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Hibernation in Turkish hamsters: effects on incisor dentin morphology and implications for studying hibernation in evolutionary and historical contexts

By

Mariska Priya Batavia

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

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in the

Graduate Division

of the

University of California, Berkeley

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Professor Irving Zucker, Chair

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Spring 2013

Hibernation in Turkish hamsters: effects on incisor dentin morphology and implications for
studying hibernation in evolutionary and historical contexts

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by Mariska Priya Batavia

Abstract

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University of California, Berkeley

Professor Irving Zucker, Chair

Mammalian thermoregulation is energetically costly, and mammals employ numerous strategies to ameliorate these costs, particularly at low ambient temperatures. This body of work is broadly focused on mammalian thermoregulation, with emphasis on the adaptive value and evolution of insulative fur and hibernation in rodents. Chapter 1 serves as a brief introduction to mammalian heterothermy, and general methods for this dissertation are discussed in Chapter 2.

Chapter 3 tested for an energetic tradeoff between growth and thermoregulation in juvenile Siberian hamsters (*Phodopus sungorus*) and the effect of an insulative pelage on intrinsic growth rate. Growth, like thermoregulation, is energetically costly, and many studies implicate an energetic tradeoff between them. Fur is known to reduce thermoregulatory costs in adult mammals, but its role in maintaining energy balance during growth is unclear. Hamsters weaned at 18 days of age and left fully furred or deprived of all dorsal fur by shaving at 20 days of age, were housed at 10°C or 23°C. Body mass, body length, and food consumption were measured until hamsters were 35 days old. Thermal challenge, whether by low ambient temperature or shaving, resulted in increased food intake and decreased efficiency at converting food into body mass – i.e., less body mass was accrued per gram of food consumed. Body mass and length were not affected by the thermal challenges. These results suggest that there is no mandatory tradeoff between growth and thermoregulation in this species, particularly when food is in abundant supply.

Chapter 4 provided a detailed descriptive account of hibernation in Turkish hamsters (*Mesocricetus brandti*), a common model organism for studies of hibernation. I employed continuous telemetric monitoring of body temperature in hibernating male and female Turkish hamsters at ambient temperatures of 5°C and 13°C to characterize torpor bout depth, duration, and frequency, as well as rates of entry into and arousal from torpor. Hamsters generated brief intervals of short (<12 h), shallow test bouts ($T_b > 20^\circ\text{C}$), followed by deep torpor bouts lasting 4-6 days at $T_a = 5^\circ\text{C}$ and 2-3 days at $T_a = 13^\circ\text{C}$. Females at $T_a = 5^\circ\text{C}$ had longer bouts than males, but maintained higher torpor T_b ; there were no sex differences at $T_a = 13^\circ\text{C}$. Neither body mass loss nor food intake differed between the two T_a s. Hamsters entered torpor primarily during the scotophase (subjective night), but timing of arousals was highly variable. Hamsters at both T_a s

generated short, shallow torpor bouts between deep bouts, suggesting that this species may be capable of both hibernation and daily torpor.

Chapters 5 and 6 made use of the fact that vertebrate dentin is deposited on a circadian basis, and daily layers manifest as bands on the medial surfaces of rodent incisors. Hibernation alters dentin deposition, and a distinct hibernation mark has been described on incisor surfaces of several rodent species. Chapter 5 tested the effects of day length, torpor expression, and ambient temperature on incisor dentin surface morphology in Turkish hamsters housed in one of four conditions: long days (LDs) at 22°C, short days (SDs) at 22°C, SDs at 5°C, and SDs at 13°C. Body temperature was monitored continuously with implanted radio transmitters, and teeth examined postmortem. Teeth of SD hamsters had narrower, less distinct circadian increments than those of LD hamsters, but the width of ultradian increments was similar in both photoperiods. Hibernation at both 5°C and 13°C was associated with very narrow, sharply defined dentin increments and increased tooth heterogeneity. Hamsters in SDs at 5°C that did not hibernate lacked characteristic hibernation increments. At 5°C, but not 13°C, the number and cumulative width of hibernation increments were related to number and cumulative duration of periodic arousals, suggesting that it may be possible to infer information about hibernation physiology by examining a deceased or extinct specimen's teeth.

Chapter 6 compared conventional histological preparation of incisor cross-sections to images obtained by hard X-ray micro-tomography, a technique similar to medical CT scanning. Six of the Turkish hamster specimens from Chapter 5 (2 each of LD controls, SD controls, and 5°C hibernators) were used; lower right incisors were prepared histologically and lower left incisors were scanned. Scanning was nearly as good as histological preparation, though increments were slightly but significantly wider when measured from scanned images. Several specimens of four hibernating sciurid species from the Museum of Vertebrate Zoology were also scanned in search of a putative hibernation mark; such a mark was present in only two individuals. Hard X-ray micro-tomography is a valuable tool for studying dentin microstructure when specimens are irreplaceable or otherwise unavailable for permanent alteration necessitated by conventional histology.

Two broad conclusions follow from this body of work. First, there are sex differences in thermoregulatory traits, which merit further investigation and may ultimately shed light on different selective pressures operating on males and females. Second, incremental dentin in rodent incisors is a viable tool for studying hibernation behavior and seasonal changes in growth rates in evolutionary and historical populations, provided that care is taken to account for inter- and intra-specific variation in dentin morphology.

To Aaron, Summer, and Violet
for making every single day beautiful

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Chapter 1

Introduction

Mammalian thermoregulation

Mammals are tachymetabolic endotherms; they have a relatively high metabolic rate (MR) compared to other vertebrates (Bennett and Dawson, 1976; White et al., 2006; Clarke and Pörtner, 2010), and can modulate metabolism for the purpose of regulating body temperature (T_b).¹ Bradymetabolic ectotherms, by contrast, have relatively low MRs and rely primarily on ambient conditions, behavior (*e.g.*, shuttling among microenvironments, changing posture, basking), and/or non-metabolic physiological responses (*e.g.*, altering peripheral blood flow and surface conductance) to control T_b (Cowles and Bogert, 1944; Ruibal, 1961; Bartholomew and Tucker, 1963; Huey, 1974; McNab, 2002).

Tachymetabolic endothermy is energetically costly – mammals and birds have field energy expenditures an order of magnitude greater than other comparably sized vertebrates (Nagy, 2005) – but conveys some important advantages. It allows mammals to maintain a relatively high, stable T_b even when ambient temperature (T_a) fluctuates. Maintenance of homeothermy, in turn, permits specialization of enzymes to operate within a narrow temperature range, which generally yields better performance as compared to enzymes that are active over a range of temperatures (Heinrich, 1977). Moreover, maintenance of homeothermy allows mammals to be active during times of the day, year, and in geographic regions that would otherwise be thermally unfavorable. Tachymetabolic endothermy may also support relatively high growth and reproductive rates (McNab, 2002) and the capacity for sustained vigorous activity (Bennett and Ruben, 1979; Ruben, 1995).

Despite the benefits of tachymetabolic endothermy, mammals employ numerous strategies to reduce the energetic costs associated with thermoregulation, particularly at low T_a . Fur and fat insulate the body and reduce heat exchange with the environment (Scholander et al., 1950; Hammel, 1955; Conley, 1986; Rosen and Renouf, 1997). Behaviors that reduce heat loss include huddling, nesting, postural and locomotor changes, and microhabitat selection (Sealand, 1952; Vogt and Lynch, 1982; Conley and Porter, 1986; Kenagy and Pearson, 2000; Kauffman et al., 2003). Finally, physiological adaptations that mitigate heat loss include vasoconstriction and heterothermy (Vogt and Lynch, 1982; Geiser and Ruf, 1995); the latter is discussed below.

Mammalian heterothermy

Heterothermy is characterized by a marked reduction in metabolic rate and a decrease in T_b (Lyman et al., 1982; Geiser and Ruf, 1995). Unlike dormancy of ectotherms, endothermic heterothermy is regulated. Endothermy is not abandoned during torpor bouts, as evidenced by the fact MR increases to maintain T_b at a species-specific minimum T_b , even when T_a falls below

¹ Birds independently evolved similar metabolic and thermoregulatory syndromes (Dawson and Whittow, 2000).

that point (Geiser and Kenagy, 1988; Buck and Barnes, 2000). Endotherms arouse endogenously from their torpid state by increasing MR. Dormant ectotherms, by contrast, can neither maintain a temperature gradient between themselves and the environment nor arouse endogenously from a dormant state (McNab, 2002).

Hibernation versus daily torpor

Before addressing heterothermic patterns in depth, a brief clarification on the word *torpor* is instructive. *Torpor* can be used as a collective term for hibernation and daily torpor, synonymous with *heterothermy*. A *torpor bout* refers to a single hypometabolic episode, irrespective of whether the species hibernates or employs daily torpor; between torpor bouts, T_b and MR return to normal levels. Similarly, when an animal is *in torpor*, this is meant to indicate that it is in the midst of a torpor bout. *Torpor* is also occasionally used as shorthand for *daily torpor*. For clarity, this dissertation will use the term *heterothermy* as a collective term for hibernation and daily torpor, and will use *daily torpor* (rather than the shorthand *torpor*).

Hibernation and daily torpor are two patterns of mammalian heterothermy. Geiser and Ruf (1995) set forth guidelines for defining hibernation versus daily torpor according to body mass, minimum torpor T_b , minimum oxygen consumption (as an index of minimum MR), and bout length. They found substantial overlap in body mass and minimum T_b , and therefore recommended that hibernation and daily torpor be distinguished primarily by minimum oxygen consumption and bout length. Most authors characterize hibernation by metabolic reduction of ~95% and torpor bouts lasting ≥ 4 days; these bouts are punctuated by arousals to normal MR and T_b . The reason for periodic arousals is unknown, but among the many proposed hypotheses are that arousals serve to eliminate metabolic waste, preserve immunocompetence, prevent memory loss, or neutralize reactive oxygen species (reviewed in Humphries et al., 2003). Daily torpor is characterized by metabolic reduction of ~70% and torpor bouts less than 24 hours. Despite the overlap between hibernation and daily torpor in minimum torpor T_b , the T_b reduction observed during daily torpor is often shallower than that during hibernation.

These criteria for distinguishing hibernation from daily torpor are not perfect. With the discovery of several tropical and subtropical heterotherms, including many members of the Afrotheria (reviewed in Lovegrove, 2012) and a tropical Malagasy primate (Dausmann et al., 2004), T_b and MR reduction have become somewhat arbitrary criteria for defining patterns of heterothermy. While torpid, mammals thermoconform above their lower critical temperature, so T_b and MR reduction in these cases is reflective of ambient conditions rather than physiological capability. Even bout length – arguably the most reliable of the original criteria – does not always clearly indicate which strategy is being used. Geiser and Ruf (1995) reported no examples of bout lengths intermediate between 24 hours and 4 days. Subsequently, examples of intermediate bout lengths have been documented in several species, including the Hottentot golden mole (*Amblysomus hottentottus longiceps*; Scantlebury et al., 2008) and the pichi armadillo (*Zaedyus pichiy*; Superina and Boily, 2007).

The original definitions of Geiser and Ruf (1995) also do not account for functional aspects of hibernation and daily torpor. Daily torpor is often used facultatively under conditions of low T_a or food scarcity, whereas hibernation is a longer-term commitment, requiring behavioral and physiological mechanisms to control, for example, pre-hibernation fattening or food storage, digestive atrophy, and timing and control of arousals (e.g., Carey, 1995; Carey et al., 2003; Dark, 2005; Malan, 2010). Thus, even when bout length exceeds 24 hours, animals

may be using heterothermy facultatively, as appears to be the case in, for example, the feathertail glider (Jones and Geiser, 1992) and golden moles (Scantlebury et al., 2008).

Even considering functional aspects of hibernation and daily torpor, it is important to note that these terms describe patterns. Grigg et al. (2004) have argued that, until we have a detailed understanding of the physiological mechanisms underlying different heterothermic strategies (see below), positively identifying these patterns and distinguishing between them is difficult and in some cases, may be arbitrary.

The physiological basis of mammalian heterothermy

Heterothermy results in substantial energy savings, both from reducing thermoregulatory costs and through the reduction of basal metabolism. It is controversial whether the reduction in basal metabolic rate results simply from a lowering of body temperature (Q_{10} effects), or if active metabolic suppression is involved. Numerous studies suggest that the latter is more likely (Wilz and Heldmaier, 2000; McNab, 2002; Dausmann et al., 2009), but the use of metabolic suppression may depend on body size and the pattern of heterothermy. Small mammals that employ daily torpor appear to rely primarily on Q_{10} effects to reduce metabolism, whereas larger mammals, and those for whom energy is most limited utilize additional metabolic suppression (Geiser, 2004).

Several explanations for metabolic suppression have been proposed. Some involve limiting substrate supply to the glycolytic pathway, Krebs cycle, and/or electron transport chain (Carey et al., 2003; Staples and Brown, 2008), and others involve reductions in energetic demand by slowing ion leaks, protein synthesis, and other energetically demanding cellular processes (Carey et al., 2003). Although supply and demand explanations are by no means mutually exclusive, mechanisms for limiting supply and demand are unknown. Respiratory acidosis, changes in protein phosphorylation states, and downregulation of key enzymes are among proposed hypotheses (Carey et al., 2003; Staples and Brown, 2008).

Much more work remains to be done on the physiological basis of heterothermy. In addition to elucidating the mechanism(s) of metabolic suppression, other questions remain unanswered. For example, the environmental and/or endogenous cues that initiate and terminate a torpor bout, as well as the mechanism of control over arousal frequency and duration, are unknown. It is also unclear whether hibernation and daily torpor are accomplished through the same physiological mechanisms.

Evolution of heterothermy

It is widely believed that heterothermy is plesiomorphic (ancestral) to mammals. Hibernation and daily torpor are widespread in all three major clades of mammals, including echidnas (Grigg and Beard, 2000) and a phylogenetically diverse range of marsupial and placental mammals (Geiser and Ruf, 1995). Although it is unknown whether the same physiological mechanisms underlie heterothermy in all mammals (see above), many aspects of heterothermy are consistent across all three groups. For example, in all cases, T_b during a torpor bout falls to within a few degrees of T_a , and prolonged periods of heterothermy are punctuated by periodic arousals to euthermia. Further evidence for a plesiomorphic origin of heterothermy comes from reptiles, many of which show daily T_b fluctuations reminiscent of mammalian heterotherms (without endogenously generated arousals to euthermia); Malan (1996) has argued

that hibernation and daily torpor may be derived from this ancient daily pattern of T_b fluctuation.

Conversely, Geiser (1994, 1998) has suggested that hibernation is relatively easy to evolve, and therefore could have arisen multiple times in mammals. He argues that at the time when the last common mammalian ancestor lived, climatic conditions would not have made heterothermy favorable (Geiser, 1994). Additional support for this hypothesis comes from the fact that eutherian and marsupial mammals differ in the timing of development of heterothermy as it relates to endothermy (Geiser, 2008), thus suggesting that heterothermy evolved separately in these two clades.

Even less is known about how the capacity for heterothermy has changed and evolved in specific clades of mammals. For example, within the Glires (Rodentia and Lagomorpha), only rodents outside of the Ctenohystrica (Blanga-Kanfi et al., 2009) utilize heterothermy (Figure 1.1). There are three equally parsimonious scenarios for the patterns of heterothermy observed: (A) The last common ancestor was not a heterotherm. Rodents gained heterothermy, but rodents of the Ctenohystrica, which do not show any heterothermy, subsequently lost it. (B) The last common ancestor was not a heterotherm, and heterothermy was gained in each of the two clades of rodents that deploy this trait (the squirrel-related clade and mouse-related clade; Blanga-Kanfi et al., 2009). (C) The last common ancestor was a heterotherm, and lagomorphs and Ctenohystrica each lost heterothermy. Knowing the ancestral condition for the Glires could shed light on the evolution of heterothermy within the larger clade Euarchontoglires (Primates, Scandentia, Dermoptera, Lagomorpha, and Rodentia; Springer et al., 2003; Wible et al., 2007).

One of the most salient problems in resolving evolutionary questions about heterothermy is the lack of available data on heterothermy in extinct taxa. As discussed below, teeth – commonly preserved post-mortem – offer a possible solution to this problem.

Rodent incisors as tools for studying hibernation

Teeth, by virtue of their incremental formation, have been shown in some cases to reflect physiological events, including hibernation (Klevezal and Mina, 1990; Klevezal, 1996; Trunova and Lobkov, 1997; Trunova and Klevezal, 1999; Selkova, 2003; Rinaldi, 1999a, 1999b; Goodwin et al., 2005; Goodwin and Ryckman, 2006; Klevezal and Lobkov, 2008). Ever-growing teeth, such as the incisors of rodents and lagomorphs, are ideal for studies of hibernation, due to their high growth rate and consequent sensitivity to physiological disruption (Klevezal, 1996).

Tooth structure and growth

Vertebrate teeth are comprised of three tissues. Enamel covers the outside of the tooth's crown and is the hardest, with >96% mineralized with hydroxyapatite. Cells called ameloblasts secrete enamel on the tooth's surface prior to eruption, but are lost as the crown emerges into the oral cavity. In teeth with determinate growth, enamel is secreted only prior to the eruption of teeth, and can therefore only serve as a recorder of physiological perturbations early in an animal's life.

Cementum covers the outside of the root in teeth with determinate growth, and covers a small area at the base of the tooth in ever-growing teeth, which are unrooted. It is the least mineralized – approximately 50% - of all the dental tissues. Cementum attaches the periodontal ligament to the tooth; the periodontal ligament, in turn, is anchored to the jaw and holds the tooth within its alveolus and withstands the forces of mastication. Cementum is deposited throughout

life, and incremental structure reflects seasonal variation in rate of deposition and mechanical stress on the teeth (Lieberman, 1993).

Dentin comprises the bulk of the tooth body. Cells surrounding the central pulp cavity – called odontoblasts – secrete the organic component of dentin (mostly collagen), which is subsequently mineralized with hydroxyapatite crystals. When mineralization is complete, dentin is approximately 70% mineralized. Odontoblasts secrete organic matrix on a circadian basis (e.g., Schour and Steadman, 1935; Rosenberg and Simmons, 1980; Erickson, 1996; Klevezal, 1996; Rinaldi, 1999a). Of the dental tissues, dentin is best suited to studies of hibernation, given its ongoing deposition throughout life, circadian periodicity, and consequent sensitivity to physiological perturbation.

It is convenient to think of the dentin component of an ever-growing rodent incisor as a stack of cones. Each day, odontoblasts lining the pulp cavity deposit a new cone of dentin at the base and inside margin of the tooth; with the addition of each layer, the entire stack is pushed forward into the oral cavity (Schour and Steadman, 1935) (Figure 1.2A). Simultaneous attrition at the incisor apex maintains the tooth's length roughly constant (Schour and Steadman, 1935).

If cut in transverse section, these circadian dentin layers are visible as a series of concentric circles around the pulp cavity, called von Ebner lines (e.g., Schour and Steadman, 1935; Rosenberg and Simmons, 1980) (Figure 1.2B). Daily layers are also visible on the medial surface of the tooth, which lacks enamel. As dentin cones are deposited, the base of each cone remains exposed on the tooth surface, and as they stack atop each other they form a series of ridges and valleys (Figure 1.2C). The grooves between layers, called periradicular bands, have been shown to correspond to von Ebner lines (Rinaldi, 1995, 1999b). The resulting surface topography, viewed under an oblique light source, is visible as alternating dark and light bands, obviating the need for destructive histological sectioning (Rinaldi 1995, 1999a, 1999b).

Evidence of hibernation in rodent incisors

Numerous studies from the last three decades have documented distinct features in the incremental dentin of rodent incisors that are attributable to hibernation (Klevezal and Mina, 1990; Rinaldi, 1999a, 1999b; Trunova and Klevezal, 1999; Trunova, 2001; Selkova, 2003; Goodwin et al., 2005; Goodwin and Ryckman, 2006; Klevezal and Shchepotkin, 2012; Klevezal et al., 2012); the specific morphology differs depending on whether the markings are viewed in cross section or on the medial tooth surface.

Trunova and Klevezal (1999) identified three “types” of hibernation marks in rodent incisor histological cross sections. Type I marks, characteristic of *Spermophilus*, *Uroditellus*, *Marmota*, and *Mesocricetus*, consist of several light and dark bands of decreasing width. Type II marks, found in several species of glirids and dipodids, have a single dark and light band. Type III marks, identified only in *Tamias*, have bands that stain the same way as normal, circadian bands, but become progressively narrower. Hibernation marks in histological cross section have also been documented in numerous other studies (Klevezal and Mina, 1990; Trunova and Lobkov, 1997; Trunova, 2001; Selkova, 2003; Klevezal and Lobkov, 2008).

Medial incisor surfaces have been studied in relatively fewer taxa, including marmots (*Marmota*), ground squirrels (*Uroditellus*), and prairie dogs (*Cynomys*) – all members of the Marmotini tribe of the family Sciuridae (Rinaldi 1999a, 1999b; Goodwin et al., 2005; Goodwin and Ryckman, 2006; Klevezal and Lobkov 2008). Hibernation is associated in these taxa with a

depression in the incisor dentin and fine, indistinct “hibernation increments” within the depression (Rinaldi, 1999a, 1999b; Goodwin et al., 2005; Goodwin and Ryckman, 2006).

The causes of formation of hibernation marks are not known; Trunova and Klevezal (1999) suggested that marked slowing of dentin deposition plays a role, and that the fine layers comprising the mark may correspond in some way to arousals or activity during the hibernation season. However, in sciurids studied to date, hibernation increments are not circadian (Rinaldi, 1999a; Goodwin et al., 2005) and do not correspond to the number of arousals (Rinaldi, 1999a); the width of the hibernation mark also does not track total time in hibernation (Goodwin et al., 2005). Although little is known about how and why the hibernation mark forms, it is a reliable indicator that hibernation has occurred, and provides an unprecedented opportunity to study the evolution of hibernation in the fossil record, as teeth are among the hard parts most commonly preserved.

All studies of hibernation marks to date have examined individuals hibernating at low winter temperatures for relatively long intervals (e.g., Klevezal and Mina, 1990; Trunova and Lobkov, 1997; Rinaldi, 1999a; Trunova and Klevezal, 1999; Selkova, 2003; Goodwin et al., 2005; Goodwin and Ryckman, 2006). However, some species, such as *Cheirogaleus medius*, the fat-tailed dwarf lemur, and *Tachyglossus aculeatus*, the short beaked echidna, hibernate under more temperate conditions (Grigg et al., 2004; Dausmann et al., 2009). There is no available information about (a) how long an individual must be torpid in order to produce a hibernation mark and (b) whether or not there is a critical minimum body temperature (or metabolic rate) that must be attained before the mark is produced. Considering that much of rodent evolution played out during the Paleocene and Eocene, which were warm and temperate relative to today’s climate (reviewed in Markwick, 1998; Huber and Nof, 2006), it is of substantial interest to know if these climatic conditions were conducive to the formation of a hibernation mark.

Overview of experiments

This dissertation is broadly focused on mammalian thermoregulation, with emphasis on the adaptive value and evolution of select thermoregulatory traits. Chapter 3 describes an experiment wherein the adaptive value of fur was tested during growth. Juvenile Siberian hamsters (*Phodopus sungorus*) were shaved or left fully furred, and were housed either at 10°C or 23°C as food intake, body mass, and length were measured. Shaving and cold exposure additively increased energetic requirements, resulting in higher food intake; no differences in somatic measures were detected. This experiment demonstrated that there is no mandatory energetic tradeoff between growth and thermoregulation when food is abundant, and although fur decreases energetic costs, it is not essential for normal growth.

The remaining chapters focus on mammalian hibernation and the use of ever-growing incisors as a potential tool to study hibernation in evolutionary and recent historical contexts. Turkish hamsters (*Mesocricetus brandti*) are a tractable model organism for studying hibernation in the laboratory, but despite decades of use (e.g., Hall and Goldman, 1980; Lyman et al., 1981; Hall et al., 1982; Hall and Goldman, 1982; Darrow et al., 1986; Goldman et al., 1986; Goldman and Darrow, 1987; Goldman, 1989; Bartness et al., 1991; Yigit et al., 2008), a detailed understanding of hibernation in this species was lacking. As such, Chapter 4 describes various hibernation parameters in Turkish hamsters, including torpor bout depth, duration, and frequency, as well as rates of entry into and arousal from torpor bouts. Hamsters were monitored using surgically implanted temperature transmitters, and were housed at either 5°C or 13°C. In

addition to reporting basic descriptive parameters, this study revealed that Turkish hamsters routinely generate short, shallow torpor bouts interspersed among more profound bouts, and that females and males differ in the length and depth of torpor bouts.

Chapter 5 explores the effects of hibernation, as well as day length and T_a , on incremental dentin in Turkish hamster incisors. T_b of hibernating hamsters at 5°C or 13°C was monitored using surgically implanted transmitters, and teeth were extracted, photographed, and analyzed following euthanasia. Teeth were compared to those of control hamsters, housed in long day or short day photoperiods at room temperature. Teeth of hibernators at both T_a s were characterized by at least one region of sharply defined, narrow increments, and the number and cumulative width of these increments were related to arousals from torpor. Short days also produced qualitative and quantitative differences in dentin increments, as compared to long day controls. This study was the first to document circadian increments and hibernation morphology on incisor surfaces in a non-sciurid rodent, the first to demonstrate a link between tooth morphology and arousals, and suggests that hibernation leaves a distinct signature on dentin regardless of minimum T_b during torpor.

Chapter 6 describes a pilot study using hard X-ray micro-tomography to image hibernation marks in rodent incisors, as a potential non-destructive tool for studying hibernation in historically collected or fossil specimens. Teeth from hibernating Turkish hamsters were scanned at Lawrence Berkeley National Laboratory's Advanced Light Source, and images obtained from these scans were compared to histological cross-sections of the same teeth. Images obtained by micro-tomography and by histological sectioning yielded comparable results. A few specimens of several sciurid species from UC Berkeley's Museum of Vertebrate Zoology were also scanned; a putative hibernation mark was identified in 2 out of 9 individuals. This technology shows promise for nondestructively studying incremental structure in teeth, particularly where specimens cannot be altered or destroyed by conventional histological preparation.

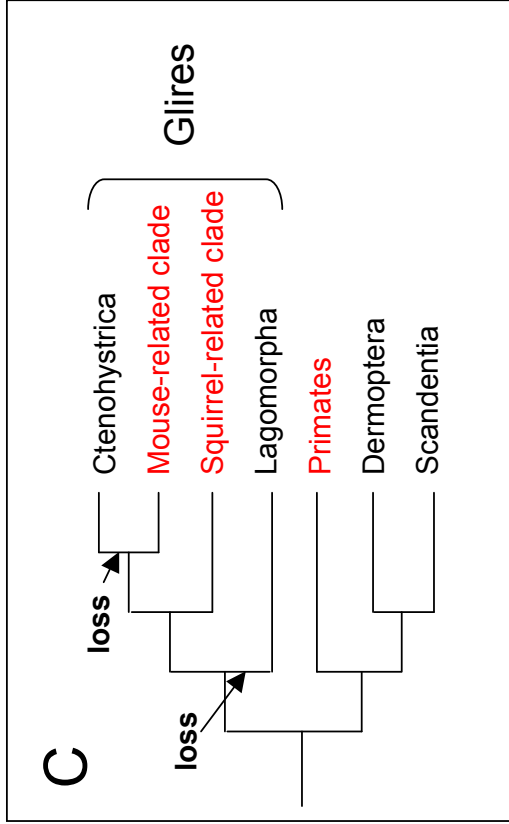
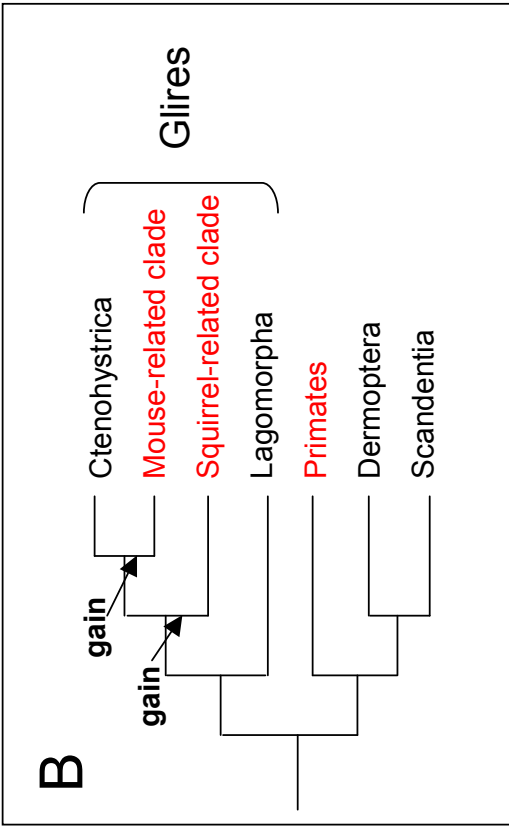
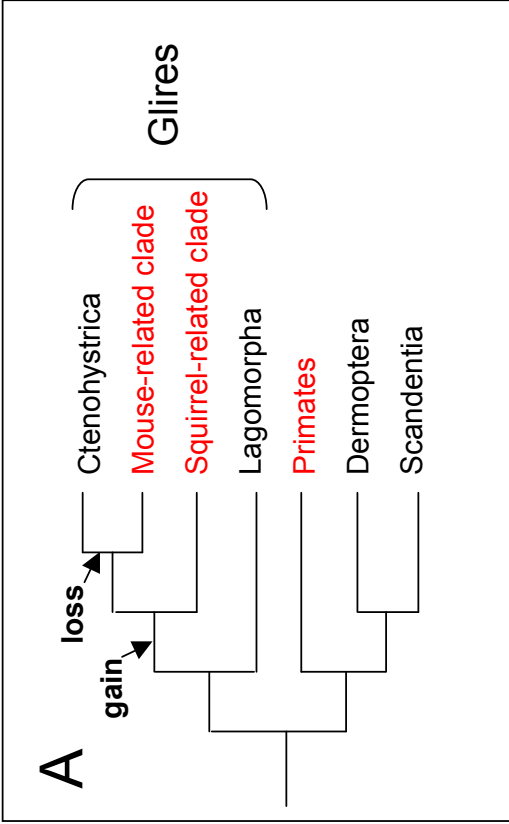


Figure 1.1. Possible scenarios of heterothermy evolution in the Glires. See text for descriptions. Clades in red include some heterothermic taxa. Families in the squirrel-related clade are Apodontidae, Sciuridae, and Gliridae. Families in the mouse-related clade are Anomaluridae, Pedidae, Castoridae, Heteromyidae, Geomyidae, Dipodidae, Spalacidae, Muridae, and Cricetidae. The Ctenohystrica include Ctenodactylidae, Hystricidae, Bathyergidae, Thryonomyidae, Petromuridae, and the Caviomorpha. Possible gains and losses of heterothermy in Primates, Dermoptera, and Scandentia are not shown. Phylogeny after Springer et al., 2003, Wible et al., 2007, and Blanga-Kanfi et al., 2009.

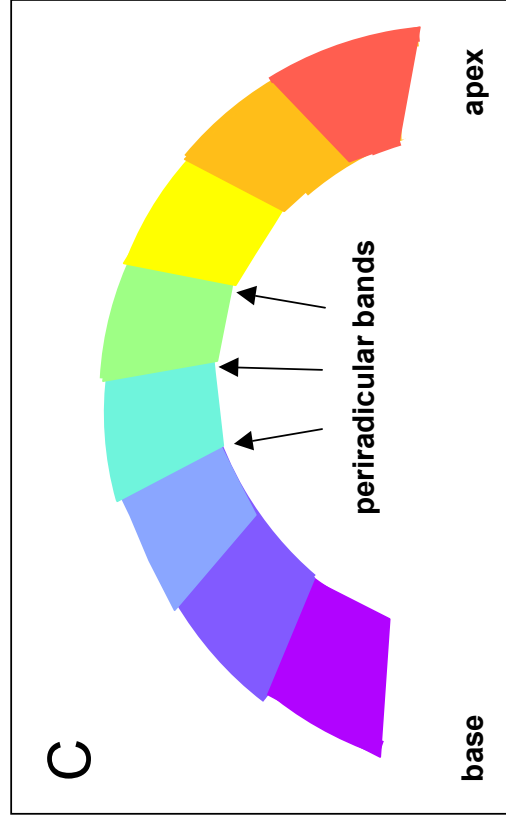
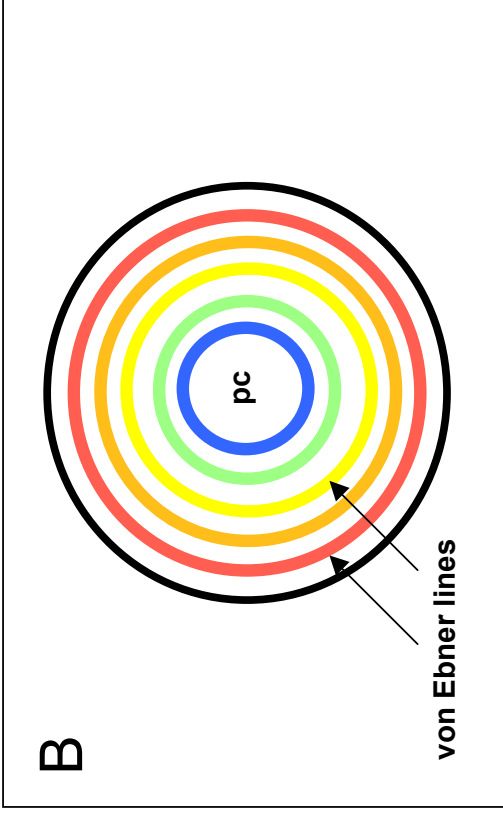
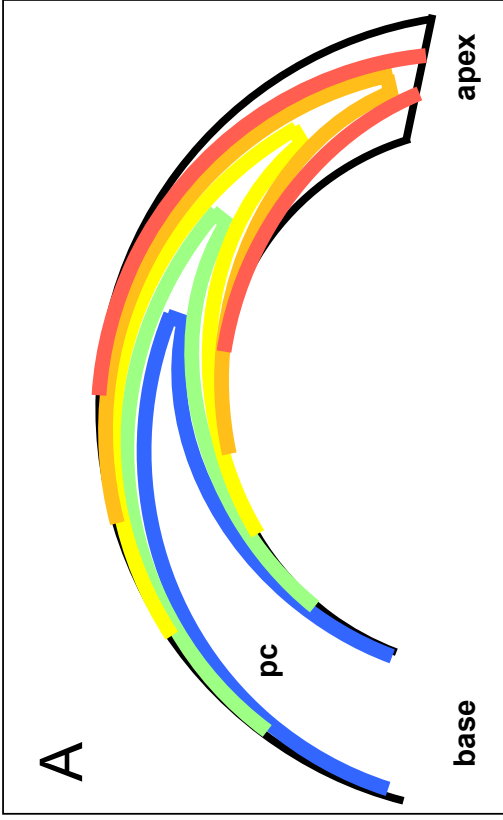


Figure 1.2. Simplified schematic showing incremental structure and growth of a rodent incisor. **(A)** An incisor cut in longitudinal section. Daily cone-shaped layers (red, orange, yellow, green, blue) are deposited at the base and inside margin of the pulp cavity (pc). **(B)** Cut in transverse section, daily layers (von Ebner lines) are visible as concentric circles around the pulp cavity. **(C)** Rims of dentin cones remain exposed on the medial surface of the incisor, forming a series of ridges and grooves; these grooves are called periradicular bands, and correspond to von Ebner lines.

Chapter 2

General Methods

Animals

All animal procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley (institutional approval # R084-0911C) and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Polypropylene cages provisioned with Tek-Fresh Lab Animal Bedding measured 27×16×13 cm for Siberian hamsters and 46×25×19 cm for Turkish hamsters. All hamsters had *ad libitum* access to food (Purina Rodent Chow 5015 for Siberian hamsters and Harlan Teklad Rodent Diet 8664 for Turkish hamsters) and water. All hamsters were euthanized by carbon monoxide inhalation followed by cervical dislocation.

Induction of reproductive quiescence and hibernation

Male and female Turkish hamsters were transferred to short days (SDs) with a 10L photoperiod (10 h light/day, lights on at 0700) at 22±2°C for 4-6 weeks; SD treatment renders females anovulatory and induces testicular regression in males (Stetson and Hamilton 1981, Hong et al. 1986, Hall et al. 1982). Reproductive quiescence, in turn, facilitates entry into hibernation (Hall and Goldman 1980, 1982, Hall et al. 1982). These hamsters were subsequently moved to cold chambers maintained at either 5±1°C or 13±1°C, with the same 10L light cycle. 5°C was selected because previous studies documented hibernation in Turkish hamsters at T_a s of 3-6°C (e.g., Lyman et al., 1981, 1983, Darrow et al. 1986, Goldman and Darrow 1987, Bartness et al. 1991); 13°C was selected because in a preliminary study it was the highest T_a at which our population would reliably enter hibernation.

Surgical implantation of radiotransmitters and monitoring of T_b

In Turkish hamsters, T_b was recorded telemetrically using radiotransmitters (model VM-FH; approx. 1.5 cm³ and 3 g; MiniMitter, Sunriver, OR) while hamsters were housed in cold chambers. Transmitters were coated in wax and calibrated using a water bath (30-38°C) prior to implantation. Hamsters were deeply anesthetized using isoflurane vapors, and transmitters implanted intraperitoneally via a single midline incision, which was closed using sterile suture. Hamsters received perioperative injections of 0.05 ml meloxicam (5mg base/mL) and 0.3 mL of dilute buprenorphine (0.3 mg base/mL diluted 1:10 in sterile saline). The same postoperative doses of analgesics were administered 8 h later and every 8 h thereafter as needed. Hamsters recovered in SD, 22±2°C conditions for at least 10 days prior to transfer to cold chambers. Once in the cold chambers, T_b data were collected every 10 min via receiver boards under each animal's cage. Data were transmitted to and stored on a computer by the program Dataquest (St. Paul, MN).

Torpor thresholds

To account for differences among individuals (Barclay et al. 2001), each Turkish hamster's mean normothermic T_b was calculated from data obtained during the first 72 h in the cold, and torpor thresholds were set 1°C below the lowest T_b exhibited by each individual during this interval. To identify the beginning and end of torpor bouts, T_b had to be at or below the threshold for three consecutive measurements (beginning of torpor), or above the threshold for three consecutive measurements (end of torpor).

Extraction of teeth

Postmortem, hamster skulls were macerated in tap water for 1-3 weeks, until incisors slid easily out of their alveoli. Teeth were dried and marked at 3-4 mm intervals with India ink to establish points of reference.

Statistics

All tests were performed using JMP 7.0 (SAS Institute Inc., Cary, NC). Differences were considered significant if $P < 0.05$. All data are presented as mean $\pm$ S.E.M.

Chapter 3

Influence of Pelage Insulation and Ambient Temperature on Energy Intake and Growth of Juvenile Siberian Hamsters¹

Introduction

Mammals deploy behavioral, physiological, and/or anatomical traits to control the rate of heat exchange with the environment, thereby reducing the energetic cost of thermoregulation at low T_a . Huddling, nesting, postural and locomotor changes, and microhabitat selection are among behaviors that reduce heat loss (Sealander, 1952; Vogt and Lynch, 1982; Conley and Porter, 1986; Kenagy and Pearson, 2000; Kauffman et al., 2003); physiological adaptations include vasoconstriction and torpor (Vogt and Lynch, 1982; Geiser and Ruf, 1995), and anatomical traits include insulation in the form of adipose tissue and fur (Scholander et al., 1950; Hammel, 1955; Conley and Porter, 1986; Rosen and Renouf, 1997). Nonetheless, maintenance of a high stable T_b by metabolic means is energetically costly, particularly when there is a wide temperature differential between an individual and its surroundings (Vogt and Lynch, 1982; Masuda and Oishi, 1988; Kauffman et al., 2001a, 2003), and numerous studies implicate an energetic tradeoff between thermoregulation and growth. For example, California mouse (*Peromyscus californicus*) pups housed with their fathers lose less weight and begin gaining weight sooner after weaning than those with absent fathers; this difference may be attributable to lower energy expenditure on thermoregulation by pups when their fathers are present (Dudley, 1974). In rabbit pups, optimal huddle size increases at low T_a (Rodel et al., 2008), indicating that small huddles or solitary pups incur higher thermoregulatory costs than larger huddles. Pups maintained alone or with few littermates have lower T_b s, are significantly less efficient at converting milk into body mass, and accrue less adipose tissue than pups raised in larger huddles (Bautista et al., 2003; Gilbert et al., 2007).

This study tested for an energetic tradeoff between growth and thermoregulation in newly weaned juvenile Siberian hamsters, and the possible adaptive role of fur in maintaining energy balance during growth. Several studies have documented the insulative properties of fur (Scholander et al., 1950; Hammel, 1955; Zhao and Cao, 2009) and its role in reducing thermoregulatory costs (Kauffman et al., 2001a, 2001b, 2004). However, the potential significance of fur during growth has received scant attention. Most rodents, including Siberian hamsters, rapidly gain body mass for at least six weeks post-weaning (Yellon and Goldman, 1984; Park et al., 2003). In the wild it is likely that *Phodopus* are solitary during this time, based on observations of aggression between same-sex conspecifics (Wynne-Edwards and Lisk, 1987) and low field population densities (Wynne-Edwards et al., 1992). Thus, contact with conspecifics during post-weaning growth is unlikely to feature in thermoregulation, and insulative fur likely becomes increasingly important for diminishing thermoregulatory costs during this energetically demanding time.

¹ Batavia, M., A. Matsushima, O. Eboigboden, and I. Zucker. 2010. Influence of pelage insulation and ambient temperature on energy intake and growth of juvenile Siberian hamsters. *Physiology and Behavior* 101: 376-380.

This experiment tested the impact of a normal insulating pelage on growth and food consumption after weaning under conditions of moderate (23°C) and lower (10°C) T_a s. I reasoned that weaned pups deprived of fur by shaving and/or housed at 10°C would respond to a cold challenge in one of two ways: they might consume the same amount of food as furred pups at 23°C and grow more slowly, an outcome consistent with a previous study (Gilbert et al., 2007), in which isolated or small huddles of rabbit pups suckled the same amount and grew less than pups maintained in larger groups. Alternatively, if food intake and assimilation are not already maximized, then cold-challenged pups might increase food intake and grow on the same trajectory as furred pups at 23°C. This outcome would be consistent with studies on adult rodents, in which increased thermoregulatory demands are accompanied by elevated food consumption and maintenance of normal body mass (Masuda and Oishi, 1988; Kauffman et al., 2001a; Paul et al., 2010). The present study assessed which of these outcomes emerged in cold-challenged newly weaned Siberian hamster pups.

Methods

Animals

Twelve breeding pairs of adult hamsters, aged 5-7 months, were reared from birth at $23\pm 2^\circ\text{C}$ and on a 14L:10D (14L) photoperiod (14 hours of light per day; lights off at 1800h PST). Litters were left undisturbed until weaning at 18 days of age, and then pups were individually housed and provisioned with enough bedding for sanitation, but insufficient for nest construction. Half of the pups were kept at $23\pm 2^\circ\text{C}$ in 14L, and the rest were transferred to a cold chamber maintained at $10\pm 2^\circ\text{C}$ and with the same 14L photoperiod.

T_a , fur removal, and somatic measures

At 20 days of age, hamsters at each T_a were assigned to one of three groups balanced with respect to body mass, sex, and natal litter. Hamsters were anesthetized by exposure to isoflurane vapors and handled for equal amounts of time. Control hamsters were left unshaved, a second group of control hamsters had a small patch of fur (approximate area 1 cm², mass 5 mg) removed from the lower back to control for the effects of the shaving procedure, and the third group had all dorsal fur removed with an electric clipper (approximate area 14 cm², mass 90 mg). Body length (nose to tail distance) was measured to the nearest mm in anesthetized hamsters (Borer and Kaplan, 1977), and re-measured on days 25, 30, and 35, followed by re-shaving on day 35. All dorsal fur was shaved in control hamsters at the conclusion of the experiment on day 35. Cold room hamsters were briefly removed to 23°C for measurements requiring anesthesia, and were completely recovered before replacement in the cold. Time outside the cold room did not exceed 10 min per measurement.

Body mass was measured to the nearest 0.1 g on days 18 and 20, and every third day thereafter until animals were 35 days old. Mass was measured only after hamster cheek pouches were emptied, if necessary. The mass of fur removed from experimental animals (90 mg) was less than the margin of error for body mass (0.1 g), and was therefore presumed negligible. Shaving and measurements were completed between 1400h and 1600h PST.

Food consumption

On the day of weaning, cages were provisioned with ~120 g of food. Pellets remaining in the food hopper were weighed on day 20, and every third day thereafter until day 35. Pellets hoarded on cage bottoms or in animals' cheek pouches were collected and included in food mass measurements. Total food remaining on a given day was subtracted from the previous value and divided by the number of days between measurements to determine mean 24-h intake. Fresh food pellets were added after each measurement to maintain an excess of food, with fresh food replacing older food once a week. Because food absorbs moisture at low temperatures, pellets were acclimated to each experimental T_a for approximately 7 days before use.

Sample sizes

Due to staggered birth dates, initial pup assignment to different treatments did not adequately balance groups with respect to day 20 body mass. Correct day 20 equilibration of groups was achieved post-hoc by deleting data from a few hamsters without regard to post-day 20 data and prior to performing statistical analyses.

As previously reported (Kauffman et al., 2001a), unshaved and small patch control groups did not differ significantly with respect to body mass, length, or food intake, and were combined into a single control group for purposes of comparison to shaved animals (Table 3.1).

Statistical analyses

Three-way ANOVAs assessed the effects of shaving treatment, T_a , sex, and interactions between these factors on mean body mass, body length, and daily food intake on each measurement day, as well as total body mass and length gains, total food intake, and total efficiency (total mass gain/ total food intake). Where more than two conditions were compared, significant results were followed by post-hoc Tukey-Kramer HSD tests.

Results

Fur mass and regrowth

Complete dorsal shaving of experimental animals on day 20 yielded approximately 90 mg of fur, and a fur density of 6 mg/cm²; re-shaving these animals yielded approximately 64 mg of fur, and a fur density of 3 mg/cm². Control animals dorsally shaved for the first time on day 35 had approximately 150 mg of fur, and a fur density of 9 mg/cm².

Body mass and length

Neither shaving treatment nor T_a affected body mass or length. Males were significantly heavier than females on all days of the experiment ($P < 0.01$) (Figure 3.1), and gained significantly more mass between days 20-35 ($P < 0.001$; Table 3.1). Males were significantly longer than females on all days of the experiment ($P < 0.01$) (Figure 3.2), and gained significantly more total length between days 20-35 ($P < 0.001$) (Table 3.1).

There were no significant interactions between shaving treatment, T_a , and sex, except on day 35, when there was a significant treatment-sex interaction effect on body length ($P < 0.03$). Subsequent post-hoc analyses, however, failed to reveal significant differences attributable to a treatment-sex interaction.

Food intake and efficiency

There were no significant differences in daily food intake on day 20. Beginning on day 23, hamsters at 10°C ate significantly more food than those at 23°C ($P < 0.001$) (Figure 3.3); this difference persisted for the duration of the experiment. Hamsters at 10°C consumed significantly more total food from days 20-35 than those housed at 23°C ($P < 0.001$) (Table 3.1).

Daily food intake of shaved hamsters was significantly higher than that of control hamsters beginning on day 26 ($P < 0.05$) (Figure 3.3), and persisted for the duration of the experiment. Shaved hamsters consumed significantly more total food from days 20-35 than control hamsters ($P < 0.01$; Table 3.1).

Sex did not affect daily or cumulative food intake. There were no significant interactions between shaving treatment, T_a , and sex, except on day 35, when there was a significant temperature-sex interaction effect on daily intake ($P < 0.05$). There were no significant differences attributable to a temperature-sex interaction in subsequent post-hoc analyses.

Males were significantly more efficient than females at converting food into body mass ($P < 0.001$) (Figure 3.4); hamsters held at 23°C were more efficient than those at 10°C ($P < 0.001$) (Figure 3.4). Shaving did not affect efficiency of food conversion into body mass gains, and there were no significant interactions between shaving treatment, T_a , and sex.

Discussion

Somatic growth of juvenile Siberian hamsters was not compromised by fur removal, exposure to low T_a , or a combination of these factors. Neither body mass nor body length differed significantly between hamsters at 23°C and 10°C, or between furred and furless hamsters. However, both fur removal and low T_a resulted in increased food intake. The highest daily food consumption occurred in shaved hamsters at 10°C, suggesting an additive effect of fur loss and low T_a . A similar additive effect of shaving and cold was reported for lactating female Siberian hamsters (Paul et al., 2010).

These results are consistent with studies on adult rodents demonstrating maintenance of body mass and increased food intake in response to thermal challenges (e.g., Masuda and Oishi, 1988; Kauffmann et al., 2001a; Paul et al., 2010), but differ from results obtained for rabbit pups, which did not increase milk intake despite higher thermoregulatory costs (Bautista et al., 2003; Gilbert et al., 2007; Rodel et al., 2008) and consequently compromised growth (Gilbert et al., 2007). A possible explanation for this species difference is that rabbit pups gain access to milk very briefly only once per day (Broekhuizen et al., 1986; Gilbert et al., 2007), and stomach capacity may limit intake, which is effectively maximized under normal conditions. The weanling hamsters in the present experiment had continuous access to food, and therefore greater opportunity to increase intake in response to thermal stress. Indeed, my data do not suggest that food intake reached a ceiling. At peak food consumption on day 35, shaved groups kept at 10°C consumed 6.4 g (females) or 7.4 g (males) per day, a modest elevation above the 4.4 g (females) or 4.3 g (males) consumed by furred animals at 23°C. By comparison, Paul et al. (2010) found

that daily consumption in shaved Siberian hamster dams nursing pups in the cold was 21.5g, compared to 4.0 g in non-lactating control females, and even this elevation was not deemed to have maximized food intake.

Thus, under conditions of unlimited food availability, my results argue against a mandatory energetic trade-off between growth and thermoregulation. Hamsters matched higher thermoregulatory demands by consuming more food and decreasing their efficiency of converting food into body mass. Energy from the additional food consumed presumably was directed toward thermoregulation, and as a result, the proportion of total energy allocated for growth changed even though absolute growth rate did not. Conditions of abundant or unlimited food are not uncommon in the natural world; over the course of the breeding season (March – October), wild Siberian hamster weanlings – particularly those weaned in the summer – may have access to virtually unlimited food (Weiner, 1987). The fact that neither intake capacity nor energy supply limits the intrinsic growth rate of this species raises the question of why these animals do not grow faster than they do, given the presumed benefits of reaching reproductive maturity and maximum size as quickly as possible (Ricklefs, 1969; Arendt, 1997). Possible explanations include limiting factors such as nutrient availability, maximum rate of cellular differentiation, or costs associated with rapid growth (e.g., less efficient use of nutrients and developmental error, as reviewed in Arendt (1997)).

Although the insulation provided by fur is not essential for supporting growth at its intrinsic rate when food is unlimited, my results show that pelage ameliorates the energetic cost of maintaining homeothermy in growing juvenile Siberian hamsters, as in adults (Kauffmann et al., 2001a, 2001b, 2004). Fur may be instrumental in allowing growth to proceed on a normal trajectory under conditions of reduced energy availability, which likely are encountered by early and late season weanlings in the wild (Weiner, 1987). In addition, I considered only the effects of fur loss on somatic growth; it remains possible that some aspect of growth and/or development not measured by this experiment was affected by the loss of insulation and the consequent increase in thermoregulatory costs. Finally, it is possible that partial re-growth of fur by day 35 reduced the strength of the thermal challenge, such that no trade-off between growth and thermoregulation was apparent.

Robust sex differences in growth and body mass of Siberian hamsters echo findings in other rodents (e.g., Kenagy, 1987; Millar, 1987; Plunkett et al., 2000; Gattermann et al., 2002). Males were heavier and longer than females throughout the experiment, without consuming more food, suggesting more efficient conversion of food into body mass by males than females. This sex difference persisted in hamsters subjected to fur removal and exposure to low ambient temperatures. Although there was no statistically significant interaction between sex, temperature, and shaving, at 23°C, females appear to have a more robust increase in food intake in response to shaving than do males. One possible explanation is that sex differences in the timing, pattern, and/or quantity of fur re-growth make females more vulnerable to thermal stress, such that a greater difference between shaved and unshaved individuals is apparent under the thermally moderate conditions of 23°C. However, this explanation is not consistent with the more robust effect of shaving on males at 10°C; thus, it seems likely that these non-significant trends are attributable to sampling error.

In conclusion, fur plays an important role in reducing the energetic costs of thermoregulation in growing Siberian hamsters, but is not essential for allowing growth to proceed at its intrinsic rate when food is in abundant supply. Scant information is available on the potential energetic trade-offs between growth and thermoregulation in mammals, and even

fewer studies address the possible evolutionary implications of such a trade-off (or lack thereof). Additional work on a phylogenetically widespread array of juvenile mammals would shed light on the energetics of growth and thermoregulation, as well as on the evolution of energy balance in growing mammals.

Table 3.1. Mean ($\pm$ S.E.M.) total mass gain, length gain, and food intake from days 20-35.

N	Sex	Treatment group	Temperature (°C)	Total mass gain (g)*	Total length gain (mm)*	Total food intake (g)†§
13	male	control	23	10.4 $\pm$ 0.7	11 $\pm$ 1	64.0 $\pm$ 1.6
13	male	control	10	9.7 $\pm$ 1.0	10 $\pm$ 1	82.7 $\pm$ 3.2
5	male	shaved	23	10.7 $\pm$ 0.2	11 $\pm$ 1	67.9 $\pm$ 1.0
6	male	shaved	10	11.1 $\pm$ 1.0	11 $\pm$ 1	93.7 $\pm$ 1.7
13	female	control	23	8.0 $\pm$ 0.7	9 $\pm$ 1	60.6 $\pm$ 2.5
8	female	control	10	8.9 $\pm$ 0.7	8 $\pm$ 1	84.7 $\pm$ 1.2
5	female	shaved	23	7.9 $\pm$ 1.0	8 $\pm$ 1	64.7 $\pm$ 3.1
5	female	shaved	10	7.7 $\pm$ 0.9	7 $\pm$ 1	91.2 $\pm$ 5.3

* Significant effect of sex (males > females; $P < 0.001$)

† Significant effect of temperature (10°C > 23°C; $P < 0.001$)

§ Significant effect of shaving (shaved > control; $P < 0.01$)

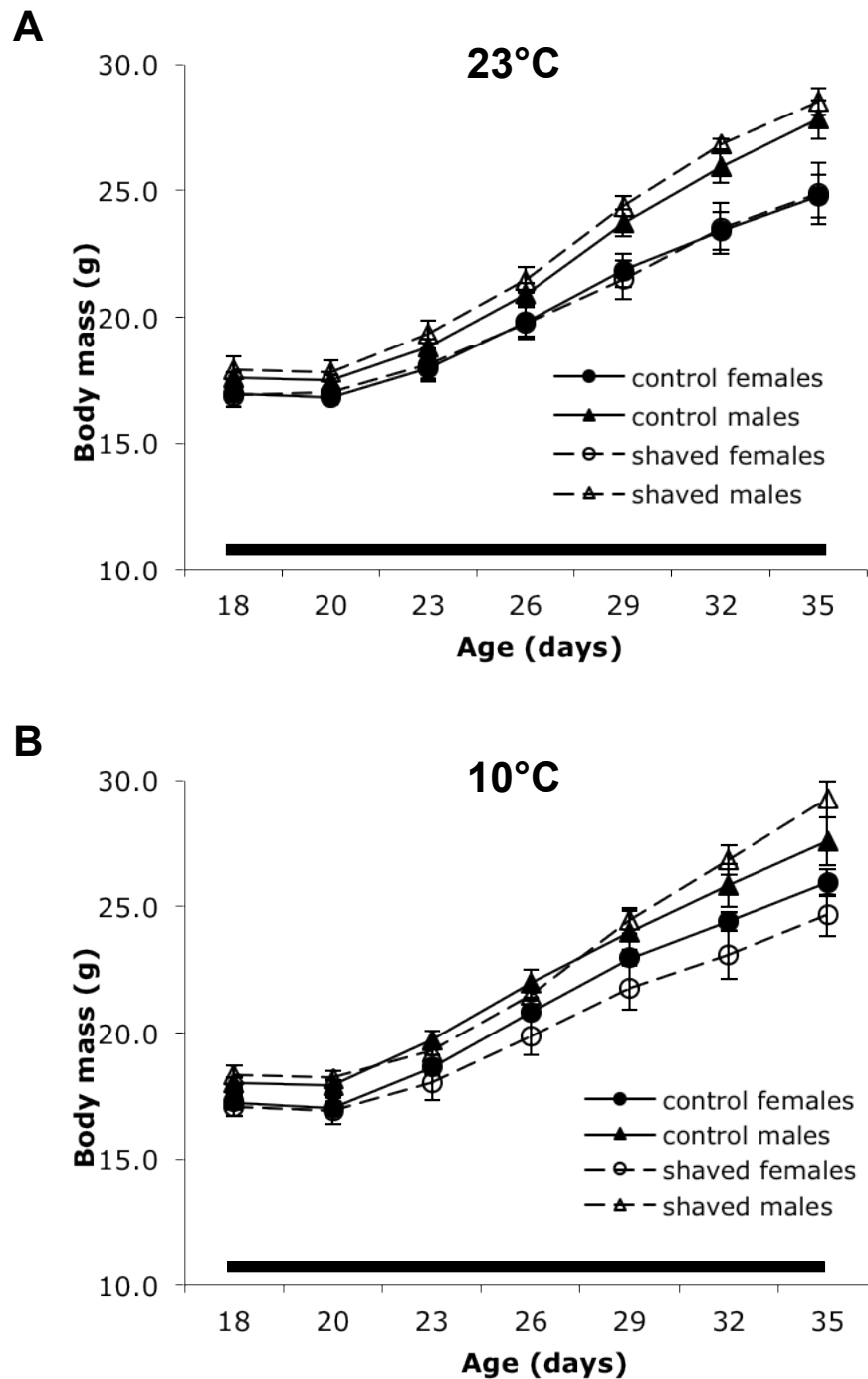


Figure 3.1. Mean ($\pm$ S.E.M.) body mass between days 18 and 35 for hamsters housed at 23°C (**A**) or 10°C (**B**). Males were significantly heavier than females on all days of the experiment ($P < 0.01$; black bar). There were no significant differences between control and shaved hamsters or between hamsters housed at 23°C and 10°C. There were no significant interactions between sex, T_a , and shaving treatment.

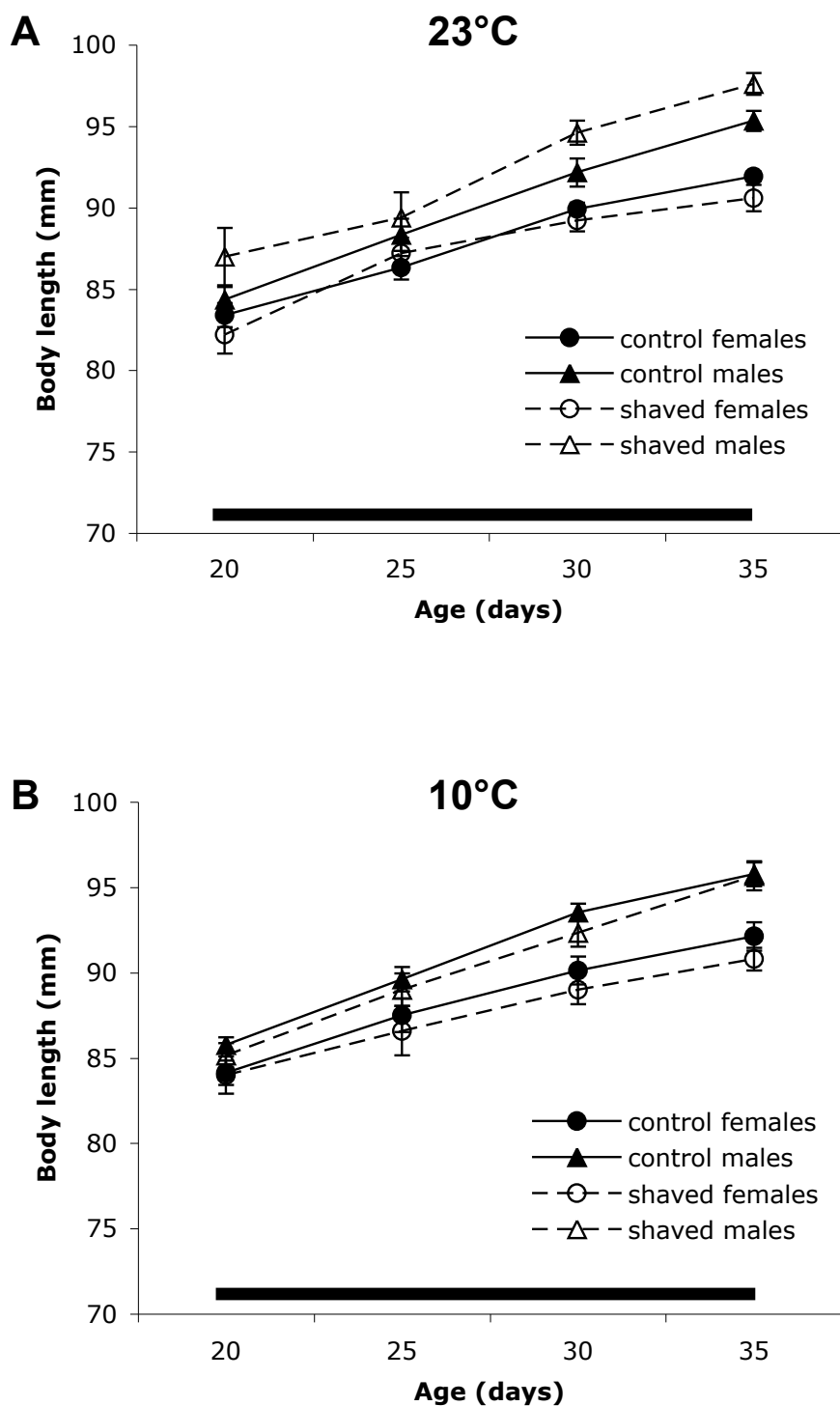


Figure 3.2. Mean ($\pm$ S.E.M.) body length between days 20 and 35. Hamsters housed at 23°C (**A**) or 10°C (**B**). Males were significantly longer than females on all days of the experiment ($P < 0.01$; black bar). There were no significant differences between control and shaved hamsters or between hamsters housed at 23°C and 10°C. There were no significant interactions between sex, T_a , and shaving treatment.

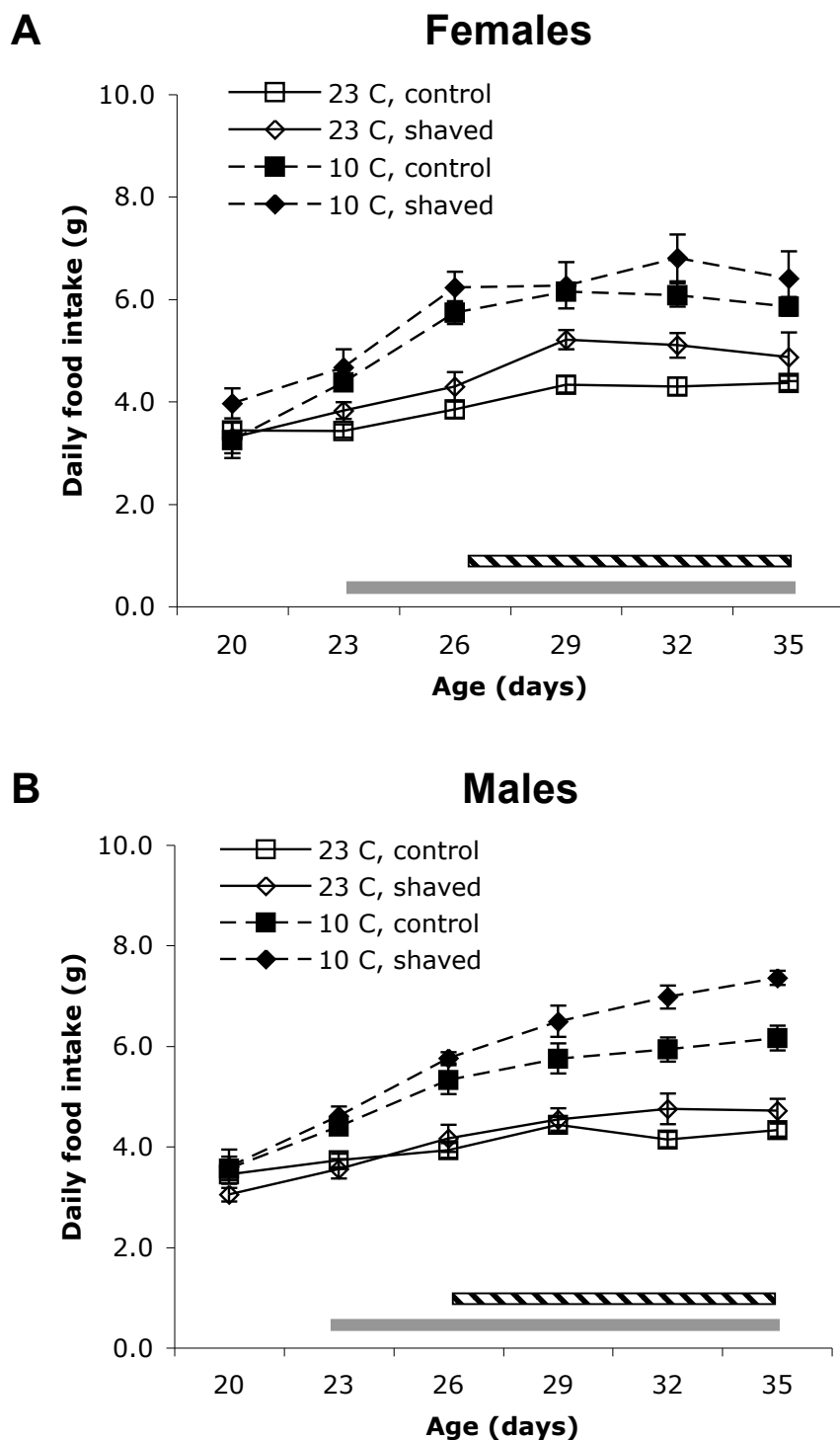


Figure 3.3. Mean ($\pm$ S.E.M.) daily food intake between days 20 and 35 in females (**A**) and males (**B**). Hamsters at 10°C consumed significantly more food per day than those housed at 23°C starting on day 23 ($P < 0.001$; gray bar). Shaved hamsters consumed significantly more food per day than controls starting on day 26 ($P < 0.05$; striped bar). There were no significant sex differences in daily food intake. There were no significant interactions between sex, T_a , and shaving treatment.

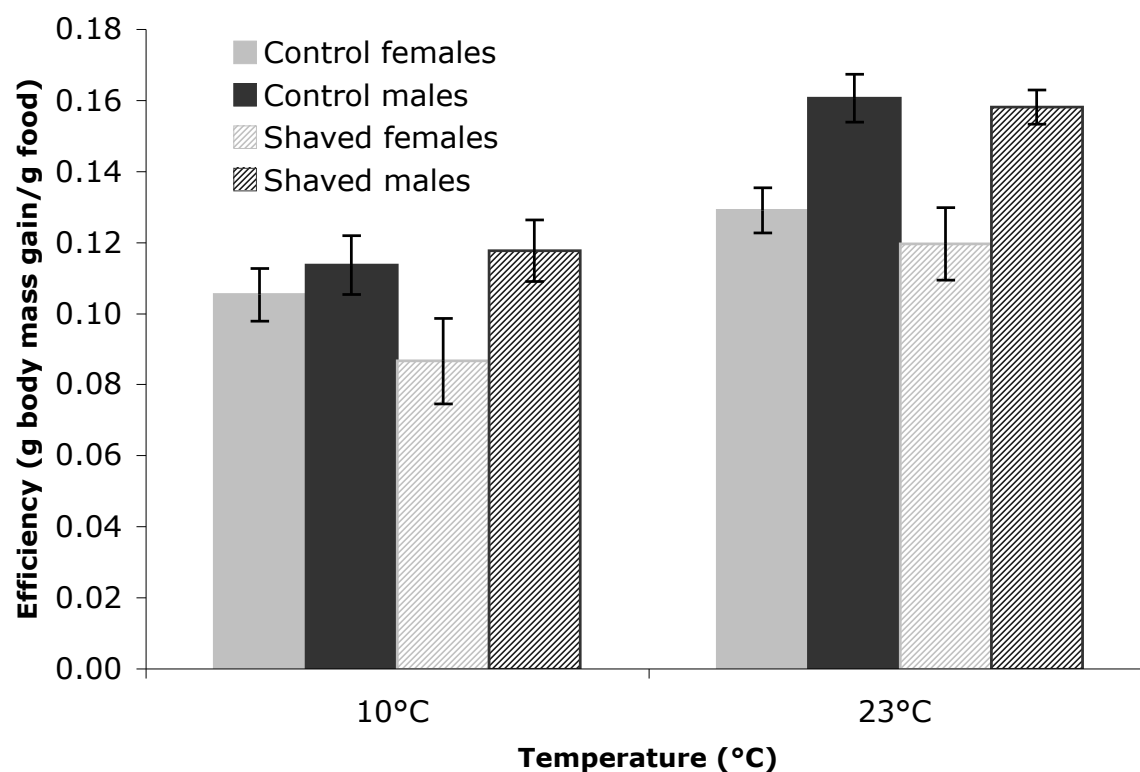


Figure 3.4. Mean ($\pm$ S.E.M.) efficiency (total mass gain/ total food consumed from days 20-35). Males were significantly more efficient than females ($P < 0.001$), and hamsters housed at 23°C were more efficient than those housed at 10°C ($P < 0.001$). There were no significant differences between control and shaved hamsters. There were no significant interactions between sex, T_a , and shaving treatment.

Chapter 4

Hibernation Patterns of Turkish Hamsters: Influence of Sex and Ambient Temperature¹

Introduction

Mammalian hibernation permits substantial energy savings under adverse environmental conditions (Lyman et al., 1982; Geiser and Ruf, 1995; Heldmaier et al., 2004), and is an important survival strategy for a phylogenetically diverse array of species (Geiser and Ruf, 1995; Lovegrove, 2012). Detailed description of heterothermic patterns provides the foundation for comparative analyses, and is a prerequisite for elaborating the evolutionary and physiological underpinnings of hibernation and daily torpor (e.g., Geiser and Ruf, 1995; Carey et al., 2003; Heldmaier et al., 2004; Lovegrove, 2012). Moreover, baseline information about typical hibernation behavior allows researchers to assess the impacts of changes in food availability, nutritional status, and/or climate change on hibernation (e.g., Lovegrove et al., 2001; Humphries et al., 2002, 2003; Angiletta et al., 2010). Finally, because hibernation affects other life history traits, including reproduction (e.g., Oxberry, 1979; Barnes et al., 1986; Turbill et al., 2011) and longevity (e.g., Lyman et al., 1981; Turbill et al., 2011, 2012), specification of hibernation patterns is essential to understanding seasonal adaptations of hibernators and provides a more comprehensive picture of a species' biology.

Turkish hamsters (*Mesocricetus brandti*) are of particular interest for studies of biological rhythms, including the circannual hibernation cycle, because they differ from virtually all other photoperiodic rodents. Most species undergo gonadal regression – a prerequisite for hibernation in males – in response to a surge in melatonin secretion, which in turn is stimulated by exposure to short days; removal of the pineal gland, which disrupts melatonin secretion, prevents gonadal regression (referenced in Butler et al., 2008). In contrast, Turkish hamsters undergo gonadal regression not only in response to long duration melatonin signals, but also in response to very short duration melatonin signals or the complete absence of melatonin; these conditions occur upon removal of the pineal gland and/or exposure to very long days (>17L) or constant light (Carter et al., 1982; Hong et al., 1986; Butler et al., 2008; Jarjisian and Zucker, 2011). Only one other species – the European hamster (*Cricetus cricetus*) – responds to pinealectomy in this manner (Masson-Pévet et al., 1987). In both species suppression of melatonin secretion in nature likely is limited to intervals during which hamsters are torpid.

Turkish hamsters have been a model organism for studies of hibernation as it relates to longevity (Lyman et al., 1981), reproductive endocrinology (Hall and Goldman, 1980; Hall et al., 1982; Hall and Goldman, 1982; Goldman et al., 1986; Goldman and Darrow, 1987), photoperiodism and melatonin (Hall and Goldman, 1982; Hall et al., 1982; Darrow et al., 1986; Goldman et al., 1986; Goldman and Darrow, 1987; Goldman, 1989), diet (Bartness et al., 1991),

¹ Batavia, M., G. Nguyen, K. Harman, and I. Zucker. 2013. Hibernation patterns of Turkish hamsters: influence of sex and ambient temperature. *Journal of Comparative Physiology B* 183: 267-277. DOI 10.1007/s00360-012-0706-3.

and oxidative stress (Yigit et al., 2008). Despite extensive use of Turkish hamsters in hibernation research, basic aspects of their hibernation behavior (e.g., torpor bout length, depth, frequency, inter-bout intervals, etc.) remain to be established.

Past estimates of hibernation characteristics for this species – derived exclusively from daily observations of posture, respiration, displacement of sawdust or oats on the dorsum, and/or responsiveness to a puff of air (e.g., Hall and Goldman, 1980; Hall et al., 1982; Lyman et al., 1983) – do not permit precise calculations of bout length, depth of torpor, or timing of entry into or arousal from torpor. Moreover, previous studies that maintained T_a as low as 3°C (Bartness et al., 1991) and as high as 10°C (Hall and Goldman, 1980, 1982; Hall et al., 1982) reported a range of estimates for bout duration, which is known to vary inversely with torpor T_b in other hibernating rodents (e.g., Twente and Twente, 1965; Geiser and Kenagy, 1988; Buck and Barnes, 2000). Some early studies of Turkish hamster hibernation conflated the effects of variable T_a s and day lengths (e.g., Lyman et al., 1983), which complicates evaluation of the relative contributions of these environmental factors to hibernation patterns.

In the present study Turkish hamsters were kept in a fixed short day length at one of two fixed T_a s (5°C and 13°C). Continuous telemetric monitoring of T_b was employed to precisely characterize hibernation behavior, and food intake was monitored as an index of energy consumption. In addition to providing detailed information on torpor bout characteristics, this study yields new insights into the effects of sex and ambient temperature on hibernation in this species.

Methods

Animals

Male (n=12) and female (n=12) Turkish hamsters from the local breeding colony (Butler et al., 2008) aged 5-12 months were maintained from birth in 16L (16h light/day, lights on at 0200) and 22±2°C. Hamsters were individually housed during the experiment. Data from three 13°C females were omitted from all analyses due to faulty transmitters (see below).

Cold chambers and recording of T_b

Hamsters were transferred to short days (10L; lights on at 0700) at 22±2°C to induce reproductive quiescence, and underwent surgical implantation of radiotransmitters. These procedures are described in detail in Chapter 2.

Hamsters were subsequently moved to cold chambers maintained at either 5±1°C or 13±1°C (males, n=6; females, n=6 at each T_a), with the same 10L light cycle. Males and females were distributed evenly within each cold chamber. Animal monitoring was carried out between 0800 and 1000 each day. Maximum and minimum cold chamber T_a s were recorded daily to the nearest 0.1 °C with a calibrated digital thermometer. Hamsters remained in cold chambers until transmitter batteries failed, at which point they were returned to 16L and T_a =22±2°C. T_b data were collected every 10 min via receiver boards under each animal's cage.

Torpor parameters

Each hamster's mean normothermic T_b and torpor thresholds were calculated as described in Chapter 2. Torpor bout duration was calculated as the amount of time spent at or below the threshold. Inter-bout interval (IBI) was defined as the time between the end of one torpor bout and the onset of the next.

Test bouts were defined as those in which the T_b decrease did not achieve a stable value. Deep torpor bouts were those wherein T_b reached a plateau a few degrees above T_a ; minimum T_b was measured at this nadir. The temperature difference between T_b and T_a ($T_b - T_a$) during deep torpor bouts was calculated using the minimum T_b and minimum T_a during each bout.

For test bout duration, minimum T_b , $T_b - T_a$, and IBI, mean values were calculated for each hamster; to avoid weighting data from individuals that generated higher than average numbers of bouts, these individual means were used in subsequent statistical analyses. Minimum and maximum values were used to generate individual ranges for test bout duration, test bout minimum T_b , and test/deep bout IBIs.

Hamsters at $T_a = 5^\circ\text{C}$ were not disturbed by experimenter presence in the cold chamber, but at $T_a = 13^\circ\text{C}$ hamsters sometimes stirred in response to the opening of the chamber door (essential for animal monitoring). Thus, times of entry into and arousal from torpor were analyzed only for hamsters held at $T_a = 5^\circ\text{C}$. Additionally, for deep bout duration, each hamster's maximum bout length was analyzed to reduce bias resulting from possible disturbance-induced arousal. Each hamster's longest deep torpor bout was also assessed to calculate its rate of entry into and arousal from deep torpor. Overall rates were calculated using normothermic T_b and minimum T_b as anchoring points. Rates of entry and arousal were also calculated over several T_b ranges, after Kauffman et al. (2001b) as described in Table 2.

Body mass and food intake

Body mass was recorded when hamsters were transferred to short days, as well as when they were placed in and removed from the cold chamber. Due to an oversight, four hamsters at $T_a = 5^\circ\text{C}$ were not weighed upon removal from the cold chamber.

Food intake was monitored as an index of energy consumption in a subset of hamsters ($n=7$) at $T_a = 5^\circ\text{C}$, and in all hamsters ($n=9$) at $T_a = 13^\circ\text{C}$. Cages were provisioned with approximately 250 g of food, and pellets remaining in the food hopper were weighed one week later. Pellets hoarded on cage bottoms were collected and included in food mass measurements. Fresh food was provided after each measurement. Because chow pellets absorb moisture at low T_a s, 250 g of food was placed in an empty cage each week in each cold chamber, and re-weighed one week later to correct for moisture-induced inflation in food weight.

Treatment groups were balanced with respect to body mass at the time of placement in cold chambers, but to avoid disturbance of torpor (particularly at $T_a = 13^\circ\text{C}$), body mass was not measured weekly. Food intake values are therefore presented as g food consumed/hour spent normothermic, rather than as mass specific values. I used telemetric data to calculate the amount of time each hamster spent normothermic over the same weekly intervals during which food intake was measured. Food intake was compared between T_a s during an initial week of normothermia, and 1, 3, and 5 weeks after the onset of deep torpor bouts.

Statistical analyses

Except where noted, pairs of means were compared using unpaired t-tests. More than two means were compared using one-way ANOVAs; significant results were followed with Tukey-Kramer HSD tests. Relationships between variables were analyzed by linear regression analyses.

Results

Normothermia and shallow torpor bouts

Normothermic T_b during the first 72 h of cold exposure at T_a s of 5 °C and 13°C was $36.9 \pm 0.1^\circ\text{C}$ (groups combined), and did not differ between the sexes or between T_a s (Figure 4.1).

At both T_a s, most hamsters initiated test bouts within 3 weeks of entry into the cold. Females initiated test bouts sooner than males (7 ± 2 days versus 18 ± 5 days at 5°C and 16 ± 1 days versus 19 ± 6 days at 13°C), but these differences were not significant. There was no significant relationship between duration of 10L exposure prior to cold and the timing of initiation of test bouts for either sex at either T_a .

Mean test bout durations, T_b minima, IBIs and ranges for each of these parameters at both T_a s are summarized in Table 4.1. There were no significant differences between sexes or T_a s.

Most hamsters generated shallow bouts for fewer than two weeks prior to the first deep torpor bout, although three individuals (one 5°C male and two 13°C females) manifested shallow bouts for more than three weeks. Even after the onset of deep torpor bouts, 9 of 12 hamsters of both sexes (75%) at $T_a=5^\circ\text{C}$ and 7 of 9 hamsters (78%) at $T_a=13^\circ\text{C}$ continued to generate shallow, short bouts (<11 h in $T_a=5^\circ\text{C}$ and <20 h in $T_a=13^\circ\text{C}$) interspersed among deep, multi-day torpor bouts (Figure 4.2).

Deep torpor

Deep torpor bouts exceeded 24 h at both T_a s. Maximum bout length at $T_a=5^\circ\text{C}$ was significantly longer for females than males; a comparable difference was not evident at $T_a=13^\circ\text{C}$ (one-way ANOVA and post-hoc Tukey-Kramer HSD tests; $P<0.05$; Figure 4.3). Hamsters of both sexes at $T_a=5^\circ\text{C}$ had significantly longer bouts than their counterparts at $T_a=13^\circ\text{C}$ (one-way ANOVA and post-hoc Tukey-Kramer HSD tests; $P<0.05$; Figure 4.3).

T_b fluctuated by $\leq 0.5^\circ\text{C}$ during deep torpor, likely reflecting minor fluctuations in T_a . At $T_a=5^\circ\text{C}$, minimum T_b during deep torpor bouts was significantly higher for females than for males (unpaired t-test; $P<0.01$; Figure 4.1). Mean minimum T_b s were $2.8 \pm 0.3^\circ\text{C}$ (females) and $0.0 \pm 0.6^\circ\text{C}$ (males) above minimum T_a s, which ranged from 4.4°C to 5.5°C . At $T_a=13^\circ\text{C}$, minimum T_b did not differ significantly between sexes (Figure 4.1). Under these conditions, mean minimum T_b s were $0.9 \pm 0.2^\circ\text{C}$ (females) and $0.6 \pm 0.1^\circ\text{C}$ (males) above minimum T_a s, which ranged from 11.4°C to 12.0°C . The T_b - T_a gradient maintained by females at $T_a=5^\circ\text{C}$ was significantly higher than that maintained by all other sex- T_a groups (one-way ANOVA and post-hoc Tukey-Kramer HSD tests; $P<0.05$).

Mean IBIs after deep bouts commenced were 22.8 ± 1.9 h and 25.0 ± 2.2 h long at $T_a=5^\circ\text{C}$ and 13°C , respectively. Mean, minimum, and maximum IBIs did not differ between sexes or T_a s.

Timing of torpor

A total of 231 torpor bouts was analyzed at $T_a=5^\circ\text{C}$ (Figure 4.4). Entry into torpor occurred significantly more often (89%) during the scotophase (subjective night) than during the photophase (subjective day) (chi-square goodness of fit test; $P<0.01$). Timing of arousal was more variable, with 59% of arousals occurring during the scotophase ($P>0.05$).

Hamsters at $T_a=5^\circ\text{C}$ showed significantly steeper overall rates of T_b decline, and cooled more rapidly from T_b s of $25\text{--}13^\circ\text{C}$ than hamsters housed at $T_a=13^\circ\text{C}$ (Table 4.2, one-way ANOVA and post-hoc Tukey-Kramer HSD tests, $P<0.05$). Cooling rates from normothermic T_b to 25°C did not differ as a function of T_a . There were no sex differences in overall rates of entry into torpor or in rates of cooling from normothermic T_b to 25°C . From T_b s of $25\text{--}13^\circ\text{C}$ and 13°C to minimum T_b , however, males at $T_a=5^\circ\text{C}$ cooled significantly more quickly than females ($P<0.05$); this difference was not apparent at $T_a=13^\circ\text{C}$ (one-way ANOVA and post-hoc Tukey-Kramer HSD tests).

Arousal was achieved more rapidly by hamsters at $T_a=13^\circ\text{C}$ than by those at $T_a=5^\circ\text{C}$ (one-way ANOVA and post-hoc Tukey-Kramer HSD tests; $P<0.05$). From T_b s of $13\text{--}25^\circ\text{C}$, however, this difference in arousal rate was significant only for males, and there was no difference between T_a s from 25°C to normothermic T_b . Neither overall rates of arousal, nor rewarming from 25°C to normothermic T_b differed between the sexes, but males at $T_a=5^\circ\text{C}$ rewarmed more rapidly than females from minimum T_b to 13°C and from $13\text{--}25^\circ\text{C}$ (one-way ANOVA and post-hoc Tukey-Kramer HSD tests; $P<0.05$).

Body mass and food intake

Initial body mass in 16L, $22\pm2^\circ\text{C}$ was 138.0 ± 5.8 g for females and 153.1 ± 4.9 for males ($P>0.05$). Male body mass decreased (-2.9 ± 1.1 g) during 6-11 weeks of housing in 10L, $22\pm2^\circ\text{C}$ (paired t-test; $P<0.05$) but mass loss was not significantly related to time spent in 10L prior to placement in cold chambers. Female body mass did not decrease during housing in 10L, $22\pm2^\circ\text{C}$. At the time of transfer to cold chambers, body mass did not differ significantly between the sexes.

Hamsters were in the 5°C chamber for a shorter time (73 ± 10 days) than those housed at 13°C , (93 ± 12 days), reflecting differences in transmitter battery lifespan. All but one hamster ($T_a=5^\circ\text{C}$) lost weight in the cold; this individual was excluded from mass loss analyses. There were no sex differences in percent mass loss. At $T_a=5^\circ\text{C}$, mass loss increased with time spent in the cold, reaching a peak of $\sim 30\%$ for individuals in the cold >80 days. At $T_a=13^\circ\text{C}$, maximum mass loss was $\sim 25\%$ in individuals in the cold for 75-95 days, and was lower for those that spent either more or less time in the cold (Figure 4.5). For hamsters exposed to cold for <100 days, percent mass loss was significantly predicted by the number of days spent in the cold at both $T_a=5^\circ\text{C}$ (% mass loss = $-3.38 + 0.38[\text{days in cold}]$, $R^2 = 0.74$, $P<0.029$) and $T_a=13^\circ\text{C}$ (% mass loss = $9.00 + 0.17[\text{days in cold}]$, $R^2 = 0.85$, $P<0.026$). Neither the slopes nor intercepts of these regression lines differed significantly between the two T_a s (ANCOVA; $P>0.05$).

Normothermic food intake could not be calculated for one individual that entered torpor within a week of placement in the cold chamber. In addition, some hamsters' transmitters failed before they had experienced 3 or 5 weeks of deep torpor, and these individuals were excluded from food intake analyses for those time points; numbers of hamsters included at each time point are indicated in Figure 4.6. Nonetheless, at each time point groups were balanced with respect to

initial body mass. Food intake did not differ significantly between T_a s at any time point; however, there were trends (unpaired t-tests; $P < 0.06$) toward higher consumption at $T_a = 5^\circ\text{C}$ during normothermia, and toward higher consumption at $T_a = 13^\circ\text{C}$ during the 5th week of deep torpor (Figure 4.6).

Discussion

Despite the extensive use of Turkish hamsters in hibernation research (Hall and Goldman, 1980; Lyman et al., 1981; Hall et al., 1982; Hall and Goldman, 1982; Darrow et al., 1986; Goldman et al., 1986; Goldman and Darrow, 1987; Bartness et al., 1991; Yigit et al., 2008), the present study is the first to describe in detail the basic torpor characteristics of this species. In contrast to studies that found variable rates (~20-30%) of non-responsiveness to short-day cold challenges (e.g., Hall and Goldman, 1980; Goldman and Darrow, 1987; Bartness et al., 1991), all the hamsters in this study hibernated readily. After brief intervals of short (generally <12 h) shallow ($T_b > 20^\circ\text{C}$) test bouts, hamsters generated multi-day deep torpor bouts. There were no differences in shallow torpor bout duration, depth, or frequency between T_a s, but deep bouts were longer at $T_a = 5^\circ\text{C}$ (4-6 days) than at $T_a = 13^\circ\text{C}$ (2-3 days). Previous studies of Turkish hamster hibernation that maintained T_a as low as 3°C (Bartness et al., 1991) and as high as 10°C (e.g., Hall and Goldman, 1980, 1982; Hall et al., 1982) reported a range of estimates for bout duration. Consistent with previous work on other hibernating rodents, which shows an inverse relationship between torpor bout duration and torpor T_b , provided that torpid animals are thermoconforming (e.g., Twente and Twente, 1965; Geiser and Kenagy, 1988; Buck and Barnes, 2000), torpor bout duration in Turkish hamsters was inversely related to T_b during torpor, except in the case of sex differences (discussed below).

Whereas females at $T_a = 5^\circ\text{C}$ maintained T_b nearly 3°C above T_a , males maintained T_b within only 1°C of T_a . This difference was not apparent at $T_a = 13^\circ\text{C}$, suggesting that females have a higher critical minimum temperature than males. Despite maintaining a higher minimum T_b , females at $T_a = 5^\circ\text{C}$ generated longer bouts than males. This difference is partially attributable to the fact that females took longer to reach T_b nadirs and longer to rewarm to normothermia, but is ultimately difficult to reconcile in light of numerous studies – including this one – indicating an inverse relationship between bout length and depth (e.g., Twente and Twente, 1965; Geiser and Kenagy, 1988; Buck and Barnes, 2000). This study is the first to report a sex difference in critical minimum temperature; the potential energetic and fitness consequences of this difference merit further investigation.

Hamsters lost up to 30% of their initial body mass during the first 3-4 months in the cold, as reported previously (e.g., Hall and Goldman, 1982; Hall et al., 1982; Goldman and Darrow, 1987). Hamsters residing in the 13°C cold chamber beyond this point had lower % mass loss; recovery of body mass was likely coincident with gonadal recrudescence (Hall and Goldman, 1982; Hall et al., 1982).

Turkish hamsters do not fatten in preparation for hibernation, unlike most other deep hibernators (Lyman and O'Brien, 1977; French, 1988), but rather hoard food and continue to eat during periodic arousals (Vander Wall, 1990). Neither body mass loss during the first 100 days in the cold, nor normothermic food intake during the first 5 weeks of deep torpor differed between T_a s. The energetic costs may be similar at both T_a s, resulting in similar patterns of food intake and mass loss. Hamsters at $T_a = 5^\circ\text{C}$ generated significantly longer torpor bouts, but inter-bout intervals were similar at both T_a s, and consequently, arousals from torpor were more

frequent at $T_a=13^\circ\text{C}$. If the higher costs of normothermia and arousal at $T_a=5^\circ\text{C}$ were offset by fewer total arousals – which account for the majority of a hibernator's winter energy expenditure (Wang, 1978; Körtner and Geiser, 2000), then the overall cost of living at $T_a=13^\circ\text{C}$ may be similar to that at $T_a=5^\circ\text{C}$. A second possibility is that digestive conversion efficiency increased with the depth and duration of torpor, so that hamsters at $T_a=5^\circ\text{C}$ offset the higher costs of normothermia and arousal by extracting more calories from the same amount of food, as has been reported for the chipmunk *Tamias striatus*, another food-hoarding species (Humphries et al., 2003). A third possibility is that limitations of sample size prevented detection of differences in food intake between T_a s; perhaps consistent with this idea, I detected only a trend ($P<0.06$) toward higher food intake at $T_a=5^\circ\text{C}$ during an initial period of normothermia. Additional work using respirometry would likely help to discriminate between these hypotheses.

Entries into torpor occurred almost exclusively during the scotophase, as also occurs in golden mantled ground squirrels (Ruby et al., 2002) and Syrian hamsters (Oklejewicz et al., 2001). It is notable that both nocturnally and diurnally active rodent hibernators initiate torpor bouts during the dark phase. Timing of arousals from torpor was variable, with no strong tendency to occur either in the photophase or scotophase. In species that employ daily torpor, timing of arousals appears to be under circadian control – often coupled to either the onset of the species' active phase or the warmest part of the day (Körtner and Geiser, 2000) – though timing of arousals is variable in Siberian hamsters (Ruby, 2003). Less work has been done on the timing of arousals in hibernators; golden-mantled ground squirrels arouse preferentially during the photophase (Ruby et al., 2002), but Syrian hamsters – closely related to Turkish hamsters – lack circadian organization in the timing of arousals (Oklejewicz et al., 2001).

Like other heterothermic species, Turkish hamsters cool most rapidly during their initial descent into torpor, and cooling slows as T_b approaches its nadir (e.g., Wilz and Heldmaier, 2000; Kauffman et al., 2001b; Lee et al., 2009). It is difficult to directly compare cooling rates between species, given that differences in T_a , body mass, nest construction, and sociality all potentially affect cooling; nonetheless, both Siberian hamsters (*Phodopus sungorus*) and edible dormice (*Glis glis*) cool at least twice as quickly as Turkish hamsters when housed at $4\text{--}5^\circ\text{C}$ (Kauffman et al., 2001b; Wilz and Heldmaier, 2000). Alaska marmots (*Marmota flaviventris*) – much larger than Turkish hamsters and exposed to lower T_a – cool more quickly upon initial descent into torpor, but subsequently cool more slowly than Turkish hamsters as they approach minimum torpor T_b (Lee et al., 2009). Among hamsters at $T_a=5^\circ\text{C}$, rewarming rates were low at the beginning of arousals and accelerated at T_b s intermediate between minimum T_b and normothermia, as described in other mammals (Hammel, 1986; Geiser and Baudinette, 1990). Overall rates of arousal in Turkish hamsters are broadly comparable to rates reported for other rodents (Geiser and Baudinette, 1990).

A final point worthy of note is that, in most hamsters, shallow torpor bouts were interspersed between deep bouts throughout the hibernation season, in contrast to shallow torpor (so-called test bouts) that many species, including Turkish hamsters, generate at the beginning and end of the hibernation season (Strumwasser 1959; Geiser 2004). Turkish hamsters may thus be capable of both hibernation and daily torpor. Shallow bouts occurred at all times of day and at both T_a s. We cannot exclude the possibility that at $T_a=13^\circ\text{C}$ some of these events may reflect premature arousal due to disturbance, but this was not a concern at $T_a=5^\circ\text{C}$, because hibernators were not disturbed by human presence in the cold chamber. Field observations would be needed to rule out the possibility that these shallow bouts are an artifact of captivity. In addition, studies integrating respirometry data would help determine whether metabolic rate during these short

bouts more closely matches the marked reduction in metabolism during bouts of hibernation, or whether the metabolic reduction is shallower, as is the case for most daily heterotherms (Geiser and Ruf, 1995; Geiser, 2004, but see Wilz and Heldmaier, 2000; Geiser and Mzilikazi, 2011). Few species are known to utilize both hibernation and daily torpor (e.g., Bartholomew and Hudson, 1960; Wilz and Heldmaier, 2000; Toussaint et al., 2010), suggesting that Turkish hamsters may be particularly valuable for studying potential physiological differences between these two patterns of heterothermy and the possibility that hibernation evolved from daily torpor.

Table 4.1. Test bout duration, minimum T_b , and inter-bout interval (IBI) at $T_a=5^\circ\text{C}$ and 13°C .

	5°C (n=12)	13°C (n=9)
Bout duration (h)	4.0 ± 0.5 (range $1.3 \pm 0.3 - 7.9 \pm 0.8$)	5.4 ± 0.7 (range $1.9 \pm 0.7 - 11.5 \pm 1.1$)
Minimum T_b (°C)	27.4 ± 0.8 (range $21.3 \pm 1.5 - 32.2 \pm 0.6$)	26.5 ± 1.5 (range $19.1 \pm 1.7 - 31.3 \pm 1.3$)
IBI (h)	32.9 ± 4.8 (range $15.6 \pm 5.0 - 53.0 \pm 5.3$)	25.2 ± 4.1 (range $7.4 \pm 3.1 - 55.0 \pm 7.7$)

Table 4.2. Rates of entry into and arousal from torpor (°C/hour) at $T_a=5^{\circ}\text{C}$ and 13°C .

	5°C males (n=6)	5°C females (n=6)	13°C males (n=6)	13°C females (n=3)
Overall rate of entry	1.46 ± 0.09 ^a	1.36 ± 0.04 ^a	1.17 ± 0.03 ^b	1.03 ± 0.11 ^b
<i>Normothermia – 25°C</i>				
	4.21 ± 0.34 ^a	3.58 ± 0.25 ^{ab}	3.20 ± 0.35 ^{ab}	2.22 ± 0.44 ^b
25 – 13°C or T_{bmin}	3.55 ± 0.21 ^a	2.75 ± 0.12 ^b	0.80 ± 0.03 ^c	0.73 ± 0.05 ^c
13°C - T_{bmin}	0.54 ± 0.04 ^a	0.42 ± 0.02 ^b	N/A	N/A
Overall rate of arousal	12.84 ± 0.43 ^a	11.35 ± 0.43 ^a	17.79 ± 1.09 ^b	16.12 ± 0.52 ^b
$T_{bmin} – 13^{\circ}\text{C}$	5.85 ± 0.45 ^a	3.87 ± 0.20 ^b	N/A	N/A
13°C or $T_{bmin} – 25^{\circ}\text{C}$	30.90 ± 2.48 ^a	22.67 ± 1.39 ^b	16.14 ± 0.72 ^c	15.62 ± 1.15 ^{bc}
25°C – <i>normothermia</i>	19.66 ± 1.89 ^a	19.74 ± 1.40 ^a	21.49 ± 3.00 ^a	16.72 ± 0.51 ^a

a,b,c – Values in the same row that do not share a common letter differ significantly ($P<0.05$).

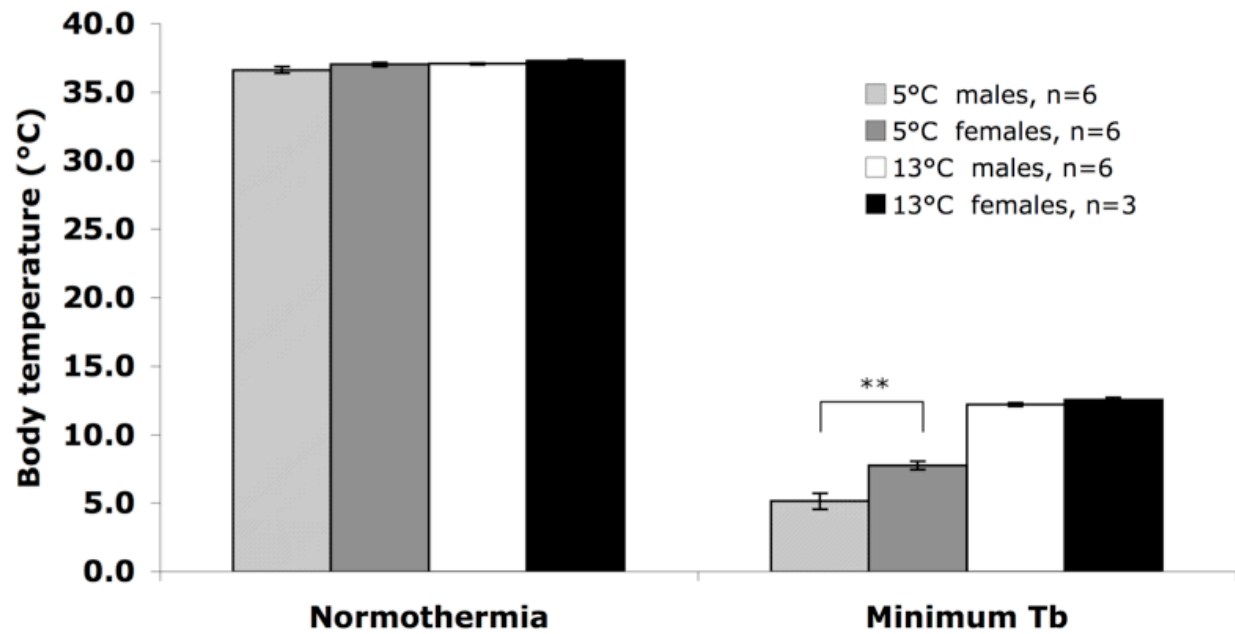


Figure 4.1. Mean T_b during the first 72h of cold exposure (normothermia) and mean minimum T_b during deep torpor (minimum T_b). Error bars represent S.E.M. ** $P < 0.01$.

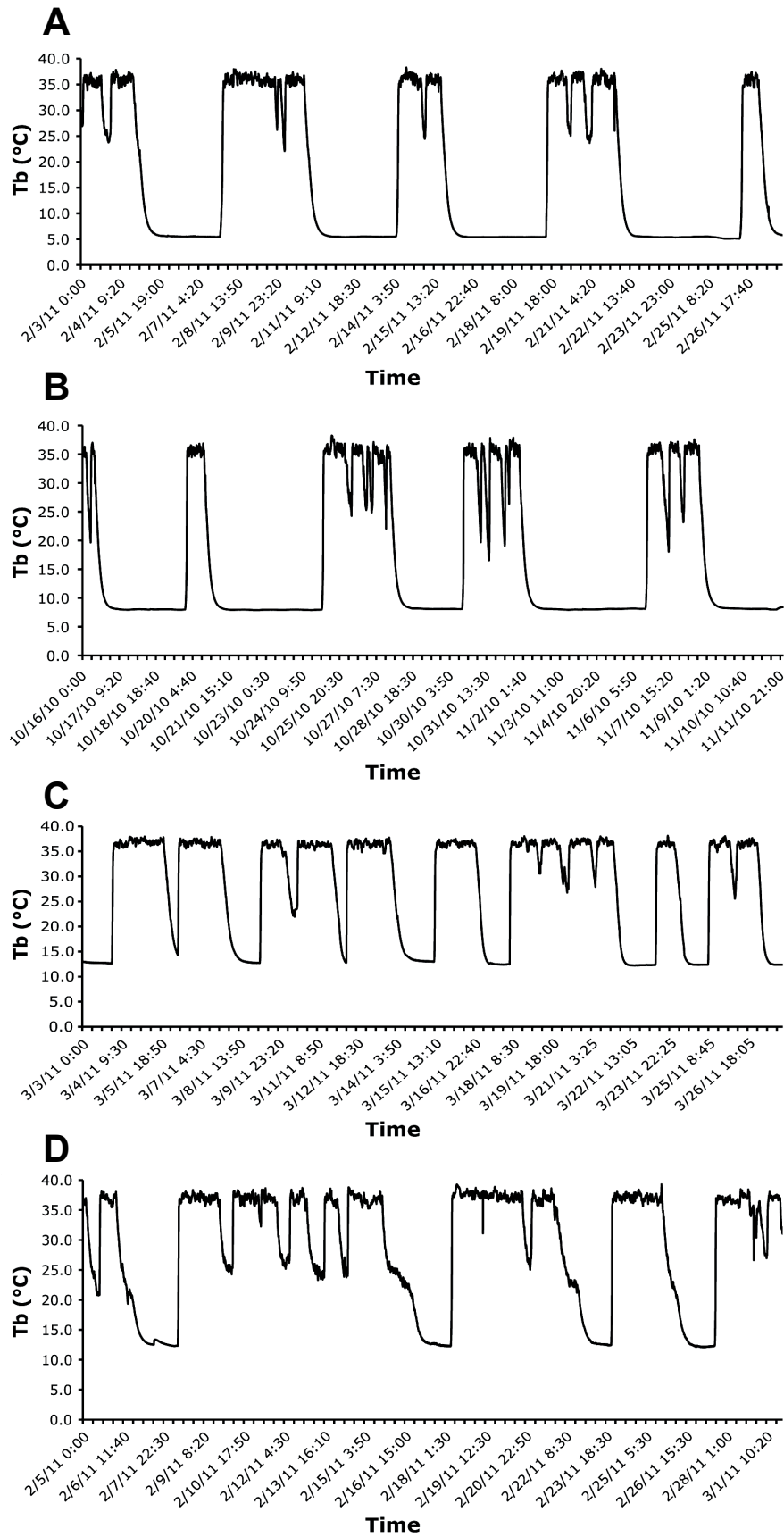


Figure 4.2. T_b for a male at 5°C (A), a female at 5°C (B), a male at 13°C (C), and female at 13°C (D) over the course of ~25 consecutive days. For hamsters at both T_a s, short, shallow torpor bouts were interspersed among deep, multi-day bouts.

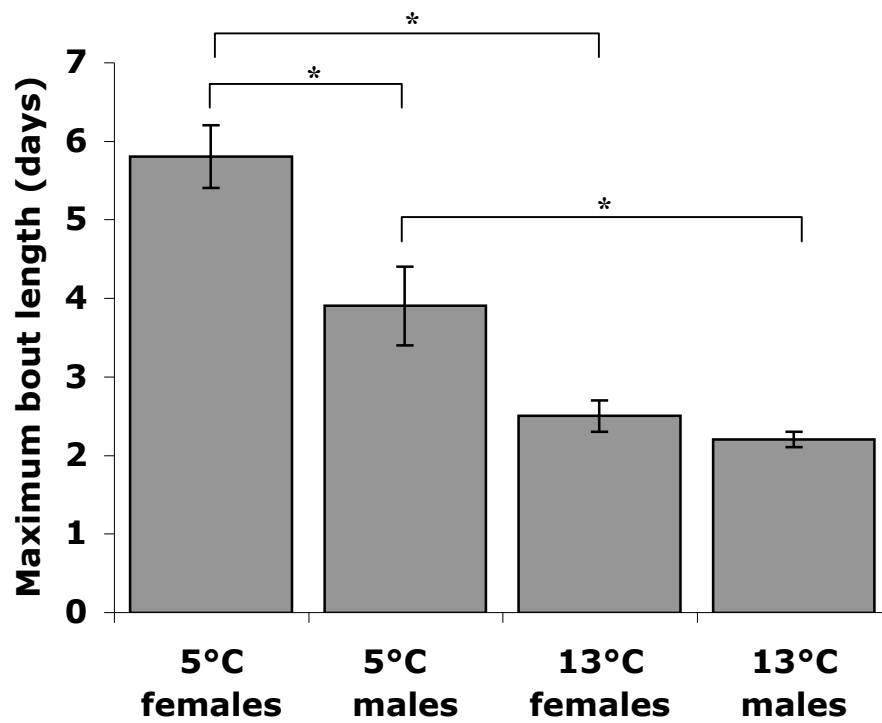


Figure 4.3. Maximum bout lengths were longer at $T_a=5^\circ\text{C}$ than at $T_a=13^\circ\text{C}$, and at $T_a=5^\circ\text{C}$ females had longer maximum bout lengths than males. * $P<0.05$.

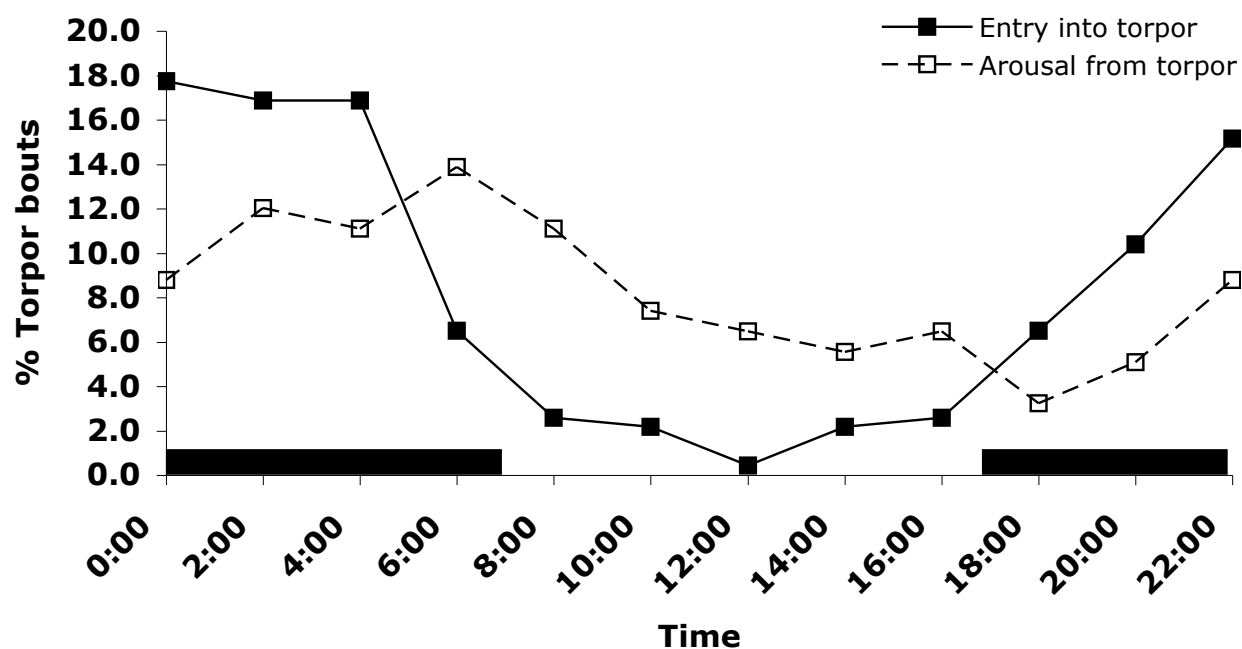


Figure 4.4. Times of entry into (solid line) and arousals from (dashed line) 231 torpor bouts at $T_a=5^{\circ}\text{C}$. Entry into torpor occurred predominantly during the scotophase (1700-0700; black horizontal bar). Timing of arousals was variable.

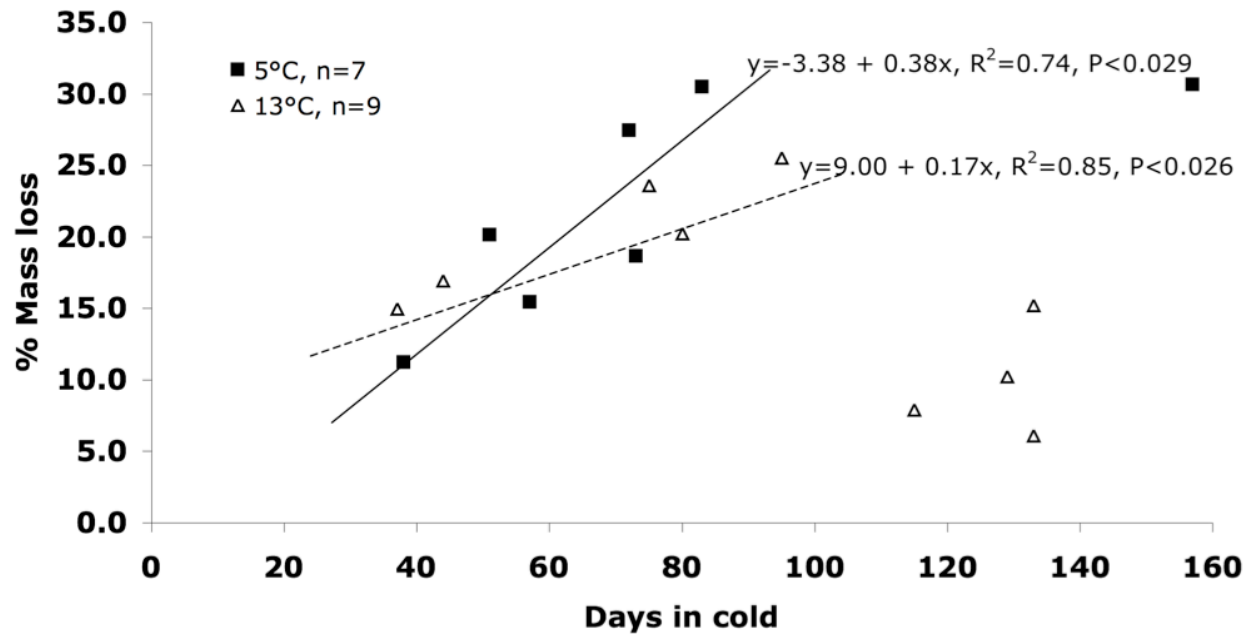


Figure 4.5. Body mass decline as a function of time in the cold. At $T_a=5^\circ\text{C}$, mass loss leveled off at ~30% for hamsters housed in the cold for >80 days. At $T_a=13^\circ\text{C}$, mass loss peaked at ~25% for hamsters housed in the cold for 75-95 days, but was lower for individuals that spent more or less time in the cold. Regression lines for time <100 days in the cold are shown for $T_a=5^\circ\text{C}$ (solid line) and $T_a=13^\circ\text{C}$ (broken line).

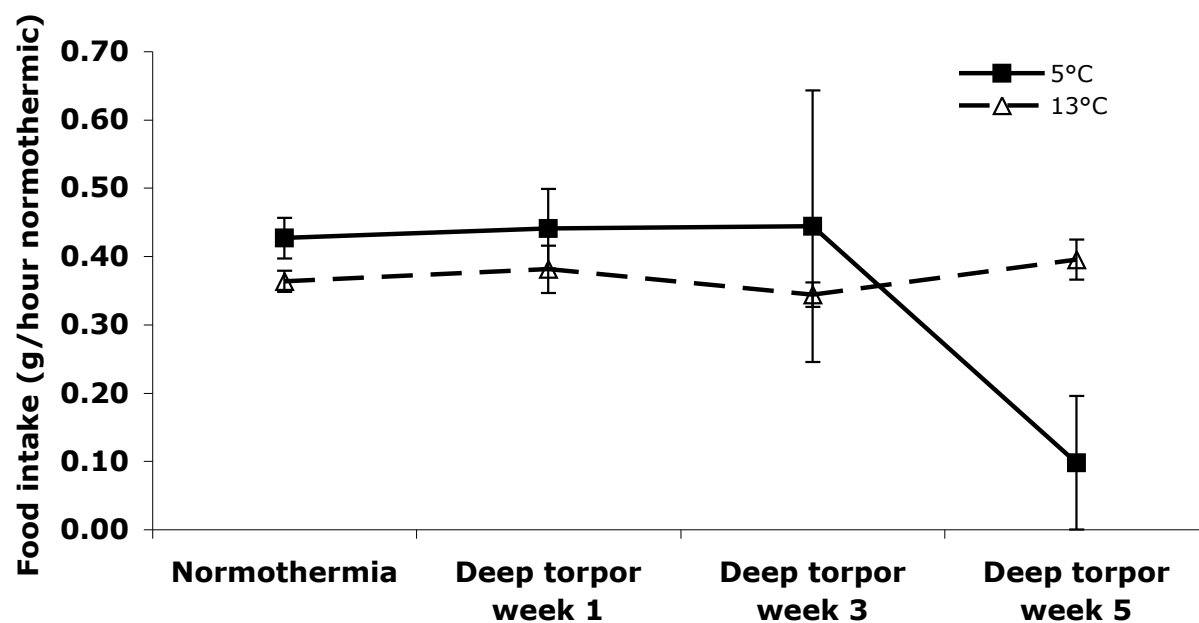


Figure 4.6. Food intake did not differ between the two T_a s during normothermia or at week 1, 3, or 5 after the onset of deep torpor. The number of individuals at each time point is in parenthesis.

Chapter 5

The Effects of Day Length, Hibernation, and Ambient Temperature on Incisor Dentin in the Turkish Hamster¹

Introduction

Little is known about mammalian hibernation in evolutionary and recent historical (within the last century) contexts, partly because evidence of physiology and behavior is rarely preserved postmortem. Evolutionary questions – such as how many times hibernation evolved and whether it is ancestral to all mammals – have been evaluated primarily with comparative physiological and phylogenetic data (Augee and Gooden 1992, Malan 1996, Geiser 1998, Grigg and Beard 2004, Grigg et al. 2004, Lovegrove 2012), but have not been corroborated with fossil evidence. Analysis of changes in hibernation behavior over the last century has been rare (but see Inouye et al. 2000), but could prove valuable in refining models that predict the effects of anthropogenic climate change on hibernating species (e.g., Humphries et al. 2002, 2004, Boyles et al. 2011).

As described in Chapter 1, incremental structure in dentin may be a useful trait for studying hibernation in both extinct species and historical populations, given that its deposition is affected by a host of environmental, life history, and physiological factors, including hibernation (Klevezal and Mina 1990, Klevezal 1996, Rinaldi 1999, Trunova and Klevezal 1999, Selkova 2003, Goodwin et al. 2005, Goodwin and Ryckman 2006). Ever-growing rodent incisors provide an especially good model system to study the effects of hibernation on dentin deposition (Goodwin et al. 2005, Goodwin and Ryckman 2006), because their high rate of continual growth makes them sensitive indicators of the physiological milieu throughout the life of the animal.

Previous studies of rodent incisor dentin have identified hibernation marks in histological cross section in members of the families Sciuridae, Gliridae, Dipodidae, and Cricetidae (Klevezal and Mina 1990, Klevezal 1996, Trunova and Lobkov 1997, Trunova and Klevezal 1999, Selkova 2003), and on medial incisor surfaces of marmots (*Marmota*), ground squirrels (*Urocitellus*), and prairie dogs (*Cynomys*) – all members of the Marmotini tribe of the family Sciuridae (Rinaldi 1999, Goodwin et al. 2005, Goodwin and Ryckman 2006, Klevezal and Lobkov 2008). The latter are characterized by a depression in the incisor dentin and fine, indistinct “hibernation increments” within the mark (Rinaldi 1999, Goodwin et al. 2005, Goodwin and Ryckman 2006). In sciurids studied to date, hibernation increments are not circadian (Rinaldi 1999, Goodwin et al. 2005) and do not correspond to the number of arousals (Rinaldi 1999); the width of the hibernation mark also does not track total time in hibernation (Goodwin et al. 2005). The factors that shape the morphology of this region thus remain obscure.

Tooth surface topography could facilitate analysis of hibernation in evolutionary and historical contexts if we better understood the factors influencing dentin morphology. With this

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ultimate goal in mind, I tested the effects of day length, hibernation, and T_a on dentin surface morphology in a non-sciurid hibernator, the Turkish hamster (*Mesocricetus brandti*).

Methods

Animals and experimental groups

Male and female Turkish hamsters (n=40) 6-20 months old from the local breeding colony (see Butler et al. 2008 for details) were maintained from birth in 16L (16h light/day, lights on at 0200h) and $22\pm 2^\circ\text{C}$. Hamsters were individually housed during the experiment.

Long-day (LD) control hamsters (n=7; 4 males and 3 females) were kept in a 16L photoperiod (16 h light/day, lights on at 0200h) and $22\pm 2^\circ\text{C}$. Short-day (SD) control hamsters (n=8; 3 males and 5 females) were housed in a 10L photoperiod and $22\pm 2^\circ\text{C}$ for 3-4 months (lights on at 0700h).

All remaining hamsters were transferred to short days (10L; lights on at 0700) at $22\pm 2^\circ\text{C}$ to induce reproductive quiescence (described in Chapter 2), and were subsequently moved to cold chambers maintained at either $5\pm 1^\circ\text{C}$ (n=15; 11 males and 4 females) or $13\pm 1^\circ\text{C}$ (n=10; 7 males and 3 females), with the same 10L light cycle. 12 hamsters in the 5°C chamber hibernated (“ 5°C hibernators”) and 3 males failed to display testicular regression or hibernation (“nonresponders”); nonresponsiveness is a well-described phenomenon in several heterothermic species and is reviewed in Prendergast et al. (2001). All hamsters (n=10) in the 13°C chamber hibernated (“ 13°C hibernators”).

Hamsters remained in the cold until transmitter batteries failed 6 to 20 weeks later (see below), at which point they were returned to 16L and $22\pm 2^\circ\text{C}$, where they recovered for 7 days prior to euthanasia.

Recording of T_b and calculation of torpor parameters

All hamsters underwent surgical implantation of radiotransmitters, and T_b of hamsters in cold chambers was monitored as described in detail in Chapter 2. LD and SD control hamsters underwent surgical procedures for control purposes, but T_b was not measured in these groups.

Torpor thresholds were determined for each individual as described in Chapter 2. Each hamster’s cumulative time torpid and time normothermic between torpor bouts were calculated by summing the durations of all torpor bouts and arousals between those bouts, respectively.

Analysis of teeth

Incisors were extracted and marked as described in Chapter 2, and examined under a dissecting microscope using reflected light. Lower right incisors were examined in all hamsters except for two, which had abnormal morphology of the right incisor; in these cases, the lower left incisor was examined instead.

Photographs capturing the relief pattern on each tooth’s surface were analyzed using the program FIJI (ImageJA 1.44b, public domain). Increments were categorized as circadian, ultradian, or hibernation increments. Those that were relatively narrow and sharply defined were identified as hibernation increments; the hibernation zone was defined as the area(s) of each tooth over which hibernation increments were observed. Increments that did not exhibit this

characteristic morphology were assumed to be circadian. Adjacent increments that were unambiguously sub-increments of a larger unit (whether it was a circadian or hibernation increment) were considered ultradian (for example, Figure 5.1C). For the purposes of comparison and analysis, the larger unit, rather than the ultradian increment, was counted and measured, except where noted.

Increments were measured to the nearest 0.01 mm, and each hamster's mean increment width was calculated. In hibernators, mean hibernation increment width was also calculated, and width of the hibernation zone was determined by summing the widths of individual hibernation increments. Where regions of hibernation increments were interrupted by normal increments, the latter were not included in calculating hibernation zone width. In all specimens, increments along the occlusal surface were excluded from analysis because they were difficult to measure accurately. Each hamster's mean ultradian increment width was calculated from the five most basal visible ultradian increments. Total tooth length was also measured. Regional heterogeneity of each tooth was quantified by counting the number of distinct regions along the length of the tooth; regional boundaries were characterized by abrupt changes in increment width and/or distinctness. All observations and measurements were completed without my knowledge of specimen treatment history.

Statistics

One-way ANOVAs compared groups in terms of tooth length, number of increments along each tooth, mean increment width, and mean ultradian increment width; significant results were followed with Tukey-Kramer HSD tests. An unpaired t-test assessed the difference between mean hibernation increment width in 5°C versus 13°C hibernator teeth. Regional heterogeneity in hibernators (5°C and 13°C combined) versus non-hibernators (LD controls, SD controls, and nonresponders combined) was assessed with the Fisher's exact test on the number of regions (<3 or ≥ 3); most individuals had either 1-2 regions or >4 regions, and thus, 3 regions was a natural dividing point. Correlation analyses tested the relationship between (1) increment count and mean increment width, (2) number of torpor bouts and cumulative time in torpor and (3) number of torpor bouts and cumulative time aroused from torpor. Regression analyses tested whether cumulative time in torpor, cumulative time aroused from torpor (normothermia), or number of torpor bouts predicted total number of increments, mean increment width, number of hibernation increments, or hibernation zone width.

Results

Qualitative differences in incisor morphology

Most LD control teeth (5 of 7; 71%) showed distinct increments that were easy to count and measure (Figure 5.1A); these increments were assumed to be circadian based on similarities to daily increments in other rodents (Rinaldi 1999, Rinaldi and Cole 2004, Goodwin et al. 2005, Goodwin and Ryckman 2006, Klevezal 2010). A few SD control teeth (3 of 8; 38%) appeared similar to LD control teeth in terms of increment clarity, but dentin of the remaining 5 teeth (63%) was more uniform, with only faintly visible increments that were difficult to visualize, count, and measure (Figure 5.1B). LD and SD control teeth had increments that were relatively uniform in width. Very narrow increments (<0.2 mm) were more often visible in LD teeth, and

were almost always clearly sub-increments organized into sets of two or three (Figure 5.1C); these increments were assumed to be ultradian.

Teeth of 5°C hibernators and 13°C hibernators were qualitatively indistinguishable. Unlike control teeth, they commonly had both regions where increments were well-defined, sometimes with visible ultradian increments, and regions where dentin was nearly uniform and increments were only faintly visible. 18 of 22 hibernators (82%) had incisors with 3 or more distinct regions; the same was true for only 4 of 18 non-hibernators (22%; Fisher's exact test; $P < 0.001$).

There was at least one region of narrow, sharply-defined increments usually lacking higher organization in 10 of 12 hibernators at 5°C (83%) and 8 of 10 hibernators at 13°C (80%) (Figures 5.1D and 5.1E). All transmitters failed while hamsters were torpid, at which point animals were transferred to 16L, $22 \pm 2^\circ\text{C}$, which induced arousal and ensured that all hamsters survived exactly 7 days after their final arousal. Among hibernators, 3 of 12 (25%) at 5°C and 5 of 10 (50%) at 13°C had a region at the base of the tooth with 4-7 increments that were wider than more apical adjacent increments (Figure 5.1F).

Nonresponders lacked the narrow, sharply-defined increments characteristic of hibernators. Two specimens most closely resembled the LD control phenotype, with relatively clear increments, and one most closely resembled the SD control phenotype, with more uniform dentin and fainter increments.

Quantitative differences in dentin increments

Within each treatment group, there were no sex differences in tooth length, number of increments, increment width, or ultradian increment width, so sexes were combined for analysis. There was also no relationship between age at euthanasia and any of these variables.

There were no differences in tooth length between groups; mean tooth length (groups combined) was 26.65 ± 0.35 mm. There were, however, significant differences in number of increments (ANOVA; $P < 0.002$; Table 5.1 and Figure 5.2). Incisors of 5°C and 13°C hibernators had more increments than did LD control hamster incisors (Tukey Kramer HSD test; $P < 0.05$); SD control and nonresponsive hamster incisors had intermediate numbers of increments that did not differ from values of LD control hamsters or either group of hibernators (Tukey Kramer HSD test; $P > 0.05$).

There were also significant differences in mean increment width (ANOVA; $P < 0.001$; Table 5.1 and Figure 5.3). LD control hamsters had wider increments than SD control hamsters, 5°C hibernators and 13°C hibernators (Tukey Kramer HSD test; $P < 0.05$). SD control and hibernator groups did not differ from each other, and nonresponders had intermediate increment widths that did not differ from those of any other group (Tukey Kramer HSD test; $P > 0.05$). There were no significant differences in ultradian increment widths between groups (ANOVA; $P > 0.05$; Table 5.1 and Figure 5.3). Widths of sharply defined hibernation increments were 0.27 ± 0.02 mm at 5°C and 0.26 ± 0.03 mm at 13°C ($P > 0.05$).

There was a significant negative correlation between number of increments and mean increment width in 5°C hibernators ($r = -0.95$; $P < 0.001$), 13°C hibernators ($r = -0.96$; $P < 0.001$), and SD control hamsters ($r = -0.96$; $P < 0.001$).

Torpor parameters and tooth morphology

There was no significant relationship between cumulative time torpid and number of arousals at either T_a , primarily attributable to variable numbers of short, shallow torpor bouts, both prior to and interspersed among deeper, longer bouts (see Batavia et al. 2013a). There was a positive correlation between cumulative time normothermic between torpor bouts and number of arousals at both 5°C ($r^2=0.77$; $P<0.004$) and 13°C ($r^2=0.86$; $P<0.002$).

Hamsters generated 19 ± 3 torpor bouts at 5°C (range 5-34 bouts), and 44 ± 8 torpor bouts at 13°C (range 20-85 bouts). Cumulative time spent torpid was 28.6 ± 9.1 days at 5°C (range 3.3-119.4 days), and 34.9 ± 6.6 days at 13°C (range 12.4-68.0 days). Cumulative time normothermic between torpor bouts was 17.6 ± 2.5 days at 5°C (range 8.2-30.0 days) and 39.7 ± 6.2 days at 13°C (range 15.4-71.7 days). Neither increment count nor increment width was predicted by cumulative time in torpor, cumulative time normothermic between torpor bouts, or number of arousals.

For hibernators at 5°C, hibernation increment count was predicted by both the number of arousals ($r^2=0.52$; $P<0.019$; Figure 5.4A) and total time normothermic between torpor bouts ($r^2=0.55$; $P<0.014$; Figure 5.4B). Hibernation zone width was also predicted by total time normothermic between torpor bouts ($r^2=0.56$; $P<0.013$; Figure 5.4C). There was no significant relationship between hibernation increment count or cumulative width and any torpor parameters at 13°C.

Discussion

This study is the first to describe dentin surface topography of Turkish hamster incisors, and to experimentally assess effects of day length, hibernation, and hibernaculum T_a on incisor dentin morphology. Dentin increments on non-hibernating Turkish hamster incisors were assumed to be circadian based on their similarity to circadian increments described on incisor surfaces in other rodents (Rinaldi 1999, Rinaldi and Cole 2004, Goodwin et al. 2005, Goodwin and Ryckman 2006, Klevezal 2010). Moreover, LD control hamster teeth were comprised of ~41 increments, consistent with the ~39 daily increments comprising lower incisors of migratory hamsters (*Cricetulus migratorius*; Klevezal 2010) and a turnover rate of 40-50 days in rats of comparable size to Turkish hamsters (Schour and Steadman 1935). The appearance of 2-3 subunits within each daily increment likely represents ultradian rhythms in deposition, as previously documented in rabbits and rodents (Rosenberg and Simmons 1980, Ohtsuka and Shinoda 1995, Goodwin et al. 2005, Goodwin and Ryckman 2006, Klevezal 2010).

Day length affected both qualitative and quantitative aspects of dentin deposition. Incisors of SD control hamsters had narrower circadian increments than those of LD hamsters; these increments were also relatively less distinct, indicating a more uniform surface topography than is typical of LD teeth, perhaps reflecting attenuation in the amplitude of the daily rhythm of collagen production or mineralization (Schour and Steadman 1935, Ohtsuka et al. 1998, Rinaldi 1995, 1999). The narrowing of daily increments in SDs is consistent with lower daily rates of dentin deposition in fall and winter (Rinaldi and Cole 2004, Goodwin and Ryckman 2006, Klevezal 2010). Rinaldi and Cole (2004) found no relationship between dentin deposition and daily fluctuations in temperature or precipitation, and attributed seasonal differences in beaver incisor growth rates to seasonal shifts in dietary preference, metabolism, and/or activity levels. Our LD and SD hamsters experienced the same T_a and diet; therefore, it is likely that seasonal

changes in dentin deposition in this species are at least partially mediated by day length, rather than the exclusive result of changes in diet, precipitation, or temperature. Whether day length influences tooth growth directly or indirectly, possibly via photoperiod-induced changes in activity, metabolism, or energy requirements (e.g., Heldmaier et al. 1989, Prendergast and Zucker 2012), is unknown.

Three male hamsters were unresponsive to short day lengths, as evidenced by failure to undergo testicular regression or generate torpor bouts. Responsiveness to SDs is mediated by the pineal hormone melatonin, which is secreted during the dark phase of the photocycle (reviewed in Goldman 2001, Bartness et al. 1993). A SD photoperiod generates a longer-duration nocturnal melatonin signal (Bartness et al. 1993), which induces reproductive quiescence (e.g., Hong and Stetson 1988, Butler et al. 2008, Jarjisian and Zucker 2011) – a prerequisite for hibernation in male Turkish hamsters (Hall et al. 1982). Several other seasonal traits – including changes in pelage, body mass, food intake, and activity (Duncan and Goldman 1984, Dark et al. 1983, Puchalski et al. 1988, Prendergast and Zucker 2012) – are mediated by the same control mechanism. Disruption of any component of this pathway can induce nonresponsiveness to short day lengths, the neuroendocrine basis for which varies by species (Prendergast et al. 2001). My results were ambiguous with respect to the pattern of dentin deposition displayed by nonresponders; two hamsters manifested the LD phenotype, and a third the SD phenotype. Larger sample sizes are needed to determine which of these patterns characterizes the majority of photononresponsive hamsters. If, as my small sample indicates, most exhibit a LD phenotype, this would implicate melatonin as the proximate factor by which day length exerts its effects on tooth growth.

Ultradian increment width did not differ significantly between LD and SD hamsters, in contrast to substantial lengthening of ultradian rhythm periods of body temperature and locomotor activity in Siberian hamsters in SDs (Heldmaier et al. 1989, Prendergast and Zucker 2012). Based on these studies, I anticipated wider ultradian increments in SD teeth, because given a constant rate of deposition, a longer period ultradian rhythm should permit more dentin to be deposited per ultradian cycle, manifesting as wider increments. A likely explanation for this discrepancy is that the lengthening of ultradian rhythm period in SDs was countered by a concomitant decrease in growth rate (see above), culminating in ultradian increments of roughly the same width in both LDs and SDs.

Hibernation markedly affected dentin deposition. A majority of hibernators at both T_{as} had incisors with at least one region of narrow, sharply defined bands; this morphology is diagnostic of hibernation in Turkish hamsters. Hibernators had more regions along each tooth than nonhibernators, suggesting that hibernation contributes to overall tooth heterogeneity. I did not observe a single, short, clearly delineated depression in medial incisor dentin, as documented in marmots (Rinaldi 1999), ground squirrels (Goodwin et al. 2005), and prairie dogs (Goodwin and Ryckman 2006). A possible explanation for this difference is that, unlike most rodent hibernators, which eat little or no food during the hibernation season, Turkish hamsters do not fatten in preparation for hibernation, but rather consume small amounts of hoarded food during periodic arousals (Vander Wall 1990, Batavia et al. 2013a). Their teeth are likely subjected to more wear than is characteristic of their fat-storing counterparts, and thus likely grow relatively more over the course of the hibernation season. The region(s) of sharply defined, narrow bands characteristic of hibernating Turkish hamster teeth may be the same as the hibernation mark in marmots, ground squirrels, and prairie dogs, but stretched over a greater length of tooth. Another, not mutually exclusive possibility, is that similarities in hibernation dentin morphology

in sciurid species studied to date, which differ from Turkish hamsters (a cricetid rodent), reflect their phylogenetic relationships. Studies of additional species may shed light on whether different hibernation dentin morphologies are characteristic of certain rodent clades, or whether they generalize to all fat- and food-storing hibernators.

Hibernating Turkish hamsters at both T_a s had more increments, and a lower mean increment width than did LD control hamsters. This difference is partly attributable to the inclusion of narrow hibernation increments in calculations of increment number and mean width; however, SD control and nonresponder hamsters had teeth with intermediate numbers of increments and increment widths, which did not differ statistically from those of hibernators. Part of the difference between hibernators and LD controls may therefore be attributed to day length and/or cold exposure. Indeed, SDs, cold exposure, and hibernation may additively increase the number of increments and decrease mean increment width.

Recovery of normal dentin deposition upon final arousal from hibernation and removal from SD cold conditions occurred in only a minority of hamsters before they were euthanized one week later. Goodwin et al. (2005) similarly reported that resumption of circadian increments in *Urocyon* (formerly *Spermophilus*; Helgen et al. 2009) did not occur until emergence aboveground, and that the number of circadian increments deposited after the hibernation mark in males underestimated time elapsed since final arousal by 4-6 days. In marmots, this error was 0-4 days (Rinaldi 1999). Collectively, these findings indicate a delay in the resumption of normal dentin deposition at the end of the hibernation season.

At 5°C, both the number of narrow, sharply defined hibernation increments and the cumulative width of these increments were positively correlated with the number of arousals and the cumulative time spent normothermic between torpor bouts. Rinaldi (1999) speculated that the number of hibernation increments should be related to the number of arousals from hibernation, but found no relationship between these variables in marmots. My results support the Rinaldi hypothesis at 5°C, but not at 13°C. At 5°C, deposition appears to be confined to normothermic intervals between torpor bouts; whether hibernation increments are deposited with a set periodicity during these intervals remains to be established. The lack of correspondence at 13°C suggests that at higher T_a s, the relationship between hibernation increments and arousals deteriorates. In a preliminary study, I determined that 13°C was the highest T_a at which Turkish hamsters would reliably enter hibernation, though they were easily roused at this T_a . I lack data comparing torpid metabolic rates at 5°C and 13°C, but expect that metabolic depression was not as extreme at 13°C (Geiser 2004); dentin deposition may therefore have continued at a reduced rate even during bouts of torpor, perhaps accelerating during periodic arousals.

Despite the lack of a relationship between arousals and hibernation increments at 13°C, incisor dentin morphology of hamsters hibernating at this T_a was qualitatively identical to that of hamsters hibernating at 5°C. Considering that much of rodent evolution played out during the Paleocene and Eocene epochs, which were warmer and more temperate than today's climate (reviewed in Markwick 1998, Huber and Nof 2006), this finding is notable, as it implies that hibernation may be diagnosable in fossils from these periods. With sufficiently large numbers of fossils and the identification of putative hibernation marks or increments, one ostensibly can determine the occurrence of hibernation in specific clades of rodents.

My findings also can inform historical studies of hibernation. Although the relationship between hibernation increments and periodic arousals varies by species, once this relationship is established in a contemporary species or population of interest (or a close relative), historically collected specimens can be compared to recent specimens for evidence of changes in frequency

and duration of arousals. Arousals are the most energetically costly part of the hibernation cycle (Wang 1978, Körtner and Geiser 2000), so changes in their frequency or cumulative duration likely have energetic, nutritional, reproductive, and survival ramifications (e.g., Reeder et al. 2012). Investigations of the effects of climate change on hibernation over the past century may help predict the fate of hibernating species in the face of future global climate change.

Table 5.1. Dentin increments on medial surfaces of lower incisors.

	N=	# Increments	Mean increment width (mm)	Mean ultradian increment width (mm)
LD controls	7	41 ± 4	0.53 ± 0.03	0.17 ± 0.01
SD controls	8	57 ± 4	0.41 ± 0.03	0.17 ± 0.01
5°C hibernators	12	62 ± 3	0.34 ± 0.02	0.16 ± 0.01
13°C hibernators	10	62 ± 3	0.32 ± 0.02	0.15 ± 0.01
Nonresponders	3	47 ± 6	0.43 ± 0.04	0.14 ± 0.02

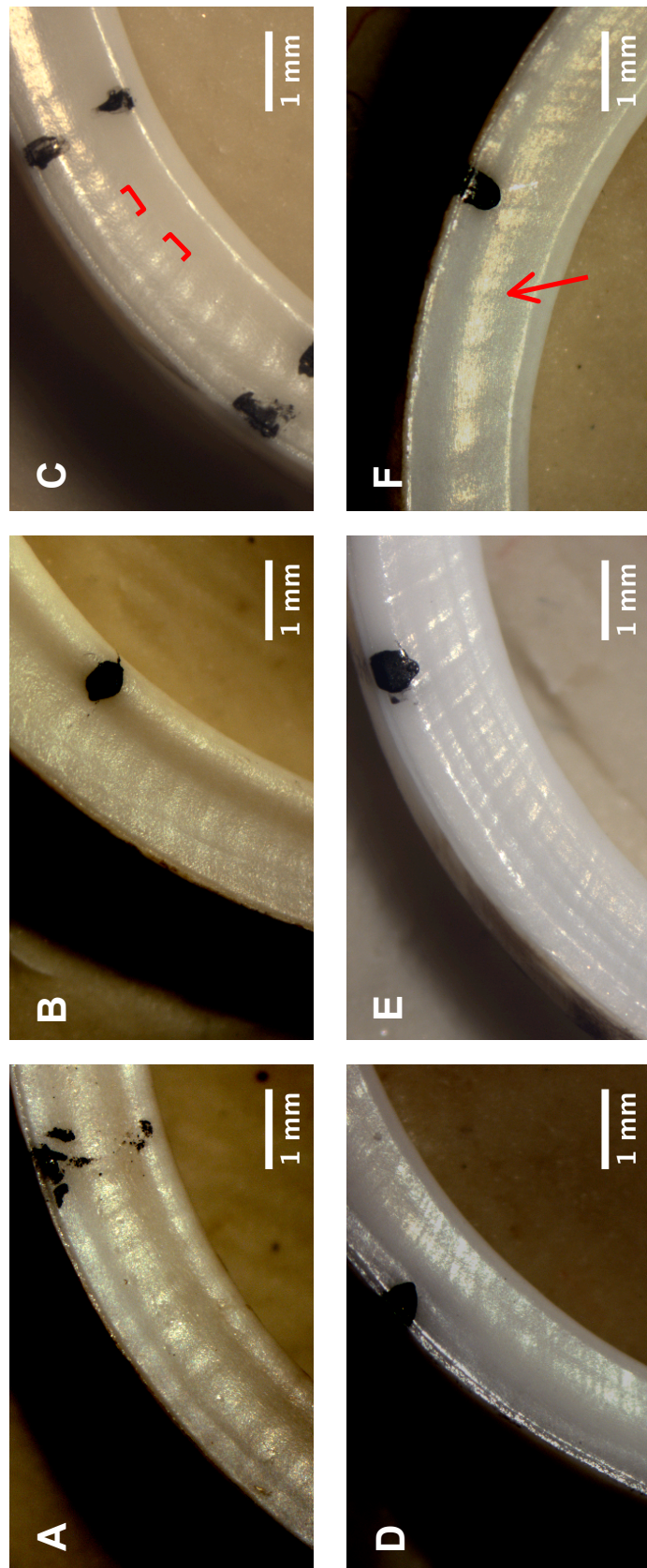


Figure 5.1. All images show medial surfaces of lower right incisors, with the tooth base to the left and the apex to the right. LD control hamster teeth (**A**) were characterized by clear, regularly spaced increments; SD control hamster teeth (**B**) had somewhat less distinct, regularly spaced increments. Ultradian increments <0.2 mm were commonly organized into doublets or triplets in both LD and SD teeth (**C**; brackets). Teeth of hibernators at 5°C (**D**) and 13°C (**E**) were characterized by regions of relatively narrow, sharply defined increments. A subset of hibernators showed clear reversion to wide, evenly spaced increments at the base of the tooth (**F**; arrow).

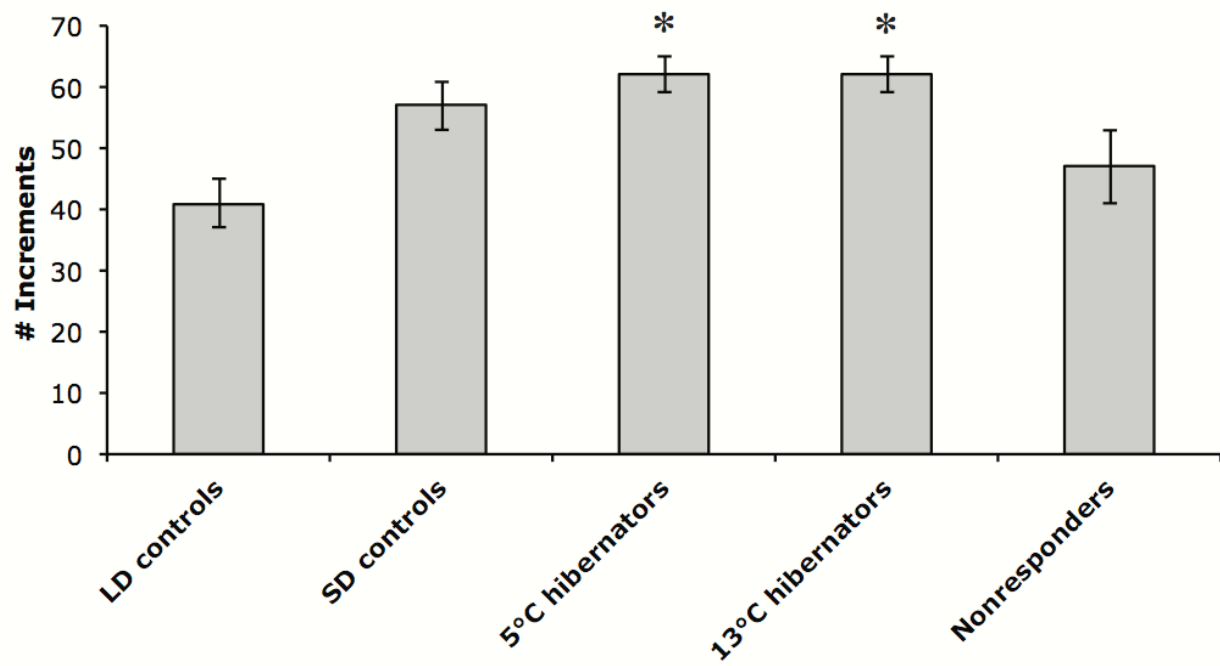


Figure 5.2. 5°C and 13°C hibernators had incisors comprised of significantly more dentin increments than LD control hamsters; SD control and nonresponsive hamsters had intermediate numbers of increments and did not differ significantly from LD controls or either group of hibernators. * $P < 0.05$ versus LD controls.

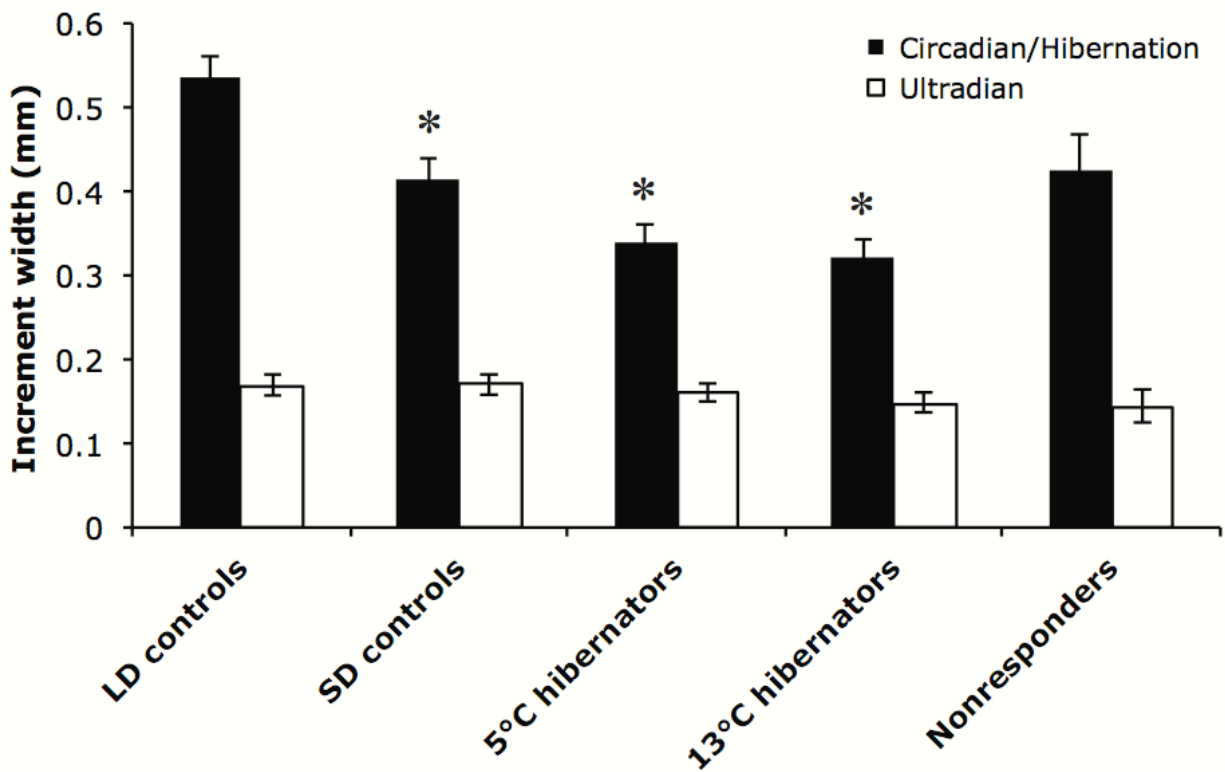


Figure 5.3. Increments (circadian and/or hibernation) were significantly wider in LD control hamsters than SD control hamsters, 5°C hibernators and 13°C hibernators. SD controls and hibernator groups did not differ from each other, and nonresponsive hamsters were not significantly different from any other group. There were no significant differences in ultradian increment widths. *P<0.05 versus LD controls.

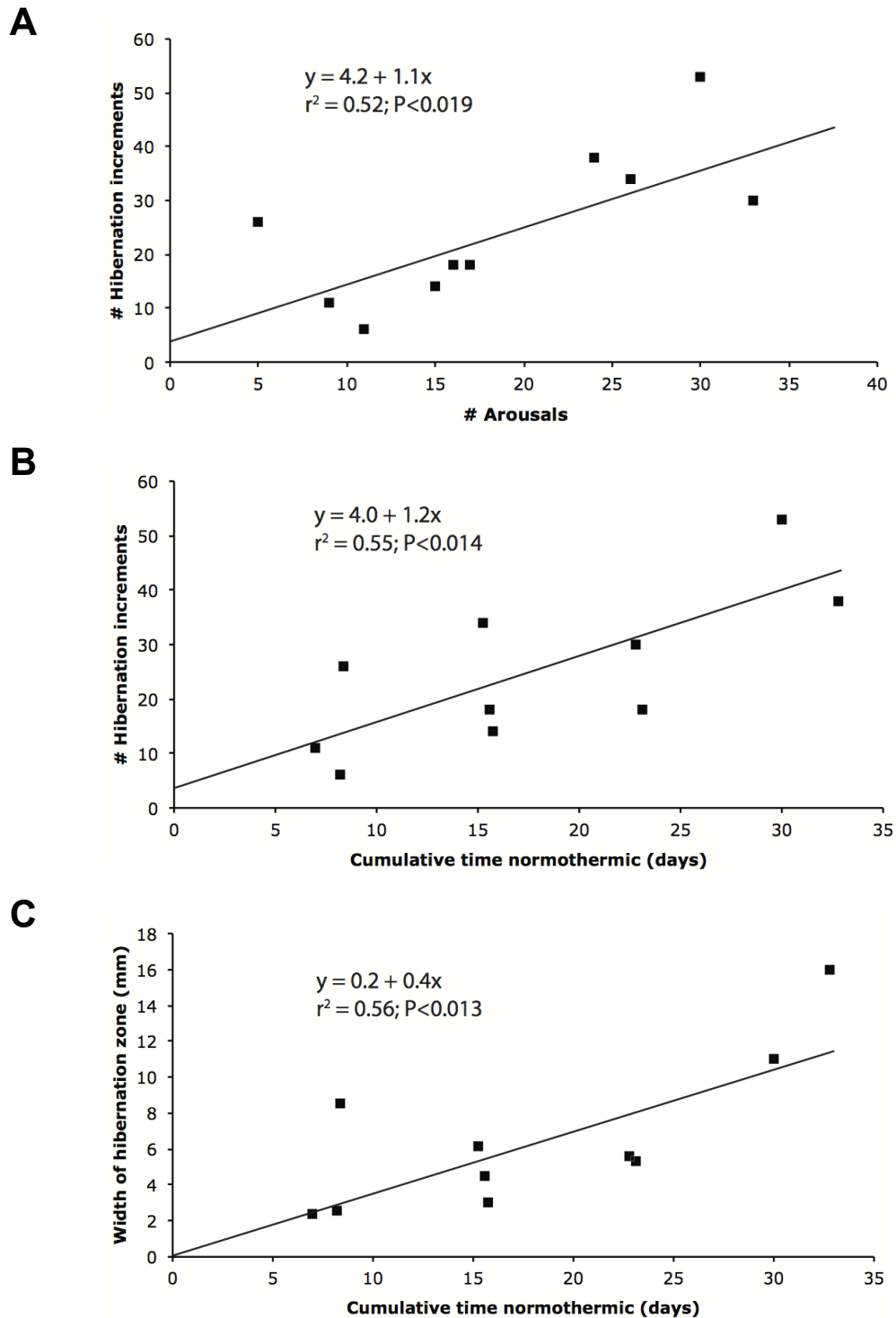


Figure 5.4. For 5°C hibernators, number of hibernation increments was significantly predicted by both number of arousals (**A**) and cumulative time normothermic between torpor bouts (**B**). Summed width of all hibernation increments (the “hibernation zone”) was also predicted by cumulative time normothermic between torpor bouts (**C**).

Chapter 6

Hard X-ray Micro-tomography as an Alternative to Conventional Histology: Implications for Studying Incremental Dentin of Historically Collected Specimens

Introduction

Incisor dentin deposition in rodents is altered during hibernation, yielding a characteristic hibernation morphology that differs by species, but which is distinct from normal circadian increments (Klevezal and Mina, 1990; Rinaldi, 1999a, 1999b; Trunova and Klevezal, 1999; Trunova, 2001; Selkova, 2003; Goodwin et al., 2005; Goodwin and Ryckman, 2006; Klevezal and Shchepotkin, 2012; Klevezal et al., 2012; Batavia et al., 2013b). Because teeth are commonly preserved post-mortem, dentin increments and hibernation marks offer the opportunity to study hibernation in historical contexts. Teeth collected and stored in museum collections can in theory be compared to recently collected specimens of the same species, to assess historical shifts in hibernation behavior. For example, it might be possible to detect longitudinal changes in the frequency or cumulative duration of arousals from hibernation (Batavia et al., 2013b), of interest to biologists studying the effects of climate change on energy expenditure of hibernating species.

Historically collected museum specimens, are, however, irreplaceable, so examining their microstructure nondestructively is of paramount importance. Information about hibernation can be gleaned either from the medial surface of the incisor (Rinaldi, 1999a, 1999b; Goodwin et al., 2005; Goodwin and Ryckman, 2006; Klevezal and Shchepotkin, 2012; Klevezal et al., 2012; Batavia et al., 2013b), which requires removal of the tooth from its alveolus, or by histological sectioning (Klevezal and Mina, 1990; Klevezal, 1996; Trunova and Klevezal, 1999; Trunova, 2001; Selkova, 2003;). Removing teeth from alveoli without damaging the surrounding mandible or maxilla is nearly impossible in historically preserved specimens (personal observation), and histology necessarily requires permanent alteration and destruction of the tooth.

The goal of the current investigation was therefore to assess high resolution X-ray micro-tomography as a nondestructive alternative to conventional histology, and to pilot its use in a few specimens from the Museum of Vertebrate Zoology (MVZ) at the University of California, Berkeley. This technique is similar to medical computed tomography (CT) scanning, except that it can image high-density materials – including bone and tooth – and can resolve features as small as a few microns. I used the Lawrence Berkeley National Laboratory Advanced Light Source's Beamline 8.3.2 to scan lower incisors of laboratory-housed control and hibernating Turkish hamsters. The product was a series of virtual "slices" through each tooth; I compared these virtual slices to actual histological sections. I also scanned a small number of MVZ specimens from four species of sciurid hibernators, in search of evidence of a putative hibernation mark. My findings indicate that images produced by high-resolution X-ray microtomography were nearly as good as those obtained from conventional histology, and that this technique offers promise for cases in which specimens cannot be damaged or replaced.

Methods

Tooth samples

Teeth from six Turkish hamsters (*Mesocricetus brandti*) described in Chapter 5 were used in this study (Table 6.1). Two hamsters – one male and one female – were selected from each of three experimental groups: long-day controls, short-day controls, and 5°C hibernators (see Chapter 5 for details of treatment groups). Each hamster's lower left incisor was scanned using high resolution X-ray micro-tomography. The lower right incisor was prepared histologically, thereby preserving the left tooth in case additional scanning became necessary.

A list of MVZ specimens scanned is shown in Table 6.2. Four species – *Uroditellus beldingi beldingi*, *Tamias speciosus frater*, *Tamias alpinus*, and *Marmota flaviventris* – were selected because they were well-represented in the MVZ's collection, which maximized the possibility of finding specimens collected shortly after emergence from hibernation. This was important because putative hibernation marks would be expected to disappear after terminal arousal from hibernation as a result of continual incisor growth and attrition at tooth apices (Schour and Steadman, 1935). In addition, although all specimens scanned in this study (except for the marmots) were collected within the last decade (Table 6.2), historical specimens of the same species from similar localities and/or elevations are also available in the MVZ's collections, and offer the potential for future study. Finally, all of these species are robust hibernators and occur in geographic areas of California known to have undergone climatic change during the last century. Individual specimens were selected on the basis of collection date and elevation – those collected from the highest elevations and earliest in the spring or summer offered the greatest chance of detecting evidence of recent hibernation. Additionally, all specimens scanned were females, which emerge from hibernation later than males (Michener, 1984), and were therefore more likely to show evidence of a hibernation mark.

Micro-tomography

Scanning was completed at the Lawrence Berkeley National Laboratory Advanced Light Source using Beamline 8.3.2. In Turkish hamsters, extracted lower left incisors were scanned at the approximate midpoint of the incisor shaft, where tooth curvature was least apparent, because final images were clearest when stacked virtual “slices” (see below) fell in the same vertical plane (i.e., where curvature was at a minimum). Lower incisors of MVZ specimens were still lodged in their alveoli, and thus curvature could not be accurately assessed. For the sake of consistency, these teeth were scanned at the point where they emerged from the mandibular alveolus. All specimens were scanned at 19 keV. Pixel size (magnification) varied due to availability of lenses and because I wanted to test the efficacy of different lenses (lower magnification lenses require shorter scans, thereby exposing specimens to less radiation); values are reported in Tables 6.1 and 6.2. Image reconstructions were performed using the program Octopus version 8.6 (inCT, Gent, Belgium). Fully reconstructed images were then stacked in FIJI (ImageJA 1.44b, public domain) to form virtual “slices” for further analysis.

Histology

Lower right incisors were individually embedded in Silmar 41 clear polyester casting resin (US Composites, West Palm Beach, FL) catalyzed at 1% with methyl ethyl ketone peroxide. Resin blocks cured for >1 week prior to sectioning with a diamond wheel (Norton, Worcester, MA) on a low speed saw (Buehler, Lake Bluff, IL). Three 1 mm sections were cut from right incisors, beginning at approximately the same point on the tooth shaft where left incisors were scanned, and moving toward the tooth base, where there was less chance of encountering secondary (non-incremental) dentin (Klevezal, 1996). I mounted the sections on frosted slides (Ward's Natural Science Establishment, Inc., Rochester, NY) using 5 minute epoxy (ITW Performance Polymers, Riviera Beach, FL) and ground them to approximately 0.25 mm thickness with a variable speed grinder-polisher (Buehler, Lake Bluff, IL) and grit paper increasing from 400 grit to 1200 grit (Buehler, Lake Bluff, IL). Cover slips (Fisher Scientific, Pittsburg, PA) were mounted with fast-hardening mounting reagent Eukitt (O. Kindler, West Germany), and slides were imaged under polarized light with a Leica DM 2500 microscope (Leica Microsystems, Wetzlar, Germany); photographs were captured with a digital camera (Nikon DS-Fi1, Nikon Instruments, Inc., Tokyo, Japan).

Image analysis

All images were analyzed in FIJI (ImageJA 1.44b, public domain). For each specimen, the image showing the highest number of clearly distinguishable increments was used for analysis; increments were counted and measured to the nearest 1 μm .

Statistics

Paired t-tests were used to compare number of countable increments and mean increment width in left (scanned) versus right (histologically sectioned) incisors. One specimen (MB12) was excluded from statistical analyses because the left and right teeth differed substantially in the size of the pulp cavity. The left, scanned tooth had a much smaller pulp cavity and consequently, more visible increments, particularly toward the center of the tooth, where increments tend to be narrower. Any difference in countable increments or mean increment width, in this case, clearly reflected idiosyncrasies of the teeth, rather than the visualization method employed.

Results

All images of scanned teeth showed so-called “ring artifacts,” an unavoidable by-product of the x-ray micro-tomography scanning process. Staff members at the scanning facility helped to reduce the presence ring artifacts by manipulating an algorithm in the reconstruction software, but some rings nonetheless remained visible in final images. With rare exceptions, ring artifacts did not obscure dentin increment morphology, nor were they easily mistaken for dentin increments.

Image quality and increment visibility were not noticeably different between specimens scanned with a 5x (0.001330 or 0.0018 mm pixel) versus 10x (0.0009 mm pixel) lens.

X-ray micro-tomography versus histology in Turkish hamster incisors

Increments were generally clearest on the medial, lateral, and lingual aspects of the tooth, whether visualized in histological section or by micro-tomography. Increments were often not visible in the region immediately surrounding the pulp cavity, possibly due to the presence of non-incremental secondary dentin (Klevezal, 1996) or because dentin layers were oriented at an angle suboptimal for cross-sectional viewing. With both methods, increments were more pronounced in teeth of hibernators than in those of LD or SD non-hibernating controls (Figures 6.1 – 6.3).

Micro-tomography scans revealed fewer increments than histological sections, but this difference was not significant ($P < 0.08$). Mean increment width was $\sim 22 \pm 5\%$ greater when measured from micro-tomography scans, and this difference was significant ($P < 0.002$). The number of countable increments and mean increment width measured in each individual by both methods are shown in Table 6.1.

Survey of MVZ sciurid hibernators

Dentin increments were visible in all three *U. beldingi beldingi* specimens (Figure 6.4). Some cracking between concentric increments was evident in at least two specimens (216239 and 216241), probably as a result of drying. Increments were clearest in the area around the pulp cavity, but tended to become fainter and fade completely toward the periphery of the tooth. Specimen 207175 had a pronounced relatively dark band encircling the pulp cavity, distinct from finer increments closer to the pulp cavity. Unfortunately the quality of images from this specimen was poor and could not be improved during the image reconstruction process.

Scans of *T. spaciosus frater* were variable. Dentin of specimen 201451 was completely homogeneous and had no discernible increments (not shown). Specimens 216325 and 216326 (Figure 6.5) showed dentin increments close to the pulp cavity, but dentin became homogeneous toward the periphery of the tooth. Both lower incisors of specimen 216326 were imaged in the same scan, revealing the pulp cavities to be of markedly different sizes. This specimen also showed several dark bands that were comprised of 2-3 finer increments (Figure 6.5, inset).

Both incisors of a single *T. alpinus* were imaged in the same scan (Figure 6.6), revealing pulp cavities of roughly the same size and shape. Banding was clearly visible in both incisors, but the image quality for this particular run was poor and could not be improved during the image reconstruction process. As with other specimens, banding was clearest around the pulp cavity, but disappeared toward the periphery of the tooth.

Incisors of *M. flaviventris* were too large to scan in their entirety, so images show only partial cross-sections of teeth (Figure 6.7). Dentin increments were clear in both specimens; unlike other species surveyed, increments were visible nearly to the periphery of the tooth. Both specimens had very small pulp cavities. Specimen 98957 had a region of narrow, prominent increments that differed from the adjacent regions of fainter increments.

Discussion

Turkish hamster incisors imaged by micro-tomography and prepared histologically yielded qualitatively similar results. Specimens that showed relatively faint increments in histological section (e.g., Figure 6.2) also had relatively fainter increments when scanned; those

that showed robust increments in histological sections had relatively distinct increments in scanned images (e.g., Figure 6.3).

Micro-tomography revealed fewer increments than histological sections, though the difference was not significant. Despite the lack of statistical significance, I believe that histology does, overall, reveal more increments than micro-tomography, particularly toward the periphery of the tooth. LD and SD control teeth consistently showed more increments when prepared histologically than when scanned (Table 6.1). In hibernating specimens, histology and scanning yielded comparable results, possibly because hibernation is associated with greater contrast between dentin layers, which are therefore more easily detected by micro-tomography. A larger sample of specimens would likely reveal a significant difference between these two methods, particularly if hibernators were excluded. Even if there were a significant difference in the number of countable increments, however, the implications would probably be inconsequential, given that there is wide variation even within a single individual, depending on where the tooth is imaged along its shaft (personal observation).

Increments measured from micro-tomography images were significantly wider than those measured from histology images. The reason for this difference is unclear, but may be due to the fact that micro-tomography and subsequent image reconstruction result in more blurring, such that it is more difficult to precisely identify borders between increments. Despite the slightly wider and possibly lower number of increments revealed by micro-tomography, this technique yields a good approximation of results obtained from histological sections and is therefore a valuable non-destructive alternative to conventional histology.

Of 9 MVZ sciurid specimens scanned, 8 showed countable increments, and 2 showed possible evidence of hibernation (see below). In most specimens increments were not visible toward the periphery of the tooth, likely as a result of the angle of dentin layers and the stacking of images during the image reconstruction process. One specimen (*T. speciosus* 216326; Figure 6.5) showed very fine, possibly ultradian increments.

Trunova and Klevezal (1999) reported that hibernation marks in incisors of *Mesocricetus*, *Marmota*, and *Uroditellus* were characterized by hyper- and hypochromatic bands (as compared to normal circadian increments) decreasing in width. One *U. beldingi beldingi* specimen (207175) in my study showed an apparent single contrasting band (Figure 6.4); hibernation marks of this description are characteristic of other rodent genera, including some glirids and dipodids. Several possible explanations exist: 1) *U. beldingi beldingi* was not included in Trunova and Klevezal's (1999) study, and may show a different type of hibernation mark from other *Uroditellus* species. 2) The results reported in Trunova and Klevezal (1999) may not represent the range of variation characteristic of hibernation marks in a given species. In support of this hypothesis, the *M. brandti* imaged in my study – known to have hibernated under controlled laboratory conditions – showed more pronounced dark and light dentin layers, but no net decrease in increment width, as previously reported. 3) The mark in *U. beldingi beldingi* may be an artifact of poor image quality for this specimen. 4) The mark may reflect a different event, such as parturition, lactation, or nutritional stress, all of which can affect dentin deposition (Klevezal and Myrick, 1984; Miller et al., 1985; Manzanilla, 1989). Without detailed information on how hibernation affects dentin deposition in this species (and the others scanned in this study), it may not be possible to definitively attribute dentin morphology to hibernation.

In addition to a possible hibernation mark in *U. beldingi beldingi* 207175, *M. flaviventris* 98957 had a region of pronounced increments similar to those of a hibernating *M. baibacina* shown in Trunova and Klevezal (1999). Again, there appears to be no net decrease in increment

width, as previously reported; in this particular specimen, it is difficult to determine whether the change in increment contrast is a scanning artifact.

Given the limited ability of field researchers to collect animals at high elevation sites early in the spring (due to limited access and snow pack), it is possible that most - if not all - specimens scanned in this study were trapped after any putative hibernation marks were grown out. Targeted collecting soon (< 1 month) after spring emergence, and subsequent imaging of incisors, would be useful to document the specific morphology of hibernation marks in these populations, as well as the approximate interval over which such marks are detectable prior to growing out. Even with this type of analysis, however, historical collections were probably affected by similar constraints on high-elevation collecting, and therefore, may not be useful in assessing longitudinal shifts in hibernation behavior. Comparisons of dentin increments in historically-collected versus modern specimens could still be of use in assessing seasonal changes in growth rates and shifts in seasonality over time. Micro-tomography would also be valuable in studies of tooth cross-sectional shape, alveolar bone structure, and incremental enamel.

In conclusion, hard X-ray micro-tomography yields a good approximation of data obtained by histological sectioning. For specimens not in short supply, histology is still preferable, given that access to equipment and technical expertise is limited, and that scanning and image reconstruction are nearly as time consuming as conventional histology. However, when specimens are irreplaceable or not otherwise available for permanent damage or alteration, micro-tomography is a viable non-destructive alternative to histology.

Table 6.1. Turkish hamster incisors prepared histologically and scanned by micro-tomography.

Specimen ID	Sex	Treatment group	Histology # increments	Histology increment width (mm)	Scan # increments	Scan increment width (mm)	Scan pixel size (mm)
210	Male	LD control	12	0.023	7	0.026	0.001330
303	Female	LD control	23	0.023	15	0.027	0.001330
MB115	Male	SD control	14	0.023	4	0.027	0.001330
415	Female	SD control	14	0.025	11	0.031	0.001330
274	Male	5°C hibernator	20	0.015	22	0.021	0.0009
MB12	Female	5°C hibernator	14	0.026	26	0.017	0.0009

Table 6.2. Incisor specimens from Berkeley's Museum of Vertebrate Zoology scanned by hard X-ray micro-tomography.

Specimen ID	Species	Collection date	Location	Elevation (m)	Scan pixel size (mm)
207175	<i>Urocitellus beldingi beldingi</i>	7/16/2004	Yosemite National Park, Tuolumne County, CA	3167	0.0018
216239	<i>Urocitellus beldingi beldingi</i>	7/24/2005	Yosemite National Park, Tuolumne County, CA	3014	0.0009
216241	<i>Urocitellus beldingi beldingi</i>	7/24/2005	Yosemite National Park, Tuolumne County, CA	3014	0.0009
216325	<i>Tamias speciosus frater</i>	5/27/2005	Yosemite National Park, Mariposa County, CA	1898	0.0018
216326	<i>Tamias speciosus frater</i>	5/27/2005	Yosemite National Park, Mariposa County, CA	1924	0.0018
201451	<i>Tamias speciosus frater</i>	6/8/2003	Yosemite National Park, Mariposa County, CA	1886	0.0009
207200	<i>Tamias alpinus</i>	7/13/2004	Yosemite National Park, Mariposa County, CA	3024	0.0018
98957	<i>Marmota flaviventris sierra</i>	6/3/1942	Onion Valley, Inyo County, CA	2743	0.0018
113686	<i>Marmota flaviventris sierra</i>	5/26/1949	Bodie, Mono County, CA	2572	0.0018

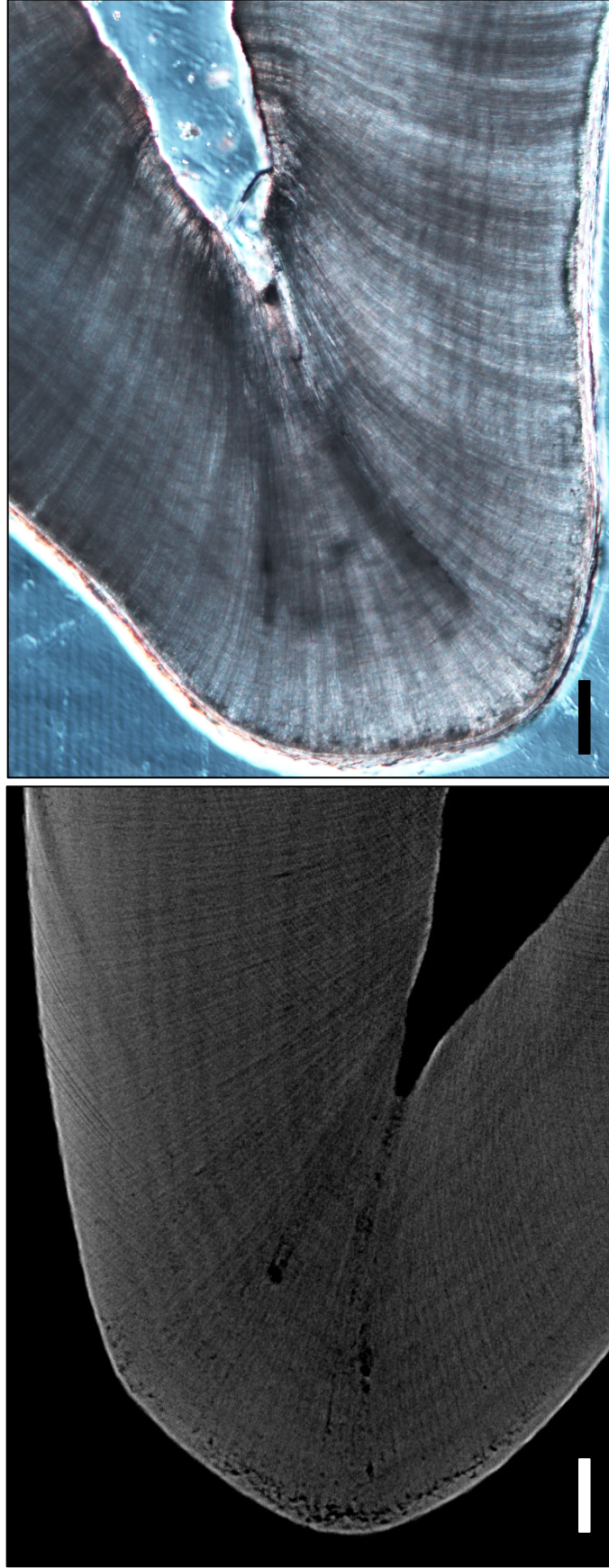


Figure 6.1. Lower incisors of a female Turkish hamster (specimen ID: 303) housed in long day conditions prior to euthanasia. The left image was generated by scanning the tooth with a 5x lens using x-ray micro-tomography; the right image was obtained by sectioning the tooth histologically and viewing under polarized light at 10x. Scale bars are 0.1 mm.

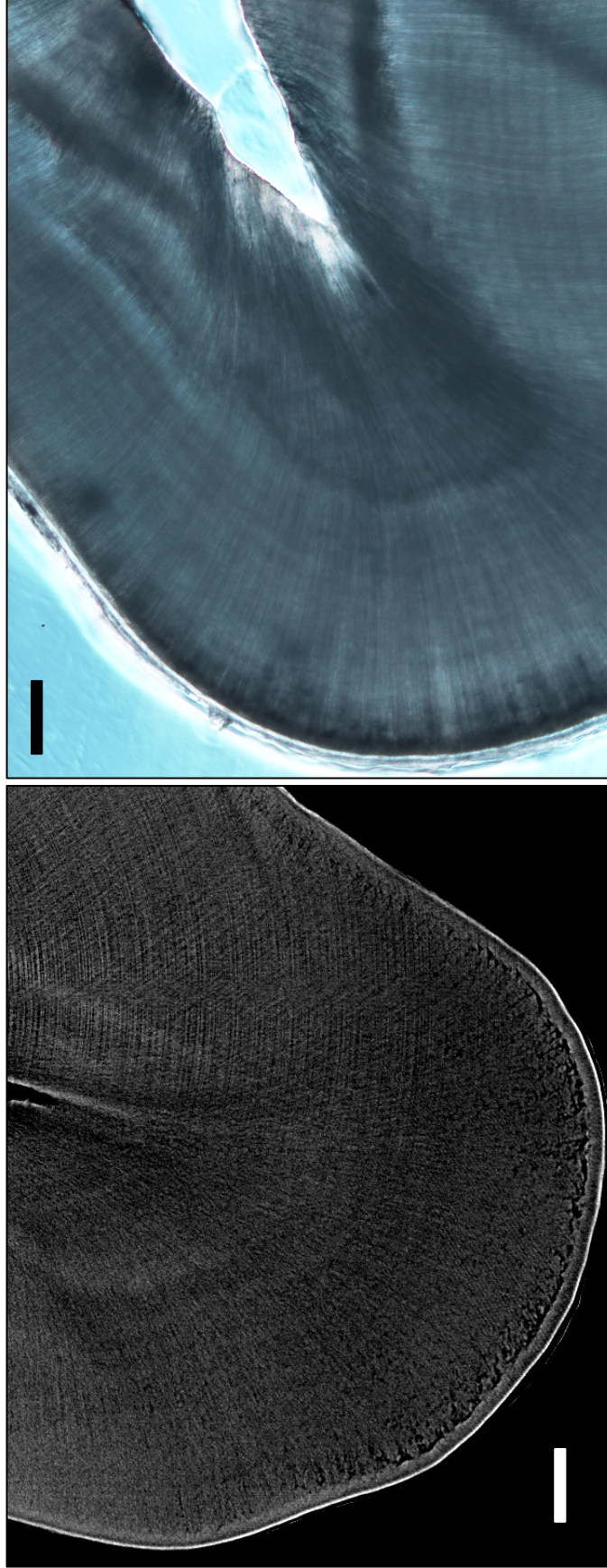


Figure 6.2. Lower incisors of a female Turkish hamster (specimen ID: 415) housed in short day conditions prior to euthanasia. The left image was generated by scanning the tooth with a 5x lens using x-ray micro-tomography; the right image was obtained by sectioning the tooth histologically and viewing under polarized light at 10x. Scale bars are 0.1 mm.

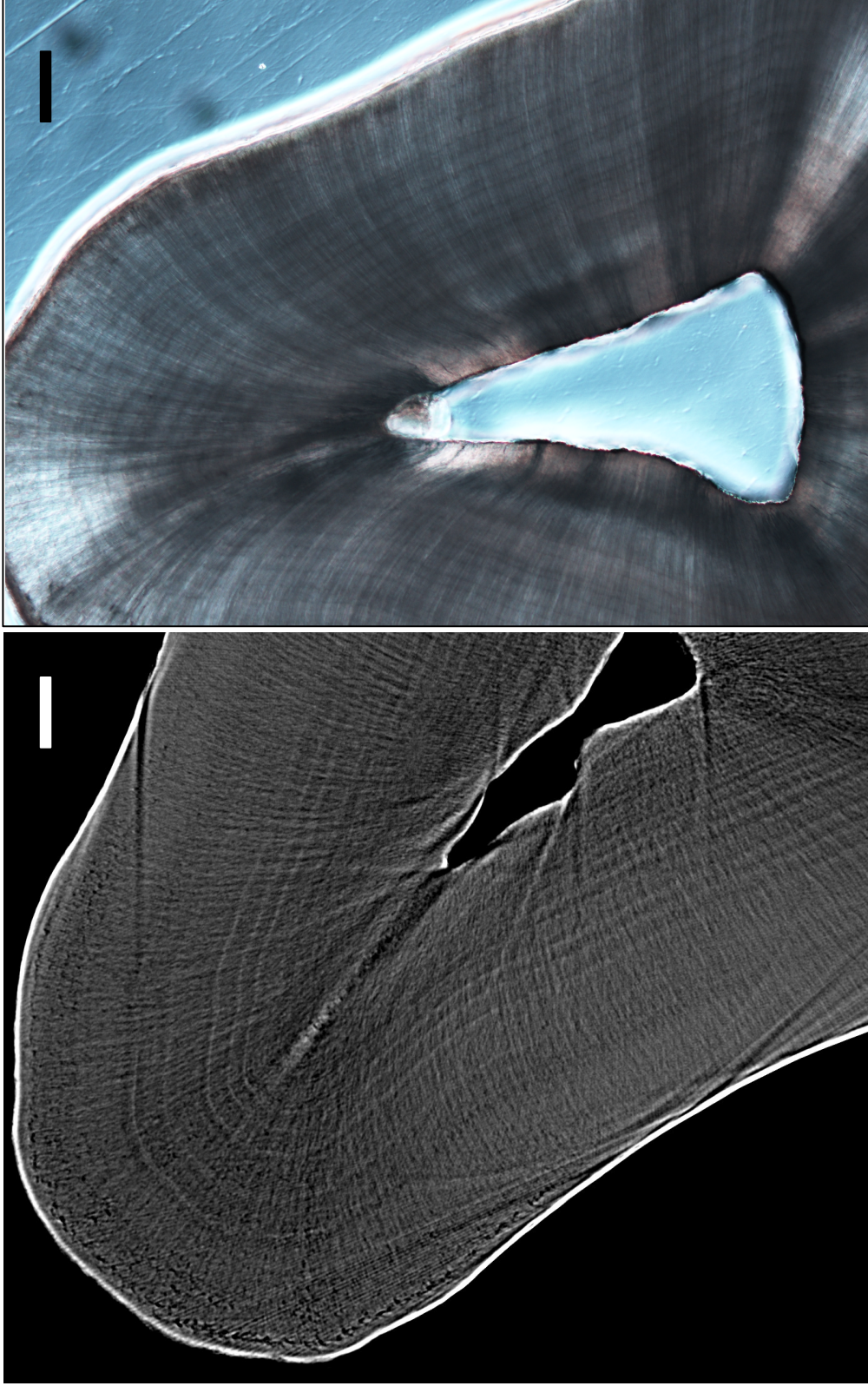


Figure 6.3. Lower incisors of a hibernating male Turkish hamster (specimen ID: 274) housed in 5C short day conditions prior to euthanasia. The left image was generated by scanning the tooth with a 10x lens using x-ray micro-tomography; the right image was obtained by sectioning the tooth histologically and viewing under polarized light at 10x. Scale bars are 0.1 mm.

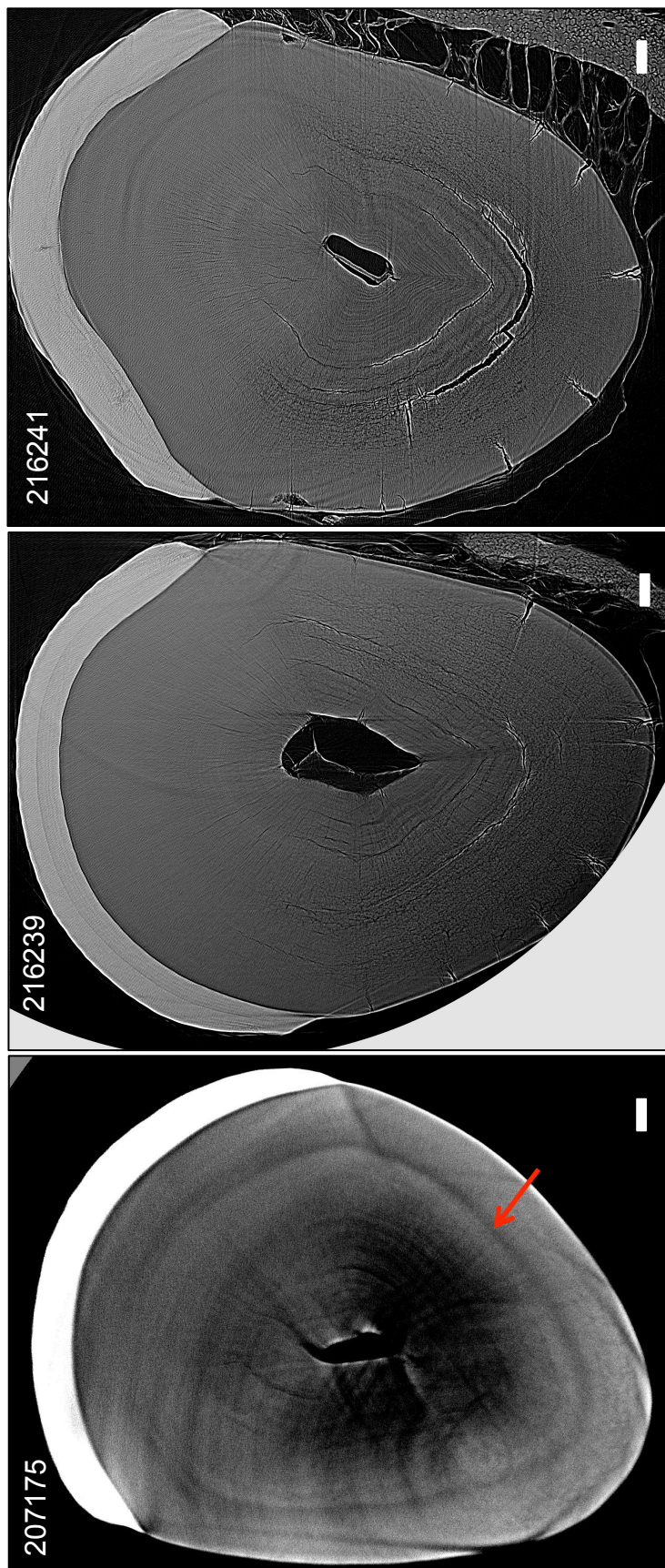


Figure 6.4. Lower incisors of female *Urocitellus beldingi beldingi* collected in July of 2004 or 2005. MVZ specimen numbers are shown in the top left corner of each image. In all three specimens increments were clearest close to the pulp cavity and faded toward the periphery of the tooth. A possible hibernation mark was visible in specimen 207175 (red arrow). Scale bars are 0.1 mm.



Figure 6.5. Lower incisors of female *Tamias speciosus frater* collected in May 2005. MVZ specimen numbers are shown in the top left corner of each image. Increments were clearest close to the pulp cavity. Lower incisors of specimen 216326 had pulp cavities of markedly different sizes. In this specimen, very fine increments (possibly ultradian increments) were discernible (inset). Scale bars are 0.1 mm.

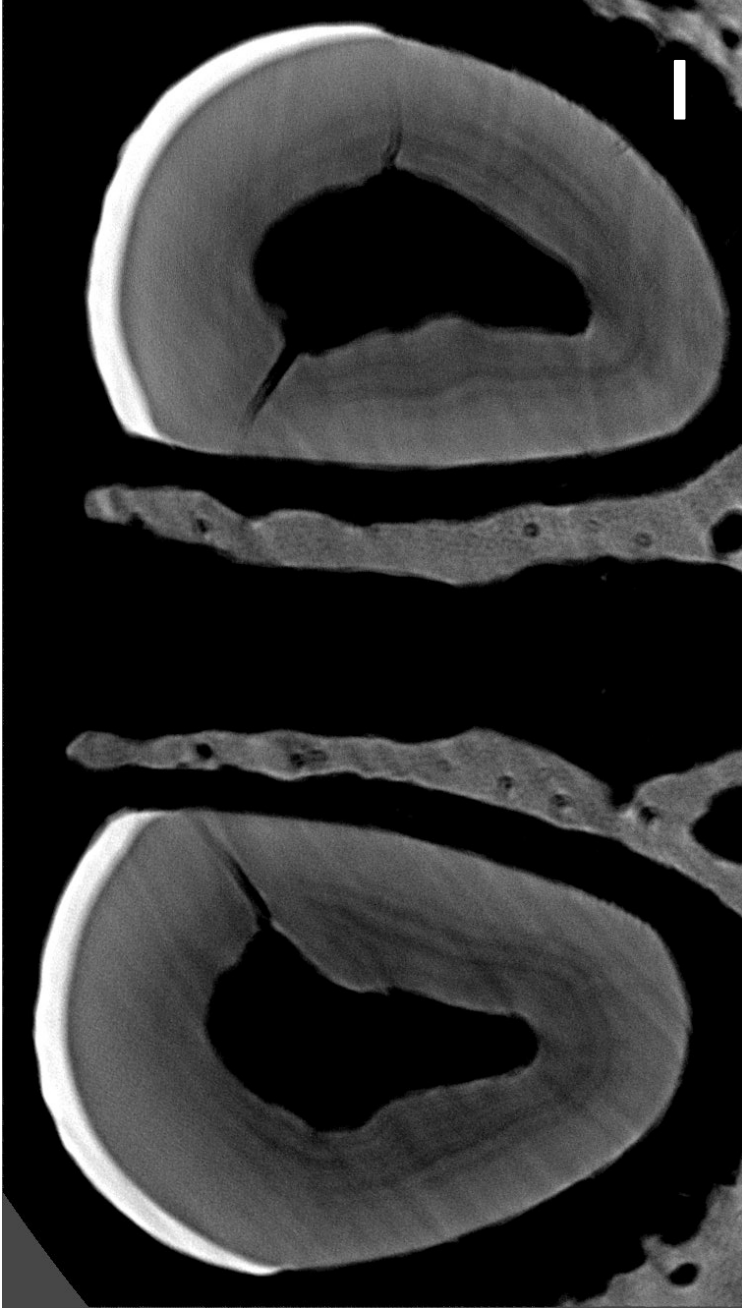


Figure 6.6. Lower incisors of female *Tamias alpinus* (MVZ specimen 207200) collected in July 2004. Teeth are shown embedded in the surrounding mandible. Despite relatively poor image quality, increments are visible in both incisors. Scale bar is 0.1 mm.

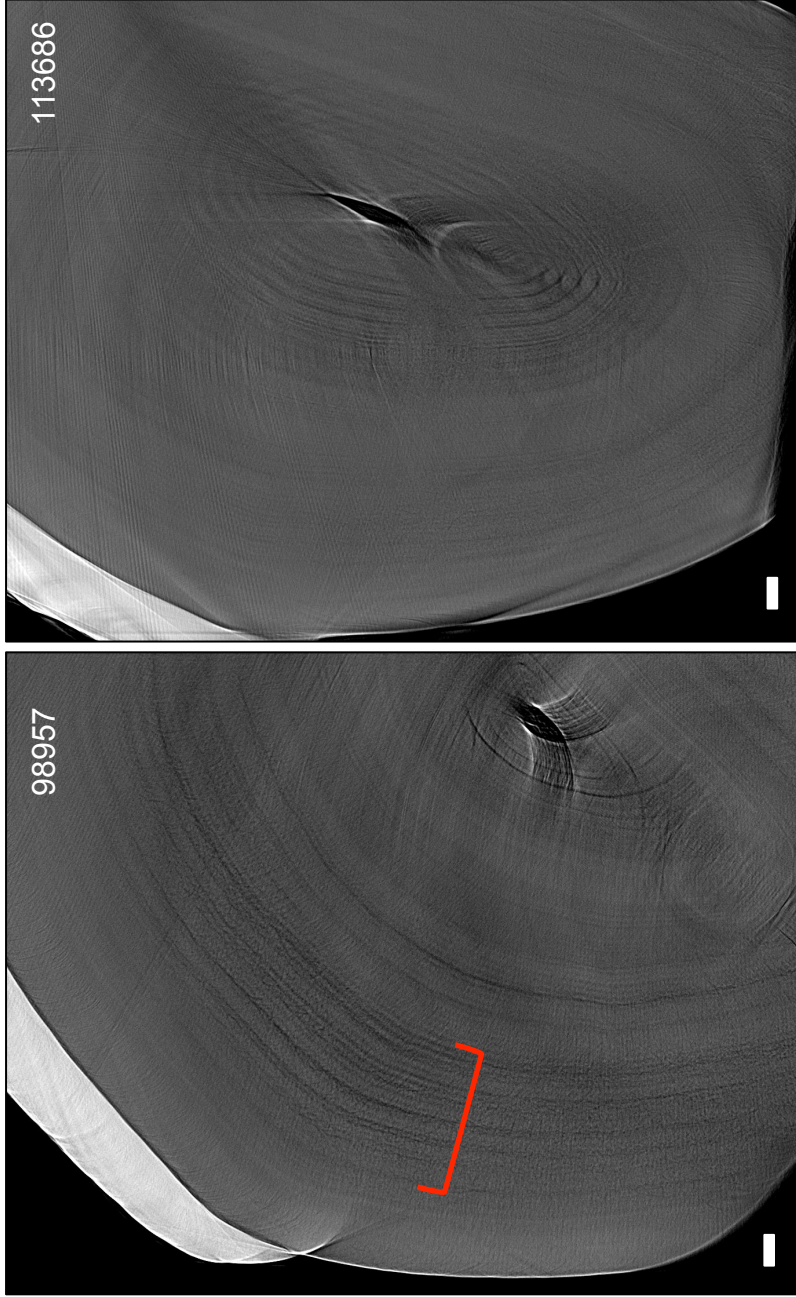


Figure 6.7. Lower incisors of female *Marmota flaviventris* collected in May 1949 and June 1942. MVZ specimen numbers are shown in the top right corner of each image. Increments are visible from the pulp cavity to nearly the periphery of the tooth. Specimen 98957 shows a region of pronounced bands of relatively narrow width (red bracket), which may be indicative of hibernation. Scale bars are 0.1 mm.

Chapter 7

Summary and Conclusions

The energetic costs of mammalian thermoregulation – and the varied physical, behavioral, and physiological adaptations that mammals employ to ameliorate those costs – are of profound importance to our understanding basic mammalian biology. This body of work addressed several specific topics relating to mammalian thermoregulation.

Chapter 3 assessed the adaptive value of fur to juvenile Siberian hamsters (*Phodopus sungorus*), and found that, although fur does reduce energetic expenditure under thermally challenging conditions, it is not essential for maintaining rapid growth, provided that food is available in unlimited supply. Chapter 4 described basic torpor parameters in the Turkish hamster (*Mesocricetus brandti*), a widely used model organism in hibernation research. Building on this work, Chapter 5 explored the effects of hibernation on incisor dentin morphology, and found that hibernation – regardless of ambient temperature – manifests as a distinct morphology on the medial surface of Turkish hamster incisors. Chapter 6 tested a new, non-destructive technique for imaging incisor dentin and hibernation marks in several hibernating rodent species. Together, Chapters 5 and 6 open the door for further exploration of hibernation marks in the teeth of fossil and historically-collected museum specimens, which in turn may bear on the evolution of and recent historical shifts in hibernation behavior.

Conclusions relevant to specific sections of this dissertation were addressed previously, but several more general observations merit brief discussion here. First, in both Chapter 3 (the adaptive value of fur in growing Siberian hamster pups) and Chapter 4 (a descriptive study on Turkish hamster hibernation), there were notable sex differences in thermoregulatory traits. In Chapter 3, males were significantly more efficient at converting consumed food into body mass than females, suggesting that females may have had to expend more energy on thermoregulation than males. Whether their costs were higher (potentially due to a difference in timing and pattern of fur regrowth) or their thermogenic response was less efficacious than that of males is unclear. Chapter 4 revealed that females had a higher critical minimum temperature while in torpor, and cooled and rewarmed more slowly than males when entering and arousing from torpor, respectively. In both species, these sex differences potentially have adaptive consequences, given that higher energetic costs for thermoregulation may affect energy reserves available for other demands, such as reproduction.

Historically, research in many biological disciplines has neglected females, failed to specify the sex of model organisms, or failed to compare sexes (Beery and Zucker; 2011). Though it has long been known that females tend to terminate hibernation later in the spring than males (Michener, 1984), studies of sex differences in torpor parameters or other thermoregulatory traits are rare (e.g., Grinevitch et al., 1995; Mzilikazi and Lovegrove, 2002; Cryan and Wolf, 2003). Even when sexes are compared, studies are almost always designed to assess the effects of reproduction or reproductive hormones, and do not address inherent differences between males and females in a non-reproductive state. This topic is ripe for further exploration, as it may highlight different selective pressures operating on males and females that are not directly tied to reproduction.

A second salient point that emerged from this body of work – specifically from Chapter 5 (effects of day length, hibernation, and ambient temperature on incisor dentin) and Chapter 6 (comparison of conventional histology and hard X-ray micro-tomography) – is that there is substantial individual variation in dentin morphology. In Chapter 5, even under controlled temperature, day length, nutritional, and social conditions, there was still variation in the qualitative properties of incisor dentin. Although long-day and short-day non-hibernating controls differed significantly from each other, within each of these groups were individuals that showed dentin morphology more consistent with the other group. Hibernation manifested clearly on a majority of hibernator teeth, but there were a few samples that either lacked the characteristic hibernation morphology or were ambiguous. In Chapter 6, there were two cases of extreme variation the size of the pulp cavity (and therefore, the amount of dentin) within a single individual.

The implications of this variation are several-fold. First, any analyses of dentin increments or putative hibernation marks in fossil or historical taxa must be performed at a population level. Based on my results, dentin morphology is simply not sufficiently straightforward to definitively diagnose day length (season) at death or hibernation in single individuals. Second, for any assessment of shifts in hibernation behavior or growth rates, it would be important to first establish the baseline morphology and seasonal changes in growth rate in a closely related population of interest, ideally under controlled conditions, before undertaking comparisons to a historical population. Third, for diagnosing hibernation in fossil specimens of extinct species, it would be prudent to examine the morphology characteristic of the closest living relatives (with the realization that dentin morphology is, evidently, a highly labile trait). Finally, it is important to recognize the caveats inherent in using dentin morphology to elucidate information about a past species' or population's physiology. It may be one of very few available tools for studying such questions, and certainly has value, but it is limited in its specificity, and quite possibly (and variably) in its accuracy. It should be used with caution, and in conjunction with other available techniques and evidence.

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