EXPRESSION AND MECHANISMS OF HIBERNATION IN THE ARCTIC:

THE ALASKA MARMOT AND ARCTIC GROUND SQUIRREL

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# EXPRESSION AND MECHANISMS OF HIBERNATION IN THE ARCTIC: THE ALASKA MARMOT AND ARCTIC GROUND SQUIRREL

A

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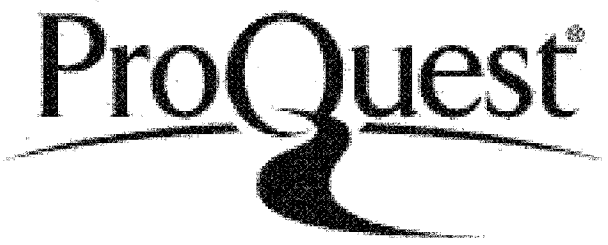


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## ABSTRACT

The Arctic is home to animals that have taken adaptations to overwintering to extremes. In this dissertation, I have investigated one of these adaptations, hibernation, in two species from the Arctic, the Alaska marmot (*Marmota broweri*) and the arctic ground squirrel (*Urocitellus parryii*). The expression of hibernation under natural conditions in these species was compared by collecting body temperature records of free-living individuals. The Alaska marmot, a highly social species, demonstrated extreme synchrony in body temperature patterns among a family group, indicating a strong reliance on social thermoregulation. In contrast, the arctic ground squirrel was confirmed to be a solitary hibernator that reduces body temperature below freezing during torpor. Both species must produce heat when soil temperatures are significantly below freezing for most of the winter. At these subfreezing ambient temperatures, the arctic ground squirrel has shown an increasing reliance on nonlipid fuel during torpor, driving a loss of lean mass during hibernation of ~20%. I calibrated deuterium dilution to repeatedly estimate body composition in this species, which dramatically changes adiposity through its annual cycle, and used this technique to quantify lean mass loss throughout hibernation in a study of tissue metabolism. I also developed and applied the natural abundance of nitrogen and carbon stable isotopes as tools for monitoring differential tissue metabolism and differentiating mixed metabolic fuel use in the arctic ground squirrel. These data clarified the mechanism of change in nitrogen stable isotopes and indicated that hibernating ground squirrels rebuild organ tissues while breaking down muscle tissue to meet energetic demands. Furthermore, I corroborated a shift in metabolic fuel use toward nonlipid sources during torpor at low ambient temperatures by using the carbon isotope ratio in exhaled breath in combination with respiratory quotient. This dissertation combines studies of hibernation patterns in free-living animals with experimental data on the tissues and fuels being catabolized at very low temperatures to broaden our understanding of how small mammals successfully hibernate in severe winter conditions. It also presents the development and use of stable isotope ratios as physiological tools in hibernating species.

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## General Introduction

The extreme environmental conditions of the Arctic present a host of challenges to the organisms that live in this region. Foremost among these is the thermal challenge of sub-freezing ambient temperatures during the winters lasting 7-8 months. The strategies animals can use to successfully overwinter in the face of low temperatures and food deprivation are determined by body size. Large mammals may migrate between areas of food availability while very small mammals utilize subnivean space and remain active. However, some mammals of intermediate body size use hibernation as a strategy to conserve energy. When hibernating, animals alternate between energy-saving torpor bouts at low body temperature and metabolic rate and brief periods of high body temperature and metabolic rate (Geiser 2004). While hibernation is not strictly an arctic phenomenon, arctic hibernators are a model of the extent of physiological adaptation that is possible in the most thermally demanding environments. The focus of this dissertation is the physiological expression of and mechanisms involved in hibernation in two sympatric species of arctic hibernators that reside north of the Brooks Range in Alaska, the Alaska marmot (*Marmota broweri*) and the arctic ground squirrel (*Urocitellus parryii*). These animals experience life at the extremes and demonstrate the current limits of hibernation physiology in mammals.

The Alaska marmot and arctic ground squirrel are related species within the family Sciuridae (Helgen et al. 2009) and have many similar life history traits; for example, they are diurnal burrowing mammals that have an annual cycle revolving around hibernation (Armitage 1981). However, they also differ in body size and key behavioral characteristics that may differentially influence hibernation patterns. Foremost among these, most marmot species display a high level of sociality while ground squirrels do not (Arnold 1993; Barash 1989). Many aspects of the arctic ground squirrel's life history and physiology, from reproductive phenology to environmental plasticity, have been revealed through hibernation body temperature records obtained from free-living animals in multiple studies (Buck et al. 2008; Sheriff et al. 2011; Williams et al. 2011b). However, very little is known about the Alaska marmot,

especially its hibernation characteristics. Obtaining body temperature records from free-living Alaska marmots is the first step to understanding the hibernation strategy of this relatively large hibernator and how its sociality influences its hibernation behavior.

The arctic ground squirrel has been a model species for studying the mechanisms of hibernation at low temperatures, in studies ranging from energetics of rewarming to metabolic suppression and oxidative stress (Jinka et al. 2011; Karpovich et al. 2009; Orr et al. 2009; Williams et al. 2011a). Studies from captive arctic ground squirrels have shown a shift from pure lipid metabolism, characteristic of temperate hibernators (Dark 2005), to increasing protein and/or carbohydrate metabolism at ambient temperatures below freezing (Buck and Barnes 2000). The source of this protein and/or carbohydrate was thought to be lean mass, and this inference was supported by field studies showing that female arctic ground squirrels lost ~20% of their lean mass over hibernation at subfreezing temperatures (Buck and Barnes 1999a, 1999b). This unique pattern of metabolic fuel use was thought to be necessary for maintaining a large thermal gradient between body temperature and ambient temperature (Buck and Barnes 1999b, 2000). However, questions remain regarding metabolic fuel use, such as which tissues are catabolized to provide nonlipid fuels and how metabolic costs are met at the lowest limits of ambient temperature. These questions have received little attention, in part due to the lack of tools for monitoring differential tissue metabolism and mixed metabolic fuel use. This dissertation proposes and evaluates two new approaches for investigating metabolism during hibernation, based on variations in natural stable isotope abundance generated by hibernation.

Differences in the natural stable isotope abundance of the light elements are emerging as useful tools in animal physiology (Gannes et al. 1998). Stable isotope ratios can be altered by physiological processes, providing a marker by which such processes can be monitored. I used the arctic ground squirrel system to explore whether these types of markers can be informative about hibernation physiology, specifically about the tissues catabolized for fuels during hibernation and the mixtures of fuels burned at extreme

temperatures. In an ancillary study, I calibrated a nonlethal isotopic method for estimating body composition in the field.

This dissertation focuses on two main themes. The first is the expression of hibernation in the Alaska marmot compared to the arctic ground squirrel, and the second is the mechanism of metabolic fuel use during hibernation in the arctic ground squirrel.

Chapter 1 presents the first body temperature record of a free-living Alaska marmot across the hibernation season, using techniques for monitoring body temperatures developed in free-living arctic ground squirrels. This preliminary finding, published in 2009 in *Ethology Ecology & Evolution*, revealed both similarities and differences with arctic ground squirrel body temperature patterns that were expanded and directly compared in Chapter 2.

In Chapter 2, I present body temperature records from a marmot family inhabiting a single hibernaculum over several years and arctic ground squirrels from two burrow sites only 5 km from the marmot hibernaculum. The Alaska marmots displayed a high degree of synchrony in body temperature changes, confirming that they hibernate communally and utilize social thermoregulation, while arctic ground squirrels hibernate individually and decrease their body temperature below 0 °C to minimize the large thermal gradient between soil temperature and body temperature.

Chapter 3 is a calibration of deuterium dilution to estimate body composition in the arctic ground squirrel. Deuterium dilution is a useful method that utilizes stable isotope tracers, but it requires species-specific calibration for accuracy. This is especially true in hibernators that span a wide range of body compositions. This calibration will contribute to other physiological studies of this species by allowing repeated estimates of body composition. The results of this calibration were published in *Rapid Communications in Mass Spectrometry* in 2011. I applied this method in Chapter 4 to estimate lean mass loss during hibernation.

Chapter 4 clarifies the physiological mechanisms involved in changes in tissue nitrogen stable isotope ratios ($\delta^{15}N$) and uses these changes to determine the metabolic activity of organs and skeletal muscles during hibernation. An extensive review of the

literature indicated that the mechanism of $\delta^{15}N$ change was not well-defined. My data supported one of two models described in the literature; specifically, $\delta^{15}N$ changes in tissue are a result of protein synthesis in addition to catabolism instead of protein catabolism alone. From this, I determined that organ tissues were continually remodeled during hibernation in arctic ground squirrels. In contrast, $\delta^{15}N$ values of skeletal muscle did not change with lean mass loss, suggesting that muscles serve as protein storage but are not rebuilt during hibernation.

Chapter 5 investigates metabolic fuel use during hibernation at ambient temperatures from -2 to -26 °C using two different metrics, the established measure of respiratory quotient and a proposed measure using carbon isotope ratios ($\delta^{13}C$) in exhaled breath. These two measures, which should each indicate lipid use, were correlated and showed a shift in fuel use away from pure lipid metabolism during torpor at ambient temperatures below those previously investigated. This confirmed that the arctic ground squirrel requires fuel sources other than lipid to successfully overwinter in its extreme environment. It is also a first demonstration that $\delta^{13}C$ in the exhaled breath of fasted animals can indicate fuel use.

In summary, this dissertation investigates hibernation under extreme conditions, both in the lab and in the field, and compares hibernation strategies between two related species with very different social systems. In addition, it refines the application of a new set of physiological tools, changes in the natural abundance of stable isotopes, to studies of hibernation physiology.

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# Chapter 1 BODY TEMPERATURE PATTERNS DURING HIBERNATION IN A FREE-LIVING ALASKA MARMOT (*MARMOTA BROWERI*)[1]


## Abstract

*Marmota broweri* is endemic to arctic Alaska. We implanted data loggers into one free-living subadult female in August 2007 to record body temperature ($T_b$). Seasonal heterothermy lasted for 224 days (10 September to 21 April). Midwinter torpor bout length (mean ± SD) was 13.91 ± 3.56 days and duration of interbout euthermy was 18.4 ± 2.6 hours. Unlike other marmot species, torpor entry during midwinter was consistently characterized by two distinct cooling rates differing by about 10-fold and separated by a transient temperature increase. Minimum hibernaculum temperature was -15.0 °C in February (7 month mean: -7.33 °C). Minimum $T_b$ was 1.0 °C when defending a 15.5 °C thermal gradient. At least six animals emerged from the hibernaculum, suggesting that communal hibernation may be a strategy to reduce metabolic costs while maintaining above-freezing $T_b$.


[1] Published as LEE T. N., BARNES B. M. & BUCK C. L. 2009. Body temperature patterns during hibernation in a free-living Alaska marmot (*Marmota broweri*). Ethology Ecology & Evolution 21:403-413.

**Introduction**

The Alaska marmot, *Marmota broweri*, was first recognized as a subspecies, *Marmota caligata broweri*, by HALL & GILMORE in 1934 from specimens collected by Native residents on the northwest coast of Alaska. Later, RAUSCH (1953) proposed a reorganization of the genus *Marmota* that described the Alaska marmot (*Marmota marmota broweri*) as a subspecies of the alpine marmot found in Europe. This reorganization was never fully accepted and the Alaska marmot was recognized as a unique species in 1965 when cytogenetic studies revealed it had 2n = 36 chromosomes compared to *M. caligata*'s 2n = 42 (RAUSCH & RAUSCH 1965) and *M. marmota*'s 2n = 38 (RAUSCH & RAUSCH 1971). Since its specific recognition, very few studies have investigated either the ecology (RAUSCH & RAUSCH 1971; ALBERT 1977; RAUSCH & BRIDGENS 1989) or the physiology (WILLIAMS & RAUSCH 1973) of this arctic species.

*M. broweri* is endemic to northern Alaska, though its range has been misconstrued due to a lack of focused efforts to describe it (RAUSCH 1953; BARASH 1989; HOFFMANN 1999). Recently, GUNDERSON et al. (2009) synthesized a current range map of *M. broweri* through analysis of museum specimens and extensive field surveys of potential habitats. Most populations of *M broweri* occur in the Brooks Range, though some populations are found in the Kokrines Hills and Ray Mountains just north of the Yukon River in Alaska, which may function as a barrier between *M. broweri* and *M. caligata* (GUNDERSON et al. 2009). Marmots are typically found on talus slopes 1000-1200 m in elevation with grassy meadows present for foraging (GUNDERSON et al. 2009). This agrees with previous descriptions of *M. broweri*'s habitat (BEE & HALL 1956) and is consistent with the preferred habitats of most species of North American mountain marmots (ARMITAGE 2003). *M. broweri* utilize multiple summer burrows among talus (RAUSCH & RAUSCH 1971) but congregate in social groups of up to 12, but usually 7-8, animals in a single hibernaculum for winter hibernation (RAUSCH & RAUSCH 1971; RAUSCH & BRIDGENS 1989). The hibernaculum is usually situated

in an exposed area of soil so the burrow plug, placed in mid September, will thaw and allow exit during late April or early May (RAUSCH & BRIDGENS 1989). Some evidence indicates *M. broweri* breed within the hibernaculum before spring emergence (RAUSCH & RAUSCH 1971; RAUSCH & BRIDGENS 1989).

Hibernation of *M. broweri* has only been cursorily investigated in three studies of captive animals. An early study revealed that a captive colony of hibernating *M. broweri* tolerated $CO_2$ levels as high as 13.5% and $O_2$ levels as low as 4%, though levels were usually below 4% and above 15%, respectively (WILLIAMS & RAUSCH 1973). During that experiment, marmots in the artificial hibernaculum experienced ambient temperatures as low as -25 °C. Marmots in an artificial den in Barrow, Alaska tolerated ambient temperatures as low as -13 °C (ALBERT 1977), and there is a report of ambient temperatures during hibernation as low as -48 °C while temperature probes in contact with the marmots frequently recorded -5 °C (FOLK 1966).

*M. broweri* is sympatric with *Urocitellus parryii* in the Alaskan Arctic. Soil temperatures of natural hibernacula of *U. parryii* decrease to as low as -23.4 °C in winter (BUCK & BARNES 1999b), and animals defend a minimum body temperature during hibernation no lower than -2.9 °C both in the field (BUCK et al. 2008) and in captivity (BARNES 1989). Although *U. parryii* are solitary hibernators (BUCK & BARNES 1999b) and *M. broweri* hibernate in social groups (RAUSCH & RAUSCH 1971; RAUSCH & BRIDGENS 1989), both species likely experience similar environmental temperatures. Here, we describe for the first time overwinter thermal conditions of a natural hibernaculum, the timing of hibernation, and pattern of body temperature regulation of a free-living *M. broweri*.

**Materials and Methods**

Two colonies of *M. broweri* near the Toolik Field Station (TFS), 68° 38' N, 149° 36' W, were targeted for this study. Tomahawk® live traps (Tomahawk Live Trap, Tomahawk, WI) approximately 23 x 23 x 66 cm were situated near burrow entrances or

where animals had disappeared under rocks. Despite extensive trapping effort in early August 2007, no animals were captured from the larger colony. One animal, a young female estimated to be 1-2 years old, was captured at the smaller colony's hibernaculum through blocking the entrance so that the animal could only exit into a trap.

The captured marmot was transported to the laboratory at TFS and anesthetized with a 3 + 40 mg/kg xylazine-ketamine cocktail injection (BEIGLBÖCK & ZENKER 2003) and maintained on a surgical anesthesia level using 1-5% isoflurane gas anesthesia. The marmot was weighed on an electronic balance to the nearest gram and 3 mL of blood was sampled from the femoral vein for other studies. Two TidBit Stowaway temperature loggers (Onset Computer Corporation, Bourne, MA), coated in Elvax (DuPont, Wilmington, DE) and weighing approximately 15 g each, were surgically implanted into the abdominal cavity (LONG et al. 2007). Two loggers were implanted to ensure that data would be collected even if one of the loggers failed. Briefly, an area approximately 15 $cm^2$ of the animal's abdomen was shaved and scrubbed. An incision of approximately 3 cm was made through the skin and subcutaneous adipose tissue was blunt dissected to reveal the linea alba where the abdominal cavity was opened with an incision and the data loggers were inserted to float freely in the peritoneal space. Muscles and skin were closed with absorbable and non-absorbable (respectively) interrupted sutures and the adipose tissue was closed with an absorbable continuous suture. Implanted loggers were pre-programmed to measure and record core body temperature ($T_b$; ± 0.2 °C) every 20 minutes. The animal was released at the site of capture within 12 hours of recovery from anesthesia.

We outfitted the hibernaculum site with two temperature-sensitive data loggers (Hobo Pro Series Temp Dual Component Temperature, Onset Computer Corporation, Bourne, MA) in late September 2007 after the burrow opening had been plugged from within with loose soil and cobble. The data loggers were pre-programmed to measure and record temperature at the depth of an attached thermistor probe and at the surface every hour (± 0.2 °C). The thermistor probes were fitted within 1.25 cm (inner diameter) cpvc pipe (BUCK & BARNES 1999b). One thermistor probe was placed into part of the

hibernaculum through a hole in the soil left by a steel rod. The second probe was placed into the frozen burrow plug.

The following spring (4-7 May 2008), five marmots including the implanted female were captured at the hibernaculum directly after emergence and were implanted with temperature data loggers (as above); loggers from the previously implanted female were explanted and replaced using a similar surgical procedure. At least one additional marmot, a yearling much smaller than the others, was seen at the burrow immediately after emergence.

The season of heterothermy, defined as the interval between when the animal first decreased $T_b < 30$ °C and last increased $T_b > 30$ °C, is separated into bouts of torpor ($T_b < 30$ °C) alternating with arousal to intervals of euthermy ($T_b \geq 30$ °C; BUCK et al. 2008). Torpor bouts of low, stable $T_b$ (< 1 °C variation over 24 hours) were considered midwinter torpor bouts and were used in analyses. Rates of cooling (°C/hr) following arousal were divided into two phases: rapid and slow. Rapid cooling rates are defined as an initial drop in $T_b$ of $\geq 3$ °C within 40 minutes from euthermic $T_b$ until a transient temperature increase, after which slow cooling rates continued until $T_b \leq 5$ °C. Rewarming rates were calculated from low, stable $T_b$ to 30 °C to allow comparison with other species and $T_b$ 10 to 30 °C to estimate maximal rewarming rates. Mean and minimum hibernaculum soil temperatures ($T_s$) were calculated from each timeframe of the associated phase of body temperature. We used linear regression to analyze relationships between marmot characteristics (dependent variables: cooling rates, rewarming rates, mean torpid $T_b$, minimum torpid $T_b$, and torpor bout duration) and soil characteristics (independent variables: mean and minimum soil temperature; $\alpha = 0.05$). Means ± SD are reported. All animal use procedures were approved by the Institutional Animal Care and Use Committee of the University of Alaska Fairbanks (Protocol 07-34).

## Results

***Patterns of Body Temperature*** The marmot first entered torpor on 10 Sept 2007 and ended heterothermy 21 April 2008 for a total heterothermic season duration of 224 days during which the animal was torpid 87.5% of the time (Figure 1.1). During midwinter, the animal displayed 11 torpor bouts averaging 13.91 ± 3.56 days and spent 95.2% of this time torpid. The 10 intervening euthermic intervals averaged 18.4 ± 2.6 hours in duration. Torpor bout duration was not related to either mean or minimum $T_s$ ($F = 0.0058$, $p > 0.05$; $F = 0.1107$, $p > 0.05$, respectively).

Both mean and minimum $T_b$ during individual midwinter torpor bouts decreased with decreasing mean $T_s$ ($F = 17.91$, $p = 0.0022$, $R^2 = 0.67$; $F = 12.96$, $p = 0.0057$, $R^2 = 0.59$, respectively) and were even more strongly related to minimum $T_s$ during the bout ($F = 23.70$, $p = 0.0009$, $R^2 = 0.72$; $F = 16.68$, $p = 0.0027$, $R^2 = 0.65$, respectively). Minimum $T_b$ recorded was 1.0 °C on February 9-10 when $T_s$ was -14.1 to -14.5 °C.

Minimum torpid $T_b$ changed dramatically throughout the heterothermic season. Midwinter torpor bouts were preceded by seven early-winter torpor bouts of decreasing minimum $T_b$ and followed by five late-winter torpor bouts of increasing minimum $T_b$. $T_b$ during these early and late-winter torpor bouts was not stable, and bouts were truncated at relatively high $T_b$ (Figure 1.1).

***Cooling and Rewarming Rates*** Entry into torpor during midwinter was consistently characterized by two distinct rates of cooling (Figure 1.2). The animal cooled rapidly until reaching $T_b$ of 21.5 ± 1.1 °C, and then cooled much more slowly after a transient increase in temperature peaked at 22.8 ± 1.2 °C. The rate of rapid cooling was not affected by mean $T_s$ ($F = 2.96$, $p > 0.05$) and averaged -5.9 ± 2.7 °C/hr (range: -1.3 to -11.2 °C/hr). Slow cooling ranged from -0.18 to -0.43 °C/hr and was significantly related to mean $T_s$ ($F = 26.00$, $p = 0.0006$, $R^2 = 0.74$).

Rates of rewarming from stable $T_b$ to 30 °C and $T_b$ 10 to 30 °C were not related to mean $T_s$ ($F = 0.24$, $p > 0.05$; $F = 0.04$, $p > 0.05$, respectively). Rewarming from stable

$T_b$ to 30 °C averaged 3.3 ± 0.5 °C/hr (range: 2.4 to 4.2 °C/hr) whereas rates from $T_b$ 10 to 30 °C averaged 6.0 ± 1.5 °C/hr (range: 4.5 to 8.6 °C/hr).

***Soil Temperatures*** Soil temperature at the hibernaculum averaged -7.3 ± 4.5 °C from 22 September 2007 to 9 May 2008. Minimum soil temperature was -15.0 °C in February. From 22 September to 20 April, the temperature of the soil plug averaged -11.8 ± 6.2 °C with a minimum of -25.4 °C. The temperature of the soil plug became erratic on 21 April, indicating the opening of the burrow and exposure to air, which was visually confirmed on 23 April (last seen closed 23 March). Three interspersed temperatures measured from the soil plug a few hours prior to exposure to air were unusually high (+8 to 11 °C) as compared to surface temperature (about -6 °C); these spikes in temperature likely reflect the influence of marmot $T_b$ as the animal(s) worked to open the plug. Temperatures at the snow-ground interface averaged -14.6 ± 10.5 °C with a minimum of -42.0 °C.

***Body Mass*** The female marmot weighed 3.094 kg on 5 August 2007 and 2.055 kg when recaptured after emergence on 7 May 2008. During this time, including 17 days of euthermy at emergence, the animal lost 1.039 kg, 33.6% of her initial body mass. Four other marmots were captured at emergence only: two non-reproductive, young males (2.22 kg and 2.77 kg), one adult breeding female (2.85 kg), and another young female (2.16 kg).

**Discussion**

Here we present the first description of overwinter body temperature patterns of a free-living *M. broweri* and associated soil temperatures of the hibernaculum, which demonstrates that *M. broweri* defended a large thermal gradient between $T_b$ and $T_s$ while maintaining above-freezing body temperatures during torpor. *M. broweri* displayed an apparently atypical change in cooling rates during torpor entrance that, for at least this marmot, is consistent throughout midwinter. Whether this pattern is manifest for others

within the species has yet to be determined. Torpid $T_b$ decreased in sequential torpor bouts in the fall and showed a mirrored increase in the spring, but the extent to which social dynamics influenced this pattern are unknown.

Most species of marmot that have been studied naturally hibernate at ambient temperatures above freezing (ARNOLD et al. 1991; FERRON 1996; FLORANT et al. 2000) and may not be capable of hibernating in sub-freezing temperatures. ORTMANN & HELDMAIER (2000) demonstrated that *M. marmota* spontaneously arouse from torpor when ambient temperatures decrease below -0.5 °C. However, *M. camtschatica* from arctic Russia, where soil temperature at nest level can decrease to -22 °C, hibernated in captivity at ambient temperatures of -5 °C and displayed minimum rectal temperatures of 0.9 °C (VASIL'EV 2000). *M. broweri* are also able to hibernate in freezing temperatures as shown by the hibernaculum soil temperatures recorded in this study. Measured $T_s$ should closely approximate ambient temperatures in the hibernaculum. We think that the thermistor probe was positioned in part of the burrow system because the steel rod used to make a hole for the probe plunged through 'open space' about 1 meter below the soil surface. This space was about 20 vertical cm and was underlain by permafrost. Thus, the patterns of $T_s$ we report probably represent ambient temperatures experienced by the marmot. Burrows of *U. parryii* are at similar depth and exhibit similar patterns of soil temperatures (BUCK & BARNES 1999b) and hibernating *M. broweri* in artificial hibernacula tolerate similar temperatures (ALBERT 1977). In our study it is unlikely the thermistor probe was in contact with the marmots as the temperature recordings show no influence of body temperature.

The young female marmot demonstrated body temperature patterns typical of deep hibernators, i.e., multi-day torpor bouts with spontaneous interbout arousal intervals, but the extent of hibernation was more extreme in ambient temperature, duration, $T_b$ depression, and torpor bout length than has been recorded in other species of marmots in the field (Table 1.1). The hibernation duration and intervals were more similar to those of *U. parryii* hibernating in the same area under similar conditions (Table 1.1).

In studies of communal hibernation in the European marmot, the energetic savings of each animal hibernating communally depends on the least efficient member of the group: synchrony of torpor bouts among members is necessary for hibernation to save enough energy for animals to survive (ARNOLD 1988). At least six *M. broweri* exited the hibernaculum in the spring, suggesting that *M. broweri* hibernate in social groups as has been previously reported (RAUSCH & RAUSCH 1971; RAUSCH & BRIDGENS 1989). Though we don't know the $T_b$ patterns of the nestmates of the study animal, arousals initiated before attaining a stable and minimum $T_b$ are likely, at least in part, a result of passive warming from other animals increasing $T_b$ during arousal. Although bouts of torpor in *U. parryii* are shorter at the beginning and end of the heterothermic season, truncated multi-day torpor bouts at high $T_b$ are not seen in *U. parryii* (BUCK et al. 2008). Short torpor bouts at relatively high $T_b$ during early hibernation in *M. broweri* may be described as "test drops" proposed to 'reset' hypothalamic $T_b$ set-points (FLORANT & HELLER 1977). This hypothesis for the functional significance of these torpor bouts, however, would not explain the mirrored pattern at the end of hibernation. In spring, the incremental increases in $T_b$ over sequential torpor bouts may be due to disturbance from a male that has attained euthermy to undergo spermatogenesis (BARNES 1996).

*M. broweri* appears to have a strategy for entering torpor that differs from the uninterrupted pattern of decrease in body temperature typical of *U. parryii* (KARPOVICH et al. 2009), *M. marmota* (ARNOLD 1988; ORTMANN & HELDMAIER 2000), *M. monax* (ZERVANOS & SALSBURY 2003), and *M. flaviventris* (WOODS et al. 2002). In *M. broweri*, $T_b$ drops rapidly (-5.9 °C/hr) from euthermic body temperatures during arousal to an average of 21.5 °C, at which point cooling slows dramatically to an ambient temperature-related rate less than -0.43 °C/hr after a small 1.2 °C temperature increase (Figure 1.2). Rapid cooling may be the result of a strategy to quickly dump heat as metabolic rate is suppressed (ORTMANN & HELDMAIER 2000; WOODS et al. 2002) and may be achieved by behavioral means, i.e., animals may be separated in the hibernaculum in a position to expose maximal surface area. As body

temperatures approach 20 °C, the marmots likely still have some motor capability (FOLK 1966), and they may then move (causing the small temperature increase) to huddle together (ARNOLD 1988), causing a decrease in cooling rates by reducing surface area for heat loss and increasing thermal mass. Alternatively, $T_b$ may fall passively with the initial drop of metabolic rate until reaching a certain temperature, at which point animals may increase metabolic rate as they initiate thermoregulatory heat production to slow the rate of $T_b$ decline. Results from captive animal studies with simultaneous measurement of $T_b$ and metabolic rate of animals are necessary to mechanistically understand the significance of the change in rate of cooling.

Marmots, like other mammalian hibernators, increase mass rapidly during the summer active season prior to hibernation (ARMITAGE 2003). *M. flaviventris* attains peak body mass in August (WARD & ARMITAGE 1981), and marmots may maintain mass up to 5 weeks before immergence (ARMITAGE 2003) as forage senesces and their gastrointestinal tract reduces in both function and mass in preparation for hibernation (HUME et al. 2002). In our study, the marmot may have been near maximal body mass when captured in the fall 36 days prior to becoming heterothermic. When captured in the spring, the animal had been euthermic for 17 days. There was no evidence of feeding during this time: snow covered possible forage, marmots did not exit the burrow between 23 April and their capture 4-7 May, and no fecal material was seen from any animal brought into the laboratory. Marmots may need at least a week after hibernation to rebuild their gastrointestinal tracts to be able to digest forage, but rebuilding in the spring also depends on ingestion of food (HUME et al. 2002). If we assume the animal was at peak body mass in August, she lost 33.6% of her body mass during hibernation, which is similar to mass losses in several other marmot species and less than losses of 46-53% in *M. caligata* and *M. olympus* (ORTMANN & HELDMAIER 2000, ARMITAGE 2003). This unexpectedly low mass loss could indicate that the animal was not at peak body mass in August as increased metabolic costs of maintaining a larger thermal gradient (BUCK & BARNES 2000) should be reflected by increased mass loss over winter. Alternatively, *M. broweri* may be more efficient during hibernation than other species.

Possible increases in efficiency could be due to increased insulation, lower metabolic rates during torpor, or a combination of factors.

*M. broweri* did not display subfreezing $T_b$ even under a 15.5 °C thermal gradient, suggesting that they may not be able to physiologically tolerate $T_b < 0$ °C. These marmots may use communal hibernation as a strategy to reduce the metabolic costs of hibernating against the large thermal gradient they experience. The distinct cooling rates should be further investigated by a combination of metabolic rate and $T_b$ measurements and behavioral observations. Also, possible means of increased efficiency of *M. broweri* should be explored to learn more about how this species can withstand the extreme environment it inhabits.

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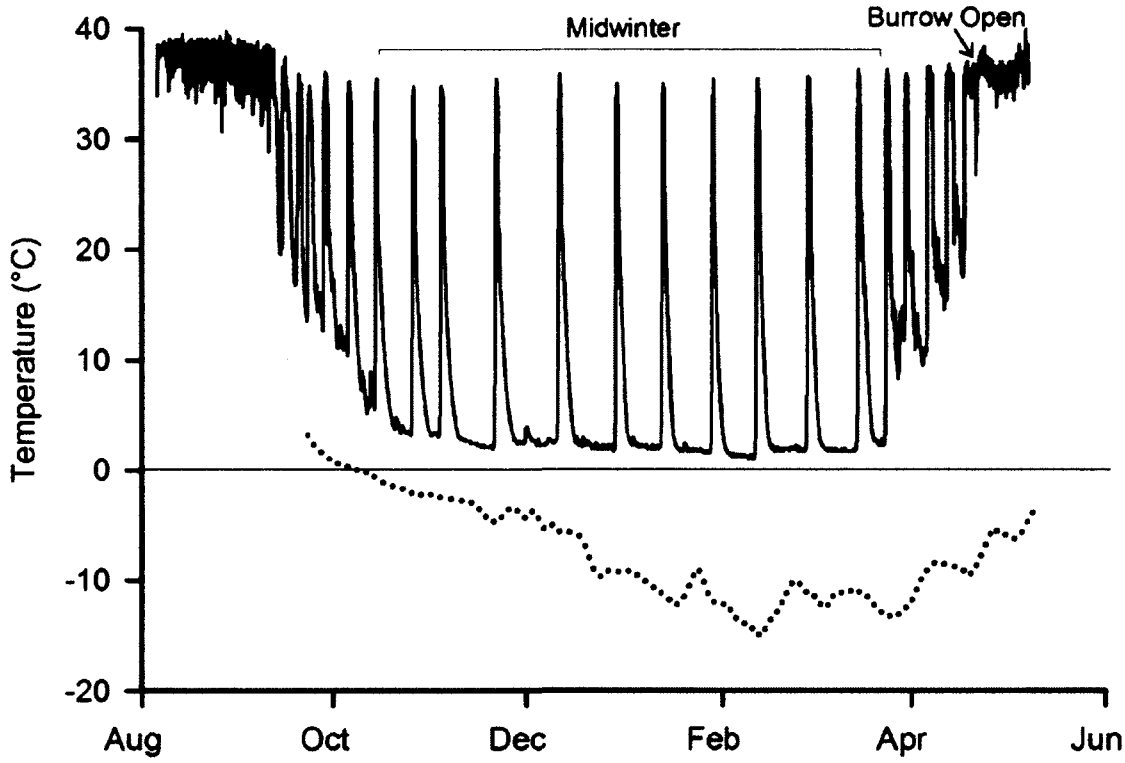


Figure 1.1 Body temperature ($T_b$, solid line) and associated soil temperature (dotted line) profile of a female Alaska marmot hibernating during winter 2007-08. Horizontal bracket shows torpor and arousal bouts considered midwinter (torpor bouts of low, stable $T_b$ with < 1 °C variation over 24 hours). Arrow indicates date burrow was opened.

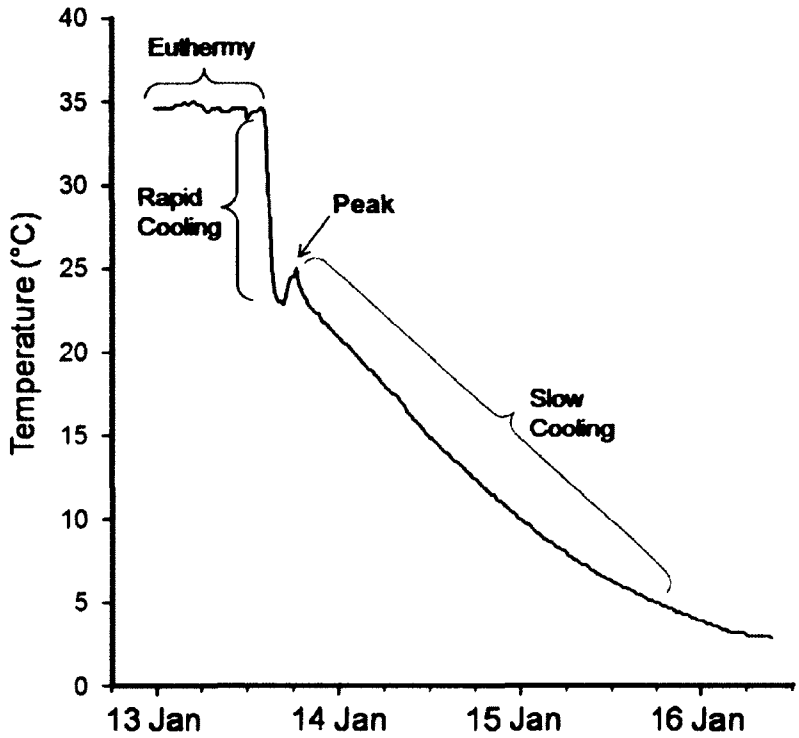


Figure 1.2. Detail of body temperature during entrance into torpor of a female Alaska marmot during January 2008 to illustrate consistent, distinct rates of cooling.

Table 1.1 Comparison of hibernation parameters among *Marmota broweri, M. monax, M. marmota*, and *Urocitellus parryii.*

| Measurement | *M. broweri* (f)[1] | *M. monax* (Penn)[2] | *M. marmota* | *U. parryii* (f) |
|---|---|---|---|---|
| Heterothermy start | 10 Sept[a] | 31 Oct- 4 Nov[a] | 24 Sept- 3 Oct[3b] | 13 Aug- 11 Sept[5b] |
| Heterothermy end | 21 April[a] | 1-5 Mar[a] | 7-13 April[3b] | 18 April- 13 May[5b] |
| Hibernation duration | 224 d[a] | 121.8 ± 1.83 d (f)[a] | 186-201 d[b] | 227 d (juvenile)[6a] |
| | | | | 245 d (adult) |
| Torpor bout length (midwinter) | 13.91 ± 3.56 d[c] | 8.04 ±0.32 d[d] | | 17.6 d[6e] |
| Interbout arousal length (midwinter) | 18.4 ± 2.6 hr[c] | 56.0 ± 7.4 hr[d] | | 15.6 hr[6e] |
| Minimum $T_b$ | 1.0 °C | 6.5 °C | ~7.8 °C[4] | -2.9 °C[7] |
| Minimum $T_a$ | -15.0 °C | 6.3 °C | > -0.5 °C[4] | -23.4 °C[8] |
| Potential thermal gradient ($T_b$ -$T_a$) | 16.0 °C | 0.2 °C | ~8.3 °C | 20.5 °C |

$T_b$ = body temperature; $T_a$ = ambient temperature; [a]Based on dates of heterothermy; [b]Based on dates of immergence and emergence; midwinter defined as [c]period of torpor bouts reaching low, stable $T_b$ with < 1 °C variation in 24 hours, [d]middle third of hibernation season, and [e]period between first and last two multi-day torpor bouts; "f" denotes measurements of females; [1]This study; [2]ZERVANOS & SALSBURY 2003; [3]ARNOLD et al. 1991; [4]ORTMANN & HELDMAIER 2003; [5]BUCK & BARNES 1999a; [6]BUCK et al. 2008; [7]BARNES 1989; [8]BUCK & BARNES 1999b.

# Chapter 2 SOCIALITY INFLUENCES HIBERNATION PATTERNS OF TWO ARCTIC SPECIES, THE ALASKA MARMOT AND ARCTIC GROUND SQUIRREL[1]

## Abstract

Sociality influences hibernation strategies in mammals. We studied 2 arctic hibernators, the Alaska marmot (*Marmota broweri*) and arctic ground squirrel (*Urocitellus parryii*) by implanting body temperature ($T_b$) data loggers in free-living animals. We recovered loggers from 3-4 marmots from the same hibernaculum in each of 3 years and from ground squirrels that hibernated at the same burrow site in 1 year. Synchrony in $T_b$ patterns in Alaska marmots indicates a dependence on social thermoregulation while lack of synchrony supports that arctic ground squirrels hibernate individually. The time between the first and last marmot to initiate an arousal was 3.71 ± 2.51 h and to recool to 30 °C was 5.67 ± 3.67 h. Minimum $T_b$ in marmots was 0.6 °C and -2.0 °C in ground squirrels. Marmots displayed an interrupted cooling pattern when entering torpor with 2 distinct cooling rates differing 21-fold. Ground squirrels cooled in a continuous pattern, initially 3-fold slower than marmots during rapid cooling but 4-fold faster during slow cooling. Alaska marmots utilize social thermoregulation to cope with arctic winter conditions while arctic ground squirrels decrease $T_b$ below freezing to further minimize the thermal gradient with their surroundings.

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**Introduction**

Marmots express different degrees of sociality, with species inhabiting the harshest environments being the most social (Barash 1974). Many species of the genus *Marmota* are found at high altitude or latitude and experience very short growing seasons, providing an abbreviated season for annual growth and reproduction (Barash 1989). All marmots hibernate to conserve energy overwinter, and some social species hibernate communally, huddling together during torpor and arousals (Armitage and Woods 2003; Arnold 1988). Huddling is a form of social thermoregulation that contributes to energy savings during hibernation (Gilbert et al. 2010). Communal hibernation and social thermoregulation may be necessary for marmots to survive long, cold winters and result in animals beginning spring activities in better body condition (Arnold 1990, 1993). However, social hibernation is not a prerequisite for overwintering in extreme environments as ground squirrels hibernate individually in similar environments that can overlap those of social marmots (Armitage 1981).

Arnold (1993) proposed 3 hypotheses for the development of the different behaviors between social marmots and solitary ground squirrels, including that marmots rely on social thermoregulation to cope with low burrow temperatures while ground squirrels have developed the ability to cool to lower body temperatures ($T_b$). The lower $T_b$ of ground squirrels, even below freezing, lowers the ambient temperature threshold at which heat production becomes necessary during torpor. The Palearctic, high altitude alpine marmot, *Marmota marmota*, has been observed huddling during hibernation in captivity (Arnold 1988), which reduces surface area and creates a larger thermal mass that is less sensitive to ambient temperature fluctuations (Gilbert et al. 2010). Social thermoregulation was confirmed in free-living alpine marmots by the determination of synchrony among individuals in the alternation between torpor and arousals to euthermia (Arnold 1993, 1995; Ruf and Arnold 2000). However, the alpine marmot cannot maintain torpor at ambient temperatures < -0.5 °C (Arnold et al. 1991; Ortmann and Heldmaier 2000), and asynchrony in socially thermoregulating animals is energetically

costly (Armitage and Woods 2003). This suggests that synchrony during hibernation may be even more critical for marmots exposed to more challenging thermal conditions. A Nearctic species occurring above the Arctic Circle, the Alaska marmot (*Marmota broweri*), experiences soil temperatures in winter as low as -15 °C (Lee et al. 2009) and appears to be highly social (Rausch and Bridgens 1989; Rausch and Rausch 1971), but little is known about its hibernation patterns. This species is sympatric with the well-studied arctic ground squirrel (*Urocitellus parryii*), which is known to sustain $T_b$ during torpor as low as -2.9 °C (Barnes 1989) at soil temperatures as low as -23 °C (Buck and Barnes 1999), yet it is presumed to hibernate individually (Armitage 1981).

We investigated patterns of body temperature change in free-living Alaska marmots trapped from the same hibernaculum and arctic ground squirrels trapped from the same burrow site in nearby populations in the Arctic. We recorded $T_b$ with implanted temperature-sensitive data loggers and measured soil temperatures at burrow depth at each hibernaculum to quantify thermal challenge. We evaluated synchrony of torpor-arousal cycles among individuals within species and differences in cooling and rewarming curves between species.

## Materials and Methods

***Animals.*** We conducted field work on populations of Alaska marmots (*Marmota broweri*) and arctic ground squirrels (*Urocitellus parryii*) living in the northern foothills of the Brooks Range, Alaska, near the Toolik Field Station of the University of Alaska Fairbanks (68° 38' N, 149° 36' W). Marmots were live-trapped at their hibernaculum, which had a single entrance, using live traps (Tomahawk Live Trap, Tomahawk, WI; approximately 23 x 23 x 66 cm) during fall 2007 and early spring 2008-2011. We barricaded the entrance of the earthen burrow with rocks and the open end of the trap so that marmots could only exit by entering the trap. Ground squirrels were captured using live traps (15 x 15 x 48 cm) baited with carrots after their emergence from hibernation in spring 2009 at 2 distinct burrow sites that had multiple openings. The marmot and

ground squirrel hibernacula were 5 km apart. Individuals were tagged with small metal ear tags and weighed. Ground squirrels captured during the active season of their birth year were considered juveniles through the end of their first hibernation. Marmot age (yearling, 2-year old, adult) was determined by body mass relative to adult body mass according to Arnold (1995). Adult female squirrels that had previously lactated showed distinctly prominent teats compared to females emerging from their first hibernation (pers. observation); marmots were assessed by the same criteria.

***Surgical Procedures.*** Trapped animals were transported to an animal holding facility at Toolik Field Station. Temperature-sensitive data loggers (custom range 32K Stowaway TidBit, Onset Computer Corporation, Bourne, MA) were calibrated at 0 °C and 37 °C (Long et al. 2007), shrink-wrapped, coated 3 times using an Elvax-Paraffin Coating Kit (Minimitter, Bend, OR), and gas sterilized. Data logger package weight was ~15 g. Marmots were anesthetized with an IM injection of 3 + 40 mg/kg xylazine-ketamine cocktail (Beiglböck and Zenker 2003) and maintained on a surgical level by gas anesthesia (isoflurane, 0.5-1.5%). Squirrels were anesthetized with gas anesthesia only (isoflurane, 3-5%). Abdominal surgery on marmots and squirrels was performed as described in Lee et al. (2009). Briefly, we shaved and scrubbed a small area of the animal's abdomen before making an incision of ~2-3 cm through the skin and peritoneum and into the abdominal cavity. After a data logger was inserted into the peritoneal space, muscle, peritoneum, and skin were closed separately with sutures. Animals were held until full recovery from anesthesia (usually overnight) and inspected for the integrity of stitching before being released at their burrow site. All animal procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (protocols 06-06, 09-16, and 07-34) and followed the guidelines of the American Society of Mammalogists (Sikes et al. 2011).

***Metrics.*** We programmed loggers to record core body temperature ($T_b$; ± 0.2 °C) every 20 (2007-2009) or 34 (2010) minutes. During heterothermy, animals alternated between torpor ($T_b$ < 30 °C) and brief interbout arousals ($T_b$ > 30 °C; Buck et al. 2008;

Karpovich et al. 2009). We defined midwinter as the time during each year when all marmots achieved steady torpor (< 1 °C variation over 24 h) and analyzed synchrony in $T_b$ during this time. For comparisons between species, we used data from torpor bouts in ground squirrels that overlapped the marmots' midwinter during that year (3 Oct 2008 to 24 Mar 2009). For all animals, we determined minimum $T_b$ during steady-state torpor, number and duration of steady torpor bouts, and the number of torpor bouts that did not reach a stable $T_b$. Animals were determined to be synchronous if all individuals in the group overlapped in time spent at high $T_b$. Synchrony of change in $T_b$ during arousal intervals was measured at 2 points as the time between the first and last animal to initiate arousal (defined as the first of 3 consecutive increases in $T_b$) and reach $T_b$ of 30 °C during re-entrance into torpor (time determined by interpolating between measurement intervals). All rates of rewarming and cooling were calculated during midwinter. Rapid cooling rates were calculated from 30 °C to 24 °C and slow cooling rates from 19 °C to 6 °C to avoid the transient temperature increases that occur in cooling marmots (Lee et al. 2009). Rewarming rates were calculated from arousal initiation to 30 °C for comparison between marmots and ground squirrels. We also calculated rewarming rates from 10 °C to 30 °C for comparison with another social species, the alpine marmot (Ruf and Arnold 2000).

***Soil Temperature.*** Soil temperature was recorded by a temperature-sensitive data logger (Hobo Pro Series Temp Dual Component Temperature, Onset Computer Corporation, Bourne, MA). The data logger was pre-programmed to measure and record soil temperature (± 0.2 °C) every hour at ~1 m (approximate burrow depth) via a thermistor probe within cpvc pipe (Lee et al. 2009).

***Statistical Analysis.*** Cooling and rewarming rates were compared between species using t-tests based on an average rate for each individual from the winter of 2008-09. One female arctic ground squirrel's data logger failed in January; only intact torpor and arousal bouts prior to logger failure are included in data presented. Values are reported as mean ± SD and alpha was set at 0.05.

## Results

***Synchrony.*** We recovered data from the 1 Alaska marmot implanted for the 2007-08 winter (a female), 4 of 5 for the 2008-09 winter (3 females, 1 male; Figure 2.1), 3 of 4 for the 2009-10 winter (3 females), and 3 of 6 for the 2010-11 winter (1 female, 2 males; 5 loggers were recovered but 2 failed). Two females were represented in 3 years; all other individuals were represented in only 1 year. Although we caught no more than 6 marmots in any year, we resighted most marked animals. A total of 12 individual marmots were sighted near the hibernaculum during the summer of 2009.

Marmots displayed a large amount of overlap in time spent at high $T_b$ and a high degree of synchrony in their patterns of $T_b$ change during cycles of torpor and arousal during midwinter each year: marmots initiated arousals within 3.7 ± 2.5 h and recooled past 30 °C within 5.7 ± 3.7 h of each other (Figure 2.1, 2.2; Table 2.1). Marmots displayed different numbers of torpor bouts at the beginning and end of seasonal heterothermy and thus synchrony could not be calculated. Animals achieved group synchrony in $T_b$ patterns 28-35 days after individuals began heterothermy. Males warmed and maintained euthermic $T_b$ (> 30 °C) for 18-32 days beginning 23 Mar- 6 Apr, but subsequently decreased $T_b$ below 30 °C 2-4 times within the last 4-19 days before ending heterothermy. While males were euthermic, females continued to be heterothermic but did not attain steady torpor (Figure 2.3). Group synchrony ceased 14-37 days before individuals ended heterothermy.

Arctic ground squirrels captured from 2 burrow sites (3 females at 1 site, 3 females and 1 male at the other) at emergence in spring 2009 displayed no synchrony in $T_b$ as they did not consistently overlap time spent at high $T_b$ (Figure 2.4 shows the most 'synchronous' arousal episode). Ground squirrels captured at the burrow site with 4 animals initiated arousals at least 100.6 h apart and recooled below 30 °C into torpor at least 102.8 h apart. The male ended heterothermy and maintained euthermic $T_b$ 20-32 days before the females. The females continued to maintain steady torpor during the period the male was at high body temperature (Figure 2.4).

***Cooling Rates.*** Alaska marmots displayed 2 distinct cooling rates (i.e., interrupted cooling) when entering into torpor (Figure 2.5, Table 2.2). An initial rapid rate of cooling from $T_b$ 30 °C to 24 °C averaged -5.2 ± 3.0 °C/h over 2.5 ± 2.1 h for all animals over all years. $T_b$ then increased briefly or remained stable before marmots resumed cooling at a slow cooling rate of -0.25 ± 0.08 °C/h. Arctic ground squirrels displayed continuous cooling and averaged -1.7 ± 0.3 °C/h over temperatures corresponding to the rapid cooling segment and -0.97 ± 0.29 °C/h from 19 °C to 6 °C, corresponding to the slow cooling segment. Rapid and slow cooling rates significantly differed between species (rapid: $t = -4.48$, $p = 0.0015$; slow: $t = 14.69$, $p < 0.0001$).

Based on her size and prominent teats, 1 female marmot for which we have data (2008-09) was likely the breeding female of the colony. This female followed the interrupted cooling pattern (i.e., rapid cooling interrupted by a transient temperature increase) typical of Alaska marmots during only 4 of 13 midwinter cooling bouts. Instead, this animal was often the last of the animals with a logger to recool and did so in a continuous cooling pattern; this female often reached low $T_b$ before or with the others in the group (Figure 2.2). One other female cooled continuously during 2 bouts in 2008-09 and a third-winter female cooled continuously once during 2009-10.

***Rewarming Rates.*** Rewarming rates significantly differed between species (rewarm to 30 °C: $t = 9.26$, $p < 0.0001$; rewarm 10-30 °C: $t = 6.54$, $p < 0.0001$). Marmots rewarmed from torpor to 30 °C at a rate of 2.9 ± 0.7 °C/h. From 10 °C to 30 °C, their warming rate was 5.9 ± 1.8 °C/h. Squirrels rewarmed from torpor at an average rate of 4.9 ± 0.9 °C/h and from 10 °C to 30 °C at 10.7 ± 1.3 °C/h.

***Characteristics of Heterothermy.*** Among the arctic ground squirrels, 3 of 6 females and the male were juveniles hibernating for the first time, but no first-hibernation marmots are represented. The beginning, end, and duration of heterothermy (~230 d) were similar for male and female marmots and female squirrels (Table 2.2). Torpor bout length for marmots averaged 13.81 ± 3.19 d for all bouts in comparison to 16.77 ± 2.86 d in ground squirrels, while interbout arousals of marmots lasted 21.2 ± 5.0 h compared to

14.2 ± 3.5 h in ground squirrels (Table 2.2). The number of stable torpor bouts and interbout arousals during midwinter were comparable between marmots and squirrels, but marmots displayed 2-10 irregular torpor bouts (i.e., animal did not reach low, stable $T_b$) at the beginning and end of heterothermy while only 1 female squirrel experienced an irregular bout at the beginning of heterothermy and another female experienced 3 irregular bouts at the end. The minimum $T_b$ recorded in an individual female and male marmot was 0.6 °C and 0.9 °C, respectively. The minimum $T_b$ in a female and male arctic ground squirrel was -2.0 °C and -1.6 °C, respectively. Minimum soil temperatures were compared with minimum $T_b$ of animals at each location during each year to create potential thermal gradients. Marmots experienced potential thermal gradients of 11.0-16.0 °C over 4 years while ground squirrels faced potential gradients of 14.8-16.3 °C during 1 year (Table 2.2).

**Discussion**

Our findings from 2 species in the same arctic environment directly support the hypothesis that the social systems of marmots and ground squirrels have led to different strategies to survive long, cold winters (Arnold 1993). Alaska marmots display a high degree of synchrony in $T_b$ change during hibernation, confirming that they huddle during communal hibernation (Arnold 1988), which minimizes heat loss through social thermoregulation (Gilbert et al. 2010). This likely contributes to successful overwintering by enabling them to maintain above-freezing $T_b$ in the face of a large thermal gradient. In contrast, arctic ground squirrels displayed no synchrony with nearby squirrels. Instead, our data support that squirrels hibernate individually and reduce the thermal gradient and heat loss by allowing $T_b$ to decrease to -2 °C. Marmots displayed an interrupted cooling pattern with 2 distinct cooling rates during entrance to torpor, as was noted previously from the record of a single individual (Lee et al. 2009). The rapid cooling phase in marmots was 3-times faster than the comparable range of cooling in squirrels, while the slow cooling phase in marmots was 4-times slower than in squirrels.

The benefits of social thermoregulation have been recognized in daily torpor in Siberian hamsters and bats (Gilbert et al. 2010; Jefimow et al. 2011) and during hibernation in alpine and black-capped marmots and bats (Arnold 1993, 1995; Gilbert et al. 2010; Vasiliev 1991), but only 1 study has quantitatively investigated synchrony of $T_b$ patterns during hibernation (Ruf and Arnold 2000). Early work on alpine marmots graphically demonstrated the synchrony of a group of marmots held in captivity (Arnold 1988), and from Figure 2 we quantified the degree of synchrony from 1 interbout arousal to compare to our study. In a group of 4 alpine marmots, including 2 juveniles, arousal to high $T_b$ was initiated 12.4 h apart, after which the later individuals tended to passively increase in $T_b$ before warming to euthermia. In contrast, 4 adult free-living Alaska marmots initiated arousal within a mean time of 5.5 h and only rarely showed evidence of potential passive warming among the animals we were able to measure (Figure 2.2). Alpine marmots were less synchronous in returning to torpor, as calculated by the synchrony of reaching a $T_b$ of 30 °C during cooling. Alpine marmots had a synchrony of 31.0 h, while Alaska marmots cooled to 30 °C within a mean of 7.3 h. Greater synchrony in Alaska marmots may be due to the larger thermal gradient (up to 16 °C) that Alaska marmots must maintain relative to alpine marmots. In contrast, alpine marmots rarely experience sub-freezing burrow temperatures (Arnold et al. 1991) and cannot maintain torpor below -0.5 °C (Ortmann and Heldmaier 2000).

Marmots lost synchrony 14-37 days before the seasonal end of heterothermy. From the 2 winters males were included in the dataset, we observed that the loss of group synchrony corresponded to males maintaining high $T_b$, which is necessary for spermatogenesis (Barnes et al. 1986). Females did not reach low, stable $T_b$ while males were at high $T_b$ for periods of 18-32 days, which could be due to the exogenous heat production of males influencing female torpid $T_b$. Interestingly, all 3 males became heterothermic again after the extended euthermic period, with 2-4 additional torpor bouts before exiting hibernation, which could suggest that these individuals were no longer reproductive upon coming above ground (Barnes et al. 1986). Because marmots have been known to breed underground (Rausch and Bridgens 1989; Rausch and Rausch

1971), this euthermic period may include both spermatogenesis and mating, after which male marmots re-enter torpor to conserve energy until emergence.

The lack of synchrony at the end of seasonal heterothermy was mirrored at the beginning: during the first 4-5 weeks of heterothermy, Alaska marmots did not show the striking levels of synchrony found during midwinter, and individuals often displayed different numbers of heterothermic bouts. Asynchrony at the beginning of heterothermy cannot be attributed to male euthermy, but it may be due to the presence of juveniles. Juvenile marmots impair synchrony, which contributes to the increased cost of hibernation in groups that include young (Arnold 1993, 1995; Ruf and Arnold 2000). Juvenile marmots may need more time to adjust to heterothermy and may consequently disturb their nestmates until they can successfully enter torpor, but the contributions of first- and second-winter Alaska marmots to synchrony is still unknown. Juvenile arctic ground squirrels (4 in this study) did not show signs of an adjustment period, but they were more developed than juvenile marmots upon entering their first hibernation. For example, female ground squirrels emerged from their first hibernation with 75 ± 2% of the average adult female mass at emergence and were ready to breed (Armitage 1981; pers. observation), while a marmot (779 g) captured in the spring after her first winter weighed only 33% of the average nonbreeding adult female mass at emergence (2,328 g). A female marmot captured after what was assumed to be her second winter weighed 1,732 g, 74% of the nonbreeding female mass, but was within the range of other females after her third winter (Table 2.3). This may indicate that Alaska marmots mature over 3 years and younger animals remain with the family group, potentially affecting group synchrony (Arnold 1995).

The differences in warming and cooling rates between arctic ground squirrels and Alaska marmots could not be contributed solely to body mass, though size should explain some of the variation. Ground squirrels rewarmed about 1.7-times faster than marmots as they heated a smaller mass, and Alaska marmots warmed from 10 to 30 °C at a rate of 5.9 °C/h, which is 2.7-times faster than the slightly larger alpine marmots warmed over the same interval at only 2.19 °C/h (Ruf and Arnold 2000). However, Alaska marmots

typically had a body mass about 3-times that of arctic ground squirrels, yet they cooled 3-times faster at the beginning of torpor entrance, indicating that marmots are enabling increased rates of heat loss at the initiation of a torpor bout while squirrels maximize insulation. After a transient temperature increase, marmots resumed cooling at 0.25-times the rate of the squirrels over the same temperature range, which would be expected considering the larger thermal mass and reduced surface area of a huddle. In contrast, huddled adult alpine marmots cooled in a continuous pattern with an overall average rate of -0.42 °C/h from 30 °C to 10 °C at ambient temperatures > 0 °C (Ruf and Arnold 2000). We previously proposed a behavioral mechanism in which Alaska marmots increased heat loss by exposing surface area before joining the huddle while still ambulatory (Lee et al. 2009). In this study, we found that the breeding female of the colony usually entered torpor last and cooled continuously instead of in the interrupted cooling pattern typical of the other marmots, indicating that interrupted cooling is not obligatory in Alaska marmots, yet she reached low $T_b$ at approximately the same time as the others. This animal may be part of regulating the synchrony of the group and may enter torpor last on the outside of the huddle to ensure that the young are thermally protected, as dominant animals have been shown to do (Arnold 1988).

We were unable to recover all loggers from all implanted marmots, and we were also unable to implant every marmot that hibernated in the hibernaculum during any given winter (Table 2.3). In particular, we have no data from any juveniles, and we were never able to confirm an individual as the territorial male. More animals were present during all years: we caught unmarked animals from the hibernaculum every spring and 12 different individuals were observed around the hibernaculum in summer 2009. Our synchrony data only includes animals from which we recovered data loggers and may thus demonstrate a higher degree of synchrony than the real synchrony of all animals in the hibernaculum. Animals during the winter from which we have 4 $T_b$ records were less synchronous than animals in years from which we have only 3 $T_b$ records, but in this case the additional animal was determined to be a breeding animal and was typically the last to enter torpor. Therefore, most other, unmarked adults may fall within the patterns of

the animals for which we have data. Our findings are also limited as they are from a single family group over multiple years and thus represent a small number of related animals.

We have shown that the high-latitude Alaska marmot, which experiences an even greater thermal challenge than the high-altitude alpine marmot, potentially demonstrates the most synchronous $T_b$ patterns known among communally hibernating animals. Synchronous change in $T_b$ is likely an adaptation to minimize energetic costs while utilizing social thermoregulation, and it contrasts with the patterns of hibernation observed in sympatric arctic ground squirrels, which appear to hibernate individually even in close proximity to conspecifics. Alaska marmots typically display a unique, interrupted cooling pattern which has not been previously observed in hibernators, but they may also cool continuously like other hibernating mammals. Further research is needed to better understand the benefits and mechanisms associated with this unique cooling pattern.

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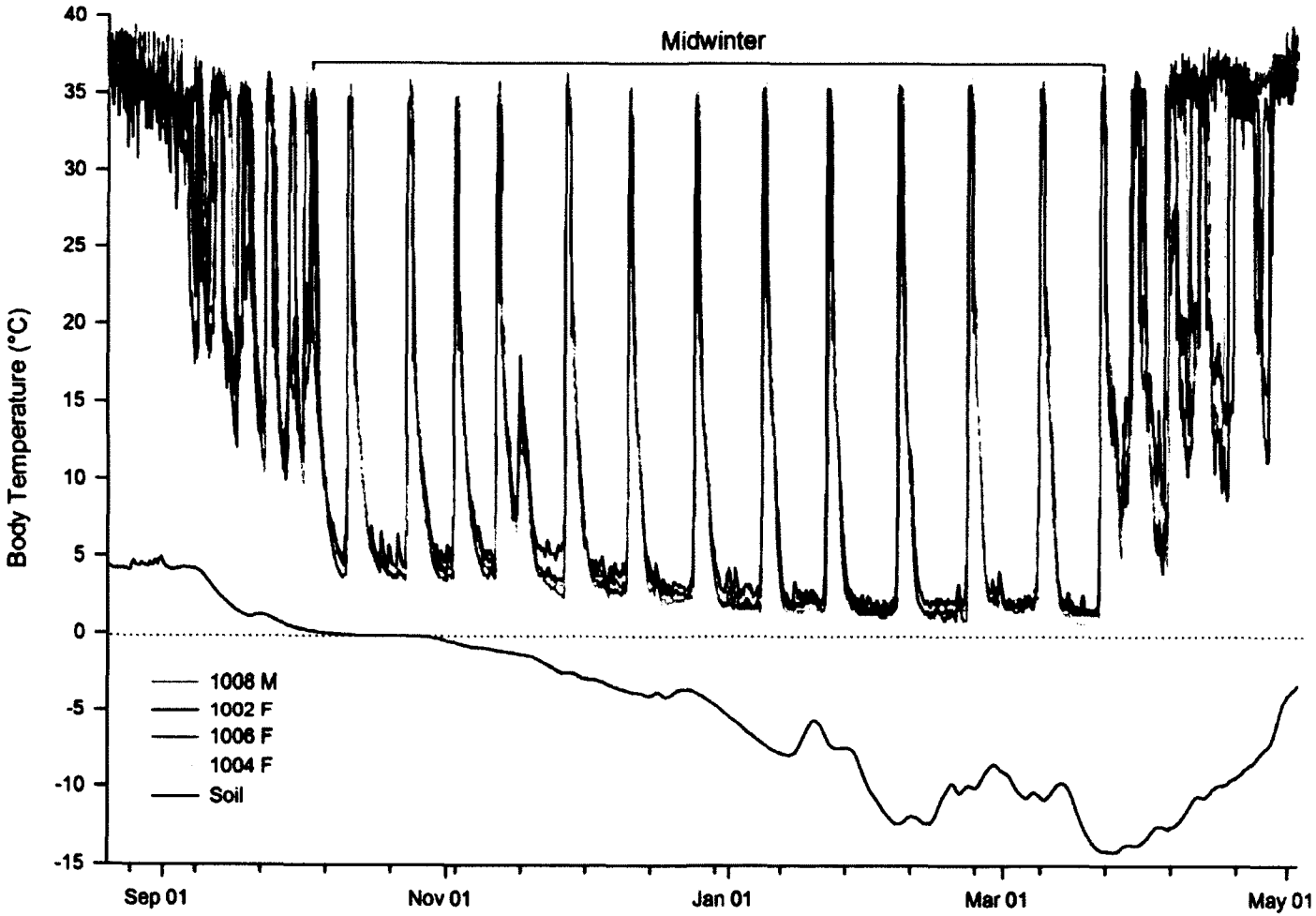


Figure 2.1. Season of heterothermy for 4 adult Alaska marmots during winter 2008-09. Midwinter is the period corresponding to low, stable $T_b$ (< 1 °C variation in 24 h).

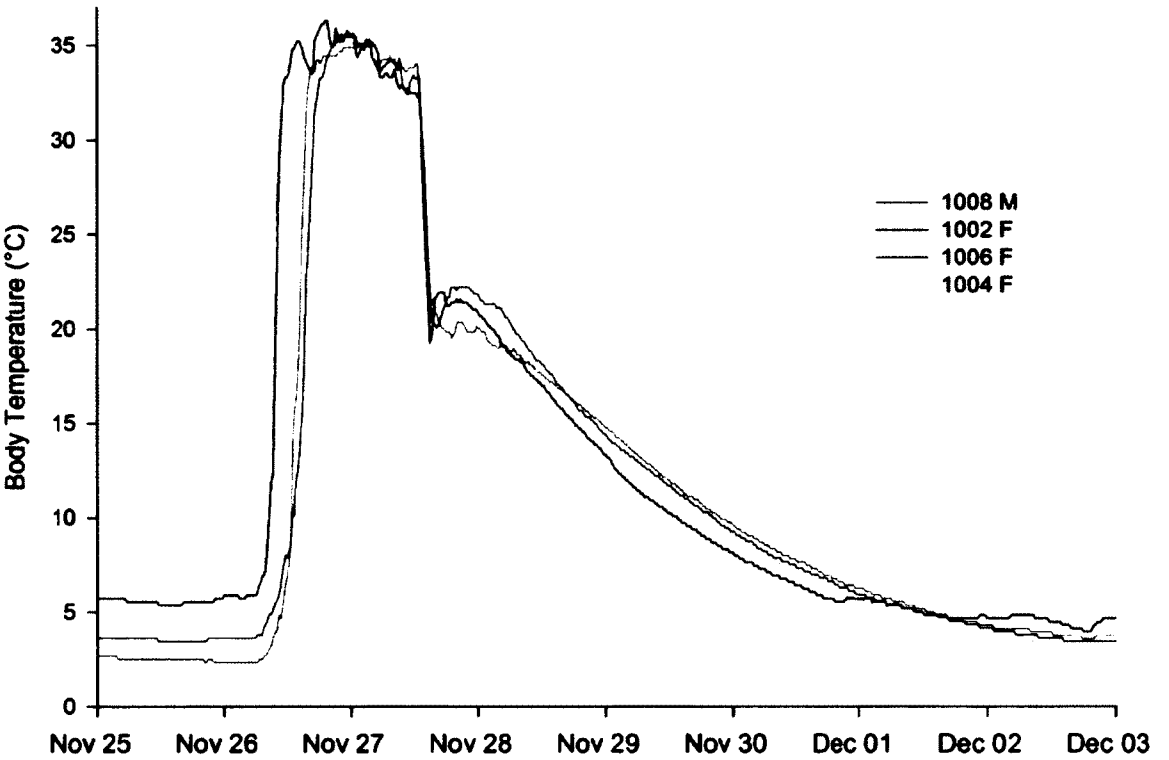


Figure 2.2. An example of synchrony among body temperatures of 4 free-living adult Alaska marmots during arousal and return to torpor in winter 2008-09. Marmot 1004 F (yellow line) was identified as the breeding female and typically cooled last in a continuous pattern, unlike the interrupted cooling curve typical of other Alaska marmots.

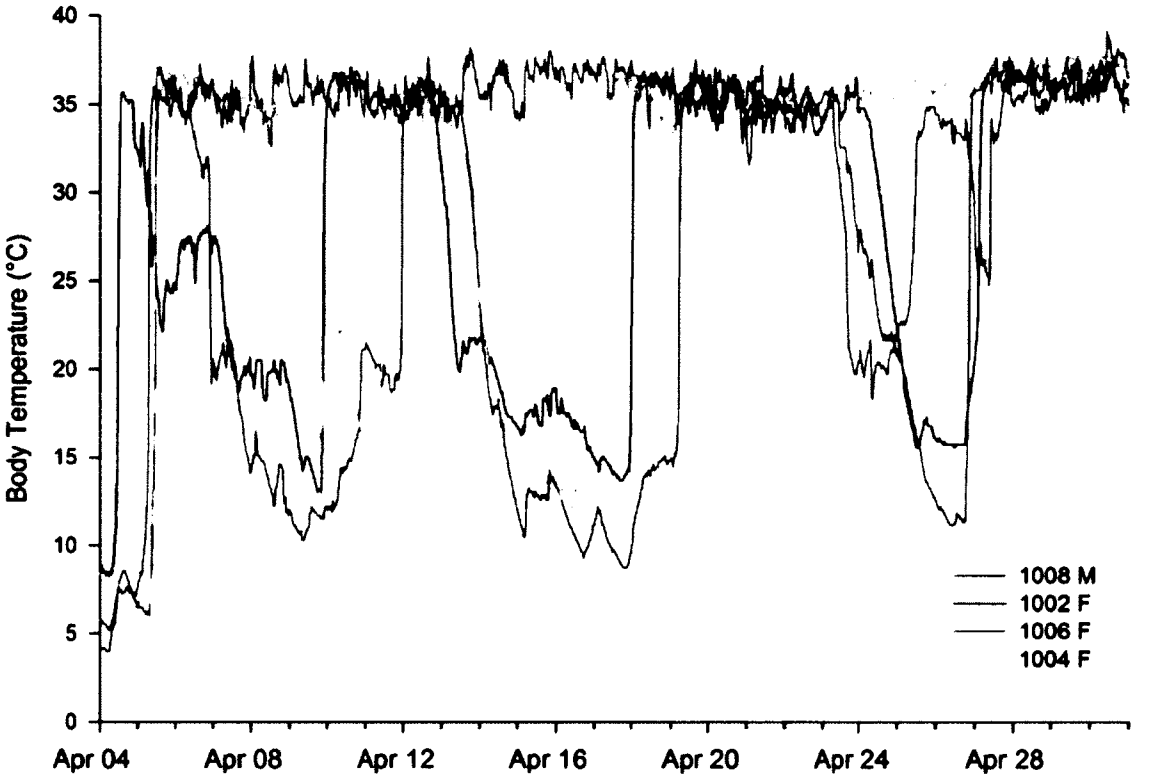


Figure 2.3. End of heterothermy for 4 Alaska marmots in spring 2009. Marmot 1008 M, a male (orange line), returned to high body temperature and females correspondingly lost synchrony and did not return to steady torpor. The male cooled below 30 °C twice after maintaining high body temperature for 18 days, but the individual assumed to be the breeding female, 1004 F (yellow line), remained at high body temperature during the other animals' last drop below 30 °C.

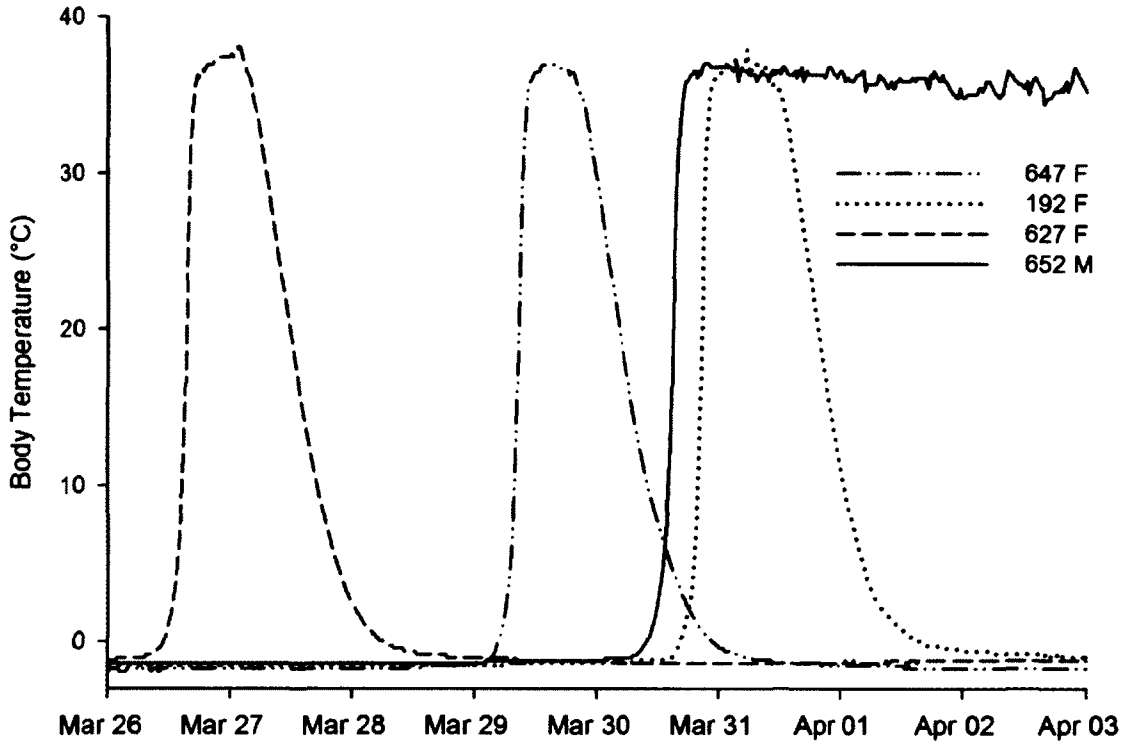


Figure 2.4. Body temperature change in 4 arctic ground squirrels captured at the same burrow site upon emergence from hibernation in spring 2009. Squirrel body temperatures are independent of each other, and the females are not affected by the male's return to high body temperature.

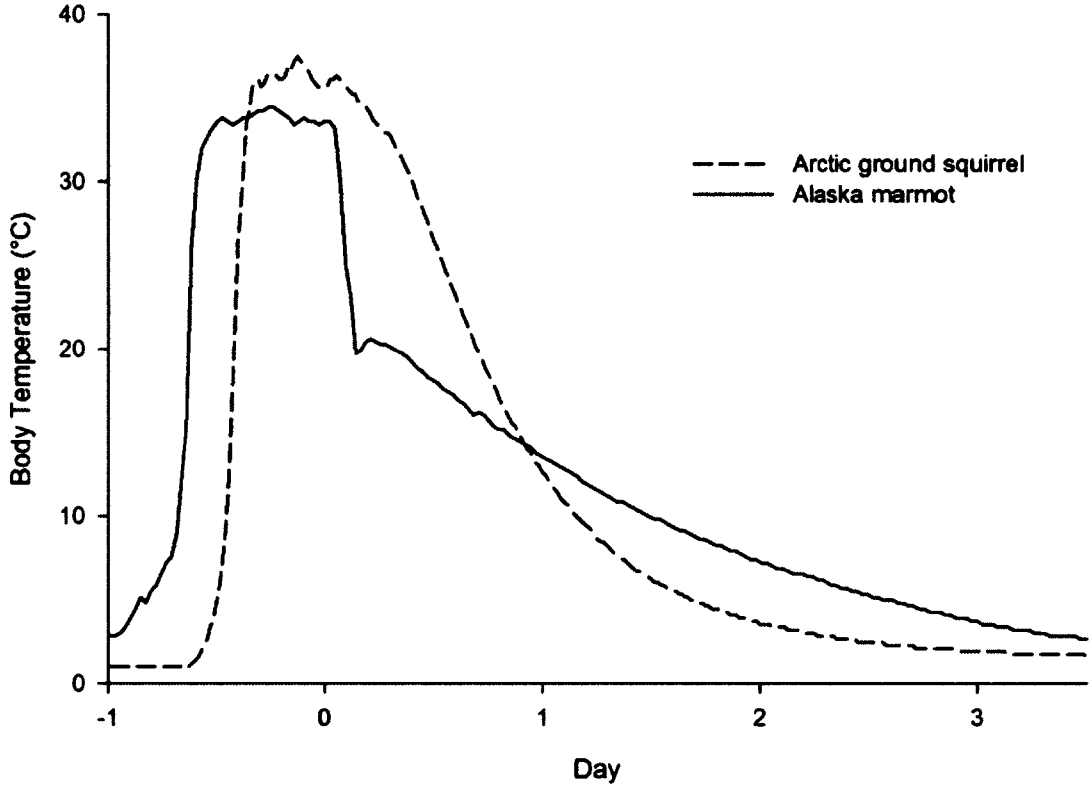


Figure 2.5. An arctic ground squirrel male and an Alaska marmot male demonstrate different cooling patterns from an interbout arousal (continuous and interrupted, respectively). Interrupted cooling of marmots during the rapid cooling phase was 21-times faster than during the slow cooling phase. Data were assigned a time of 0 at the beginning of cooling during the arousal.

Table 2.1. Parameters of heterothermy of Alaska marmots during midwinter.

| Parameter | 2008-09 (3F, 1M) | 2009-10 (3F) | 2010-11 (1F, 2M) |
|---|---|---|---|
| # Torpor bouts* | 13 | 11 | 11 |
| # Interbout arousals* | 12 | 10 | 10 |
| Torpor bout length* (d) | 12.23 ± 0.06 | 15.59 ± 0.02 | 14.49 ± 0.14 |
| Interbout arousal (h) | 20.5 ± 0.7 | 21.0 ± 0.3 | 23.5 ± 2.8 |
| Synchrony- Cooling at 30 °C (h) | 7.3 ± 2.6[x] | 3.8 ± 2.5 | 5.6 ± 4.9 |
| Synchrony- Warming from torpor (h) | 5.5 ± 3.0 | 3.1 ± 1.6 | 2.2 ± 1.0 |

Note: Animals were considered in torpor when body temperature ($T_b$) < 30 °C and in interbout arousal when $T_b$ > 30 °C. Synchrony is the time between the first and last loggered animal to reach a defined threshold. F indicates females and M indicates males. Means are expressed ± SD.

*midwinter: defined in marmots as period when $T_b$ reaches low and stable levels

[x]does not include first cooling into midwinter: marmots had not coordinated cooling yet (synchrony = 30.5 h)

Table 2.2. Characteristics of heterothermy in Alaska marmots and arctic ground squirrels hibernating in the Arctic.

| | Alaska Marmots | | Arctic Ground Squirrels | |
|---|---|---|---|---|
| | Females (n = 8) | Males (n = 3) | Females (n = 6) | Male (n = 1) |
| Heterothermy start† | 4 Sept (24 Aug-10 Sept) | 9 Sept (24 Aug-17 Sept) | 9 Sept (29 Aug-22 Sept) | 3 Oct |
| Heterothermy end† | 16 Apr (10-27 Apr) | 9 May (27 Apr-12 May) | 28 Apr (19 Apr-1 May) | 30 Mar |
| Heterothermy duration (d) | 228.0 ± 6.2 | 230.7 ± 7.6 | 229.7 ± 6.5 | 177.7 |
| Torpor bout length* (d) | 13.87 ± 3.09 | 13.65 ± 3.49 | 16.74 ± 2.70 | 16.98 ± 3.82 |
| Interbout arousal* (h) | 20.6 ± 3.7 | 22.9 ± 7.3 | 13.2 ± 2.0 | 20.3 ± 4.5 |
| # Torpor bouts* | 11-13 | 11-13 | 10-11 | 10 |
| # Interbout arousals* | 10-12 | 10-12 | 9-10 | 9 |
| # Irregular bouts, start of heterothermy† | 7 (5-10) | 4 (4-8) | 0 (0-1) | 0 |
| # Irregular bouts, end of heterothermy† | 3.5 (2-5) | 4 (4-5) | 0 (0-3) | 0 |
| Minimum $T_b$ (°C) | 1.2 ± 0.4 | 1.1 ± 0.2 | -1.7 ± 0.3 | -1.6 |
| Minimum $T_{soil}$ (°C)ˣ | -12.4 ± 2.5 | -12.2 ± 2.8 | -17.3 ± 0.7 | -16.7 |
| Maximum gradient ($T_b$-$T_{soil}$, °C) ‡ | 13.5 ± 2.1 | 12.6 ± 2.1 | 15.4 ± 0.6 | 15.1 |
| Rapid cooling rate (30 to 24 °C; °C/h) | -5.1 ± 3.2 | -5.5 ± 2.5 | -1.8 ± 0.2 | -1.5 ± 0.2 |
| Slow cooling rate (19 to 6 °C; °C/h) | -0.26 ± 0.07 | -0.25 ± 0.08 | -1.00 ± 0.29 | -0.79 ± 0.25 |
| Rewarming rate (Torpor to 30 °C; °C/h) | 2.8 ± 0.7 | 2.9 ± 0.7 | 5.0 ± 0.9 | 4.4 ± 0.3 |
| Rewarming rate (10 to 30 °C; °C/h) | 5.8 ± 1.7 | 6.3 ± 2.0 | 10.5 ± 1.3 | 11.8 ± 0.9 |

Note: Heterothermy began with first body temperature ($T_b$) < 30 °C and ended with final return to $T_b$ > 30 °C; torpor duration is days $T_b$ < 30 °C, interbout arousal duration is hours $T_b$ > 30 °C. Means are expressed ± SD.

†expressed as median (range); *midwinter: defined in marmots as period when $T_b$ reaches low and stable levels; defined in arctic ground squirrels as the same timeframe within hibernation as marmots for the year squirrels were captured (includes torpor bouts spanning from 3 Oct- 24 Mar); ˣmarmots: 4 years at 1 burrow; squirrels: 1 year at 2 burrows. ‡calculated as mean of (minimum $T_b$ of an individual - minimum $T_{soil}$ at that individual's burrow that year)

Table 2.3. Alaska marmot body mass (kg) at first capture in spring.

| | **Females** | | breeding | young | | **Males** | | | |
|---|---|---|---|---|---|---|---|---|---|
| Year | 1002 | 1006 | 1004 | 1010 | 1016 | 1000 | 1008 | 1012 | 1014 |
| 2008 | 2.156 | 2.055 [d] | 2.847 | | | 2.770 | 2.222 | | |
| 2009 | 2.365 [d] | 2.210 [d] | | 1.732 | | | 2.402 [d] | | |
| 2010 | 2.497 [d] | 2.285 [d] | 2.834 [d] | 2.171 [d] | | | | 2.630 | 2.130 |
| 2011 | 2.568 [d] | 2.642 [f] | 2.815 [f] | | 0.779 | | | 2.504 [d] | 2.853 [d] |

[d] data recovered at this capture

[f] failed $T_b$ logger recovered

# Chapter 3 ESTIMATING LEAN MASS OVER A WIDE RANGE OF BODY COMPOSITION: A CALIBRATION OF DEUTERIUM DILUTION IN THE ARCTIC GROUND SQUIRREL[1]


## Abstract

Calculating body water through isotope dilution has become a useful, nondestructive way to estimate body composition in many species. The most accurate estimates using this method require calibration against proximate chemical analysis of body composition for individual species, but no studies to our knowledge have calibrated this method on a hibernating mammal that seasonally undergoes dramatic changes in body composition. We use deuterium oxide to estimate total body water in captive arctic ground squirrels, *Urocitellus parryii*, and compare two approaches of calculating lean mass from total body water, both calibrated against lean mass based on proximate analysis. The first method uses a single tissue hydration constant to calculate lean mass from total body water; the second method uses a predictive equation to calculate lean mass from total body water and body mass. We found that the predictive equation performs better over the large range of body composition common to this species. Distillation of blood samples did not affect lean mass estimates from either calculation method. These findings indicate that isotope dilution using a predictive equation should work well as an alternative to destructive methods in other small mammals that undergo radical changes in body composition across their annual cycle.


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**Introduction**

Body composition of birds and mammals is a key metric in many physiological and ecological studies. The most accurate method for measuring body composition, proximate analysis, directly determines the mass of body water, fat, and lean dry mass by chemical extraction;[1] however, this method is destructive and precludes repeated sampling. Many methods have been developed to estimate body composition nonlethally and even noninvasively, including dual energy X-ray absorptiometry (DXA) which directly estimates fat and lean mass content.[2] In contrast to DXA, several of these methods depend on the difference in water content (hereafter, "hydration") between lipid and lean tissue, including bioelectrical impedance analysis (BIA), total body electrical conductivity (TOBEC), and isotope dilution. Of these, stable isotope dilution is particularly attractive because, unlike other methods, it is insensitive to the position of the animal during measurements and does not require technical equipment in the field. Here we present a brief description of the method and some outstanding issues underlying its current application across different taxa.

Estimation of body composition through isotope dilution involves injecting a known quantity of water labeled with either deuterium ($^2H$) or $^{18}O$ into the body water pool and measuring the dilution of the label after mixing. This estimates the total body water (TBW) pool, which is then used to estimate lean mass under the assumption that lipid contains essentially no water. Fat mass may be estimated by subtracting lean mass from total body mass, although we note that these estimates may have low accuracy when fat stores are very small. While these procedures are straightforward, there are different approaches to converting estimates of TBW to lean mass. One approach assumes that the hydration of lean mass is constant;[3] however, subsequent studies have shown that hydration can range from 62% to 74% among bird and mammal species.[1] Hydration can also vary among individuals with stage of growth, season, reproductive status, and capture stress.[1, 4] This lack of a universal hydration of lean tissue poses one of the

biggest problems with using the isotope dilution method to estimate body composition,[5] particularly for animals exhibiting a wide range of body composition.[6]

A newer approach to body composition estimation through isotope dilution can potentially resolve this issue while taking into account wide ranges of fat content. This approach involves calibrating a predictive equation based on isotope dilution and morphometric data against direct measure by proximate analysis. Some studies have derived useful predictive equations for related taxa (i.e., pinnipeds[7] and ursids[8]), but the most accurate predictive equations are species-specific.[9, 10] Studies have calibrated this approach against proximate analysis in seals (Antarctic fur seals[10] and gray seals[11]), domestic dogs,[12] barnacle geese,[13] and Boer goats.[9] Layton et al.[14] found this approach to be an effective way to deal with changing hydration during the growth of young American mink, and it may also be useful in animals that dramatically alter body composition across seasons. For example, hibernating mammals undergo extreme shifts in body composition as they deposit lipid in preparation for, and deplete lipid during, hibernation.[15] However, we are not aware of any studies from small hibernators that calibrated predictive equations for estimating lean mass from isotope dilution.

In addition to issues surrounding lean mass calculation, sample preparation should also be considered.[12, 16] Most stable isotope dilution studies distill water from blood samples before analysis by isotope ratio mass spectrometry (IRMS).[5, 13] However, distillation requires specific equipment such as a vacuum distillation line and may introduce more error via extra handling steps or incomplete recovery of sample water. It may also preclude the use of very small samples resulting from minimally invasive techniques for sampling blood (e.g., from clipping a claw). Increasing use of high temperature TCEA (thermal conversion elemental analysis) to introduce oxygen and hydrogen isotopes in a continuous flow into an IRMS may relax the requirement for sample distillation.[17] To our knowledge, no studies have systematically examined whether distillation of blood samples is necessary with these methods, or the extent of its effect on isotopic measurements and estimates of lean mass.

In this study we calibrated the use of isotope dilution as a nonlethal method of estimating lean mass in a hibernator against proximate chemical analysis. The arctic ground squirrel, *Urocitellus parryii*,[18] dramatically changes body composition through its annual cycle[19, 20] and is thus particularly well suited for investigating whether isotopic methods of lean mass estimation are robust over large ranges of lean and fat mass. Because arctic ground squirrels exhibit the most extreme physiology (minimum body temperature, thermoregulatory capacity, etc.) described for any hibernator, it is also the subject of ongoing ecological and physiological research that would benefit from an accurate and nondestructive method of estimating body composition.[21-23] In calibrating isotope dilution, we evaluated whether distillation with a simple technique affected hydrogen isotope ratios in blood samples and whether measured effects were sufficient to substantially alter estimates of lean mass. We also compared the use of a single hydration constant to estimate lean mass from TBW with the use of a linear, predictive equation based on both TBW and body mass. We evaluated each combination of these methods of sample preparation and approaches to estimation in individuals spanning a wide range of body composition.

## Material and Methods

***Animals*** We selected 11 animals (8 females and 3 males), including one juvenile, from a colony of wild-caught arctic ground squirrels maintained at the University of Alaska Fairbanks on a standard diet of Mazuri Rodent Chow. Animals were selected for calibration of isotope dilution based on variation in body mass, which ranged from 534-1335 g at the time of sampling.

Prior to calibration of isotope dilution, we determined the appropriate dosage of deuterium and sampling times for *U. parryii* by using three additional individuals (2 females, 1 male) from the colony. To achieve our target enrichment (40 ppm above background) with our enriched injectate (30,000 ppm, 3%), we calculated the dosage of deuterium to administer using Equation 12.1 from Speakman.[24] We anesthetized each

animal by gas anesthesia (isoflurane, 3-5%) and clipped a claw at the distal edge of the quick to collect a background sample of free-flowing blood into 2-3 heparinized Natelson capillary tubes (~100 μL per tube), which were immediately flame-sealed, centrifuged to separate plasma and red blood cells, and kept upright to prevent mixing of blood fractions in cool storage. We injected the animals intraperitoneally with a 3% deuterium solution and repeatedly anesthetized animals and sampled blood as described above every ~20-30 min from 40-150 min. Concentrations of deuterium observed between 40 and 150 min after injection did not significantly differ ($n = 3$, RM-ANOVA, $F_{2,4} = 1.51$, $p = 0.29$), and we selected 60 min post-injection as our target for collection of the diluted sample. Results indicated a sufficient dose was administered according to the equation:

$$\text{injection volume (mL)} = \text{body mass (g)} * 0.000867 \quad (3.1)$$

For calibration of isotope dilution estimates of body composition, the 11 squirrels were anesthetized, weighed, sampled, and injected as described above. After 60 (± 1) min following deuterium injection, each animal was anesthetized again and another blood sample was taken. Animals were then euthanized by sodium pentobarbital overdose (390 mg/kg) and frozen at -20 °C until analyzed for body composition by proximate analysis. All animal use procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee under protocol 06-40.

***Stable Isotope Analysis*** For each squirrel and sampling time, we selected one of a matching pair of capillary tubes of blood to be distilled to water while the other was kept as undistilled plasma for analysis. Water was distilled from blood samples in flame-sealed, long-tipped Pasteur pipettes on hot plates (Figure 3.1). Samples of the enriched injectates were diluted to approximately 200‰ (target squirrel enrichment) and analyzed to characterize the actual isotopic composition of labeled water injected. Sample $\delta^2H$ values were measured at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks by continuous flow isotope ratio mass spectrometry (IRMS), using a ThermoElectron high temperature conversion elemental analyzer (TCEA) coupled to a Thermo-Finnigan Delta V Plus isotope ratio mass spectrometer via the Conflo III

interface (both instruments: Thermo Fisher Scientific, Bremen, Germany). We manually injected samples in triplicate into the TCEA for analysis. All isotope values are expressed in delta notation as $\delta X = ((R_{sample} - R_{standard}) / R_{standard}) * 1000‰$, where X = the heavy isotope (here, $^2H$), R = the ratio of heavy to light isotope, and the standard is Vienna Standard Mean Ocean Water (V-SMOW).

***Proximate Analysis*** Proximate analysis of whole squirrels was performed according to modifications of the standard procedure described by Mason et al.[25] Briefly, individual squirrel carcasses were thawed, cut into sections, and passed through a meat grinder three times. Three subsamples of the resulting homogenate (~2.0 g) were dried to a constant mass. Water content was determined by subtraction of dry mass from initial mass of the homogenate and varied among triplicate subsamples by ± 0.86% of wet mass. Two subsamples were dried and lipid extracted in petroleum ether using a modified Soxhlet procedure. Lipid content was determined gravimetrically and varied between replicated analyses by ± 0.83% of wet mass. Lean mass was determined as the sum of the lipid free dry mass and the water content (g). All portions were then extrapolated to the mass of the whole animal to calculate total masses of water, lipid, and lean mass.

***Total Body Water Estimates*** To generate lean mass estimates from isotope dilution, we first estimated TBW of the animals according to the methods of Speakman.[24] Briefly, moles of water in the animal ($mol_A$) were estimated according to the equation:

$$mol_A = (mol_I * (Sample_E - Injectate_E)) / (Sample_B - Sample_E) \quad (3.2)$$

where $mol_I$ is moles of injectate, $Sample_E$ and $Sample_B$ are concentrations of deuterium in enriched and background samples (respectively) in ppm, and $Injectate_E$ is the concentration of deuterium in the enriched injectate in ppm. Moles of water in the animal were then converted to an estimate of total body water by isotope dilution ($TBW_{ISO}$) by the following equation:

$$TBW_{ISO} = mol_A * mass_W \quad (3.3)$$

where $mass_w$ is the molecular mass of the water of the animal determined empirically (see [24] for detailed discussion).

***Lean Mass Estimates***

From Hydration Constant. We calculated the mean hydration constant (HC) derived from proximate analysis according to the equation:

$$HC = TBW_P / LM_P \quad (3.4)$$

where $TBW_P$ is the total body water determined by proximate analysis and $LM_P$ is the lean mass determined from proximate analysis. We then used this hydration constant approach to estimate lean mass (LM) using the equation:

$$LM = TBW_{ISO} / HC \quad (3.5)$$

From Predictive Equation. We also used lean mass determined by proximate analysis to parameterize a predictive equation of lean mass based on body mass and $TBW_{ISO}$. We evaluated the extent to which the predictive equation improved upon the performance of the hydration constant approach by determining how well each approach recovered actual lean mass of individuals across a wide range of body mass and body composition.

***Statistical Analysis*** We assessed agreement between estimates of lean mass using isotope dilution and lean mass measured by proximate analysis following Bland and Altman:[26] agreement between methods is greatest when the standard deviation of the differences between methods is smallest. We compared distilled and undistilled plasma samples with a paired t-test within background and enriched samples. Enriched samples from one animal were compromised and eliminated from analysis, restricting lean mass calculations to 10 animals. We used multiple regression analysis of $TBW_{ISO}$ and body mass (BM) against lean mass (LM, from proximate analysis) to generate a predictive equation. All $R^2$ values reported are adjusted for the number of explanatory terms in the model and $\alpha$ was set at 0.05.

**Results**

***Distilled vs. Undistilled*** There was no difference in $\delta^2H$ value between distilled and undistilled enriched samples ($n = 10$, $t = -0.48$, $p = 0.64$). Values of $\delta^2H$ for distilled background samples were on average 4.9‰ higher than undistilled samples ($n = 11$, $t = -5.82$, $p = 0.0002$). However, lean mass estimates generated from distilled and undistilled samples agreed equally well[26] with proximate analysis when calculated from either approach (Table 3.1).

***Generating Lean Mass Estimators*** Arctic ground squirrels in this study had an average lean tissue hydration of 0.686 ± 0.022 (SD; range: 0.652 - 0.719; n = 11). This value was used in Equation 3.5 to estimate lean mass from $TBW_{ISO}$ (hydration constant approach).

To generate a single predictive equation applicable to both distilled and undistilled samples, a single distilled or undistilled estimate of TBW was randomly assigned for each squirrel (n = 5 distilled, 5 undistilled). Multiple regression analysis generated the following predictive equation ($n = 10$, $F_{2,7} = 208$, $p < 0.0001$, $R^2_{adj} = 0.98$).

$$LM = 1.1535 * TBW_{ISO} + 0.1481 * BM + 6.75 \quad (3.6)$$

The standard errors of the parameters in this expression were as follows: $TBW_{ISO}$ SE = 0.112 and BM SE = 0.026. Fat mass can be estimated by subtracting calculated lean mass from total body mass; these estimates of fat mass will have the same error (g) as lean mass estimates (Table 3.1).

***Comparing Techniques for Lean Mass Estimation*** As expected, the hydration constant approach provided a close match between estimated and actual lean mass at the mean body composition, but over- or underestimated lean mass at the extremes; in contrast, using a predictive equation provided accurate estimates of lean mass across the full range of body composition, which spanned an eight-fold range of percent body fat (6.5-52%; Figure 3.2). Estimates of lean mass based on the predictive equation had two-fold better agreement with proximate analysis than estimates based on the mean

hydration, using the standard deviation of the residuals as a measure of error (± 16 g vs. 37 g, Table 3.1).[26]

**Discussion**

This study demonstrated that isotope dilution is an effective method for estimating lean mass (and thereby fat mass) of a hibernator over an eight-fold range in percent body fat. Only among unenriched background samples did sample preparation affect $\delta^2H$ values: those of distilled samples were elevated over undistilled samples by < 5‰. However, this effect was small relative to other sources of error and thus did not result in significant differences in estimated lean mass compared with proximate analysis. While the prevalence of distillation in body composition studies implies that distilled samples are more accurate, little attention has been given to this question. Distillation by evaporative freeze capture has been argued to yield more precise estimates of TBW than direct serum counts of tritium, but unfortunately comparisons could not be made to empirically derived TBW to determine accuracy of the two methods and the results could have been affected by factors that do not apply to TCEA IRMS.[16] Distillation has also been found to be unnecessary for measuring $\delta^2H$ values in urine by isotope ratio infrared spectroscopy (IRIS).[27] In light of our findings, we generated a predictive equation for lean mass that accounted for variation in hydration and can be used with distilled and undistilled samples. Estimates generated from this predictive equation agreed with proximate analysis much better than did estimates from the hydration constant approach, even though we calculated a hydration constant specific to *U. parryii* (0.686) rather than using the standard constant of 0.732.[3] This difference was particularly apparent at the extremes of body condition, where a constant hydration factor failed to accurately reflect lean mass. Because *U. parryii* naturally varies widely in body composition (from 7% to 42% fat within a few months of the active season),[20] a species-specific predictive equation is particularly appropriate.

We found that including body mass in the predictive equation for lean mass estimation improved its explanatory power ($R^2 = 0.98$ vs. 0.90 without body mass). Body mass was strongly correlated with percentage body fat ($n = 10$: $F_{1,8} = 40.74$, $p = 0.0002$, $R^2_{adj} = 0.82$), which was negatively (although nonsignificantly) related to hydration of lean mass ($F_{1,8} = 3.92$, $p = 0.083$, Figure 3.3). This indirect association between tissue hydration and body mass (via percentage body fat) may account for why predictive models were improved when body mass was included, especially at extremes of body composition. A negative relationship between tissue hydration and percent body fat[6; this study] might occur if lipid becomes integrated into muscle tissue during fattening and thus precludes a portion of water space, but more studies are needed to determine the mechanism involved.

Isotope dilution using a predictive equation is an effective method to estimate lean mass in *U. parryii* and should also work well in other small mammals that undergo radical changes in body composition across their annual cycle. Sample distillation did not significantly affect estimates of lean mass; however, the parameters of the predictive equation varied slightly if generated exclusively from distilled or undistilled samples. We based our reported predictive equation on TBWs randomly assigned from distilled or undistilled samples to give it the broadest generality. Ideally, these approaches to lean mass estimation would be validated on an independent sample of animals. However, we would not expect such a validation to change our conclusion that the predictive equation performs better than the hydration constant, and that sample distillation is not necessary for evaluation of body composition.

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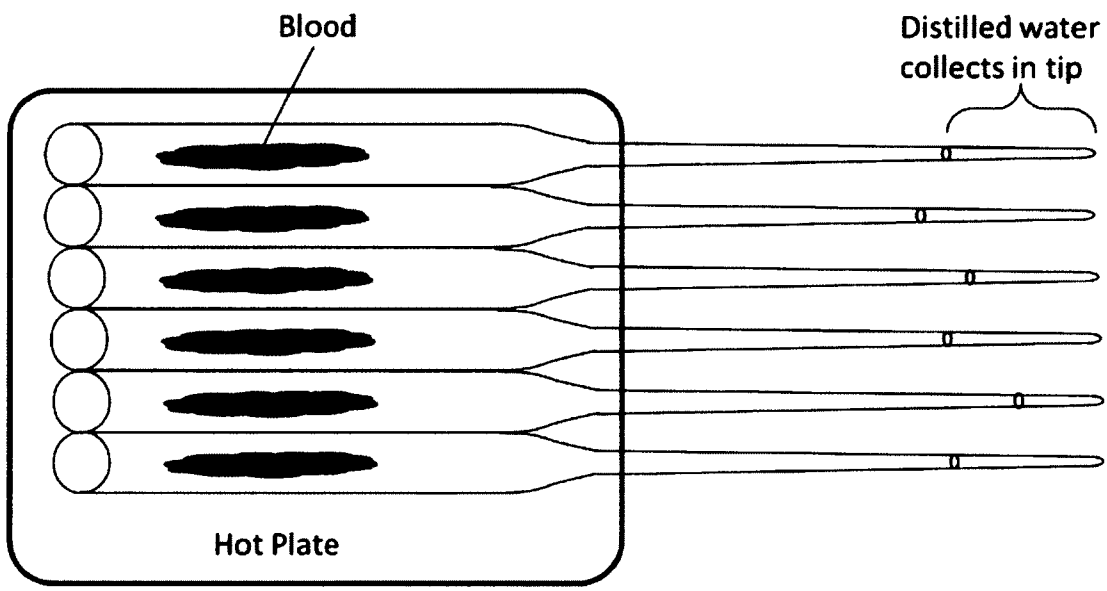


Figure 3.1. Setup for distillation of blood samples. Samples were placed into 23-cm Pasteur pipettes and flame-sealed on both ends. As the sample was heated on the hot plate (< 100 °C), water vapor condensed in the narrow tip extending beyond the hot plate. Once distillation was complete, the sample was flame-sealed into the tip until analysis.

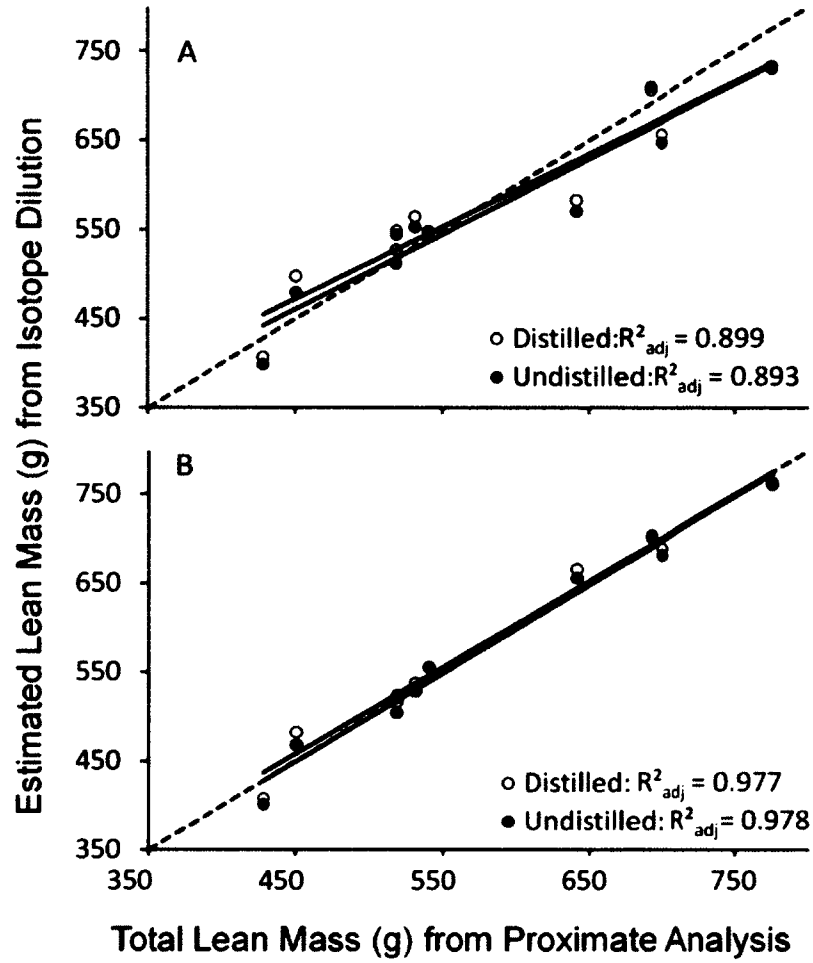


Figure 3.2. A comparison of lean mass of arctic ground squirrels determined from proximate analysis with estimates made from isotope dilution. Open symbols indicate estimates based on distilled samples, and filled symbols indicate estimates based on undistilled samples. The dotted line represents a 1:1 agreement between estimates and lean mass. Estimates from isotope dilution were generated using A) a species-specific average hydration constant and B) a multiple regression equation including body mass. $P < 0.0001$ in all cases.

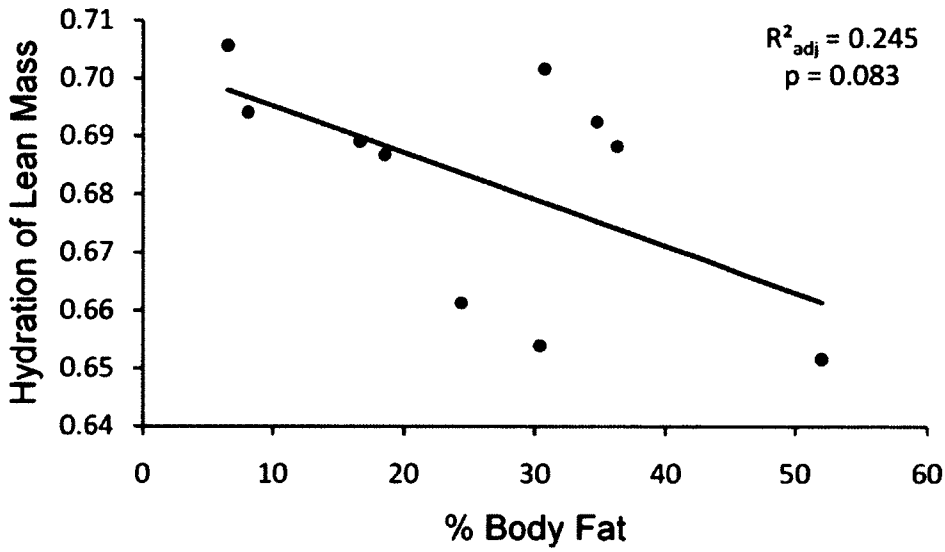


Figure 3.3. Hydration of lean mass of arctic ground squirrels tended to decrease as a function of percentage body fat. Because growing animals tend to have higher water content in their lean tissue,[1] the single juvenile squirrel was eliminated from this analysis.

Table 3.1. Accuracy and precision of lean mass estimates based on two calculation types and two sample preparation methods

| Sample Preparation | Calculation Type | Mean Difference from $LM_P$ | | SD from $LM_P$ | |
|---|---|---|---|---|---|
| | | g | (%) | g | (%) |
| Distilled | Hydration Constant | -2.11 | (-0.25%) | ± 36.47 | (± 6.37%) |
| Undistilled | Hydration Constant | -10.08 | (-1.28%) | ± 36.39 | (± 6.09%) |
| Distilled | Predictive Equation | 4.73 | (0.91%) | ± 16.57 | (± 3.28%) |
| Undistilled | Predictive Equation | -1.58 | (-0.30%) | ± 16.16 | (± 3.13%) |

Note. Difference (i.e., error) calculated as Lean $Mass_{Proximate}$ ($LM_P$) - Lean $Mass_{Isotope}$ based on sample preparation and method of calculation.

# Chapter 4 STABLE NITROGEN ISOTOPES IN HIBERNATING ARCTIC GROUND SQUIRRELS: A TEST OF ALTERNATE MODELS FOR INCREASED $\delta^{15}N$ VALUES IN TISSUES DURING FASTING[1]

## Summary

We describe two models explaining the increase in tissue nitrogen isotope ratios ($\delta^{15}N$) that occurs during fasting in animals. The catabolic model posits that protein breakdown selectively removes the lighter isotope of nitrogen ($^{14}N$) from catabolized tissues, leaving behind a proportional increase in the heavy nitrogen isotope ($^{15}N$). The anabolic model posits that tissue synthesis during fasting results in elevated $\delta^{15}N$ values, as the unreplaced loss of $^{14}N$ to urea causes an increase in the proportion of $^{15}N$ in the residual plasma amino acids used for protein synthesis. We effected a range of lean mass loss in arctic ground squirrels (*Urocitellus parryii*) fasting during hibernation and collected organ and muscle tissues for analysis of $\delta^{15}N$ values. The catabolic model predicts an increase in $\delta^{15}N$ values in muscle, which undergoes little protein synthesis and significant catabolism. The anabolic model predicts no change in muscle, but an increase in $\delta^{15}N$ values in liver, which continues protein synthesis even during significant catabolism. We found a significant increase in liver $\delta^{15}N$ values with lean mass loss while muscle $\delta^{15}N$ values remained unchanged, a result that supports the anabolic model. Other organs (heart, small intestine, and brown adipose tissue) increased in $\delta^{15}N$ values, indicating protein synthesis in these tissues during hibernation. Urine was 3.8‰ lighter than plasma, and both urine and plasma increased in $\delta^{15}N$ values with lean mass loss. This study helps clarify the mechanisms causing $\delta^{15}N$ change during nutritional stress, thus increasing its utility for physiological research and reconciling previously contradictory results.

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## Introduction

The ecological and physiological strategies by which animals cope with adaptive fasting or starvation stress are subjects of enduring interest in physiological ecology (Mrosovsky and Sherry, 1980; Castellini and Rea, 1992; McCue, 2010). Some animals undergo prolonged fasting as part of their annual cycle, including hibernating mammals, long distance migrants, and birds that incubate eggs continuously. These animals anticipate fasting by building up large fat stores to support metabolic costs, but they may also catabolize significant amounts of lean mass during the fast (Hobson et al., 1993; Buck and Barnes, 1999). Other animals exposed to unpredicted decreases in food availability may have insufficient fat stores and rely on catabolism of lean mass for energy to an even greater extent. Loss of lean mass during either fasting or starvation can impact survival or subsequent reproductive success of animals, but the importance or consequences of the use of lean tissue is difficult to investigate without repeated captures and analyses of body condition.

Increase in the nitrogen stable isotope ratio ($\delta^{15}N$) of tissues may serve as an indicator of lean mass loss during nutrient deprivation. The physiological responses to anticipated fasting and unanticipated starvation are similar, but our focus is fasting and we use this term to encompass other forms of nutrient deprivation, including protein deficiency. Several studies have shown increases in $\delta^{15}N$ values in tissues during fasting or nutritional stress (Hobson et al., 1993; Scrimgeour et al., 1995; Fuller et al., 2005; Boag et al., 2006; Alamaru et al., 2009). However, a growing number of studies have failed to observe an increase (Castillo and Hatch, 2007; Kempster et al., 2007; McCue and Pollock, 2008; Tranquilla et al., 2010; Mayor et al., 2011) or only detected it in specific tissues (Doucett et al., 1999; Gloutney et al., 1999; Cherel et al., 2005; Guelinckx et al., 2007). Reconciling these apparently contradictory results will require a more complete understanding of the mechanisms underlying changes in $\delta^{15}N$ values in fasting animals (Gannes et al., 1997; Martinez del Rio et al., 2009).

Animal nitrogenous waste has a low $\delta^{15}N$ value relative to body nitrogen (Steele and Daniel, 1978; Minagawa and Wada, 1984; Sponheimer et al., 2003); therefore, the

whole animal $\delta^{15}N$ value is expected to increase when urinary loss is not replaced by dietary nitrogen (Gannes et al., 1997). However, it is less clear how individual tissues are affected by the fasting body's net loss of light nitrogen. Previous studies have suggested two mechanisms to explain increases in tissue $\delta^{15}N$ values during fasting. The first mechanism we term the 'catabolic' model, in which a disproportionate loss of $^{14}N$-containing amino acids during protein breakdown causes an increase in the residual $\delta^{15}N$ value of any tissue undergoing catabolism (Gannes et al., 1997; Gloutney et al., 1999; Martinez del Rio and Wolf, 2005; Gaye-Siessegger et al., 2007; Lohuis et al., 2007; McCue, 2008; Tranquilla et al., 2010). This model derives from the results of Hobson et al. (1993) in which fasting geese lost significant mass from both liver and muscle (58% and 44%, respectively), and both of these tissues increased in $\delta^{15}N$ values. However, several authors have advocated an alternative 'anabolic' model in which protein synthesis, not breakdown, leads to increases in tissue $\delta^{15}N$ values (Scrimgeour et al., 1995; Focken, 2001; Fuller et al., 2005; Boag et al., 2006; Wolf et al., 2009; Habran et al., 2010). In this model, loss of lighter nitrogen from the free amino acid pool during excretion causes the $\delta^{15}N$ value of residual amino acids to increase when not replaced by exogenous sources (Sick et al., 1997), and these 'heavy' amino acids are incorporated into tissues during protein synthesis. Here, the terms anabolic and catabolic refer to which of the two concurrent metabolic processes is the proximate cause of increasing tissue $\delta^{15}N$ values and do not imply a net metabolic state of the tissue. Understanding which mechanism is at work is critical to accurately interpreting $\delta^{15}N$ values in investigations of animal physiology during nutritional deprivation. For example, if the catabolic model is correct, $\delta^{15}N$ values would increase in tissues in proportion to their use as catabolic protein stores. However, if the anabolic model is correct, increasing $\delta^{15}N$ values would identify tissues maintained by protein synthesis.

Protein catabolism and anabolism are generally thought to be balanced during protein turnover at steady states in feeding animals (Waterlow, 2006). During fasting, however, catabolism and anabolism become unbalanced to an extent that differs among

tissues. In muscle, for example, catabolism greatly exceeds anabolism during fasting, and protein synthesis effectively ceases (Cherel et al., 1991; Waterlow, 2006). In contrast, protein synthesis in the liver continues at levels just below normal or increases with catabolism, imposing a demand for amino acids (Garlick et al., 1975; Cherel et al., 1991; Waterlow, 2006). These differences in protein turnover in muscle and liver tissue during fasting lead to different predictions of isotopic change by the catabolic and anabolic models in these tissues.

Arctic ground squirrels (*Urocitellus parryii*, Richardson, 1825) present a useful model of natural fasting to distinguish between the anabolic and catabolic models. These small mammals (~800 g) hibernate for 7-8 months every year without eating or drinking, but they usually excrete nitrogen as urea after periodically arousing from torpor to high body temperature. In addition, these animals lose a significant fraction of lean mass during natural hibernation at low ambient temperatures (Buck and Barnes, 1999). Although arctic ground squirrels readily tolerate body temperatures below 0°C during torpor under natural conditions (Buck et al., 2008), they must proportionally increase metabolism to prevent freezing as ambient temperatures decrease further (Buck and Barnes, 2000). Thermogenesis at sub-zero ambient temperatures appears to rely on a shift in metabolic fuels from strictly fat to increased use of carbohydrates generated from gluconeogenesis, the substrates of which are thought to be derived in part from protein breakdown (Buck and Barnes, 2000). The variability in lean mass loss under conditions to which arctic ground squirrels are naturally and periodically exposed generates a range of catabolism that should be sufficient to differentiate between the catabolic and anabolic models.

We evaluated the validity of the anabolic and catabolic models by comparing the predictions of change in $\delta^{15}N$ values from the models with measured changes in tissue $\delta^{15}N$ values in hibernating arctic ground squirrels. To generate variability in lean mass loss, we exposed squirrels to two temperature treatments that induced differential metabolic rates and fuel use for different durations of hibernation (Buck and Barnes, 2000). We then collected samples of liver, muscle, urine, plasma, and other tissues. The

differential use and turnover of tissues during fasting generates alternative predictions from the two models of increasing tissue $\delta^{15}N$ values. Specifically, if protein breakdown directly causes an increase in $\delta^{15}N$ values as required by the catabolic model, muscle $\delta^{15}N$ values should increase. We expect this because muscles are the largest reservoir of protein in the body and generally exist in a catabolic state during fasting. However, if protein synthesis is required for tissue $\delta^{15}N$ values to increase, as in the anabolic model, liver $\delta^{15}N$ values will increase and muscle $\delta^{15}N$ values will not. Both models predict that urine and plasma $\delta^{15}N$ values will increase with increasing lean mass loss. Based on these outcomes, we will use the supported model to interpret changes in $\delta^{15}N$ values in other tissues of interest during hibernation (heart, small intestine, and brown adipose tissue).

**Materials and Methods**

***Animals*** This study took place in the fall and winter of 2007-08 and used 40 arctic ground squirrels (*Urocitellus parryii,* family Sciuridae) from a captive colony at the University of Alaska Fairbanks. Both male and female, non-growing squirrels were used. Squirrels were either captured near the University of Alaska Fairbanks' Toolik Field Station (68° 38' N, 149° 36' W) in the Alaskan Arctic or born in captivity. The most recently captured squirrels used in this study had been in captivity for 4 months prior to the start of the experiment. Squirrels in captivity were maintained on a standard diet of Mazuri Rodent Chow ($\delta^{15}N = 2.7 \pm 1.2‰$, $\delta^{13}C = -20.9 \pm 0.8‰$). Females with pups were provided with Purina Cat Chow ($\delta^{15}N = 2.9 \pm 0.6‰$, $\delta^{13}C = -16.2 \pm 0.3‰$) as a supplement to support the increased protein demands of lactation from 2 weeks after birth (Vaughan et al., 2006). This supplement was removed from females at weaning and from juveniles 8 weeks prior to the beginning of the experiment. Animals were maintained on a 19.5L:4.5D light cycle at an ambient temperature of 18-22°C until 13 August 2007 when they were moved to an 8L:16D photoperiod at +2°C. As animals

began to hibernate, they were sampled as described below and moved to a quiet chamber without food to continue hibernation. We placed wood shavings on the backs of torpid animals and noted an arousal interval when shavings had been disturbed (Pengelley and Fisher, 1961). All animal use procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee, Protocol 06-40.

***Study Design*** As animals began to hibernate, we randomly assigned them to treatment groups. Control animals (n = 5) were sampled after 3-8 d of hibernation at +2°C. Experimental animals were assigned to two temperature treatments (+2°C or -10°C) and three hibernation durations at each temperature (45, 68, or 90 d) for a total of six treatment groups (n = 5 per group, n = 30 total). Because squirrels in captivity under constant conditions may shorten their annual cycle, leading to decreased hibernation duration (Pengelley et al., 1976), we selected relatively short durations and assigned additional squirrels to each temperature treatment. One of these extra animals was allowed to hibernate for 115 d at -10°C and four squirrels at +2°C were sampled after naturally terminating hibernation after 82, 139, 177, and 232 d. Thus, the total sample size was n = 40.

***Estimation of Lean Mass Through Isotope Dilution*** We used isotope dilution to estimate lean mass using a calibration equation based on chemical analyses of body composition (Lee et al., 2011). We estimated initial lean mass of all squirrels except the control group on the second day of hibernation and final lean mass of all animals prior to euthanasia. In all cases (except the four squirrels that naturally terminated hibernation), we induced the squirrel to arouse to high body temperature by handling. Once the animal had achieved high body temperature, it was anesthetized with gas anesthesia (isoflurane, 3-5%) and a claw was clipped to collect a background sample of blood into heparinized capillary tubes that were then either flame-sealed for cool storage (deuterium analyses) or clay-sealed and frozen at -20°C (carbon and nitrogen analyses). A 3% deuterium injection was administered intraperitoneally (IP) according to the equation: injection volume (ml) = mass (g) * 0.000867, and the animal was allowed to recover from

anesthesia. After 61.4 (± 2.4 s.d.) min, the animal was anesthetized again and a sample of deuterium-enriched blood was taken by claw clip. Experimental squirrels were then placed in the chamber at their assigned ambient temperature where they returned to torpor within 1-3 d.

***Euthanasia and Sample Collection*** Animals were moved to a warm room (18-22°C) and induced to arouse. We monitored rectal temperature during arousal via a wax-tipped thermocouple inserted approximately 2.5 cm into the rectum; 9 h after the rectal temperature reached 30°C, we anesthetized the animal and repeated isotope dilution as above to estimate lean mass. After 1 h, the animal was anesthetized again and the second, enriched, isotope sample was taken by cardiac puncture, during which we also collected blood (2 mL) into heparinized Vacutainers for carbon and nitrogen isotope analysis of plasma and red blood cells. The animal was then euthanized while still under anesthesia by an overdose of sodium pentobarbital injected into the heart. Tissues (heart; liver; small intestine; brown adipose tissue; subcutaneous and abdominal white adipose tissue; and gastrocnemius, quadriceps, abdominal, and scapular skeletal muscles) were removed and frozen at -20°C for isotope analysis. Urine samples were collected directly from the bladder with a syringe when possible (n = 33) and frozen in cryovials at -20°C. Squirrels that terminated hibernation naturally were anesthetized and sampled as above on their third day at high body temperature.

***Stable Isotope Analysis*** Stable isotope samples were analyzed at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks. For simultaneous carbon and nitrogen analysis, plasma (5.5 μL) and red blood cells (1.8 μL) were pipetted into tin capsules, which were then dried, weighed, and crimp-sealed for analysis (0.1-0.3 mg dry weight). Organ tissue, muscle tissue, and food samples were dried, powdered, homogenized, and weighed out to 0.2-0.3 mg into tin capsules; brown adipose tissue was analyzed in dried fragments of 0.2-0.3 mg. Because white adipose tissue contains little nitrogen, these tissues were prepared in dried fragments of 0.4-1.2 mg for carbon analysis and 4-8 mg for nitrogen analysis during separate analyses. For urine sample preparation,

10 μL of 37% HCl was added to a small, muffled glass microfiber filter prior to addition of 10 μL of urine to prevent loss of N by ammonification. These samples were dried in an oven (60°C) and placed into tin capsules for analyses. For deuterium ($^2H$) analysis, water was distilled from blood samples in flame-sealed Pasteur pipettes on hot plates and analyzed (Lee et al., 2011). Undistilled plasma from samples that were too small to distill was manually injected into the instrument for analysis. Previous work has shown no difference in lean mass calculated from analysis of distilled blood and undistilled plasma samples (Lee et al., 2011). Several blood samples in flame-sealed Natelson capillary tubes were lost during storage; matching samples from clay-sealed and frozen microhematocrit capillary tubes were analyzed in place of lost samples when available. Matching samples from each type of handling were analyzed to ensure that there was no difference in $\delta^2H$ values ($t < 1.64$, $p > 0.05$).

Sample isotope ratios were analyzed at the Alaska Stable Isotope Facility by continuous flow isotope ratio mass spectrometry. The carbon and nitrogen isotope ratios of solid samples were analyzed using a Costech ECS4010 elemental analyzer (Costech Scientific Inc., Valencia, CA, USA) interfaced to a Finnigan Delta$^{Plus}$ XP isotope ratio mass spectrometer (IRMS) via the Conflo III interface (Thermo Fisher Scientific, Bremen, Germany). The hydrogen isotope ratio of plasma was analyzed using a ThermoElectron high temperature conversion elemental analyzer interfaced to a Finnigan Delta V Plus IRMS via the Conflo III interface (both instruments: Thermo Fisher Scientific, Bremen, Germany). All isotope values are expressed in delta notation as $\delta X = ((R_{sample} - R_{standard}) / R_{standard}) * 1000‰$, where X = the heavy isotope, R = the ratio of heavy to light isotope, and the standards are as follows: N = atmospheric nitrogen ($N_{air}$); C = Vienna PeeDee belemnite (V-PDB); and H = Vienna standard mean ocean water (V-SMOW). Laboratory reference materials ($\delta^{15}N = 7.0‰$, $\delta^{13}C = -15.8‰$) run concurrently with samples had $\delta^{15}N$ and $\delta^{13}C$ values of $7.0 \pm 0.2‰$ and $-15.8 \pm 0.1‰$, respectively ($n = 104$). We corrected $\delta^2H$ values based on standard calibration.

***Statistical Analysis*** Estimates of lean mass were calculated from $\delta^2H$ values according to Lee et al. (2011), and loss could only be calculated on individuals with all four isotope dilution samples intact (n = 32 out of 35). Control animals (n = 5) were assigned 0 g lean mass loss for analyses. We compared $\delta^{15}N$ and $\delta^{13}C$ values of different tissues using ANOVA, and we used a t-test to compare all torpor bout lengths between temperature treatments. We used regression analysis to determine how tissue $\delta^{15}N$ and $\delta^{13}C$ values varied as a function of lean mass loss (% total lean mass lost during experiment) after removing outliers ($\delta^{15}N$: n = 10, $\leq$ 3 per tissue from 7 of 13 tissues; $\delta^{13}C$: n = 14, $\leq$ 2 per tissue from 10 of 13 tissues) identified by Mahalonobis distances. We performed all statistical analyses with JMP 8.0 (SAS Institute, Cary, NC, USA) using $\alpha$ of 0.05 and reported all means ± s.d.

**Results**

Arctic ground squirrels exposed to the experimental regimes of differing hibernation durations and ambient temperatures of +2°C and -10°C exhibited a wide range of loss of total lean mass of 8-49%. By 90 d, squirrels that hibernated at -10°C had lost twice as much lean mass as squirrels hibernating at + 2°C (40.3 ± 5.4% vs. 19.1 ± 9.5%). The extent of lean mass loss increased with hibernation duration at -10°C (0.19% per day, $F_{1,14} = 6.03$, $p = 0.028$), but not at +2°C ($F_{1,14} = 0.13$, $p = 0.726$; Figure 4.1). Squirrels hibernating at +2°C lost on average 22.7 ± 7.1% lean mass regardless of hibernation duration. Lean mass loss (%) was positively correlated with the number of times squirrels aroused to high body temperature during hibernation (n = 32, $R^2_{adj} = 0.508$, $F_{1,30} = 32.97$, $p < 0.0001$). Duration of torpor between arousals to high body temperature was significantly shorter in squirrels hibernating at -10°C (10 ± 3 d, n = 15) than at +2°C (13 ± 3 d, n = 15; t = -3.18, p = 0.004).

Nitrogen isotope ratios differed among tissues of control animals at the beginning of hibernation ($F_{11,46} = 14.37$, $p < 0.0001$), as did carbon isotope ratios ($F_{12,52} = 13.16$, $p < 0.0001$, Table 4.1). As squirrels lost lean mass, $\delta^{15}N$ values of plasma and urine

increased (Figure 4.2, Table 4.2). Plasma $\delta^{15}N$ values were consistently elevated relative to urine throughout the range of lean mass loss (mean difference between paired samples: $3.8 \pm 0.9‰$). As predicted by the anabolic model, $\delta^{15}N$ values in liver tissue increased with increasing lean mass loss while $\delta^{15}N$ values in skeletal muscle tissue showed no change. Heart, brown adipose tissue, and small intestine also increased in $\delta^{15}N$ values as the amount of lean mass lost increased (all $p < 0.05$, see Table 4.2, Figure 4.3). White adipose tissue (subcutaneous and abdominal) and red blood cell $\delta^{15}N$ values did not change with lean mass loss (Figure 4.4, Table 4.2). Carbon isotope ratios did not change with lean mass loss in any tissue except plasma, in which $\delta^{13}C$ values increased with increasing lean mass loss ($F_{1,33} = 4.70$, $p = 0.038$; all other tissues $p > 0.15$).

**Discussion**

Our results support the anabolic model as the primary mechanism by which nitrogen stable isotope signatures change in animal tissues during fasting. The anabolic model requires that urea synthesis selectively remove light isotopes from the plasma amino acid pool, causing the residual amino acid pool to increase in $\delta^{15}N$ value. Results in fasting arctic ground squirrels supported this expectation: urine $\delta^{15}N$ values were on average 3.8‰ lighter than plasma, and both urine and plasma $\delta^{15}N$ values increased linearly with increasing lean mass loss. More critically, we observed increasing $\delta^{15}N$ values in the liver, which is expected to continue synthesizing proteins even during extreme fasting, but values did not change in four different skeletal muscles, which are expected to undergo protein breakdown but little, if any, synthesis. These results are contrary to the catabolic model that predicts that tissues that are broken down during fasting increase in $\delta^{15}N$ values, which would have resulted in increased skeletal muscle $\delta^{15}N$. Figure 4.5 summarizes the key requirements of the anabolic model, which are crucial to our understanding of the physiology that causes changes in $\delta^{15}N$ values among tissues during fasting.

The anabolic model posits that proteins synthesized during fasting and lean mass loss should incorporate amino acids with elevated $\delta^{15}N$ values. Using this model, we interpret increased $\delta^{15}N$ values in heart, brown adipose tissue, and small intestine to indicate that ground squirrels are actively synthesizing proteins in these tissues during the season of hibernation. Many organs function at full or even enhanced levels during periodic arousals to high body temperature, and most also continue to function on a reduced level during torpor; for example, the heart beats 5-10 times/min (Storey, 2010). In small hibernators, periodic rewarming during arousals creates a heavy workload on the heart as it circulates the blood heated by non-shivering thermogenesis in brown adipose tissue (Storey, 2010). Indeed, the relative mass of heart and brown adipose tissue is greater in hibernating ground squirrels than in summer ground squirrels (Wickler et al., 1991). The digestive activity of the gastrointestinal tract, including the small intestine, is significantly reduced in animals that fast during hibernation (Hume et al., 2002), but evidence is emerging that the small intestine may stay active in other roles, such as immune function (Kurtz and Carey, 2007). The idea of small intestine function during hibernation is supported by our results. Finally, plasma $\delta^{13}C$ values increased with lean mass loss, suggesting a shift in mobilized fuels from lipid, which is isotopically light (DeNiro and Epstein, 1977), to protein and carbohydrate (isotopically heavy) as described previously (Buck and Barnes, 2000).

Discussions of increased tissue $\delta^{15}N$ values during fasting are commonly framed in the context of the catabolic model (Gannes et al., 1997; Gloutney et al., 1999; Kelly, 2000; Doi et al., 2007; Gaye-Siessegger et al., 2007; Lohuis et al., 2007; McCue, 2008; Tranquilla et al., 2010). We conducted a review of studies citing Hobson et al. 1993, arguably the classic paper demonstrating increased tissue $\delta^{15}N$ values with nutritional stress, and noted whether studies invoked a version of the catabolic or anabolic model when ascribing a mechanism to this effect (Figure 4.6). We found that isotope ecologists tend to invoke the catabolic and anabolic models with approximately equal frequency (Figure 4.6C). Nutritional stress is not always clearly defined, so we have used the term nutritional status to also include studies in which animals may not have reached

physiological levels of stress (Figure 4.6A) and classified these according to study type (Figure 4.6B). Of the studies we reviewed that had original data relevant to nutritional status, about half provided sufficient information for us to evaluate consistency with the anabolic or catabolic model; all of these were consistent with the anabolic model. Many previous results that have been interpretted as contradictory can be reconciled by the anabolic model, especially those studies that failed to find changes in $\delta^{15}N$ values in catabolic tissues (Pfeiler et al., 1998; Doucett et al., 1999; Gloutney et al., 1999; Tranquilla et al., 2010). Perhaps the most difficult to reconcile is the finding of Hobson et al. (1993) that muscle $\delta^{15}N$ values of geese increased during fasting. However, further work on that population showed no change in muscle $\delta^{15}N$ values during incubation and attributed the previously reported change to physiological changes during egg laying (Gloutney et al., 1999).

Another limitation of the catabolic model is the lack of a physiological mechanism of fractionation involved in catabolism. A systematic isotope effect during catabolism would require isotopic selectivity by the enzymes that mark proteins for breakdown, such as in the ubiquitin proteasome system active in muscle during fasting (Jagoe and Goldberg, 2001). However, there are a number of other factors that influence the selection of proteins for degradation during fasting, including which enzymes are activated (Jagoe and Goldberg, 2001). It is possible that such selection criteria eliminate the possibility of isotopic selectivity or obscure isotope effects. Though the enzymes that hydrolyse dipeptides (dipeptidases) show a preference for $^{14}N$ (Silfer et al., 1992), digestion of dipeptides is the final step of protein breakdown and should proceed to completion, masking any isotope effect.

Based on our findings and review of the literature, we recommend clarification in the way isotope ecologists understand and use changes in $\delta^{15}N$ values. Strictly speaking, the $\delta^{15}N$ values of an animal's tissues do not change with nutritional stress or even catabolism. Changes in tissue $\delta^{15}N$ values are a consequence of protein synthesis from amino acids derived from catabolism during loss of light nitrogen isotopes to waste. Not all nutritionally stressed animals reuse amino acids from catabolism. For example,

growing animals incorporate lighter dietary nitrogen into new tissues in spite of nutritional stress (Kempster et al., 2007; Williams et al., 2007; Sears et al., 2009) and will only show increased $\delta^{15}N$ values if the stress is sufficient to necessitate breaking down and reincorporating endogenous stores (Hobson et al., 1993). In addition, the terms 'catabolic' and 'anabolic,' when used as a general description of a whole animal, are not informative regarding changes in $\delta^{15}N$ values that occur on a tissue level. Critical tissues, such as the liver (Waterlow, 2006), and tissues being formed, such as feathers (Cherel et al., 2005) and hair (Fuller et al., 2005), continue significant levels of protein synthesis while the whole animal may be considered catabolic. Thus, it is critical to consider metabolic processes at the tissue level before making general conclusions regarding the animal's overall physiological state based on $\delta^{15}N$ values (Ben-David et al., 1999; Gloutney et al., 1999; Gomez-Campos et al., 2011).

We have used the sum of torpor bouts and arousal intervals during hibernation as a model of fasting, but we recognize that these phases have very different metabolic states. While protein synthesis is minimal in all tissues during torpor (Zhegunov et al., 1988; Gulevsky et al., 1992), it increases by >20-fold during arousal intervals to levels near or above pre-fasting values in the liver (Zhegunov et al., 1988; Gulevsky et al., 1992). In muscle, however, protein synthesis increases during arousal intervals to levels only ~1/10 of those in liver (Gulevsky et al., 1992), making hibernation a legitimate model for our purposes. However, we encourage more work in the changes that occur in $\delta^{15}N$ values during fasting, especially studies that measure protein synthesis and catabolism rates in various tissues.

In summary, we have shown that $\delta^{15}N$ values increase with lean mass loss in plasma, urine, and organ tissues, but do not change in muscle or red blood cells during hibernation in the arctic ground squirrel. In Hobson et al.'s (1993) classic work, the authors recognized that metabolism associated with nutritional stress caused tissue $\delta^{15}N$ values to increase and proposed multiple processes that could be involved in this effect, including catabolism, protein mobilization, or protein synthesis (which they termed "redeposition processes"). Our data support the anabolic model, which articulates that

the observed increase of $\delta^{15}N$ values during nutritional stress is associated with protein synthesis.

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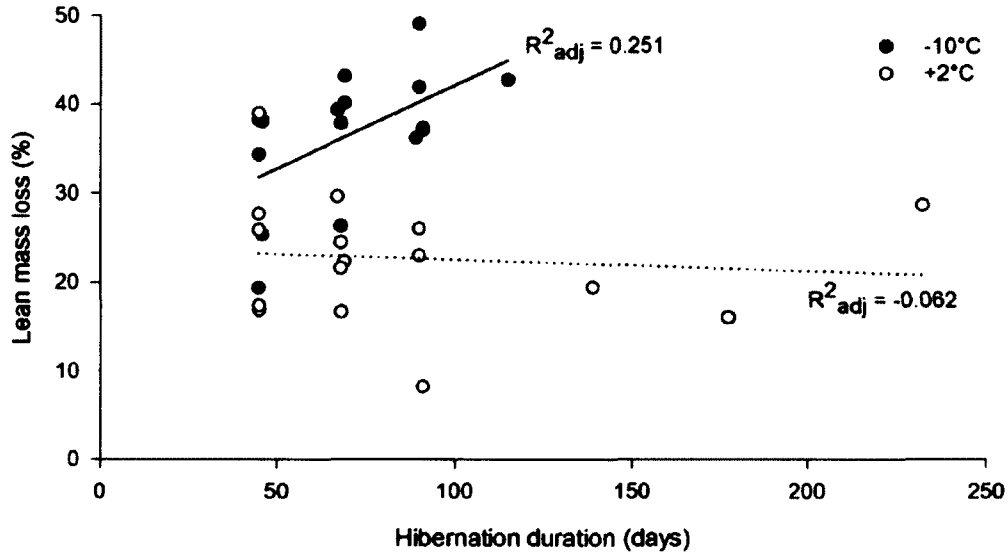


Figure 4.1. Lean mass loss (%) as a function of hibernation duration (days) in arctic ground squirrels. The relationship was significant for animals hibernating at -10°C (closed circles: n = 16), but there was no relationship for animals hibernating at +2°C (open circles: n = 16).

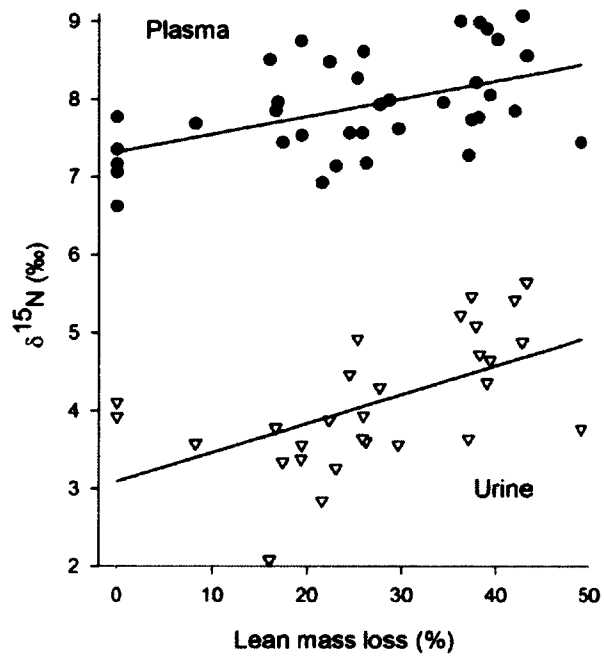


Figure 4.2. Plasma and urine significantly increased in $\delta^{15}N$ value as lean mass loss (%) increased during hibernation in arctic ground squirrels (plasma: closed circles; urine: open triangles; Table 4.2). Plasma had consistently heavier $\delta^{15}N$ values than urine.

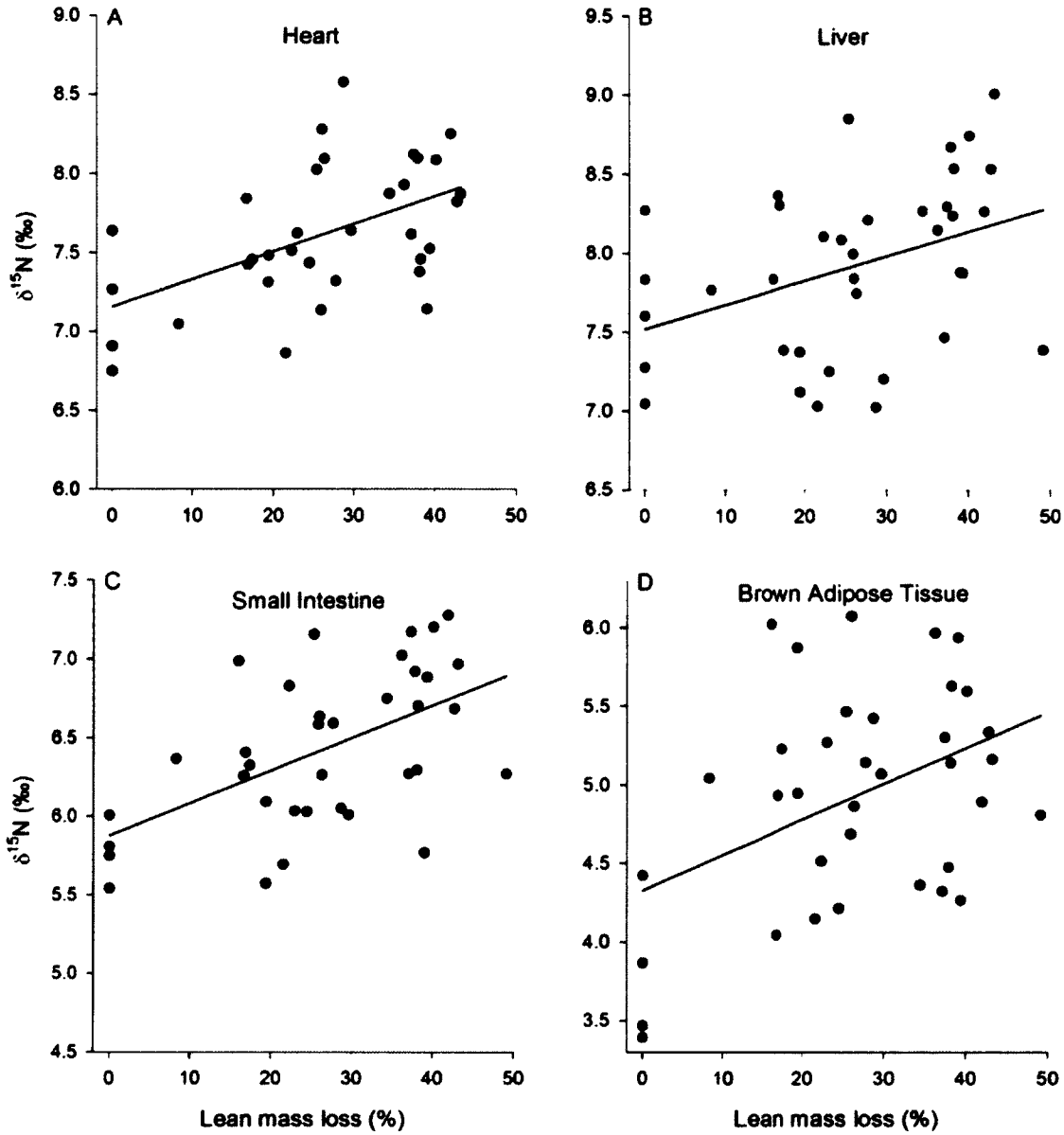


Figure 4.3. Organ $\delta^{15}N$ value as a function of lean mass loss (%) in hibernating arctic ground squirrels. A) heart, B) liver, C) small intestine, and D) brown adipose tissue (Table 4.2).

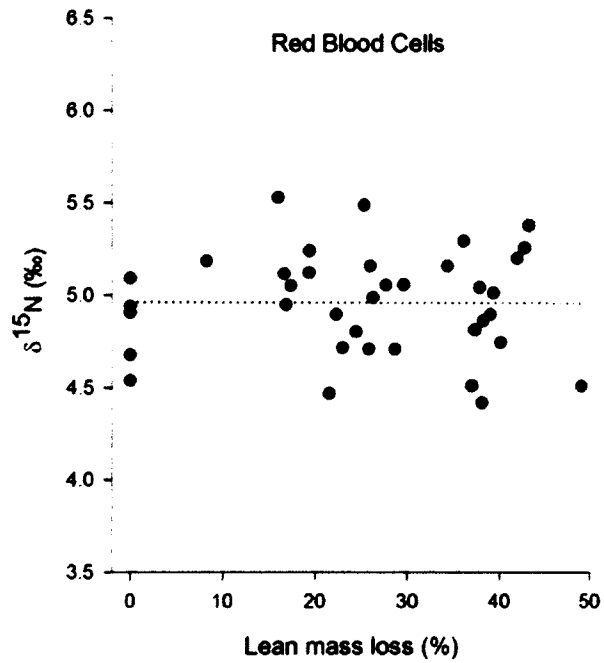


Figure 4.4. Red blood cell $\delta^{15}N$ values are not related to lean mass loss (%) and exemplify the result found in skeletal muscles (Table 4.2).

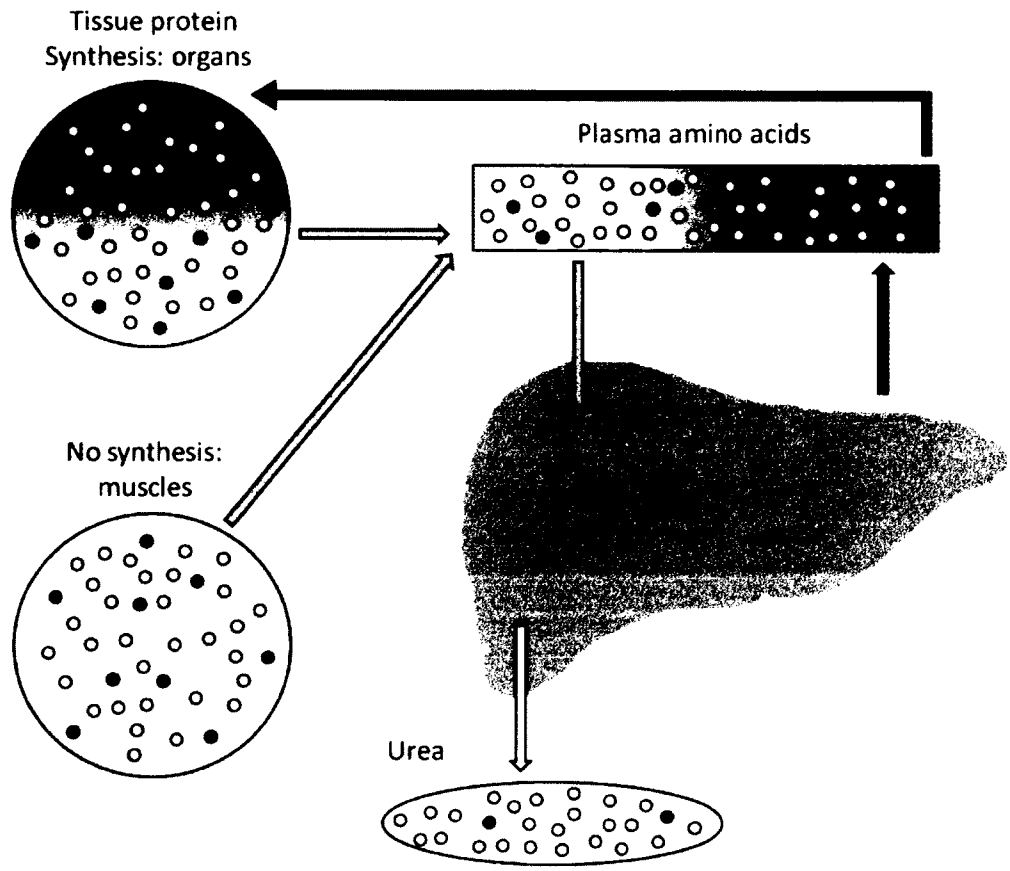


Figure 4.5. A conceptual anabolic model of increasing $\delta^{15}N$ values of tissue during fasting. Dark circles and shading indicate heavy isotopes and increases in tissue $\delta^{15}N$ values, respectively; abundance of heavy isotopes is grossly exaggerated for graphical purposes. Light circles and shading indicate light isotopes and no increase in tissue $\delta^{15}N$ values, respectively. We assume that amino acids from protein catabolism become part of the free amino acid pool in the plasma. Lighter amino acids are degraded to urea in the liver and excreted, while the remaining heavier amino acids are returned to the pool of amino acids in the plasma, increasing the $\delta^{15}N$ values of the plasma. Proteins formed from these heavier amino acids will have higher $\delta^{15}N$ values, as will the organs that are actively synthesizing protein and incorporating the amino acids with increased $\delta^{15}N$ values. The free amino acid pool carried in the plasma should demonstrate increases in $\delta^{15}N$ values proportional to the unreplaced loss of nitrogen from breakdown of body proteins.

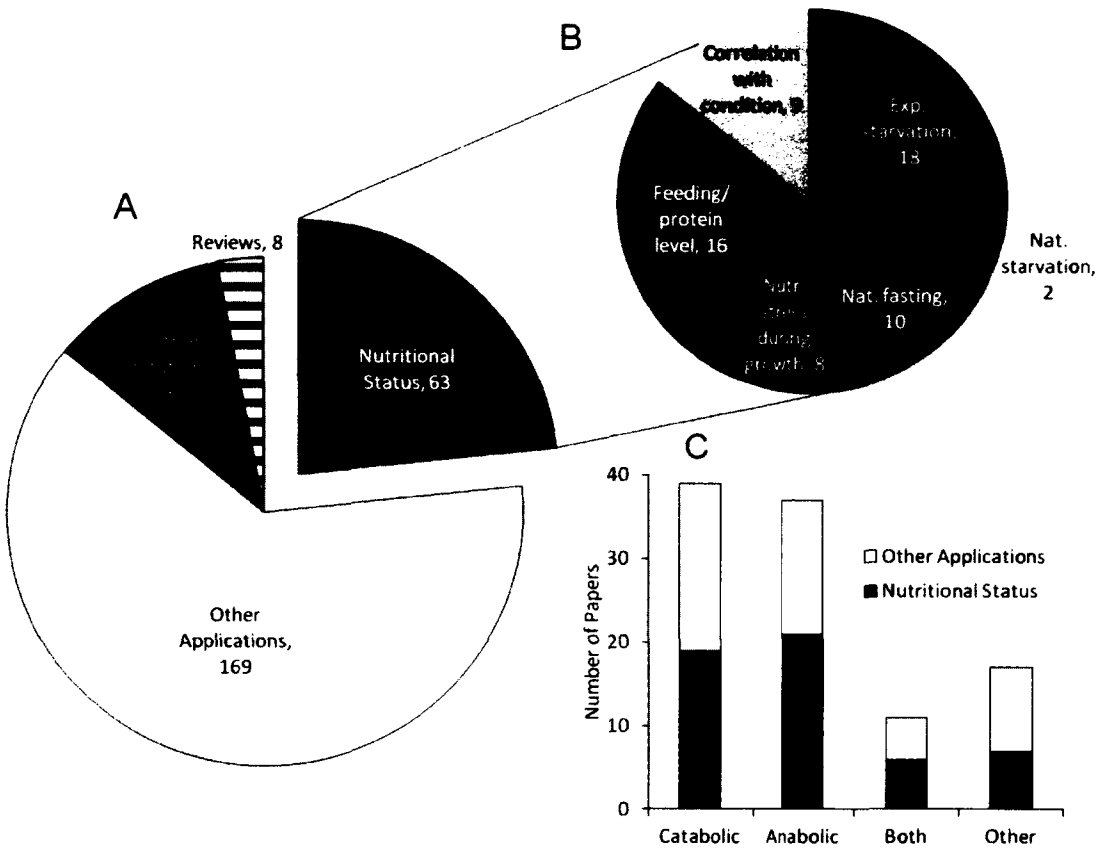


Figure 4.6. A review of 270 papers that cited Hobson et al., 1993 indexed by ISI Web of Knowledge on Aug. 4, 2011. (A) Papers were categorized as data related to changes in $\delta^{15}N$ from physiological mechanisms associated with nutritional status (Nutritional Status), papers that acknowledged that nutritional status could influence $\delta^{15}N$ values during other applications of $\delta^{15}N$ values (Other Applications), papers that cited Hobson et al., 1993 in ways not related to nutritional status (Other Citations), and reviews and a model that discussed $\delta^{15}N$ values in context of nutritional status (Reviews). (B) The study types of the data related to nutritional status are also presented in a smaller pie chart. Studies were classified as those that were experimental (Exp.) or natural observations (Nat.). Starvation was not part of the animal's life cycle while fasting was. Some studies evaluated effects of nutritional stress (Nutr. stress) during growth, effects of different feeding or protein levels on $\delta^{15}N$ values, or tested for correlations between $\delta^{15}N$ values with measures of body condition. (C) Papers in Other Applications and Nutritional Status were categorized according to the framework of discussion of $\delta^{15}N$ value changes. Studies followed the catabolic model (Catabolic), the anabolic model (Anabolic), both models (Both), or a different mechanism (Other). Several studies (n = 118 in Other Applications, n = 10 in Nutritional Status) did not discuss the mechanism.

Table 4.1. Values of $\delta^{15}N$, $\delta^{13}C$, and C:N from all tissues in arctic ground squirrels sampled at the beginning of hibernation (control group, n = 5). Subcutaneous and abdominal white adipose tissue (WAT) sample sizes are reduced because some samples had too little nitrogen present for a reliable analysis.

| Tissue | Mean $\delta^{15}N$ (SD) | Mean $\delta^{13}C$ (SD) | Mean C:N (SD) |
|---|---|---|---|
| Plasma | 7.2 (0.4) | -22.3 (0.9) | 5.5 (2.2) |
| Heart | 6.9 (0.6) | -22.5 (0.7) | 4.5 (0.2) |
| Liver | 7.6 (0.5) | -22.6 (0.3) | 5.8 (0.5) |
| Small Intestine | 5.6 (0.4) | -22.2 (0.5) | 4.3 (0.2) |
| Brown Adipose Tissue | 4.2 (1.0) | -24.6 (0.3) | 18.6 (4.9) |
| Quadriceps Muscle | 5.5 (0.4) | -21.9 (0.5) | 4.3 (0.6) |
| Gastrocnemius Muscle | 5.7 (0.3) | -21.5 (0.5) | 3.7 (0.2) |
| Scapular Muscle | 5.9 (0.5) | -22.2 (0.5) | 4.6 (0.7) |
| Abdominal Muscle | 5.6 (0.6) | -21.6 (0.8) | 3.7 (0.3) |
| Red Blood Cells | 4.8 (0.2) | -21.5 (0.3) | 3.3 (0.1) |
| Urine | 5.0 (1.2) | -22.6 (2.1) | 1.4 (0.9) |
| Subcutaneous WAT | 4.4 (0.5)* | -24.8 (0.3) | 371.2 (111.8)* |
| Abdominal WAT | ** | -24.9 (0.2) | ** |

* n = 3

** n = 0

Table 4.2. Statistics from regression analyses of tissue $\delta^{15}N$ value against lean mass loss (%) in arctic ground squirrels sampled throughout hibernation. Subcutaneous and abdominal white adipose tissue (WAT) sample sizes are reduced because some samples had too little nitrogen present for a reliable analysis. Significant results are presented in bold.

| Tissue | N | $R^2_{adj}$ | F | df | P |
|---|---|---|---|---|---|
| Plasma | 37 | 0.226 | 11.53 | 1,35 | **0.002** |
| Urine | 29 | 0.281 | 11.97 | 1,27 | **0.002** |
| Heart | 34 | 0.251 | 12.03 | 1,32 | **0.002** |
| Liver | 37 | 0.129 | 6.34 | 1,35 | **0.017** |
| Small Intestine | 36 | 0.285 | 14.92 | 1,34 | **<0.001** |
| Brown Adipose Tissue | 36 | 0.167 | 8.00 | 1,34 | **0.008** |
| Quadriceps Muscle | 37 | -0.015 | 0.45 | 1,35 | 0.506 |
| Gastrocnemius Muscle | 36 | -0.028 | 0.04 | 1,34 | 0.849 |
| Scapular Muscle | 36 | -0.012 | 0.57 | 1,34 | 0.454 |
| Abdominal Muscle | 35 | -0.027 | 0.10 | 1,33 | 0.756 |
| Red Blood Cells | 37 | -0.028 | 0.00 | 1,35 | 0.957 |
| Subcutaneous WAT | 17 | -0.009 | 0.85 | 1,15 | 0.370 |
| Abdominal WAT | 7 | -0.185 | 0.06 | 1,5 | 0.810 |

**Appendix 4.1** References for studies categorized by study type according to Figure 4.6.

EXPERIMENTAL STARVATION (18)

**Gorokhova, E. and Hansson, S.** (1999). An experimental study on variations in stable carbon and nitrogen isotope fractionation during growth of *Mysis mixta* and *Neomysis integer*. *Can. J. Fish. Aqua. Sci.* **56**, 2203-2210.

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**Gaye-Siessegger, J., Focken, U., Abel, H. and Becker, K.** (2007b). Starvation and low feeding levels result in an enrichment of $^{13}C$ in lipids and $^{15}N$ in protein of Nile tilapia *Oreochromis niloticus* L. *J. Fish Biol.* **71**, 90-100.

**Guelinckx, J., Maes, J., Van Den Driessche, P., Geysen, B., Dehairs, F. and Ollevier, F.** (2007). Changes in $\delta^{13}C$ and $\delta^{15}N$ in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Mar. Ecol. Prog. Ser.* **341**, 205-215.

**McCue, M. D.** (2007). Western diamondback rattlesnakes demonstrate physiological and biochemical strategies for tolerating prolonged starvation. *Physiol. Biochem. Zool.* **80**, 25-34.

**Reitzel, A. M. and Miner, B. G.** (2007). Reduced planktotrophy in larvae of *Clypeaster rosaceus* (Echinodermata, Echiniodea). *Mar. Biol.* **151**, 1525-1534.

**Kaufman, M. R., Gradinger, R. R., Bluhm, B. A. and O'Brien, D. M.** (2008). Using stable isotopes to assess carbon and nitrogen turnover in the Arctic sympagic amphipod *Onisimus litoralis*. *Oecologia* **158**, 11-22.

**McCue, M. D.** (2008). Endogenous and environmental factors influence the dietary fractionation of $^{13}C$ and $^{15}N$ in hissing cockroaches *Gromphadorhina portentosa*. *Physiol. Biochem. Zool.* **81**, 14-24.

**McCue, M. D. and Pollock, E. D.** (2008). Stable isotopes may provide evidence for starvation in reptiles. *Rapid Commun. Mass Spectrom.* **22**, 2307-2314.

**Alamaru, A., Yam, R., Shemesh, A. and Loya, Y.** (2009). Trophic biology of *Stylophora pistillata* larvae: evidence from stable isotope analysis. *Mar. Ecol. Prog. Ser.* **383**, 85-94.

**Mayor, D. J., Cook, K., Thornton, B., Walsham, P., Witte, U. F. M., Zuur, A. F. and Anderson, T. R.** (2011). Absorption efficiencies and basal turnover of C, N and fatty acids in a marine Calanoid copepod. *Funct. Ecol.* **25**, 509-518.

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# Chapter 5 STABLE ISOTOPE ANALYSIS OF $CO_2$ IN BREATH INDICATES METABOLIC FUEL SHIFTS IN TORPID ARCTIC GROUND SQUIRRELS[1]


## Abstract

Stable carbon isotopes in breath show promise as an indicator of immediate fuel utilization in animals because tissue lipids have a lower carbon isotope ratio ($\delta^{13}C$) than carbohydrates and proteins. Respiratory quotient (RQ) has a lipid and carbohydrate endpoint and does not differentiate between mixed fuel and protein catabolism, but stable carbon isotopes in breath should have a distinct lipid signature and may help resolve metabolic fuel use. We measured breath $\delta^{13}C$ and RQ concurrently in torpid arctic ground squirrels (*Urocitellus parryii*) at ambient temperatures ranging from -2 to -26 °C. As predicted, we found a correlation between RQ and breath $\delta^{13}C$ values; however, the range of RQ in this study did not reach intermediate levels to allow further resolution of metabolic substrate use with the addition of breath $\delta^{13}C$ measurements. Our data suggest that breath $\delta^{13}C$ values are depleted by 1.1‰ relative to lipid tissue during pure lipid metabolism. From RQ, we determined that arctic ground squirrels rely on nonlipid fuel sources for a significant portion of energy during torpor (up to 37%). The shift toward nonlipid fuel sources may be influenced by adiposity of the animals in addition to thermal challenge.


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## Introduction

Animals use three primary metabolic fuels to meet energy demands: lipid, carbohydrate, and protein. Lipid is the most energy-efficient fuel, providing 8 to10-times more energy on a wet mass basis than carbohydrates and proteins (McWilliams et al. 2004). Carbohydrates are often directly metabolized for immediate energy, but are not stored in large quantities in mammals (Vock et al. 1996). Protein is typically reserved for structural and functional roles and does not contribute significantly to energy metabolism during periods of energy balance (Robbins 2001). Metabolic fuel selection, often shifting between lipid and carbohydrate, depends on several factors, including exercise intensity and training status (surveyed in Table 2 in Holloszy et al. 1998). Fasting and starvation demonstrate a well-defined pattern of metabolic fuel selection, with timing of shifts between phases based on amount of lipid stored and protein catabolism as a last resort (Robbins 2001).

Hibernation is a strategy used by a variety of animals to conserve energy in anticipation of or during periods of insufficient food resources. During hibernation, animals often do not eat for months and rely entirely on endogenous stores of metabolic fuel. Some species can conserve as much as 90% of the energy they would otherwise use by spending the majority of the hibernation season in torpor, the low-metabolism, energy-saving phase of hibernation (Karpovich et al. 2009; Wang and Wolowyk 1988). Most hibernators support their metabolic costs from large lipid stores which are accumulated during the pre-hibernation fattening period (Dark 2005), but this appears insufficient for animals hibernating in extreme environmental conditions (Buck and Barnes 2000).

Arctic ground squirrels (*Urocitellus parryii*) are the most northern hibernators in North America and experience hibernacula temperatures averaging -8.9 °C and as low as -23 °C during hibernation (Buck and Barnes 1999b). Given that arctic ground squirrels can maintain a minimum body temperature of -2.9 °C for weeks at a time during torpor (Barnes 1989), free-living animals must be continuously thermogenic to maintain the

thermal gradient between their core and ambient soil temperatures across the hibernation season, which lasts 7-8 months (Buck et al. 2008). In the weeks prior to entering hibernation, arctic ground squirrels increase lipid (Buck and Barnes 1999a) and lean mass (Boonstra et al. 2011), a significant proportion of which is used over winter (Buck and Barnes 1999b). Use of lean mass during hibernation is indicated by a shift toward increased reliance on mixed fuel metabolism during torpor at decreasing ambient temperatures ($T_a$), as indicated by respirometry (Buck and Barnes 2000). Carbohydrates are needed to facilitate lipid oxidation associated with brown adipose tissue thermogenesis (Cannon and Nedergaard 1979; Vallerand et al. 1990) as well as fuel certain tissues such as the brain and kidneys (Berg et al. 2002). The primary precursor to glucose in most hibernators is glycerol, a byproduct of catabolism of triglycerides (Galster and Morrison 1975; Staples and Hochachka 1998), but it has been suggested that the increasing demand for carbohydrates by thermogenesis surpasses the supply of glucose formed from glycerol and requires breakdown of proteins for gluconeogenesis (Buck and Barnes 2000; Galster and Morrison 1975; Krilowicz 1985). This hypothesis is supported by dramatic upregulation during hibernation of the gene *PCK1*, which codes for a crucial enzyme in gluconeogenesis from pyruvate, lactate, or amino acid precursors (Williams et al. 2011).

Respirometry is the traditional method used to differentiate metabolic fuel use, but it cannot differentiate between protein and mixed fuel catabolism. The respiratory quotient (RQ), the ratio of carbon dioxide ($CO_2$) produced to oxygen ($O_2$) consumed (Kleiber 1961), is approximately 0.7 during lipid oxidation while carbohydrate oxidation results in an RQ of 1.0. Oxidation of proteins yields an intermediate RQ of 0.83 (Kleiber 1961), similar to that expected from mixed lipid and carbohdrate metabolism. Alternative measures of fuel use in addition to RQ may help better distinguish the proportions of metabolic contribution among carbohydrate, protein, and lipid.

Stable carbon isotope measurements are becoming more common in studies of substrate use, as respired $CO_2$ is a direct product of the animal's immediate metabolism (Hatch et al. 2002; Voigt et al. 2008a). Lipid naturally has a lighter carbon isotope ratio

($\delta^{13}C$) than other metabolites (DeNiro and Epstein 1977). Animals that are fasting demonstrate a shift toward lighter $\delta^{13}C$ values in respired $CO_2$ (Perkins and Speakman 2001; Schoeller et al. 1984; Voigt et al. 2008a), which is consistent with an increase in the proportion of lipid utilization during food deprivation (Robbins 2001). Investigations of metabolism in plants have shown a strong correlation between RQ and $\delta^{13}C$ values in respired $CO_2$ (Pataki 2005; Tcherkez et al. 2003), but few studies in animals have combined measurements of RQ and $\delta^{13}C$ breath values from naturally distinct substrates (Schoeller et al. 1984).

Our first objective was to determine whether RQ and $\delta^{13}C$ values covary in an animal system utilizing substrates with naturally distinct $\delta^{13}C$ signatures. To address this objective, we concurrently measured RQ and breath $\delta^{13}C$ values in hibernating arctic ground squirrels, using $T_a$ to induce shifts in fuel use. Previous work on arctic ground squirrels has shown a robust and linear increase in RQ as $T_a$ decreases below 0 °C (Buck and Barnes 2000) and found a clear difference in $\delta^{13}C$ values between tissue lipids and lean mass (Chapter 4). Our second objective was to determine whether using two 2-endpoint measurements, RQ and $\delta^{13}C$ breath values, could help resolve fuel use in torpid arctic ground squirrels. Specifically, if RQ values are intermediate and $\delta^{13}C$ values are intermediate, we would conclude that squirrels are using a mix of lipid and other fuels. However, if RQ values are intermediate and $\delta^{13}C$ values are heavy, we would conclude the animals are using a fuel based on proteins and/or carbohydrates but utilizing little, if any, lipid.

## Materials and Methods

***Animals*** Arctic ground squirrels (*Urocitellus parryii*) were captured near Toolik Field Station (68° 38' N, 149° 36' W) in the Alaskan Arctic in fall 2008 and summer 2009 and maintained on Mazuri Rodent Chow in captivity at the University of Alaska Anchorage. Each animal had a temperature-sensitive radiotransmitter (~7 g; Data

Sciences International, St. Paul, MN, USA) surgically implanted in its abdomen (see Karpovich et al. 2009 for methods). Squirrels were initially held at room temperature on a 18L:6D light cycle in 48 x 32 x 32 cm hanging metal cages and were provided with ample cotton material from which they constructed nests. They were then moved into cold chambers at +2 °C on a 9L:15D light cycle. When an animal began hibernating (body temperature $\leq$ 30 °C; Buck et al. 2008), it was transferred to a plastic metabolic chamber with wire lid and placed on a receiver linked to an automated data collection system (Data Sciences International, St Paul, MN, USA) that recorded core abdominal temperature every 10 min.

***Treatment Groups*** Once a sufficient number of animals began hibernating, they were divided into two treatment groups that followed different schedules of controlled temperature reduction. The temperature within Chamber 1 (n = 8 animals, 5 males and 3 females) was decreased from +2 to -20 °C in 2-degree increments. The temperature within Chamber 2 (n = 9 animals, 6 males and 3 females) was decreased from +2 °C to 0 °C, -10 °C, and -20 °C, at which point the temperature was lowered in 2-degree increments until -26 °C. At each $T_a$, RQ for each animal was recorded during steady-state torpor, and breath was sampled simultaneously. Once all animals in the environmental chamber had been sampled, all were induced to arouse to euthermic body temperature by handling, weighed, and returned to the chamber set at the next $T_a$. All animal use procedures were approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (protocol 150918).

***Respirometry*** Open-system respirometry was used to measure RQ of four animals simultaneously during steady-state torpor. Data collection began at least 7 h after body temperature decreased below 0 °C. For all experimental animals, body temperature fluctuated less than 0.2 °C over the 6 h duration of data collection. Metabolic chambers (43 x 27 x 19 cm, 22,059 $cm^3$) were sealed with weatherstripping on weighted lids. Incurrent and excurrent ports were in the chamber lid; however, excurrent air was sampled from the bottom of the chamber to ensure air mixing. Ambient air was

drawn through the chamber at 200 ml/min or 2,500 ml/min, depending on the metabolic rate of the animal: when the animal depleted the $O_2$ level to 19%, high flow was enabled as a safety mechanism to ensure sufficient $O_2$ was present to sustain higher metabolic rates during interbout arousal episodes. Excurrent air was analyzed for $CO_2$ concentrations by two 1-channel CA10A $CO_2$ analyzers and $O_2$ concentrations by a 2-channel Oxzilla $O_2$ analyzer (Sable Systems, Las Vegas, NV, USA). Integrity of the respirometry system was checked by periodic ethanol burns of known RQ. Each metabolic trial was preceded by manual span and zero gas calibrations of each instrument. LabGraph (Tøien 1992) also recorded span and zero gas values every 3 h during a trial to allow correction. After sufficient time had elapsed for several complete air changes within the metabolic chambers (6 h at low flow, 2 h at high flow), RQ was calculated from the final 6 h of a trial as the average $CO_2$ produced/$O_2$ consumed.

***Breath Sample Collection and Analysis*** Excurrent air from the metabolic chambers and reference ambient air pulled directly from the cold chamber were subsampled upstream of the analyzers via 4-way stopcocks (product #30600-03, Cole Parmer, Vernon Hills, IL, USA) into evacuated glass Exetainers (12 mL, LabCo Limited, Buckinghamshire, UK). $CO_2$ concentrations of the samples at the time of sampling were measured by the $CO_2$ analyzers and corrected for any drift or offset by LabGraph.

Excurrent air samples were analyzed within 7 weeks by the Alaska Stable Isotope Facility at the University of Alaska Fairbanks for $\delta^{13}C$ values via continuous flow isotope ratio mass spectrometry using a ThermoElectron GasBench II interfaced to a Finnigan Delta$^{Plus}$ XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). All isotope values are expressed in delta notation as $\delta X = ((R_{sample} - R_{standard}) / R_{standard}) * 1000‰$, where X = the heavy isotope, R = the ratio of heavy to light isotope, and the standard is Vienna PeeDee Belemnite. We used a basic mixing model equation to account for $CO_2$ present in ambient air:

$$\delta^{13}C_{breath} = (\delta^{13}C_{sample} - (f_{ambient} * \delta^{13}C_{ambient})) / (1 - f_{ambient}) \quad (5.1)$$

where $f_{ambient}$ is the fraction of ambient air included in the sample. We calculated $f_{ambient}$ as the concentration of $CO_2$ in ambient air divided by the concentration of $CO_2$ within the metabolic chamber. A large number of samples from animals held at ambient temperatures > -2 °C had too little $CO_2$ contributed by squirrel metabolism for confident determination of squirrel breath $\delta^{13}C$ values (i.e., $f_{ambient} > 0.525$, Welch et al. 2006); therefore, RQ and $\delta^{13}C$ values from samples from 0 °C and +2 °C were not included in the analysis.

Expected lipid $\delta^{13}C$ values for arctic ground squirrels used in Figure 5.2 are from Chapter 4 (-24.8‰ based on the average of two white adipose tissues from five animals, n = 10 samples). Expected muscle $\delta^{13}C$ values were based on lipid-extracted skeletal muscle samples (four muscles from five animals, n = 20 samples, $\delta^{13}C$ = -21.6‰, Chapter 4).

***Statistical Analysis*** We determined the relationship between RQ and $\delta^{13}C$ breath values with a linear mixed model with $\delta^{13}C$ values as the dependent variable, RQ as the independent variable, and individual as a random effect. In each chamber, we compared mean RQ and breath $\delta^{13}C$ values at each ambient temperature using a mixed-model ANOVA with individual as a random effect. Four outliers, which were identified as data points > 3 SD from the mean, were eliminated from analysis. To determine the proportions of different endogenous fuels being used from RQ, we calculated the proportion of carbohydrate as fuel with an equation from Kleiber (Appendix 13, 1961) assuming protein-free metabolism:

$$f_{carb} = 1 - [38 * (1-RQ)] / [(59.5 * RQ) - 30.9] \quad (5.2)$$

**Results**

***Breath $\delta^{13}C$ Values*** Breath $\delta^{13}C$ values and RQ were correlated over the range of values found in this study ($F_{1,103.5} = 36.97$, $R^2_{adj} = 0.34$, $p < 0.0001$, Figure 5.1), although RQ explained relatively little of the variation in breath $\delta^{13}C$ values. Mean $\delta^{13}C$ values in

squirrel breath during torpor at each ambient temperature ranged from -25.6 to -21.8‰ and significantly varied with temperature within each chamber (Chamber 1: $F_{9,68} = 28.78$, $p < 0.0001$; Chamber 2: $F_{4,34} = 15.24$, $p < 0.0001$, Figure 5.2A).

***Respiratory Quotient*** RQ ranged from a minimum mean of 0.73 at -10 °C to a maximum mean of 0.76 at -2 °C during steady-state torpor. Mean RQ differed by temperature in each chamber (Chamber 1: $F_{9,68} = 11.81$, $p < 0.0001$; Chamber 2: $F_{4,34} = 9.07$, $p < 0.0001$; Figure 5.2B). The percentage of carbohydrate fuels utilized, as calculated by Equation 5.2, ranged from 18-37% with the maximum at -2 °C and minimum at -10 °C.

**Discussion**

Carbon isotope ratios of breath $CO_2$ during torpor varied widely between the endpoints of lipid and lipid-free tissue from arctic ground squirrels. RQ and $\delta^{13}C$ breath values were correlated during steady-state torpor over ambient temperatures from -2 to -26 °C. This suggests that $\delta^{13}C$ breath may be a useful tool to investigate endogenous fuel use due to the distinct $\delta^{13}C$ values between lipid and nonlipid fuels. In contrast to the findings of Buck and Barnes (2000), we found that temperature effects on RQ were relatively small, and therefore, RQ did not span the range needed to further resolve fuel use with the addition of breath $\delta^{13}C$ values.

This is the first study to document a correlation between RQ and breath $\delta^{13}C$ values during metabolism of purely endogenous stores in animals. Other animal studies that have combined measurements of RQ and breath $\delta^{13}C$ values have used endogenous $\delta^{13}C$ signatures and RQ to quantify a shift to isotopically distinct exogenous fuel (Welch et al. 2006; Welch et al. 2008) or used RQ to estimate fuel contributions to metabolism, which were then used to predict breath $\delta^{13}C$ values from values of plasma macronutrients (Schoeller et al. 1984). However, RQ in this study only explained about a third of the variation in breath $\delta^{13}C$ values. This could be due in part to the fact that proteins and

carbohydrates do not have consistent $\delta^{13}C$ values relative to lipid. Fasted rats demonstrated $\delta^{13}C$ values of plasma proteins that were intermediate between plasma glucose and lipid, though closer to glucose, while human plasma glucose was intermediate between plasma proteins and lipids (Schoeller et al. 1984). Previous work on arctic ground squirrels found similar $\delta^{13}C$ values among organ and muscle tissues (sources of protein and carbohydrates) that were distinct from signatures of adipose tissues (Chapter 4), but we did not directly measure $\delta^{13}C$ values of proteins and carbohydrates in this study. Therefore, while RQ and $\delta^{13}C$ both have minimal values for lipid, the correlation in animals may break down with a shift toward the other macronutrients.

Applying isotopic measurements of breath in studies of animal physiology is a relatively new development (Hatch et al. 2002) and discrimination factors, resulting from isotopic selectivity in metabolic processes, have not yet been thoroughly defined between fuel sources and exhaled $CO_2$. Several controlled experimental studies have found differences between $\delta^{13}C$ breath values and $\delta^{13}C$ diet values (surveyed in Table 2 in Voigt et al. 2008a), but the number of metabolic steps that exogenous and endogenous substrates go through before utilization are different and thus have different potential for fractionation. In addition, fractionation in the breakdown of endogenous protein and lipid stores likely varies, leading to different discrimination factors. Both positive and negative discrimination factors have been reported between breath and macronutrients in tissues and diet (Podlesak et al. 2005; Schoeller et al. 1984; Voigt et al. 2008a). Therefore, our ability to extrapolate an expected breath $\delta^{13}C$ value based on measured tissue macronutrient $\delta^{13}C$ values is still limited.

Many studies have noted a decrease in breath $\delta^{13}C$ values in conjunction with fasting (Perkins and Speakman 2001; Schoeller et al. 1984; Voigt et al. 2008a; Voigt et al. 2008b; Welch et al. 2006). While it is clear that a shift toward light, endogenous lipid sources is occurring in these situations, the potential for discrimination between endogenous lipid and breath $CO_2$ has not been explored. Our data included breath $\delta^{13}C$

values that were more depleted than endogenous lipid values, suggesting a negative fractionation between tissue lipid sources and $CO_2$ in breath. We used the predictive equation from the correlation of RQ and breath $\delta^{13}C$ values in our study and the RQ value of pure lipid catabolism (0.707, Kleiber 1961) to generate a breath $\delta^{13}C$ value for arctic ground squirrels to indicate pure lipid catabolism. This value, -25.9‰, is 1.1‰ lower than the mean $\delta^{13}C$ value of white adipose tissue in arctic ground squirrels (Chapter 4). Our data also suggest that metabolism of carbohydrate or protein to breath $CO_2$ may have a positive discrimination factor: when we adjusted mean $\delta^{13}C$ values of four lipid-extracted skeletal muscles (-21.6‰) down by 1.1‰, some of the $\delta^{13}C$ breath values exceeded the expected value for purely protein or carbohydrate metabolism.

Previous investigation of metabolism of arctic ground squirrels during torpor reported a dramatic, linear increase in RQ with decreasing $T_a$ (Buck and Barnes 2000), suggestive of a 76% reliance on nonlipid fuels at a $T_a$ of -16 °C compared to only 28% at a $T_a$ of 0 °C. In the current study, RQ suggested a maximum reliance on nonlipid fuels of 37% at a $T_a$ of -2 °C and a minimum of 18% at a $T_a$ of -10 °C followed by an increase at $T_a$ below -10 °C. We are unsure why animals in the current study did not exhibit a similar dramatic increase in RQ with decreased $T_a$, although it might be related to differences in body composition of the study animals. The animals in the previous study were sampled over different years and at different times during a season, while the animals in the current study were sampled repeatedly within the same year and sequentially across the first few months of the hibernation season. Animals in the previous study may have been measured at low lipid masses, potentially making them more reliant on nonlipid sources (Caloin 2004; Cherel et al. 1993; Cherel et al. 1992). Animals in the current study would have had the largest lipid reserves at the beginning and sequentially depleted the reserves throughout the relatively short term of the study as $T_a$ also decreased. Thus, temperature effects on metabolic fuel selection may have been confounded by differences in adiposity, as it also influences the ratio of lipid to protein utilization (Caloin 2004).

Another factor contributing to the differences in fuel use between the current study and that of Buck and Barnes (2000) may be the physiological adjustments needed for the larger changes in $T_a$ (4 °C in previous study vs. 2 °C in current). This might have increased reliance on nonlipid fuels in a manner similar to strenuous exercise before training causing increased carbohydrate use (Holloszy et al. 1998). In support of this, squirrels experiencing a 10 °C change at $T_a$ of -10 °C in the current study had more variable RQ and higher $\delta^{13}C$ signatures compared with squirrels experiencing a 2 °C change. A similar effect after a 10 °C decrease to -20 °C may have been masked as values were relatively high at this $T_a$ in both chambers. Differences in the magnitude of $T_a$ change could help explain the less dramatic response seen in the current study while body composition may account for the increase in nonlipid fuel use occurring at lower $T_a$ compared with the previous study.

This study demonstrates for the first time that $\delta^{13}C$ breath values can be used to identify metabolic shifts between endogenous substrates with naturally distinct $\delta^{13}C$ signatures in animals. The correlation between $\delta^{13}C$ values in breath and RQ provides a foundation for using $\delta^{13}C$ breath values as a more sensitive index of endogenous fuel use. The breath $\delta^{13}C$ value extrapolated from our data to coincide with pure lipid metabolism was 1.1‰ more depleted than lipid stores, which suggests fractionation may occur during the catabolism of endogenous fuel stores. Further work is needed to confirm this and delineate discrimination factors resulting from metabolic pathways between endogenous substrates and respired $CO_2$.

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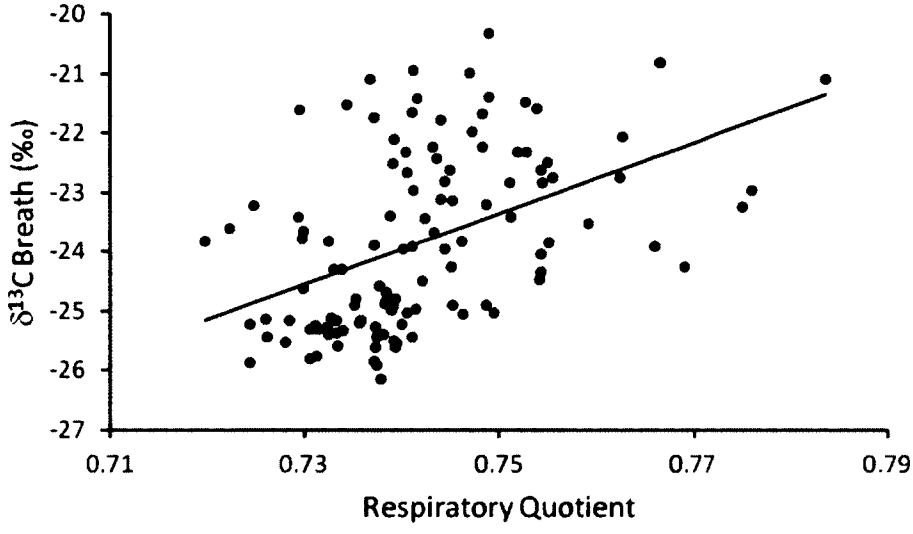


Figure 5.1. Relationship between respiratory quotient (RQ) and $\delta^{13}C$ breath values in arctic ground squirrels in steady torpor at ambient temperatures from -2 to -26 °C (n = 17 repeatedly measured; $R^2_{adj} = 0.34$, $p < 0.0001$).

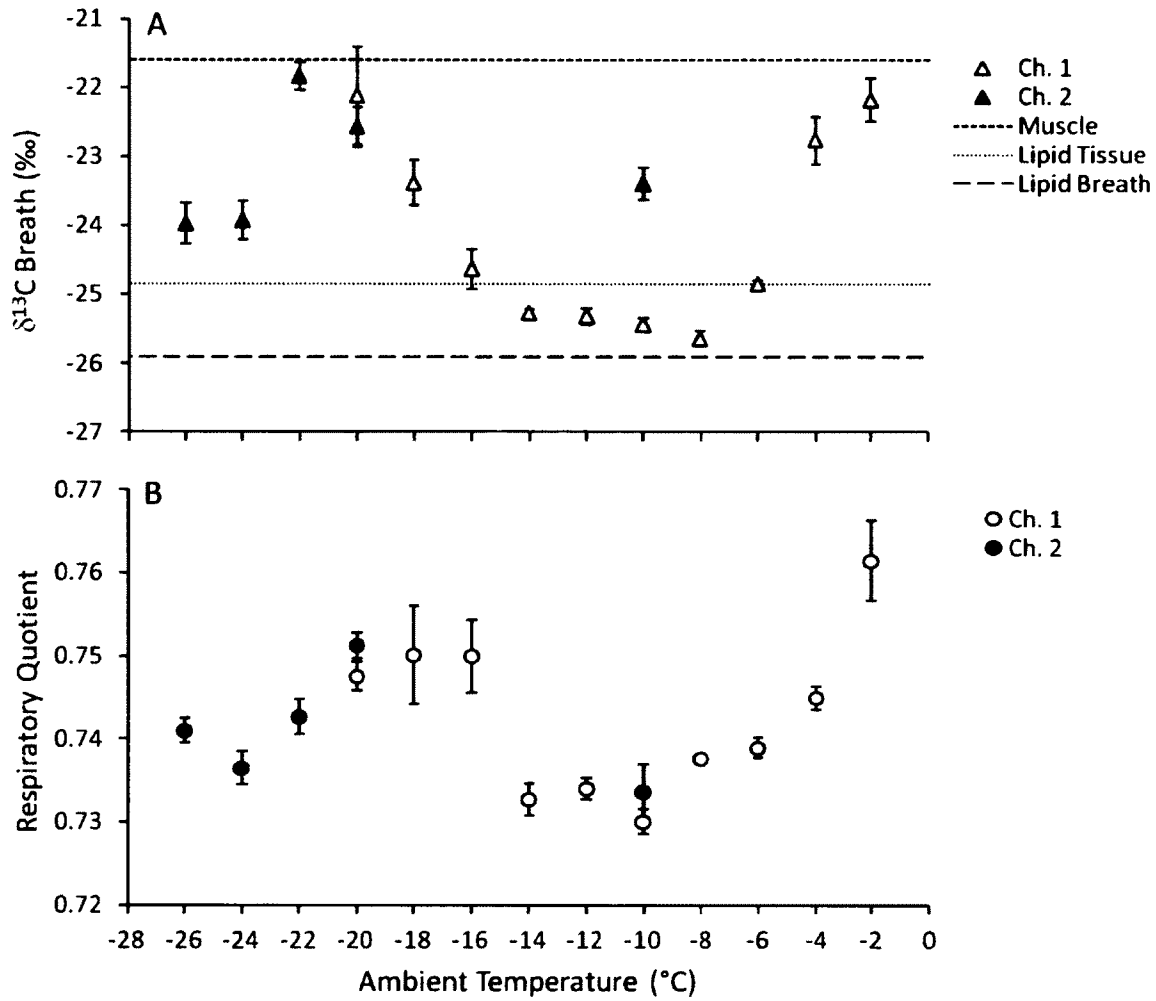


Figure 5.2. $\delta^{13}C$ values of expired breath (A) and respiratory quotient (B) from torpid arctic ground squirrels repeatedly measured at different ambient temperatures. Squirrels in different environmental chambers experienced different cooling regimes and are represented by different fill (Ch. 1, open; Ch. 2, filled). In A, the top dotted line represents the $\delta^{13}C$ values of lipid-extracted muscle from hibernating arctic ground squirrels while the middle dotted line (Lipid Tissue) represents $\delta^{13}C$ values from white adipose tissues of hibernating arctic ground squirrels (Chapter 4). The lowest dotted line (Lipid Breath) represents the predicted breath $\delta^{13}C$ value from pure lipid catabolism based on lipid tissue $\delta^{13}C$ values adjusted down by 1.1‰ as the potential discrimination between endogenous lipid and breath $CO_2$.

## General Conclusion

This dissertation investigates the expression of different strategies of hibernation in the Arctic and develops stable isotopes as a tool to study the underlying physiological mechanisms of metabolic fuel use during thermogenesis. It combines comparative, observational studies of hibernation in free-living animals with new, experimental approaches to examining fuel use at the tissue and metabolic level. The focal species include the well-known arctic ground squirrel (*Urocitellus parryii*) and the little-studied Alaska marmot (*Marmota broweri*), related species that differ in social structure.

Chapter 1 presented the first record of body temperature patterns during hibernation in the Alaska marmot. Like arctic ground squirrels, the Alaska marmot exhibits deep torpor with periodic, brief arousals to euthermy. However, I did not observe body temperatures below 0 °C, as is common for sympatric arctic ground squirrels. The pattern of cooling exhibited by the Alaska marmot was unique, characterized by a period of very rapid cooling followed by a brief increase in temperature, and then a period of slow cooling. This pattern has not been previously described in hibernating mammals. In Chapter 2, I built upon this work to show that Alaska marmots sharing a hibernaculum demonstrate extreme synchrony of alternations between torpor and brief bouts of high body temperature. These findings indicate that marmots practice extensive social thermoregulation, most likely to reduce the cost of heat production in an environment that is < 0 °C for most of the hibernation season. I also found that the interrupted cooling pattern observed in Chapter 1 was used widely but not exclusively during cooling by Alaska marmots, raising questions about its function and regulation. In Chapter 2, I also studied patterns of body temperature in ground squirrels from the same burrow sites, which therefore had the greatest potential to utilize social thermoregulation. Arctic ground squirrel hibernation is well studied, but patterns of body temperature from individuals in close proximity had not previously been compared. These animals displayed a complete lack of synchrony in body temperature patterns, supporting observations that this species hibernates individually.

Experimental studies have quantified the degree to which arctic ground squirrels must be thermogenic during torpor at ambient temperatures well below freezing (Buck and Barnes 1999, 2000). Arctic ground squirrels rely on a significant proportion of their lean mass to fuel this increased energetic demand (Buck and Barnes 1999), even increasing lean mass during preparation for hibernation (Boonstra et al. 2011). In Chapter 3, I calibrated a deuterium dilution method for measuring body composition in arctic ground squirrels over a wide range of fat and lean masses in order to repeatedly estimate body composition. This method facilitated a detailed investigation of lean mass use and tissue metabolism during hibernation (Chapter 4) and will be useful in future field and lab investigations of arctic ground squirrels.

In Chapter 4, I present data that show that the lean mass utilized during hibernation likely comes from skeletal muscle. In contrast, organ tissues are maintained through remodeling during hibernation. The requirement for carbohydrates to facilitate lipid utilization as thermogenic demand increases is thought to exceed the supply from glycerol, a product of lipid catabolism (Buck and Barnes 2000; Galster and Morrison 1975). To meet this need for carbohydrates, it appears that protein from the breakdown of muscle is converted to carbohydrates. Chapter 5 confirms the shift in metabolic fuel use in torpid arctic ground squirrels, but demonstrates that there are factors in addition to thermal challenge that influence the reliance on carbohydrates.

The insights into metabolic fuel use during hibernation have been made possible by the development and application of changes in natural abundance of stable isotopes. The work in this dissertation advances the growing body of knowledge of applications of stable isotope techniques to animal physiology, most significantly by clarifying the mechanism involved in the change of nitrogen stable isotope ratios ($\delta^{15}N$) during fasting. This is an important contribution because the mechanism has not been well defined in the literature and $\delta^{15}N$ change has been interpreted in different, sometimes contradictory, ways. Chapter 4 reviews this literature and offers a consistent model for $\delta^{15}N$ change supported by the results of Chapter 4, which will improve the use of this powerful tool in physiological studies. In Chapter 5, I reported a correlation between the carbon isotope

ratio ($\delta^{13}C$) of breath and respiratory quotient (RQ) in animals. This has previously been shown in plants, but utilization of this tool is just beginning in animal physiology. The results from Chapter 5 will contribute momentum to the field and also give direction for future studies to investigate discrimination factors in carbon metabolism.

In summary, this dissertation contributes to our understanding of the mechanisms of hibernation in the Arctic, as well as adding to our knowledge of the life history of the Alaska marmot, a mammal endemic to Alaska. It also contributes significant knowledge to the growing field of stable isotope ecology by developing tools for monitoring differential tissue metabolism and differentiating mixed metabolic fuel use in animals.

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