes rather than a sole effort to rewarm as rapidly as possible. If increasing  $T_b$  too rapidly is detrimental, animals may regulate rewarming rates to be less than the maximum possible, which is consistent with my data.

During natural arousals, the greatest warming rates are often clustered together, giving the first derivative plot a clean peak (Figure 10B). Frequently, peak warming rates are not maintained as uniformly during induced arousals. Induced arousals tend to be more erratic and disordered than natural arousals. Oftentimes during induced arousal, a “boom and bust” pattern is present wherein

the peak rates are scattered and may be followed by decreases in body temperature (Figure 10B). This disorder in the rate of change in  $T_b$  may be the result of poor control of peripheral circulation. In marmots, redistribution of blood flow to peripheral circulation has been shown to slow rates of rewarming (Phillips and Heath, 2004).

The increased variability and greater rewarming rates associated with induced arousal may be explained by considering the mechanisms of heat production and distribution. Brown adipose tissue (BAT) and skeletal muscle are the major sites of heat production during arousal (Carey et al., 2003; Fons et al., 1997; Genin et al., 2003 and references therein). For a typical arousal, initial increases in  $T_b$  appear to be due to BAT activity with shivering thermogenesis beginning to contribute heat midway through the arousal (Fons et al., 1997). In two species of ground squirrels, the onset of shivering thermogenesis varies from when  $T_b > 12^\circ\text{C}$  (housed at  $5^\circ\text{C}$ ) to  $T_b > 15^\circ\text{C}$  (housed at  $2^\circ\text{C}$ ; taken from Tøien et al., 2001; Phillips and Heath, 2004). Appropriate cardiovascular system function underlies the heat generation and dissipation that occur during arousal. Brown adipose tissue and skeletal muscle must be supplied with adequate metabolic fuels in order to generate heat (Cannon and Nedergaard, 2004; Horwitz et al., 1968; Fons et al., 1997; Genin et al., 2003). This heat must then be conducted into other tissues via the cardiovascular system. Regional blood flow is regulated during arousal. Blood flow posterior to the diaphragm is restricted in arousing thirteen-lined ground squirrels until the thoracic temperature is  $\sim 25^\circ\text{C}$  (Bullard and Funkhouser, 1962). Such restrictions of

peripheral blood flow are common in other species of hibernators. Blood flow to the hind foot is markedly reduced during early arousal in hamsters (Osborne et al., 2005). In the big brown bat, anterior organs receive increasing fractions of cardiac output while posterior organs receive decreasing fractions of blood after the initiation of arousal (Rauch and Beatty, 1975).

The disorder present in induced arousals may indicate that an animal's ability to regulate peripheral blood flow is impaired; such an impairment might lead to inconsistencies in heat generation and heat dissipation similar to the patterns seen in my data (Figures 10B and 11; Table 2). It is plausible that the "boom and bust" pattern of increasing body temperature and the increased variance associated with induced arousal are a result of cardiovascular dysregulation. Induced arousal was previously found to alter heart rate and blood pressure (Tähti and Soivio, 1977; 1978). If blood flow is erratic during induced arousal, increased variability consistent with my data might be expected. I plan to focus future investigations on further evaluating cardiovascular system function during natural and induced arousal from torpor.

Prematurely inducing arousal may alter other phases of a torpor bout, namely the interbout arousal (IBA). The duration of the IBA period was reduced ~ 25% following induced arousal from torpor (Figure 17). The similarity between natural and induced arousal for parameters such as time to maximum rewarming rate and its associated body temperature is consistent with the idea of a common neural program (Figures 12 and 15). However, the responsiveness to the induced arousal stimulus is influenced by the amount of time spent in torpor

(Figure 18). It seems plausible that if there is a consistent arousal program, the ability of an animal to successfully execute this program is impaired following a prematurely induced arousal.

Natural and induced arousals have historically been regarded as synonymous events. This assumption should be reevaluated. I found that inducing a premature arousal from torpor alters key aspects of rewarming. The variability for changes in body temperature is increased (Table 2). The maximum rewarming rate is increased (Figure 11). Further, these increased rewarming rates occur with sporadic frequency and may be followed by decreases in  $T_b$  (Figure 10B). Arousal duration is increased (Figure 13). The duration of the interbout arousal (IBA) period is decreased (Figure 17). Also, the responsiveness of animals to the induced arousal stimulus is influenced by the amount of time spent in torpor (Figure 18). I recommend that careful consideration be given to experimental design and data interpretation for experiments utilizing animals that were prematurely induced to arouse from torpor.

## CHAPTER 4

### METABOLIC COSTS AND PHYSIOLOGICAL CONSEQUENCES OF NATURAL VERSUS INDUCED AROUSAL

#### Abstract

Previous studies have shown that arousal is an intricate and well regulated process wherein multiple cellular and systemic physiological activities are rapidly reengaged. Although animals naturally arouse from torpor with highly predictable timing, numerous investigations have utilized animals that were induced to arouse prematurely. I have previously demonstrated that key aspects of rewarming are altered during induced arousal. Now I address the metabolic cost of natural versus induced arousal from torpor. Metabolic activity is estimated through indirect calorimetry and assays of metabolites in blood plasma. Indicators of oxidative damage to lipids and proteins in heart, liver, kidney, and brain tissues were measured to investigate potential physiological consequences of these two events. Metabolic activity differs during early arousal, and hibernators appear to be well protected against oxidative damage.

#### Introduction

The dormancy of a hibernation season is punctuated by periods of intense metabolic activity. During arousal from torpor, hibernators experience rapid increases in body temperature ( $T_b$ ) and metabolic activity. In only 2 to 3 hours, an animal can experience the elevation of body temperature by more than 30° C (Figures 1, 3, 8, 10, and 13). Oxygen consumption increases nearly 100 fold

during the process of arousal (Wang, 1979; Wang and Lee, 2000). Brown adipose tissue and skeletal muscle generate the heat that drives these increases in body temperature and oxygen consumption (Cannon and Nedergaard, 2004; Horwitz et al., 1968; Fons et al., 1997; Genin et al., 2003). Early in arousal blood flow is restricted to the core of the animal however, midway through an arousal blood supply to peripheral tissues resumes (Bullard and Funkhouser, 1962; Osborne et al., 2005; Rauch and Beatty, 1975).

When hibernating ground squirrels are induced to arouse prematurely, many aspects of the rewarming process are altered. Maximum rewarming rate, the variability of changes in body temperature, arousal duration, and IBA duration all differ following an induced arousal (Figures 11, 13, 17; Table 2). Further, there is a lag period between the application of the arousal stimulus and the initiation of arousal (Figure 18). The duration of this lag period is influenced by the amount of time spent in torpor (Figure 18). It seems feasible that when animals are induced to arouse prematurely, their ability to regulate the rewarming process is impaired. It is possible that in addition to rewarming dynamics, metabolic activities could also be altered when animals are prematurely induced to arouse from torpor. To investigate metabolic activity, oxygen ( $O_2$ ) consumption and carbon dioxide ( $CO_2$ ) production were measured during natural and induced arousals. The concentrations of glucose, free fatty acids, and lactate in blood plasma were also evaluated.

While torpid, hibernators rely almost entirely on fatty acid metabolism (Lyman et al., 1982). However during arousal, metabolism of glucose is restored (Dark



and Miller, 1997; Westman and Geiser, 2004). When glucose oxidation is inhibited, squirrels take ~ 3 times as long to warm from 10 to 15° C (Dark and Miller, 1997). Given the delayed onset of  $T_b$  increases during induced arousals (Figure 18), it is plausible that the ability to switch fuel sources is impaired during induced arousal. Heart rate, blood pressure, and ventilation are altered during induced arousal (Tähti and Soivio, 1977, 1978). If oxygen supply is limited, lactate, a hallmark of anaerobic metabolism, might be expected to increase during induced arousal (Livingstone, 1983).

The rate of change in  $T_b$  during a natural arousal appears well ordered, indicating coordination between heat generation and heat dissipation systems (Utz et al., 2007; Figure 10). During arousal, brown adipose tissue and skeletal muscle must be supplied with adequate metabolic fuels in order to generate heat (Cannon and Nedergaard, 2004; Horwitz et al., 1968; Fons et al., 1997; Genin et al., 2003). This heat must then be conducted into other body tissues via the cardiovascular system. Changes in  $T_b$  during an induced arousal are frequently more variable than for natural arousal (Figure 10; Table 2). This disorder is consistent with the notion that an animal's ability to balance heat generation and heat dissipation may be impaired during induced arousals. Cardiovascular parameters may be altered during induced arousal (Tähti and Soivio, 1977, 1978). In addition to altered cardiovascular activity, the oxygenation status of the blood may differ as well since ventilatory parameters are also altered during an induced arousal (Tähti and Soivio, 1977). Perhaps the disorder present during

induced arousal is due to an impaired ability to maintain appropriate cardiovascular system activity.

If blood flow during induced arousal is erratic, one might expect that various body tissues would experience periods of inadequate blood supply followed by periods where blood flow is restored. Restricted blood supply, also known as ischemia, followed by reperfusion, the return of blood supply, often leads to reperfusion injury (Allen and Bayraktutan, 2009; Klune and Tsung, 2010; Vardanian et al., 2008). Reperfusion injury leads to inflammation and oxidative stress (Allen and Bayraktutan, 2009). Rapid reintroduction of oxygen can lead to oxidative damage to cellular macromolecules. Oxidative damage to proteins can lead to the formation of carbonyl groups, and oxidative damage to lipids can form peroxides (Murray et al., 2008; Eschwege et al., 1999). These indicators of oxidative damage to proteins and lipids were assessed in heart, liver, kidney, and brain tissues to investigate putative oxidative damage following natural and induced arousals.

## Materials and Methods

### Animals

Golden-mantled ground squirrels (*Spermophilus lateralis*) were live trapped from local populations in Southern Nevada, Southern California, or Southern Utah. Prior to the beginning of the hibernation season, temperature sensitive radiotelemeters (model VM-FH disc; Mini Mitter, Sun River, OR) were surgically implanted into the abdominal cavity. These radiotelemeters allow for precise

measurement of  $T_b$  throughout the hibernation season. Body temperature was recorded every minute to the hundredth of a degree. Animals were housed in an environmental chamber at an ambient temperature of 4°C during the hibernation season. The University of Nevada, Las Vegas Institutional Animal Care and Use Committee approved all procedures.

### Respirometry

Five animals were allowed to proceed through a natural arousal. Subsequently, these same animals were induced to arouse prematurely by mild shaking for 30 seconds. Animals were induced to arouse when they had completed approximately half of the prior torpor bout duration. The initiation and termination of an arousal were determined based on the rate of change in body temperature (Utz et al., 2007). Animals were weighed then placed into an airtight container. Oxygen ( $O_2$ ) consumption and carbon dioxide ( $CO_2$ ) production were estimated using a Sable Systems FoxBox II gas analyzer set to a flow rate of 300 ml/min (Sable Systems Inc., Las Vegas, NV). A flow through setup was utilized, and background levels of  $O_2$  and  $CO_2$  were obtained for 1 to 2 hours prior to placing an animal into the respirometry chamber.

### Plasma Metabolites

For both natural and induced arousals, animals were sacrificed at body temperatures of 10, 20, and 30° C. In addition to arousal, samples from late torpor (LT), and interbout aroused (IBA) animals are included for comparison. Whole blood samples were collected immediately and treated with EDTA (ethylenediaminetetraacetic acid) to prevent clotting. Samples were centrifuged

at 1,500 RCF (relative centrifugal force) for 10 minutes to obtain plasma. Plasma samples were frozen at -80° C. Immediately after thawing, plasma levels of lactate, glucose, and free fatty acids were obtained using commercially available kits or reagents (Pointe Scientific Lactate (Liquid) Reagent Set, Pointe Scientific Liquid Glucose (Oxidase) Reagent Set, Molecular Probes, Inc. ADIFAB Free Fatty Acid Indicator) according to the manufacturer's instructions.

#### Oxidative Damage Indicators

For both natural and induced arousals, animals were sacrificed at body temperatures of 10, 20, and 30° C. Brain, heart, liver, and kidney tissues were collected immediately and snap frozen in liquid nitrogen. In addition to arousal, tissue samples from summer active (SA), late torpor (LT), and interbout aroused (IBA) animals are included for comparison. Tissues were pulverized in liquid nitrogen prior to homogenization.

Samples used in analysis of lipid peroxidation were homogenized on ice in a solution of 20 mM TRIS, pH 7.4 [tris(hydroxymethyl)aminomethane] and 5 mM BHT (butylated hydroxytoluene). Homogenates were centrifuged at 4° C for 10 minutes at 3,000 RCF, separated into aliquots for determination of protein concentration and lipid peroxidation, then frozen at -80° C. Samples were adjusted to 5 mg/ml following measurement of protein concentration. Lipid peroxidation reactions were run in triplicate and assembled as follows: 40 µl of sample, 130 µl of 10 mM 1-methyl-2-phenylindole in acetonitrile/methanol (3:1), and 30 µl of concentrated methanesulfonic acid containing 34 µM Fe(III) were added to a microcentrifuge tube and incubated for 30 minutes at 45° C. Following

incubation, the reaction mixture was centrifuged at room temperature for 10 minutes at 15,000 RCF. One hundred and fifty  $\mu$ l of supernatant was transferred to a 96-well plate, and absorbance at 586 nm was measured. The compound 1,1,3,3-tetramethoxypropane (TMOP) was used to generate a standard curve, and sample lipid peroxide content was calculated from a linear equation derived from the TMOP standards. Each 96-well plate utilized was loaded with all the samples of one tissue type and the TMOP standards. These procedures were modified from Gérard-Monnier et al., 1998 and the BIOMOL ALDetect Lipid Peroxidation Assay Kit.

Samples used in analysis of protein carbonylation were homogenized on ice in 50 mM HEPES, pH 7.2 [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 5 mM EDTA, and 2X protease inhibitor cocktail (Thermo 78410). Homogenates were centrifuged at 4° C for 15 minutes at 10,000 RCF. Supernatants were transferred to a clean tube and incubated with 1% streptomycin sulfate to remove nucleic acid contaminants. Samples were centrifuged at 4° C for 10 minutes at 10,000 RCF, separated into aliquots for determination of protein concentration and protein carbonylation, then frozen at -80° C. Samples were adjusted to 5 mg/ml following measurement of protein concentration. Protein carbonylation reactions were run in triplicate; a sample blank was run in parallel for each sample. Reactions were assembled as follows: 40  $\mu$ l of sample and 160  $\mu$ l of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl (hydrochloric acid) were added to each reaction tube; 40  $\mu$ l of sample and 160  $\mu$ l of 2.5 M HCl were added to each sample blank. All tubes were incubated in the dark at room

temperature for 1 hour; tubes were vortexed every 10 minutes. Tubes were incubated on ice for 5 minutes following the addition of 200  $\mu$ l of 20% TCA (trichloroacetic acid). Samples were centrifuged at 4° C for 10 minutes at 10,000 RCF. Supernatants were removed, and the protein pellet was washed with 200  $\mu$ l of 10% TCA. Pellets were washed twice in 200  $\mu$ l of ethanol/ethyl acetate (1:1) then centrifuged at 4° C for 10 minutes at 10,000 RCF. Finally, pellets were dissolved in 200  $\mu$ l of 8 M guanidine HCl. One hundred and fifty  $\mu$ l of supernatant was transferred to a 96-well plate, and absorbance at 360 nm was measured following a final 10 minute centrifugation at 4° C, 10,000 RCF. Protein carbonyl content was calculated by subtracting the absorbance of the sample blank from the sample. Each 96-well plate was loaded with all the samples from a single stage of torpor as well as a reference sample; reference sample absorbance varied by  $\pm$  3% between plates (data not shown). These procedures were modified from Levine et al., 1990 and the Cayman Protein Carbonyl Assay Kit.

### Statistical Analyses

Respirometry data were subjected to a paired t-test. Plasma metabolite data were subjected to ANOVA. Data from analysis of oxidative damage indicators were subjected to ANOVA. All analyses were conducted with Statview 4.1 software. A p value < 0.05 was considered significant for all analyses.

## Results

### Respirometry

Animals were allowed to proceed through a natural undisturbed arousal. Midway through the following torpor bout, these same animals were subjected to 30 seconds of mild shaking to induce a premature arousal. Oxygen (O<sub>2</sub>) consumption and carbon dioxide (CO<sub>2</sub>) production were measured. The respiratory quotient (RQ) was calculated as the ratio of CO<sub>2</sub> production/ O<sub>2</sub> consumption. Figure 19 depicts a representative trace of O<sub>2</sub> consumption, CO<sub>2</sub> production, and RQ values for a natural and induced arousal from one animal. Increases in O<sub>2</sub> consumption and CO<sub>2</sub> production are typically delayed during the onset of an induced arousal (Figure 19).

Mean rates of O<sub>2</sub> consumption, CO<sub>2</sub> production, and RQ values at the initiation of arousal are depicted in Figure 20. Mass specific O<sub>2</sub> consumption was  $0.00379 \pm 0.001 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for natural arousal and  $0.00347 \pm 0.001 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for induced arousal. Type of arousal did not affect initial rates of oxygen consumption ( $p > 0.05$ ). Mass specific CO<sub>2</sub> production was  $0.000769 \pm 0.0002 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for natural arousal and  $0.000152 \pm 0.00005 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for induced arousal. Initial RQ values were  $0.27 \pm 0.07$  and  $0.073 \pm 0.04$  for natural and induced arousals, respectively. Type of arousal had a significant effect on initial rates of CO<sub>2</sub> production and RQ values ( $p < 0.05$ ).

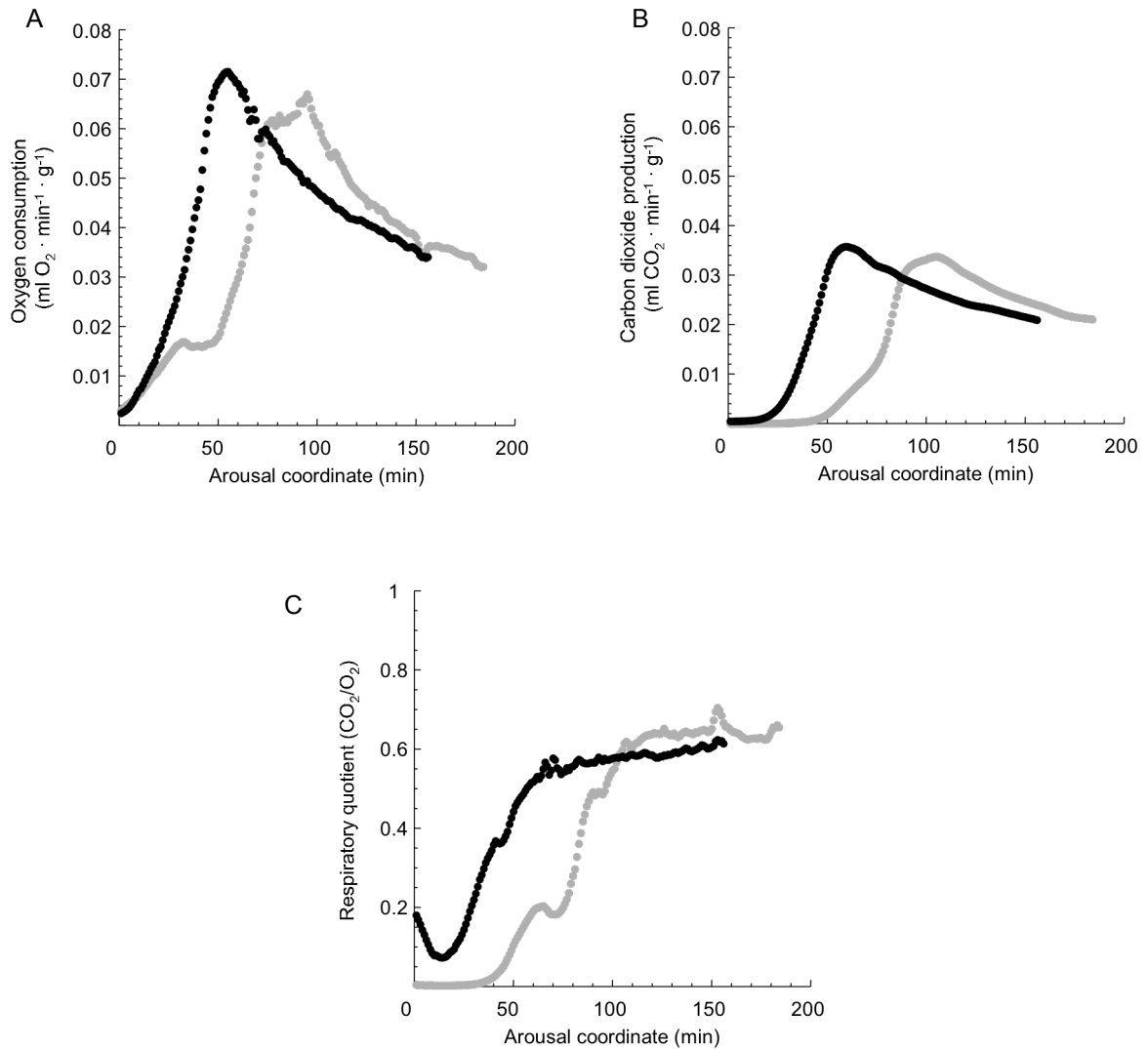


Figure 19. Representative traces of oxygen consumption, carbon dioxide production, and respiratory quotient values for natural and induced arousals from one individual. Mass specific rates of O<sub>2</sub> consumption (A), mass specific rates of CO<sub>2</sub> production (B), and RQ values (C) for natural (black) and induced (gray) arousal from one individual housed at 4° C.



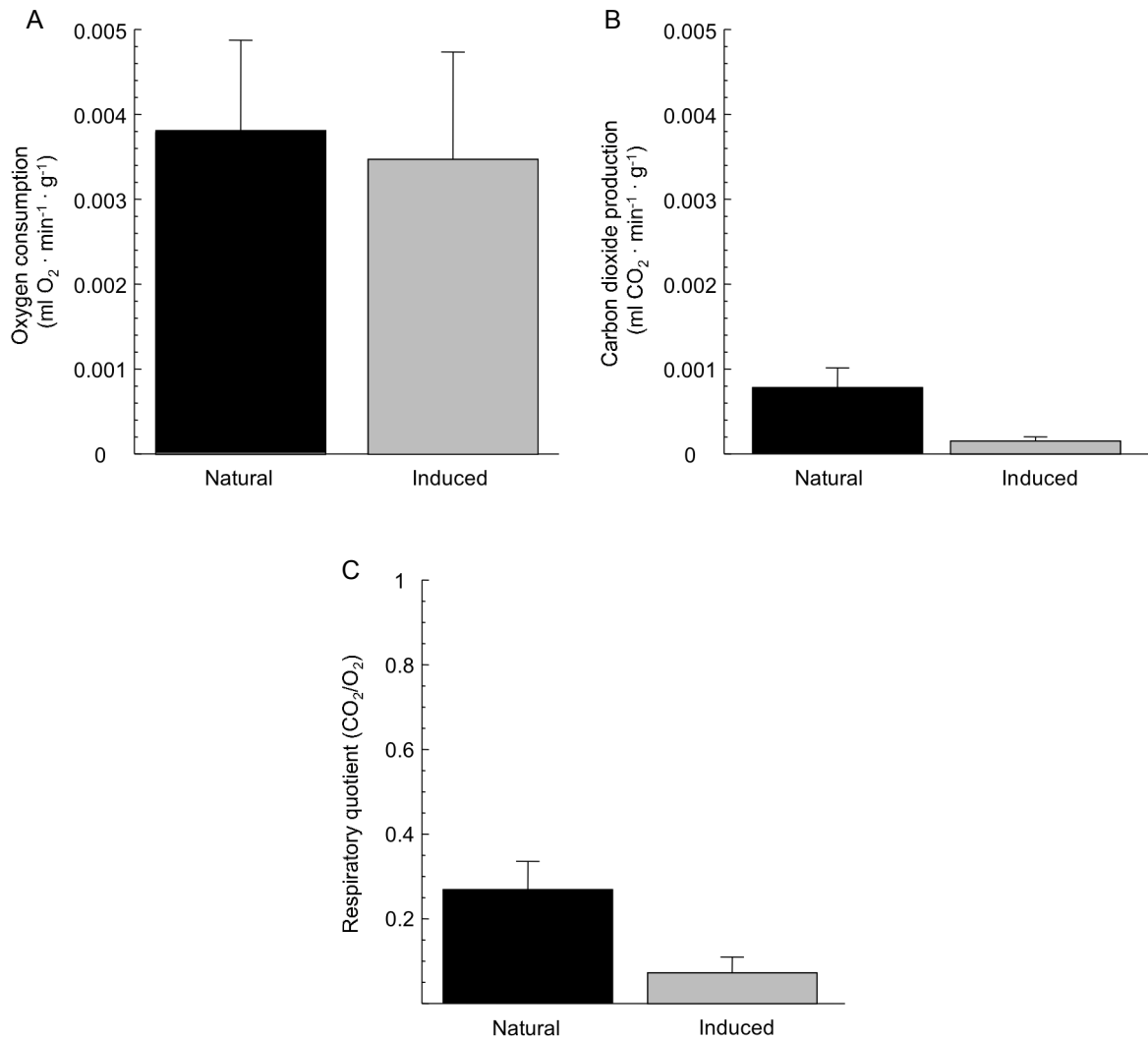


Figure 20. Oxygen consumption, carbon dioxide production and respiratory quotient values at the initiation of arousal. Bars represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . All animals were housed at 4° C. Arousal type does not have a significant affect on (A) mass specific O<sub>2</sub> consumption ( $p > 0.05$ ). Type of arousal significantly affected (B) mass specific CO<sub>2</sub> production and (C) RQ values ( $p < 0.05$ ).

Figure 21 illustrates mean rates of O<sub>2</sub> consumption, CO<sub>2</sub> production, and RQ values at the termination of arousal. For both natural and induced arousal, mass specific O<sub>2</sub> consumption increased  $\sim 11$  fold from the initiation to the termination of arousal. Mass specific O<sub>2</sub> consumption at the termination of arousal was

$0.0431 \pm 0.005 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for natural arousal and  $0.0382 \pm 0.002 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for induced arousal. Type of arousal did not significantly affect rates of oxygen consumption at the end of arousal ( $p > 0.05$ ).

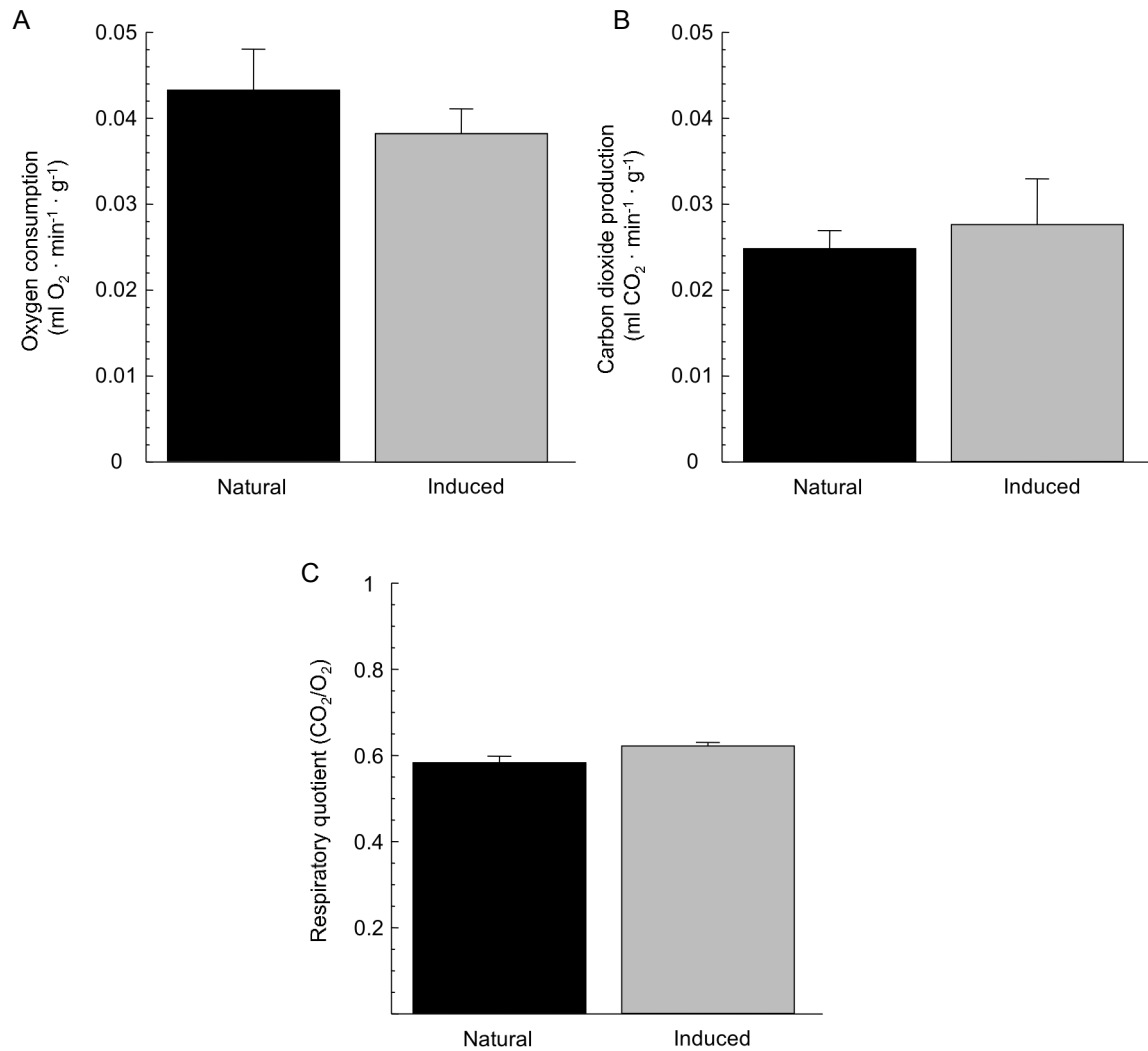


Figure 21. Oxygen consumption, carbon dioxide production and respiratory quotient values at the termination of arousal. Bars represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . All animals were housed at  $4^\circ \text{C}$ . Arousal type does not have a significant affect on (A) mass specific  $\text{O}_2$  consumption or (B) mass specific  $\text{CO}_2$  production ( $p > 0.05$ ). Type of arousal has a significant effect on (C) RQ values ( $p < 0.05$ ).

Mass specific CO<sub>2</sub> production increased ~ 32 fold from initiation to termination for natural arousals whereas the low initial rates for induced arousal led to ~ 181 fold increase. Mass specific CO<sub>2</sub> production at the end of arousal was  $0.0247 \pm 0.002 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for natural arousal and  $0.0276 \pm 0.005 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  for induced arousal. Type of arousal did not have an affect on CO<sub>2</sub> production ( $p > 0.05$ ). RQ values at the end of arousal were  $0.58 \pm 0.02$  and  $0.062 \pm 0.01$  for natural and induced arousals, respectively. Type of arousal had a significant effect on RQ values at the end of arousal ( $p < 0.05$ ).

The body temperature associated with maximum rates of increase in oxygen consumption and carbon dioxide production was measured (Figure 22). Animals experienced a maximum rate of change in O<sub>2</sub> consumption at a T<sub>b</sub> of  $8.96 \pm 0.89^{\circ} \text{C}$  for natural arousals and  $9.06 \pm 0.75^{\circ} \text{C}$  for induced arousals. Animals experienced a maximum rate of change in CO<sub>2</sub> production at a T<sub>b</sub> of  $8.80 \pm 0.46^{\circ} \text{C}$  for natural arousals and  $11.4 \pm 2.24^{\circ} \text{C}$  for induced arousals. It is interesting to note that during natural arousal, animals experience maximum rates of change in O<sub>2</sub> consumption and CO<sub>2</sub> production when T<sub>b</sub> is ~ 9° C. Whereas during induced arousal the T<sub>b</sub> associated with maximum increases in CO<sub>2</sub> production is generally elevated and more variable than for corresponding natural arousals. Type of arousal did not affect the body temperature associated with a maximum rate of change in O<sub>2</sub> consumption or CO<sub>2</sub> production ( $p > 0.05$ ).

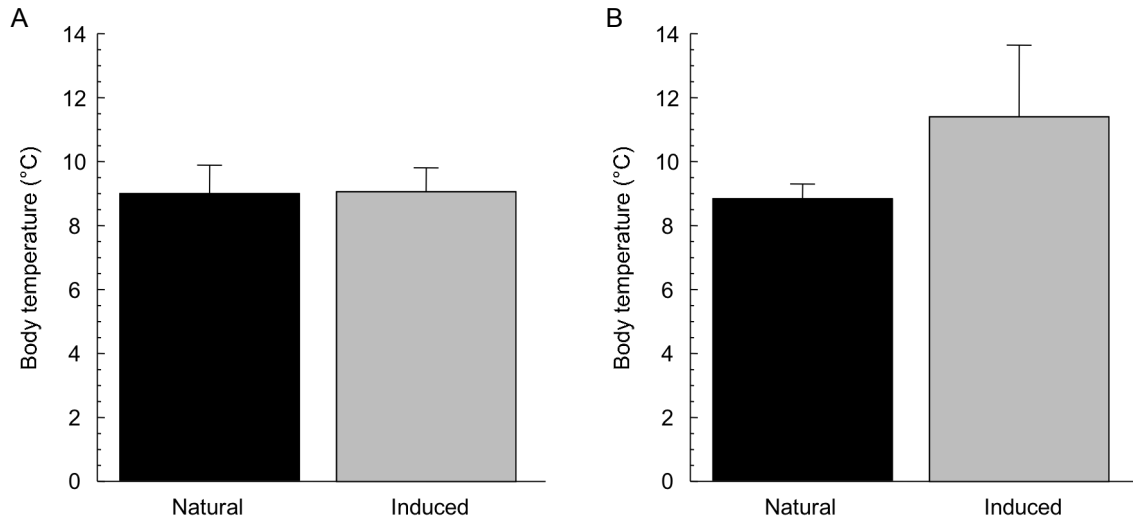


Figure 22. Body temperature associated with maximum rates of increase in oxygen consumption and carbon dioxide production. Bars represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 5$ . All animals were housed at 4° C. Arousal type does not have a significant affect on the  $T_b$  associated with maximum rates of increase in (A) O<sub>2</sub> consumption or (B) CO<sub>2</sub> production ( $p > 0.05$ ).

### Plasma Metabolites

During torpor, hibernators rely almost entirely on fatty acid metabolism, however during arousal metabolism of glucose is restored (Lyman et al., 1982; Dark and Miller, 1997; Westman and Geiser, 2004). The concentration of glucose in blood plasma was measured in animals representing natural arousal, induced arousal, interbout aroused (IBA), and late torpor states (Figure 23). Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C. Plasma glucose concentration during late torpor was  $106 \pm 43$  mg/dl. Glucose concentration fell to  $58 \pm 2$  mg/dl for naturally arousing animals at 10° C. Values were comparable for natural and induced arousal; glucose concentrations increased  $\sim 3$  fold as animals warmed from 10 to 30° C. The highest glucose

concentration measured was  $233 \pm 11$  mg/dl, which occurred during the IBA period. Hibernation state did not affect plasma glucose concentration ( $p > 0.05$ ).

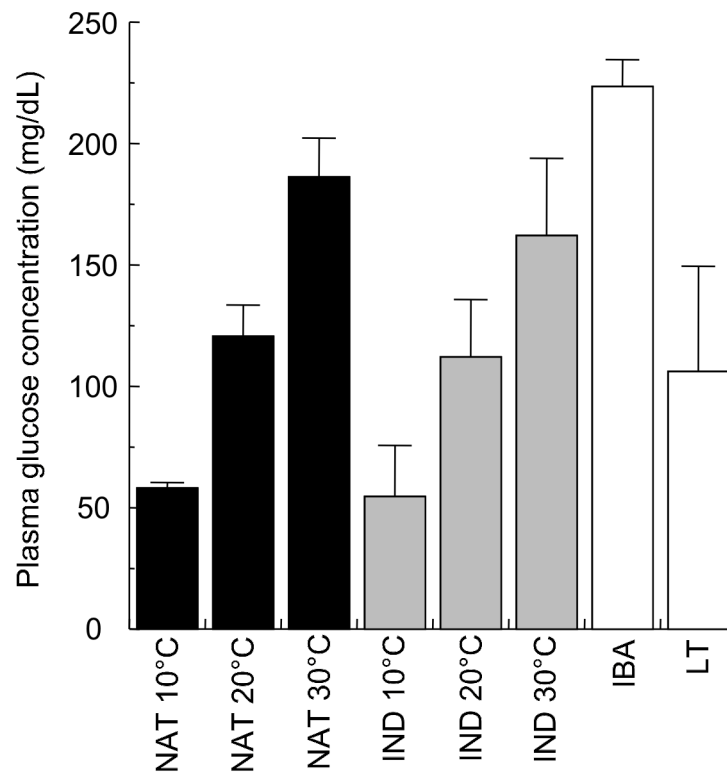


Figure 23. Concentration of glucose in blood plasma. Bars represent means  $\pm$  SE for natural arousal (black), induced arousal (gray), interbout aroused (IBA; white), and late torpor (LT; white) animals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C;  $n = 3$  for all groups except IND 10° C where  $n = 2$ . Hibernation state did not significantly affect glucose concentration ( $p > 0.05$ ).

Plasma levels of free fatty acids (FFA) were investigated across multiple stages of a torpor bout, including natural and induced arousal (Figure 24). Late torpor free fatty acid levels were  $122 \pm 7$   $\mu\text{g}/\mu\text{l}$ . FFA levels declined slightly to  $99 \pm 26$   $\mu\text{g}/\mu\text{l}$  during natural arousal at 10° C. Values were comparable for natural

and induced arousals at 10° C. FFA levels approximately doubled between body temperatures of 10 and 20° C for both types of arousal, and these elevated levels were maintained at 30° C and during the IBA period. Hibernation state significantly affected FFA levels ( $p < 0.05$ ). Free fatty acid levels were significantly greater at body temperatures of 20 and 30° C compared to 10° C for both natural and induced arousal ( $p < 0.05$ ).

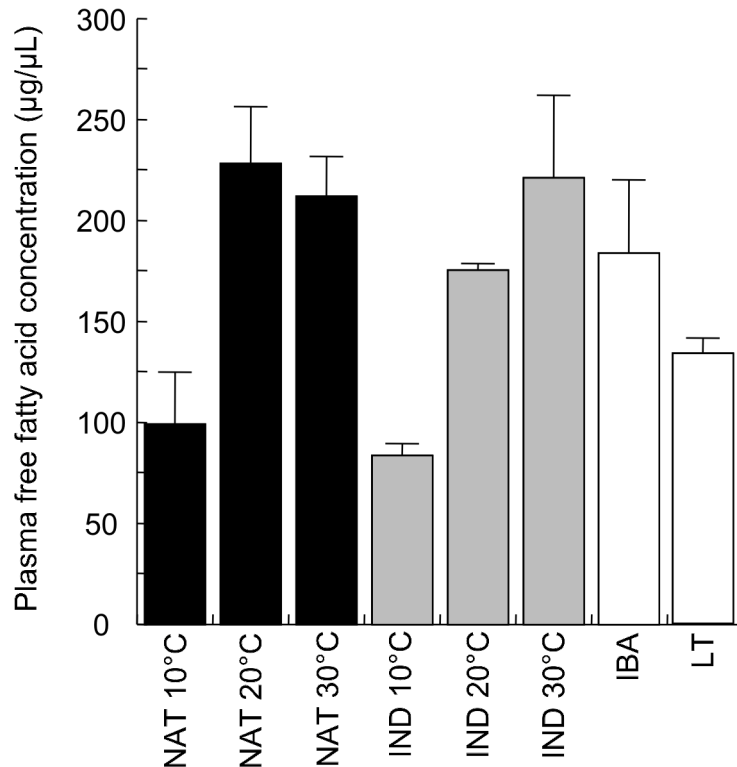


Figure 24. Concentration of free fatty acids in blood plasma. Bars represent means  $\pm$  SE for natural arousal (black), induced arousal (gray), interbout aroused (IBA; white), and late torpor (LT; white) animals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C;  $n = 3$  for all groups except IND 10° C where  $n = 2$ . Hibernation state has a significant effect on free fatty acid levels ( $p < 0.05$ ). Values at 20° C, 30° C, and during IBA are significantly greater than during arousal at 10° C for both natural and induced arousal ( $p < 0.05$ ).

Lactate, a hallmark of anaerobic metabolism, was measured for natural and induced arousal from torpor (Figure 25). During late torpor, plasma lactate

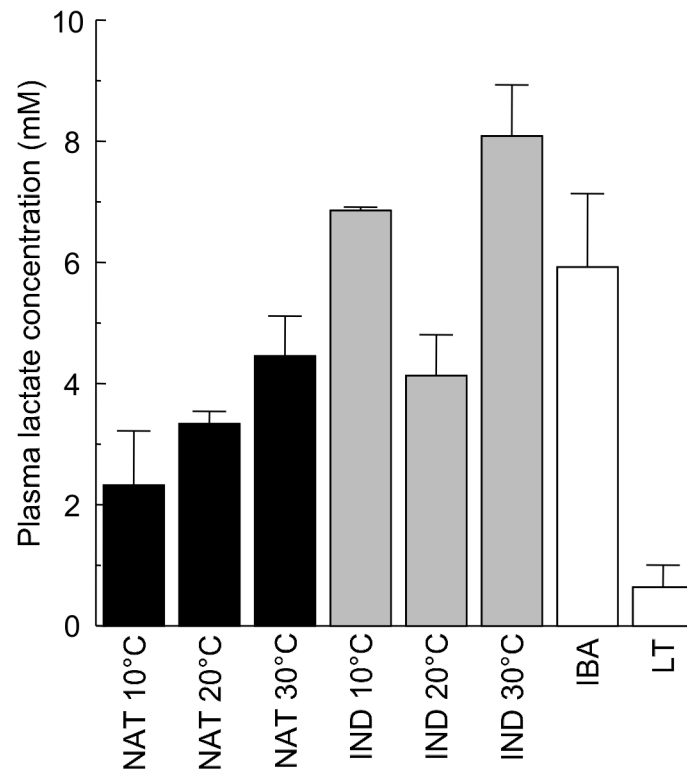


Figure 25. Concentration of lactate in blood plasma. Bars represent means  $\pm$  SE for natural arousal (black), induced arousal (gray), interbout aroused (IBA; white), and late torpor (LT; white) animals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C;  $n = 3$  for all groups except IND 10° C where  $n = 2$ . Hibernation state had a significant effect on plasma lactate concentrations ( $p < 0.05$ ). Lactate concentrations from all three groups of induced arousal animals were significantly greater than late torpor values ( $p < 0.05$ ). Lactate concentrations are significantly different between natural and induced arousal animals at body temperatures of 10 and 30° C ( $p < 0.05$ ).

concentration was  $0.64 \pm 0.36$  mM. During natural arousal, lactate progressively increased to a peak value of  $4.5 \pm 0.66$  mM, corresponding to a body temperature of 30° C. During induced arousal at 10° C, lactate concentration was

6.8 ± 0.05 mM; this value is ~ 3 fold greater than the corresponding value for natural arousal. The highest lactate concentration measured was 8.1 ± 0.85 mM, corresponding to induced arousal at 30° C. Lactate levels for induced arousals at 30° C were more than 12 fold greater than during late torpor. Lactate levels remained elevated during the IBA period. Hibernation state had a significant effect on plasma lactate concentrations ( $p < 0.05$ ). Lactate concentrations from all three groups of induced arousal animals were significantly greater than late torpor values ( $p < 0.05$ ). During natural arousal, a significant difference was only observed when animals reached a  $T_b$  of 30° C. Lactate concentrations are significantly different between natural and induced arousal animals at body temperatures of 10 and 30° C ( $p < 0.05$ ).

Figure 26 presents summary plasma metabolite data for natural and induced arousal to facilitate a direct comparison of these two states. Overall, plasma concentrations of glucose and free fatty acids are similar ( $p > 0.05$ ). In contrast, plasma lactate concentrations are significantly increased during induced arousal ( $p < 0.05$ ).



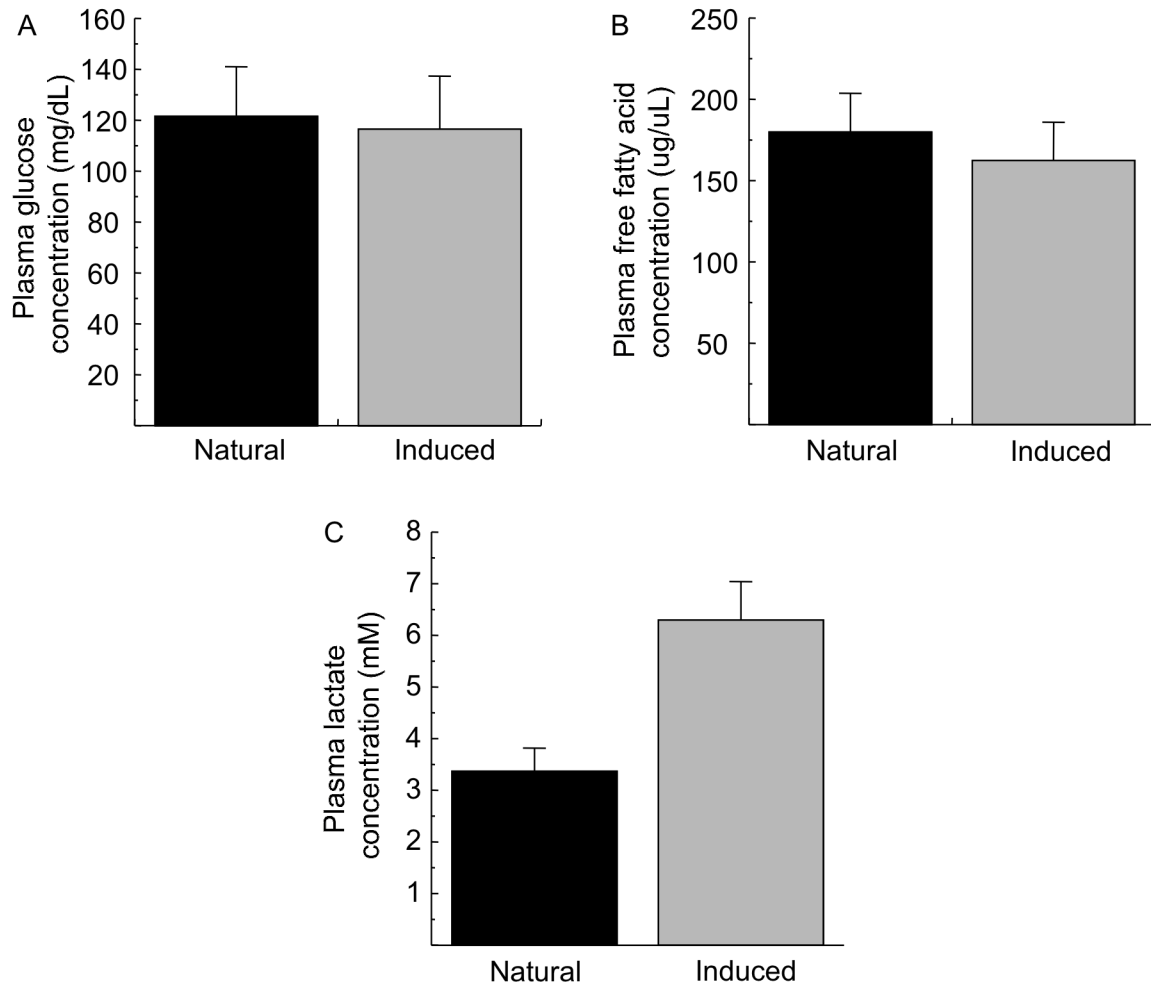


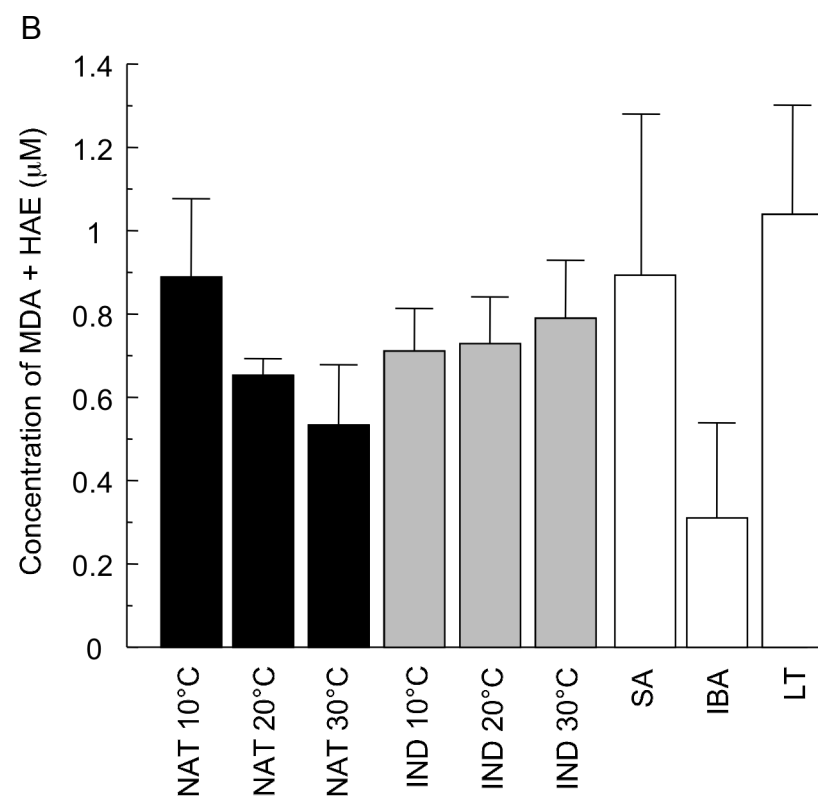
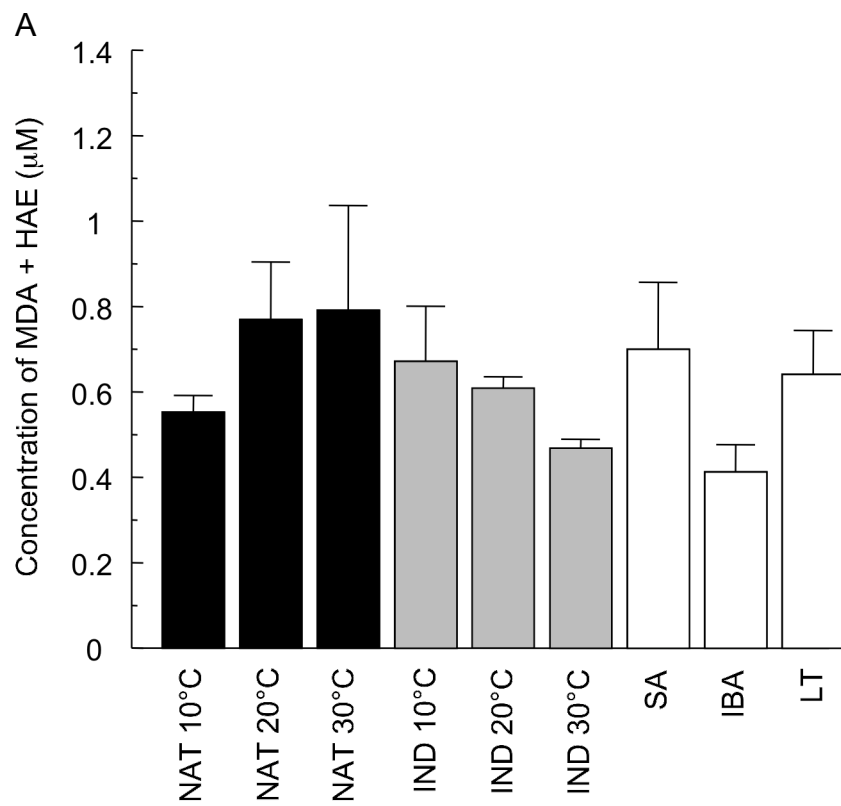
Figure 26. Summary data for plasma concentrations of glucose, free fatty acids, and lactate during natural and induced arousals. Bars represent means  $\pm$  SE for natural (black) and induced (gray) arousal;  $n = 9$  for natural arousal,  $n = 8$  for induced arousal. Concentrations of glucose (A) and free fatty acids (B) are comparable ( $p > 0.05$ ). Type of arousal has a significant effect on plasma lactate (C) concentration ( $p < 0.05$ ).

### Oxidative Damage Indicators

Previous studies have shown that cardiovascular and ventilatory system activities are altered during induced arousal (Tähti and Soivio, 1977,1978). To investigate the possibility of reperfusion injury during arousal, oxidative damage to cellular macromolecules was assessed. Lipid peroxides decompose into

various compounds including reactive aldehydes. The lipid peroxidation assay detects the decomposition products malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). Lipid peroxidation was assessed in heart, liver, kidney, and brain tissue for both natural and induced arousals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C. Tissues from summer active, late torpor, and interbout aroused (IBA) animals are included for comparison.

The concentration of MDA and HAE in heart tissue ranged from  $0.410 \pm 0.06$   $\mu\text{M}$  for IBA animals to  $0.792 \pm 0.25$   $\mu\text{M}$  for natural arousal at 30° C (Figure 27A). Hibernation state did not affect lipid peroxidation in heart ( $p > 0.05$ ). Values for the liver ranged from  $0.311 \pm 0.23$   $\mu\text{M}$  for IBA animals to  $1.040 \pm 0.26$   $\mu\text{M}$  for LT animals (Figure 27B). Hibernation state did not affect lipid peroxidation in liver ( $p > 0.05$ ). For the kidney, the concentration of MDA and HAE ranged from  $0.209 \pm 0.07$   $\mu\text{M}$  for IBA animals to  $0.877 \pm 0.09$   $\mu\text{M}$  for LT animals (Figure 27C). Hibernation state had a significant effect on kidney peroxide levels ( $p < 0.05$ ). Lipid peroxide levels were significantly greater during late torpor than all other states ( $p < 0.05$ ). Lipid peroxide levels in the brain were less than in the other tissues. Values for the brain ranged from  $0.061 \pm 0.04$   $\mu\text{M}$  for induced arousal at 30° C to  $0.206 \pm 0.01$   $\mu\text{M}$  for LT animals. Hibernation state did not affect lipid peroxidation in brain (Figure 27D).



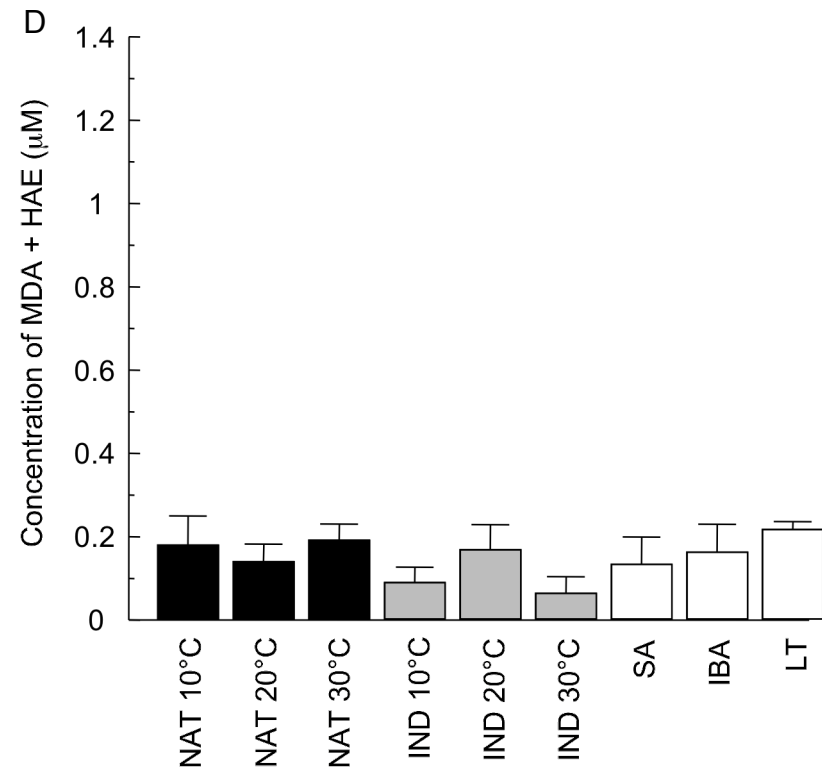
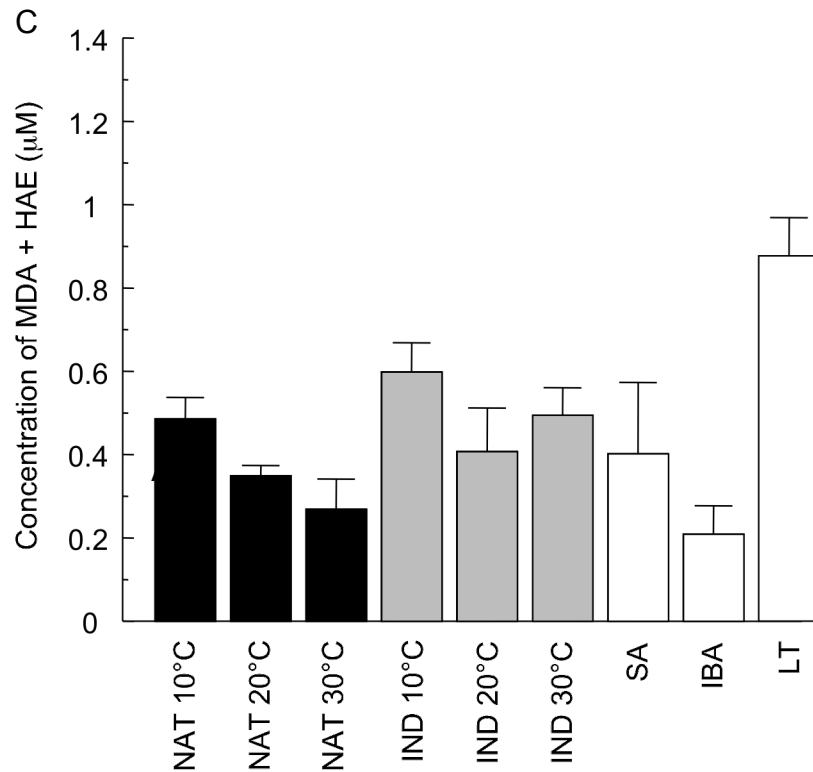
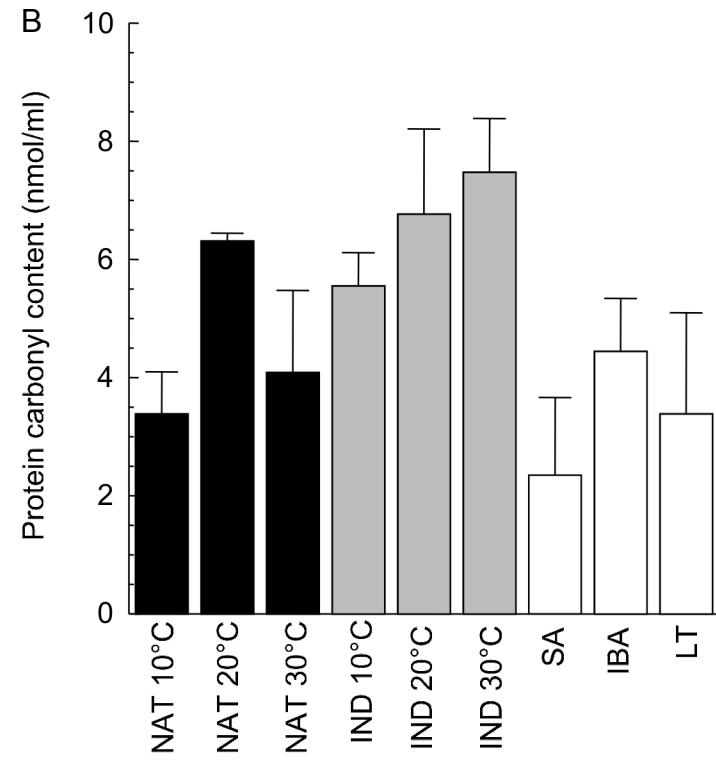
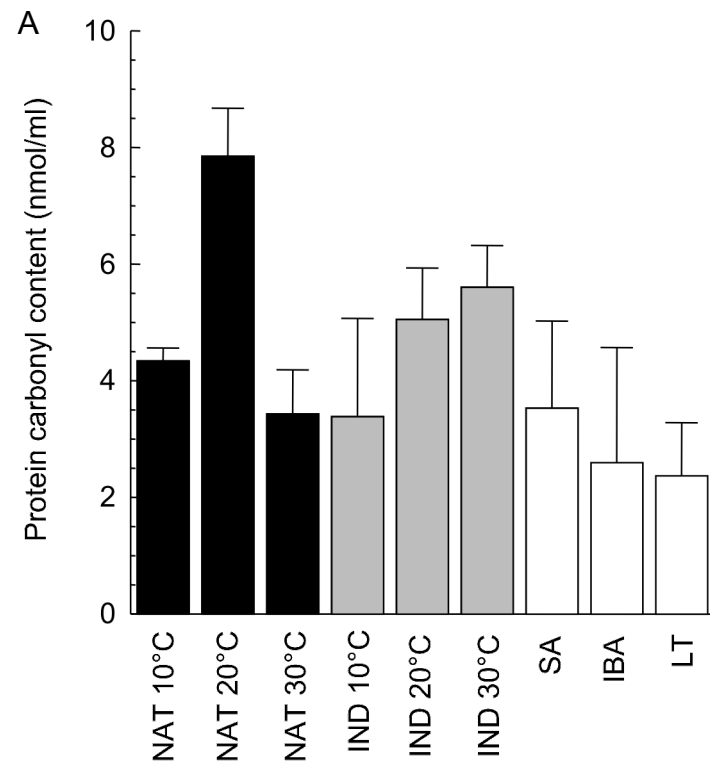


Figure 27. Lipid peroxidation levels for several stages of hibernation. Lipid peroxidation was assessed in (A) heart, (B) liver, (C) kidney, and (D) brain. Bars represent means  $\pm$  SE for natural arousal (black), induced arousal (gray), summer active (SA; white), interbout aroused (IBA; white), and late torpor (LT; white) animals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C;  $n = 3$  for all groups. Hibernation state did not affect lipid peroxidation in heart, liver, or brain ( $p > 0.05$ ). Hibernation state had a significant effect on lipid peroxidation in the kidney; levels were greater during late torpor than all other states ( $p < 0.05$ ).

In addition to lipids, proteins can also be oxidatively damaged, and protein oxidation may be associated with the formation of carbonyl groups. Protein carbonyl content was measured in heart, liver, kidney, and brain tissues for several stages of hibernation (Figure 28). In heart samples, carbonyl content ranged from  $2.374 \pm 0.91$  nmol/ml for LT animals to  $7.854 \pm 0.82$  nmol/ml for natural arousals at 20° C (Figure 28A). In the liver carbonyl content ranged from  $2.348 \pm 1.31$  nmol/ml for SA animals to  $7.475 \pm 0.91$  nmol/ml in induced arousals at 30° C (Figure 28B). Values for kidney ranged from  $1.970 \pm 0.68$  nmol/ml to  $5.707 \pm 0.70$  nmol/ml for SA and induced arousal 30° C animals, respectively (Figure 28C). Hibernation state did not affect protein carbonyl content for heart, liver, or kidney samples ( $p > 0.05$ ). Carbonyl content in brain tissue ranged from  $1.313 \pm 0.33$  nmol/ml to  $4.596 \pm 0.66$  nmol/ml for natural arousals at 10° C and induced arousals at 30° C, respectively (Figure 28D). Hibernation state had a significant effect on brain carbonyl content ( $p < 0.05$ ). Values for induced arousals at 30° C were greater than other states, except IBA and NAT 20° C.



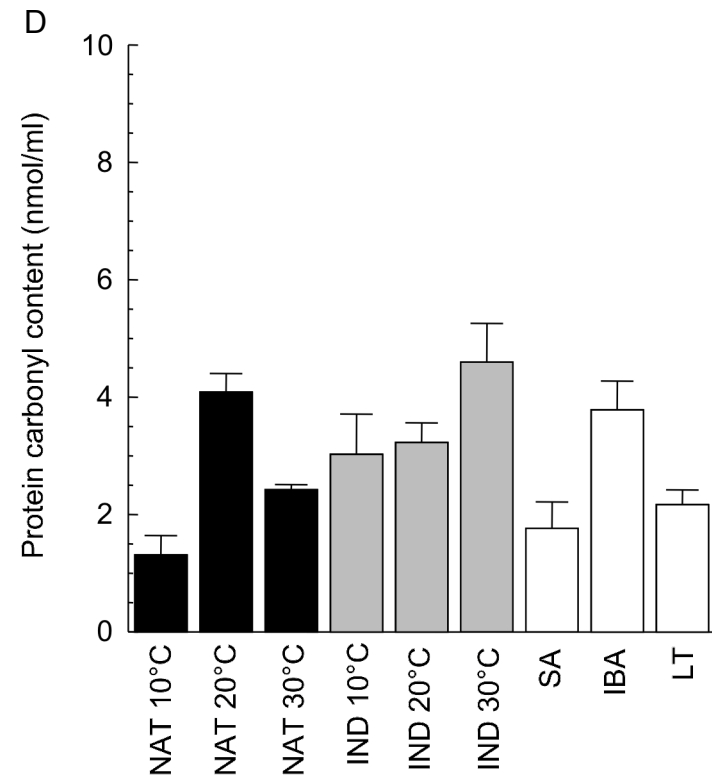
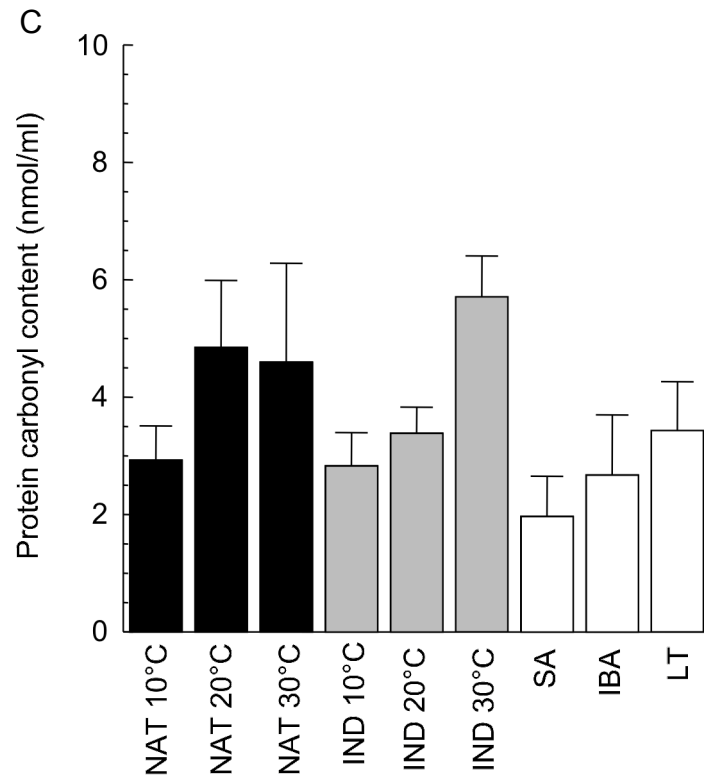


Figure 28. Protein carbonyl levels for several stages of hibernation. Protein carbonylation was assessed in (A) heart, (B) liver, (C) kidney, and (D) brain. Bars represent means  $\pm$  SE for natural arousal (black), induced arousal (gray), summer active (SA; white), interbout aroused (IBA; white), and late torpor (LT; white) animals. Arousing animals were sacrificed at body temperatures of 10, 20, or 30° C;  $n = 3$  for all groups. Hibernation state did not affect lipid peroxidation in heart, liver, or kidney ( $p > 0.05$ ). Hibernation state had a significant effect on protein carbonyl content in brain ( $p < 0.05$ ). Carbonyl content for induced arousals at 30° C was greater than other states, except IBA and NAT 20° C.

## Discussion

Although little attention has previously been paid to the distinctions between natural and induced arousals, documentation of differences between these two states is accumulating. Initially, differences in heart rate, blood pressure, and ventilatory patterns were documented by Tähti and Soivio (1977; 1978). A previous investigation of rewarming dynamics demonstrated differences for the maximum rate of rewarming, arousal duration, and IBA duration following prematurely induced arousals (Figures 11, 13, and 17). Further, responsiveness to the induced arousal stimulus was significantly influenced by the amount of time spent in torpor (Figure 18). Given these changes in cardiovascular and rewarming dynamics, it seems feasible that metabolic parameters may also be altered during induced arousal.

An indirect calorimetry system was utilized to estimate metabolic rate. Oxygen ( $O_2$ ) consumption and carbon dioxide ( $CO_2$ ) production were measured. The respiratory quotient (RQ) was calculated as the ratio of  $CO_2$  produced to  $O_2$  consumed. The representative traces of  $O_2$  consumption,  $CO_2$  production and RQ values depicted in Figure 19 demonstrate the delayed onset common during induced arousal. Although initial rates of  $O_2$  consumption are similar, rates of  $CO_2$  production and therefore RQ values differ between natural and induced arousals (Figure 20). Rates of  $CO_2$  production are approximately 5 times greater in the first minutes of a natural arousal (Figure 20).  $O_2$  consumption and  $CO_2$  production rates are comparable by the end of arousal, however RQ values still differ (Figure 21). Due to the extremely low initial rates of  $CO_2$  production, during



induced arousal animals experience ~ 181 fold increase in the rate of CO<sub>2</sub> production; during natural arousal the increase is ~ 32 fold. Body temperature (T<sub>b</sub>) has been indicated as an important regulatory cue for other aspects of rewarming and was therefore included in the respirometry analysis (Utz et al., 2007). For natural arousal, maximum rates of change in O<sub>2</sub> consumption and CO<sub>2</sub> production occurred at a T<sub>b</sub> of ~ 9° C (Figure 22). During induced arousal the T<sub>b</sub> for O<sub>2</sub> consumption was similar; the value for CO<sub>2</sub> production was ~ 11.5° C, though this difference was not found to be statistically significant (Figure 22).

Hibernators rely almost entirely on fatty acid metabolism during torpor, however metabolism of glucose is restored during arousal (Lyman et al., 1982; Dark and Miller, 1997; Westman and Geiser, 2004). When glucose oxidation is inhibited, squirrels take approximately 3 times as long to warm from 10 to 15° C (Dark and Miller, 1997). Given the delayed onset of T<sub>b</sub> increases during induced arousals (Figure 18), perhaps prematurely inducing arousal impairs the ability to switch fuel sources. To address availability of fuels and metabolites, blood plasma levels of glucose, free fatty acids (FFA), and lactate were measured. Glucose concentration progressively increased during both natural and induced arousals; levels increased ~ 3 fold as animals warmed from 10 to 30° C. Glucose levels remained elevated during the interbout aroused (IBA) period (Figure 23). Release of glucose into the blood does not appear to be impaired (Figures 23 and 26). Free fatty acid levels were comparable for arousing animals at 10° C. FFA concentrations doubled between 10 and 20° C for both types of arousal and remained elevated at 30° C and during the IBA (Figure 24). Release of FFA into

the blood does not appear to be impaired during induced arousal from torpor (Figures 24 and 26). At 10° C plasma lactate levels were ~ 3 fold greater for induced arousal (Figure 25). Further, lactate levels for induced arousals at 30° C were more than 12 fold greater than the values associated with late torpor. Lactate levels differ markedly for natural versus induced arousal (Figures 25 and 26).

Lactate is often associated with anaerobic metabolism, however lactate oxidation also plays a role in maintaining redox balance within cells (Brooks, 2002). Increased lactate levels may be the result of limited oxygen supply however increased lactate levels can also facilitate an increase in  $\text{NAD}^+$  pools (Brooks, 2002). Although lactate increases during induced arousal, cellular fuels, namely glucose and FFA do not appear limited (Figures 23 through 26). Perhaps the ability of cells to oxidize these fuels is impaired. Limited oxygen delivery to cells would be consistent with the metabolite data. Even if ventilation is normal, aberrant circulation may lead to insufficient oxygen delivery at the tissue level. Respirometry data indicate the amount of  $\text{O}_2$  going into the animal is the same, however the amount of  $\text{CO}_2$  coming out is reduced during induced arousal (Figure 20). These data are consistent with induced arousal leading to insufficient oxygen delivery or impaired ability of cells to oxidize metabolic fuels. Investigation of anaerobic metabolic activity during natural and induced arousal should be illuminating.

In addition to metabolic sources,  $\text{CO}_2$  can also be released due to pH regulation. As temperature is increased, there is a decrease in blood pH. The

reverse relationship is also true; decreases in temperature lead to an increase in blood pH. This relationship between blood pH and temperature is due to the acid-ionization constants ( $pK_a$ ) of buffers such as bicarbonate in the blood.

Hibernators can experience more than 30° C changes in body temperature. As body temperature decreases, pH would be expected to increase due to the aforementioned temperature effects. If blood pH is maintained while temperature decreases, there must be an acidification. As hibernators enter torpor, blood pH is maintained near euthermic values, indicating the occurrence of a respiratory acidosis (Malan et al., 1985, 1988). During arousal, this excess CO<sub>2</sub> is expelled (Malan et al., 1988). The decreased levels of CO<sub>2</sub> production during the initial period of an induced arousal may indicate impaired pH regulation.

Many cardiovascular and respiratory parameters are altered during induced arousal, and the ability of an animal to precisely regulate increases in body temperature seems impaired (Figures 10, 11, 13, 17, 18, 19, 20, and 26; Tähti and Soivio, 1977; 1978). These observations are consistent with the notion of aberrant circulation during induced arousal. If blood flow during induced arousal is erratic, one might expect that various body tissues would experience periods of inadequate blood supply followed by periods where blood flow is restored. Such ischemia reperfusion events might lead to reperfusion injury and oxidative damage to cellular macromolecules (Allen and Bayraktutan, 2009; Klune and Tsung, 2010; Vardanian et al., 2008). Oxidative damage to proteins can lead to the formation of carbonyl groups, and oxidative damage to lipids can form peroxides (Murray et al., 2008; Eschwege et al., 1999). These indicators of

oxidative damage to proteins and lipids were assessed in heart, liver, kidney, and brain tissues to investigate putative oxidative damage following natural and induced arousals.

Hibernation state did not affect the prevalence of lipid peroxides or protein carbonyls in most tissues (Figures 27 and 28). Previous studies have indicated signs of oxidative stress and activation of stress-related signaling molecules during hibernation (Carey et al, 2000; Lee et al., 2002). Other groups have reported elevated antioxidant defense systems during hibernation (Tøien et al, 2001; Drew et al., 2002). Thus it appears that hibernators are experiencing stress and engaging protective mechanisms. However, the effectiveness of these protective mechanisms has not been as well studied. The low levels of lipid peroxides and protein carbonyls indicate that hibernators are well protected from oxidative damage during both natural and induced arousal as well as throughout the other periods of a torpor bout (Figures 27 and 28).

As hibernators progress through the winter season, they experience a myriad of physiology. Arousal from torpor is a multifaceted event, and its intricacies are currently being elucidated. Many investigations have utilized animals that were induced to arouse prematurely. Induced arousals differ from natural arousals in several important ways. It has previously been demonstrated that prematurely inducing arousal alters the rewarming rates, arousal duration, and IBA duration. Herein I have shown that in addition to rewarming dynamics, metabolic activity is also altered. Rates of CO<sub>2</sub> production and RQ values are markedly reduced during the first minutes of induced arousal (Figure 20). Although rates of O<sub>2</sub>

consumption and CO<sub>2</sub> production are equivalent for natural versus induced arousals by the final minutes, RQ values remain decreased during induced arousal (Figure 21). Plasma levels of glucose and free fatty acids are equivalent, but plasma lactate levels increase significantly during induced arousal (Figures 23 through 26). These metabolic alterations may indicate issues with fuel oxidation and/or pH regulation. Even so, hibernators appear well protected against oxidative damage as lipid peroxides and protein carbonyls remain low during both types of arousal (Figures 27 and 28).

## CHAPTER 5

### SUMMARY, SIGNIFICANCE, AND FUTURE STUDIES

At a basal level of understanding, a hibernation season seems to be comprised of two distinct and dissimilar physiological states: torpor and euthermia. Upon closer inspection, one gains an appreciation for the intricacies of the transition phases, entrance and arousal. Even finer queries have yielded insights into the spectrum of physiological states experienced throughout a single torpor bout. Yet a deep understanding of the process of arousal has been lacking. It was previously thought that animals should arouse as rapidly as possible to minimize energetic expenditure (Figure 2; Stone and Purvis, 1992).

Investigation of natural arousals at varying ambient temperatures indicated that arousal is a more constrained, regulated process than previously expected. The data indicate the dynamics of rewarming are affected by both time and body temperature (Utz et al., 2007). Maximum rate of rewarming (RRW) and the time required to reach maximum RRW decrease with increasing  $T_a$ , the  $T_b$  associated with maximum RRW increases with increasing  $T_a$ , and all animals reached their maximum rewarming rates when they had generated 30 to 40% of the heat required to reach a euthermic  $T_b$  (Utz et al., 2007).

The question of how an animal arouses from torpor becomes important when one considers the multifaceted nature of this transition period. Many investigations have utilized animals that were induced to arouse prematurely (Table 1). However direct comparisons between natural and induced arousal are extremely limited (Table 1). When dynamics are compared for natural versus

induced arousal, many aspects differ. When animals are prematurely induced to arouse from torpor, the variability for changes in body temperature is increased (Table 2). The maximum rewarming rate is increased (Figure 11). Further, these increased rewarming rates occur with sporadic frequency and may be followed by decreases in  $T_b$  (Figure 10B). Arousal duration is increased (Figure 13). The duration of the interbout arousal (IBA) period is decreased (Figure 17). Also, the responsiveness of animals to the induced arousal stimulus is influenced by the amount of time spent in torpor (Figure 18). These alterations are consistent with the notion that induced arousal impairs an animal's ability to regulate the rewarming process, particularly the cardiovascular and circulatory systems.

Given altered rewarming dynamics during induced arousals, metabolic activity and the potential for reperfusion injury and associated oxidative damage to cellular macromolecules were also investigated. The representative traces of  $O_2$  consumption,  $CO_2$  production and RQ values depicted in Figure 19 demonstrate the delayed onset common during induced arousal. Initial rates of  $O_2$  consumption are similar (Figure 20). However, initial rates of  $CO_2$  production and therefore RQ values differ between natural and induced arousals (Figure 20). Rates of  $CO_2$  production are approximately 5 times greater in the first minutes of a natural arousal (Figure 20).  $O_2$  consumption and  $CO_2$  production rates are comparable by the end of arousal, however RQ values still differ (Figure 21). Levels of glucose and free fatty acids in blood plasma are similar for natural and induced arousal, but plasma lactate levels are significantly elevated during

induced arousal (Figures 23 through 26). These metabolic alterations may indicate issues with fuel oxidation and/or pH regulation.

Previous studies have indicated signs of oxidative stress, activation of stress-related signaling molecules as well as elevated antioxidant defense systems during hibernation (Carey et al, 2000; Lee et al., 2002; Tøien et al, 2001; Drew et al., 2002). Thus it appears that hibernators are experiencing stress and engaging protective mechanisms. However, the effectiveness of these protective mechanisms has not been as well studied. Low levels of lipid peroxides and protein carbonyls in heart, liver, kidney, and brain tissues indicate that hibernators are well protected from oxidative damage during both natural and induced arousal as well as throughout other periods of a torpor bout (Figures 27 and 28).

It was previously expected that animals would warm as quickly as possible to minimize energetic expenditures (Stone and Purvis, 1992). However, my work indicates that arousal is a more complex process involving coordination of many cellular and systemic physiological processes (Utz et al., 2007). The widespread use of induced arousals throughout the hibernation community may have led to some misperceptions about hibernation physiology. For example, Lee et al. report a 2.5 fold increase in lactate concentrations in bat brain in a study of stress signaling molecules (2002). However, these animals were induced to arouse prematurely, so is this increase in lactate truly an aspect of arousal or an artifact of how the animals were handled? Knowledge of the differences in rewarming dynamics and respiratory activity during induced arousals may be useful for



enhancing the understanding of hibernation. A uniform and accurate classification of torpor states will benefit the field immensely.

Many aspects of arousal are yet to be investigated. Although several observations are consistent with altered circulation during induced arousal, direct measures are lacking. Direct measurements of blood gasses and blood flow during natural and induced arousals would be illuminating. Currently, we have virtually no knowledge of the role of anaerobic metabolism during a torpor bout. Direct calorimetry experiments, coupled with available respirometry data, would allow analysis of the relative contributions of anaerobic metabolism throughout a torpor bout. It would be particularly interesting to compare the putative role of anaerobic metabolism in fueling natural versus induced arousals. Although much attention has been directed towards measuring oxidative damage associated with free radical production, there is an alternative “redox hypothesis” of oxidative stress (reviewed in Jones, 2008). According to the redox hypothesis, oxidative stress causes disruption of thiol redox circuits that normally control cell-signaling pathways (Jones, 2008). These thiol systems are sensitive to 2-electron oxidants. In other words, this is a unique mechanism when compared to free-radical oxidations (Jones, 2008). Investigation of the redox status of these thiol systems across a torpor bout and during arousal would be fascinating.

The hibernation season is dynamic; animals are constantly oscillating between the dissimilar states of torpor and interbout arousal (IBA). However, as investigations have proceeded with finer time scales, intricate spectrums of physiology have been revealed. The transition from torpor to IBA is a

multifaceted, regulated process. Early models of rewarming dynamics have been refuted. One must now consider that the nature of an arousal, whether it is natural or prematurely induced, will influence the process of arousal itself as well as the following IBA phase. Although induced arousals often occur in laboratory settings, premature arousal may occur in response to temperature changes, an ecologically relevant stimulus. Animals will often arouse according to the “original” timing following an induced arousal. Therefore, prematurely inducing arousal shortens the amount of time spent in torpor and increases the amount of time spent at euthermic body temperatures. The result of increased periods of euthermia is more rapid depletion of fat stores, which compromises an animal’s ability to survive the winter season. Warmer, more variable winters may lead to increased instances of mortality. It is valuable to remember that the road traveled matters, both for accurate data collection and survival in changing climates.

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