FACTORS CONTROLLING THE PHENOLOGY

AND LIMITS OF HIBERNATION IN A SCIURID

By

Melanie M. Richter

RECOMMENDED:

Alexander Kitaysky, Ph.D.
Advisory Committee Member

Kelly Drew, Ph.D.
Advisory Committee Member

Michael Harris, Ph.D.
Advisory Committee Member

Brian M. Barnes, Ph.D.
Advisory Committee Co-Chair

C. Loren Buck, Ph.D.
Advisory Committee Co-Chair

Diane Wagner, Ph.D.
Chair, Department of Biology and Wildlife

APPROVED:

Paul Layer, Ph.D.
Dean, College of Natural Science and Mathematics

John Eichelberger, Ph.D.
Dean of the Graduate School

31 July 2015
Date

FACTORS CONTROLLING THE PHENOLOGY AND LIMITS OF HIBERNATION IN A SCIURID

A

DISSERTATION

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Melanie M. Richter, B.S.

Fairbanks, AK

August 2015

ABSTRACT

Animals that live in seasonal environments have a variety of adaptations to survive periods of low to no food availability. One such adaptation is hibernation, which is characterized by profound decreases in activity, metabolic rate, and in most cases, body temperature. Among animals that hibernate, only two species are known to maintain low tissue temperature while defending significant temperature gradients, and the best studied of these is the arctic ground squirrel (*Urocitellus parryii*). In the first chapter, we determine the lower ambient temperature limit of hibernation for an arctic ground squirrel (-26°C), and that a maximum torpid metabolic rate exists (0.37 mL O_2/g*h). This maximum torpid metabolic rate allows animals to defend a ~26°C temperature gradient between their core and their environment. In this chapter we also demonstrate that another, temperate, hibernating species, the golden-mantled ground squirrel (*Callospermophilus lateralis*), is capable of continuing hibernation at sub-freezing temperatures and can defend a temperature gradient of at least 9°C. Due to the extreme environment that arctic ground squirrels inhabit, they have a very short growing season (~3-7 months) during which they must reproduce, grow, and accumulate energy stores prior to hibernation onset. In the second chapter we investigate the roles androgens play in hibernation phenology and male aggressive behavior. We use plasma samples collected from free-living animals and radioimmunoassays to determine circulating androgen levels. We then match the peaks in androgens to the timing of the two periods of male-male aggression (testosterone in the spring and dehydroepiandrosterone in the late summer/fall). We also present evidence to support testosterone as the main factor determining the timing of spring euthermy and emergence among reproductively mature males. In the third chapter we utilize captive animals to determine the importance of a cache to male reproductive development. Using three separate experiments, we show that while the accumulation of a cache in the late summer/fall may increase the likelihood of a male undergoing reproductive development, it alone may not be enough to ensure reproductive development. Additionally, we demonstrate that simply having access to *ad libitum* food in the spring is not enough to ensure reproductive development, nor is a restricted spring ration enough to prevent it.

DEDICATION PAGE

THIS DISSERTATION IS DEDICATED TO THE SQUIRRELS.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ACKNOWLEDGEMENTS

I would like to first acknowledge, with much gratitude, the sources of funding that made this work possible: the National Science Foundation and the US Army Medical Research and Material Command. I would also like to thank Alaska INBRE for granting me a graduate student fellowship for one year, the Institute of Arctic Biology for granting me a summer fellowship and the Biology and Wildlife Department at the University of Alaska Fairbanks and the Biology Department at the University of Alaska Anchorage for hiring me as a teaching assistant. I would also like to acknowledge Drs. Eric Bortz and Fred Rainey for hiring me as a research technician enabling me to finish my last year of writing.

I want to acknowledge the fantastic lab mates I have been privileged to work with over the last 8 years. They have been a great source of assistance and commiseration on both a personal and professional level. In the same vein, the staff and researchers at Toolik Field Station made my very long field seasons more of a vacation than work and I am forever indebted to them for that. I would also like to thank my co-advisors, Drs. Loren Buck and Brian Barnes and my committee for helping to develop me into the researcher I hope to be. Science is a collaborative discipline that does not exist in a vacuum but rather in a community. For that reason, I use "we" in each chapter to refer to not only myself, but the co-authors that aided each chapter's progress. From guidance on writing and statistical analysis, to research techniques and data collection, every co-author aided me in producing each manuscript.

Lastly, and most importantly, I would like to acknowledge my parents, without whom I never would have been able to finish such a grueling, but rewarding, degree.

GENERAL INTRODUCTION

Hibernation is a pleisiomorphic trait that is present in a great variety of mammalian taxa and is characterized by profound decreases in metabolic rate, activity, and typically, body temperature (Geiser 1998, McKechnie 2014). The significantly decreased body temperature hibernators experience during torpor is not due to the abandonment of homeothermy, but rather is achieved by adjusting the hypothalamic set-point (Mills and South 1972). The new set-point is then guarded throughout the torpor cycle such that a temporarily (1-2 hours) cooled hypothalamus at any time-point initiates an increase in metabolic rate and heat production, while a temporarily (1-2 hours) heated hypothalamus results in a depressed metabolic rate (Heller and Hammel 1972; Heller and Colliver 1974; Florant and Heller 1977). The decrease in metabolic rate, however, is not due solely to the reduced body temperatures of torpor.

There are two main ways in which mammals are able to accrue metabolic savings during the hibernation season. First, the decreased enzymatic activity associated with the Q_{10} effects of decreased body temperature enable metabolic rate to fall as body temperature decreases (Geiser 2004). Secondly, temperature-independent metabolic suppression is utilized and enables metabolic rate to be reduced independently of body temperature, which is particularly important during entry into torpor (Geiser 2004). This second method is essential for large hibernating animals, such as the black bear (*Ursus americanus*). Due to their large size and low surface area to volume ratio, black bear have a low thermal conductance, referred to as thermal inertia in Chapter 1, which results in a slow rate of heat loss. Thermal conductance is defined as the rate at which heat is lost by an organism and is a physical process that includes conduction, convection, radiation and evaporative modes of heat transfer. In a hibernating mammal, thermal conductance is most influenced by levels of insulation (fur), blood flow to the periphery, and body size, with larger animals having lower thermal conductivities due to their lower surface to volume ratios and thicker fur. Due to these body size dependent differences in thermal conductance, rates of basal metabolism in euthermic mammals do not scale linearly with body mass, but rather to the 0.66 or 0.75 power (for a review and discussion: Schmidt-Nielsen 1984). The low thermal

conductivity of a bear means that they must rely on temperature-independent metabolic suppression as the main source of metabolic savings, with only minor supplementation from the small decreases in body temperature (~7°C from euthermic levels, Tøien et al. 2011). Smaller hibernators, on the other hand, are able to use temperature-independent metabolic suppression during entry into torpor, and then maximize metabolic savings by utilizing Q_{10} effects while torpid (Ruf and Geiser 2014).

Hibernators spend the majority of their hibernation season in torpor, at low body temperature and at minimal metabolic rate, but intermittently arouse, return to high, or euthermic, body temperature and high metabolic rate, and remain there for <24 hours before resuming torpor (Carey et al., 2003). The arousal process can consume 86% of the entire overwinter energy budget for a hibernator when ambient temperatures remain at or above the hypothalamic set-point (Wang 1978). While arousals are undoubtedly necessary, the factors driving the timing and ultimate cause of an arousal are not yet fully understood. There is evidence that spontaneous arousals can be driven by the accumulation, or loss, of metabolites over a torpor bout (Epperson et al., 2011; Jinka et al., 2012). In addition to spontaneous arousals, hibernators can undergo induced, or alarm, arousals caused by environmental disturbances such as temperature changes or physical agitation (Pengelley and Fisher 1968, Twente and Twente 1968). Spontaneous and induced arousals differ from each other in a number of ways including arousal duration and maximum rewarming rate (Utz and van Breukelen 2013). During an arousal, the initial source of heat comes from the actions of norepinephrine, released from activated sympathetic neurons, on brown adipose tissue and uncoupling protein-1 (Cannon and Nedergaard 2004; 2011) as shivering is not considered effective for heat generation at low body temperature (Kitao and Hashimoto 2012). This same non-shivering thermogenesis mechanism is presumably what allows some hibernators to remain torpid at ambient temperatures below their hypothalamic set-point and defend significant temperature gradients (Buck and Barnes 2000). One example of this is the arctic ground squirrel (*Urocitellus parryii*). Arctic ground squirrels utilize non-shivering thermogenesis to undergo thermogenic torpor, which occurs when a significant gradient (we defined here as >2.0°C) between body temperature and ambient temperature is maintained in steady-state torpor, to survive the Arctic winter.

The arctic ground squirrel is the northern-most hibernator studied in the laboratory. This species experiences some of the coldest overwinter temperatures on record (minimum: -23.4, mean: -15.8; Buck and Barnes 1999). Due to their distribution, the arctic ground squirrel undergoes a very long hibernation season, lasting from 200-250 days depending on sex and reproductive state (Sheriff et al. 2011). The arctic ground squirrel also survives and maintains the lowest recorded body temperature of any mammal (-2.9°C, Barnes 1989). While they sustain a below freezing body temperature, it is still significantly warmer than the soil in which they are hibernating. Having to defend a substantial temperature gradient (2°C or greater) while torpid is an unusual circumstance for the majority of hibernators (reviewed in Karpovich et al. 2009 and Chapter 1, Table 1.1) and the ability to elevate their torpid metabolic rates is under studied.

Studies of hibernating mammals have frequently been limited to temperatures at which animals are able to thermally conform to their environments and allow body temperature to match ambient, thereby maximizing their metabolic savings. In the first chapter, we investigate whether thermogenic torpor is limited to a species that evolved in environments where conditions include frequent exposure to sub-zero temperatures (the arctic ground squirrel) or if, as we predicted, it is a more universal ability and shared by another species of ground squirrel. Using a temperate zone species, the golden-mantled ground squirrel (*Callospermophilus lateralis*), we demonstrate this species' ability to survive prolonged periods of torpor at below freezing temperatures by increasing its torpid metabolic rate, an ability only extensively measured in arctic ground squirrels. While golden-mantled ground squirrels have previously been shown to respond to moderate subzero temperatures (-1 and -2; Wit and Twente 1983; Geiser and Kenagy 1988) with increased metabolism, these temperatures are only slightly (1-2°C) below their maintained body temperature (0°C; Healy et al. 2012) and these temperatures were lethal for a subset of tested animals (Wit and Twente 1983). We hypothesized that (I) the main difference between these two species' ability to remain torpid at very low ambient temperatures is due to their different body sizes, and therefore different thermal conductivities, rather than due to an inherent physiological differences and (II) that the larger size, and thus lower thermal conductance, of the arctic ground squirrel would enable them to remain torpid at lower temperatures than the smaller golden-mantled ground squirrel. In this research we also attempt to identify the lower ambient temperature limit of hibernation for both the arctic and golden-mantled ground squirrel, predicting that below this temperature animals would be unable

to maintain low body temperature for prolonged periods due to an inability to increase torpid metabolic rate above a set level; determining this set level also allowed us to define the maximum torpid metabolic rate for the arctic ground squirrel.

Hibernation is directly followed by reproductive development, and then pre-hibernatory fattening in obligate hibernators, such as many ground squirrel species, and these events occur with a circannual rhythm that persists under continuous temperature and photoperiod (Pengelley and Fisher, 1963; Ruf and Geiser 2014). Similar to other biological rhythms, the timing of events in the circannual hibernation cycle can be entrained/modified by environmental factors such as soil temperature (Barnes and Ritter 1993). It has previously been established that reproductive development is linked to the timing of hibernation in male ground squirrels (Barnes 1996); males that do not undergo testicular development hibernate longer than their reproductively competent cohorts and females enter hibernation earlier and end hibernation later than mature males, resulting in the longest hibernation seasons (reviewed in Michener 1984). Studies have shown that exposure to exogenous testosterone, but not estradiol, can inhibit ground squirrels of both sexes from entering torpor (Lee et al. 1990). In the second chapter, we hypothesize that (III) there is a link between circulating androgens, specifically testosterone and dehydroepiandrosterone, behaviors, and hibernation phenology in free-living male arctic ground squirrels. We predicted that high levels of circulating testosterone would only be found in reproductively mature males in the spring, coincident with the mating season and associated male-male aggression; this peak in testosterone should be brief with mature males' levels returning to baseline once the mating season ends. The late summer period of measured aggression (Buck and Barnes 2003) should coincide with elevated levels of the adrenal androgen dehydroepiandrosterone due to its association with non-mating season aggression in other species (reviewed in Soma et al. 2015). If testosterone plays a role in the timing of spring phenology, castrated males should have hibernation seasons that begin in the fall and end late in the spring, similar to those of immature males; if testosterone also plays a role in controlling hibernation onset then castrated males should enter hibernation early, similar to females. Reproductive status, and therefore hibernation phenology, may be influenced by endogenous androgens, but it can additionally be influenced by environmental conditions, such as food availability (Barnes 1984).

Sexually mature male arctic ground squirrels emerge from hibernation having recovered the body mass and condition lost over winter (Sheriff et al. 2013) by feeding from the caches that they accumulate in the fall (Buck and Barnes 1999). A varying proportion of males "opt out" of the reproductive season each year, and therefore do not develop scrotal, spermatogenic testes, and emerge in the spring at a significantly lower body mass than they attained before hibernation (Buck and Barnes 1999). The factors that determine which males participate in mating, and which do not, has not been determined. In the third chapter we hypothesize (IV) that access to a cache will impact the decision to undergo reproductive development. Here, we use the availability of food in the spring and the ability to accumulate a cache in the fall to determine whether food availability is the dominate factor in determining which males undergo reproductive development in the spring. We predicted that males able to establish a cache would develop scrotal testes, whereas males that were unable to cache would have longer hibernation seasons and not reproductively mature.

Literature Cited

Barnes, B. (1989). "Freeze avoidance in a mammal: Body temperatures below 0 degrees Celsius in an arctic hibernator." Science 244(4912): 1593.

Barnes, B. M. (1984). "Influence of energy stores on activation of reproductive function in male golden-mantled ground squirrels." Journal of Comparative Physiology B 154(4): 421 425.

Barnes, B. M. (1996). Relationships between hibernation and reproduction in male ground squirrels. Adaptations to the Cold: Tenth International Hibernation Symposium. F. Geiser, A. J. Hulbert and S. C. Nicol. Armidale, University of New England Press.

Barnes, B. M. and D. Ritter (1993). "Patterns of body temperature change in hibernating arctic ground squirrels." Life in the cold: ecological, physiological, and molecular mechanisms (C. Carey, GL Florant, BA Wunder, and B. Horwitz, eds.). Westview Press, Boulder, Colorado: 119-130.

Buck, C. L. and B. M. Barnes (1999). "Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter." Journal of Mammalogy 80(4): 1264-1276.

Buck, C. L. and B. M. Barnes (2000). "Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator." American Journal of Physiology: Regulatory, Integrative & Comparative Physiology 48(1): R255.

Buck, C. L. and B. M. Barnes (2003). "Androgen in free-living arctic ground squirrels: seasonal changes and influence of staged male-male aggressive encounters." Hormones & Behavior 43(2): 318.

Cannon, B. and J. Nedergaard (2004). "Brown adipose tissue: function and physiological significance." Physiological Reviews 84(1): 277-359.

Cannon, B. and J. Nedergaard (2011). "Nonshivering thermogenesis and its adequate measurement in metabolic studies." The Journal of Experimental Biology 214(2): 242 253.

Carey, H. V., M. T. Andrews and S. L. Martin (2003). "Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature." Physiological Reviews 83(4):1153-1181.

Epperson, L. E., A. Karimpour-Fard, L. E. Hunter and S. L. Martin (2011). "Metabolic cycles in a circannual hibernator." Physiological Genomics 43(13): 799-807.

Florant, G. and H. Heller (1977). "CNS regulation of body temperature in euthermic and hibernating marmots (*Marmota flaviventris*)." American Journal of Physiology Regulatory, Integrative and Comparative Physiology 232(5): R203-R208.

Geiser, F. (1998). "Evolution of daily torpor and hibernation in birds and mammals: Importance of body size." Clinical & Experimental Pharmacology & Physiology 25(9): 736.

Geiser, F. (2004). "Metabolic rate and body temperature reduction during hibernation and daily torpor." Annual Review of Physiology 66(1): 239-274.

Geiser, F. and G. Kenagy (1988). "Torpor duration in relation to temperature and metabolism in hibernating ground squirrels." Physiological Zoology: 442-449.

Healy, J. E., K. A. Burdett, C. L. Buck and G. L. Florant (2012). "Sex differences in torpor patterns during natural hibernation in golden-mantled ground squirrels (*Callospermophilus lateralis*)." Journal of Mammalogy 93(3): 751-758.

Heller, H. and G. Colliver (1974). "CNS regulation of body temperature during hibernation." American Journal of Physiology 227(3): 583-589.

Heller, H. C. and H. T. Hammel (1972). "CNS control of body temperature during hibernation." Comparative Biochemistry and Physiology Part A: Physiology 41(2): 349-359.

Jinka, T. R., B. T. Rasley and K. L. Drew (2012). "Inhibition of NMDA-type glutamate receptors induces arousal from torpor in hibernating arctic ground squirrels (*Urocitellus parryii*)." Journal of Neurochemistry 122(5): 934-940.

Karpovich, S. A., Ø. Tøien, C. L. Buck and B. M. Barnes (2009). "Energetics of arousal episodes in hibernating arctic ground squirrels." Journal of Comparative Physiology B: Biochemical, Systemic & Environmental Physiology 179(6): 691-700.

Kitao, N. and M. Hashimoto (2012). "Increased thermogenic capacity of brown adipose tissue under low temperature and its contribution to arousal from hibernation in Syrian hamsters." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 302(1): R118-R125.

Lee, T. M., K. Pelz, P. Licht and I. Zucker (1990). "Testosterone influences hibernation in golden-mantled ground squirrels." American Journal of Physiology 259(4 Pt 2): R760 767.

McKechnie, A. E. (2014). "The ecology and evolution of mammalian heterothermy in a changing world." Journal of Zoology 292(2): 71-73.

Michener, G. (1984). Age, sex, and species differences in the annual cycles of ground-dwelling sciurids: implications for sociality. The Biology of Ground-dwelling Squirrels: Annual cycles, behavioral ecology, and sociality. J. Murie and G. R. Michener. Lincoln Nebraska, University of Nebraska Press: 81-107.

Mills, S. H. and F. E. South (1972). "Central regulation of temperature in hibernation and normothermia." Cryobiology 9(5): 393-403.

Pengelley, E. T. and K. C. Fisher (1963). "The effect of temperature and photoperiod on the yearly hibernating behavior of captive golden-mantled ground squirrels (*Citellus lateralis tescorum*)." Canadian Journal of Zoology 41(6): 1103-1120.

Pengelley, E. T. and K. C. Fisher (1968). "Ability of the ground squirrel, *Citellus lateralis*, to be habituated to stimuli while in hibernation." Journal of Mammalology 49(3): 561-562.

Ruf, T. and F. Geiser (2014). "Daily torpor and hibernation in birds and mammals." Biological Reviews.

Schmidt-Nielsen, K. (1984). Scaling: why is animal size so important?, Cambridge University Press.

Sheriff, M., R. Fridinger, Ø. Tøien, B. Barnes and C. Buck (2013). "Metabolic rate and prehibernation fattening in free-living arctic ground squirrels." Physiological and Biochemical Zoology 86(5): 515-527.

Sheriff, M. J., G. J. Kenagy, M. Richter, T. Lee, Ø. Tøien, F. Kohl, C. L. Buck and B. M. Barnes (2011). "Phenological variation in annual timing of hibernation and breeding in nearby populations of Arctic ground squirrels." Proceedings of the Royal Society B: Biological Sciences 278(1716): 2369-2375.

Soma, K. K., N. M. Rendon, R. Boonstra, H. E. Albers and G. E. Demas (2015). "DHEA effects on brain and behavior: insights from comparative studies of aggression." The Journal of Steroid Biochemistry and Molecular Biology 145: 261-272.

Tøien, Ø., J. Blake, D. M. Edgar, D. A. Grahn, H. C. Heller and B. M. Barnes (2011). "Hibernation in black bears: independence of metabolic suppression from body temperature." Science 331(6019): 906-909.

Twente, J. W. and J. A. Twente (1968). "Effects of epinephrine upon progressive irritability of hibernating *Citellus lateralis*." Comparative Biochemistry and Physiology 25(2): 475 483.

Utz, J. C. and F. van Breukelen (2013). "Prematurely induced arousal from hibernation alters key aspects of warming in golden-mantled ground squirrels, *Callospermophilus lateralis*." Journal of Thermal Biology 38(8): 570-575.

Wang, L. C. (1978). "Energetic and field aspects of mammalian torpor: the Richardson's ground squirrel." Journal of Thermal Biology 3(2): 87.

Wit, L. C. and J. W. Twente (1983). "Metabolic responses of hibernating golden-mantled ground squirrels *Citellus lateralis* to lowered environmental temperatures." Comparative Biochemistry and Physiology Part A: Physiology 74(4): 823-827.

Chapter 1 THERMOGENIC CAPACITY AT SUB-ZERO TEMPERATURES: HOW LOW CAN A HIBERNATOR GO?[1]

Abstract

Hibernation in mammals is a physiological and behavioral adaptation to survive intervals of low resource availability through profound decreases in metabolic rate (MR), core body temperature (T_b), and activity. Most small mammalian hibernators thermo-conform, with T_b approximating ambient temperature (T_a); arctic species are an exception since they must actively defend what can be large thermal gradients between T_b and T_a. Here we compare the thermogenic capacity of the arctic ground squirrel (*Urocitellus parryii*) to that of the golden-mantled ground squirrel (*Callospermophilus lateralis*), a temperate zone montane hibernator. We allowed animals to re-enter torpor at sequentially lower T_a's and found that arctic ground squirrels maintained steady-state torpor at T_a's as low as -26°C through a 37-fold increase in torpid MR (TMR), compared to their minimum TMR exhibited at T_a 0°C. Golden-mantled ground squirrels are able to maintain steady-state torpor at T_a's at least as low as -8°C through a 14.5-fold increase in MR compared to their minimum TMR at T_a 2°C. In a second experiment, torpid animals were exposed to continuously decreasing T_a's (0.25°C/30min); individuals of both species increased their metabolism while remaining torpid at low T_a's (as low as -30°C for arctic ground squirrels and -10°C for golden-mantled ground squirrels). Although the capacity to hibernate at sub-freezing T_a's is not unique to arctic ground squirrels, their large body size, greater torpid metabolic scope, and previously ascribed capacity to supercool allow them to occupy much colder hibernacula for prolonged seasons of hibernation.

[1] Published as: Richter, M.M., C.T. Williams, T.N. Lee, Ø. Tøien, G.L. Florant, B.M. Barnes and C.L. Buck. 2015. Thermogenic Capacity at Subzero Temperatures: How Low Can a Hibernator Go? Physiological and Biochemical Zoology 88(1): 81-89.

Introduction

Seasonality of environments, which encompasses annual cycles of temperature, precipitation and resource availability, is a selective force that has led to the evolution of a variety of molecular, physiological and behavioral adaptations in indigenous species. One of the most intriguing adaptations of animals to seasonal environments is mammalian hibernation, characterized by extremely reduced metabolic rate (MR; as low as 1-5% of basal, Geiser and Ruf 1995), body temperature (T_b) and activity (reviewed in Boyer and Barnes 1999; Storey 2000; Carey et al., 2003; Geiser 2004). Mammalian hibernation is a geographically and taxonomically widespread phenomenon that allows animals to survive periods of low resource availability by providing significant savings in energy use as they subsist on hoarded and/or endogenous energy stores (Geiser 2004; McKechnie 2014).

A majority of hibernation research has been conducted on sciurid ground squirrels and marmots held in the laboratory and exposed to above-freezing T_a's (reviews: Davis 1976; Geiser and Baudinette 1990; Geiser 2004) with relatively few published investigations examining the physiology of hibernation at T_a's lower than 0°C (Geiser and Kenagy 1988; Barnes 1989; Buck and Barnes 2000; Karpovich et al., 2009). Hibernators rely on temperature-independent metabolic inhibition, in addition to the temperature-dependent or Q_{10} effects associated with decreased T_b, to reduce MR during torpor (reviewed in Geiser 2004). In most species hibernating at $T_a \geq T_b$ set-point, torpid MR (TMR) decreases with decreasing T_a in a Q_{10} dependent manner (Hammel et al., 1968; Geiser and Kenagy 1988; Snyder and Nestler 1990; Geiser 2004), although arctic ground squirrels (*Urocitellus parryii*) also utilize temperature-independent metabolic inhibition enabling them to maintain a constant and low TMR over a T_a and T_b range of 0 to 16°C (Buck and Barnes 2000). This relationship of decreasing TMR with decreasing T_b holds true for most hibernators so long as the animal's T_b remains near T_a. However, as T_a approaches and decreases below the T_b set-point, the animal must either increase its TMR to maintain $T_b \geq T_b$ set-point, arouse from torpor or, failing these, the animal will die (Geiser and Kenagy 1988; Geiser et al., 1990; Arnold et al., 1991; Buck and Barnes 2000).

Although sciurids have been used extensively to study hibernation in the laboratory, comparatively little is known about hibernation and hibernacula conditions of sciurids in the wild (literature summary in Table 1). Of these studies, the majority include soil temperatures from a

single season and one small section of the species' overall distribution; only two sciurid species have been shown to experience overwinter soil temperatures ≤ -5°C: the arctic ground squirrel and the Alaska marmot (*Marmota browerii*). These two species are also the only representatives of the Arctic, where continuous permafrost constrains the depth of hibernacula such that these animals predictably experience sub-freezing soil temperatures across much of their hibernation season (Carl 1971; Buck and Barnes 1999*b*; Lee et al., 2009). Given the limited information about hibernaculum conditions available on species living in the temperate zone, it is unclear whether they are routinely subjected to sub-zero soil temperatures for extended periods of time within their current range distributions.

Since few species are known to hibernate at subzero T_a's, very few captive studies of hibernators have challenged animals with T_a's substantially below freezing and analyzed their corresponding torpid thermogenic capacity. Laboratory studies of arctic ground squirrels reveal that they have a tremendous capacity to increase metabolism and generate heat during torpor and arousals at low T_a (Buck and Barnes 2000; Karpovich et al., 2009). Field measurements of arctic ground squirrel hibernacula have shown that T_a during winter can decrease to as low as -23.4°C (Buck and Barnes 1999*b*) which is lower than T_a's that hibernators have been subjected to in captive investigations (-16°C; Buck and Barnes 2000). Thus, the maximum MR arctic ground squirrels can maintain during steady-state torpor remains unknown. Laboratory studies conducted at sub-zero T_a's on hibernating golden-mantled ground squirrels (*Callospermophilus lateralis*), a temperate zone species, have produced conflicting results with respect to their thermogenic capacity during torpor. Geiser and Kenagy (1988) found that all golden-mantled ground squirrels increase metabolism and generate heat to prevent the potential deleterious effects associated with T_b falling below T_b set-point, whereas Wit and Twente (1983) found that not all individuals were able to increase torpid thermogenesis and, when exposed to sub-freezing T_a's (-1 to -2°C), a subset of animals either aroused or died. Arctic ground squirrels and golden-mantled ground squirrels are two sciurid species that have demonstrated some capacity for thermogenic torpor, making these species good candidates for an analysis of thermogenic capacity during torpor.

Here we report the thermogenic capacity of hibernating arctic ground squirrels during steady-state torpor and compare their response to that of golden-mantled ground squirrels that

may only rarely be exposed to sub-freezing T_a's. We anticipated that both species would be capable of defending large thermal gradients between T_b and T_a. We hypothesized that differences between species in their capacity to maintain a thermal gradient should principally be a function of differences in size and thus thermal inertia such that the smaller golden-mantled ground squirrels should exhibit a greater increase in MR with more moderate decreases in T_a but with both species exhibiting similar maximum TMRs and similar metabolic scopes. Given their larger size, more northerly distribution, and use of sub-zero hibernacula, we predicted that arctic ground squirrels would be able to maintain torpor at lower T_a's than golden-mantled ground squirrels. To test our hypotheses, we utilized standardized protocols whereby we exposed animals to progressively lower T_a's while concurrently measuring both MR and T_b.

Material and Methods

Study species and husbandry.

The arctic ground squirrel is distributed from northeastern Russia throughout Alaska and northwestern Canada (Iwen 1999). Eighteen (5 female, 13 male) arctic ground squirrels (*Urocitellus parryii*) were either live-trapped north of the Brooks Range, AK near the Atigun River (68°27'N, 149°21'W, elevation 812m) and transported to the University of Alaska Anchorage vivarium or were born in captivity to mothers that were live-trapped near the Atigun River. The golden-mantled ground squirrel (*Callospermophilus lateralis*) lives in the temperate zone (between the tropic of Cancer and the Arctic Circle in the Northern Hemisphere) and its range includes the montane regions of western North America, and south through southern New Mexico (Howell 1938). Eight (4 female, 4 male) golden-mantled ground squirrels were live-trapped for this experiment in Larimer County, Red Feather Lakes, CO (40.8°N, 105.59°W, elevation 2,531m) and transported to the University of Alaska Anchorage.

Prior to experiments, all animals were maintained individually in metal cages (48x31x30cm, UnifabCages, Kalamazoo, MI, USA) on a 12L:12D photoperiod and at T_a of 20 ± 2°C. Animals were provided cotton batting for nesting (Perfect Fit, McDonald, Tukwila, WA, USA), food (Mazuri Rodent Chow, Brentwood, MO, USA) and water ad libitum. In the fall of each experimental year we moved animals into environmental chambers maintained at an T_a of 2

± 1°C with a 8L:16D photoperiod. As animals began to exhibit bouts of torpor, determined during daily observation, we transferred them and their nests into plastic tubs (43x27x19cm for arctic ground squirrels, 41x25x18cm for golden-mantled ground squirrels; Nalgene, Rochester, NY, USA), removed all food and water and allowed them to resume torpor. All work was approved by the University of Alaska Anchorage IACUC, protocol number 175424-2.

Body temperature (T_b).

To record T_b, we surgically implanted temperature-sensitive radiotransmitters (~7g, model TA10TA-F40-LF, Data Sciences International, St Paul, MN, USA) into the peritoneal cavity of animals at least two months before initiation of metabolic measurements. Briefly, animals were anesthetized using isoflurane and under aseptic conditions a 3 cm incision was made along the animal's midline through the cutaneous and muscle layers; the gas-sterilized transmitter was placed inside the peritoneal cavity. The muscle and subcutaneous layers were closed using chromic gut and polydioxanone sutures, respectively and the skin subsequently glued (Vetbond, 3M, St. Paul, MN, USA). After surgery animals were returned to their wire cages at T_a 20 ± 2°C where they remained until moved to an environmental chamber (2 ± 1°C). Prior to surgery, all transmitters were calibrated to 0.1°C with a mercury thermometer at 0.0°C, 35°C and 39°C. Transmitters were activated once hibernation began and animals were moved to tubs. T_b's were recorded every ten minutes.

Respirometry.

Rates of oxygen consumption were recorded concurrently from four animals using an automated 2-channel system that alternated between channels every 5 minutes (adapted from Tøien 2013). During measurements, the tubs housing animals were covered with closed-foam gasket sealed Polycarbonate lids. Ex-current air was drawn from the chambers through flow meters, after which a sub-sample passed through a dual gas flow multiplexer (a modified RM-8, Sable Systems International, Las Vegas, NV, USA) that switched air streams between a pair of animals and calibration gases. A sub-sample was then dried using Nafion dryers (Perma Pure, Toms River, NJ, USA) in a reflux mode, prior to being analyzed for O_2 and CO_2 content using an Oxilla II dual channel O_2 analyzer and two CA-10A CO_2 analyzers (Sable Systems International, Las Vegas NV, USA). Immediately prior to each recording, the CO_2 and O_2 analyzers were span

and zero calibrated (using the standard of 20.94% O_2 in air, soda lime, and a calibration gas with 0.5% CO_2 in air). At the beginning of each recording, and every hour throughout, a reference air sample was collected from inside the animal holding chamber. Samples of zero CO_2 air and span gas were automatically collected every three hours. Rate of oxygen consumption was calculated according to the principles of the Haldane transformation (Haldane and Graham 1912) with corrections as outlined in Tøien (2013). The rate of chamber air flow was measured and maintained at either 200 ± 10 ml/min (low flow) or 2500 ± 10 ml/min (high flow) with mass flow controllers (Flowbar8, Sable Systems International, Las Vegas, NV, USA and Brooks 5850E, 5 l/min range, Coastal Instruments, Burgaw, NC, USA, respectively). Computer controlled base-lining units (Sable Systems International, Las Vegas, NV, USA) were used to automatically switch from low to high chamber flow when animals aroused and from high to low flow when animals went into torpor based on O_2 depletion thresholds of 1.3% and 0.08%, respectively (as diagramed in Figure 15, Tøien 2013). All respirometry data were collected, corrected for drift and analyzed using LabGraph (Tøien 2013). Efficacy of system performance was assessed by burning a known mass of ethanol within the respirometry chamber before measurements began, halfway through the temperature protocols, and again after completion of experiments.

Steady-state torpor.

In the fall, at the beginning of the hibernation season, all animals (18 arctic ground squirrels, 8 golden-mantled ground squirrels) were moved into environmental chambers and respirometry measurements were initiated after all animals had exhibited at least one bout of torpor. To ensure that animals were in steady-state torpor, we conducted all measurements between days 1 and 18 of a torpor bout (T_b remained constant (± 0.5°C) for 2 or more hours prior to commencing recording). At low flow, data were not used until after 6 hours (3.26 complete air changes) to ensure measured gas concentrations accurately reflected animal metabolism; at high flow the duration was reduced to 2 hours (13.6 complete air changes). Mean rates of oxygen consumption were determined for individual animals during steady-state torpor over 6-hour periods.

Between experimental temperatures, all animals were weighed to the nearest gram (CW11-2EO, Ohaus Co., Pinebrook, NJ, USA) and physically disturbed to induce an arousal

such that all animals were euthermic prior to exposure to the following T_a. Arctic ground squirrels were randomly divided into two groups, each of which experienced a different T_a exposure protocol: protocol 1 included T_a's ranging from 2 to -20°C in 2°C increments; animals in protocol 2 were exposed to T_a's of 2°C and 0°C before we decreased the T_a to -10°C and then -20°C, following which T_a was decreased in 2°C increments until animals either failed to enter torpor, were unable to maintain low T_b, or until a maximum MR was obtained (as indicated by successive T_a's eliciting the same MR). Golden-mantled ground squirrels were exposed to the temperatures from protocol 1 after their initial torpor bout; however, in accordance with our animal care protocol, golden-mantled ground squirrels were removed from the cold room after measurements were made at -8°C. Animals were at each T_a for at least 24 h prior to being aroused for the next experimental T_a.

Ramping.

All ramping experiments were conducted in the year following steady-state torpor experiments (i.e., in the subsequent hibernation season). Once all animals had undergone at least one bout of torpor at 0°C, we began the ramping protocols as follows. Seven arctic ground squirrels (6M, 1F) were aroused from torpor at 0°C, weighed and then placed at -20°C where they again entered steady-state torpor. While simultaneously recording T_b and MR these animals were then subjected to gradually decreasing T_a's (set point decreased by 0.25°C every 30 min, except for at -25.5°C which was maintained for an hour to allow animals to acclimate) until animals exhibited an arousal. We defined arousal as an increase in MR of 0.1 ml O_2/g*h for 5 consecutive decreases in T_a. This definition allowed us to differentiate between incremental increases in MR associated with the progressively increasing temperature gradients and the actual attempt to arouse from low T_b while excluding the MR 'overshoots' (when an animal increased MR for a brief period of time that did not manifest as an increase in T_b) that the animals exhibited during the protocol. We followed a similar protocol with four golden-mantled ground squirrels (2M, 2F) in steady-state torpor at 0°C. The starting T_a's for both species were chosen based on the results obtained from our steady-state torpor work. Mean MRs for ramping were selected from the last 10 min prior to the set-point again being adjusted. The T_b and T_a we report for the ramping experiment are the last temperatures recorded prior to the change in T_a.

Data analysis.

All data presented are means ± SEM, unless otherwise noted. We used linear mixed effects models in R (REML function in lmerTest package; RStudio, version 3.0.1) with individual ID included as a random effect to examine the rate at which steady-state mass-specific torpid MR changed in response to changing T_a. For arctic ground squirrels we included only data collected between 0°C and -24°C in our model (the linear portion of the response; see results) whereas for golden-mantled ground squirrels we included data collected between 2°C and -8°C. To determine if torpid MR of arctic ground squirrels had reached a plateau, we compared MR at -24°C and -26°C using a paired T-test. We compared the maximum torpid MR between species using a Student's T-test. For all tests, we concluded results to be statistically significant when $P \leq 0.05$.

Results

Steady-state torpor.

Ground squirrels used in these experiments were able to maintain a low, constant T_b indicative of steady-state torpor at all temperatures tested. In response to exposure to incrementally decreasing sub-zero T_a's, torpid squirrels of both species increased MRs and continued to defend an increasingly large thermal gradient between T_a and T_b (Figure 1.1).

Arctic ground squirrels maintained low T_b's and steady-state torpor at T_a's to as low as -26°C (4 of 5 animals exposed to -26°C). At this T_a, the four animals maintained a TMR of 0.36 ± 0.01 ml O_2/g*h, a value not significantly different from the TMR of six animals torpid at -24°C (0.37 ± 0.01 ml O_2/g*h; paired t-test, P=0.3, Figure 1.1). Arctic ground squirrels displayed a 37-fold increase in TMR from 0°C to -24°C (0.01 ± 0.00 ml O_2/g*h at 0°C to 0.37 ± 0.01 ml O_2/g*h at -24°C). The relatively high TMR at -26°C (0.36 ± 0.01 ml O_2/g*h) enabled these arctic ground squirrels to defend a ~25.5°C thermal gradient and maintain a mean core T_b of -0.5 ± 0.3°C. The change in TMR from -24°C to 0°C was -0.014 ml O_2/g*h per °C. Of the 5 animals exposed to -26°C, one animal entered torpor briefly (2.6 h, minimum T_b: -0.4°C) before initiating an arousal; this animal was unable to fully arouse and died during the attempt (maximum T_b

achieved: 3.0°C). As a result of this fatality, no animals were exposed to T_a's < -26°C during the steady-state torpor measurements.

Golden-mantled ground squirrels maintained steady-state torpor at T_a's to -8°C, the lowest T_a they were subjected to given the constraints of our animal care protocol. The highest TMR recorded from golden-mantled ground squirrels occurred in animals torpid at -8°C (0.29 ± 0.01 ml O_2/g*h). This TMR is significantly lower than the maximum TMR recorded from arctic ground squirrels at -26°C (Students T-test, $P = 0.002$); but the TMR of golden-mantled ground squirrels had not yet plateaued. The golden-mantled ground squirrels were able to elevate their MR by 14.5-fold (0.02 ± 0.001 ml O_2/g*h at +2°C to 0.29 ± 0.01 ml O_2/g*h at -8°C) as they maintained above-freezing T_b at the sub-zero T_a's tested. Golden-mantled ground squirrels exhibited their lowest T_b (0.0 ± 0.2°C) when hibernating at -4°C, thus establishing a ~4°C temperature gradient between their core and the environment. However, while they reached their T_b nadir at -4°C, the largest gradient golden-mantled ground squirrels defended occurred at T_a -8°C (9.0 ± 0.3°C). The change in MR from T_a's of -8°C to -2°C was -0.024 ml O_2/g*h per °C.

Animals were weighed before the steady-state recordings had begun and during every induced arousal. For arctic ground squirrels undergoing the first steady-state protocol (T_a's from 2 to -20°C in 2°C increments), 11 arousals were induced and animals lost 154 ± 11 g of initial mass (Fall: 750 ± 32 g, Spring: 596 ± 30 g, a loss of ~21%) over the 104 days of the experiment. Over the same time course, arctic ground squirrels in the second protocol (T_a's of 2°C, 0°C, -10°C, -20°C, -22°C, -24°C, -26°C) underwent six induced arousals and lost 220 ± 23 g of initial body mass (Fall: 783 ± 19 g, Spring: 563 ± 23 g, a loss of ~28%). The two golden-mantled ground squirrels that were subjected to all T_a's in the steady-state protocol lost a total of 85 and 55 g (Fall: 247 and 222 g, Spring: 162 and 167 g, a loss of ~34 and ~25% of initial body mass, respectively) over five induced arousals and 84 days of the experiment.

Ramping.

Individuals from both species were able to defend a thermal gradient between torpid T_b and T_a without immediately arousing when challenged with incrementally lower, sub-zero T_a's. Prior to the metabolic measurements for the ramping experiment, arctic ground squirrels spontaneously entered torpor at 0°C; these animals were aroused, weighed and re-entered torpor

at -20°C immediately before the ramping protocol began. The mean T_a that arctic ground squirrels alarm aroused was -25.9 ± 1.1°C (range of -23.0 to -30.0°C). The average TMR just prior to arousal was 0.29 ± 0.02 ml O_2/g*h, ranging from 0.20 to 0.38 ml O_2/g*h (Table 1.2). The golden-mantled ground squirrels entered torpor at T_a of 0°C were then exposed to progressively lower T_a's; the mean T_a that induced arousal was -6.3 ± 1.8°C (range of -3.0 to -10.0°C). TMRs just prior to arousal averaged 0.12 ± 0.06 ml O_2/g*h, and ranged from 0.01 to 0.26 ml O_2/g*h (Table 1.2).

Discussion

In this study we investigated the thermogenic responses of two hibernating ground squirrel species exposed to sub-freezing temperatures during steady-state torpor. We found that arctic ground squirrels exhibited a 37-fold increase in TMR as they defended a thermal gradient of ~25.5°C between T_b and T_a, at T_a as low as -26°C, below the lowest published soil/hibernacula temperature for this species (Table 1.1). We suggest that the maximum TMR we measured in arctic ground squirrels was very close to their maximum TMR they are capable of as MR exhibited an abrupt plateau between -24 and -26°C. This was supported by our finding that torpid squirrels subjected to steadily decreasing T_a aroused at -25.9 ± 1.1°C, although one individual continued to hibernate at T_a = -30.0°C. The maximum TMR of 0.37 ± 0.01 ml O_2/g*h is close to their basal MR (0.4 to 0.61 ml O_2/g*h, Scholander et al., 1950; Withers et al., 1979) but is lower than the 0.51-0.84 ml O_2/g*h reported for resting MR of wild-caught arctic ground squirrels (Sheriff et al., 2013). We also found that golden-mantled ground squirrels are capable of increasing their TMR by at least 14.5-fold as they defend a thermal gradient of ~9°C between T_b and T_a while maintaining steady-state torpor at T_a's as low as -8°C (the lowest T_a tested). The highest TMR measured for the golden-mantled ground squirrels, like that of the arctic ground squirrels, was also well below their published basal MR (0.29 ± 0.01 ml O_2/g*h vs. 0.73 ml O_2/g*h, Snapp and Heller 1981). Arctic ground squirrels, however, exhibited lower minimum T_b, lower minimum TMR, a greater torpid metabolic scope, a smaller increase in TMR with decreasing T_a, and the capacity to maintain torpor at significantly lower T_a's compared to golden-mantled ground squirrels.

The golden-mantled ground squirrels utilized in our study maintained T_b above 0°C while hibernating at sub-freezing temperatures, contrary to Wit and Twente (1983). Animals were able to remain in steady-state torpor at T_a's as low as -8°C, and one animal only aroused when T_a reached -10°C; this value is substantially lower than the T_a -2°C that resulted in death for a subset of animals in Wit and Twente (1983). Likely, the discrepancy between our findings and previously published results is the difference in protocols utilized and not a real difference in thermal tolerance of the animals themselves. Our steady-state protocol allowed animals to fully arouse before subjecting them to a lower T_a, and our ramping protocol was much more gradual than what animals underwent in previously published work, where torpid animals were moved directly from an above zero T_a to below freezing T_a (Wit and Twente 1983).

Although both arctic ground squirrels and golden-mantled ground squirrels maintained torpid T_b's within a fairly narrow range throughout the experiments, the two species differed slightly in response to sub-zero T_a's. While arctic ground squirrels' T_b during torpor remained relatively constant with decreasing T_a, abdominal T_b in golden-mantled ground squirrels increased by ~0.9°C between T_a -4 and -8°C (-0.08 ± 0.16°C, 0.95 ±0.25°C, respectively, P = 0.08). Throughout the hibernation cycle, T_b is closely monitored and regulated by the hypothalamus; as the hypothalamus is heated or cooled, MR of the animal is suppressed or increased, ensuring that hypothalamic temperature does not deviate significantly from its set-point (Heller and Hammel 1972; Mills and South 1972; Florant and Heller 1977). However, thermogenic animals exhibit regional heterothermy during torpor (Barnes 1989) and therefore it is unclear whether the observed difference in abdominal T_b reflects a difference in hypothalamic set-point or was due to differences in heat transfer from the brown adipose tissue in the thoracic region. The arctic ground squirrels in our experiment maintained a slightly higher minimum abdominal T_b (-0.94 ± 0.11°C) than previously published for captive animals hibernating in outdoor enclosures (-2.9°C, Barnes 1989) or for free-living animals hibernating in the wild (-2.0°C – 0.9°C, Buck et al., 2008; Williams et al., 2012); however, our values are similar to those reported by Barnes (1989) for a single captive animal hibernating in environmental chambers (-1.3°C).

We presume that the increase in MR with decreasing T_a observed in the present study directly reflects an increase in rates of non-shivering thermogenesis. In hibernators, non-

shivering thermogenesis is activated via norepinephrine's effects on uncoupling protein-1 (UCP-1) in brown adipose tissue (Cannon and Nedergaard 2004; 2011). Animals hibernating at temperatures below their hypothalamic set-point may be particularly dependent on heat generated by brown adipose tissue. While EMG activity from shivering has been observed at T_b's as low as 4°C (Tøien et al., 2001) shivering is not thought to be an effective heat generator at this temperature (Kitao and Hashimoto 2012). Brown adipose tissue on the other hand up-regulates UCP1 upon cold exposure, and the highest levels are found in hibernators that are actively generating heat (Barger et al., 2006) indicating functionality at very low temperatures.

In this study we established an upper limit to the TMR for the arctic ground squirrel of 0.37 ± 0.01 ml O_2/g*h (37-fold increase from lowest TMR, Figure 1.1). Evidence supporting this as a maximum TMR in this species comes from the apparent plateau in MR between animals torpid at T_a -24 and -26°C. Interestingly, this maximum TMR is substantially lower than what these animals are capable of generating during an arousal (Karpovich et al., 2009). We were fortuitously able to record MR during arousals from two animals that were torpid at T_a -26°C; these animals demonstrated maximum arousal MRs of 3.24 and 3.44 ml O_2/g*h (a 324- and 344-fold increase, respectively, over the lowest mean TMR). These maximum arousal MR's are very similar to what Karpovich et al. (2009) found for arctic ground squirrels arousing from torpor at -12°C (3.40 ± 0.18 ml O_2/g*h) as well as being similar to peak levels resulting from stimulated arousals of arctic ground squirrels torpid at 2°C (Tøien et al., 2001) indicating that this is the maximum MR for thermogenesis during arousal in this species. It is possible that the discrepancy between maximum TMR and the MRs achieved during arousals is due to a threshold effect, i.e., animals may be able to increase their TMR only so much before the norepinephrine concentrations elicit an arousal. Once norepinephrine levels enabling non-shivering thermogenesis reach the threshold an arousal is initiated, the T_b set-point of the hypothalamus is reset to euthermic levels (~37°C), and MR increases accordingly (Florant and Heller 1977). Thus, alarm arousals might simply be a consequence of the elevated norepinephrine levels in both the hypothalamus and the brown adipose tissue associated with increased thermogenesis during torpor at low T_a. While this is a speculative hypothesis, there is some evidentiary support in that injections of norepinephrine directly into the hypothalamus or into the periphery both induce arousal from torpor (intrahypothalamic injection: Beckman and Satinoff 1972; intraperitoneal injection: Twente and Twente 1978).

We observed differences in the TMRs generated from each protocol; the steady-state torpor protocol resulted in higher TMRs compared to those from the ramping protocol (Figure 1.1 and Table 1.2). Interestingly, we did see a convergence in the data from both approaches onto a single value for the lower T_a limit of hibernation for each species. For the arctic ground squirrel, both experiments found a T_a limit of close to -26°C; the maximum MR of torpor plateaued between T_a -24 and -26°C (0.37 ± 0.01 ml O_2/g*h, Figure 1.1) in the first experiment and animals aroused at a mean T_a of -25.9 ± 1.1 °C during the ramping experiment (Table 1.2). The golden-mantled ground squirrels were more responsive to decreasing T_a and aroused from torpor at a mean T_a of -6.3 ± 1.8°C (range: -3 to -10°C, Table 1.2) during the ramping experiment, while the steady state protocol was terminated at -8°C. The difference in lower T_a limit between species might reflect the thermal inertia gained from the larger body mass of the arctic ground squirrels, which were 316% heavier than golden-mantled ground squirrels at peak adiposity. Thermal inertia may also help explain why TMR was lower in the ramping experiment as this inertia could result in a lag between changes in T_a and hypothalamic T_b and thus delay the metabolic response.

In addition to the increased thermogenic load animals incurred due to sub-freezing T_a's in our study, they also underwent frequent, induced arousals. This combination of elevated TMR and induced arousals resulted in a significant body mass loss (17-33%) over 104 days of hibernation for the arctic ground squirrels. For a comparison, free-living, adult female arctic ground squirrels lose 30% of their body mass over the course of the 237 ± 2.2 day hibernation season (Buck and Barnes 1999*a*) which includes ~15 spontaneous arousals (Buck et al., 2008). The two golden-mantled ground squirrels that were exposed to the full range of T_a's in the steady-state protocol lost 34.4 and 24.8% of their fall body mass over 5 induced arousals and 84 days of hibernation; this compared to ~29% body mass loss over ~232 days of hibernation and ~20 arousals for a free-living individual (Healy et al., 2012). The rapid body mass loss our animals experienced over a short period of time is indicative of the cost of arousals for a hibernator in addition to the increased TMR incurred at low T_a's. At T_a's above T_b set-point 86% of overwinter energy expenditure is accounted for by arousals (Wang 1978); as T_a decreases, the relative cost of arousals actually decreases due to the increased metabolic load of thermogenic torpor (Karpovich et al., 2009). Although soil temperatures measured in the Arctic can be as low as -23.4°C, soils at most hibernacula typically do not freeze solid at a depth of ~1 m until late

October (Buck and Barnes 1999*b*); therefore, arctic ground squirrels need only be thermogenic during deep torpor for a portion (5-7 months, Buck et al., 2008) of the hibernation cycle.

Hibernation functions as an energy conservation strategy that enables survival during periods of low resource availability. Arctic ground squirrels were able to maintain torpor at T_a's as low as -26°C, which is only ~3°C colder than what they are known to experience in the field (-23.4°C, Table 1). Although golden-mantled ground squirrels have a temperate zone distribution, we found that they remain at T_b ~ 0°C and have the capacity to increase MR and maintain steady-state torpor in T_a's at least as low as -8°C. Interestingly, this is also only a few degrees cooler than the minimum winter soil temperatures these animals are known to experience in the wild (-4.9°C, Table 1). Our results are consistent with the hypothesis that the physical environment plays an important role in shaping the hibernation phenotype of hibernating sciurids. However, we recognize the severe limitations of inferring adaptation based on two-species studies (reviewed in Garland and Adolph 1994) and we encourage further study on the thermogenic capacity of hibernators during deep torpor so that multi-species comparisons can be made.

Acknowledgements

We would like to thank F. Kohl, T. Stevenson, and B. Salli for their assistance with animal care duties. We would also like to thank K.L. Drew and the two anonymous reviewers for their constructive comments on this manuscript. This study was supported by funding from the National Science Foundation to CLB (EF-0732763 & IOS-1147187), CTW (IOS-1147187) and BMB (EF-0732755 & IOS-1147232) and from the Lipid Foundation to GLF (645848). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. Animals were maintained according to the Animal Care and Use Committee regulations of the University of Alaska Anchorage.

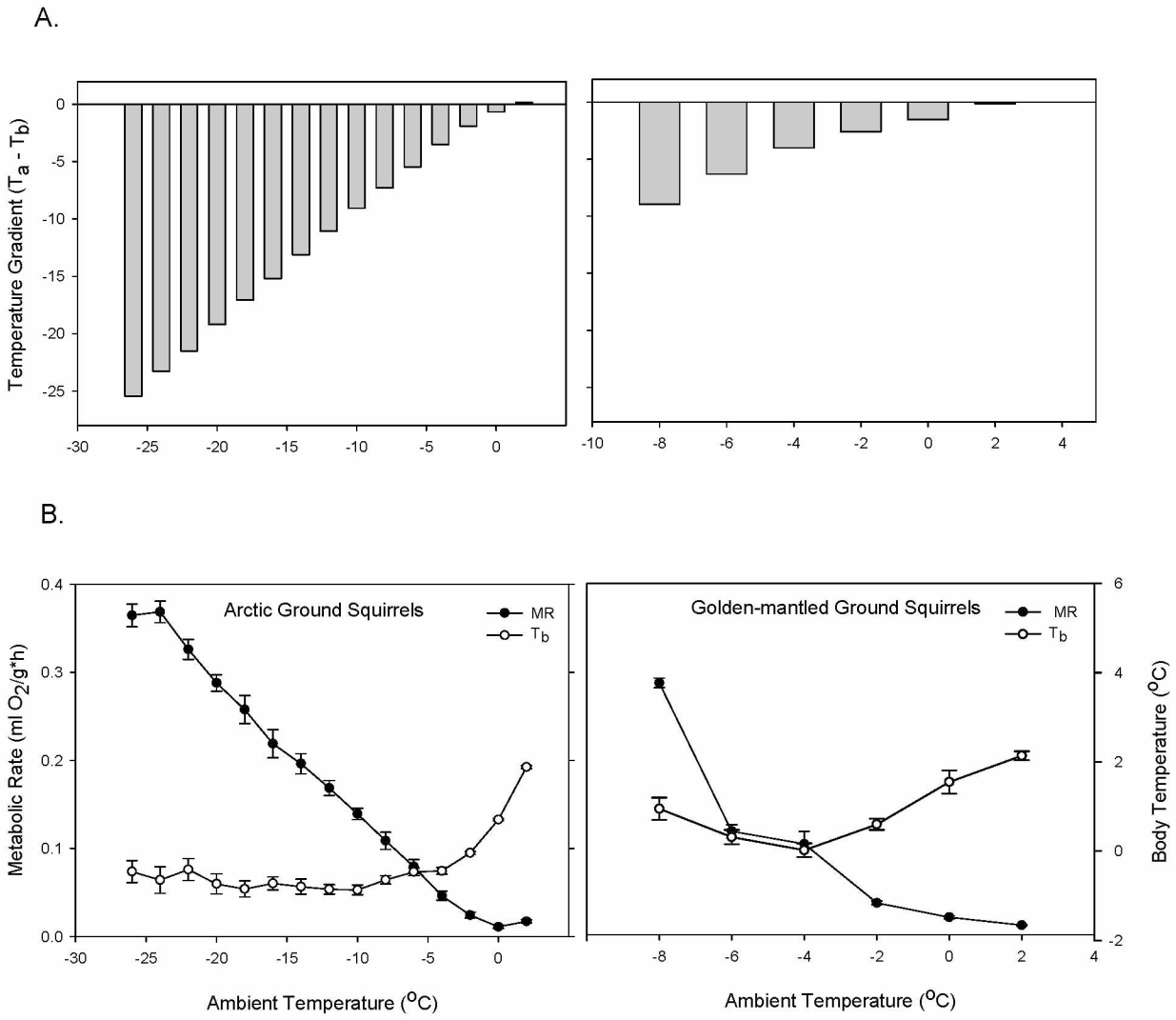


Figure 1.1: Panel A: Mean temperature gradients of animals in steady-state torpor at experimental ambient temperatures (T_a, °C). Panel B: The effect of T_a on steady-state torpid metabolic rate (mean ± SEM) in ground squirrels. Number of arctic ground squirrels ranges from 4 to 18 individuals at each temperature. Number of golden-mantled ground squirrels ranges from 2 to 8 individuals at each temperature.

Table 1.1: Published values for burrow/soil temperature and minimum torpid metabolic rate. Temperatures in °C, torpid metabolic rate (TMR) in mL O_2/g*h.

Species	Burrow/Soil Temperature	Minimum TMR (mL O_2/g*h)	Sources
Callospermophilus lateralis	-2°C-4.9°C	0.045	Healy et al., 2012; C.L. Frank, pers comm.; Snapp and Heller 1981
Callospermophilus saturatus	2°C	0.017	Kenagy et al., 1989; Geiser et al., 1990
Ictidomyes tridecemlineatus	-1°C	0.02	Kisser and Goodwin, 2012; C.L. Buck, pers comm.
Marmota browerii	Min.: -15.0°C, Mean: -7.3°C	N/A	Lee et al., 2009
Marmota marmota	0°C	0.013	Arnold et al., 1991
Marmota flaviventris	5-7°C	0.022	Florant and Heller 1977; Florant et al., 2000
Marmota monax	1.9°C	0.032	Ferron, 1996; Lyman 1958
Urocitellus columbianus	-2°C	N/A	Young, 1990
Urocitellus parryii	Min: -23.4°C, Mean: -8.9°C	0.01	Buck and Barnes, 1999*b*; Buck and Barnes 2000
Urocitellus richardsonii	-2.6°C	0.02	Michener 1992; Wang 1979

Table 1.2: Results from ramping experiment

GMGS#	T_a of arousal	MR at arousal	AGS#	T_a of arousal	MR at arousal
GMGS 1	-10°C	0.26	10-17	-23°C	0.20
GMGS 2	-3°C	0.02	10-04	-25°C	0.23
GMGS 3	-9°C	0.18	08-15	-24°C	0.34
GMGS 4	-3°C	0.01	09-13	-23°C	0.29
			08-34	-28°C	0.38
			09-06	-28°C	0.30
			08-28	-30°C	0.32
Means:	-6.3±1.8	0.12±0.10		-25.9±1.1	0.29±0.02

Maximum metabolic rates (reported as oxygen consumption in ml O_2/g*h ± SEM) from minimum ambient temperature (T_a) at which animals continued to exhibit torpor as determined via a temperature-ramping experiment that involved exposing animals to progressively decreasing T_a's during a single torpor bout. Individual golden-mantled ground squirrels are presented as their animal number (GMGS#) as are the arctic ground squirrels (AGS#).

Literature Cited

Arnold W., G. Heldmaier, S. Ortmann, H. Pohl, T. Ruf and S. Steinlechner. 1991. Ambient temperatures in hibernacula and their energetic consequences for alpine marmots *Marmota marmota*. Journal of Thermal Biology 16(4): 223-226.

Barger J. L. B. M. Barnes and B. B. Boyer. 2006. Regulation of UCP1 and UCP3 in arctic ground squirrels and relation with mitochondrial proton leak. Journal of Applied Physiology 101(1): 339-347.

Barnes, B. 1989. Freeze avoidance in a mammal: Body temperatures below 0°C in an arctic hibernator. Science 244(4912): 1593.

Beckman A. L. and E. Satinoff. 1972. Arousal from hibernation by intrahypothalamic injections of biogenic amines in ground squirrels. The American Journal of Physiology 222(4): 875-879.

Boyer B. B. and B. M. Barnes 1999. Molecular and metabolic aspects of mammalian hibernation. Bioscience 49(9): 713-724.

Buck C., A. Breton, F. Kohl, Ø. Tøien, B. Barnes, B. 2008. Overwinter body temperature patterns in free-living Arctic squirrels (*Spermophilus parryii*). In Lovegrove and A. McKechnie, eds. Hypometabolism in animals: torpor hibernation and cryobiology. University of KwaZulu-Natal, Pietermaritzburg: 317-326.

Buck C. L. and B. M. Barnes. 1999*a*. Annual cycle of body composition and hibernation in free-living arctic ground squirrels. Journal of Mammalogy 80(2): 430.

----- 1999*b*. Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter. Journal of Mammalogy 80(4): 1264.

Buck C. L. and B. M. Barnes. 2000. Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator. American Journal of Physiology: Regulatory, Integrative & Comparative Physiology 48(1): R255.

Cannon B. and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. Physiological Reviews 84(1): 277-359.

-----. 2011. Nonshivering thermogenesis and its adequate measurement in metabolic studies. The Journal of Experimental Biology 214(2): 242-253.

Carey H. V., M. T. Andrews and S. L. Martin. 2003. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiological Reviews 83(4): 1153-1181.

Carl E. A. 1971. Population control in arctic ground squirrels. Ecology 52(3): 395 413.

Davis D. E. 1976. Hibernation and circannual rhythms of food consumption in marmots and ground squirrels. Quarterly Review of Biology: 477-514.

Ferron J. 1996. How do woodchucks (*Marmota monax*) cope with harsh winter conditions? Journal of Mammalogy: 412-416.

Florant G. and H. Heller. 1977. CNS regulation of body temperature in euthermic and hibernating marmots (*Marmota flaviventris*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 232(5): R203-R208.

Florant, G. L., V. Hill and M. D. Ogilvie (2000). Circadian rhythms of body temperature in laboratory and field marmots (*Marmota flaviventris*). Life in the Cold: Eleventh International Hibernation Symposium. Springer, Berlin.

Garland T. and S. C. Adolph. 1994. "Why not to do two-species comparative studies: limitations on inferring adaptation." Physiological Zoology: 797-828.

Geiser F. 2004. Metabolic rate and body temperature reduction during hibernation and daily torpor. Annual Review of Physiology 66(1): 239-274.

Geiser F. and R. Baudinette. 1990. The relationship between body mass and rate of rewarming from hibernation and daily torpor in mammals. Journal of Experimental Biology 151(1): 349-359.

Geiser F., S. Hiebert and G. Kenagy. 1990. Torpor bout duration during the hibernation season of two sciurid rodents: interrelations with temperature and metabolism. Physiological Zoology: 489-503.

Geiser F. and G. Kenagy. 1988. Torpor duration in relation to temperature and metabolism in hibernating ground squirrels. Physiological Zoology: 442-449.

Geiser F. and T. Ruf. 1995. Hibernation versus daily torpor in mammals and birds: physiological variables and classification of torpor patterns. Physiological Zoology 68(6): 935-966.

Haldane J. S. and J. I. Graham. 1912. Methods of air analysis. Griffin and Co. Ltd. J.B. Lippincott Co. Philidelphia, PA, USA.

Hammel H., T. Dawson, R. Abrams and H. Andersen. 1968. Total calorimetric measurements on *Citellus lateralis* in hibernation. Physiological Zoology: 341-357.

Healy J. E., K. A. Burdett, C. L. Buck and G. L. Florant. 2012. Sex differences in torpor patterns during natural hibernation in golden-mantled ground squirrels (*Callospermophilus lateralis*). Journal of Mammalogy 93(3): 751-758.

Heller H. C. and H. T. Hammel. 1972. CNS control of body temperature during hibernation. Comparative Biochemistry and Physiology Part A: Physiology 41(2): 349-359.

Howell A. H. 1938. Revision of the North American ground squirrels, with a classification of the North American Sciuridae. North American Fauna: 1-256.

Iwen F. 1999. Arctic ground squirrel, *Spermophilus parryii*. Washington, D.C., Smithsonian Institution Press.

Karpovich S. A., Ø. Tøien, C. L. Buck and B. M. Barnes. 2009. Energetics of arousal episodes in hibernating arctic ground squirrels. Journal of Comparative Physiology B: Biochemical, Systemic & Environmental Physiology 179(6): 691-700.

Kenagy G. J., S. M. Sharbaugh and K. A. Nagy. 1989. Annual cycle of energy and time expenditure in a golden-mantled ground squirrel population. Oecologia 78(2): 269-282.

Kisser B. and H. T. Goodwin. 2012. Hibernation and overwinter body temperatures in free-ranging thirteen-lined ground squirrels, *Ictidomys tridecemlineatus*. American Midland Naturalist 167(2): 396-409.

Kitao N. and M. Hashimoto 2012. Increased thermogenic capacity of brown adipose tissue under low temperature and its contribution to arousal from hibernation in Syrian hamsters. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 302(1): R118-R125.

Lee T. B. Barnes and C. Buck. 2009. Body temperature patterns during hibernation in a free-living Alaska marmot (*Marmota browerii*). Ethology Ecology & Evolution 21(3-4): 403-413.

Lyman, C. P. 1958. Oxygen consumption, body temperature and heart rate of woodchucks entering hibernation. American Journal of Physiology--Legacy Content 194(1): 83-91.

McKechnie A. E. 2014. The ecology and evolution of mammalian heterothermy in a changing world. Journal of Zoology 292(2): 71-73.

Michener G. R. 1992. Sexual differences in over-winter torpor patterns of Richardson ground-squirrels in natural hibernacula. Oecologia 89(3): 397-406.

Mills S. H. and F. E. South. 1972. Central regulation of temperature in hibernation and normothermia. Cryobiology 9(5): 393-403.

Scholander P., R. Hock, V. Walters and L. Irving. 1950. Adaptation to cold in arctic and tropical mammals and birds in relation to body temperature, insulation, and basal metabolic rate. The Biological Bulletin 99(2): 259-271.

Sheriff M. J., R. W. Fridinger, Ø. Tøien, B. M. Barnes and C. L. Buck. 2013. Metabolic rate and prehibernation fattening in free-living arctic ground squirrels. Physiological and Biochemical Zoology 86(5): 515-527.

Snapp B. D. and H. C. Heller. 1981. Suppression of metabolism during hibernation in ground squirrels (*Citellus lateralis*). Physiological Zoology: 297-307.

Snyder G. K. and J. R. Nestler. 1990. Relationships between body temperature, thermal conductance, Q_{10} and energy metabolism during daily torpor and hibernation in rodents. J Comp Physiol B 159(6): 667-675.

Storey, K. B. 2000. Turning down the fires of life: metabolic regulation of hibernation and estivation. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology 126: S90-S90.

Tøien, Ø. 2013. Automated open flow respirometry in continuous and long-term measurements: design and principles. Innovative Methodology 114: 1094-1107.

Tøien, Ø., K. L. Drew, M. L. Chao and M. E. Rice. 2001. Ascorbate dynamics and oxygen consumption during arousal from hibernation in Arctic ground squirrels. American Journal of Physiology: Regulatory, Integrative & Comparative Physiology 50(2): R572.

Twente, J. W. and J. Twente. 1978. Autonomic regulation of hibernation by *Citellus* and *Eptesicus*. Strategies in Cold: Natural Torpidity and Thermogenesis. L.C.H. Wang and J.W. Hudson. New York, Academic Press: 327-373.

Wang, L. C. 1978. Energetic and field aspects of mammalian torpor: the Richardson's ground squirrel. Journal of Thermal Biology 3(2): 87.

Wang, L. C. (1979). "Time patterns and metabolic rates of natural torpor in the Richardson's ground squirrel." Canadian Journal of Zoology 57(1): 149-155.

Williams, C. T., B. M. Barnes and C. L. Buck. 2012. Daily body temperature rhythms persist under the midnight sun but are absent during hibernation in free-living arctic ground squirrels. Biology Letters 8(1): 31-34.

Wit L. C. and J. W. Twente. 1983. Metabolic responses of hibernating golden-mantled ground squirrels *Citellus lateralis* to lowered environmental temperatures. Comparative Biochemistry and Physiology Part A: Physiology 74(4): 823-827.

Withers P. C., T. M. Casey and K. K. Casey. 1979. Allometry of respiratory and haematological parameters of arctic mammal. Comparative Biochemistry and Physiology Part A: Physiology 64(3): 343-350.

Young P. 1990. Hibernating patterns of free-ranging Columbian ground squirrels. Oecologia 83(4): 504-511.

Appendix 1.1 Co-Author Consent

I hereby give my approval for this manuscript [Richter, M. M., C. T. Williams, T. N. Lee, Ø. Tøien, G. L. Florant, B. M. Barnes and C. L. Buck (2015). "Thermogenic Capacity at Subzero Temperatures: How Low Can a Hibernator Go?" Physiological and Biochemical Zoology 88(1): 81-89] to be included in the dissertation of Melanie M. Richter.

Cory Williams

Cory Williams

Digitally signed by Cory Williams
DN: cn=Cory Williams, o=University of Alaska Anchorage, ou=Dept. of Biological Sciences, email=cwilliams@uaa.alaska.edu, c=US
Date: 2015.06.00 16:05:11 -0700'

Trixie Lee

Trixie Lee

Digitally signed by Trixie Lee
DN: cn=Trixie Lee, o=Harding University, ou=Biology Department, email=tnlee@harding.edu, c=US
Date: 2015.06.22 11:59:46 -05'00'

Greg Florant

Gregory L Florant

Digitally signed by Gregory L. Florant
DN: cn=Gregory L. Florant, o=Colorado State University, ou=Biology Dept, email=Florant@Colostate.edu, c=US
Date: 2015.09.10 14:15:07 -06'00'

Øivind Tøien

Øivind Tøien

Digitally signed by Øivind Tøien
DN: cn=Øivind Tøien, o=University of Alaska Fairbanks, ou=Institute of Arctic Biology, email=otoien@alaska.edu, c=US
Date: 2015.09.10 14:45:59 -08'00'

Appendix 1.2 IACUC Approval

University of Alaska Anchorage

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ASSURANCE OF ANIMAL CARE FORM

Office Use Only:

IACUC PROTOCOL NO 2009Buck3 USDA PAIN CLASSIFICATION: C (D) E (CIRCLE ONE)

DATE RECEIVED: 7-6-2009 APPROVAL DATE: 7-23-2009

RENEWAL MONTH: July FIRST ANNUAL RENEWAL DATE: 7-23-2010

SECOND ANNUAL RENEWAL DATE: 7-23-2011

Title of Project/Course (Include Course Number): Hibernation at Extreme Low Temperatures

☒ NEW SUBMISSION
☐ THREE YEAR MANDATORY RE-WRITE
☐ MAJOR MODIFICATION TO EXISTING PROTOCOL #

Approx. Starting Date: 8/09 Completion Date: 08/10 ☐ Ongoing

Name of Funding Source NSF OPP IPY Grant Deadline (if applicable):

I. DECLARATION: The information on this Assurance of Animal Care Form is an accurate description of my animal care and use protocol(s). All people using animals have been properly trained to use appropriate methods and have read and agree to comply with this protocol. All individuals working under this Assurance will comply with the procedures and methods outlined in NIH Guide for the Care and Use of Laboratory Animals, as well as PHS Policy, The Animal Welfare Act, and applicable University Policies. All field research will be carried out in accordance with the principles outlined in Acceptable Field Methods of Mammalogy, Guidelines for the Use of Wild Birds in Research, Guidelines for the Use of Fishes in Field Research, and/or Guidelines for the Use of Live Amphibians and Reptiles in Field Research. All use of animals in agricultural research or teaching will comply with the procedures and methods outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. All work proposed herein is the most refined possible to avoid or minimize discomfort, distress, and pain to the animals; does not unnecessarily duplicate previous experiment; and non-animal alternatives have been considered.

C. Loren Buck Digitally signed by C. Loren Buck DN: cn=C. Loren Buck, o=US, o=UAA, ou=Dept of Biology, email=loren@uaa.alaska.edu Date: 2009.07.23 13:02:08 -08'00'

Principal Investigator or Course Director Date

II. APPROVAL:

Final Approval - Chairperson, Institutional Animal Care and Use Committee

Eric S. Murphy Date 7-23-2009

1

Chapter 2 THE INFLUENCE OF ANDROGENS ON HIBERNATION PHENOLOGY OF FREE-LIVING MALE ARCTIC GROUND SQUIRRELS[1]

Abstract

Many free-living ground squirrel species are sexually dimorphic in hibernation phenology. The underlying causes of these differences are not yet known. Androgens, testosterone (T) in particular, inhibit hibernation. To determine the influence of endogenous androgens on annual timing of hibernation and aggression, we first measured circulating levels of T and dehydroepiandrosterone (DHEA), an adrenal androgen implicated in non-mating season aggression in other species, in free-living male arctic ground squirrels (*Urocitellus parryii*, AGS). We also manipulated endogenous androgen levels by surgical castration, and consequently compared body temperature records from intact (n = 24) and castrated (n = 9) males to elucidate the influence of endogenous androgens on annual body temperature rhythms. Unsurprisingly, the highest T levels (0.69 ± 0.20 ng/mL) were found among reproductively mature male AGS in spring, whereas, both immature males in spring and all males in late summer had T levels an order of magnitude lower (0.06 ± 0.00 and 0.07 ± 0.01 ng/mL, respectively). DHEA levels were higher in males during the late summer compared to reproductively mature males in spring (124.6 ± 20.8 and 40.6 ± 4.2 pg/mL, respectively). Eliminating gonadal androgens via castration resulted in males remaining heterothermic significantly later in spring (Julian date 112.1 ± 2.9) than reproductive males (87.1 ± 3.9) but did not change the timing of hibernation onset (castrate: 284.8 ± 1.0 vs. intact: 276.2 ± 3.1). We conclude that while androgens play a significant role in spring hibernation phenology of males, their role in fall hibernation onset is unclear.

Key words: hibernation phenology; androgen; arctic ground squirrel

[1] Prepared for submission to Hormones and Behavior as M.M. Richter, B.M. Barnes, K.M. O-Reilly, A.M. Fenn, C.L. Buck. The influence of androgens on hibernation phenology of free-living male arctic ground squirrels.

Introduction

Arctic ground squirrels (*Urocitellus parryii*, AGS), the northernmost hibernator, experience the most extreme winter conditions and have only a very short season in which to mate, rear offspring, and prepare for the following winter (Buck and Barnes 1999b, Carl 1971). In early spring in preparation for the mating season, adult males end heterothermy (period of hibernation when animals undergo torpor-arousal cycles) and remain sequestered below ground (Williams et al. 2012b). This pre-emergent period is characterized by 10-27 days of euthermic but arrhythmic body temperature (T_b; Williams et al. 2012a) and is required for testicular recrudescence and spermatogenesis (Barnes et al. 1987). During this pre-emergent euthermic period males draw from a food cache to regain lost body mass (Buck and Barnes 1999a). In addition to the males that engage in the mating season, a varying proportion forgo mating opportunities, continue hibernating through the mating season (Bronson 1979; Buck and Barnes 1999b; Schwagmeyer and Brown 1983; Sheriff et al. 2011; Slade and Balph 1974), and neither undergo testicular maturation nor have a significant pre-emergent euthermic period (Williams et al. 2012b). Unlike the differences found in timing of spring emergence, timing of entrance into hibernation is nearly synchronous among males (Sheriff et al. 2011). Female AGS begin hibernation significantly earlier, emerge from hibernation significantly later than reproductive males, and exhibit no pre-emergent euthermic period (Buck and Barnes 1999a; Sheriff et al. 2011; Williams et al. 2012b). The modulator(s) of sex differences in hibernation phenology is as yet unknown.

In the early spring, male AGS have high levels of circulating androgens (Boonstra et al. 2001; Buck and Barnes 2003) coincident with reproductive development and behaviors, male-male aggression and mate guarding (Carl 1971; Buck and Barnes 2003). At the conclusion of the breeding season, testes atrophy (Barnes and York 1990; Buck and Barnes 1999a), mating season aggression ends and circulating androgen levels decline (Buck and Barnes 2003). In the late summer, males accumulate a cache to be utilized in the following spring; this cache, and the surrounding territory, is defended against other males in the weeks prior to the start of hibernation (Carl 1971; Buck and Barnes 1999a; Buck and Barnes, 2003). The hormonal correlate of this second period of male-male aggression, associated with the defense of territory and caches, is not known.

In other animal species, non-mating season aggression can be influenced by an adrenal androgen, dehydroepiandrosterone (DHEA). Among many song birds, red squirrels (*Tamiasciurus hudsonicus*), and dwarf hamsters (*Phodopus sungorus*), non-mating season aggression coincides with increased concentrations of circulating DHEA, rather than testosterone (T) (Soma et al. 2008 (a review); Boonstra et al. 2008; Scotti et al. 2008). It is possible that male AGS may utilize the same non-testicular androgen during the late summer, when their testes are regressed and quiescent (Barnes and York 1990) and that its actions may contribute to the late hibernation onset observed in males compared to females.

The differences in hibernation patterns observed between reproductively mature males, immature males, and females combined with the known inhibitory effects of T on hibernation (Darrow et al. 1988; Goldman et al. 1986; Lee et al. 1990; Smit-Vis 1972; Vitale et al. 1985) strongly imply an important phenological role for endogenous androgens. To test this role of androgens we castrated males, to remove the main source of endogenous T, and compared T_b patterns between intact and manipulated males. We also measured plasma T and DHEA concentrations in samples collected from intact males across the active season and correlated the two discrete periods of male-male aggression with increased androgens, T in the spring, and DHEA in the late summer.

Methods and Materials

Animals and Blood Sampling:

Free-living adult and juvenile AGS were live-trapped using Tomahawk traps (Tomahawk Live Trap, Tomahawk, WI, USA) baited with carrot north of the Brooks Range, AK near the Atigun River (68°27'N, 149°21'W, elevation 812m). Animals were trapped during the active season (April to September) between 2008 and 2013 and transported by truck to the nearby Toolik Field Station where they were anesthetized by a 3-5 min exposure to isoflurane vapors. On first capture, animals were uniquely tagged (Monel no. 1 ear tags, National Brand & Tag Company, New Port, KY, USA and AVID MUSICC passive integrated transponder [PIT] tags, Norco, CA, USA), weighed, and assessed for sex. Males were scored for reproductive state (spring captures only, score of 0-3; 0: no palpable testes; 3: fully enlarged, scrotal testes), and

sampled for blood (1-2 mL) via cardiac puncture. The blood sample was immediately added to vials containing EDTA, centrifuged, the plasma drawn off and frozen at -80°C until time of assay. Animals were held in their traps overnight in the laboratory, provided rodent chow ad libitum (Mazuri Rodent Chow, Brentwood, MO, USA) and fresh carrots, and released at site of capture the following morning.

Hormone Assays:

Plasma T was measured in randomized batches between 2010 and 2012. Samples were analyzed in duplicate using a commercially available RIA kit (ImmunoChem™ Double Antibody RIA cat. No. 07189102; MP Biomedicals, Santa Ana, CA, USA). The antibody used in this kit is highly specific (highest cross-reactivity with 5α-dihydrotestosterone at 3.40%) and the assay has a reported sensitivity of 0.03 ng/mL. We followed the manufacturer's recommended protocol with the following modifications: all volumes were halved and we added an additional standard at 0.05 ng/mL by diluting the 0.1 ng/mL 1:1 with the provided steroid diluent. Using this protocol provided inter- and intra-assay coefficient of variations (CV's) of 13.6% and 14.1 ± 8.8%, respectively. We measured inter-assay CV's using a pooled sample run in each assay; we used results of this same pooled sample assayed at the beginning and end of each assay to calculate intra-assay CV's. The assay was validated with both an analysis of standard addition and a test of parallelism. Samples with measured levels of T below the lower detectable limit for this assay were assumed at the level of detectability (0.05 ng/mL; 241 from intact males, 31 from castrated males, 43 from females). Two out of the 419 samples from intact males were determined to be statistical outliers, based on a box-plot (higher than the upper quartile + 3*inner quartile range), and therefore omitted from analysis and presentation; none of the 44 samples collected from female animals were considered outliers.

Plasma DHEA was measured in randomized batches in 2013. We measured the active form of DHEA instead of its inactive form, DHEA-S. 100μL samples were assayed in duplicate using a commercially available RIA kit (DSL8900, BeckmanCoulter, Pasadena, CA, USA). The antibody used is highly specific to DHEA with extremely low cross-reactivity (highest with isoandrosterone at 0.733%) and the sensitivity of the assay, as stated by the manufacturer, is 9.0 pg/mL. The manufacturer's protocol was used with the following modification: standards were created at 26 pg/mL, 53 pg/mL, 105 pg/mL, 210 pg/mL, 325 pg/mL, 650 pg/mL and 1650 pg/mL

by diluting the provided calibrators with 0 ng/mL calibrator. Inter-assay CV, measured using a single pooled sample run in each assay, was 3.6%, and intra-assay CV, calculated using the pooled sample assayed at the start compared to end of each assay, was 15.7 ± 7.8%. Prior to analysis, samples were extracted using methylene chloride, dried under nitrogen gas, and re-suspended in phosphate-buffered saline with glucose. Other extraction methods using other solvents were tested, but this method gave the most reproducible results. Extraction efficiencies were calculated for each sample and resulted in a mean extraction efficiency of 33.1 ± 0.01%. However, values are presented without correction since there was little variation in extraction efficiencies. These methods were validated for use in this species using both an analysis of standard addition and a test of parallelism. Samples found to be below the lower detectable limit for this assay (25.5 pg/mL; 62 from intact males, 3 from castrates) were listed at that lower limit for presentation and analysis. One sample out of the 224 analyzed was determined to be a statistical outlier, based on a box-plot, and therefore omitted from analysis and presentation.

Body temperature (T_b) measurement:

One of three different temperature loggers (modified TidBit Stowaway model TBICU32-05+44, (14g, ±0.3°C), Onset Computer Corp, Bourne MA, USA; iButton DS1922L and DS1921G (both 3g, ±0.5°C), Maxim Integrated, San Jose, CA, USA), programmed to recorded T_b every 20-120 min (interval dependent on type of logger), was implanted in animals (total of 24 males, 17 females) for semi-continuous measurement of T_b and subsequent analysis of hibernation phenology. Prior to implantation, loggers were calibrated, coated in Elvax (DuPont, Wilmington, DE, USA), and gas-sterilized. Briefly, animals were anesthetized using isoflurane, then under aseptic conditions, a 3-5 cm incision was made along the animal's midline through the linea alba and the logger was placed inside the peritoneal cavity. The muscle and subcutaneous layers were closed using chromic gut and polydiozanone sutures, respectively, and the skin subsequently glued (Vetbond, 3M, St. Paul, MN, USA). After surgery animals were returned to their traps and provided with fresh carrots and rodent chow, and held overnight in the laboratory before being released at site of capture the following morning. Another group of 14 males underwent all the procedures described above, and, additionally, were castrated. Of the manipulated animals, 9 castrates were recovered and their loggers downloaded. The animals

without recovered loggers were all recaptured at least once after surgery and may have been lost to predation or dispersal/loss of territory.

Characteristics of T_b regulation across hibernation were analyzed as described in Buck et al. 2008. Heterothermy start date was defined as first day T_b decreased below 30°C and heterothermy end date was defined as the last time T_b increased above 30°C. Mean torpor bout length was calculated for the duration that T_b was $\leq$ 30°C for each bout of torpor. The length of the pre-emergent euthermic period was determined as the duration prior to the resumption of strong diel rhythms in T_b indicative of aboveground activity (Williams et al. 2012b). We use hibernation to refer to the period that animals remain below ground, including the pre-emergent euthermic period, and heterothermy to refer to the period of time when animals are undergoing alternating cycles of torpor and arousal.

Behavioral Observations:

Five free-living, intact adult male AGS were observed within a 4.2 hectare grid during the 2008 active season. The grid was separated into 5 equal sections (0.84 hectares) with each section observed, weather permitting, every 5 days. Observations were made from a fixed location on the grid each day at a distance of 5-10 m from the animal of interest using binoculars (10X x 42). Behavioral observations were conducted from 09:30 – 17:00 from 4 July – 4 August, 2008. The four weeks of focal observations were pooled as follows: Week 1 = 4 – 12 July (Standardized day 106 – 114), Week 2 = 13 – 19 July (Standardized day 115 – 121), Week 3 = 20 – 26 July (Standardized day 122 – 128), and Week 4 = 27 July – 4 August (Standardized day 129 – 137). The weeks are unevenly divided due to inconsistencies with weather and the number of days animals were observed. When precipitation occurred for >50% of the day, animals showed very little activity (Williams et al. 2014a) and therefore these days were deemed inadequate for observation and omitted from analysis.

Behavior was tracked and recorded using a computer program (Behavior Tracker #158503, version 1.5). Behaviors monitored included foraging, aggressive interactions, grooming, alarm-calling, time below ground, and caching; however, only foraging, aggressive interactions and caching are reported here since these are the behaviors we hypothesize are under the influence of late summer androgens. Behaviors were analyzed for total time in addition to

the number of times the behavior took place. All behaviors were standardized by the total amount of time each animal was observed as this substantially varied from animal to animal (0.2 – 4.0 h per week). The ratio of time spent engaged in each behavior/total time observed is presented.

All procedures used were designed to minimize any pain and discomfort to the animals, are in accordance with NIH standards, and were approved by the University of Alaska Fairbanks' and Anchorage's Institutional Animal Care and Use Committees (Protocol #'s: 340270-33, 148893-1, 130316-31, 160426-4).

Statistical Analysis:

All data presented are means ± SEM, unless otherwise noted. Comparisons were considered significant when $p < 0.05$. Timing of blood sampling, and thus hormone concentrations reported, are standardized to the mean date of the end of heterothermy of reproductively competent males in the year the sample was collected. This method accounts for year to year variations in circannual timing such that Standardized Date 1 is the mean date reproductively mature males ended heterothermy and Standardized Date 50 would be fifty days after. Each hormone was analyzed separately and grouped into Spring (Standardized Date 1 to 50), and Late Summer (Standardized Date 100 to 200) sampling bins. Hormone data was analyzed using a Kruskal-Wallis One Way Analysis of Variance (ANOVA) on ranks with a Dunn's pairwise comparison and a linear regression in SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). Behavior data and associated DHEA values were analyzed using linear regressions; prior to analysis, percent foraging data underwent an arc-sin transformation. To decrease the pseudo-replication associated due to some repeated measures, multiple samples in the same bin from the same individual were analyzed as a mean instead of individually. Characteristics of hibernation phenology were analyzed for differences using a one-way ANOVA with LSD multiple comparisons correction using SPSS Statistics 17.0 (IBM Armonk, NY, USA).

Results

Plasma androgen levels across the active season fluctuated as predicted and are presented as Figures 2.1 and 2.2. In the spring, reproductively mature males exhibited the highest levels of

plasma T of all groups (0.6 ± 0.09 ng/mL, $p < 0.05$). Plasma DHEA levels were highest in the late summer, but only significantly higher than those of reproductively mature spring males (171.9 ± 97.1 ng/mL and 42.9 ± 3.3 pg/mL, respectively, $p < 0.05$). Using samples analyzed for both androgens and linear regression analysis we found no relationship between T and DHEA levels across the entire active season (n= 66, $p = 0.481$), in the spring (n = 6, $p = 0.349$), or in the late summer/fall (n = 57, $p = 0.672$). Females were found to have T levels below the detectable limit across the entire active season (< 0.05 ng/mL, n = 44; data not shown).

The results of the castrations are presented in Table 2.1. The start and end date of heterothermy, duration of hibernation, number of torpor bouts and mean torpor bout length were equivalent in castrates and intact, reproductively immature males. The castrates only differed from intact, reproductively immature males in the duration of the penultimate arousal bout, which was significantly longer in castrates than any other group (75.8 ± 11.4 h, $p \leq 0.033$). Castrated males had a pre-emergent euthermic period of 6.7 ± 2.4 days, which was intermediate between that of intact, reproductively immature males (3.2 ± 2.3 days) and reproductively mature males, which had significantly longer pre-emergent euthermic periods than any other group (16.6 ± 2.1 days, $p \leq 0.023$). Castrates (112.1 ± 2.9), intact, reproductively immature males (103.3 ± 6.0), and females (107.2 ± 1.4) all ended heterothermy on similar dates and significantly later than reproductively competent males (174.8 ± 4.5, $p \leq 0.001$).

The results of a month-long observational period are summarized in Fig 2.3. We observed a progressive decline in the percentage of time males spent foraging (74.5 ± 13% in Week 1 to 65.2 ± 7.2% in Week 4, however, this did not reach significance ($r^2 = 0.0784$, $p = 0.294$), possibly due to low power (0.178), Fig 2.3a). Males also increased both the observed mean number of aggressive encounters per hour (Week 1: 8, Week 4: 26, $r^2 = 0.265$, $p = 0.041$; Fig 2.3b) and incidences of caching per hour (Week 1: 0, Week 4: 12, $r^2 = 0.399$, $p = 0.007$; Fig 2.3b) during this relatively short period in the late summer. In addition to these measured changes in behaviors, the authors, and other researchers (Carl 1971; Buck and Barnes 2003), have observed that both the number and intensity of aggressive encounters and instances of caching continue after the period for which measurements were recorded. Though the correlation is not tightly linked in our data, due to the short period of behavioral observations, the increased

instances of caching and aggression occur as levels of DHEA begin to increase (one-way ANOVA, $F(6, 123) = 3.51$; $p = 0.027$).

Discussion

In multiple species of ground squirrels, adult males end heterothermy and emerge earlier than females in the spring (reviews: Michener 1984, Williams et al. 2014b); however, the endocrine mechanisms of sexual differences in hibernation phenology are unknown. We propose that the differences may be driven, at least in part, by circulating androgens. The objective of this study was to determine the role of endogenous androgens in annual timing of hibernation in free-living male AGS. We monitored the active season cycles of plasma T and DHEA using RIAs. We established that T levels are highest among reproductive males in the early spring, during the mating season (0.6 ± 0.09 ng/mL), and then quickly decline to baseline levels once the mating season ends (females pregnant by 7 May (Standardized Date 40); Sheriff et al., 2011) and the testes regress. Males maintain low concentrations of T (0.07 ± 0.01 ng/mL) for the remainder of the active season (Fig 2.1 and 2.2). We found that plasma DHEA levels remain low throughout the active season until late summer/fall when males display a second aggressive period (Buck and Barnes 2003) that coincides with an increase in DHEA levels (Fig 2.1 and 2.2). Using castration to remove the significant source of endogenous T levels, we established a link between T and the early end of the heterothermic season in male AGS (Table 2.1). The similarity between the timing of hibernation onset in the fall in intact and castrated male AGS suggests that gonadal androgens do not play a role in prolonging euthermy in males.

There are numerous functional implications of increased T in reproductively viable males in the spring. The most obvious function of high T in the spring is to initiate reproductive development and spermatogenesis in those males that undergo testicular maturation. Another closely related function of high spring T is to support the behaviors and territoriality associated with the male-male aggression observed in male AGS during the spring (Buck and Barnes 2003). The data presented here are unique in that they show that the specific androgen responsible for these behaviors is gonadally derived T (Fig 2.1, Table 2.1). Additionally, a hypothesized function for spring T is to end heterothermy in males undergoing reproductive development (Barnes 1996), since T inhibits hibernation (Lee et al. 1990). Support for this hypothesis comes

from the increased circulating T and follicle stimulating hormone found in males just prior to the cessation of torpor-arousal cycles (Barnes et al. 1988) and spring-gonadectomized males re-entering torpor-arousal cycles (Dark et al. 1996). The data presented here also support this argument, as castrated males end heterothermy and emerge significantly later in the spring compared to males that undergo reproductive development (Table 2.1).

The elevated levels of circulating DHEA found in late summer animals occurs over a longer period of time than the T peak found in reproductive spring males (Fig 2.1 and 2.2). We postulate two functions of this late summer androgen surge. We hypothesize that one possible action of the increased circulating DHEA is to prevent males from entering torpor at an earlier time point, when females immerge (Table 2.1). A second action of increased late summer DHEA is to facilitate the aggression observed during the caching period, prior to the onset of hibernation (Fig 2.3; Carl 1971; Buck and Barnes 2003). Non-mating season aggression has been linked to increased levels of DHEA in a number of other species, both mammalian and avian (red squirrels (*Tamiasciurus hudsonicus*): Boonstra et al. 2008; dwarf hamsters (*Phodopus sungoras*): Scotti et al. 2008; review: Soma et al. 2008, 2015), which supports this possible function. From observations made in the field it appears that from the beginning of July through August, when the behavioral study ended, male AGS tend to spend a decreasing proportion of their time above ground foraging (from 75.5 ± 13.5% of their time foraging in the first week of July to 65.2 ± 7.2% of their time foraging the first week of August). During this same period we also observed an increase in the number of aggressive interactions (from 8 to 26), and instances of caching (from 0 to 12). Previous work in this population has determined that during this same time period free-living males are still depositing fat, but have already recovered their lean mass (Sheriff et al. 2013).

Boonstra et al. (2011; 2014) have proposed an anabolic function and need of circulating androgens in AGS in late summer. They propose that elevated androgens and the localization of androgen receptors enable AGS to increase their lean mass in preparation for hibernation. This increased lean mass is subsequently drawn upon to support the mixed fuel metabolism of thermogenesis during hibernation (Buck and Barnes, 2000). Although compelling, in our population of free-living AGS, we find a temporal mismatch between the period of lean mass accretion (Sheriff et al. 2013) and elevated late summer DHEA concentration (Fig 2.1 and 2.2).

Free-living AGS in this population complete lean mass growth by 12 August (Sheriff et al. 2013), well before the rise in DHEA, but after the high levels of T found in spring. Thus, in Arctic Alaska, the late summer increase in DHEA levels likely do not significantly facilitate anabolism, but rather functions to influence increased rates of aggression and caching behavior among males observed late in the active season. Further, the function of elevated late season DHEA concentrations observed in males could be to delay the onset of hibernation as compared to females in the population.

Conclusions

In summary, we were able to outline the cycles of two androgens, T and DHEA, in free-living male AGS (Fig 2.1 and 2.2). We were also able to show that changing levels of these two hormones coincide with possible trends in changing behaviors such as caching and aggression (Buck and Barnes 2003, Fig 2.1, 2.2, 2.3), as well as to spring hibernation phenology (Table 2.1). Castrated male AGS displayed similar hibernation characteristics to reproductively immature males, demonstrating the important role endogenous androgens, namely T, play in influencing the timing of heterothermy end and pre-emergent euthermy, but not the timing of hibernation onset (Table 2.1). Further work is required to determine what role, if any, androgens play in influencing the timing of hibernation onset.

Acknowledgements

We would like to thank the staff of Toolik Field Station for their support during our years spent collecting the data for this study. We would also like to thank F. Kohl, C.T. Williams, M.J. Sheriff, T.N. Lee, T. Stevenson and B. Salli for their assistance in capturing animals in the field. This work was supported by funding from the US Army Medical Research and Materiel Command to B.M.B (05178001). Additional support was provided by the National Science Foundation to C.L.B. (EF-0732763 and IOS-1147187) and B.M.B (EF-0732755 and IOS-1147232). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. Additional support for the research reported in this publication was provided by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 5P20GM103395. The content

is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH.

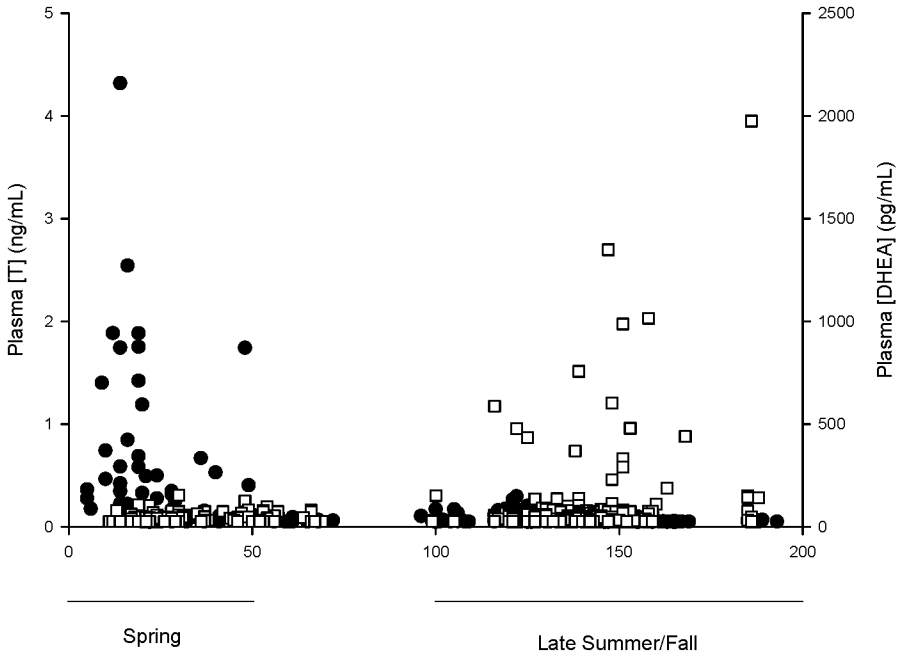


Figure 2.1: Scatter-plot of individual plasma samples collected from free-living male AGS from 2008 to 2013. Each point represents a single assayed sample. Individual animals are represented from one to ten times, depending on the number of times the animal was captured for subsequent sampling (total individuals for T = 143, for DHEA = 129). Closed circles represent plasma testosterone concentrations (ng/mL) and open squares represent plasma DHEA concentrations (pg/mL). All samples found to be below the lower detectable limit of the assay have been adjusted to that lower detectable limit. Sample dates have been standardized to the mean date of euthermy for reproductive males in the year the sample was collected. Total number of samples assayed for testosterone concentration is 406. Total number of samples assayed for DHEA concentration is 223.

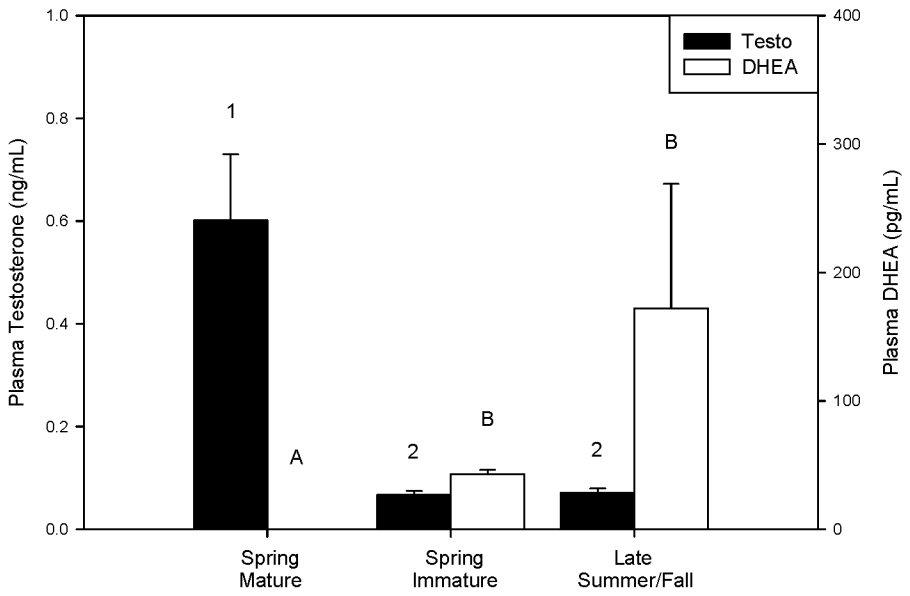


Figure 2.2: Seasonal and reproductive status effects on plasma androgen concentrations. Reproductive status was determined at time sample was collected. Numbers (T) and letters (DHEA) indicate statistical significance between groups ($p < 0.05$) as determined by a Kruskal-Wallis One Way Analysis of Variance. Values presented are means ± SEM. Number of samples per group for testosterone, from left to right: 91, 17, and 182. Number of samples per group for DHEA, from left to right: 57, 8, and 98.

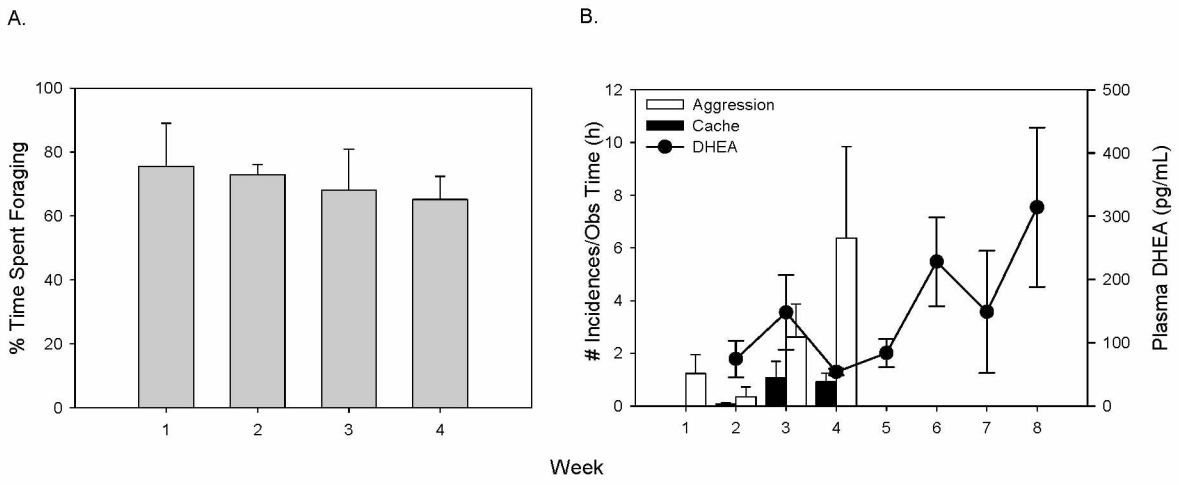


Figure 2.3: Summary of behavior observations of free-living male AGS. Left-hand panel displays the decreased percentage of time spent foraging over the course of the observational study. Right panel shows the increased incidences of observed caching and aggression per hour of observation. Week 1: 4 – 12 July (Standardized Date 106 – 114, n = 4), Week 2: 13 – 19 July (Standardized Date 115 – 121, n = 5), Week 3: 20 – 26 July (Standardized Date 122 – 128, n = 3), and Week 4: 27 July – 4 August (Standardized Date 129 – 137, n=4). The line presents plasma DHEA concentrations (means ± SEM; pg/mL) during and after the observational study (n = 0, 19, 9, 32, 35, 23, 10, 2).

Table 2.1: The results of the body temperature (T_b) data collected over three consecutive hibernation seasons (2009 to 2012), presented as means ± SEM. Heterothermy start date = first day T_b fell below 30°C and heterothermy end date = last time T_b increased above 30°C. Mean torpor bout length = time T_b remained ≤30°C. The length of the pre-emergent euthermic period = the time prior to the resumption of strong circadian T_b rhythms, as defined by Williams et al. 2012b. Data were analyzed using a one-way ANOVA with LSD multiple comparison correction. Letters indicate groups that are statistically different ($p < 0.05$).

	Heterothermy Start (Julian Date)	Heterothermy End (Julian Date)	Heterothermy Duration (Days)	Number of Torpor Bouts	Mean Torpor Bout Length (Days)	Penultimate Arousal Bout Length (Hours)	Length of Pre-Emergent Euthermic Period (Days)
Repro (n=18)	276.2 ± 3.1^{A}	87.1 ± 3.9^{A}	174.8 ± 4.5^{A}	8.8 ± 0.6^{A}	14.1 ± 0.7^{A}	39.0 ± 7.7^{A}	16.6 ± 2.1^{A}
Non-Repro (n=6)	270.2 ± 5.7^{A}	107.3 ± 6.0^{B}	201.2 ± 7.4^{B}	11.2 ± 0.9^{B}	16.3 ± 0.7^{B}	34.4 ± 9.7^{A}	$3.2 \pm 2.3^{B,C}$
Castrates (n=9)	284.8 ± 1.0^{A}	112.1 ± 2.9^{B}	191.3 ± 7.1^{B}	$10.7 \pm 0.8^{A,B}$	$14.9 \pm 0.7^{A,B}$	75.8 ± 11.4^{B}	6.7 ± 2.4^{C}
Adult Females (n=15)	230.0 ± 3.8^{B} (n=17)	107.2 ± 1.4^{B}	241.0 ± 3.5^{C}	14.5 ± 0.5^{C}	16.1 ± 0.5^{B}	27.2 ± 2.3^{A}	0.4 ± 0.2^{B}

Literature Cited

Barnes, B. M., P. Licht, P., Zucker, I., 1987. Temperature-dependence of invitro androgen production in testes from hibernating ground-squirrels, *Spermophilus lateralis*. Canadian Journal of Zoology 65(12): 3020-3023.

Barnes, B.M., 1996. Relationships between hibernation and reproduction in male ground squirrels. In F. Geiser, A. J. Hulbert & S. C. Nicol (Eds.), Adaptations to the Cold: Tenth International Hibernation Symposium. Armidale: University of New England Press.

Barnes, B. M., York, A.D., 1990. Effect of winter high temperatures on reproduction and circannual rhythms in hibernating ground squirrels. Journal of Biological Rhythms 5(2): 119-130.

Boonstra, R., A. J. Bradley, A.J., Delehanty, B., 2011. Preparing for hibernation in ground squirrels: Adrenal androgen production in summer linked to environmental severity in winter. Functional Ecology 25(6): 1348-1359.

Boonstra, R., Hubbs, A.H., Lacey, E.A., McColl, C.J., 2001. Seasonal changes in glucocorticoid and testosterone concentrations in free-living arctic ground squirrels from the boreal forest of the Yukon. Canadian Journal of Zoology 79(1): 49-58.

Boonstra, R., Lane, J.E., Boutin, S., Bradley, A., Desantis, L., Newman, A.E.M., Soma, K.K., 2008. Plasma DHEA levels in wild, territorial red squirrels: Seasonal variation and effect of ACTH. General and Comparative Endocrinology 158(1): 61-67.

Boonstra, R., Mo, K., Monks, D.A., 2014. Managing anabolic steroids in pre-hibernating arctic ground squirrels: obtaining their benefits and avoiding their costs. Biology letters 10(11): 20140734.

Bronson, M. T. 1979. Altitudinal variation in the life history of the golden-mantled ground squirrel (*Spermophilus lateralis*). Ecology 60(2): 272-279.

Buck, C., Breton, A., Kohl, F., Tøien, Ø., Barnes, B., Lovegrove, B.,. McKechnie, A., 2008. Overwinter body temperature patterns in free-living Arctic squirrels (*Spermophilus parryii*). Hypometabolism in Animals: Torpor Hibernation and Cryobiology: 317-326.

Buck, C.L., Barnes, B.M., 1999a. Annual cycle of body composition and hibernation in free living arctic ground squirrels. Journal of Mammal. 80(2): 430-442.

Buck, C.L., Barnes, B.M., 1999b. Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter. Journal of Mammal. 80(4): 1264-1276.

Buck, C. L. and B. M. Barnes (2000). "Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator." American Journal of Physiology: Regulatory, Integrative & Comparative Physiology 48(1): R255.

Buck, C.L., Barnes, B.M., 2003. Androgen in free-living arctic ground squirrels: seasonal changes and influence of staged male-male aggressive encounters. Hormones & Behavior 43(2): 318.

Carl, E.A., 1971. Population control in arctic ground squirrels. Ecology 52(3): 395-413.

Dark, J., Miller, D.R., Zucker, I. 1996. Gonadectomy in the spring reinstates hibernation in male golden-mantled ground squirrels. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 270(6), R1240-R1243.

Darrow, J.M., Duncan, M.J., Bartke, A., Bonagallo, A., Goldman B.D., 1988. Influence of photoperiod and gonadal-steroids on hibernation in the European hamster. J.of Comp. Phys. a-Sensory Neural and Behav. Phys. 163(3): 339-348.

Goldman, B.D., Darrow, J.M., Duncan, M.J., Yogev, L., 1986. Photoperiod, reproductive hormones, and winter torpor in three hamster species. Living in the Cold: Physiological and Biochemical Adaptations: 341-350.

Lee, T.M., Pelz, K., Licht, P., Zucker, I., 1990. Testosterone influences hibernation in golden mantled ground squirrels. American Journal of Physiology 259(4 Pt 2): R760-767.

Michener, G.R. 1984. Age, sex, and species differences in the annual cycles of ground-dwelling sciurids: implications for sociality. In J. Murie & G. R. Michener (Eds.), The Biology of Ground-dwelling Squirrels: Annual cycles, behavioral ecology, and sociality: 81-107.

Schwagmeyer, P.L., Brown, C., 1983. Factors affecting male-male competition in thirteen-lined ground squirrels. Behavioral Ecology and Sociobiology 13(1): 1-6.

Scotti, M.A.L., Belén, J., Jackson, J.E., Demas, G.E., 2008. The role of androgens in the mediation of seasonal territorial aggression in male Siberian hamsters (*Phodopus sungorus*). Physiology & Behavior 95(5): 633-640.

Sheriff, M.J., Fridinger, R.W., Tøien, Ø., Barnes, B.M., Buck, C.L., 2013. Metabolic rate and prehibernation fattening in free-living arctic ground squirrels. Physiological and Biochemical Zoology 86(5): 515-527.

Sheriff, M.J., Kenagy, G.J., Richter, M., Lee, T., Tøien, Ø., Kohl, F., Buck, C.L., Barnes, B.M., 2011. Phenological variation in annual timing of hibernation and breeding in nearby populations of arctic ground squirrels. Proceedings of the Royal Society B: Biological Sciences 278(1716): 2369-2375.

Slade, N.A., Balph, D.F., 1974. Population ecology of Uinta ground squirrels. Ecology: 989 1003.

Smit-Vis, J.H, 1972. The effect of pinealectomy and of testosterone administration on the occurrence of hibernation in adult male golden hamsters. Acta Morphol Neerl Scand 10(3): 269-282.

Soma, K.K, Rendon, N.M., Boonstra, R., Albers, H.E., & Demas, G.E. 2015. DHEA effects on brain and behavior: insights from comparative studies of aggression. The Journal of Steroid Biochemistry and Molecular Biology, 145, 261-272.

Soma, K.K., Scotti, M.A.L., Newman, A.E.M., Charlier, T.D., Demas, G.E., 2008. Novel mechanisms for neuroendocrine regulation of aggression. Frontiers in Neuroendocrinology 29(4): 476-489.

Vitale, P., Darrow, J., Duncan, M., Shustak C., Goldman, B., 1985. Effects of photoperiod, pinealectomy and castration on body weight and daily torpor in Djungarian hamsters (*Phodopus sungorus*). Journal of Endocrinology 106(3): 367-375.

Williams, C.T., Barnes, B.M., Buck, C.L., 2012a. Daily body temperature rhythms persist under the midnight sun but are absent during hibernation in free-living arctic ground squirrels. Biology Letters 8(1): 31-34.

Williams, C.T., Barnes, B.M., Richter, M., Buck, C.L., 2012b. Hibernation and circadian rhythms of body temperature in free-living arctic ground squirrels. Physiological & Biochemical Zoology 85(4): 397-404.

Williams, C.T., Wilsterman, K., Kelley, A.D., Breton, A.R., Stark, H., Humphries, M.M., McAdam, A.G., Barnes, B.M., Boutin, S., Buck, C.L., 2014a. Light loggers reveal weather-driven changes in the daily activity patterns of arboreal and semifossorial rodents. Journal of Mammalology 95(6): 1230-1239.

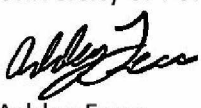
Williams, C.T., Barnes, B.M., Kenagy, G.J., Buck, C.L. 2014b. Phenology of hibernation and reproduction in ground squirrels: integration of environmental cues with endogenous programming. Journal of Zoology 292:112-124.

Appendix 2.1 Co-Author Consent

I hereby give my approval for this manuscript [M.M. Richter, B.M. Barnes, K.M., O'Reilly, A.M. Fenn, C.L. Buck. The influence of androgens on hibernation phenology of free-living male arctic ground squirrels. Prepared for submission to Hormones and Behavior] to be included in the dissertation of Melanie M. Richter.

Kathleen M. O'Reilly

University of Portland, June 9, 2015



Ashley Fenn

Appendix 2.2 IACUC Approval

UAA UNIVERSITY of ALASKA ANCHORAGE

University of Alaska Anchorage
Institutional Animal Care and Use Committee

Memo

To: Dr. C. Loren Buck

From: Eric S. Murphy, Chair, IACUC

Date: 7/3/2009

Re: IACUC protocol # 2009Buck 2

The Institutional Animal Care and Use Committee at the University of Alaska Anchorage met on 6/24/2009 and reviewed your protocol "The Role of Androgens in the Phenology of Hibernation," that you submitted to us on 6/10/2009. Upon receiving your revisions on 7/2/2009, I am pleased to approve your protocol. Please note that this approval is contingent upon your compliance with all relevant University, city, state, and federal regulations, and requires that you possess all relevant permits before work is initiated. Your protocol ID number is **2009Buck2**. Your approval is good for a period of 3 years, and will expire on **7/3/2012**. You are required to submit an annual report of your activities prior to 7/3 in each of the next two years. This form is available at http://www.uaa.alaska.edu/research/ric/iacuc/forms.cfm .

We remind you that all changes in personnel and animal handling protocols must be submitted to the committee prior to such changes taking place. In addition, should you experience any unexpected animal mortalities, illnesses, or injury (to animals or personnel involved with the project), you are required to report such to the IACUC immediately.

Thank you for your support of animal care guidelines. We hope that your research goes well.

Eric S. Murphy

Eric S. Murphy, Chair
UAA IACUC

3211 Providence Drive * Anchorage, AK 99508 * Phone: (907) 786-1626 * Fax: (907) 786-4898

(907) 474-7800
(907) 474-5993 fax
uaf-iacuc@alaska.edu
www.uaf.edu/iacuc

Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

February 24, 2015

To: Brian Barnes
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [130316-32] Extremes of Hibernation Physiology (Field)

The IACUC has reviewed the Progress Report by Administrative Review and the Protocol has been approved for an additional year.

Received:	February 20, 2015
Initial Approval Date:	April 1, 2009
Effective Date:	February 24, 2015
Expiration Date:	April 1, 2016

This action is included on the March 12, 2015 IACUC Agenda.

PI responsibilities:

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*

Chapter 3 DOES THE AVAILABILITY OF A FOOD CACHE AFFECT TIMING OF HIBERNATION AND SPRING REPRODUCTIVE STATUS IN MALE ARCTIC GROUND SQUIRRELS (UROCITELLUS PARRYII)?[1]

Abstract

It has previously been reported that a varying proportion of male ground squirrels forgo reproductive development their first spring. The factors that influence this variation have yet to be defined. We set out to determine whether food availability plays a role in timing of hibernation and reproductive development. We used three approaches: In the first experiment, 12 captive animals were allowed to establish a food cache in their cages in autumn; after the onset of hibernation, the caches were removed from half the animals. In the second experiment, captive animals were not allowed to cache in the fall; in the spring, prior to the end of hibernation, 8 were provided with ad libitum access to food, while the other 7 were kept on a restricted ration. In the third experiment, field-caught males were allowed to dig burrows and nest in outdoor dirt-filled enclosures; 7 of these were provided with cacheable food prior to hibernation and 7 were provided food that could not be cached. Animals in all three experiments were assessed for timing of hibernation end, testicular development, and plasma testosterone levels after hibernation. In the first experiment, all 12 animals underwent reproductive development; in the second experiment 6 of 8 males provided with ad libitum food in the spring, and 3 of 7 kept on a restricted ration, underwent reproductive development; in the third experiment 5 of 7 males that could cache underwent reproductive development, while only 2 of 7 that could not cache did. In all experiments, the animals that underwent reproductive development had higher levels of testosterone. While none of our experiments were conclusive as to the role food availability plays in male testicular development, they all suggest that building a cache in the fall improves the chances of spring development. One clear result we did find was that, much like what has been observed in free-living animals, males that undergo reproductive development hibernate for a shorter period of time, compared to their reproductively naïve cohorts under similar conditions.

[1] Prepared for submission to the *Canadian Journal of Zoology* as M.M. Richter, B.V. Gagliotti, C.L. Buck, B.M. Barnes. Does the availability of a food cache affect timing of hibernation and spring reproductive status in male arctic ground squirrels (*Urocitellus parryii*)?

Introduction

Arctic ground squirrels (*Urocitellus parryii*, AGS) are the farthest north hibernators under extensive study. The AGS habitat is characterized by continuous permafrost that constrains the depths at which animals can burrow thus ensuring their exposure to subzero temperatures for the majority of their hibernation season (Carl 1971, Buck and Barnes 1999b). This extreme environment has resulted in hibernating AGS displaying uncommon adaptations including the use of mixed fuel metabolism (Buck and Barnes 2003) to supply the necessary energy to maintain extremely high metabolic rates while torpid at sub-zero temperatures (Richter et al. 2015). In addition to the high over-winter energy demands of living in the Arctic, male AGS arouse and emerge into a snow covered environment with little to no food availability (Hock 1960). The short growing season severely constrains the timing and availability of food for the reproduction, growth, and fattening that are required prior to the onset of the next hibernation season. This constraint affects males leading into, and during, the spring mating season, which occurs prior to the start of the growing season (Hock 1960), and this constraint may lead to some males forgoing reproduction during some years.

Among ground squirrels, only a subset of yearling males sexually mature and develop descended, spermatogenic testes (Schwagmeyer and Brown 1983, Slade and Balph 1974, Bronson 1979) with as many as 25-60% of first year males delaying reproductive development until their second, or even third, spring (Bronson 1979; Buck and Barnes 1999a). This is different from females that almost all attempt to reproduce each year of their life (Bronson 1979, Michener 1983, Buck and Barnes 1999, Sheriff et al. 2011). The observed yearly variations in proportion of males undergoing reproductive development suggest that age at first reproduction in male ground squirrels is likely influenced by environmental and body conditions, and/or population structure.

Age at first reproductive maturation in male ground squirrels can be influenced by their energetic status prior to, and immediately following, hibernation (Holmes 1988). Manipulating food available at the start through the end of hibernation (Barnes 1984) or surgically removing fat deposits from (Forger et al., 1986) male golden-mantled ground squirrels (*Callosperomphilus saturatus* and *lateralis*, respectively) results in either a failure to undergo, or delay in,

reproductive development. Reproductive development can also be affected by quality of diet; male ground squirrels fed lower fat containing diets having less testosterone and smaller testes compared to those fed a high fat diet who exhibit accelerated reproductive development (Dark et al., 1992). The above studies hypothesized that smaller males with less fat at the end of hibernation do not reproductively mature because they lack the energy reserves to maintain high body temperature over the prolonged interval that is necessary for gonadal maturation.

We utilized a number of approaches to determine how the availability of a food cache, its continued presence during hibernation, and availability in spring affects the timing and pattern of hibernation and the spring reproductive status of male AGS. In males held captive in cages, we tested the hypotheses that (1) removing a cache during hibernation will result in males foregoing gonadal development and ending hibernation later in spring compared to males with intact caches; (2) males entering hibernation without cached food will undergo gonadal development if fed *ad libitum* after heterothermy ends; and (3) that males held in outdoor enclosures and fed food that could be cached in self-dug burrows would end heterothermy earlier and undergo gonadal development unlike males fed food that could not be cached. In these experiments we also measured seasonal changes in body mass and circulating levels of testosterone in spring as additional measures of spring reproductive condition.

Methods and Materials

Spring Food Availability

Fifteen juvenile male arctic ground squirrels (*Urocitellus parryii*, AGS) were caught in Tomahawk live-traps near Toolik Field Station on the North Slope of Alaska (68°27' N, 149°21' W, elevation 812 m) in late July and early August 2008. Animals were transported to the University of Alaska Fairbanks where they were housed in hanging wire cages (49x30.5x20.3 cm, Acme Metal, Statesville, NC, USA) for 6 weeks in a warm room (~22°C, photoperiod 12L:12D) and provided with cotton batting for nesting material (Perfect Fit, McDonald, Tukwila, WA, USA), *ad libitum* food (Mazuri Rodent Chow) and water. In September, animals were moved into an environmental chamber (2 ± 2°C, 8L:16D) and assigned at random to one of two groups: Group one, CACHE, n = 8, was provided with a 200g cache of rodent chow after at least one full bout of torpor. Group two, NO CACHE, n = 7, had all food removed from their cages after torpor was first observed. Animals were then allowed to hibernate without interruption.

Torpor bout length and the occurrence of arousal episodes were monitored daily using the sawdust method (wood shavings were placed on the back of torpid animals; if the shavings were absent on a subsequent day, the animal was presumed to have aroused and return to torpor since the previous check, Pengelley and Fisher 1961). In spring, males were considered to have ended hibernation if they remained active at high body temperature for four consecutive days. On the fourth day active, animals were briefly anesthetized using isoflurane vapors weighed, assessed for reproductive state (see below) and a 0.5 mL blood sample was collected via cardiac puncture. The cache of CACHE animals was weighed every 3-5 days and the mass recorded as an estimate of the food being consumed before the cache was replenished to 200g. NO CACHE animals were treated as above; however, they were provided with the minimum amount of food (~3-6g daily) required for them to retain their post-hibernation body mass (as determined on euthermia day 4), ± 10%. These procedures were repeated every 3-5 days for 20 days after hibernation had ended (total of 4 blood draws).

Cache vs. Cache Removed

Nine pregnant female AGS were caught in Tomahawk live-traps in April 2010 near Toolik Field Station on the North Slope of Alaska (68°27' N, 149°21' W, elevation 812 m) transferred to the University of Alaska Anchorage, and gave birth in captivity. Juvenile males (n=12) were weaned and housed individually in hanging wire cages (48x31x30 cm, custom made by UnifabCages, Kalamazoo, MI, USA). In July 2010, squirrels were moved into plastic tubs (38 x 56 x 20 cm, Nalgene, Rochester, NY, USA) and provided with pine shavings and cotton batting for nesting material, rodent chow, sunflower seeds and water *ad libitum*. Animals were allowed to cache the rodent chow and sunflower seeds in their cages. Food intentionally removed from the food dish was considered cached and not removed from the cage during cleanings. An exception was when the chow became wet/spoiled, in which case it was removed from the cage (this amount never exceeded 1% of the total cache at a time).

Animals were moved into an environmental chamber at an ambient temperature of 0°C ± 2°C and constant darkness in November. Animals were allowed to continue eating and caching until they spontaneously began hibernating. After animals had under-gone at least one complete bout of torpor (as determined by the sawdust method), they were separated into two treatment groups. Half of the animals (n = 6) were allowed to retain their cache of food (CACHE), while

the other half had all food removed from their cages while they were in torpor (CACHE REMOVED). Torpor bouts were monitored using the sawdust method. In spring, after four consecutive days of activity, animals were considered to have ended hibernation and removed from their cages, weighed, anesthetized with isoflurane, reproductive state evaluated, and a 0.8mL blood sample was collected via cardiac puncture. This process was repeated once weekly for 28 days. During this time, animals were housed in a warm room (22 ± 2°C). Animals from the cache removed group (CACHE REMOVED) were fed a minimal amount (~3-6 g) of rodent chow to maintain their post-hibernation weight ± 10%; animals in the CACHE group were allowed to feed from their caches during this time.

Semi-Natural Enclosures

Pre-Hibernation Procedures:

Fourteen male arctic ground squirrels were collected from the North Slope as juveniles in summer, 2008. Animals were implanted with gas sterilized body temperature loggers (15g, modified TidBit Stowaway model TBICU32-05+44, Onset Computer Corp.) programmed to record body temperature (± 0.2°C) every 20min (Long et al. 2007). Unpotted loggers were cased in plastic heat-shrink before coating with biologically inert plasticized wax (Elvax, DuPont, Wilmington, DE, USA) and calibrated at 0.0 and 40.0°C in a temperature controlled bath. Animals were anesthetized using isoflurane, a 3-4cm incision made along the midline, the logger was placed amid the abdominal fat, and the incision closed (*linea alba* using chromic gut, subcutaneous tissue with Dexon, and the skin with Prolene). This procedure was repeated to remove the loggers the following spring. After 10-15 days of recovery, animals were moved to outdoor, earth-filled enclosures beginning mid-July, 2009. Each enclosure housed one animal. Enclosures were 0.9 x 0.9 meters made of wire mesh (~2.5cm^2) that extended 1.8 meters underground to allow burrowing in a contained area (described in Barnes and Ritter 1993). Animals immediately began burrowing upon release and were provided hay for use as nest material. For the remainder of the 2009 active season, half of the animals were fed a diet that was cacheable, while half were fed the same diet, but in a non-cacheable form to test how the ability to cache food affected timing of hibernation and reproductive status the following spring. Each animal in the CACHE group (n = 7) was given 70 ± 25g of rodent chow and whole, shelled sunflower seeds daily. Each animal in the NO CACHE group (n = 7) was given equivalent

amounts of rodent chow and ground sunflower seeds, combined with water, creating a porridge consistency. Animals were weighed once every 7-10 days while active after being trapped in small Tomahawk traps (Tomahawk, WI, USA) using peanut butter as bait. An animal was deemed hibernating when they were not seen above ground for seven days.

Post-Hibernation Procedures:

Enclosures were viewed twice a week starting March, 15th 2010 to inspect for the first appearance through the snow of emerging animals. After emergence, the animals were trapped and moved into a shelter where they were weighed, anesthetized with isoflurane, reproductive state was evaluated, and a 0.8mL blood sample was collected via cardiac puncture every seven days for 35 days after first emergence. During the post-hibernation season all animals were fed ad libitum rodent chow and sunflower seeds.

Reproductive State

For all three experiments, reproductive state was determined visually and manually. Males were considered "non-reproductive" if they retained non-descended testes and a non-pigmented scrotum. "Reproductive" males included males that had testes that could be palpated easily in the abdomen, males that had scrotal testes that were easily palpated but did not fill the scrotum and a pigmented scrotum, as well as males with fully enlarged testes and a pigmented scrotum.

Plasma Testosterone

Blood samples (0.5 to 1.5mL) were collected into EDTA coated tubes. The blood was centrifuged to separate the plasma, which was stored at -80°C until assayed for testosterone content. Plasma was assayed using a commercially available RIA kit (ImmunoChem™ Double Antibody RIA cat. No. 07189102; MP Biomedicals, Santa Ana, CA, USA). The antibody used in this kit is highly specific (highest cross-reactivity with 5α-dihydrotestosterone at 3.40%) and has a sensitivity of 0.03ng/mL. Using the manufacturer recommended protocol we maintained interassay and intraassay coefficient of variances (CV's) of 13.6% and 14.1 ± 8.8%, respectively. We measured interassay CV's using a single pooled sample run in each assay. We measured intraassay CV's by comparing results of this same pooled sample assayed at the beginning and

end of each assay. This protocol was validated for use in this species using both an analysis of standard addition and a test of parallelism. Samples found to be below the lower detectable limit for this assay (0.05 ng/ml), were listed at that lower limit for presentation and analysis.

Statistics

All data presented are means ± SEM, unless otherwise noted. Differences between reproductive and non-reproductive ratios were analyzed using a Fisher's Exact Test. Differences between groups were determined using a Student's T-test. Testosterone data were analyzed using linear mixed models with repeated measures and Bonferroni corrections. For the model, plasma testosterone was the dependent variable, factors included reproductive status and experimental group and all inherent interactions, and animal ID was the random variable. Differences were considered significant when $p < 0.05$. All analyses were done in SPSS Statistics 17.0 (IBM Armonk, NY, USA).

Results

Spring Food Availability

Six of eight juvenile males hibernating indoors in the CACHE group, given *ad libitum* access to food after ending heterothermy, underwent reproductive development after ending hibernation, while three of seven animals fed a restricted ration (NO CACHE) became reproductive in the month after ending hibernation ($p = 0.315$). There was no significant difference in pre-hibernation body mass between animals in the NO CACHE and those in the CACHE group (780.8 ± 34.4g vs. 782.6 ± 24.9g respectively, $p = 0.97$) and no significant difference between groups in spring body mass (575.3 ± 46.5g vs. 654.1 ± 20.0g, $p = 0.13$). A comparison of the body masses of animals that became reproductive to those that did not was also not significant in pre-hibernation (803.6 ± 61.6g, 753.7 ± 83.8g, $p = 0.23$) nor immediately post-hibernation (638.3 ± 93.7g, 569.2 ± 103.2g, p=0.20). Animals in both the NO CACHE and the CACHE group began hibernation (Julian Dates: 213.1 ± 54.3 (Aug 1 ± 54.3 days) vs. 294.5 ± 41.1 (Oct 22 ± 41.1 days), respectively, $p = 0.25$) and ended hibernation on similar dates (91.6 ± 12.9 (Apr 2 ± 12.9 days) vs. 76.6 ± 14.2 (Mar 18 ± 14.2 days), $p = 0.46$). However, the duration of the hibernation season was significantly longer in animals that did not develop reproductively

than in animals that underwent reproductive development (184.8 ± 14.2 days vs. 79.67 ± 13.9 days, $p < 0.01$). Plasma testosterone concentration was similar between the CACHE and NO CACHE groups ($F(1, 10.854) = 4.205$, $p = 0.65$), but was significantly different between reproductive and non-reproductive males ($F(1, 10.854) = 12.641$, $p = 0.005$; Fig 3.1a and 3.1b). The week the sample was collected also played a significant role in plasma testosterone ($F(3,23.007) = 4.779$, $p = 0.01$; Fig 3.1a and 3.1b), as well as the interaction between the week and reproductive status being significant ($F(3, 23.007) = 3.342$, $p = 0.037$).

Cache vs. Cache Removed

All (12 of 12) captive animals that were allowed to establish a cache in their cages underwent reproductive maturation even if that cache was removed after torpor began (CACHE REMOVED). Animals in the CACHE group attained the same pre-hibernation body weight (1010.3 ± 83.7g) as the CACHE REMOVED animals (960.8 ± 52.5g, $p = 0.61$). Animals in both groups ended heterothermy at mean body masses that did not significantly differ (806.7 ± 113.1g for CACHE and 704.3 ± 48.9g for CACHE REMOVED, $p = 0.39$). After the month-long monitoring period directly following the end of heterothermy, the CACHE group attained a significantly greater body mass than the CACHE REMOVED animals (818.4 ± 25.7g vs. 590.6 ± 44.6g, $p < 0.01$). Animals in both groups started, ended and hibernated for the same duration (CACHE: 54.7 ± 7.9 days, CACHE REMOVED: 66.5 ± 7.1 days, $p = 0.29$). Plasma testosterone concentrations were not different between groups ($F(1, 15.040) = 0.50$, $p = 0.827$, Fig 3.1c), but which week after hibernation the sample was collected was a significant factor ($F(3, 22.080) = 3.940$, $p = 0.022$, Fig 3.1d).

Semi-Natural Enclosures

Despite entering hibernation at comparable weights (CACHE: 774.1 ± 22.9g, NO CACHE: 753.4 ± 29.9g, $p = 0.53$), males hibernating outdoors in enclosures in the CACHE group emerged from hibernation at a significantly larger body masses than the NO CACHE animals that were unable to cache food (657 ± 31.7g vs. 543.6 ± 28.1g, respectively, $p = 0.02$). Two of seven NO CACHE animals underwent reproductive development in the month following emergence, whereas five of seven CACHE animals underwent reproductive development ($p = 0.286$). The animals that reproductively matured combined between groups emerged at a

significantly larger body mass (660.7 ± 31.8g) than those that did not (539.9 ± 25.3g, $p = 0.01$). Males that reproductively matured spent significantly less time heterothermic (201.5 ± 4.2 days, $p < 0.01$) than males that did not undergo reproductive development (251.3 ± 5.5 days). Males that did not undergo reproductive development had significantly shorter pre-emergent euthermic periods (3.17 ± 0.7 days) than males that utilized this time to feed from their caches and undergo reproductive development (13.67 ± 1.5 days, $p = 0.0003$) Males that became reproductively mature began torpor later (Aug 20 ± 7.2 days) and ended earlier (May 9 ± 5.8 days) than males that did not (Jul 29 ± 2.7 days, $p = 0.182$ and Apr 6 ± 2.9, $p = 0.0014$, respectively). The only significant factor affecting plasma testosterone was reproductive state ($F(1, 8.691) = 5.714$, $p = 0.041$; Fig 3.1e, 3.1f).

Discussion

We found that there are a number of factors and complex interactions that affect reproductive development in males. Here we demonstrate that the decision to undergo reproductive maturation is not as straightforward as the presence or absence of food upon resumption of high body temperature in the spring. Our spring food availability study demonstrated that the mere presence of *ad libitum* food in the spring was not enough to ensure that all males underwent reproductive development; nor was the absence of *ad libitum* food in the spring enough to prevent reproductive maturation. This also held true for animals in the outdoor enclosures that were provided with cacheable food, not all of which underwent reproductive maturation. However, having built up a substantial cache in the late summer/fall did result in all laboratory males becoming reproductively mature, despite half of those males being put on a restricted ration upon termination of heterothermy. Combined, these experiments show that while having access to *ad libitum* food in the spring can aid a male AGS in undergoing reproductive development, the formation of a cache prior to entering the heterothermic season may be just as important as what is actually available in spring.

The least surprising result of these studies is that reproductively mature males had higher levels of plasma testosterone than non-reproductive males (Spring Food Availability $p = 0.005$ and Semi-Natural Enclosures $p = 0.041$). A much more interesting result was the role time played in the patterns of testosterone expression. Both indoor experiments, Spring Food

Availability and CACHE vs. CACHE REMOVED, showed week as a significant factor in testosterone level ($p = 0.01$ and 0.022, respectively); whereas week was not a significant factor for reproductively mature males in the Semi-Natural Enclosures ($p = 0.621$). The sampling period for both indoor experiments occurred immediately after resumption of euthermy, whereas the males in the Semi-Natural Enclosures could not be sampled until they came above ground, ~2 weeks after resumption of euthermy. The higher levels of testosterone immediately post-heterothermy occur after peak follicle stimulating hormone and coincide with the peak in luteinizing hormone, and combined most likely initiate the reproductive development of the testes (Barnes 1986). After the initialization of spermatogenesis, plasma testosterone levels can be maintained at a lower level throughout the mating season before declining to baseline for the remainder of the active season (Barnes 1986, Richter et al., 2015).

The one consistent result across all of our experiments is that the males that became reproductively mature spent a shorter time heterothermic and in hibernation than those that did not. This result is unsurprising due to results from golden-mantled ground squirrels (*Callospermophilus saturatus*) whose hibernation length was correlated to fall body mass; Barnes (1984) additionally found that spring body mass was affected by food availability in the fall. For males to undergo reproductive development, a 1-3 week process (Barnes 1996), they must maintain high body temperature and fuel the development in one of two ways: 1. with endogenous fat stores that were not spent during hibernation, or 2. by eating. The AGS lives in an environment that remains below freezing throughout the majority of the winter (Buck and Barnes 1999a, 1999b), devoid of plant growth and frequently snow covered in the spring (Hock 1960). This environment forces males of this species to end heterothermy in poor body condition and provides little to no food on the surface when they come to high body temperature, thus leaving a previously acquired cache as the only possible source for the energy required for spermatogenesis as well as the euthermy required to undergo it.

Only male ground squirrels are known to accumulate caches (Krog 1954, Carl 1971, McLean and Towns, 1981, Gaglioti et al. 2011), further supporting their importance to only that sex. The establishment of a substantial cache in the late summer/fall may thus be a prerequisite for spring reproductive development; the inability to establish one may be the driving factor behind why a varying proportion of first year males do not attempt reproduction in a given year.

In addition to enabling reproductive development, a cache allows male AGS to regain the body mass lost over winter; its presence also explains how mature males can emerge from hibernation at the same body mass they attained in the fall (Buck and Barnes 1999a). The ability to recuperate body mass and increase body size prior to the mating season is directly linked to reproductive success, with larger males more successfully competing for mates than smaller individuals (Schwagmeyer and Brown 1983).

In addition to these effects of energy status, population structure has been shown to influence the proportion of reproductive development in male ground squirrels. Male European ground squirrels (*Spermophilus citellus*) living in populations with high male densities opt to forgo reproduction their first spring, likely to avoid the stress and violent social interactions that can be deadly during the mating season (Strauss et al., 2007). Slade and Balph (1974) found that the removal of part of the population of Uinta ground squirrels (*Urocitellus armatus*), therefore reducing density, results in a greater proportion of yearling males becoming reproductively mature. Changes in population structure and density could result in differences in male reproductive development either directly through social interactions or indirectly due to effects on the availability of food resources and thus the ability of males to fuel euthermia and gonadal development. Coincident with the caching period, male AGS display increasing amounts of male-male aggression to protect both their caches and their territories (Buck and Barnes 2003). Therefore, in order for a juvenile male to successfully undergo reproductive development in the spring, they will first have to disperse from the natal burrow to their own territory, grow and fatten, accumulate a substantial cache, and defend it from the larger, adult males in their population.

As the climate changes, it could have a significant impact on the mating success of males. Current predictions include a prolonged fall period, therefore a lengthened growing period (Serreze and Francis 2006, Post et al., 2009). If this does occur, it could enable juvenile males to grow larger and possibly establish a cache, allowing them to undergo reproductive development and successfully compete with older males their first spring. Unlike other arctic animals that are experiencing an environmental mismatch that has actually decreased their reproductive success (Post and Forchhammer 2008), this prolonged fall might increase the fitness of many AGS males similar to what has been observed among yellow-bellied marmots (*Marmota flaviventris,* Ozgul

et al. 2010). However, climate change predictions also suggest that there may be delayed springs due to increased winter snowfall (Wheeler and Hik 2013) which could have the opposite effects on AGS males. Early emerging males will have to balance the increased cache availability of food in the fall with possibly delayed green-up and snow melt both of which are already known to cause high mortality (Morton and Sherman 1978).

Acknowledgements

The authors would like to thank F. Kohl and T. Stevenson for assistance with animal husbandry. This work was supported by funding from the US Army Medical Research and Materiel Command to B.M.B (05178001) Additional support was provided by the National Science Foundation to C.L.B. (EF-0732763 and IOS-1147187) and B.M.B (EF-0732755 and IOS-1147232). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. Additional support for the research reported in this publication was provided by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 5P20GM103395. The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH.

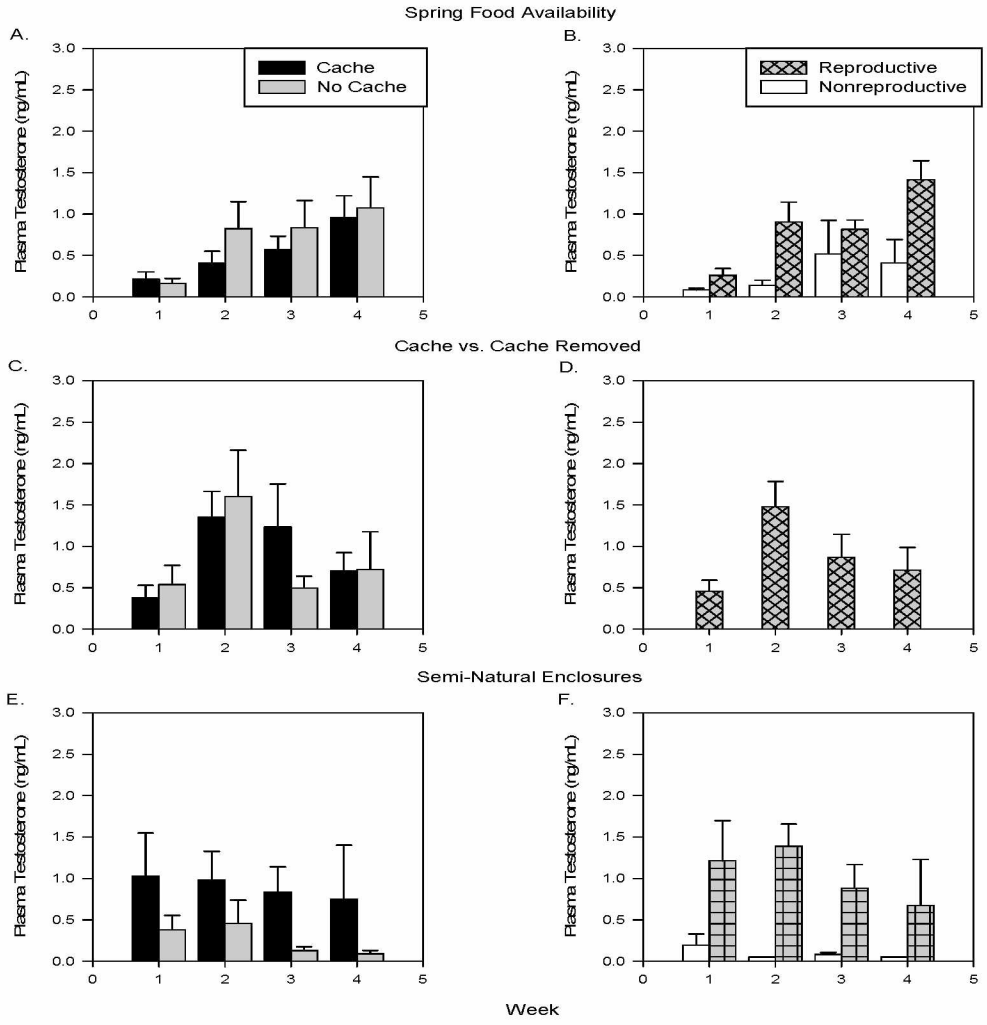


Figure 3.1: Means ± SEM of plasma testosterone RIAs for each experiment. Panels A and B: plasma testosterone from the Spring Food Availability experiment. Reproductive state (p = 0.005), week (p = 0.01) and the interaction (p = 0.037) were all significant factors in the linear mixed model. N's of each group: Cache = 8, No Cache= 7; Non-reproductive males = 6, Reproductive males = 9. Panels C and D: plasma testosterone from the Cache vs. Cache Removed experiment. Week was the only significant factor (p = 0.022). N's for weeks 1-3: Cache = 6, Cache Removed = 6; Reproductive = 12; N's for week 4: Cache = 4, Cache Removed = 6; Reproductive = 10. Panels E and F: plasma testosterone results from the Semi-Natural Enclosures experiment. The only significant factor from the linear mixed model analysis was reproductive status (p = 0.041). N's for week 1-3: Cache = 7, No Cache = 7; Non-reproductive = 7, Reproductive = 7. N's for week 4: Cache = 6, No Cache = 4, Non-reproductive = 3, Reproductive = 7.

Table 3.1: Summary table of experimental results. Values presented are means, ± SEM. *denotes significant difference between the two groups ($p < 0.05$).

	Spring Food Availability	Cache vs. Cache Removed	Semi-Natural Enclosures
Cache, Fraction Repro	6/8	6/6	5/7
No Cache, Fraction Repro	3/7	6/6	2/7
Cache, Fall Body Mass	782.6 ± 24.9 g	1010.3 ± 83.7g	774.1 ± 22.9g
No Cache, Fall Body Mass	780.8 ± 34.4g	960.8 ± 52.5g	753.4 ± 21.9g
Cache, Days Torpid	102.0 ± 21.4 days	54.7 ± 7.9 days	213.2 ± 7.3 days
No Cache, Days Torpid	144.3 ± 25.6 days	66.5 ± 7.1 days	235.9 ± 12.3 days
Repro, Fall Body Mass	803.0 ± 21.8g	985.6 ± 44.8g	776.3 ± 21.5g
Non-repro, Fall Body Mass	753.7 ± 34.2g	N/A	751.3 ± 22.9g
Repro, Days Torpid	78.7 ± 13.9 days	60.6 ± 5.4 days	201.5 ± 4.2 days
Non-repro, Days Torpid	184.8 ± 14.2 days*	N/A	251.3 ± 5.5 days*

Literature Cited

Barnes, B. M. (1984). "Influence of energy stores on activation of reproductive function in male golden-mantled ground squirrels." Journal of Comparative Physiology B 154(4): 421 425.

Barnes, B. (1986). "Annual cycles of gonadotropins and androgens in the hibernating golden mantled ground squirrel." General and Comparative Endocrinology 62(1): 13-22.

Barnes, B. M. (1996). Relationships between hibernation and reproduction in male ground squirrels. Adaptations to the Cold: Tenth International Hibernation Symposium. F. Geiser, A. J. Hulbert and S. C. Nicol. Armidale, University of New England Press.

Bronson, M. T. (1979). "Altitudinal variation in the life history of the golden-mantled ground squirrel (*Spermophilus lateralis*)." Ecology 60(2): 272-279.

Buck, C. and B. Barnes (1999a). "Annual cycle of body composition and hibernation in free living arctic ground squirrels." Journal of Mammalogy 80(2): 430-442.

Buck, C. L. and B. M. Barnes (1999b). "Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter." Journal of Mammalogy 80(4): 1264-1276.

Buck, C. L. and B. M. Barnes (2003). "Androgen in free-living arctic ground squirrels: seasonal changes and influence of staged male-male aggressive encounters." Hormones & Behavior 43(2): 318.

Carl, E. A. (1971). "Population Control in Arctic Ground Squirrels." Ecology 52(3): 395-413.

Dark, J., N. F. Ruby, G. N. Wade, P. Licht and I. Zucker (1992). "Accelerated reproductive development in juvenile male ground squirrels fed a high-fat diet." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 262(4): R644-R650.

Forger, N. G., J. Dark, B. M. Barnes and I. Zucker (1986). "Fat ablation and food restriction influence reproductive development and hibernation in ground squirrels." Biology of Reproduction 34(5): 831-840.

Gaglioti, B. V., B. M. Barnes, G. D. Zazula, A. B. Beaudoin and M. J. Wooller (2011). "Late Pleistocene paleoecology of arctic ground squirrel (*Urocitellus parryii*) caches and nests from Interior Alaska's mammoth steppe ecosystem, USA." Quaternary Research 76(3): 373-382.

Hock, R. J. (1960). "Seasonal variations in physiologic functions of arctic ground squirrels and black bears." Bulletin of the Museum of Comparative Zoology at Harvard College 124: 155-171.

Holmes, W. G. (1988). "Body fat influences sexual maturation in captive male Belding's ground squirrels." Canadian Journal of Zoology 66(7): 1620-1625.

Krog, J. (1954). "Storing of food items in the winter nest of the Alaskan ground squirrel, *Citellus undulatus*." Journal of Mammalogy: 586-586.

Long, R. A., R. A. Hut and B. M. Barnes (2007). "Simultaneous collection of body temperature and activity data in burrowing mammals: A new technique." Journal of Wildlife Management 71(4): 1375-1379.

McLean, I. G., Towns, A.J. (1981). "Differences in weight changes and the annual cycle of male and female arctic ground squirrels." Arctic 34(3): 249-254.

Morton, M. L. and P. W. Sherman (1978). "Effects of a spring snowstorm on behavior, reproduction, and survival of Belding's ground squirrels." Canadian Journal of Zoology 56(12): 2578-2590.

Michener, G. R. (1983). "Spring emergence schedules and vernal behavior of Richardson ground-squirrels - Why do males emerge from hibernation before females." Behavioral Ecology and Sociobiology 14(1): 29-38.

Ozgul, A., D. Z. Childs, M. K. Oli, K. B. Armitage, D. T. Blumstein, L. E. Olson, S. Tuljapurkar and T. Coulson (2010). "Coupled dynamics of body mass and population growth in response to environmental change." Nature 466(7305): 482-485.

Pengelley, E. and K. Fisher (1961). "Rhythmical arousal from hibernation in the golden-mantled ground squirrel, *Citellus lateralis tescorum*." Canadian Journal of Zoology 39(1): 105 120.

Post, E. and M. Forchhammer (2008). "Climate change reduces reproductive success of an Arctic herbivore through trophic mismatch." Philosophical Transactions of the Royal Society B: Biological Sciences 363(1501): 2367-2373.

Post, E., M. C. Forchhammer, M. S. Bret-Harte, T. V. Callaghan, T. R. Christensen, B. Elberling, A. D. Fox, O. Gilg, D. S. Hik and T. T. Høye (2009). "Ecological dynamics across the Arctic associated with recent climate change." Science 325(5946): 1355-1358.

Richter, M. M., C. T. Williams, T. N. Lee, Ø. Tøien, G. L. Florant, B. M. Barnes and C. L. Buck (2015). "Thermogenic capacity at subzero temperatures: How low can a hibernator go?" Physiological and Biochemical Zoology 88(1): 81-89.

Schwagmeyer, P. L. and C. Brown (1983). "Factors affecting male-male competition in thirteen lined ground squirrels." Behavioral Ecology and Sociobiology 13(1): 1-6.

Serreze, M. and J. Francis (2006). "The Arctic amplification debate." Climatic Change 76(3-4): 241-264.

Sheriff, M. J., G. J. Kenagy, M. Richter, T. Lee, Ø. Tøien, F. Kohl, C. L. Buck and B. M. Barnes (2011). "Phenological variation in annual timing of hibernation and breeding in nearby populations of Arctic ground squirrels." Proceedings of the Royal Society B: Biological Sciences 278(1716): 2369-2375.

Slade, N. A. and D. F. Balph (1974). "Population ecology of Uinta ground squirrels." Ecology: 989-1003.

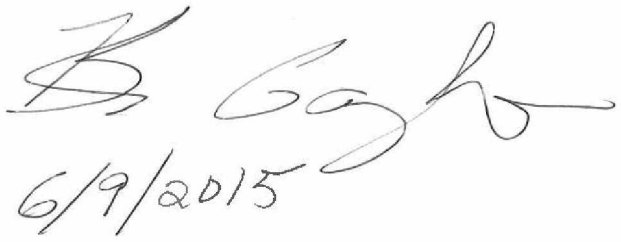
Strauss, A., I. Hoffmann and E. Millesi (2007). "Effects of nutritional factors on juvenile development in male European ground squirrels (*Spermophilus citellus*)." Mammalian Biology 72(6): 354-363.

Wheeler, H. and D. Hik (2013). "Arctic ground squirrels *Urocitellus parryii* as drivers and indicators of change in northern ecosystems." Mammal Review 43(3): 238-255.

Appendix 3.1 Co-Author Consent

I hereby give my approval for this manuscript [M.M. Richter, B.V. Gaglioti, C.L. Buck, B.M. Barnes. Does the availability of a food cache affect timing of hibernation and spring reproductive status in male arctic ground squirrels (*Urocitellus parryii*)? Prepared for submission to Canadian Journal of Zoology] to be included in the dissertation of Melanie M. Richter.

Ben Gaglioti



6/9/2015

Appendix 3.2 IACUC Approval

UNIVERSITY OF ALASKA FAIRBANKS

(907) 474-7800
(907) 474-5444 fax
fyiacuc@uaf.edu
www.uaf.edu/iacuc

Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

July 23, 2008

To: Brian Barnes, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Assurance Application

The University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) reviewed the following Assurance at their July 22, 2008, meeting. This Assurance was approved pending receipt of a revised assurance addressing the committee's questions. The assurance received on July 22, 2008 was determined to be satisfactory; therefore I am pleased to issue approval.

Protocol#: 08-55

Title: *The significance of prehibernation status for reproduction in yearling male Arctic Ground Squirrels*

Received: July 15, 2008 (orig)
July 22, 2008 (rev)

Approved: July 23, 2008

Review Due: July 23, 2009

The PI is responsible for acquiring and maintaining all required permits and permissions prior to beginning work on this assurance. Failure to obtain or maintain valid permits is considered a violation of an IACUC assurance, and could result in revocation of IACUC approval.

UNIVERSITY OF ALASKA FAIRBANKS

(907) 474-7800
(907) 474-5444 fax
fyiacuc@uaf.edu
www.uaf.edu/iacuc

Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

February 10, 2009

To: Brian Barnes, PhD
Principal Investigator

From: Erich H. Follmann, PhD
IACUC Chair

Re: IACUC Assurance Application

The University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC) reviewed the following Assurance at their November 2, 2008, meeting. This Assurance was approved pending receipt of a revised assurance addressing the committee's questions. The assurance received on January 29, 2009 was determined to be satisfactory; therefore I am pleased to issue approval.

Protocol#:	08-70
Title:	*The Effect of Cache Presence on Spring Reproductivity and Hormone Levels in Juvenile Male Arctic Ground Squirrels*
Received:	November 29, 2008 (orig) January 29, 2009 (rev)
Notes:	Since this project is partially funded by DoD, the PI must verify that they do not need to review it; correspondence confirming this should be forwarded to the IACUC. Despite visual cues not being a target of this research project, researchers should consider this effect when interpreting results.
Approved:	February 10, 2009
Review Due:	February 10, 2010

The PI is responsible for acquiring and maintaining all required permits and permissions prior to beginning work on this assurance. Failure to obtain or maintain valid permits is considered a violation of an IACUC assurance, and could result in revocation of IACUC approval.

Research & Graduate Studies
University of Alaska Anchorage

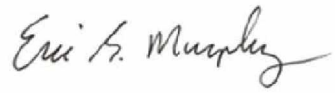
3211 Providence Drive
Anchorage, Alaska 99508-4614
T 907.786.1099, F 907.786.1791
www.uaa.alaska.edu/research/ric

DATE:	July 29, 2010
TO:	C. Loren Buck, PhD
FROM:	University of Alaska Anchorage IACUC
STUDY TITLE:	The Effect of the Presence of a Cache on the Phenology of Hibernation and Reproduction in Captive Juvenile Arctic Ground Squirrels (Urocitellus parryii)
IRB REFERENCE #:	177790-1
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	July 9, 2010
EXPIRATION DATE:	July 9, 2013
REVIEW TYPE:	Full Committee Review

The Institutional Animal Care and Use Committee at the University of Alaska Anchorage met on July 9, 2010, and approved your protocol [177790-1] *The Effect of the Presence of a Cache on the Phenology of Hibernation and Reproduction in Captive Juvenile Arctic Ground Squirrels (Urocitellus parryii)*, that you submitted to us on June 23, 2010. Please note that this approval is contingent upon your compliance with all relevant University, city, state, and federal regulations, and requires that you possess all relevant permits before work is initiated. Your protocol **ID number is 177790**. Your approval is good for a period of 3 years, and will expire on **July 9, 2013**. You are required to submit an annual report of your activities prior to July 9 in each of the next two years. This form is available in the Forms and Reference Library on IRBNet.

We remind you that all changes in personnel and animal handling protocols must be submitted to the committee prior to such changes taking place. In addition, should you experience any unexpected animal mortalities, illnesses, or injury (to animals or personnel involved with the project), you are required to report such to the IACUC immediately.

Thank you for your support of animal care guidelines. We hope that your research goes well.



Eric S. Murphy, Ph.D.

Chair, UAA IACUC

GENERAL CONCLUSION

This dissertation uses the arctic ground squirrel as a model species to investigate the limits of hibernation, the roles of androgens in behaviors and phenology in free-living individuals, and the importance of resource availability and timing on reproductive development. Here, we demonstrate the limits of the hibernation phenotype and determine the maximum torpid metabolic rate. We also show the influence androgens have on hibernation phenology in males, and how this can be manipulated by a castration. Lastly, we explore the impact food availability in both the spring and fall has on the reproductive development of males.

From very limited studies of free-living hibernators and their environments (Chapter 1, Table 1.1), as well as most laboratory experiments (review: Ruf and Geiser 2014) it may appear that only animals that live in the Arctic are capable of surviving a hibernation season at temperatures significantly below the hypothalamic set-point by remaining thermogenic at low tissue temperature. However, there is also no evidence that these species have unique physiologies that are not shared with other hibernators. Arctic ground squirrels evolved to survive and thrive in significantly subzero overwinter temperatures (Buck and Barnes 1999b). The golden-mantled ground squirrel is smaller than the arctic ground squirrel and evolved to live under much milder overwinter conditions (Healy et al., 2012). Despite their different life histories, we correctly predicted that both species of ground squirrel would be capable of defending significant temperature gradients while remaining torpid.

In the first chapter we hypothesized that (I) the main differences between these species in their capacity to maintain a thermal gradient would be due to their body size and that the larger size, and thus lower thermal conductivity, of the arctic ground squirrel would allow that species to maintain torpor at lower ambient temperatures. While we did not directly test the effects of body mass independently of species differences, our data do demonstrate a species and, indirectly, a body size difference in metabolic rate increases over the same ambient temperature range (Fig 4.1). Figure 4.1 shows that despite having similar metabolic rates at 2, 0, and -2°C, when both species are thermally conforming to their environment, there are significant differences between the species' metabolic rates as ambient temperature decreases and animals need to utilize thermogenic torpor to defend body temperature. Both species demonstrate an increase in metabolic rate with ambient temperature reductions ($F(5, 11) = 140.846$, $p < 0.001$)

and yet there was a significant interaction between species ($F(5, 11) = 28.308$, $p < 0.001$) illustrating that golden-mantled ground squirrels had a greater elevation in metabolic rate over the temperature range studied. On a gram-specific basis golden-mantled ground squirrels have a higher torpid metabolic rate than arctic ground squirrels at the same temperature, and this supports our hypothesis that the observed difference is due to total body size, and the associated higher thermal conductance of the golden-mantled ground squirrels.

The shallower slope of metabolic rate increase observed in the arctic ground squirrel (Fig. 4.1) is likely due to the lower thermal conductance (as exemplified by Fig 4, Cannon and Nedergaard 2011, Appendix 4.1), and therefore lower rate of heat loss, their larger body size provides them. Additional support for the importance of the environment's influence on body mass comes from the differences in body size with increasing latitude observed among arctic ground squirrels; animals in the Kluane National Park (~400g; 60.6194° N, 138.3310° W) are much smaller than those found in the Alaska Range (~600g; 63°04′10″N 151°00′26″W), which are smaller than those just south of the Brooks Range (~750g; 65.4900° N, 148.5467° W), which are still smaller than those found north of the Brooks Range (800-1000g; 68° 38' N, 149° 36' W) (Wheeler and Hik 2013; B.M. Barnes pers. comm.). The increase in body size, and thus lower thermal conductance, with increasing latitude, and therefore colder conditions and longer hibernation seasons, favors an environmental effect on hibernation phenotype. It has been shown in woodchucks (*Marmota monax*) that latitude, and the associated environmental conditions, can affect the hibernation patterns of a species in the field (Zervanos et al. 2010) and that some of those same latitudinal differences are maintained when animals are brought into the laboratory and experience identical overwinter conditions (Fenn et al. 2009). As we hypothesized, (II) the arctic ground squirrel was able to remain torpid at lower ambient temperatures (Chapter 1, Table 1.2). Additionally, we were able to determine that the arctic ground squirrel has a maximum torpid metabolic rate of 0.37 ± 0.01 mL O_2/g*h, which is near their basal metabolic rate (0.4 to 0.6 mL O_2/g*h, Scholander et al., 1950; Withers et al., 1979).

The factors controlling this maximum torpid metabolic rate are not known, but research into the limiting factors would be beneficial to the field. In Chapter 1, we proposed that there could be a threshold level of norepinephrine, the neurally-released molecule responsible for non-shivering thermogenesis during torpor via downstream actions on uncoupling protein-1 (Cannon

and Nedergaard 2004; 2011), that elicits an arousal. The logic behind this proposed mechanism was that as ambient temperatures decrease, a torpid squirrel must produce more heat and therefore might require increases in sympathetic activation of non-shivering thermogenesis; however, once the threshold level of activation is reached, instead of remaining torpid and thermogenic, the animal initiates an arousal. This proposed mechanism could explain why animals cannot continue to defend significantly greater thermal gradients at low tissue temperature without returning to euthermy. A possible twist on this proposed mechanism could be the recruitment of additional brown adipose depots that, once activated by the sympathetic nervous system in response to continued heat loss, support an arousal in the animal. We refute the implication in our chapter that increases in norepinephrine at the level of the brown adipose tissue stimulates arousals since it is the activation of non-shivering thermogenesis by the sympathetic nervous system, rather than an increase in circulating norepinephrine concentrations, that result in thermogenesis. In addition to determining the mechanisms of torpid thermogenesis vs. arousal thermogenesis, future studies should include more species to determine if the ability to remain thermogenic and defend a temperature gradient while torpid is truly widespread amongst ground squirrel species.

Studying free-living arctic ground squirrels enables investigations into what influences their circannual, or yearly, rhythms. Male and female arctic ground squirrels display different hibernation phenologies (Sheriff et al., 2011) and in the second chapter we demonstrate, as we hypothesized (III), that the differences are due, at least in part, to circulating androgen levels. The increase in circulating testosterone levels observed in males drives their early arousal and resumption of euthermy in the spring time. However, why males remain active on the surface after females have entered hibernation cannot be due to circulating testosterone levels since they are very low, and castrates also remain on the surface similar to their intact cohorts. Instead, I propose that the increase in circulating dehydroepiandrosterone, an adrenal androgen, is responsible for the late season aggression observed at this time as well as the delayed onset of hibernation. In addition to the males that come to high body temperature early and undergo reproductive development, a subset of males do not but rather continue hibernating and do not emerge until the mating season begins and do not undergo reproductive development.

Every year there are a varying proportion of first year males that do not participate in the mating season, and do not develop scrotal testes (Buck and Barnes 1999a). These males do not spend a prolonged period at high body temperature prior to emergence (Williams et al., 2012) and do not recover the body mass lost over winter by feeding from a cache, unlike the males that mature (Buck and Barnes 1999a). Unlike other species of sciurid that seemingly rely on endogenous energy stores, which can be manipulated before (Forger et al., 1986) or after (Holmes 1988) hibernation to alter reproductive development, arctic ground squirrels seem mainly reliant upon a cache to determine reproductive status. Male arctic ground squirrels that do not mature do not recover the body mass lost over winter, unlike reproductively mature males (Buck and Barnes 1999a). As we hypothesized in the third chapter (IV), the ability to accumulate a substantial cache in the late summer/fall seems to be important for a male to undergo reproductive development. However, *ad libitum* access to food in the spring can also lead to reproductive development, though it is not a guarantee.

The importance of studying animals that live in the arctic and what controls their phenologies is particularly important now in light of the predictions from climate change models. The models predict numerous changes including a lengthened growing period (Serreze and Francis 2006, Post et al., 2009) which could increase the proportion of juvenile males that are able to attain sufficient size and caches to undergo reproductive development their first spring. However, if the arctic ground squirrel cannot utilize this change in growing season, they, like certain other species, may experience mismatches with their environment that result in decreased reproductive success (Post and Forchhammer 2008). Climate change also predicts the northward movement of shrubs and forbes which, in addition to increasing food resources, may increase predation on the arctic ground squirrel (Wheeler et al., 2015).

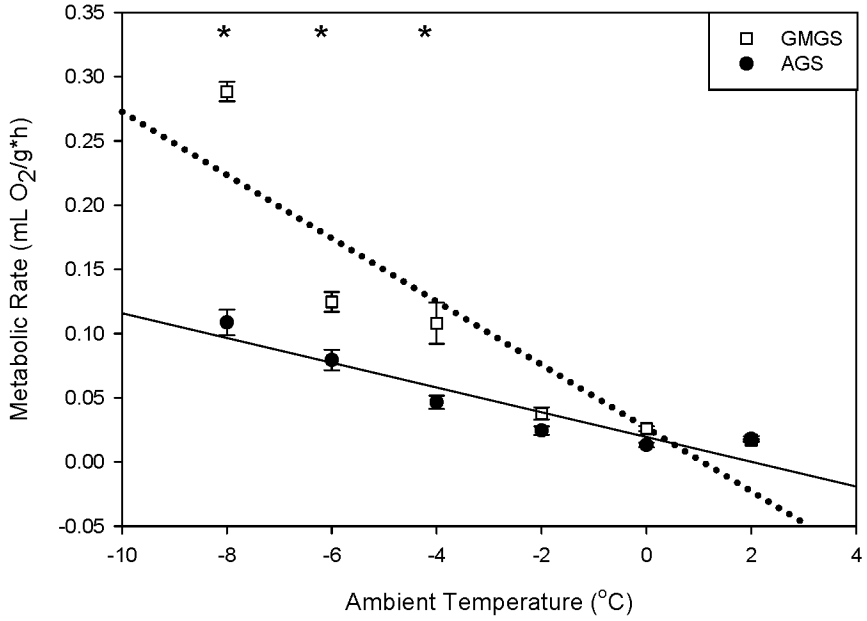


Figure 4.1: Mean torpid metabolic rates (±SEM) across the same ambient temperatures for golden-mantled ground squirrels (GMGS, open squares) and arctic ground squirrels (AGS, closed circles). The dotted line is the linear regression for GMGS, the solid line for AGS. The stars indicate significant differences between species (ambient temperatures -4 to -8°C, 2-way ANOVA; $p < 0.001$).

Literature Cited

Buck, C. and B. Barnes (1999a). "Annual cycle of body composition and hibernation in free living arctic ground squirrels." Journal of Mammalogy 80(2): 430-442.

Buck, C. L. and B. M. Barnes (1999b). "Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter." Journal of Mammalogy 80(4): 1264-1276.

Cannon, B. and J. Nedergaard (2004). "Brown adipose tissue: function and physiological significance." Physiological Reviews 84(1): 277-359.

Cannon, B. and J. Nedergaard (2011). "Nonshivering thermogenesis and its adequate measurement in metabolic studies." The Journal of Experimental Biology 214(2): 242-253.

Fenn, A. M., S. M. Zervanos and G. L. Florant (2009). "Energetic relationships between field and laboratory woodchucks (*Marmota monax*) along a latitudinal gradient." Ethology Ecology & Evolution 21(3-4): 299-315.

Forger, N. G., J. Dark, B. M. Barnes and I. Zucker (1986). "Fat ablation and food restriction influence reproductive development and hibernation in ground squirrels." Biology of reproduction 34(5): 831-840.

Healy, J. E., K. A. Burdett, C. L. Buck and G. L. Florant (2012). "Sex differences in torpor patterns during natural hibernation in golden-mantled ground squirrels (*Callospermophilus lateralis*)." Journal of Mammalogy 93(3): 751-758.

Holmes, W. G. (1988). "Body fat influences sexual maturation in captive male Belding's ground squirrels." Canadian Journal of Zoology 66(7): 1620-1625.

Post, E. and M. Forchhammer (2008). "Climate change reduces reproductive success of an Arctic herbivore through trophic mismatch." Philosophical Transactions of the Royal Society B: Biological Sciences 363(1501): 2367-2373.

Post, E., M. C. Forchhammer, M. S. Bret-Harte, T. V. Callaghan, T. R. Christensen, B. Elberling, A. D. Fox, O. Gilg, D. S. Hik and T. T. Høye (2009). "Ecological dynamics across the Arctic associated with recent climate change." Science 325(5946): 1355-1358.

Ruf, T. and F. Geiser (2014). "Daily torpor and hibernation in birds and mammals." Biological Reviews.

Scholander, P., R. Hock, V. Walters and L. Irving (1950). "Adaptation to cold in arctic and tropical mammals and birds in relation to body temperature, insulation, and basal metabolic rate." The Biological Bulletin 99(2): 259-271.

Serreze, M. and J. Francis (2006). "The Arctic amplification debate." Climatic Change 76(3-4): 241-264.

Sheriff, M. J., G. J. Kenagy, M. Richter, T. Lee, Ø. Tøien, F. Kohl, C. L. Buck and B. M. Barnes (2011). "Phenological variation in annual timing of hibernation and breeding in nearby populations of Arctic ground squirrels." Proceedings of the Royal Society B: Biological Sciences 278(1716): 2369-2375.

Wheeler, H. and D. Hik (2013). "Arctic ground squirrels *Urocitellus parryii* as drivers and indicators of change in northern ecosystems." Mammal Review 43(3): 238-255.

Wheeler, H. C., J. D. Chipperfield, C. Roland and J. C. Svenning (2015). "How will the greening of the Arctic affect an important prey species and disturbance agent? Vegetation effects on arctic ground squirrels." Oecologia: 1-15.

Williams, C. T., B. M. Barnes, M. Richter and C. L. Buck (2012). "Hibernation and circadian of body temperature in free-living arctic ground squirrels." Physiological & Biochemical Zoology 85(4): 397-404.

Withers, P. C., T. M. Casey and K. K. Casey (1979). "Allometry of respiratory and haematological parameters of arctic mammal." Comparative Biochemistry and Physiology Part A: Physiology 64(3): 343-350.

Zervanos, S. M., C. R. Maher, J. A. Waldvogel and G. L. Florant (2010). "Latitudinal differences in the hibernation characteristics of woodchucks (*Marmota monax*)." Physiological and Biochemical Zoology 83(1): 135-141.

Appendix 4.1 Figure 4 from Cannon and Nedergaard 2011

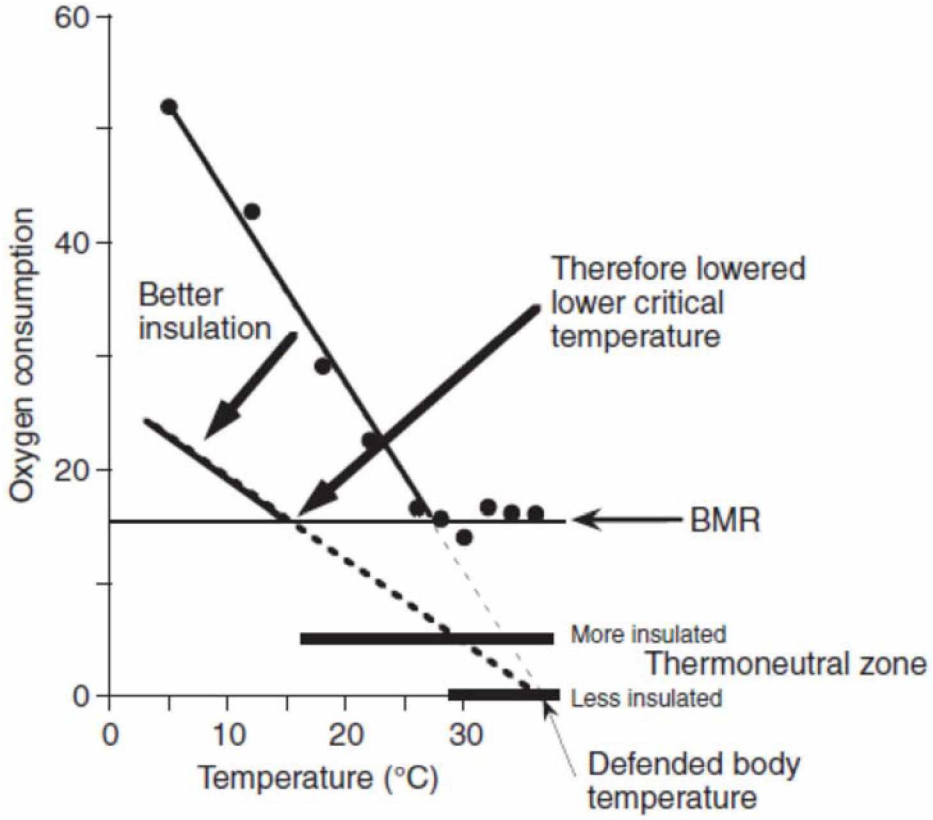


Fig. 4. The effect of increased insulation on the thermoneutral zone. As seen, animals with a better insulation (a smaller slope of the line) must necessarily also obtain a broader thermoneutral zone, because the line must extrapolate to the same defended body temperature.