

Factors affecting the hibernation in European ground squirrel

Spermophilus citellus

Physiological, behavioral and ecological aspects

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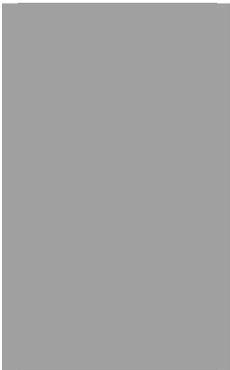


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Introduction and thesis overview

Hibernation is one of the most mysterious behavioral patterns and a dramatic example of phenotypic plasticity in endotherms. In hibernation, animals spend a considerable amount of time well below euthermic body temperatures. This low body temperature state, called torpor (see Box 1), is a hypothermic state which, in contrast to pathological hypothermia, is entered into voluntarily and can be terminated by the animal itself. The animal does not temporarily lose its capability for thermoregulation, rather during the state of torpor, thermoregulation and many other homeostatic regulatory processes are still functional. (Willis 1982a). Ultimately, the primary function of hibernation is to save energy via substantial reduction in metabolic activity (Geiser 2004).

The phenology and taxonomy of hibernation

Many endotherms have evolved the ability to escape from unfavorable environmental conditions by using dormancy. Three major forms of dormancy have been classified: hibernation, aestivation and daily torpor (Heldmaier et al. 1989). Certain endotherm species or entire taxons could be sorted into a particular type of dormancy, mainly due to their physiological (body size, fat storing ability) and ecological (climate) characteristics (Heldmaier et al. 2004).

Hibernation is a well known strategy among mammalian heterotherms which range between 0.05 and 10 kg, to temporarily escape the winter season. These species come from all three mammalian subclasses such as Prototheria (*Tachyglossus aculeatus*: Grigg and Beard 2000), Metatheria (*Dasyuromorphia*, *Didelphimorpha*: Cooper et al. 2005; Cooper et al. 2003; Cooper and Withers 2004; Körtner and Geiser 1998; Körtner and Geiser 2000b; Song et al. 1997) and many orders of Placentalia. Representatives of hibernators can be found among insectivores (*Tenrecidae* and *Erinacidae*: Lyman 1982b), rodents (*Cricetinae*, *Marmotinae*: Lyman 1982b) and bats (*Myotis lucifugus*: Thomas and Cloutier 1992), however in spite of general belief, carnivores are not true hibernators.

Box 1

Definitions of hibernation

Before detailed discussion of the different aspects of hibernation begins, the author would like to clarify the terminology of hibernation. This could be valuable for readers as some perceptible confusion may still exist as there are different usages of the terminology in commonly used peer review literature still today. Henceforth, the introduced phrases and definitions of hibernation are based on the terminology by Lyman et al. (1982).

The word “torpor” (also referred to as “deep hibernation” by some authors) is used to describe a physiological state involving a controlled lowering of the thermoregulatory set point, resulting in a drop in body temperature and metabolic rate (Barclay et al. 2001). The origin of this idiom is the Latin “torpeo”, which means being motionless and lethargic, which mostly refers to the behavior of the animals during torpor state. The opposite of “torpor” is the phrase “arousal”. Arousals are short euthermic phases that regularly interrupt torpor states during hibernation.

The words “euthermia” and “euthermic” are used to describe the warm blooded or active state, whether the animals are asleep or awake. Euthermic means “promoting warmth” and in this sense is preferable to “normothermic” which suggests that this is the normal condition and hence torpor is abnormal. The words “homeothermia” and “homeothermic” are often used to describe this condition, but these imply a steady body temperature, and animals in torpor may maintain a steady, controlled body temperature, therefore these words are not sufficiently descriptive. The word “hypothermia” is used to refer to a depressed body temperature whether it is developed under a physiological or a pathological condition. Finally, “poikilothermia” is used to denote a condition in which the body temperature is dependent on ambient temperature.

Carnivores exhibit rather a kind of so called “carnivorian lethargy” during winter as was suggested by Lyman (*Ursidae, Procyonidae* and *Mustelidae*: 1982b). The entire hibernation season of these animals is characterized by a sequence of prolonged torpor bouts. Their duration may vary with ambient temperature and season as well as the species, but average duration of deep torpor bouts lasts about two weeks (French 1982; Pengelley and Fisher 1961; Twente and Twente 1965; Twente and Twente 1968; Wang 1973). Physiological characteristics of hibernation are a reduction of body temperature near to ambient temperature, a markedly reduced metabolic rate and spontaneous arousals by activation of heat producing mechanisms (Carey et al. 2003; Geiser 2004; Wang 1979b; Willis 1982a). Hibernating animals retreat into a hibernaculum and stay there until the next spring. During this time animals live off of their body fat stores or food accumulated during the summer season (Humphries et al. 2003).

Similar behavior with a sequence of prolonged bouts of dormancy may also be observable in summer and is called aestivation. Such behavior is exhibited by some species of rodents inhabiting the arid areas of the Holarctics (*Spermophilus, Peromyscus*: Wade 1930).

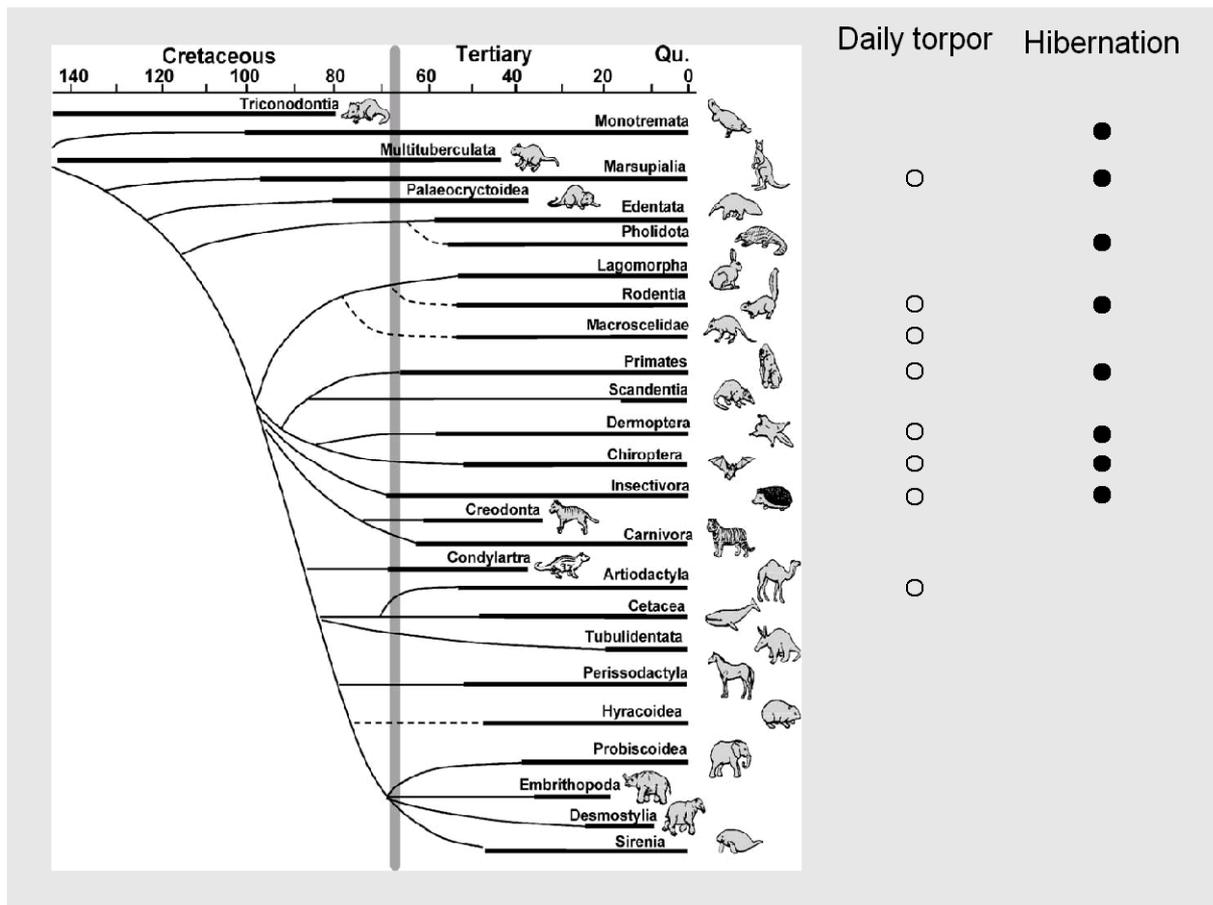


Figure 1.1 Occurrence of hibernation (●) and daily torpor (O) in mammals. Mammalian orders are labeled by symbols when at least one species has been reported to show either daily torpor or hibernation. In some groups like marsupials, chiroptera and rodents, a large number of species show either one or both of these hypometabolic behaviors. Figure is based on Heldmaier et al. (2004).

Aestivation is further characterized by a reduction of body temperature and metabolic rate at moderate ambient temperatures (~ 20°C: Wade 1930; Wilz and Heldmaier 2000).

The third form of dormancy is shallow or daily torpor. It has been observed in small mammals such as bats and some hamsters (*Phodopus sungorus*: Heldmaier et al. 1999), uniquely in primates (*Microcebus murinus*: Schmid and Kappeler 1998), but eminently in bird orders such as *Apodiformes* (hummingbirds and swifts) and *Caprimulgiformes* (nightjars, nighthawks and poorwills, Brigham et al. 2000; Kortner et al. 2000; Lyman 1982b). It is characterized by torpor bout duration of less than a day. Further characteristics include a reduced metabolic rate and a minimum body temperature between 10°C and 25°C approximately (Barclay et al. 2001; Körtner and Geiser 2000a).

The evolutionary history of hibernation

Hibernation, daily torpor and aestivation have been observed in a large number of small mammals. Thus the behavior is not limited to one or few mammalian orders, instead in most mammalian orders a few species, genera or families are known as hibernators or torpidators (see figure 1.1, Lyman 1982b). This indicates that the ability for torpor is widespread and it is often debated whether hypometabolism is a basic property of mammalian physiology as a plesiomorphic or an apomorphic trait (Carey et al. 2003; Geiser 2008; Grigg et al. 2004; Heldmaier et al. 2004).

It is necessary to discuss the evolution of hibernation along with the appearance of the ability to maintain a constant and high body temperature, endothermy. Warm blooded organisms (mammals and birds) generate a large amount of body heat, even at rest (McNab 2008; McNab 2009). Thus endothermy allows maintaining internal temperature at that desirable level where physical performance of the animal is optimal. Hence endotherms rapidly dispersed all over the world during their more than 60 million year long history, especially in temperate and cold environments where poikilotherm animals failed to establish themselves (McNab 1978). However, these physiological and ecological advantages of endothermy are achieved only at the price of high energy costs. The life of endotherms depends upon a continuous supply of food and energy wasted as heat. As metabolic rate in endotherms is allometrically related to body size (Heldmaier et al. 2004), small animals use proportionally more energy than larger ones because of a high surface to body mass ratio resulting in great loss of heat during cold exposure and an elevated mass specific metabolic rate (McNab 2008). Consequently, in small endotherms, the energetic cost of maintenance of homeothermy becomes prohibitively high, especially during cold exposure or food shortage. Therefore the ability to step back to ancestral heterothermy, was advantageous and dormancy/torpor/hibernation appeared among relatively small sized animals, in spite of 60 million years of evolution. The phylogenetic distribution of these animals is diverse, encompassing several independent lineages in which many heterotherms are closely related to non-heterotherms and in which there is no identifiable heterothermic common ancestor (Harris et al. 2004). Based upon these facts, some authors argue that heterothermy among endotherms is polyphyletic (Davis 1976; Geiser 1988; Grigg et al. 2004) and not a retained ancestral trait.

Geiser recently summarized the evolution of heterothermy in endotherms (Geiser 2008). The author suggests that in placental hibernators, heterothermy is a derived trait that evolved in

certain taxa secondarily after a homeothermic phase, perhaps as a response to an energetic challenge. As mammals and birds arose from different reptilian lineages, endothermy probably evolved separately in the two classes and given that the development of torpor during ontogeny differs between marsupials and placentals, torpor seems to have evolved on at least three lineages.

Energetics and physiology of hibernation

Endothermy has many advantages over heterothermy, but it is very costly to continuously maintain. The energy content of food consumed by endotherms is mostly spent on maintaining the constant elevated body temperature (McNab 2008; McNab 2009).

As prolonged periods of high metabolic heat production require high food intake and food availability in the wild often fluctuates, the cost of thermoregulation may become prohibitively high. This is one of the reasons why not all mammals and birds are permanently euthermic, but during certain times of the day or year enter a state of torpor (Lyman et al. 1982). Torpor in these heterothermic endotherms is characterized by a controlled reduction of body temperature, metabolic rate and other physiological functions (Carey et al. 2003; Geiser 1988; Geiser 2004). Body temperature during torpor falls from high euthermic values of ~ 32 to 42 °C to values between -3 to < 30 °C, metabolic rate is on average only 6 % of basal metabolic rate (Geiser 1988; Geiser 2004) and is often less than 1% of the resting metabolic rate in some species at low ambient temperature (Song et al. 1997).

Although metabolic rate during torpor may be a fraction of that in euthermic individuals, regulation of body temperature during torpor is continuous (Florant and Heller 1977). Body temperature is maintained at or above a species or population specific minimum by a proportional increase in heat production that compensates for heat loss (Florant and Heller 1977). During entry into torpor, the hypothalamic set point for body temperature is regulated downwards. Only when body temperature reaches the low set point during torpor is metabolic heat production used to maintain body temperature at or above this minimum body temperature. Therefore the metabolic rate in heterothermy as a function of ambient temperature, is species specific and typically „U” shaped (see figure 1.2, Song et al. 1997).

The traditional view is that as body temperature and metabolic rate fall together at torpor entry and because the Q_{10} (the change in rate over a 10°C increment) between torpor metabolic rate and body temperature often approximates 2 (which is typical of biochemical reactions), the

Chapter 1

metabolic rate reduction during torpor below basal metabolic rate is explained by temperature effects (Geiser 2004). Because unexpectedly high Q_{10} (> 3) values have been observed in some species during torpor entry and at high body temperature during torpor, it has been proposed that a physiological inhibition, in addition to temperature effects, must be involved in the reduction of metabolic rate (Carey et al. 2003; Geiser 1988; Song et al. 1997).

Torpor involves a global suppression of physiological processes; there are essentially no processes that continue at normal, euthermic rates during the low body temperature values of deep torpor. Yet, certain critical functions must continue at physiologically relevant levels during torpor and be precisely regulated even at body temperature values close to or below 0°C .

There are processes suppressed at low temperature and reactivated during arousal such as transcription (van Breukelen and Martin 2002), translation (Frerichs et al. 1998; van Breukelen and Martin 2001), mitochondrial respiration (Barger et al. 2003), digestive function, renal function (Moy 1971; Passmore et al. 1975; Zatzman 1984) and immune function (Luis and Hudson 2006; Prendergast et al. 2002). Other processes continue functioning at low temperature and are therefore likely to be of high physiological relevance for survival during torpor. Notable among these are the central nervous system (Florant and Heller 1977; Kondo et al. 2006; O'Hara et al. 1999; Ruby et al. 1998), cardiac and respiratory functions (Milsom and Reid 1995; Ormond et al. 2003), and lipolysis in adipose tissue (Buck et al. 2002; Dark et al. 1989; Heldmaier et al. 1999).

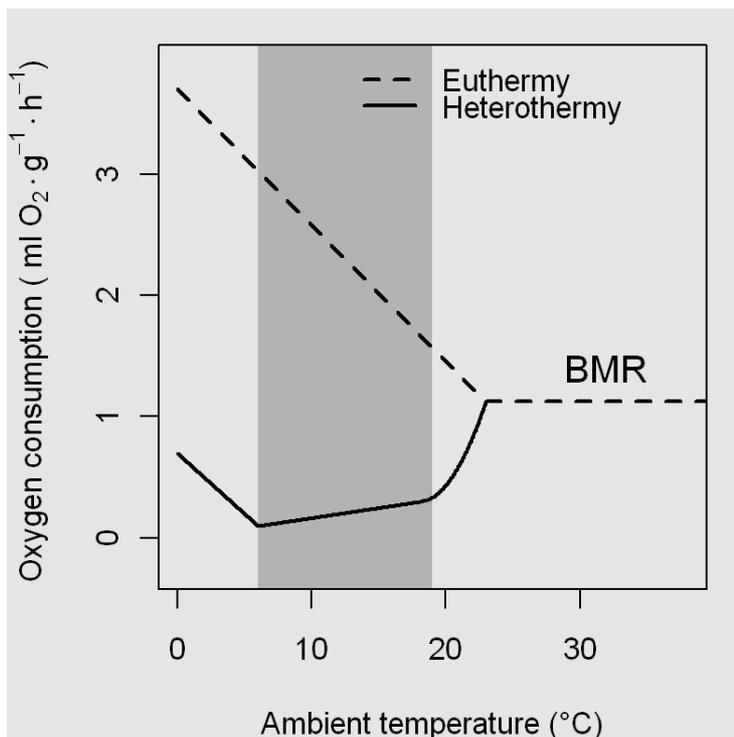


Figure 1.2 Relationship between metabolic rate and ambient temperature in an average hibernator. Metabolic rate is expressed as oxygen consumption per gram body tissue, during euthermy (dashed line) and heterothermy (solid line). In the temperature range in which animals are thermoregulating during euthermy and heterothermy, the metabolic rate increases with decreasing ambient temperature to compensate for heat loss. In the temperature range in which torpid animals are thermoconforming (grey stripe), the metabolic rate decreases curvilinearly with ambient temperature and thus body temperature. Figure based on data in Song et al. (1997).

Biological rhythm and regulation of hibernation

Hibernation is clearly not incorporated into the normal daily activity-rest cycle (Carmichael and Zucker 1986). The yearly organization of hibernation is reflected by a strong circannual rhythm of hibernation and activity in many long lived hibernators. In ground squirrels, marmots, and other hibernators, physiological and morphological variables such as reproduction, activity, food consumption, body mass and torpor follow a circannual cycle even under constant photoperiod and temperature (Davis 1976; Mrosovsky and Fisher 1970; Pengelley et al. 1978). Hibernation patterns also vary seasonally, torpor bouts are longest and deepest during the middle of the hibernation season, whereas bouts at the beginning and end of the hibernation season tend to be short (see figure 1.3, Lyman et al. 1982; Pengelley and Fisher 1961; Pengelley and Fisher 1963). Although the lengthening in bout duration in the autumn follows the normal seasonal decline of ambient temperature in underground hibernacula (Hut et al. 2002; Wang 1973), the change also persists under constant laboratory conditions, indicating involvement of an endogenous circannual cycle (Németh et al. 2009; Pengelley and Fisher 1961; Zivadinovic et al. 2005). This suggests that either all variables are under circannual control or that one or some of these variables are controlled by a circannual rhythm which thereby affects the others in turn.

Furthermore, a circannual rhythm can persist even after the ablation of the supra-chiasmatic nucleus, whereas the circadian rhythm is abolished (Lee and Zucker 1991; Ruby et al. 1996; Ruby et al. 1998). However, the long term timing and structure of the circannual cycle, including hibernation, is altered after supra-chiasmatic nucleus ablation. It appears therefore, that the brain area responsible for circadian rhythms is also involved in circannual rhythms, but it seems to have only a modulating function on circannual rhythms (Ruby et al. 1998).

It also appears that in some species hibernation follows a strong circannual rhythm, whereas in others, it is a function of environmental variables. The circannual rhythm of hibernators such as ground squirrels and marmots shows remarkably little response to photoperiod which functions as the main Zeitgeber for the circadian system and is also responsible for seasonal acclimation in many endotherms (Davis 1976; Pengelley and Fisher 1963). While the effect of photoperiod as a Zeitgeber appears to be weak and possibly limited to a short period during the summer, ambient temperature can have a strong influence on the circannual cycle (Drescher 1966). Moreover, changes in ambient temperature can act directly as a Zeitgeber for the circannual rhythm (Körtner and Geiser 2000a).

The mystery of periodic arousals.

Torpor bouts during hibernation extend over days, weeks, or even months (Körtner and Geiser 2000a), but never span the entire hibernation season. All hibernators that have been studied to date, arouse from torpor at regular intervals (Lyman 1982a). As metabolic rate during torpor in hibernators is extremely low, stored energy would last for very long time periods if they remained at these low levels throughout the hibernation season. Therefore, it appears counterproductive that hibernators arouse at regular intervals and use energetically wasteful endogenous heat production. Although many attempts have been made, the enigma of periodic arousals has not been resolved (Lyman et al. 1982).

Several hypotheses have been put forward. One proposes that accumulation or depletion of certain materials (metabolites or water) during torpor requires periodic euthermic periods for reestablishing homeostatic balance (Galster and Morrison 1970; Németh et al. 2010; Pengelley and Fisher 1961; Strijkstra 1999; Thomas and Geiser 1997; Willis 1982b). A variation of the metabolite hypothesis is based on the observation that some hibernators sleep for much of the intermittent euthermic periods. It has been proposed that an accumulation of a sleep deficit induces arousals (Daan et al. 1991).

Nevertheless, the assumed positive aspect of prolonged euthermia during hibernation has yet to be identified, whereas the negative energetic aspects are obvious. Arousals and euthermic periods during hibernation are clearly energetically expensive and account for about 70 % of the energy expenditure during the hibernation season in ground squirrels (Wang 1979b).

Behavioral, life history and ecological aspects of hibernation

Hibernation is a behavioral and physiological adaptation to survive seasonally appearing harsh environmental conditions such as cold weather and food shortage. Hibernators mainly depend on cached fuel stores (fat or food) to sustain their overwintering for six to eight months until spring (Humphries et al. 2003). Hibernation affects longevity as well (Speakman 2005). The life spans of hibernating animals are longer compared to non-hibernating animals of similar size. However, as Milton Friedman wrote “There is no such a thing as a free lunch” (Friedman 1975). Positive effects of hibernation on one hand involve serious costs on the other.

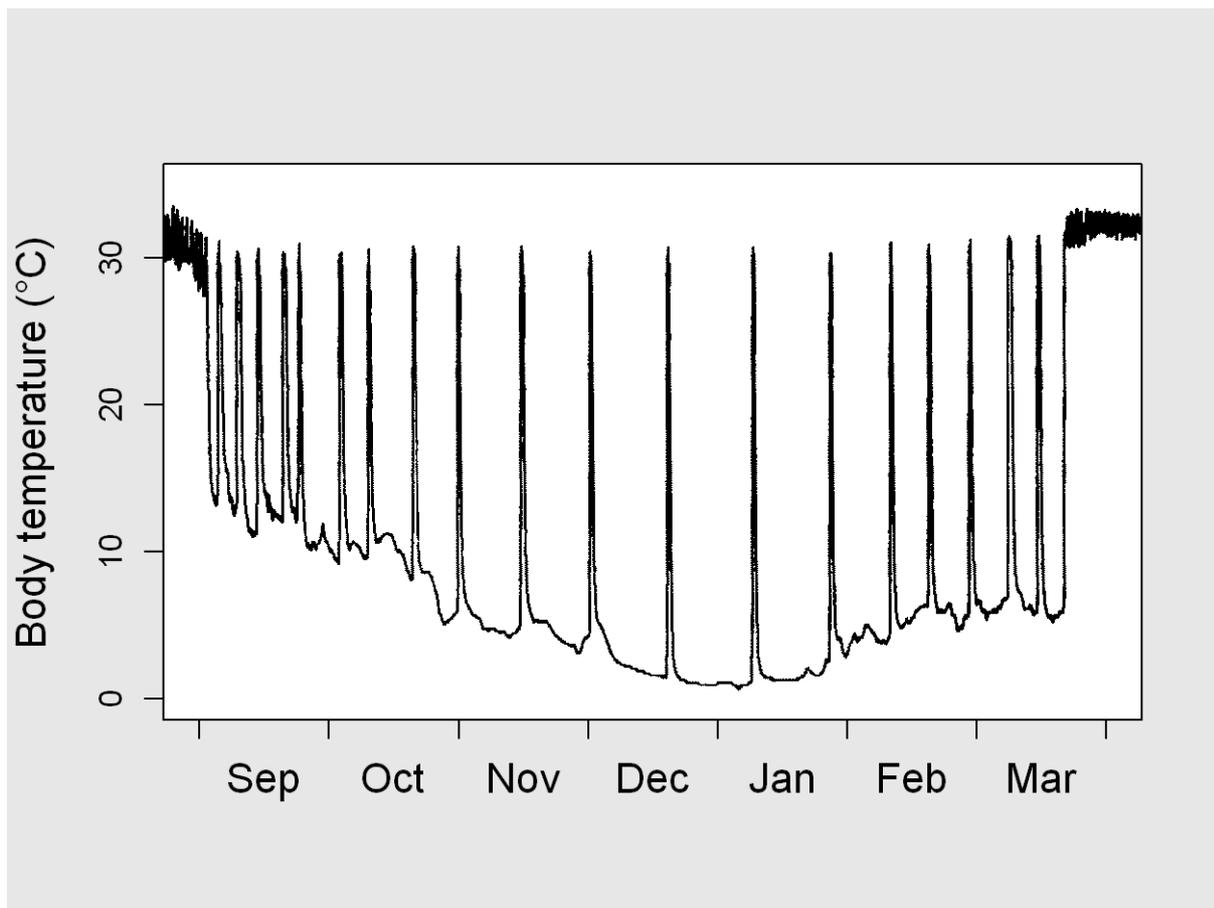


Figure 1.3 Body temperature as a function of time in ground squirrels. Typical body temperature pattern for a hibernating animal was plotted during hibernating season. Arousals are readily recognizable as spikes between two consecutive torpor bouts. Figure based on data in Hut et al. (2002).

Hibernation enhances survival by reduced energy expenditure and predation risk, but consequently hibernation takes a lot of time. The active season in many species is very short (~6-7 month, *Spermophilus citellus*: Millesi et al. 1999b), it is just long enough to ensure reproduction and survival. The active season requires mating just after emergence, reproduction and preparation for subsequent hibernation. Therefore the timing of reproduction and energy allocation has to be rigidly controlled.

An additional cost is the adverse effect of hibernation on brain function (see Box 2: Härtig et al. 2007). During hibernation, animals develop a serious impairment of spatial memory (Millesi et al. 2001) and social recognition (Mateo and Johnston 2000). This is a hidden cost of hibernation: relearning some behaviors and the spatial relations of the environment takes time and elevates the risk of predation.

Box 2.

Neurodegenerative disorders and hibernation

Results from research on developed memory impairment turned the attention of neurophysiologists and related clinicians to hibernation research recently. This newly generated interest probably originated from the remarkable similarities between the state of ground squirrels just after hibernation and human patients with severe neurodegenerative disorders such as Alzheimer's disease. Similarities have been observed on the behavioral level as an obvious lack of memories about the formerly well known surroundings and relatives, as well as on the neural level, in the brain tissue. Alzheimer's disease is a dementia neurodegenerative disorder characterized by extracellular neuritic plaques, composed of certain proteins. Neurofibrillar degeneration, i.e. neuronal death associated with pathological function of these proteins, typically affects areas of the brain involved in the cortical processing of memory function (Selkoe 2001).

It has been shown that during torpor, synaptic contacts between mossy fibers and hippocampal pyramidal neurons undergo dramatic regressive changes that are rapidly and fully reversible during euthermia in hibernating mammals (Popov and Bocharova 1992; Popov et al 1992). Recently Arendt et al. demonstrated that this rapid, reversible, and repeated regression of synaptic and dendritic components of neurons is associated with a reversible dysfunction of the mentioned proteins during the same time period (Arendt et al 2003). As hibernation in mammals is associated with a high degree of structural neuronal plasticity, it might be a model suitable to analyze cellular aspects of selective neuronal vulnerability potentially linked to neurodegenerative human disorders (Härtig et al 2007).

As mentioned above, the energy expenditure during hibernation determines body mass at emergence and emergence body mass determines, for a large part, the subsequent fate of the individuals. Body mass is directly related to overwinter survival (Murie and Boag 1984), timing of maturity (Barnes 1984; Bushberg and Holmes 1985) and reproductive success for both genders (Millesi et al. 1998; Millesi et al. 1999a). Reproductive males have to be on the surface before the females emerge to ensure their seniority in courtship (Michener 1983). Scramble competition polygyny is widespread among hibernating species, especially among ground squirrels (Millesi et al. 1998; Murie and Michener 1982). These activities entail high costs appearing as elevated body mass loss, delay in timing of molt and onset of hibernation (Millesi et al. 1998). These high costs cause a prolonged active season and increase the risk of predation.

As energetics of hibernation are in tight relationship with ambient temperature (see section Energetics and physiology of hibernation), ecological factors such as environmental temperature, or climate in a broader sense, greatly influence the life of hibernating animals. Differences in microclimate affect the post-hibernation body mass of ground squirrels (Buck and Barnes 1999b). On a higher, geographical scale, changing (warming) climate affects the distribution and phenology of the animals. A shift to the north has been observed in the distribution of

hibernating bats in the northern hemisphere due to warming environment (Humphries et al. 2002). Elevating ambient temperature has been coupled with an advance in the timing of the onset of the active season of marmots (Inouye et al. 2000).

The European ground squirrel

The European ground squirrel is a member of Sciuridae. The species was originally referred to as *Citellus citellus* (Linnaeus 1766) and more recently as *Spermophilus citellus*. The distribution of the European ground squirrel is limited to open non-agricultural vegetation in central Europe, varying from the open planes to grassy slopes of low mountain areas. Ground squirrels have a pronounced endogenous annual cycle, in which hibernation during autumn and winter is followed by reproduction (one litter) in early spring. The summer season is used for fattening in preparation for the following 6-7 months of hibernation, starting at the end of the summer (Millesi et al. 1999b). As a result, body mass shows a pronounced annual cycle and varies, when calculated as a population average, between 280 to 400 g in adult males and 200 to 300 g in adult females (Millesi et al. 1999b). The animals thrive well under controlled conditions in the laboratory. Ground squirrels are strictly diurnal, large enough to be readily visible and often present in open habitats (Murie and Michener 1982). All of the above mentioned characteristics make them eminently suitable for field and laboratory studies on hibernating mammals.

Chapter 1

Thesis overview

This thesis describes a series of investigations concerning hibernation in the European ground squirrel. The first part of the thesis is concerned with the function and mechanism of periodic euthermy. In the second part, studies are presented on the effect of nesting behavior on hibernation and the effect of hibernation on the post-emergence mating behavior of the ground squirrel. Finally, the third part is concerned with ecological consequences, in terms of the effect of climate change, on energy expenditure during hibernation and its relevance on populations of year round hibernating mammals.

Part one – timing of euthermic periods

Three hypotheses on the function and mechanism of periodic euthermy were considered.

In Chapter 2, an experiment is described in which the possible involvement of developing water deficiency during torpors in the timing of periodic euthermy was tested. To test whether a critically unbalanced water economy may contribute to the induction of arousals, we artificially induced animals to lose more water via elevated levels of urination. If water balance is involved in the triggering mechanism, torpor bouts were expected to be shorter following diuretic treatment.

In Chapter 3, an experiment was designed to investigate the effect of environmental temperature and seasonal timing on the appearance of arousals. To evaluate these two factors in the regulation of torpor bout lengths, we kept groups of European ground squirrels under three separate and constant thermal conditions corresponding to the natural range of minimal temperatures of their natural hibernacula. We expected a linear effect of temperature treatment on lengths of torpor bouts and euthermic phases and a shortening in torpor bout lengths toward the end of hibernation (seasonal timing effect).

As it was mentioned earlier, circannual timing of many morphological and physiological variables is rigidly controlled among annual hibernators. In Chapter 4, effect of body mass on the timing of arousals was evaluated. We hypothesized a possible feedback mechanism between actual body mass state in the animals and the timing of arousals. We expected that torpor bout lengths of animals with experimentally elevated body mass would be shorter (the animals becoming euthermic more frequently) due to extra fat stores far above the set point.

Part two – Behavior before and after hibernation

The effects of nesting behavior on energy expenditure were investigated in Chapter 5. Nest building behavior is undoubtedly adaptive. Nevertheless, the relevance of insulation under hibernation comes into question due to the decreased thermal gradient between the animals' body and the environment during the majority of time spent in hibernation. Data are presented on energy expenditure in terms of body mass change to reveal the consequence of overwintering in nests with different insulation capacity within the range of natural variability.

To evaluate consequences of hibernation on reproductive behavior, it is necessary to investigate animals in their natural environment where their behavior has been shaped by natural selection. We conducted field work (Chapter 6) to obtain indication of the costs and benefits of hibernation in field conditions. Within a population of European ground squirrels, we gathered correlative evidence on the effects of mating behavior and emergence timing on fitness related terms, such as reproduction and body mass.

Part three – Hibernation and climate change

There is growing evidence which shows that global climate change has an impact across natural systems. We investigated the possible effects of global warming during the forthcoming decades on hibernating ground squirrels in terms of energy expenditure during hibernation (Chapter 7). We modeled *in silico* body mass change during hibernation under three possible climate scenarios. We assumed that a biologically meaningful effect of climate change is likely, due to the known fact that environmental temperature affects the energetics of hibernation.

Finally in Chapter 8, the main findings of the work are summarized along with its limitations and potential implications. To finish, some remarks have been provided on the envisaged direction of further hibernation research.

Diuretic treatment affects the length of torpor bouts in hibernating European ground squirrels (*Spermophilus citellus*)

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ABSTRACT

During the hibernation season, hibernating mammals show a sequence of torpor bouts that are interrupted by periodic arousals and brief normothermic periods. The functional significance of periodic arousals is still uncertain. We hypothesized that the imbalances in water economy may play a role in the timing of periodic arousals in hibernating species.

We applied furosemide, a diuretic drug, to assess whether hibernating European ground squirrels respond to elevated urine production by shortening their torpor bouts. Urine production in the treated squirrels increased and led to more frequent arousals, presumably to restore water balance by recovery of lost water from blood and tissues.

The length of the subsequent normothermic phase was not affected by the diuretic treatment. Body mass change correlated primarily with the amount of voided urine. Although our study did not identify the underlying mechanism, our results support the view that water economy, and water loss may play a role in the timing of periodic arousals.

INTRODUCTION

Mammalian hibernation is an adaptation to unfavorable environmental conditions, such as cold or food shortage. Many hibernating species, such as ground squirrels, use internally stored energy supplies, mainly fat, to survive the hibernation season. However the energy content of stored fat does not cover the required amount of energy to maintain normothermic body temperature (T_b) throughout hibernation. The resolution for this conflict is that when they enter torpor, hibernators allow T_b to decrease substantially often close to ambient temperature (T_a), which, together with an inhibition of metabolism, causes a reduction in energy consumption (Willis 1982b). Hibernating mammals do not remain continuously in this torpor state, but arouse from torpor and become normothermic at regular intervals, ranging from about 2 to 30 days, depending on the species. During these normothermic periods (arousals) they sustain elevated T_b usually for less than a day, after which they return to hibernation (Geiser et al. 1990; Pengelley and Fisher 1961). Approximately 80 % of hibernating mammal's winter energy expenditure is consumed during these short periodic arousals, which lasts for less than 10 % of the whole duration of the hibernation season (Geiser 1988; Wang 1979b).

A number of hypotheses have been proposed that suggest that (i) arousals are required to clear metabolic waste (Fisher 1964), (ii) replenish blood glucose (Galster and Morrison 1970; Strijkstra 1999), (iii) initiate immune responses (Prendergast et al. 2002), or (iv) eliminate sleep deprivation (Daan et al. 1991). Each of these hypotheses have been challenged or rejected and the function of periodic arousals still remains unclear.

Imbalances in water economy arising during torpor bouts have been suggested as an alternative physiological explanation as to why hibernators must rewarm periodically (Thomas and Cloutier 1992). Possible pathways of water loss during hibernation are evaporative water loss (EWL) via pulmonary function, through the skin and from urine production (Thomas and Cloutier 1992; Thomas and Geiser 1997). This water balance theory argues that hibernators progressively lose body water through EWL and excreted urine until some critical threshold is crossed, forcing animals to arouse (Buffenstein 1984; Thomas and Geiser 1997). The possible link between the occurrences of arousals and water loss has been previously investigated on bats and ground squirrels (Thomas and Geiser 1997). In their studies the authors showed that there is a negative relationship between EWL and the torpor bouts length (TBL). However, Thomas and Geiser (1997) used T_a to determine the magnitude of EWL, which complicates the separation of the effects of EWL from the effects of temperature on TBL (Németh et al. 2009).

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Nevertheless, as for many other physiological processes, the function of the renal system is reduced, and although its rate is low, urine production is continuous in many torpid rodent species (Carey et al. 2003; Prendergast et al. 2002; Zatzman 1984; Zatzman and South 1975). We therefore hypothesized that it should be possible to manipulate water economy, independent of temperature, by experimentally changing urine production.

To test this hypothesis, we assessed whether hibernating European ground squirrels respond to an elevated urine production induced by a local acting, diuretic drug (Furosemide). As a standard loop diuretic, Furosemide inhibits the Na-K-Cl co transporter in the thick ascending limb of the loop of Henle, arrests the generation of hypertonic renal medulla and ultimately causes increased urine production (Malnic et al. 1965). Thus the urine production can be modified without chronic intervention (e.g. infusion) or drugs that act on the central nervous system (ADH related vasoactive drugs). We further hypothesized that the experimentally increased urine production will lead to more frequent arousals, to restore water balance by recovery of lost water from blood and tissues.

MATERIAL AND METHODS

Animals and housing.

The nine male European ground squirrels used in this study were live trapped in late August 2005, from a free ranging population living on a grassy airport field in Hungary (N 47°36'43", E 19°08'40") and were transferred to a climate controlled room in Eötvös Loránd University, Budapest, Hungary. Permission from local authorities and the Ethical Committee for Animal Experiments was obtained before the experiment began. The animals were housed individually in plastic containers sized 36 × 21 × 15 cm. The containers were open at the top, but covered with removable wire mesh. Barrel shaped (d16 × 8 cm) opaque plastic boxes with a removable top were used in every container as nest boxes. The animals could enter the nest box through an 'L' shaped tunnel (d6 cm) attached to the side of the box. Food (rabbit chow, Galgavit Kft., Gödöllő, Hungary) and water were given ad libitum in the prehibernation period. Grass was provided as raw material for nest construction. Constructed nests were not removed throughout the entire hibernation season. The animals were kept at $20 \pm 1^\circ\text{C}$ T_a and LD cycle of 10 : 14 (short photoperiod conditions) until mid September when T_a was gradually decreased to $10 \pm 1^\circ\text{C}$ and LD cycle turned to DD (continuous red dim light). At this point food and water were removed to facilitate the beginning of hibernation. Food and water withdrawal were maintained during the entire period of the experiment (10th February 2006 – 5th March 2006).

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Experimental design

The experimental design was a self controlled, randomized, two period, two way crossover study. Animals received either one dose of 0.4 mg / 100 g Furosemide (Sanofi-Synthelabo, Hungary) in a saline solution (1 mg/ml) during the treatment period or the equivalent volume of saline as a vehicle control during the control period. The animals were randomly assigned to one of the two treatment groups in the first period and during the second period each animal was given the opposite treatment. The applied amount of Furosemid was 1 : 20000 of the published LD50 values for laboratory rodent species, rats and mice (Malnic et al. 1965). However, furosemide has affects on other tissues besides the kidney (Humphreys 1976), it is likely that its predominant effect in vivo at the concentrations used in our experiments was to stimulate water excretion in the animals (Jackson 2006). Injections were administered subcutaneously in the middle part of the animals back. The handling of animals was carried out during the initial part of each torpor phase, 8 - 10 hours after the given torpor phase had begun, as determined from nest temperature (T_n see below). Although it is well known that animals are sensitive to physical disturbances that induce arousals during the torpor phase (Lyman 1982a), our decision was influenced by the short half life of the administered drug (rats: 2.7 h, Kim et al. 1993). We wanted to avoid the majority of the drug being cleared from the animal's system before the torpor period began. We assumed that the elimination half life of the drug is increased under low T_b , when rate of distribution (by decreased blood flow) and activity of metabolic enzymes are reduced (Ballard 1974). The two treatment periods were separated by one complete, undisturbed torpor phase to avoid carry over effects.

Measured variables

Animals were regularly weighed to the nearest 1.0g (Philips HR 2393 01) at the time of treatment. To evaluate the volume of urination, we covered the bottom of the plastic containers with impregnated blotting paper. Impregnation was made by 0.1N KCl - phenolphthalein solution. When urine contacts the surface, the paper readily absorbs it and the area of the visible patch is proportional to the amount of urine excreted. Using this method the size of the patch does not change, even when the urine evaporates. By adding a series of different amounts of liquid (water) to such paper sheets and measuring the surface size of resulting urine patches we obtained a calibration curve for determining the urine production of the squirrels.

T_b fluctuations were measured indirectly via T_n using a computerized temperature recording system. The definitions and calculations of torpor (torpor bout length, TBL) and

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arousal (arousal length, AL) durations were the same as described in our earlier study (Németh et al. 2009 see below).

To trace the changes in the T_b of the animals, we recorded the T_n as this was found to be dependent on the presence of arousals (Oklejewicz et al. 2001; Strijkstra 1999). To record T_n , we prepared the nest boxes with temperature sensitive resistors (KTY 21-5, Conrad Electronics). The sensor was attached to the inside of the removable top, approximately 2 - 3 cm from the animal's body surface. To restrict thermal convection between the sensor and the outer environment, the top of the nest box was insulated by a 1 cm thick foam plate. The signals from the thermistors were received with a multimeter (Metex, M-4640A) via a channel multiplexer controlled by a PC. Finally the multimeter was interfaced to a computer system for data acquisition which was recorded in 10 - min intervals. Before measurement, the sensors were calibrated to the nearest 0.1 °C against a precision mercury thermometer between the temperatures of melting ice (0 °C) and a 36 °C water bath.

TBL and AL were measured using the visual inspection of the time – T_n profiles (see Figure 2.1). TBL was defined as the period of time (h) beginning at the end of the previous arousal (i.e. at torpor entry) to the onset of the subsequent arousal. AL was defined as a period of time (h) that started at the beginning of rewarming and lasted until the beginning of the cooling phase, which was characterized by a moderate, but continuous decline in T_n (the visually estimated onset of the cooling curve). Rewarming period was defined as a period of time that started at the first visually detectable increase in T_n and lasted until T_n reached the characteristic normothermic temperature level. To facilitate the repeatability of the time point estimation we applied a one degree threshold in the temperature decline or incline after visually detected rewarming / cooling inflexion points. We estimated the random error (within observer reliability) of arousal timing determination. This random error was normally distributed and possessed similar magnitude as sampling interval (SD = 11 min).

Activity of squirrels out of the nest box was continuously recorded by a commercial surveillance system (2 pics / min; DCVideo System 2.0, Dir-Con Ltd., Hungary). Latency between first urination out of the nest box (Latency, LAT) and the beginning of the given arousal were estimated by comparison of T_n and the visually recorded data.

Statistical analysis

To assess the effect of diuretic treatment, the data obtained were statistically analyzed using a mixed-effects modeling. The analyses were performed on LAT, TBL, AL, rewarming period, weight change and amount of urine production, at the alpha level of 0.05, with

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treatment (TRT: vehicle control “V” or diuretic “D”), period (PER: first or second) and sequence (SEQ: V / D or D / V) considered as fixed effects and subject considered as random effect. The inter-subject variability was also estimated by the calculation of coefficient of variation (CV %). We tested the normality of standardized residuals by Shapiro-Wilk normality test. Generally, descriptive statistics are presented as mean \pm standard deviation (SD). All analyses were carried out with R (version 2.9.1), a free software program for statistical computing (R Development Core Team 2009).

RESULTS

The average body mass of the animals was 249.8 ± 16.5 g at their time of capture (August 28th). The animals’ body mass decreased during hibernation continuously, hence the average body mass of the animals at the beginning of the experiment was 154.7 ± 13.7 g.

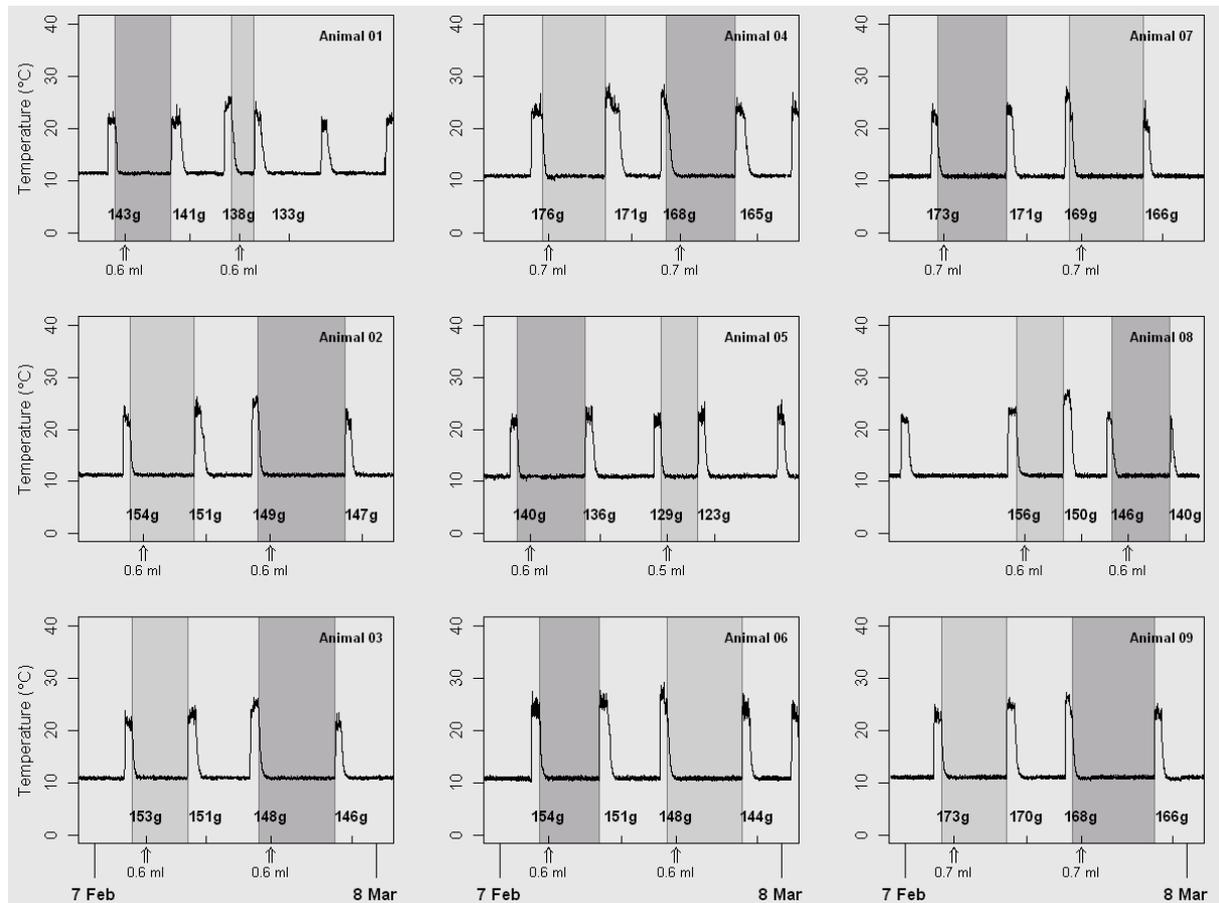


Figure 2.1 Individual nest temperature–time profiles and the schedule of the experiment. Color filled bands represent those torpor periods when animals were treated either with diuretic (light gray) or vehicle control (dark gray) subcutaneous injections. Arrows indicate the time of sc. injections and the corresponding volume. Body weight data and weighing time point were depicted above time axis. The plotted time frame is the same for all subjects (7. February–8. March).

The dosage of diuretic drug was proportional to body mass, with the given volumes of injections between 0.5 – 0.7 ml. The handling of animals was not followed by an arousal, at least within 12 h, on any occasion which could have been induced by the physical disturbance (Figure 2.1).

Diuretic treatment effect

All animals urinated during each arousal (one urination record was lost due to urine paper destruction). Between the two treatment periods, animals were not treated with vehicle control or diuretic injection; after the torpor phases the model estimated amount of urination was 0.8 ± 0.1 ml for an average animal. The estimated SD of the subject effect was 0.11 ml volume, which means there was a 12.9 % between individual variability in the estimated amount of urination without any external interference.

The estimated amount of urination increased by 72.6 % during diuretic treatment compared to vehicle control (CI: 28.3 % – 116.8 %). The amounts of urination were 1.3 ± 0.4 and 0.7 ± 0.2 ml during diuretic and vehicle control treatment, respectively (Figure 2.2). Urination was significantly affected by the applied diuretic treatment ($F_{1,6} = 17.07$, $p < 0.01$). Neither the period, nor the sequence had significant effect on urination (PER: $F_{1,6} = 0.04$, $p = 0.86$; SEQ: $F_{1,7} = 0.04$, $p = 0.86$). According to model results, diuretic treatment caused 0.5 ± 0.1 ml increase in the amount of estimated urination ($t_6 = 4.01$, $p < 0.01$).

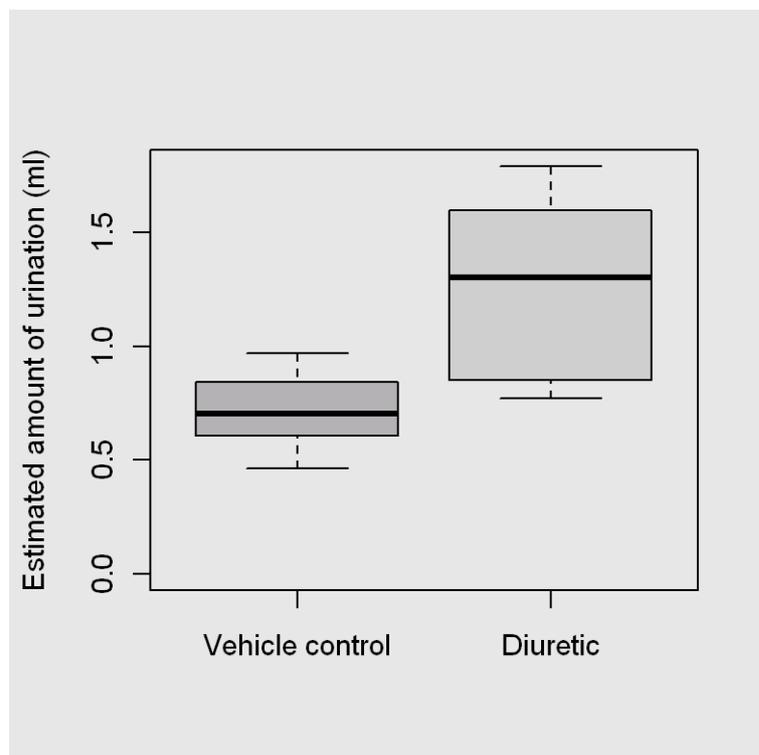


Figure 2.2 The estimated amount of urine voided during arousals after the diuretic and vehicle control treatments at the previous torpor phase. The difference between vehicle control (dark gray) and diuretic (light gray) periods is significant ($p < 0.01$). The data are presented as minimum–maximum (whiskers), upper and lower quartiles (boxes) and median (thick line).

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The estimated SD of the subject effect was 0.17 ml volume, which means a 21.8 % between individual variability in the estimated amount of urination.

Torpor bout length

TBL was 120 ± 19 h when animals were treated neither with vehicle control nor with furosemide injection. According to the model results the TBL was significantly increased by 51 ± 13 h when only saline (without diuretics) was subcutaneously injected ($F_{1,15} = 15.73$, $p < 0.01$).

The TBL was changed to 138 ± 43 h and 172 ± 27 h during diuretic and vehicle control treatment, respectively (Figure 2.3). The TBL decreased by 22.2 % during diuretic treatment compared to vehicle control (CI: 0.0 % – 44.4 %). Diuretic treatment resulted in a significant shortening of TBL (TRT: $F_{1,7} = 5.91$, $p < 0.05$). Neither the period, nor the sequence had significant effect on TBL (PER: $F_{1,7} = 0.21$, $p = 0.66$; SEQ: $F_{1,7} = 1.29$, $p = 0.29$). According to model results, TBL became shorter by 33.2 ± 14.0 h under diuretic treatment ($t_7 = 2.36$, $p < 0.05$). The estimated SD of the subject effect was 20.3 h, which means a 13.6 % between individual variability in TBL.

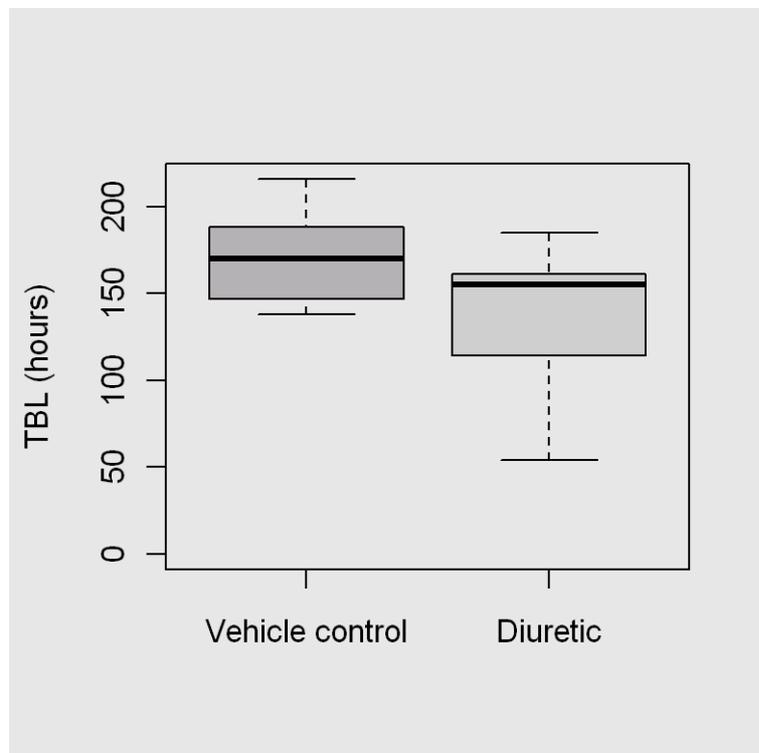


Figure 2.3 Effect of diuretic treatment on the ground squirrel's torpor bout length (TBL). The TBL was significantly shorter (approx. 1.5 day) if animals were treated with Furosemide which stimulates renal water excretion. The data are presented as minimum–maximum (whiskers), upper and lower quartiles (boxes) and median (thick line).

Arousal length

ALs were 18.6 ± 6.2 and 16.3 ± 5.8 h during diuretic and vehicle control treatment, respectively. The length of subsequent arousals was not affected by diuretic treatment (TRT:

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$F_{1,7} = 1.72$, $p = 0.23$). The period had a significant effect on AL, but the sequence did not (PER: $F_{1,7} = 7.52$, $p < 0.05$; SEQ: $F_{1,7} = 0.13$, $p = 0.73$). The estimated SD of the subject effect was 4.5 h, which means a 19.6% between individual variability in the AL.

The lengths of the rewarming period (from T_b level in torpor to normothermic level) were 0.8 ± 0.1 and 0.8 ± 0.1 h during diuretic and vehicle control treatment, respectively. The length of rewarming was not affected significantly by diuretic treatment (TRT: $F_{1,7} = 0.16$, $p = 0.69$). Neither the period, nor the sequence had significant effect on the length of the rewarming period (PER: $F_{1,6} = 0.20$, $p = 0.66$; SEQ: $F_{1,7} = 0.08$, $p = 0.79$).

Latency of voiding out of the nest

The latencies of urination were 1.5 ± 0.7 h and 7.1 ± 5.6 h during diuretic and vehicle control treatment, respectively (Figure 2.4). The latency decreased by 65.2 % during diuretic treatment compared to vehicle control (CI: not computed). Diuretic treatment resulted in a significant shortening of latency (TRT: $F_{1,7} = 7.94$, $p < 0.05$). Neither the period, nor the sequence had significant effect on latency (PER: $F_{1,7} = 0.19$, $p = 0.67$; SEQ: $F_{1,7} = 0.06$, $p = 0.81$).

According to model results, latency of urination markedly shortened (5.7 ± 2.0 h) when animals were treated with the diuretic ($t_7 = 2.84$, $p < 0.05$). The estimated SD of the subject effect was less than 0.0 h, due to high and probably over-estimated residual SD (4.2 h).

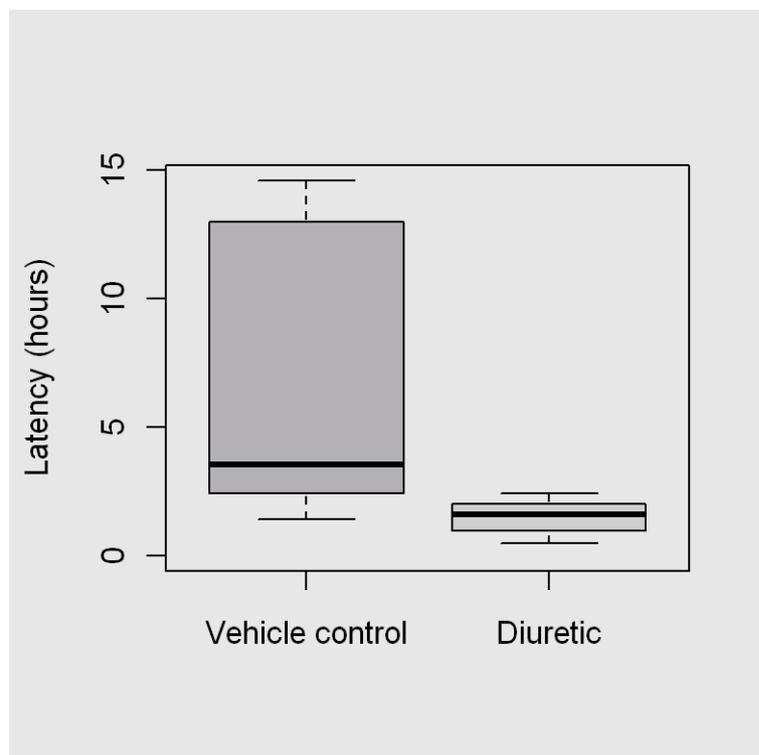


Figure 2.4 Effect of diuretic treatment on the latency of first urination relative to the beginning of the given arousal. Due to the Furosemide treatment, the latency considerably shortened while the variance strongly decreased ($p < 0.05$). The data are presented as minimum– maximum (whiskers), upper and lower quartiles (boxes) and median (thick line).

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Body mass change

Loss of body mass was 4.2 ± 1.3 and 2.8 ± 1.4 g during diuretic and vehicle control treatment, respectively. Body mass loss was enhanced by 56.0 % during diuretic treatment compared to vehicle control (CI: 25.0 % – 87.1 %). Body mass change was significantly affected by diuretic treatment. (TRT: $F_{1,7} = 16.96$, $p < 0.01$). Neither the period, nor the sequence had significant effect on body mass change (PER: $F_{1,7} = 2.52$, $p = 0.16$; SEQ: $F_{1,7} = 0.06$, $p = 0.81$).

According to model results, body mass loss increased (1.3 ± 0.3 g) when animals were treated with diuretic ($t_7 = 4.27$, $p < 0.01$). The estimated SD of the subject effect was 1.3 g, which means a 58.7% between individual variability in the body mass loss.

The treatment might have affected the body mass change through the amount of voided urine. Urination was the significant predictor of body mass loss when it had been included into the applied mixed-effects model as a covariant (Urination: $F_{1,5} = 12.86$, $p < 0.05$; TRT: $F_{1,5} = 0.13$, $p = 0.74$; PER: $F_{1,5} = 1.32$, $p = 0.30$; SEQ: $F_{1,5} = 0.01$, $p = 0.93$). According to model results, every 1 ml estimated amount of urination caused 1.6 ± 0.9 g body mass loss ($t_7 = 1.87$, $p = 0.12$). In this model, the estimated SD of the subject effect was 1.0 g, which means there was a high level of between individual variability, 91.6 %.

DISCUSSION

The hibernation pattern of European ground squirrels was modified by introducing a diuretic to increase urine production. Our results support the hypothesis that body water imbalances may play a role in the timing of periodic arousals (Thomas and Cloutier 1992; Thomas and Geiser 1997). The treatment used in our study has the ability to modify the water balance via increased urine production. The advantage of this treatment over the investigation of the effect of changing EWL, is that our procedure does not interact with the effect of T_a on TBL (Németh et al. 2009; Thomas and Geiser 1997).

Species which do not cache food as fuel for the hibernation season, store energy mainly as fat in white adipose tissue (Willis 1982a). One of the by-products of fat catabolism is metabolic water. The amount of metabolic water production (MWP) is nominally equivalent to the mass of utilized fat (Muchlinski and Carlisle 1982). EWL and MWP can account for up to 70% of water flux among mammalian species (Nagy 2004). In our experiment the water loss was increased by increased urine production under constant environmental temperature condition, without changing EWL. Although the method of induced water loss is different, the

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consequence may be similar as it would be from EWL changes (Thomas and Geiser 1997). The need to recover the lost water appears sooner by elevated EWL or by diuretic treatment. The importance of water loss in the timing of arousal was supported by comparing the TBL of injected and uninjected periods, as well. It seems, that the injected extra water was able to increase the TBL by itself and the diuretic treatment as a counteracting effect decreased the TBL towards the baseline.

Renal blood flow (RBF), glomerular filtration rate (GFR), and urine production are diminished or cease in hibernators during torpor (Carey et al. 2003; Moy et al. 1972). Pengelley and Fischer (1961) found urine in the bladders of ground squirrels (*Spermophilus lateralis*) at various times during torpor, but the volume of the urine did not show any accumulation over time. The suggestion that urine in the bladder is a residuum of the produced urine at the previous arousal was made by Pengelley and Fischer (1961), but other authors also adopted this concept (Moy 1971).

However, other studies on different hibernating species showed that urine production is reduced but continuous during hibernation and the urine in the bladder is basically not a residuum of previously produced urine. In these later studies, direct measurements of urine flow and renal function were analyzed using bladder canulation, renal clearance estimation and ultra structural microscopy (Brown et al. 1971; Zancanaro et al. 1999; Zancanaro et al. 1993; Zatzman and South 1975). Zatzman and South used bladder-cathetered animals to study urine production of hibernating marmots and found that the usual range of urine flow during torpor was 0.05-0.1 ml/day (*Marmota flaviventris*, Zatzman and South 1975). This amount corresponds with the volumes described by Pengelley and Fischer in Golden-mantled ground squirrels (*Spermophilus lateralis*, 1961). Arousing squirrels begin to enhance their urine production 90-135 min after the onset of arousal or later, mainly because of increasing perfusion of their kidneys (Moy 1971). The average time of the latency of first urination was 1.5 hours when animals were treated with Furosemide injection. Although we have no data about the actual urine production rate, only the time of first urination relative to the start of rewarming, the increasing urine formation at rewarming could contribute to the amount of urine voided after 1.5 h from the onset of rewarming, but the rate of production at this time probably does not explain the whole amount of voided urine (0.04ml/h for 150 g body weight: Moy et al. 1972).

The efficacy of applied Furosemide treatment in the elimination of cortico-medullary gradient may also be questioned. However, the mechanism of potassium cotransport blocking can be still effective in spite of the low T_b because a specific cold adaptation in ground

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squirrels sustains the function of the K-Na-Cl cotransport system that is the target of the diuretic treatment (Becker 1987). If cotransport blocking worked even under low temperatures, Furosemide treatment in our experiment could have caused increased urine production in comparison to the control period.

Alternative explanations of shortening in the TBL could come from consideration of the side effects of the applied treatment. Such a side effect can be that Furosemide causes a certain degree of hypokalaemia via its diuretic effect (Malnic et al. 1965). In former studies the potassium ion (K^+) was a possible candidate as a factor in the timing of periodic arousals, mainly because of its role in the functioning of excitatory cells such as neurons and muscle cells. These cells lose K^+ at low temperatures and elevate the level of K^+ in the blood temporarily. This extra potassium accumulates in the kidneys until its saturation (Willis et al. 1971). In an experimental study the effect of K^+ on timing of arousal was proven. A mixture of KCl and NaCl solutions were injected to raise the extracellular K^+ concentration, which caused more frequent arousals than the same volume of pure NaCl solution (Fischer and Mrosovsky 1970). These studies suggest that hypokalaemia should cause elongated torpor bout duration, hence it is unlikely that this side effect of the applied treatment would explain the shortening in the torpor duration in our study. Nevertheless, as hibernating species are highly adapted against the disruption of plasma membrane ion gradient under low T_b condition, maintaining electrolyte balance is obviously a sensitive component in their adaptation to cold (Carey et al. 2003). In this sense, diuretic treatment might have certain side effects through electrolyte balance that can cause changes in the function of the nervous system.

It is likely that the factors which are responsible for initiating arousals have a certain priority level in each given part of the hibernation season. This priority can change over the course of hibernation. For example, gonad maturation of the male ground squirrels can be responsible for the timing of the arousal at the end of the hibernation period, but not in earlier periods (Barnes et al. 1986; Barnes et al. 1988). Similarly, other species-specific physiological processes can be responsible for timing of arousals at other predetermined times, causing annually changing TBL (Németh et al. 2009; Zivadinovic et al. 2005). Although our study did not identify the underlying mechanism, our results support the view that water economy may play a role in the timing of periodic arousals (Thomas and Geiser 1997).

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Ambient temperature and annual timing affect torpor bouts and euthermic phases of hibernating European ground squirrels (*Spermophilus citellus*)

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ABSTRACT

The low body temperature state of hibernating mammals is interrupted regularly by short euthermic phases. The frequency of these euthermic phases changes according to the phase of hibernation. This typical pattern, common among ground squirrels (genus *Spermophilus* F. Cuvier, 1825), can be regulated by internal factors and affected by outside temperature. To evaluate the effects of internal annual timing and the ambient temperature, we monitored nest temperatures of overwintering European ground squirrels (*Spermophilus citellus* (L., 1766)) under three temperature conditions during late hibernation (9 °C, 5 °C, 0 °C). Our results showed that in spite of the constant thermal conditions, an annual timing effect changed torpor bout length (TBL) at 9 °C and 5 °C but not at 0 °C. Ambient temperature had an independent influence on TBL and euthermic phases. The loss in body mass was higher at higher ambient temperatures and was affected by TBL and euthermic phases. Thus, the hibernation pattern of European ground squirrels is regulated by both an annual timing pattern and the ambient temperature in the hibernacula.

INTRODUCTION

Hibernating mammals reduce their energy requirement to cope with low ambient temperatures (T_a) and reduced food availability (Körtner and Geiser 2000a). During the autumn and winter months, several sciurid rodents conserve energy by reducing metabolism with a fall in body temperature (T_b) that often approximates the T_a of the hibernaculum (Carey et al. 2003; Willis 1982a). Many physiological and cellular functions such as respiration, function of gonads and immune system are also reduced during hibernation (Barnes et al. 1988; Buck and Barnes 2000; Prendergast et al. 2002).

The rate of metabolism during deep torpor is typically $< 3\%$ of euthermic rates at the same T_a , which ensures considerable energy savings (Geiser 1988). Ground squirrel (genus *Spermophilus* F. Cuvier, 1825) species hoard little or no food during the pre-hibernation period; their principal storage form is fat (Humphries et al. 2003). These species increase their body mass in the active season, but lose mass dramatically during hibernation (Davis 1976; Humphries et al. 2003; Millesi et al. 1999b). They interrupt their low temperature torpid states by short arousal phases, and temporarily achieve normothermic T_b values at intervals of 2-20 days (Hut et al. 2002). Generally, arousals last less than a day, but the majority of winter energy expenditure (70%) occurs during these periodic events (Wang 1979b). Obviously, from an energy conservation point of view, it would be advantageous if animals remained torpid throughout hibernation.

Many studies have shown that interval between periodic arousals is not constant. Torpor bouts are initially short, increase in length at mid-hibernation and decrease toward the end of the hibernation period (Wang 1973; Young 1990). Data collected under semi-natural conditions also show that changes in torpor bout length (TBL) in fall and early spring correspond with the normal seasonal decline and incline of T_a in the underground hibernacula (Hut et al. 2002; Wang 1973). Experimental data show that temperature has an independent effect on the timing of periodic arousals, as well (Buck and Barnes 2000; Pengelley and Fisher 1963). Moreover, seasonal changes also persist under constant laboratory conditions, indicating the involvement of an endogenous circannual cycle (Körtner and Geiser 2000a; Pengelley and Fisher 1961).

These relationships also appear in the hibernating behaviour of the European ground squirrel (*Spermophilus citellus* (L., 1766)). The abovementioned characteristic seasonal pattern in the frequency of arousals was observed by Hut et al. (2002). In Hungary, the temperature at the depth of the hibernaculum reaches the minimum level in the soil during the months of January and February in its year round cycle (~ 80 cm, 5°C : Bacsó 1973). In this period, the

torpor phases are the longest, being approximately two times longer than during the early- and late-hibernation phases when soil temperature is higher by 5°C compared with during the mid-hibernation phase. Presumably, the hibernating animals detect the change of T_a (soil) as a timing cue rather than the natural light/dark cycle during their hibernation (Ruby 2003). T_a affects directly the appearance of arousals in the European ground squirrel as well (Strijkstra et al. 1999). Temperature thus may be hypothesized to act as both a factor (on physiology) and a timing cue (of the internal annual clock) at the same time.

To evaluate these two possible roles of T_a in the regulation of TBL and euthermic phases, we kept groups of European ground squirrels under three separate and constant thermal conditions corresponding to the natural range of minimal temperatures of their natural hibernacula in late hibernation and monitored their hibernation pattern from the mid- to the late-hibernation phases. We expected a linear effect of temperature treatment on TBL and euthermic phases and no effect on the annual internal timing mechanism – at lower temperatures TBL is relatively long but shorter torpor bouts during late hibernation would also occur at all temperatures.

MATERIALS AND METHODS

Animals and Housing

The 15 yearling males of European ground squirrel used in this study were live-trapped in late August from a free-ranging population living on a grassy airport in Hungary (N 47°36'43", E 19°08'40"), and were transferred to a climate-controlled room at Eötvös Loránd University, Budapest. The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The animals were housed individually in plastic containers 36 cm long × 21 cm wide × 15 cm high. The containers were open at the top but covered with removable wire mesh. Barrel-shaped (16 cm diameter × 8 cm height) opaque plastic boxes were used in every container as nest boxes with a removable top. The animals could enter the nest box through a knee-shaped tunnel (6cm diameter) attached to the side of the box. Food (rabbit chow; Galgavit Kft., Gödöllő, Hungary) and water were given ad libitum in this period. The animals were kept at 20 ± 1 °C and at a 10 h light (L) : 14 h dark (D) cycle (short photoperiod conditions) until mid September when T_a was decreased to 15 ± 1 °C and the 10 h L : 14 h D cycle turned to 24 h darkness. At this point, food and water were removed to facilitate the beginning of hibernation.

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Experimental Procedure

We kept the three separate groups ($n=5$) on different constant temperatures during the treatment (0 ± 1 °C; 5 ± 1 °C; 9 ± 1 °C). The treatment lasted from 1 January to 10 April 2003, when we gradually increased the T_a and reverted to the 10 h L : 14 h D cycle. The chosen date corresponded to emergence timing at the grassy airport where the individuals were caught. Two phases of late hibernation were distinguished. Where it was possible we randomly selected three torpor and euthermic phases out of five after 1 January for mid-hibernation phase, and also randomly selected three out of five before the end of hibernation for late-hibernation phase. The mid-hibernation phase lasted from 21 January \pm 5 days to 14 February \pm 8 days (mean \pm SD) and the late-hibernation phase lasted from 16 March \pm 9 days to 2 April \pm 7 days. To decrease the dependency of data, there had to be one unused torpor period between mid-hibernation (67 days) and late-hibernation (38 days) phase.

To track the movements of the animals we recorded the nest temperature (T_n), because it was found to be dependent on the presence of arousals (Oklejewicz et al. 2001; Strijkstra et al. 1999). To record T_n , we prepared the nest boxes with temperature-sensitive resistors (KTY 21-5; Conrad Electronics, Hirschau, Germany). The sensor was attached to the inside of the removable top, at about 2-3 cm from the body surface of the animal. To restrict thermal convection between the sensor and the outer environment, the top of the nest box was insulated by a 1 cm thick foam plate. The signals from the thermistors were received with a model multimeter (M-4640A; Metex Instruments, Seoul, South-Korea) via a channel multiplexer controlled by a PC. Finally, the multimeter was interfaced to a computerized system of data acquisition that recorded in 10 min intervals. The sensors were previously calibrated to the nearest 0.1 °C against a precision mercury thermometer between the temperatures of melting ice (0 °C) and a 36 °C water bath.

Hibernation Characteristics and Nest Temperature

TBL and arousal length (AL), were measured using the visual inspection of the x - y graphs where nest temperature (y) was plotted against time (x). TBL was defined as a period of time (h) that started at the end of the previous arousal and lasted until the onset of the subsequent arousal. AL was defined as a period of time (h) that started at the beginning of rewarming, and lasted until the beginning of cooling phase, which had a moderate but continuous decline in T_n (the visually estimated onset of cooling curve).

To facilitate the repeatability of the time-point estimation, we applied a 1 °C threshold in the temperature decline or incline after visually detected rewarming or cooling inflexion points.

We estimated the random error (within observer reliability) of the determination of the arousal timing. This random error was normally distributed and possess a similar magnitude as the sampling interval (SD = 11 min).

Minimum nest temperature (minimum T_n) was measured to estimate differences in heat production during the torpor phases and was defined as the lowest attained T_n .

Measurements of Body Mass

To minimize disturbance, body mass was recorded in 6 to 14 day intervals, when all animals were in the state of torpor. All arousals included in this analysis occurred at least 24 h after weighing. Besides the loss of body mass, we calculated the mass-specific rate of loss (MSRL) as described by Michener and Locklear (1990).

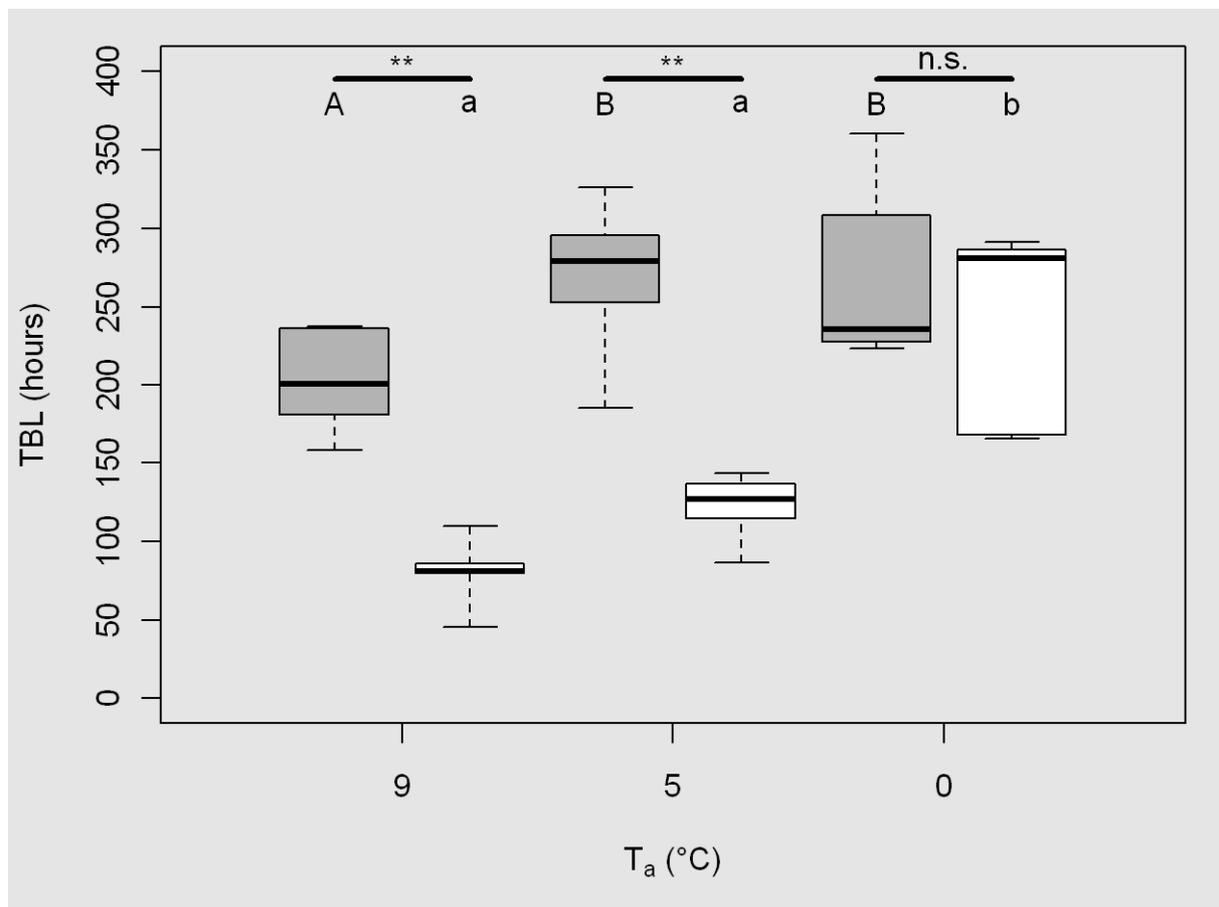


Figure 3.1 The effects of ambient temperature (T_a) and hibernation period on torpor bout length (TBL). The torpor bouts are longer in mid- (grey boxes) than in late (empty boxes) hibernation period except for animals kept at constant 0°C. The period effect is not significant in this group, hence torpors did not get shorter in late hibernation unlike in the other two groups. The post-hoc effect of the period is marked by commercial asterisk, and the effect of temperature is marked by different letters (boxes: mean±SE, whiskers: mean±SD).

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Data Analysis and Statistics

We used the mean of three arousal and torpor events in mid- and late hibernation to test our hypothesis. One-way ANOVA was used to compare differences among groups. STATISTICA version 7.1 (StatSoft Inc. 2007) was used for data analysis. Where a significant difference was revealed, the groups were compared with LSD post hoc tests. Generally, the data are presented as means \pm SD. To determine changes over time among the treated groups, comparisons made from each individual were tested by mixed effect general linear model (GLM) with body mass as a covariate. Model selection was based on Bayesian information criterion (BIC, with smaller values being better). GLM multiple regression models with stepwise method were built for best predictor selection in case of MSRL. Multicollinearity of used predictors was estimated by variance inflation factor (VIF). If VIF was greater than $1/(1-R^2)$, multicollinearity was considered as statistically significant (Klein 1962). For brevity, only the final models are presented in the Results.

RESULTS

During the experiment, European ground squirrels entered torpor 6-10 times. Animals at higher temperatures had more torpor phases than those kept at lower T_a . This difference was independent of the duration of the hibernation (Table 3.1).

Emergence

Although the temperature treatment lasted for the same time for each group, the timing of emergence differed among the treated groups ($F_{2,12} = 15.51$, $p < 0.001$). Groups at 9 and 5 °C emerged earlier, but at the same time (9°C: 5 April \pm 2 days; 5°C: 7 April \pm 2 days), while the group at 0 °C emerged later (26 April \pm 9 days).

Torpor

TBL did not differ significantly among the groups before the beginning of the treatment when all animals were kept at T_a of 15 °C ($F_{2,12} = 2.45$, $p = 0.12$; 9 °C: 203.6 ± 65.79 h; 5 °C: 299.5 ± 60.64 h; 0 °C: 240.1 ± 79.71 h). European ground squirrels at higher temperature spent less time in torpor during both mid-hibernation and late-hibernation phases, but the length of torpor states changed during the season even though temperature was held constant (mixed GLM; treatment: $F_{2,12} = 17.88$, $p < 0.001$; period: $F_{1,12} = 29.98$, $p < 0.001$; interaction: $F_{1,12} = 3.56$, $p = 0.06$; BIC = 265.1; Figure 3.1). There were less occurrences of shorter torpor phases

in the late-hibernation phase of European ground squirrels kept at 0 °C (1 of 5 showed shorter TBL: -194.5 ± 30.42 h; $t_3=6.39$, $p < 0.01$) The length of torpor phases did not differ between late-hibernation phase and the post-treatment for European ground squirrels kept at 0 °C (-35.2 ± 28.9 h; $t_{26}= 1.16$, $p = 0.25$). Body mass was excluded by the final model because of the nonsignificant effect of size and the poor model fit (BIC = 268.8).

Arousals

AL did not differ significantly among the groups before the beginning of the treatment ($F_{2,12} = 0.53$, $p = 0.60$; 9 °C: 17.1 ± 4.85 h; 5 °C: 15.0 ± 3.28 h; 0 °C: 15.21 ± 2.15 h). AL was affected by the temperature treatment, and it was about 3 h shorter in the mid- hibernation phase compared with the late-hibernation phase (treatment: $F_{2,12} = 9.19$, $p < 0.01$; period: $F_{1,12} = 4.08$, $p = 0.06$; interaction: $F_{1,12} = 0.008$, $p = 0.99$; BIC= 84.6; Figure 3.2). The lower the T_a , the shorter the ALs. Body mass was excluded by the final model, like in TBL, because of the nonsignificant effect of size and the poor model fit (BIC= 87.1).

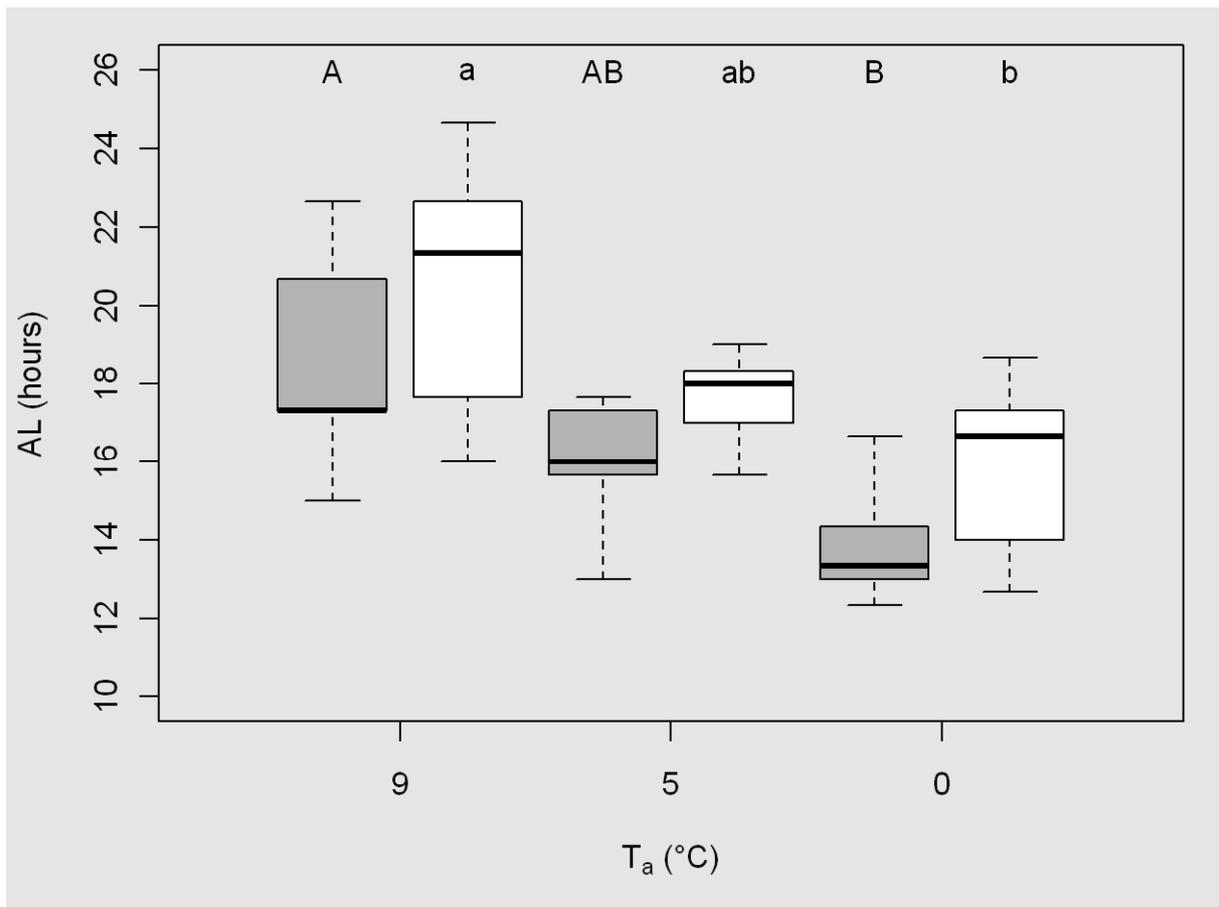


Figure 3.2 The effect of ambient temperature and hibernation period on arousal length (AL). Average arousal length did not differ between mid (grey boxes) and late (empty boxes) hibernation; however there were considerable tendencies. The post-hoc effect of temperature is marked by different letters (boxes: mean±SE, whiskers: mean±SD).

Body mass

Body mass decreased continuously during the treatment period. After about 3 months (72 ± 10 days) body mass fell by 40.2 ± 10.71 g, which is 18.4 ± 8.94 % of the body mass of European ground squirrels measured at the start of the treatment. Loss in body mass was highest at a T_a of 9°C (52.8 ± 2.75 g) followed by a T_a of 5°C (40.0 ± 7.44 g) and a T_a of 0°C (30.2 ± 3.83 g). The differences in loss of body mass between the three T_a groups were significant ($F_{2,12} = 22.82$, $p < 0.001$). The changing of mean body mass of the T_a groups were also significantly different (treatment: $F_{2,12} = 0.86$, $p = 0.44$; period: $F_{1,12} = 184.16$, $p < 0.001$; interaction: $F_{1,12} = 8.48$, $p < 0.01$; Figure 3.3).

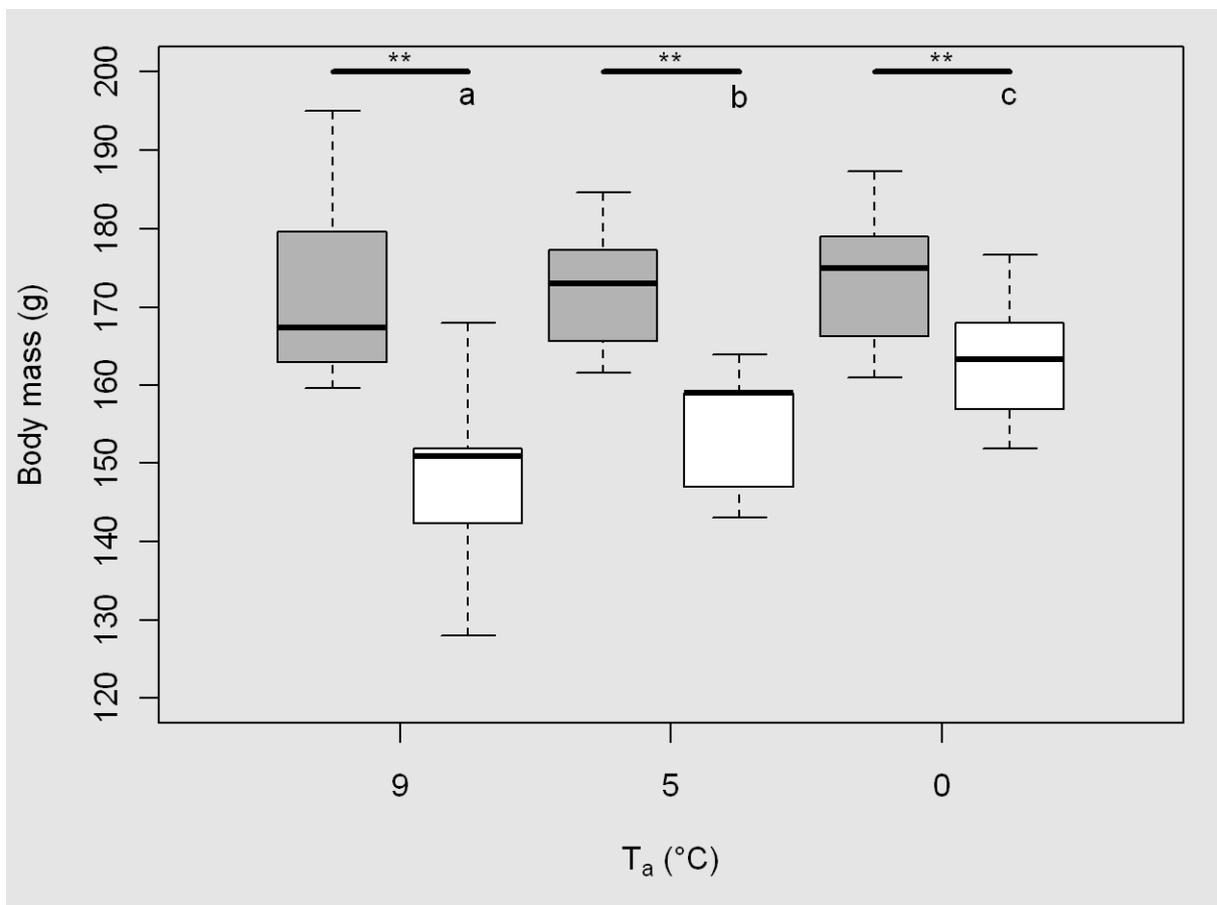


Figure 3.3 The effect of ambient temperature and hibernation period on body mass change. Although there was no significant difference among groups in mid-hibernation (grey boxes), the difference became significant in late (empty boxes) hibernation period. The post-hoc effect of the period is marked by commercial asterisk, and the effect of temperature is marked by different letters (boxes: mean \pm SE, whiskers: mean \pm SD).

Hibernation pattern and change in body mass

AL and TBL were found to be independent, continuous predictors in a stepwise regression model that could explain the variances in the MSRL for the three T_a groups.

Table 3.1

Detailed data* of examined periods of hibernation

Period of hibernation	10 °C			5 °C			0 °C		
	Mid	Late	Mid	Late	Mid	Late	Mid	Late	
Mean AL	17.3 (15-22.6)	21.3 (16-24.6)	16 (13-17.6)	18 (15.6-19)	13.3 (12.33-16.6)	16.6 (12.6-18.6)			
Mean maximum AL	25 (19-30)	29 (21-30)	18 (17-20)	22 (21-23)	16 (13-18)	21 (15-22)			
Median number of torpor bouts	6 (5-7)	6 (4-8)	3 (3-6)	5 (4-8)	3 (3-4)	3 (3-5)			
Mean maximum TBL	223 (176-251)	113 (76-164)	303 (223-382)	172 (98-249)	315 (268-375)	283 (202-312)			

*: Ranges are in parentheses. AL: arousal length. TBL: torpor bout length

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Multicollinearity of predictor variables was excluded in both models. The VIFs in the model of mid-hibernation phase were 1.48, 1 and 1.05 for treatment, AL and TBL, respectively. None of them exceed the criterion of significance ($1/(1-R^2) = 3.28$). The VIFs in the model of late-hibernation phase were 2.53, 1.13 and 1 for treatment, AL and TBL, respectively. None of them exceeded the criterion of significance ($1/(1-R^2) = 3.22$). Temperature treatment was excluded as a factor by the model for both phases. The stepwise regression model procedure resulted in a period-dependent predictor, which was AL in the mid-hibernation phase ($R^2 = 0.608$, $R = 0.779$, $F_{1,13} = 20.177$, $p < 0.001$; Figure 3.4a) and TBL in the late-hibernation phase ($R^2 = 0.695$, $R = 0.834$, $F_{1,13} = 29.761$, $p < 0.001$; Figure 3.4b) that explained the variance in MSRL. As seen in Figures 4a and 4b, the relationship between TBL and MSRL was less clear during the mid-hibernation phase ($R^2 = 0.086$, $R = 0.294$, $F_{1,13} = 1.2346$, $p = 0.286$), and the relationship between AL and MSRL could only be explained weakly during the late-hibernation phase ($R^2 = 0.192$, $R = 0.439$, $F_{1,13} = 3.1048$, $p = 0.101$).

Nest temperature

We predicted that residual heat production during the torpor phase would be reflected in the nest temperature. Minimum temperature of nests (minimum T_n) differed by treatment but did not change during the season (treatment: $F_{2,12} = 4.26$, $p < 0.001$; period: $F_{1,12} = 0.55$, $p = 0.46$; interaction: $F_{1,12} = 1.32$, $p = 0.30$), so we pooled that data from the two hibernation phases. The mean minimum T_n was higher than the T_a , but was indistinguishable from the T_a for the group kept at 9 °C (9.5 ± 0.7 , $t_4 = 1.53$, $p = 0.19$). We had the biggest temperature difference in the group kept at 0 °C (3.4 ± 1.2 , $t_4 = 6.26$, $p < 0.01$). The group kept at 5 °C showed a smaller difference in temperature (6.3 ± 0.8 , $t_4 = 3.38$, $p < 0.05$). The mean minimum T_n predicted the mean torpor length during the entire treatment period (Figure 3.5).

DISCUSSION

By keeping animals in a controlled laboratory setting and exposing sets of animals to constant but different temperature regimes, we attempted to separate the internal and external factors determining the hibernation pattern of European ground squirrels. The pattern of hibernation was shown to be dependent on temperature and on the timing of the hibernation season. Even though the temperature was held constant, the seasonal change in the pattern indicated that appearance of periodic arousals is also determined by other internal factors. The contribution of such endogenous annual timing patterns (circannual rhythms) has already been

documented in other species (Pengelley and Fisher 1961; Wang 1973 :Golden mantled ground squirrels (*Spermophilus lateralis* (Say, 1823)) and Richardson's ground squirrel (*Spermophilus richardsonii* (Sabine, 1822))) as well as in European ground squirrels (Zivadinovic et al. 2005). Although the TBLs became shorter in the majority of the treated animals by the end of the season, this effect was not observed in those animals that were kept at the lowest T_a , indicating that the interaction between internal and external factors could be equally important as factors per se (see Figure 3.1).

The TBL was shorter in animals that were kept at higher than at lower temperatures. Apparently this effect was more pronounced during the late-hibernation phase, but this difference was caused by the unchanged seasonal pattern of hibernation under the 0 °C thermal condition (see below). We found a strong negative relationship between the T_n and the TBL (Figure 3).

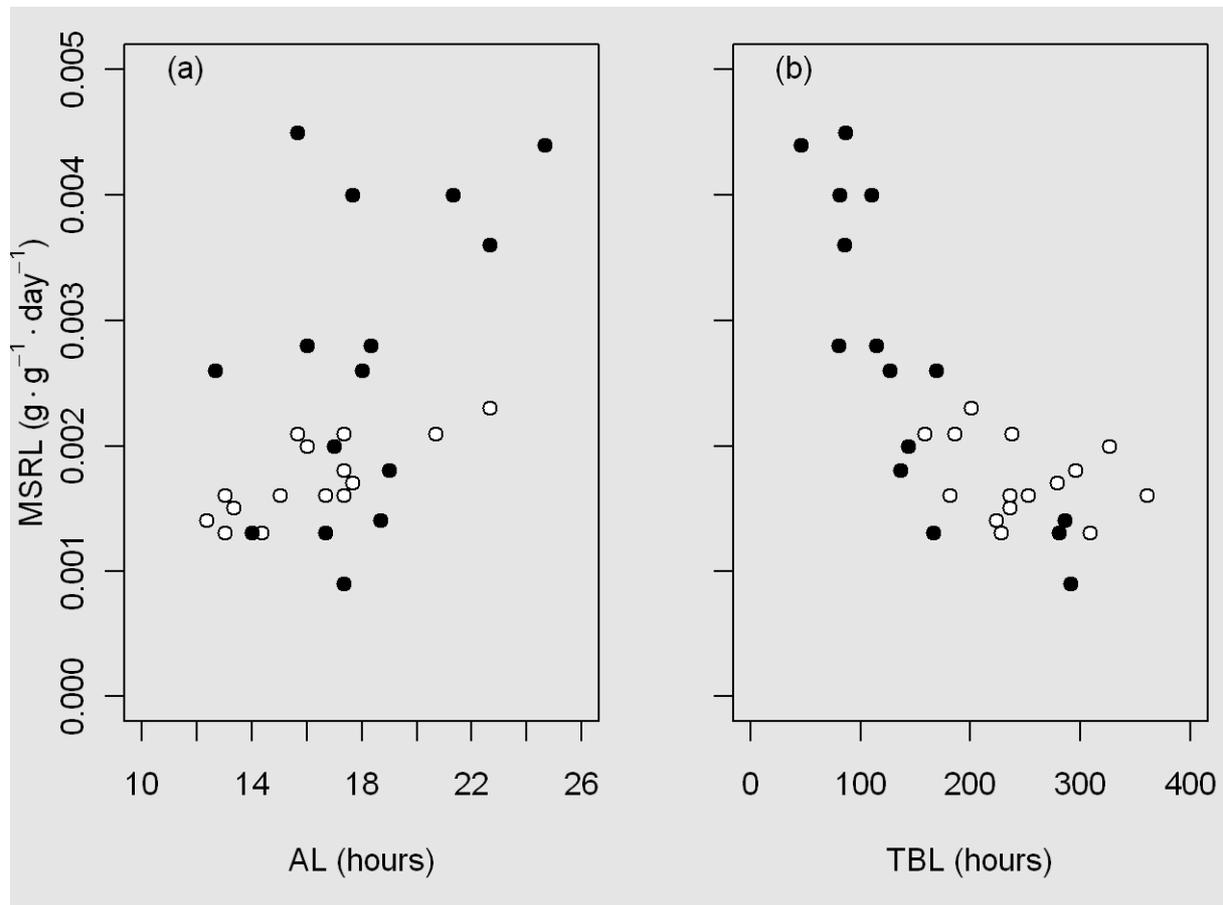


Figure 3.4 Body weight change was affected more by TBL (Fig 4b) than AL (Fig 4a). The best predictor variable of MSRL was period dependent. According to our models, variance of MSRL was explained best by the AL in mid-hibernation period (open circles). However TBL was the best predictor of variance of MSRL in late hibernation period (full circles).

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The longer TBL under colder environmental conditions could be potentially explained by differences in the rate of metabolism (Geiser 2004; Willis 1982a).

Our results show similarities, as well as some remarkable differences, compared with other studies on the same species. Hut et al. (2002) collecting data under semi-natural conditions, found longer TBL during mid-hibernation and shorter TBL during late-hibernation (see Table 3.1). The sex and age-specific differences may explain the different outcome, as the results were much more similar when we restricted the analysis to males in our groups. According to results on Columbian ground squirrels (*Spermophilus columbianus* (Ord, 1815)), the age and the gender of animals affect significantly the pattern of hibernation (Young 1990). Juvenile males have the shortest TBLs during mid-hibernation, and the shortening of TBL is less apparent at the end of hibernation in juvenile males compared with adult males.

The hibernation pattern under free running conditions showed characteristic changes in TBL during hibernation (Körtner and Geiser 2000a). These endogenous circannual rhythms are common even without external cues such as photoperiod or thermal changes among hibernator species (see Davis 1976). Keeping the animals at a constant temperature throughout the hibernation did not eliminate this specific pattern. Our results for European ground squirrels were similar to those found by others for other species (Pengelley and Fisher 1961; Twente and Twente 1965; Wang 1973; Zivadinovic et al. 2005).

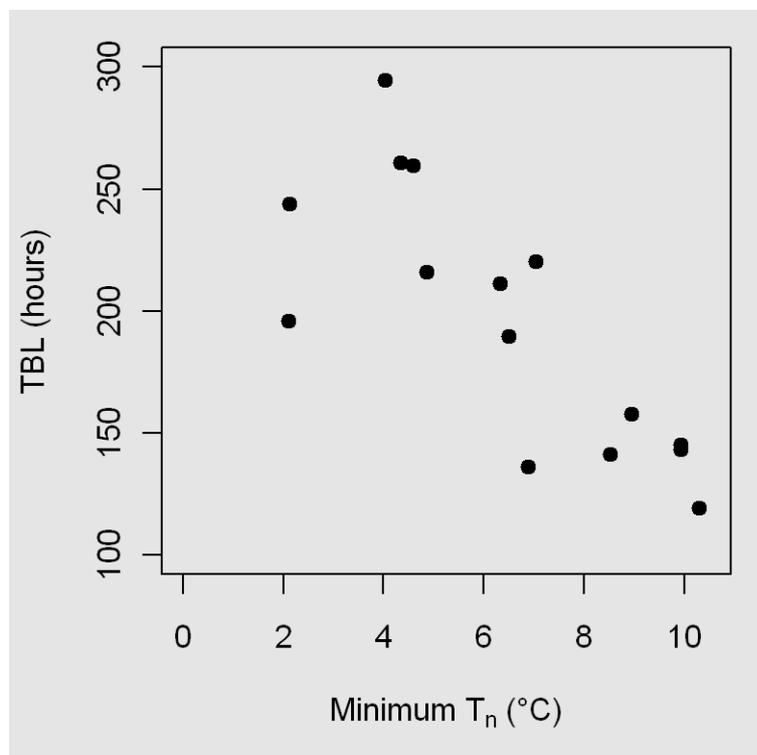


Figure 3.5 The average torpor durations are related to the minimum T_n s. The stepwise regression model excluded the treatment as factor from the final model. Best predictor was the average minimum torpor temperature for the average TBL. Furthermore, the effect of minimum T_n did not differ among the groups.

As with our studies, the groups held at T_a s of 9 °C and 5 °C had considerably shorter torpor length toward the end of the season. This pattern seemed to disappear in animals kept at T_a of 0°C, which emerged about 20 days later than European ground squirrels kept at higher T_a (see Figure 3.1). During this post-treatment period animals had 2-3 torpor phases, which were slightly but not significantly shorter than the torpor phases during the late-hibernation phase. Thus, it is unlikely that the nonshortened TBL under a T_a of 0 °C was simply caused by early termination of treatment. Animals kept at lower temperature have extended hibernation; this phenomenon refers to findings that highlight the lack of function of the master clock during deep torpor, which may contribute to changes in the annual timing as well (Oklejewicz et al. 2001; Revel et al. 2007; Ruby 2003).

AL was affected by the temperature treatment during the overall period (mid-hibernation and late-hibernation combined). As suggested by French (French 1982), the effect of temperature on arousal duration is possibly linked to the accumulation of metabolic imbalances during torpor, with homeostasis restored during the subsequent arousal phase. This suggests that the shorter the torpo, the shorter the following euthermic phase needs to be. Our results seemingly contradict this, as ALs were negatively correlated to the TBLs (see Figure 3.1 and 3.2.). The TBLs became shorter during late hibernation, whereas the ALs did not get shorter but became longer. A similar pattern was found in the hibernation of golden-mantled ground squirrels by Barnes et al. (1986).

We estimated the metabolic consequences of temperature treatment by monitoring body mass. T_a treatment affected the loss in body mass by our animals (see Figure 3.3). Both the direction and the magnitude of change fit to former results, as the smaller amount of loss in body mass at lower temperature could be the consequence of slower metabolism at lower temperatures (Buck and Barnes 2000). A constant decline in body mass was observed during hibernation among fat storing hibernators (Humphries et al. 2003). The loss in mass was due to the decrease of fat stores, while fat-free tissue mass remained constant (Dark et al. 1989). The loss in body mass was not only affected directly by metabolism, but strongly depended on the endogenous circannual rhythm (Mrosovsky and Fisher 1970). According to their results the decline in body mass was stable over the hibernation period. In our experiment, this decline appeared to be affected by T_a (see Figure 3.5).

Modifying hibernation patterns would result in a change in mass based on differences in energy expenditures between torpor and arousal phases (Wang 1979b). The results of regression models on MSRL supported this hypothesis. The longer TBL seemed to reduce the rate of loss in body mass, corresponding with the energy-conservation role of the torpor phase.

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But this relationship disappeared when the longest TBLs appeared during the mid-hibernation. Taking the two phases together along the same axis, we could recognize a negative correlation with a bottom threshold (see Figure 3.4a). This lowest energy expenditure zone, where the elongated TBL was not associated with decreased MSRL, may somehow correspond with the circannual cycle of body mass set-point (Mrosovsky and Fisher 1970).

In conclusion, we found the appearance of arousals during hibernation to be affected by both the T_a and the internal timing, but the latter seemed to be affected by the T_a conditions. This synergistic effect manifested on energy expenditure as a differential change in body mass during hibernation.

ACKNOWLEDGEMENTS

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The role of body mass in the timing of periodic euthermy in European ground squirrels (*Spermophilus citellus*)

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Submitted

ABSTRACT

Two groups of European ground squirrels with experimentally modified pre-hibernation body mass were used to test whether body mass has an effect on the timing of periodic euthermy during hibernation. Animals in the elevated body mass group display periodic euthermy more frequently, as well as shortened torpor bout lengths. Body mass difference was permanent throughout the investigated period, but was less pronounced at the end than at the beginning of this period. Although there was no difference between groups in overall time spent in euthermy, the extraordinarily high cost of rewarming at the beginning of the arousals can explain the differing body mass loss of the treated groups. These results confirm our cost-benefit based hypothesis on the role of body mass in the timing of periodic euthermy. Individuals in good condition (body mass) display costly but advantageous behavior (periodic euthermy) more frequently than individuals in poor condition.

INTRODUCTION

Hibernation, independently of the species, consists of two, distinct and periodically alternating states. The torpor state is a heterothermic state (Willis 1982a), when body temperature traces the ambient temperature in a wide range (Song et al. 1997). In this state animals substantially reduce their energy requirements by lowering body temperature and metabolic rate (Geiser 2004). This low body temperature state is periodically interrupted by high body temperature, approximately one day long periods when animals return to euthermia (Pengelley and Fisher 1961; Twente and Twente 1968; Willis 1982b). The main purpose of torpor is to reduce energy expenditure during seasonally appearing harsh periods when food availability is low and low temperature conditions cause elevated energy expenditure to maintain euthermic body temperature (Geiser 1988; Geiser 2004; Körtner and Geiser 2000a). To explain the appearance of costly periodic euthermia, many hypotheses have been proposed to reveal the advantage gained by the exhibition of costly arousals (sleep deprivation: Daan et al. 1991; accumulation of metabolic waste: Fisher 1964; depletion of blood glucose: Galster and Morrison 1970; water loss via urination: Németh et al. 2010; insufficient immune response: Prendergast et al. 2002; water loss via evaporation: Thomas and Geiser 1997). These hypotheses approach the question from different points of view of the physiology of hibernation, however, all of them agree in that arousals provide some kind of advantage and have to be adaptive (Carey et al. 2003; Willis 1982b).

From an energetic point of view, the appearance of arousals or, in a broader sense the pattern of hibernation, basically determines the overall energy expenditure of hibernation. Metabolic rate in torpor can be decreased by 90-95 % in comparison to the euthermic phase (Geiser 2004). Ninety percent of energy consumed in hibernation was spent on maintaining euthermia in arousal states (Wang 1979b). Nevertheless, the time spent in euthermia is less than 10% of the whole hibernation period (Körtner and Geiser 2000a; Wang 1973). In brief, the more arousals appear, the more expensive it is to hibernate. According to the theory of optimal behavior, individuals who possess much more of certain resource can allocate more energy for costly but advantageous behavior (Parker and Maynard Smith 1990).

Based on the above, we hypothesized that individuals that have extra resources, like body mass, should display costly but obviously advantageous behavior, like periodic euthermia, more frequently. In this sense we assume that actual body mass might affect the hibernation pattern in a feed back loop. To test this hypothesis we carried out an experiment on hibernating European ground squirrels. The emergence body mass of the animals was experimentally

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manipulated by providing food with different energy content. One group of ground squirrels were fed high energy food (H(igh)-E(nergy)-D(iet)), the other group were fed low energy food (L(ow)-E(nergy)-D(iet):). We expected that the animals in group HED would have higher body mass at the onset of their hibernation, would be in euthermia more frequently and their average torpor bout length (TBL) will be shorter than that of animals in group LED.

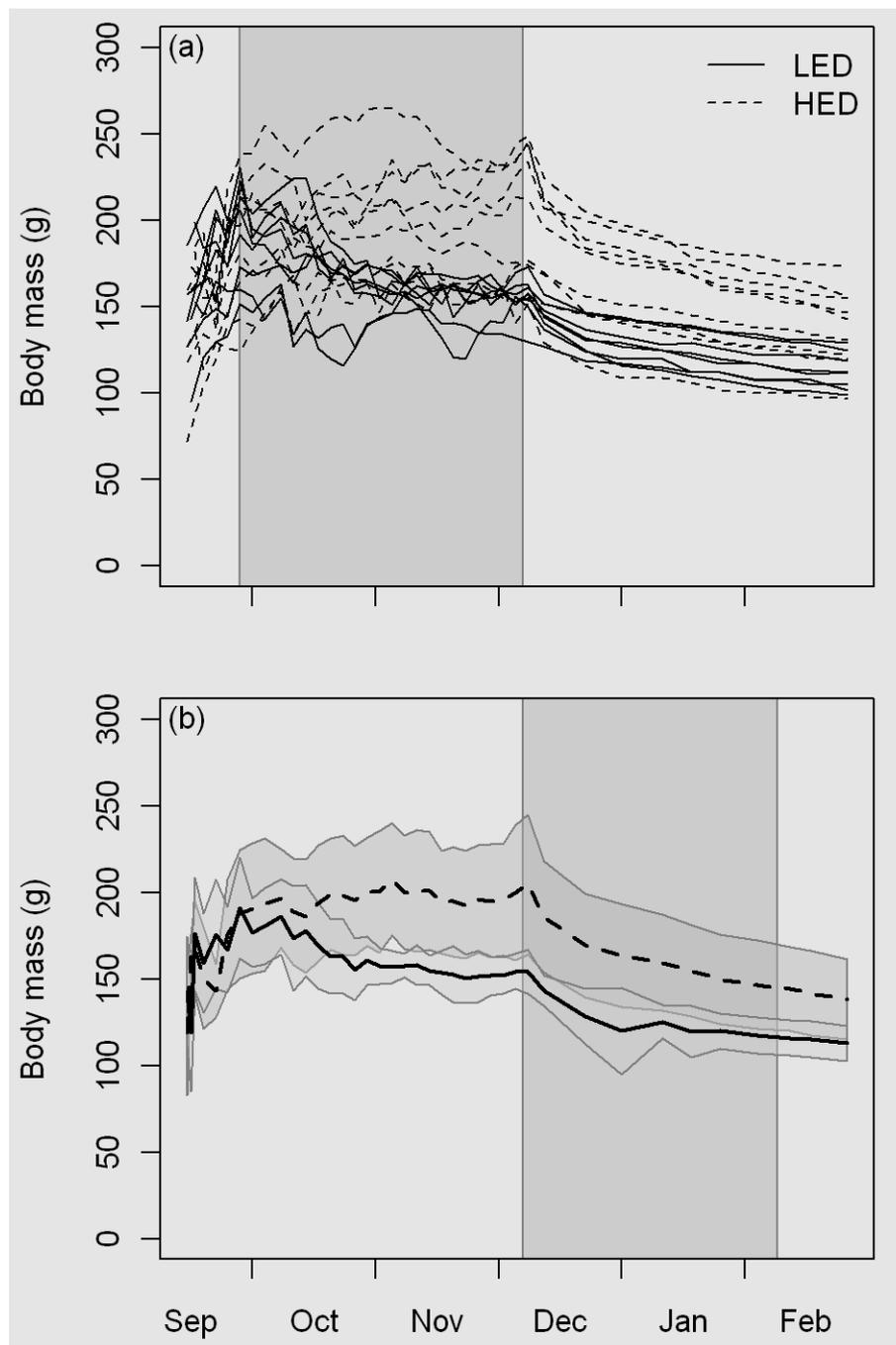


Figure 4.1 The changing of body mass during the experiment. Individual body mass – time profiles were presented (subfigure a), and marked separately by groups. Standard deviation (grey area; subfigure b) over the mean trends (thick lines) were shown separately by groups on subfigure b. Time frame of preparation (27 September 2007 – 05 December 2007, subfigure a) and investigation period (05 December 2007 – 03 February 2008, subfigure b) were depicted by grey stripes.

MATERIALS AND METHODS

Animals and housing conditions

Eighteen European ground squirrels, *Spermophilus citellus*, were trapped in Hungary by a living capture method in the autumn of 2007, and arrived in the laboratory on September 14, 2007. The animals were housed individually in plastic containers sized 36×21×15 cm. The containers were open at the top, but covered with removable wire mesh. Barrel shaped (d16×8 cm) opaque plastic boxes with a removable top were used in every container as nest boxes. The animals could enter the nest box through an 'L' shaped tunnel (d6 cm) attached to the side of the box.

Until November 14, 2007 animals were kept at room temperature, about 20°C, and exposed to natural photoperiods (short photoperiod; 10 light : 14 dark). After November 14, the room temperature was gradually lowered in ten days from room temperature to 10 ± 1 °C. This temperature was held constant until the end of the experiment. Natural photoperiod was turned to a continuous red dim light on the same day.

The animals were all weighed at the same time, approximately once a week, to the nearest 1 g (Philips HR 2393 01).

Schedule of feeding treatment

Immediately after their placement, ground squirrels were randomly assigned to two treatment groups (HED and LED). From September 14 to September 27, both groups were kept on a high calorie diet (ALTROMIN, Maintenance diet – rats and mice No. 1320). After this time, ground squirrels assigned to HED group (n=9) continued on the high calorie diet (ALTROMIN, Maintenance diet – rats and mice No. 1320), while those (n=9) assigned to LED group were kept on a low calorie diet (Galgavit Kft., Rabbit chow). This preparation period lasted until mean body weight difference reached 60g, which was judged significant in evoking the desired effect. At this point, food was withdrawn completely from all animals in both groups. The investigation period would last for the next two months (60 days) from the time point of food deprivation. This time period was judged long enough to obtain an appropriate amount of data for analysis.

Hibernation and torpor-arousal cycles

To trace the animals' body temperature, a formerly published method was used (Németh et al. 2009; Németh et al. 2010). Nest temperature (T_n) was recorded as it was found to be

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dependent on the presence of arousals (Oklejewicz et al. 2001; Strijkstra et al. 1999). To record T_n we prepared the nest boxes with temperature-sensitive resistors (KTY 21-5, Conrad Electronics). The sensor was attached to the inside of the removable top, about 2-3 cm from the animal's body surface. To restrict thermal convection between the sensor and the outer environment, the top of the nest box was insulated with a 1 cm thick foam plate. The signals from the thermistors were received by a model multimeter (Metex, M-4640A) via a channel multiplexer controlled by a PC. Finally, the multimeter was interfaced to a computerized system of data acquisition and it recorded in 10 min intervals. The sensors were previously calibrated to the nearest 0.1°C against a precision mercury thermometer between the temperatures of melting ice (0°C) and a 36°C water bath.

Torpor bout length (TBL) and arousal length (AL) were determined by visual inspection of the individual nest temperature - time profiles. TBL was defined as the period of time (days) starting at the end of the previous arousal and lasting until the onset of the subsequent arousal. Arousal length was defined as the period of time (hours) starting at the beginning of rewarming and lasting till the beginning of the cooling phase, which is a moderate but continuous decline in T_n (the visually estimated onset of the cooling curve).

The onset of the hibernation period (day month year) was defined by the beginning of the first torpor bout in the season.

Statistical analysis

Repeated measurements of the animals (TBL and AL and body mass) were analyzed using linear mixed effect models, entering differences between individual intercepts as the random effect. Averaged TBL and AL were tested for the effect of treatment. To analyze variation in body mass after the feeding experiment, we tested for the effects of feeding and time of hibernation. Normality assumption of the model residuals were tested with quartile-quartile plots. Further comparisons on treatment effect were carried out with Student's t-test. In case of failed homoscedasticity, Satterthwaite method was used for the computation of the degrees of freedom. Arousal numbers was analyzed using nonparametric Wilcoxon-test. All statistical analyses were carried out with the statistical package R version 2.9.1 (R Development Core Team 2009).

RESULTS

Body mass

The body mass of the animals upon their enrollment in the study was on average 145 ± 34 g. At this time there was no difference between the two treatment groups (LED: 145 ± 34 g, HED: 146 ± 37 g; $t_{16} = 0.060$, $p = 0.953$). After the change in diet on September 27, 2007, a difference in the body mass of the animals in the two groups gradually developed during the preparation period (Figure 1a). The pre-specified difference in average body mass was attained on December 5, 2007 (Figure 1b). This difference between the average body mass of the two treatment groups did not differ significantly from 60g (LED: 154 ± 13 g, HED: 205 ± 40 g; H_0 : difference=60, $t_{9.56}=0.684$, $p = 0.510$, $CI_{95\%}$: 20.4-80.3 g).

The subsequent change of body mass during the investigational period (December 5, 2007 - February 3, 2008) was investigated in time, by ANOVA with treatment and time as fixed, and subject as random factor. According to the results of the analysis, both the fixed factors and their interaction significantly explained the variability (Treatment: $F_{1,16} = 12.439$, $p < 0.01$; Time: $F_{1,177} = 425.985$, $p < 0.0001$; Treatment \times Time: $F_{1,177} = 27.733$, $p < 0.0001$). Animals lost 0.7 g body mass a day in group HED ($t_{177} = 0.712$, $p < 0.0001$), but animals in group LED lost significantly less, 0.4 g body mass per day ($t_{177} = 0.300$, $p < 0.0001$), in terms of least squares parameter estimations.

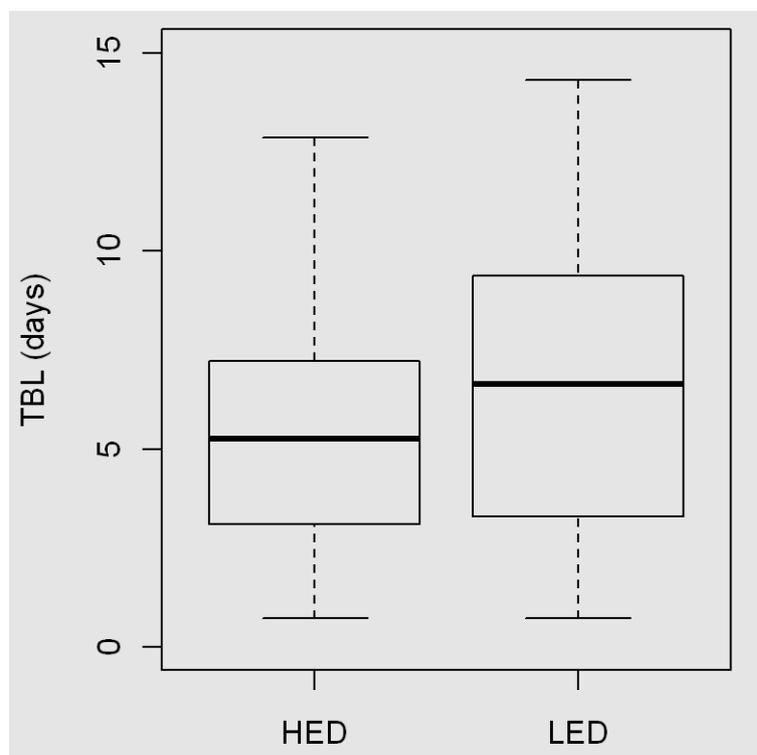


Figure 4.2 Torpor bout length (TBL) of ground squirrels after experimental body mass manipulation. Animals kept on high energy diet (HED) had significantly shorter torpor bouts ($p < 0.05$) than animals kept on low energy diet (LED). Box and whiskers represent range (min-max), quartiles (25 – 75%) and medians of the data.

BODY MASS AND AROUSAL TIMING

In spite of this difference in the rate of body mass loss, body mass of the animals in the two groups was still different at the last measured time point (February 22, 2008; LED: 113 ± 10 g, HED: 138 ± 23 g; $t_{10,97} = 3.018$, $p < 0.05$), but the difference was less pronounced ($CI_{95\%}$: 6.9-43.8 g).

Hibernation and torpor-arousal cycles

The onset of hibernation did not differ between animals in groups LED and HED ($t_{14} = 0.197$, $p = 0.847$). Animals, on average, entered hibernation on December 7, 2007 and December 8, 2007 in groups HED and LED, respectively. Nevertheless, the first animals had already hibernated on November 19, 2007 in group HED and on November 16, 2007 in group LED.

Average TBL during the investigated period was 5.6 ± 2.7 and 6.8 ± 3.4 days long for HED and LED groups, respectively (Figure 2). The difference between the groups was significant ($F_{1,14} = 4.791$, $p < 0.05$). Considering that some animals had hibernated before the investigated period, we further analyzed the data. When TBL data obtained before December 5, 2007 were also incorporated in the analysis, the statistical inference on the effect of the treatment did not change ($F_{1,14} = 4.954$, $p < 0.05$).

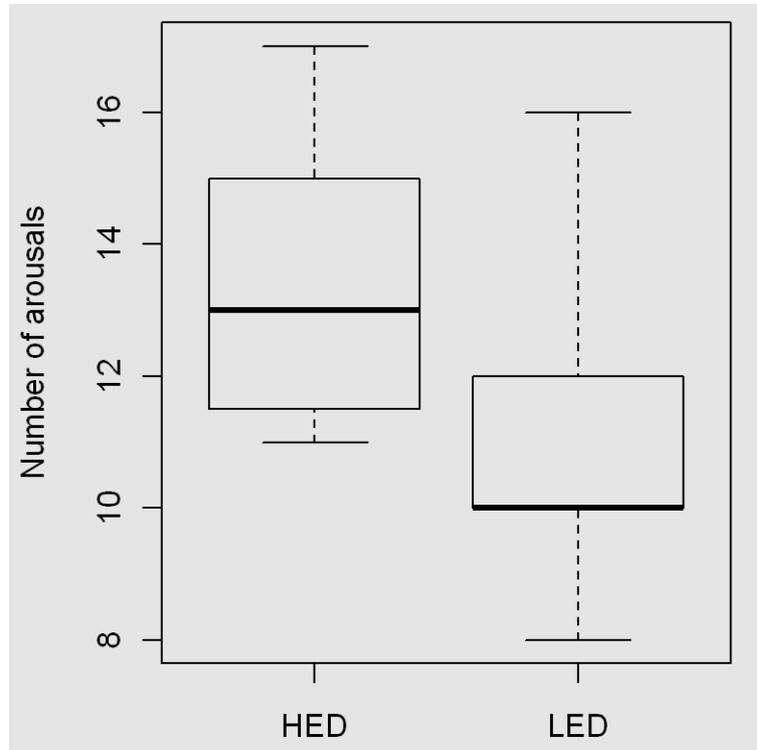


Figure 4.3 Number of arousals of ground squirrels during the investigated part of hibernation period after experimental body mass manipulation. Animals kept on high energy diet (HED) became euthermic more frequently ($p < 0.05$) than animals kept on low energy diet (LED). Box and whiskers represent range (min-max), quartiles (25 – 75%) and medians of the data.

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Animals in group HED entered euthermia more frequently than animals in group LED (Figure 3; $W = 50.5$, $N = 16$, $p < 0.05$). The effect of the treatment has no influence either on the average length of arousal periods ($F_{1,14} = 0.018$, $p = 0.895$) or on the overall time spent in euthermia ($t_{14} = 1.558$, $p = 0.142$).

DISCUSSION

This study demonstrates that body mass affects the timing of periodic euthermia. The frequency of arousals was higher in the elevated body mass group (HED) than in the group with lower body mass (LED), which entailed significantly shorter torpor bout lengths. These results are in accordance with and confirm our initial hypothesis, which assumed a relationship between the state of energy stores and the exhibition of periodic euthermia with high energy requirements.

So far, two major hypotheses have been proposed to explain the relationship of periodic euthermia to food consumption. The first hypothesis is in connection with the early finding that revealed that there is a set-point in body mass which continuously changes during the year, and reaches its lowest point in the middle of the hibernation season (Mrosovsky and Fisher 1970). This autonomic year round cycle of body mass change is very rigid and difficult to reset by environmental cues such as a change in temperature or photoperiod (Pengelley et al. 1978). Several species of hibernators hardly eat anything during the hibernation season (Pengelley and Fisher 1963). This change in the appetite approximately follows the year round cycle of the body mass set point. In accordance with metabolic waste product hypotheses (Willis 1982b), high food intake was hypothesized to be incompatible with prolonged torpor (Mrosovsky and Barnes 1974). According to the authors, an animal that is eating large quantities of food, and so producing additional sources of metabolic waste products, will not be able to hibernate as long as an anorexic animal in which the concentration of the waste products will take longer to reach critical levels triggering arousals (Mrosovsky and Sherry 1980).

The second hypothesis is in connection with the cold adaptation hibernating animals show on the cytological level. Polyunsaturated fatty acids (PUFA) are essential in the diet of mammals. Experimental manipulation of dietary lipids in hibernating and non-hibernating rodents alters the composition of body fats and cell membranes, and this is further associated with changes in the thermal properties of membranes (Carey et al. 2003). In an experiment where animals were fed with high or low PUFA containing foods, the authors demonstrated the profound effect of dietary lipids on the duration of torpor and the minimum body temperature

in a hibernating rodent (Geiser and Kenagy 1987). This result was consistent with previous correlations of high levels of membrane lipid unsaturation with ability to function at low temperatures (Cossins and Prosser 1978).

Our proposed explanation for food consumption and body mass effect on arousal timing can be considered as a third hypothesis, partially related to the former two hypotheses (animal anorexia and PUFA). Our approach was based on the relationship between the energetic cost and metabolic benefits of periodic euthermy. This hypothesis is able to alternatively explain the results obtained in studies in which body mass was also changed experimentally via various treatments (Davis 1976; Mrosovsky 1986; Mrosovsky and Barnes 1974; Mrosovsky and Fisher 1970; Mrosovsky and Sherry 1980). According to the results in the present study, the body mass difference per se, was able to influence the timing of periodic euthermy.

As per the study plan, the ambient temperature was lowered and the photoperiod was changed on November 14, 2007 to continuous darkness. Although two and five days later, one animal in group LED and one animal in group HED started the first torpor bout in their hibernation, the majority of the animals started hibernation after food withdrawal (December 5). This delay in the onset of hibernation may reflect the order of environmental cues in the initialization and maintenance of hibernation. Hibernation is rigidly controlled by innate rhythm generators (Davis 1976; Pengelley et al. 1978), but there are many external environmental timing cues (Zeitgeber) to which hibernating animals respond by adjusting their pattern of hibernation (Körtner and Geiser 2000a). There are species specific differences in these factors, but temperature, photoperiod, and food availability are the three most conspicuous factors. In captive Golden mantled ground squirrels (*Spermophilus lateralis*), hibernation could not be induced by the removal of food alone, but was greatly prolonged if food was not present (Pengelley and Fisher 1963). In the same experiment, the onset of hibernation could be changed only slightly by changing the photoperiod, but by the manipulation of the exposure to low ambient temperature it was possible to have animals hibernating in summer and active in winter. In a controlled laboratory experiment on Arctic ground squirrels (*Spermophilus parryii*), photoperiod seemed to have as great an influence on the initiation and maintenance on hibernation as did temperature (Drescher 1966). In fat mouse (*Steatomys pratensis*) the deprivation of food or water, or food alone always initiated torpor, whereas water deprivation in itself was only partially successful in the induction of torpor (Perrin and Richardson 2004). The majority of European ground squirrels entered hibernation after food deprivation. Although temperature and photoperiod change also adjusted the timing

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of hibernation in some animals, these environmental cues seemed to be less important in the initiation of hibernation.

The body mass loss rate of the two groups was clearly different in this experiment. Animals in both groups lost weight progressively during their hibernation, but animals with higher body mass (HED) lost more body mass in the same time. Thus the body mass difference between the groups continuously decreased during the investigational period. This difference cannot be explained by the body mass effect on metabolic rate because this effect should cause the opposite result, independently of the manner of thermoregulation; metabolic reduction increases with body mass during heterothermy (Geiser and Ruf 1995) and basal metabolic rate decreases with body mass in euthermy (McNab 2008). Although we did not find a significant difference in the overall time spent in euthermy, more frequent arousals might be able to explain this result. The metabolic rate during arousals is not constant. There is a high peak in oxygen consumption as well as in energy consumption at the onset of arousals (Song et al. 1997; Wang 1979b). Generally, this extra energy consumption period lasts only less than half hour and is followed by a return to normal level, but this is the most expensive part of the arousal. Although there was no difference in overall time spent in euthermy, the higher number of arousals could cause an elevation in overall energy consumption.

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Effect of nest insulation on body mass loss during hibernation (*Spermophilus citellus*)

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Submitted

ABSTRACT

Nest construction is an important part of the thermoregulatory behavior of many small endotherms. Hibernating fossorial rodent species in the Holarctic spend long periods of time in their nests during the winter months close to the freezing point. Hibernating species allow their body temperature to drop to save considerable energy; therefore the benefit of a well insulated nest is not obvious. Our study aimed to reveal the possible advantage of nest construction for hibernating animals, in terms of saved energy.

Animals were allowed to construct nests under laboratory conditions, to determine the relationship between morphology and insulation capacity of nests in European ground squirrels (*Spermophilus citellus*). The energy saving function of the nest constructed was tested by assessing the relationship between nest insulation and body mass loss during hibernation.

The nests constructed showed high inter-individual variability both in physical structure and insulation capacity. The insulation depended only on the wall thickness. The insulation of the nest significantly influenced the body mass loss during hibernation. The importance of insulation, in terms of energy saving, is probably more pronounced during periodic euthermic phases than during heterothermy, when the temperature gradient between the animal's body and the outer environment is much lower.

INTRODUCTION

During the autumn and winter months, small hibernating mammals such as the European ground squirrel (*Spermophilus citellus*) and other sciurid rodents conserve energy by reducing metabolism and decreasing body temperature (T_b) close to ambient temperature (T_a) (Hut et al. 2002; Wang 1979b; Willis 1982a). Many physiological and cellular functions, including respiration, reproduction, cardiac function, digestion, DNA transcription, and immune function, cease or are substantially reduced during this hypothermic state, commonly called “torpor” (Barnes et al. 1986; Carey et al. 2003; Prendergast et al. 2002; van Breukelen and Martin 2002).

The rate of metabolism during torpor is typically less than 3% of euthermic rates at the same T_a , which ensures considerable energy savings (Geiser 1988). The ground squirrel species (*Spermophilus spp.*) hoards little or no food during the pre-hibernation period; they survive the winter primarily by extracting fuel from stored body fat (Humphries et al. 2003). Ground squirrels increase their body mass in the active season and lose weight progressively during hibernation (Dark et al. 1989; Millesi et al. 1999b).

European ground squirrels, in common with all other mammalian hibernators, do not remain torpid continuously; they arouse repeatedly throughout the hibernation season and temporarily achieve normothermic T_b values at intervals of 2–10 days (Geiser et al. 1990; Körtner and Geiser 2000a; Németh et al. 2009). However these arousals last less than a day; up to 70% of a hibernating mammal’s winter energy expenditure occurs during these periodic arousals (Geiser et al. 1990; Körtner and Geiser 2000a; Wang 1979b).

Mammalian nests ameliorate the energetic challenges of winter. The primary function of nests constructed by small endotherms appears to be for the reduction of heat loss (Casey 1981; Ellison 1995). Insulation plays a significant role in conserving energy during hibernation by retarding loss of physiologically produced heat and reducing costs of maintaining normothermic T_b values during short arousal states (Geiser 1988; Kauffman et al. 2004). The insulation of nests is related to several factors, but primarily depends on nest structure, thickness and raw material (Mertens 1977; Redman et al. 1999; Szentirmai et al. 2005).

As animals construct better nests that provide better insulation, these thermal benefits are reflected in decreased energy consumption due to reduced heat loss. This assumption predicts that individual differences in nest construction can cause individual differences in energy consumption and ultimately in weight loss during hibernation.

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The primary aim of this study was to measure the physical properties (mass, wall thickness and nest material composition) of nests built by the European ground squirrel and to relate these to direct measurements of nest insulation. The second aim was to quantify the energetic benefits that ground squirrels gain by constructing nests, in terms of their body mass.

MATERIALS AND METHODS

Animals and housing

Ten juvenile male European ground squirrels (*Spermophilus citellus*) were captured at a grass field airport in middle Hungary (N47° 36' 53", E19° 8' 36"), during August 2005. The animals were brought to a climate controlled room at Eötvös Loránd University, Budapest. Permission was obtained from local authorities and the Ethical Committee for Animal Experiments before the experiment had begun. The experiments were carried out in accordance with ASM guidelines (Gannon and Sikes 2007).

The animals were housed individually in plastic containers sized 36 × 21 × 15 cm which contained wood shavings. The containers were open at the top but covered with removable wire mesh. Barrel shaped (16 cm diameter × 8 cm high) opaque plastic boxes were used in every container as nest boxes with a removable top. The animals could enter the nest box through an "L" shaped tunnel (diameter: 6 cm) attached to the side of the box. Food (rabbit chow, Galgavit Kft., Gödöllő, Hungary), water and fescue grass (*Festuca spp*) collected by hand for use as raw material for nest construction were given ad libitum during the pre-hibernation period.

The animals were kept at 20 ± 1 °C with a LD cycle of 10:14 (short photoperiod conditions) until mid September when T_a was decreased to 10 ± 1 °C and the LD cycle changed to DD. At this point food, water and nest material were withdrawn from animals. Wood shavings were replaced with blotting paper that was changed regularly

Nest characteristics

Nest boxes with the nests constructed by the squirrels were replaced with new nest boxes with artificially constructed nests, when all animals had hibernated for at least two months, on December 30th. After the removal of nest boxes the following measurements were carried out on the nests in the subsequent chronological order; (1) estimation of the insulation capability, measurement of (2) wall thickness and (3) nest material composition and weight of

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components. Additionally, the measurements of insulation and wall thickness of the nests were carried out with maximal care to maintain the original structure of the constructed nests.

(1) Insulation was estimated from cooling curves of the nests based on the approach of Szentirmay et al. (2005). The relationship between time and nest temperature (T_n) is expressed as an exponential function with two parameters A and k_{CR} :

$$T_{diff} = A \cdot e^{-Time \cdot k_{CR}},$$

where, T_{diff} is the difference between the actual T_n and T_a , A is the starting temperature relative to T_a and k_{CR} represents the cooling rate constants which express the ratio of heat lost during a specified time unit (h^{-1}) under the assumptions of the model (T_{diff} asymptotically approximates zero). The set up of the experimental device to measure k_{CR} for the nests constructed by the ground squirrels is based on the procedure published by Redman et al. (1999). Accordingly, 150 ml glass bottles were filled with hot (50 ± 1 °C) water which were then inserted into each squirrel nest kept at room (ambient) temperature (25 ± 2 °C). Each bottle was closed with a rubber lid, through which a temperature sensitive probe was inserted to record T_n . A second probe was used to measure the T_a of the climate chamber. The probes were previously calibrated to the nearest 0.1 °C against a precision quicksilver thermometer in a water bath between 0 and 40 °C. The signals from the probes were received with a model multimeter (M-4640A; Metex Instruments, Seoul, South Korea) via a channel multiplexer controlled by a PC.

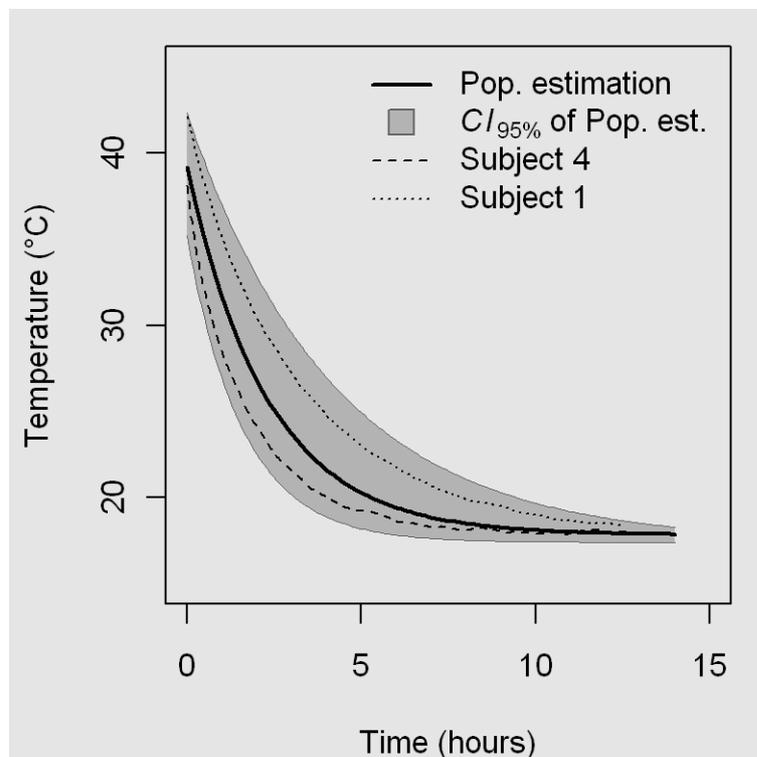


Figure 5.1 Non linear mixed effects modeling estimation for cooling curves of the nests constructed by European ground squirrels. Marginal estimation for population ($k_{CR}=0.43$; solid line) with corresponding 95% confidence interval (CI95% $k_{CR}=0.24-0.62$; grey area) are depicted. To facilitate a clear overview only data points for two individual cooling curves were plotted, one with the highest (Subject 4: 0.57; dashed line) and one with lowest (Subject 1: 0.31; dotted line) k_{CR} value.

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The multimeter was interfaced to a computerized system of data acquisition that recorded data in 2 min intervals.

(2) The wall thickness was measured at 16 points for each nest to the nearest 1.0 mm with calipers (INOX, MB-3425). According to the shape of the nest boxes, measurements were made at 45° angles (8 points) both on the bottom and the sides of the boxes.

(3) Finally, the nests were removed from the nest box and were subsequently disassembled and weighed to the nearest 1.0 g (Philips HR 2393 01) by nest material type (wood shavings and fescue).

Body weight measurement

The body weight loss of the animals during the study period was estimated via the difference in body weight between time points of November 4th and December 30th (November 4th was the first day with weight data when all the animals hibernated). All animals were weighed to the nearest 1.0 g (Philips HR 2393 01) on the same days.

Statistics and estimations

Individual and population parameter estimates of cooling curves were carried out with non-linear mixed effect modeling (Pinheiro and Bates 2002). Using this method we were able to estimate expected values at the population level and inter-individual variability as well (Pinheiro et al. 2008). General linear model procedures were used for further data analyses. Normality assumption of model residuals were tested with the Shapiro-Wilk test. In general, descriptive statistics for variables was presented as mean \pm SD. All modeling and statistical analyses were performed with R version 2.9.1, an open source software for statistical computing and modeling (R Development Core Team 2009).

RESULTS

Nest building

Complexity varies among nests constructed by ground squirrels and implies high inter-individual variability among juvenile males without earlier experience in hibernacula construction. The materials used for nest construction were the fescue provided and the wood shavings from the bottom of the plastic containers. The average nest weight was 75g with high inter-individual variability (60.8 %). The weight of the nests depended primarily on the amount of wood shavings used (a) and not on the fescue (b) (a: $F_{1,7} = 61.99$, $p < 0.001$; b: $F_{1,7} = 2.08$, p

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= 0.19). The weight ratio of wood shavings/fescue built into the nest varied from 0.00 to 17.75. The average wall thickness of the nests was 36.4 ± 10.89 mm. Wall thickness depended neither on nest mass itself ($F_{1,8} = 0.45$, $p = 0.52$) nor on wood shavings weight ($F_{1,8} = 0.16$, $p = 0.69$) nor on fescue ($F_{1,8} = 2.48$, $p = 0.15$). Thus ground squirrels appear to be able to increase the wall thickness of their nests not only by incorporating more material into their nests.

Nest attributes affecting nest insulation

Some data were missing because of technical difficulties during temperature recording; but the nonlinear modeling of cooling curves used is able to handle the missing data (Figure 5.1). The population parameter estimation obtained for k_{CR} was 0.43 ± 0.028 h^{-1} ($t_{3033} = 15.44$, $p < 0.0001$) with high (46.4 %) inter-individual variances.

We entered variables of nest physical properties (nest weight, fescue weight, wood shavings weight and wall thickness) as independent predictors in a linear model analysis and only wall thickness had a significant effect on nest insulation ($F_{1,5} = 10.58$, $p < 0.05$). When wall thickness was the single predictor, it explained 53.7 % of the variation in nest insulation ($F_{1,8} = 9.28$, $p < 0.05$; Figure 5.2). Nest insulation is thus a linear function of wall thickness.

Effect of nest insulation on short term changes in body mass – The average weight of the animals was 178.9 ± 13.45 g at the beginning of the study period (November 4th) and this value decreased to 133.5 ± 15.68 g when they were removed from their nests (December 30th). The average weight loss was 45.4 ± 22.10 g during the 56 day long study period.

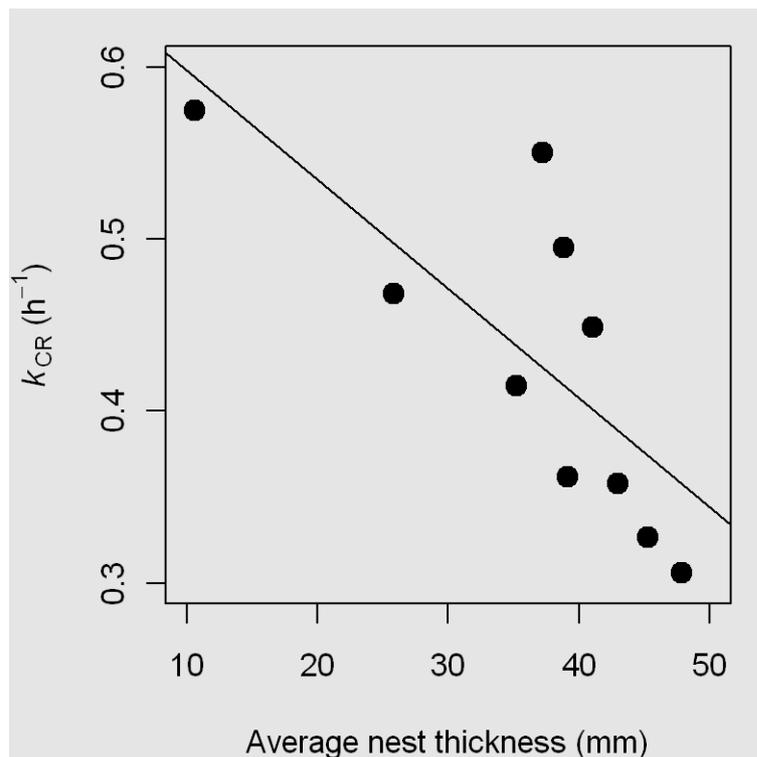


Figure 5.2 The relationship between wall thickness and the insulation capability (k_{CR}) of the nests constructed by European ground squirrels. The solid line represents the result of a fitted linear regression.

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There was a significant effect of nest insulation on the body mass change of the ground squirrels ($F_{1,8} = 10.93$, $p < 0.05$; Figure 5.3). Weight loss is thus a linear function of nest insulation. This effect was significant when the possible effect of starting body mass was also taken into consideration ($F_{1,8} = 10.41$, $p < 0.05$).

DISCUSSION

Nest construction is a widespread behavior found in representatives from most of the vertebrate groups. Small endotherms such as songbirds and rodent species create the most intricate nest structures. The most probable reason for this is that these animals have high surface to volume ratios and combined with their high T_b , they have relatively high heat loss compared to larger animals (McNab 2008; McNab 2009).

Many of the studies that have examined nesting behavior of rodents estimated the insulation quality of their nests by measuring the quantity of nesting material used (Lynch and Possidente 1978). However some studies have found that animals constructed warmer nests with less material built into the nest (Ellison 1995; Sealander 1952). In the present study, there was no correlation between the quantity of raw material used for nest construction by ground squirrels and the thermal conductance of their nests, which indicates that nest size was not a reliable measure of nest quality.

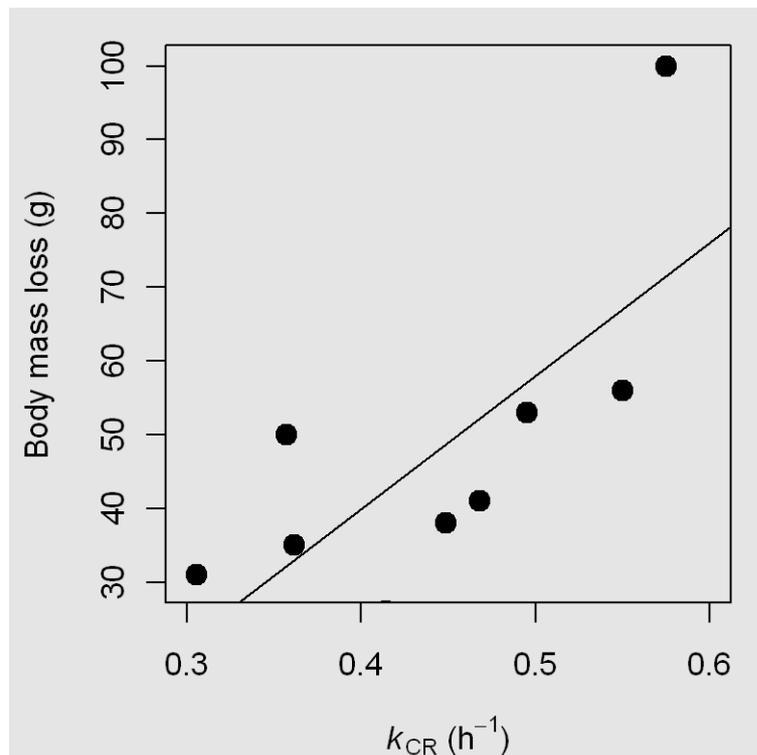


Figure 5.3 The relationship between individual body mass loss during the period of hibernation studied and the insulation capability (k_{CR}) of the nest used. The solid line represents the result of a fitted linear regression.

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Nest thickness was the best predictor of insulation capacity among the physical properties examined.

Similarly, Redman et al. (1999) found that nest thickness correlated highly with insulation in the case of short tailed field voles (*Microtus agrestis*), but, contrary to our results, voles increase the wall thickness of their nests only by incorporating more material into their nests. The ground squirrels increased the wall thickness and also the insulation by manipulating the structure of their nests. Nest structure can be manipulated by modifying the proportion of material used (McGowan et al. 2004) or by modifying the physical complexity of the homogeneous nest material. It seems that European ground squirrels are able to manipulate both the physical complexity of the nest structure (Gedeon et al. in press) and the ratio of materials built into the nest.

Successful thermoregulation in small mammals requires physiological and behavioral adjustments which either reduce heat loss or increase heat production. The latter response has been reported for many species but is metabolically very costly (Scholander et al. 1950a; Scholander et al. 1950b). The possibilities for decreasing heat loss in a cold environment by hibernating mammals include both a regulated drop in T_b and nest construction at the same time. Preventing heat loss is the primary advantage of nest construction for hibernating rodents. Nevertheless, the benefits of the nest probably are different between the periodically changing hetero- and normothermic states during hibernation (Song et al. 1997). The comparison of nest insulation between a deep hibernator, a daily heterotherm and a non-hibernating species highlights the fact that the construction of a well insulated nest differs in its energetic advantage between the hetero- and normothermic state. Buck and Barnes (1999a) found that the role of the nest in energy conservation is minor during heterothermy in a deep hibernator, the Arctic ground squirrel (*Spermophilus parryii*). The main emphasis in the authors' argumentation was that the small temperature difference between the environment and T_b did not allow much energy to be saved via enhanced insulation capacity. In contrast with deep hibernation, the role of the nest progressively increases in magnitude from daily heterotherm to non-hibernating species. A large T_b -to- T_a gradient is associated with higher metabolic rates in daily heterotherm and non-hibernating species where heat loss is greater, and insulation evidently is more critical (Kauffman et al. 2004; Kreith 1973). The white footed mouse (*Peromyscus leucopus*) and long tailed vole (*Microtus longicaudus*) are microtine rodents fairly similar in size (Lackey et al. 1985; Smolen and Keller 1987). White footed mice used more energy during their daily torpors without a nest than with a nest. Additionally, this difference increased at lower environmental temperatures (Vogt and Lynch 1982). Similar results were

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observed in non-hibernating species of long tailed voles: under low environmental temperature, animals without a nest spent even more energy maintaining euthermic T_b than did daily heterotherms (Beck and Anthony 1971). In summary, when the environmental temperature is low, the effect of the nest is not significant or is minor on energy saving in a deep torpor state, but the effect of a nest on energy saving can be definitive during euthermia. The majority of the hibernation period is spent in torpor, when the effect of a nest is minor (Buck and Barnes 1999b), but the portion of euthermia on overall energy expenditure is much higher (~70 %, Wang 1979b). The argument above also provides a good explanation of our findings, where the better insulated nests decreased the body mass loss during hibernation, via decreased energy consumption during arousals. Although conditions in our study do not precisely match the winter subterranean environment, we might conclude that there is a substantial energetic advantage to ground squirrels which produce better insulated nests (Figure 5.3). Increased energy expenditure does not allow retaining sufficient white adipose tissue stores to extend the hibernation season and to meet energy demands of post-emergence reproductive events (Boonstra et al. 2001; Michener 1983). Additionally, elevated energy consumption during hibernation can cause premature termination of hibernation (Mrosovsky and Fisher 1970) which has serious consequences (Morton and Sherman 1978).

Nest construction behavior is under high natural (Bult and Lynch 1996; Bult and Lynch 1997; Bult and Lynch 2000; Casey 1981; Yom-Tov and Hilborn 1981) or sexual selection (Szentirmai et al. 2005) in many vertebrate species. Our results suggest that there is sufficient energy savings to provide a strong selective pressure for nest building by ground squirrels during the winter, when the soil temperature at the depth of the hibernaculum is below the thermo neutrality range (4-10 °C). Selection pressure produces a decreased variability in the trait concerned through high heritability (Zhang and Hill 2005). Therefore, the fairly high variability among the nests constructed in our study and field observations (high proportion of fresh herbs; Gedeon, unpublished data) suggests other possible functions of the nest in ground squirrels, e.g. anti-microbial and water condensation functions (Gwinner and Berger 2005; Thomas and Geiser 1997).

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Factors affecting mating investment and the cost of reproduction in male European ground squirrels (*Spermophilus citellus*)

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Submitted

ABSTRACT

Our field study was carried out on a natural population of male European ground squirrels to explore the possible effects of the emergence timing and post-hibernation body mass on mating behavior.

Spring emergence date of males was positively correlated with their body mass and preceded emergence of females. Maturation at emergence depended primarily on emergence body mass. Home ranges of individuals substantially overlapped one another, and the number of females within these home ranges varied among males, along with the male's emergence date. Agonistic interactions frequently occur during the mating period with a remarkable increase at the time of mass emergence of the females. Body weight change of the males was in negative relationship with the number of females in their home range and was significantly dependent on the frequency of agonistic interactions.

These results suggest that larger males can emerge earlier, withstanding the severe environmental conditions and food shortage, and invest more into mating than smaller males. This finding can be interpreted as an example of a costly phenological strategy of the mate limited sex (male) to be present on the breeding ground when the mate limiting sex (female) appears on the ground.

INTRODUCTION

Ground squirrels (*Spermophilus spp*) represent a large group of rodents. This species richness might have arisen from the widespread distribution and adaptation to many different ecological environments. Accordingly, the reproductive systems of these species are also similarly various due to adaptations of inhabited environments from deserts to arctic (Clutton-Brock 1989; Hayssen 2008a; Hayssen 2008b). In general, that ground dwelling sciurid rodents typically show male biased sexual size dimorphism and various polygynous mating system. However, the degree of polygyny is wide among ground squirrels, but there are some typical elements that are generally common among them in relation with their hibernating life history, this commonality has long been debated (Michener 1983).

As in other hibernating ground squirrels, the reproduction of European ground squirrels (*Spermophilus citellus*) is limited to a short period in the active season. Adult males emerge from hibernation 2 to 4 weeks before the females, with fully developed testes and elevated plasma testosterone levels (Millesi et al. 1998). The mating period starts with the appearance of the first female and lasts for about 3 weeks. The mating system of *S. citellus* resembles scrambling competition polygyny in that males try to locate females in estrous (Millesi et al. 1998). The energetic costs for males are high and have been documented in the proportion of body mass loss in individual males, with subsequent carryover effects, delaying the onsets of molt and prehibernatory fattening. In line with this mating system, male behavior changes dramatically with the emergence of the first female (Millesi et al. 1998). Aggressive interactions among males and scent marking are frequent and male home ranges increase. The predictable female emergence date (Millesi et al. 1999b) and perhaps some specific female cues may be key stimuli for male behavioral changes. Testicular regression of individual males starts at the end of the mating period. It is accompanied by a cessation of aggressive behavior and a decrease in home range size (Millesi et al. 1998).

The timing of emergence of male European ground squirrels is sequential and primarily depends on the maturity and body condition of an individual (Millesi et al. 1998). This relationship is generally observable among ground squirrel species and several hypotheses were formed to explain the advantage of an early arrival to the breeding ground and its relationship to body condition (Forstmeier 2002; Michener 1983; Möller 1994). To explore the effect of the timing of emergence and post hibernation body mass on behavioral and hormonal states of mating male European ground squirrels, we analyzed data recorded in a free ranging population.

MATERIAL AND METHODS

Study site

The study was conducted at Kab-Hill, Bakony-Mountains, Hungary (47°01'N, 17°36'E; 280 m.s.l.). The 4-ha study site was an intensively grazed pasture. Sheep flocks grazed the site at least twice a day (morning and afternoon). The flocks generally consisted of app. 400-700 individuals.

Vegetation at the study site consisted of several types of grasses and plants. The most important grass species were Old World bluestem (*Bothriocloa ischaemum*) and fescue (*Festuca rupicola*). The main plant species were burr medic (*Medicago minima*), thyme (*Thymus sp.*) and yarrow (*Achillea sp.*). The study site border to the north was open-shrubbery meadow, mixed with mostly hawthorn species (*Crataegus monogynea* and *C. europeus*) and juniper (*Juniperus communis*). The east side of the study site was closed by moderate climate oak forest (*Quercetum petraeae-cerris*). Important forest species included turkey oak (*Quercus cerris*), european hornbeam (*Carpinus betulus*), sessile oak (*Quercus petraea*), wild services tree (*Sorbus torminalis*) and hedge maple (*Acer campestre*). The south side of the study site consisted of similar types of vegetation as was found in the study site. To the west of the study site was cultivated land.

Trapping

Animals were trapped using a live trapping method (hand made, copper wire snare). Traps were set in the morning before the animals became active and were closed at sunset. Trap checking depended on the time of day and weather conditions, but usually was carried out every half hour.

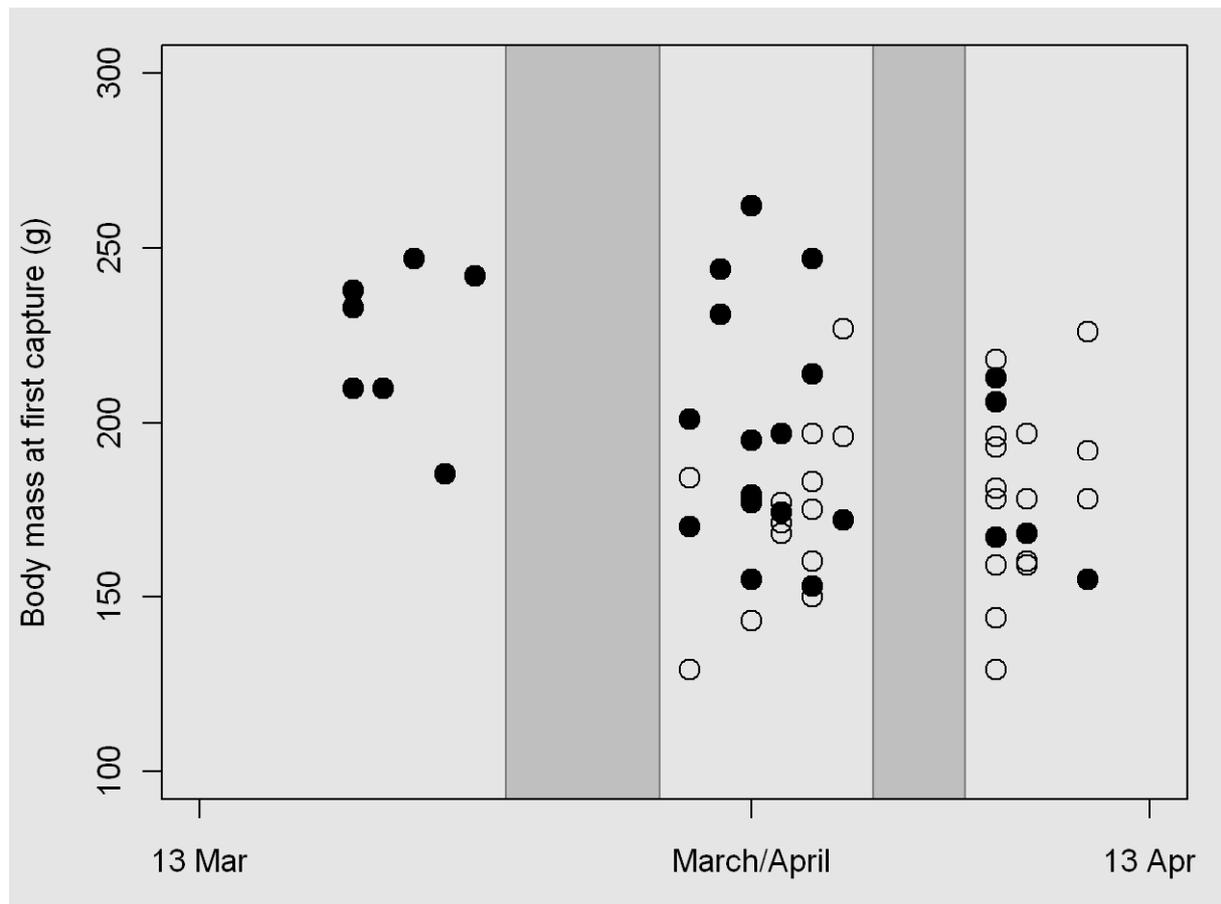
Captured animals were placed into a black linen sack immediately after they were removed from the trap. All captured animals were weighed to the nearest 1.0 g (Philips HR 2388,) and their skull length was measured with a caliper (INOX, MB-3425, 0.1 cm). Reproductive status of males and females (mature / immature) was estimated on the basis of the scrotal pigmentation and testis size and development of nipples and vulva (Millesi et al. 1998). We estimated the testis size by measuring the testis length and width with a caliper (INOX, MB-3425, 0.1 cm).

Behavioral observation and location

The animals were marked permanently during their first capture with a subcutaneous transponder (Trovan, ISO FDX-B type) and with a commercial hair dye for facilitating visual observations. Hair dyeing was repeated as needed to ensure the remote identification of the animals.

Animals were observed from two elevated (~ 2.5 m above ground) observation platforms. The observation period lasted from 19 March, 2004 when the first ground squirrel appeared on the surface to 2 May, 2004. Twenty minute long, focal animal, instantaneous sampling was used for behavioral recordings (Martin and Bateson 1993). The agonistic interactions were included into analyses as a sum of events during the observations.

For determining the position of the animals, the distance (Leica Rangemaster LRF 800, 1.0 m) and the divergence angle to the north (Bresser Nautic 7x50 GAL, 1.0 °) were recorded, from one of the observation platforms.



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To avoid temporal auto-correlation of location data, a 2 hour threshold was applied as a minimum interval time to be spent between two successive localizations.

We used the number of females, irrespective of their actual maturation state, within the males' home range as a surrogate marker to estimate the reproductive success of males. As benefit was estimated by a surrogate marker, similarly cost was also estimated in terms of energy balance using daily rate of body mass change (DBMC, g^{-day}) during the study period. The DBMC values were obtained by linear regressions fitted on individual body mass - time profiles.

Hormone assay

The correlation between measured amounts of testosterone in feces and blood concentration was determined previously, to ensure the relevance of testosterone content of collected fecal samples to the corresponding testosterone level in blood. Blood was collected from the femoral vein and fecal samples were collected directly from the animals. The blood samples were drawn in heparinized PE tubes. The plasma samples were stored at approximately -20 °C after being processed/spun in the centrifuge (Micro-haematocrit centrifuge; Hawksley and sons Ltd., 5 min). The fecal samples were stored in 96% ethanol in PE tubes at room temperature until analysis.

The validated method to determine testosterone content of samples was developed and conducted by the Laboratory of Reproductive Biology at the Faculty of Agricultural and Environmental Sciences of Szent István University, Gödöllő Hungary (Kelemen et al. 2003).

Data analysis

All analyses were carried out by using R 2.9.1, an open source software for statistical analysis and computing (R Development Core Team 2009). Home ranges during the mating period were determined by classical kernel method in the means of "adehabitat" package of R software (Calenge 2006). The estimated home ranges were based on 90% of the utilization distribution. To ensure the reliability of estimated home range sizes, only animals with more than 20 location points were included into further analyses of behaviors.

To discover relationships among variables, linear mixed effects modeling was used (LME), in which individuals were designated as a random factor (Pinheiro and Bates 2002). Normality of model residuals was tested with Shapiro-Wilk tests. Binomial response variables were analyzed by generalized linear models (GLM).

RESULTS

Timing of emergence and body mass

The date when the first male and female appeared on the surface was March 18 and March 29, 2004, respectively. There were two short periods when severe weather conditions (~ 10 days) with recurrent snow coverage and air temperature below zero, interrupted the continuous trapping (Figure 6.1). During these periods the visually detectable above ground activity of ground squirrels was almost non-existent.

The body mass of the males was significantly higher with 26.5 ± 7.72 g than females at emergence ($F_{1,55} = 11.77$, $p < 0.01$). The mean body mass of males and females was 201.7 ± 32.78 g and 175.2 ± 25.31 g, respectively. Emergence body mass depended significantly on the corresponding date of the males but not the females (gender: $F_{1,53} = 5.93$, $p < 0.05$; date: $F_{1,53} = 10.51$, $p < 0.01$; interaction: $F_{1,53} = 7.70$, $p < 0.01$; Figure 6.1). Males that emerged earlier were heavier than males that emerged later in the season. Males that emerged one day earlier than others were heavier with 2.1 g, however there was a considerable variability in emergence body weight between individuals regardless of the gender of the animals (25.3 g). We recorded the presence of a copulation plug in the genitals of two females (on 1st and 3rd of April) during the capture period.

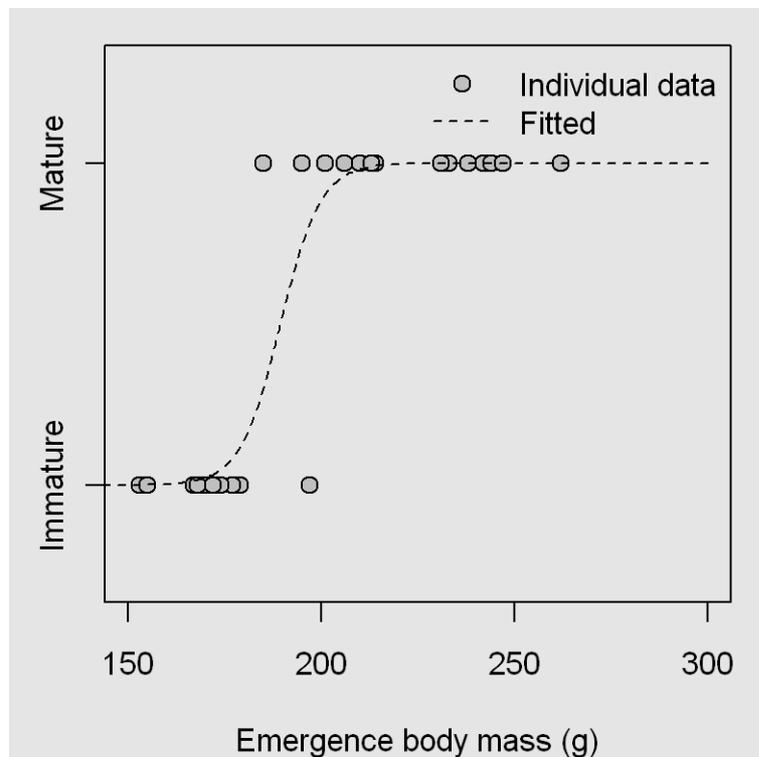


Figure 6.2 Maturity versus emergence body mass of male European ground squirrel. Model estimated logistic response curve (dashed line) beside individual data (grey circles) were plotted. Effect of emergence body mass on maturation of male ground squirrels was significant. Judgment of maturity was based on the presence of scrotal testes and pigmentation.

Body size, maturation and androgens

According to the previously completed validation process to determine the plausibility of testosterone in feces to blood level, the amount (ng/mg) in fecal samples was reliable as estimated by the concentration (ng/ml) in the blood sample ($F_{1,13} = 14.63$, $p < 0.01$). Thus it was concluded that obtained values from fecal samples are suitable to characterize, in terms of the androgens, the hormonal state of the animals. By using the following obtained equation, $C_{blood} \text{ (ng/ml)} = a + b \cdot C_{feces} \text{ (ng/mg)}$ where $a = 0.502$, ($t_{13} = 5.28$, $p < 0.001$) and $b = 0.0024$ ($t_{13} = 3.82$, $p < 0.01$) corresponding concentrations in blood were back calculated for males. The calculated mean testosterone concentration of the blood at emergence was 3.47 ± 2.07 ng/ml, and varies from 1.06 up to 7.40 ng/ml.

The relationship between emergence body mass and hormone levels was analyzed to obtain data about the relationship between emergence timing and reproductive maturation. According to our results males with higher emergence body mass had higher testosterone level at emergence ($F_{1,20} = 21.74$, $p < 0.001$).

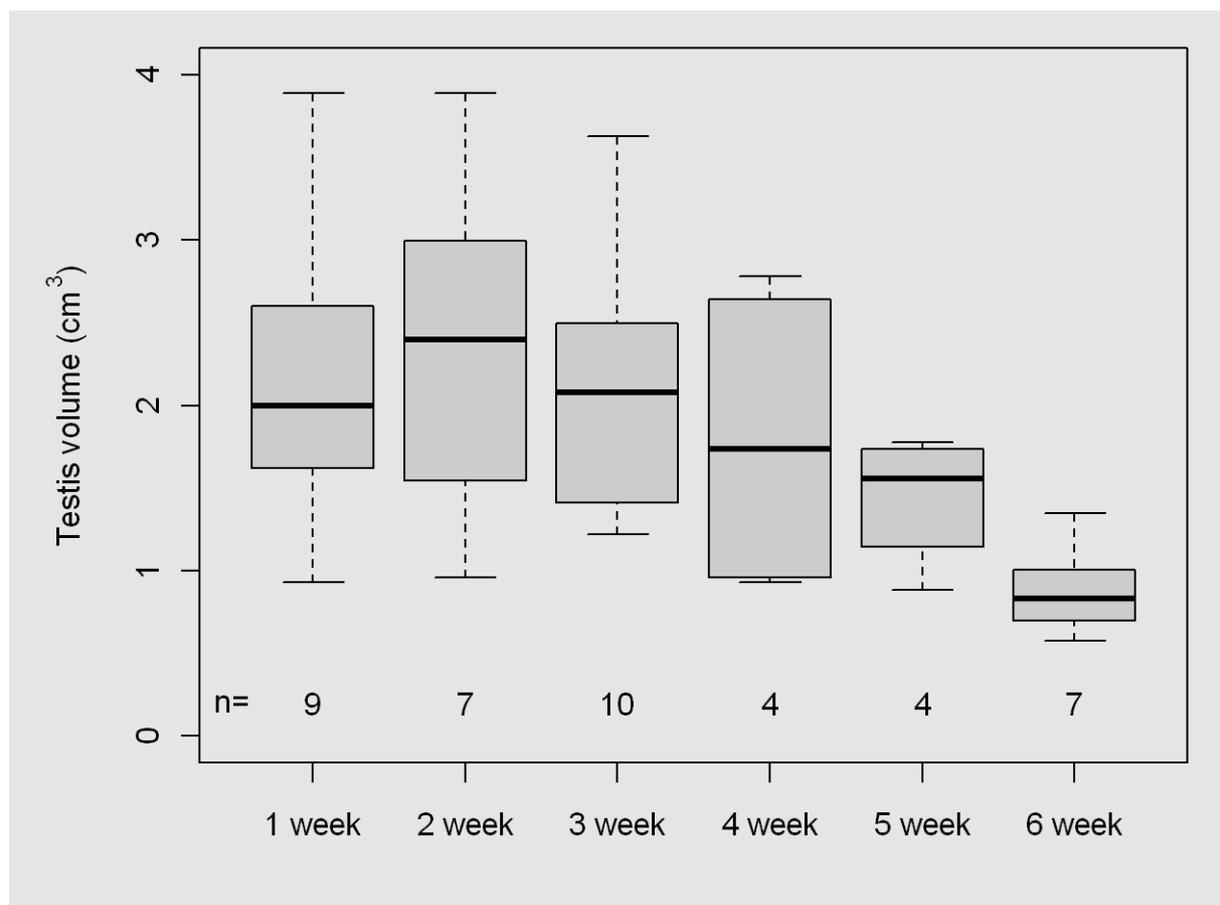


Figure 6.3 The changing of testis volume during the six week course of mating. Grouped individual testis volumes were plotted weekly. Box plots represent the range (minimum - maximum), the lower and upper quartile (25 % - 75 %) and the median of corresponding datasets.

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Maturity of males at emergence varied. 59.2 % (16/27) of the males were reproductively active at emergence. According to results of logistic regression analysis (GLM), immature males emerged later in the season ($z_{25} = 2.19$, $p < 0.05$). Maturity of the males was in relation with emergence body mass ($z_{25} = 2.27$, $p < 0.05$; Figure 6.2) rather than testosterone content of the blood ($z_{25} = 1.9$, $p = 0.06$). The testicular development varied throughout the mating period. The maximal testis sizes were attained during the second week after the first male appeared on the surface (Figure 6.3). The estimated amount of blood testosterone content was in significant relationship with the volume of testes ($F_{1,31} = 10.45$, $p < 0.01$).

Behavioral analysis of mating

The 20 males which fulfilled the pre-determined criteria regarding the minimum number of location points to determine home range size, were involved in the behavioral analysis.

The home ranges of animals substantially overlapped each other and were highly variable in size and morphology (Figure 6.4). The mean home range size was 1.2 ± 1.42 ha and varied between 0.2 – 5.5 ha.

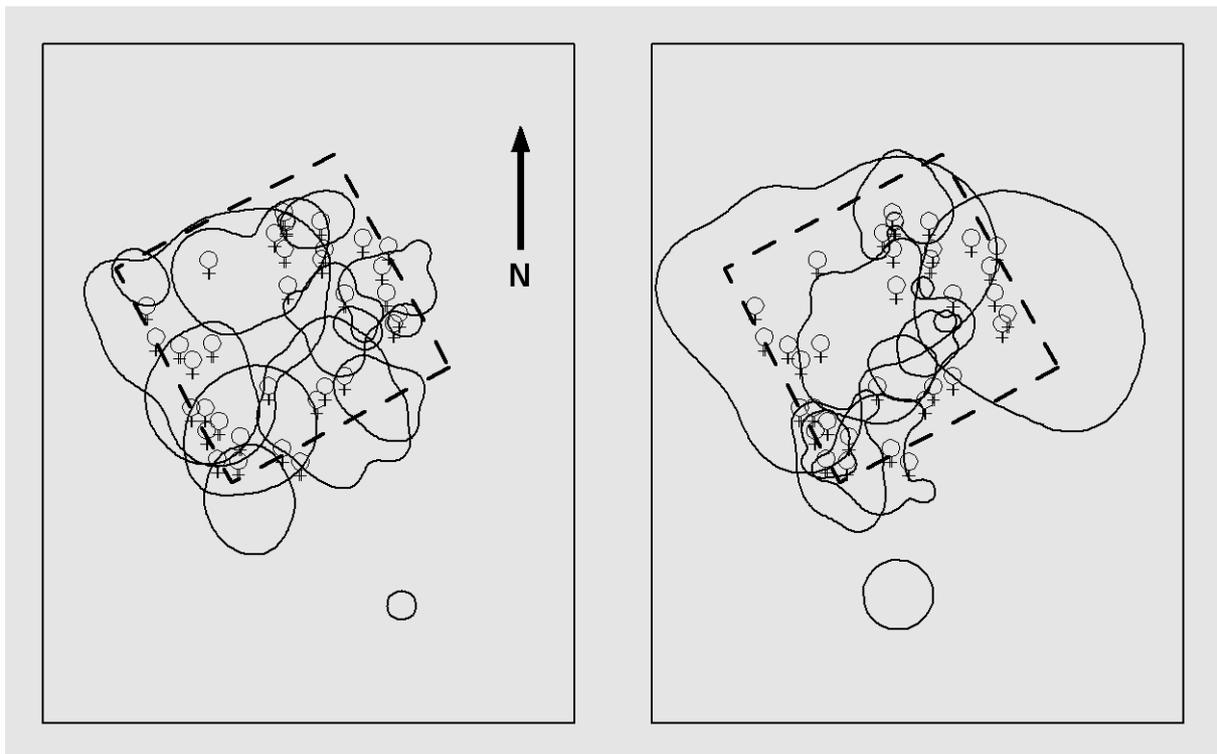


Figure 6.4 Spatial representation of area used by male European ground squirrels. Home ranges were estimated by adaptive kernel method. The 90 % utilization distribution areas (solid lines) were plotted relative to the four ha study site (dashed rectangle). Females (♀) were depicted at their first capture point. To facilitate lucidity, home ranges of ten and ten individuals were plotted on both sub-figures.

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There was a fatal consequence of combat on only one occasion, the victim was bitten on its neck and severe loss of blood was the probable cause of his death.

Surrogate markers for costs and benefits

The chosen variables to describe cost (DBMC) and benefits (number of females) of males mating behaviors were in significant relationship with each other ($F_{1,17} = 9.93$, $p < 0.01$). Males with higher number of females within their home range gained significantly less mass than those with lower number of accessible females (-0.6 ± 0.21 g-day/female; $t_{17} = 3.15$, $p < 0.01$; Figure 6.7a).

To reveal the most costly behavior(s), backward model reduction process was used to choose the best predictor set of DBMC. As a result, the best predictor set contained only the cumulative number of agonistic behaviors that had significantly explained the body mass change during the studied period ($F_{1,17} = 22.78$, $p < 0.001$). The more frequent was the agonistic behavior, the less was the gained body mass during the mating period ($t_{17} = 4.77$, $p < 0.001$; Figure 6.7b).

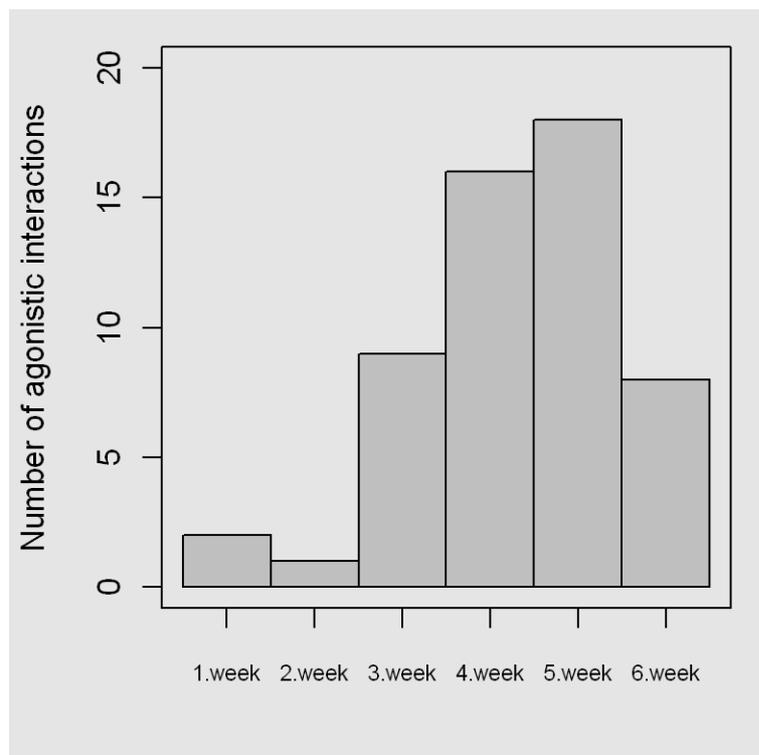


Figure 6.6 The frequencies of agonistic interactions during the six week course of mating. Weekly grouped sums of observed combats regardless of attendees were plotted.

DISCUSSION

We found evidence in European ground squirrels that both the timing of emergence and post-hibernation body mass are in relationship with male's mating behavior. These behaviors, especially the combats, which acquire copulation with receptive females, were costly for males

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in terms of energy expenditure (body mass loss) and resulting injuries, but effectively enhanced opportunity for successive mating events during the mating period. Below we address several assumptions upon which our results rest.

Many studied populations of the ground squirrel family, besides the present study (~ males : females; 1 : 0.75), show considerable male-biased sexual size dimorphism (Bartels and Thompson 1993; Michener 1998; Michener and Koepl 1985; Millesi et al. 1999b). We interpret body mass as body size because in rodents, body mass was found to be in close relationship with the principal component of body size measured on a multivariate scale (Iskjaer et al. 1989). In mammals male-biased sexual size dimorphism is usually attributed to a polygynous mating system, in which large males achieve greater mating success than small males because of success in contest competition (Andersson 1994; Weckerly 1998). The mating system of *S. citellus* was defined as a scramble competition polygyny in that males try to locate and combat for copulation rights with females (Millesi et al. 1998). However, as there were no data in literature about mating with multiple males, or the mate guarding behavior of males, and the incidence of copulation plugs in our study raised the suspicion of the presence of sperm competition, where the sperm from males competes within the female's reproductive tract to fertilize egg(s) (Birkhead and Parker 1997).

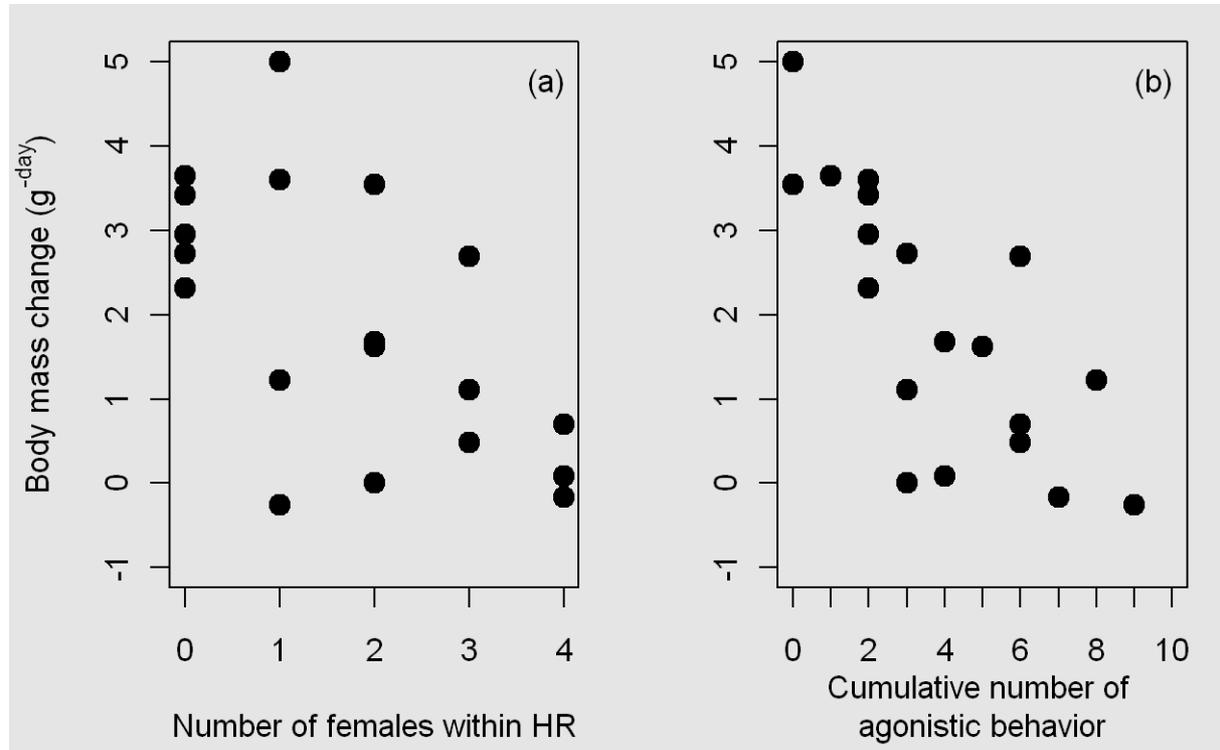


Figure 6.7 Costs and benefits of male mating behaviors. Daily body mass change versus number of acquirable females (subfigure a) and versus numbers of agonistic behavior (subfigure a). The more females within the home range, the more weight was lost during the mating period (a). The main predictor of daily weight change was the frequency of fights between males.

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Sperm competition is a widespread form of the intra-sexual competition among rodents, where the size of copulatory plug is found to vary with sperm competition level (Ramm et al. 2005). In comparison with rodent species the expectable ratio of multiple paternity litters among European ground squirrels would be over 70 % based on relative testes size to body size (Kenagy and Trombulak 1986; Ramm et al. 2005). However, this estimation provides good ground for suspecting sperm competition but probably overestimates the occurrence of multiple paternity in European ground squirrels. Characteristic space usage of male European ground squirrels (substantially overlapped home ranges) resembles to those ground squirrel species where sperm competition and multiple paternity has already been proven, where territory ownership would be associated with first access to estrous females. However the majority of females observed throughout estrus also mated with at least one other male, indicating that territory ownership was not associated with exclusive access to females (Boellstorff et al. 1994; Hanken and Sherman 1981; Lacey and Wiczorek 2001; Schwagmeyer 1988; Schwagmeyer and Foltz 1990; Schwagmeyer and Parker 1987; Schwagmeyer and Parker 1990). In contrast, territory ownership is associated with first access to estrous females (Lacey et al. 1997; Schwagmeyer and Foltz 1990; Schwagmeyer and Parker 1987). In this regard, male mating behavior parallels that of socially monogamous birds in which territorial defense by male's functions to deter extra – pair copulations by females (Dickson 1997; Lacey and Wiczorek 2001; Lacey et al. 1997). Although space usage represents an important component of male reproductive success in ground squirrels, other aspects of male behavior (e.g., the ability to dominate agonistic interactions during the time of a female's estrus) are also critical.

Variation in testis size is often interpreted in the light of sperm competition. Nevertheless, high variability in testes size and its relation to body mass, androgens and mating success implies complex, multiple level relationships as has been found in other rodent species (Schulte-Hostedde et al. 2003; Schulte-Hostedde et al. 2005). Males with higher post hibernation fat reservoirs have much more utilizable raw materials to produce androgens and to spend extra energy keeping their gonads in the appropriate state to produce both hormones and reproductive cells (Schulte-Hostedde et al. 2005). The testes size of seasonally reproductive hibernators correlates with the androgen content of the blood during the mating season (Barnes et al. 1988). As larger testes can produce more androgens (Adam and Findlay 1997; Kaplan and Mead 1994), this in turn enhances the growth of muscle mass (Schulte-Hostedde et al. 2003), larger body mass might be advantageous in mate searching activities or in male-male combats. Combats between males frequently occur in European ground squirrels and the males

engage in injury-producing fights which occasionally causes death (present study, Millesi et al. 2002; Millesi et al. 1998).

As ground squirrels inhabited the entire Holarctic region, they had to adapt to various environment from the deserts to the arctic. In the course of their adaptation to various environments, similar variability could be also appearing in their behavior - hormone relationship. The role of androgens in modifying mating behavior changes from species to species. Many males of different species change their behavior in response to experimentally changed testosterone level (Place 2000; Saino and Moller 1994; Wingfield and Hahn 1994), and others not (Demas et al. 1999). Inter-species variability in hormone related behavior is often mentioned from the side of the interaction between the physiology and the ecological constraints of the species (Wingfield and Hunt 2002). The fact that we did not find any remarkable relationship between the level of androgens and behavior is not surprising in the light of former findings that were contrary with results on closely related species (Boonstra et al. 2001). Similarly to our results, Millesi et al, had not found any significant increases in testosterone levels that could account for the behavioral changes during mating season (Millesi et al. 1998), but their latter study revealed that rather the GnRH release behaviors in male European ground squirrels is dependent with emergence of females, such as elevated intra-sexual aggression, increasing home range-size and frequent scent marking behavior (Millesi et al. 2002).

Time and energy expenditure limitation on mating enforces the effect of sexual selection in hibernating mammals as well as in migratory birds. Similarities between the life history of hibernating mammals and migratory birds also appeared in their common responses to climate change (Inouye et al. 2000). There is a crucial period in both cases that requires a preceding period when animals get ready to fulfill the requirements of a successive migration (Bortolotti et al. 2002; McNab 2009) or hibernation (Davis 1976; McNab 2008). All of these time requirements lead to extreme limitation in length of reproductive seasons and enhanced intensity of competition for mating opportunity. As in migratory birds, the individual variation in arrival time can be explained by phenotype-dependent cost and benefit functions of early arrival (Möller 2001), condition dependent variation in emergence can also be explained by the cost – benefit differences for males with different post-hibernating conditions. The costs should be particularly high under severe environmental conditions that frequently occur when birds arrive to the breeding ground or mammals emerge to the surface and lead to a strong negative relationship between arrival / emergence date and phenotypic quality (Möller 1994). The cost of early emergence can be well explained by investigating the effect of sudden severe weather

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conditions on a ground squirrel population. A snowstorm, which began 8 days after the start of the emergence period (day of first male on the ground) and lasted for 27 days, had serious consequences both on survival and reproduction of Belding's ground squirrels (*Spermophilus beldingi*: Morton and Sherman 1978). The number of missing individuals increased two times and the number of weaned litters was at least halved in comparison to other years, due to the effect of severe weather conditions. Although the above ground activity had almost ceased, the animals were still active below ground, therefore the depletion of fat stores and starvation occurred. Similar drop in surface activity was observable in our study during the period when there was recurrent snow coverage and extraordinarily cold weather as well.

If the cost can be so high during the “poor” year, then the benefit would exceed the cost during the ordinary years. The difference in time of emergence by male ground squirrels might be interpreted as an outcome of inter-sexual selection and as an example of the phenological strategy of the mate – limited sex, maximizing the reproductive success by being on the breeding ground by the time the mate limited sex appears (Michener 1983).

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Submitted

ABSTRACT

A hibernating lifestyle makes animals sensitive to the changing of environmental temperature. Therefore the effect of currently ongoing climate change can be considerable on these species. To assess this effect, we estimated the body mass change during hibernation under three different climate scenarios (PRUDENCE project), using computational modeling. The final model was tested by field data obtained during a cold and an extraordinarily warm winter.

Results of modeling predict a 4-6 % decrease in spring emergence body mass, independently of climate scenario. According to field data, there was no difference between a warmer and colder winter in emergence body mass. The modeled emergence body mass was fairly similar to data measured in the field. Nevertheless, modeling predicted a small but significant difference between the warmer and colder winter.

Short time disturbances in body mass cycle of hibernating animals can be compensated by the animals themselves or by the advantageous side effects of climate change (extended active period, enhanced primer production), but these perturbations, along with other disturbances by human activities, might have serious consequences on population dynamics on a longer time scale.

INTRODUCTION

In recent years numerous studies support the ecological impact of current climate change (Parmesan and Yohe 2003; Walther et al. 2002). Changes induced by global warming are apparent at all levels of ecological organization, they appear in life history and phenology, population and community structure and in geographical distribution of species (McCarty 2001). While some studies document and report the ongoing changes, others try to describe the potential outcome of the predicted climate change in the future (Barbraud and Weimerskirch 2001; Inouye et al. 2000; Kausrud et al. 2008). Several of the latter studies use the relationship between ecology and physiology for their forecast (Barbraud and Weimerskirch 2001; Humphries et al. 2002; Landry-Cuerrier et al. 2008). These eco-physiological modeling studies make use of the fact that certain life history or physiological properties sensitize organisms to effects of climate and therefore these species have increased responsiveness to climate change (Hodkinson 1999).

As there is a close relationship between energy expenditure and environmental temperature, this makes animal species that hibernate the most sensitive and vulnerable to climate change (Buck and Barnes 2000; Frank 2002; Geiser 2004). Decreased energy expenditure during hibernation affects the survivorship, maturation and fecundity, via the exhaustible energy stores (Barnes 1984; Millesi et al. 1999a). Hibernating animals partially give up their control over their body temperature (Kortner and Geiser 2000). On one hand it is favorable, e.g. to save energy, on the other hand, partial control offers only partial response to changing environment. Fat storing hibernators are aphagic or severely hypophagic during the hibernation season (Davis 1976) and unresponsive to energy challenges that at other times of the year promote an increase in body mass (Dark et al. 1984; Dark et al. 1989).

Hibernation consists of two fundamentally different states. The heterothermic state, commonly called torpor, is the energy conservation state which generally lasts for 3-14 days. In torpor state the body temperature of animals approaches the ambient temperature as long as this lowering of body temperature does not threaten the integrity of the organism (Barnes 1989; Carey et al. 2003). Via this mechanism a considerable amount of energy can be saved and the consumed energy does not exceed ten percent of energy consumed in the normothermic state (Geiser 1988; Wang 1979a). Heterothermic states are regularly interrupted by short (~ a day long) euthermic states (arousal) when animals recover their normal body temperature and normothermic metabolic rates. Both states are in close and clear relationship with ambient temperature, both in terms of energetics and phenology (Buck and Barnes 2000; Németh et al.

2009). This feature of hibernation renders hibernating animals a convenient subject for eco-physiological modeling studies (Landry-Cuerrier et al. 2008).

Here we propose an eco-physiological model which estimates population parameters of the physiological background of hibernation, as well as individual deviations. Our study aims at (1) fitting and parameterizing the model on previously measured laboratory data on European ground squirrels (Németh et al. 2009); (2) making predictions about future consequences of global warming on hibernating animals based on published local climate change scenarios (Bartholy et al. 2008); (3) testing the model by comparing the predictions of the model with field data collected during two consecutive winters (one normal and one extraordinarily warm). Methodologically we also aim to show that non-linear mixed effects modeling (Pinheiro and Bates 2002), which is a widely used method in pharmacokinetics to estimate population and individual parameters of a certain physiological process (Mortensen et al. 2007), is applicable for solving eco-physiological problems.

MATERIALS AND METHODS

Time series data in lab and field

Fifteen European ground squirrels were live trapped in late August 2002 from a free ranging population living on a grass field airport in Hungary (N 47°36'43", E 19°08'40"), and were transferred to a climate controlled room. The animals were housed individually in plastic containers measuring 36×21×15 cm. Food (rabbit chow, Galgavit Kft., Gödöllő, Hungary) and water were provided ad libitum. The animals were kept at $20 \pm 1^\circ\text{C}$ and an LD cycle of 10:14 (short photoperiod conditions) until mid September 2002 when ambient temperature (T_a) was decreased to $15 \pm 1^\circ\text{C}$ and the LD cycle turned to DD. At this time food and water were removed to facilitate the beginning of hibernation. We kept three separate groups ($n = 5$ each) at different constant temperatures during the treatment (0 ± 1 , 5 ± 1 , and $9 \pm 1^\circ\text{C}$). The treatment lasted from January 1st to April 10th 2003, when we gradually increased the T_a and recovered the LD cycle. The body temperature of the animals was traced with a computerized data acquisition system (Németh et al. 2009). Body mass was recorded in 6-14 day intervals, when all animals were in the state of torpor.

A field study was conducted from 2006 to 2007 at the grass field airport in Pécs-Pogány, Hungary (N 45° 59' 50.09", E 18° 14' 7.50"). The study site covers 6 ha of grassy meadow. During the active season of both years, live trapping was conducted using cage traps. Each animal was weighed, sexed and permanently marked with an individual subcutaneous

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transponder. In 2005-07 from August (week 35) to April (week 18) we gathered weekly soil temperature data from the onsite station of the National Meteorological Service at a depth of 1 m, which is similar to reported depths of the hibernacula of ground squirrels (Hut and Scharff 1998).

Modeling body mass change during hibernation

Body mass change during hibernation was estimated through modeling of hibernation energetics. The conversion constant between energy consumption and body mass change was assumed to be 37 kJ/g (Robbins and Cunha 1993). Energy expenditure (EE_T) of endotherms in hibernation (i.e. in torpor state) varies with ambient temperature and body mass (BM). This can be expressed as:

$$EE_T = MR_{INT} - MR_T + T_a \cdot \alpha_2$$

Where MR_{INT} represents a hypothetic basal metabolic rate which can be expressed as a function of body mass ($MR_{INT} = BM \cdot \alpha_1$) and MR_T is a constant representing the amount of saved energy in torpor state. The third part of the expression involves the effect of the ambient temperature in a presumed positive relationship (Geiser 2004). α_j s are the estimable population parameters.

All hibernators arouse from torpor at regular intervals during hibernation and remain euthermic for a short period of time before reentering torpor. The energy expenditure during these arousals (EE_A) is a similar simple function as in the case of torpor:

$$EE_A = MR_{INT} + MR_A - T_a \cdot \alpha_3$$

Where MRA is a constant representing the amount of invested extra energy in arousal state relative to the hypothetic basal metabolic rate. The effect of the ambient temperature is included in a presumed negative relationship (Geiser 2004).

Population stochastic modeling (PSM) was used to describe individual time series of body mass data (Mortensen and Klim 2008). PSM estimates non-linear mixed-effects models using stochastic differential equations and also handles multidimensional input (see Appendix: Mortensen et al. 2007). Fit of the model was assessed by posterior predictive check and graphical illustration of individual estimations and the range containing 90 % of 1000 iterations (as described in Gelman et al. 1996).

Parameter estimation of simulated torpor and arousal cycles

Torpor-arousal cycles were generated with parameters estimated by non-linear mixed effect modeling of individual binomial time series data. Data for estimation originated from lab experimental data and were recoded as 0/1 denoting torpor day/arousal day.

Two factors were used to describe the torpor-arousal cycle: time and T_a . Arousals appear more frequently at the beginning and at the end of the hibernation period and are governed by internal timing mechanisms (Körtner and Geiser 2000a). For the description of this innate rhythm, a cosine function was used to describe the change in the probability of arousal appearance during the hibernation period. The effect of temperature (Németh et al. 2009) was included linearly in the model (see Appendix).

Simulating body mass change under different climate scenarios

Fitted model parameters together with different initial body mass values were used to simulate body mass change over a hypothetical hibernation period by PSM. Daily temperature data (at +2 m) for projected (2070-2100) and control (1961-1990) periods were gained from PRUDENCE project public data (<http://prudence.dmi.dk/>). We have chosen three scenarios on the basis of the published information about the region of interest, the Carpathian basin (Bartholy et al. 2008) and run our model with these scenarios, ranging from conservative to extreme predictions. The chosen scenarios were developed by the Danish Meteorological Institute (DMI HS1 and DMI HC1), the Swiss Federal Institute of Technology (ETH HC A2 and ETH HC CTL) and The Royal Netherlands Meteorological Institute (KNMI HA2 and KNMI HC1), respectively. Air temperature data were used to compute average daily soil temperature data using a formerly published method (Strijkstra et al. 1999). Soil temperature data were approximated by sinusoid functions and parameters of these functions were used to test differences between periods and among scenarios (see Appendix: Pinheiro and Bates 2002; Pinheiro et al. 2008).

Simulated hibernation periods lasted from the beginning of October till the end of February, which means 150 days (every month consisting of 30 days). For simulating body mass changes under different climate scenarios and periods (control or projected) the immergence body mass ($BM_{t=0}$) was set to 400 g according to literature (Millesi et al. 1999b). The emergence body mass ($BM_{em} = BM_{t=150}$) was chosen as the target variable and 1000 iterations were done for each scenario and period. GLM was used to compare control and projected periods among different scenarios, also including interaction in the model. Overall

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effect of temperature (average temperature of a given year during hibernation) was tested separately, without any other factor with linear regression.

All modeling and statistical analyses were performed with R, an open source software for statistical computing and modeling (R Development Core Team 2009). Descriptive statistics are presented as mean \pm SD, unless otherwise stated.

Comparing model simulation results with field data

The model described above was also run with measured weekly soil temperature data at Pécs-Pogány during the winters of 2005-06 and 2006-07. Daily data was derived by the multiplication of weekly average data. As we did not possess body mass data at immergence time, $BM_{t=0}$ was set to 400 g (Millesi et al. 1999b). The simulated hibernation period lasted from the beginning of October (week 42) until the middle of March (week 11), i.e. it was 23 weeks (161 days) long. Results of 5000 iterations were analyzed. BM_{em} data was used as a target variable. Between-year comparisons were performed with mixed effect linear models, separately on field and simulated data. Formal comparison of simulated and measured BM_{em} was not carried out, because the assumption stating $BM_{t=0} = 400g$ could not be checked.

RESULTS

Modeling body mass change during hibernation

Estimation of body mass time profiles for ground squirrels kept at three different ambient temperatures (0, 5 and 9 °C) was carried out by population stochastic modeling (detailed in Appendix). The individual smoothed estimates of body mass together with the 95% confidence intervals are depicted in Figure 7.1. The width of the confidence bands does not vary or depend on the data. Thus the uncertainty of body mass does not increase with the distance to the sample point and is therefore not dependent on the sampling rate. This is a desirable property. The assumption of additive Gaussian noise was tested by the distribution of residuals which did not deviate from the Gaussian normal distribution ($W = 0.9924$, $p = 0.7586$) and showed no tendency with the observed values. We assessed the model fit with posterior predictive check (PPC). According to the PPC analysis after 1000 iterations there was no difference between individual simulations and observations ($p = 0.428$).

Parameter estimation for torpor-arousal cycles was carried out using binomial distribution (see Appendix). The probability of the appearance of arousals (0,1) was modeled as a sinusoid function of time elapsed in hibernation.

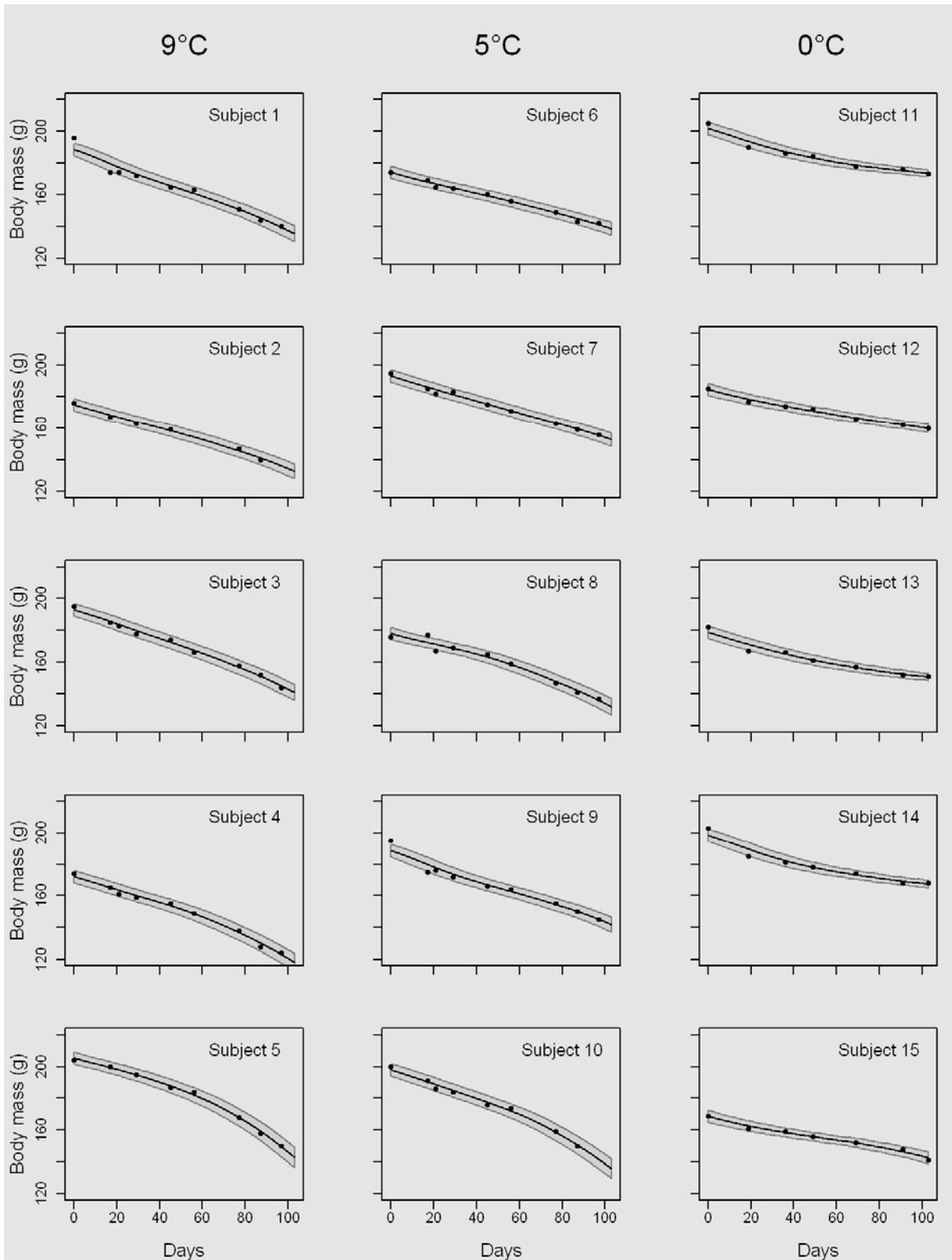


Figure 7.1 Visual predictive check (VPC) of developed stochastic population model for individuals kept under 9, 5 and 0°C ambient temperature conditions during hibernation. Measured body mass data (closed circles) plotted with individual body mass – time profile estimations (black line) with its 95% confidence intervals (grey area). Estimation was based on observed torpor-arousal cycles. Posterior predictive check (PPC) showed no difference between predicted and observed individual data after 1000 simulations (PPC: $p=0.428$).

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The probability of the appearance of arousal on a given day was 0.24 ± 0.14 on average. According to the model at the beginning and at the end of the hibernation the probability of arousal appearance varies between 0.5 and 0.33, but in the middle of the hibernation it falls as low as 0.05. Each degree of elevation in ambient temperature increases the probability of arousal appearance by $\sim 1\%$.

Simulating body mass change under different climate scenarios

Soil temperature data for control (1961 - 1990) and projected (2071 - 2100) periods for three different scenarios were derived from air temperature data gained from PRUDENCE project. Year round oscillation of soil temperature was approximated with a sinusoid non-linear function, to assess the effect of period and scenarios on soil temperature (Figure 7.2). The fixed factors have effect on T_{mean} and T_{amp} parameters but their magnitude varied (Table 7.1). The overall effect of period on T_{amp} is comparable with the effect of scenarios (period: $F_{1,64782} = 488.3$, $p < 0.0001$; scenario: $F_{2,64782} = 5382.9$, $p < 0.0001$). Approximately, both factors have a 10% effect on the parameter compared to its base line value ($9.8\text{ }^{\circ}\text{C}$). Hence the year round temperature swing varies from $16.0\text{ }^{\circ}\text{C}$ (KNMI 1961-1990) to $20.2\text{ }^{\circ}\text{C}$ (DMI 2071-100). The effect of period and scenario was more obvious on year-round mean temperature (T_{mean}), according to non-linear modeling (period: $F_{1,64782} = 104122$, $p < 0.0001$; scenario: $F_{2,64782} = 54349$, $p < 0.0001$). Soil temperature was approximately $4.0\text{ }^{\circ}\text{C}$ higher in the 2071-2100 period than during the period of 1961-1990 (period: $4.03 \pm 0.02\text{ }^{\circ}\text{C}$ $t_{64783} = 205.0$, $p < 0.0000$). This increase in temperature was fairly similar among scenarios. Predicted rise in soil temperature was 4, 3.6 and $3.4\text{ }^{\circ}\text{C}$ for the DMI, KNMI and ETH scenarios respectively. These results implied moderate differences among scenarios.

Table 7.1

Predicted temperature ($^{\circ}\text{C}$) ranges of three tested scenarios at the control and at the projected period according to the sinusoid non-linear modeling of soil (-1m) temperature data.

	Scenario		
Period	DMI	ETH	KNMI
Control	11.4 (1.6 - 21.2)	10.7 (1.4 - 20)	7.2 (-0.8 - 15.2)
Projected	15.4 (5.3 - 25.5)	14.1 (4.1 - 24.1)	10.8 (2.7 - 18.9)

Average temperature (T_{mean}) and range between brackets (T_{amp})

Our model predicted that BM_{em} of populations was significantly affected by projected climate change ($F_{1,173994} = 575.4$, $p < 0.0001$, Figure 7.3). This change did not differ between applied scenarios ($F_{2,173994} = 0.26$, $p = 0.77$), but there were significant differences among scenarios in expected BM_{em} ($F_{2,173994} = 347.6$, $p < 0.0001$). The prediction of GLM for population mean of simulated emergence body mass for period 1961-90 was $182 \pm 1g$, $186 \pm 1g$ and $197 \pm 1g$ for the DMI, EHT and KNMI scenarios respectively. This expected population BM_{em} decreased by $12 \pm 1 g$ at period 2071-100 in comparison to period 1961-90 with minor differences among scenarios.

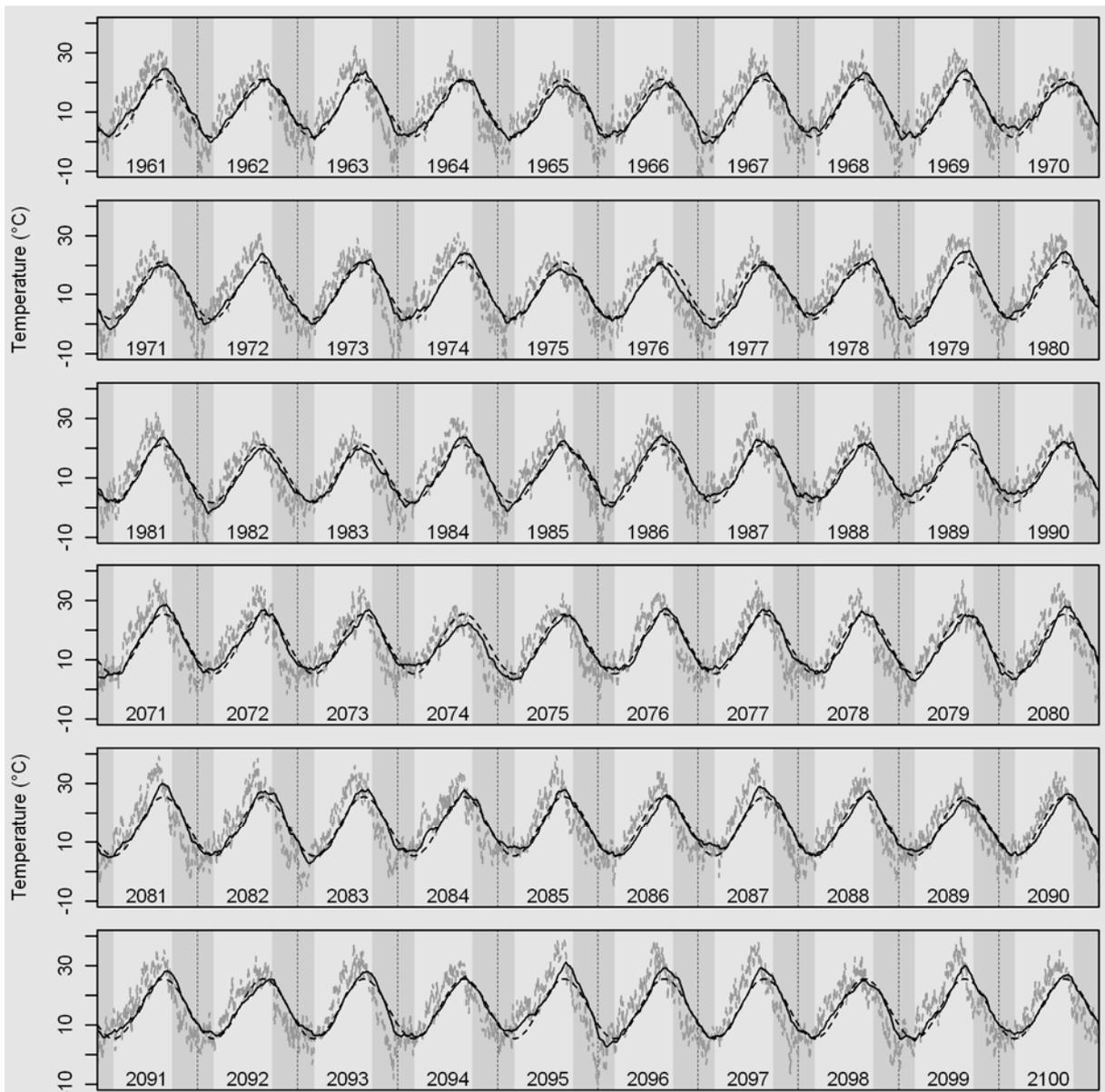


Figure 7.2 Example (scenario: DMI) for air (grey dashed line) and soil (black line) temperature - time profiles during the control (1961-1990) and projected (2071-2100) periods. Non linear model was fitted to assess the effect of period (control vs. projected) and scenario (DMI, ETH, KNMI) on year round change of soil temperature (black dashed line). Grey stripes mark periods that were used in PSM simulations as temperature inputs.

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When the effect of temperature was analyzed by itself, the model explained variance was significant ($F_{2,173998} = 1303.1$, $p < 0.0001$). Every degree of increase in soil temperature caused an approximately 3 g decrease in body mass at emergence ($-3.1 \pm 0.09\text{g}$, $t_{173998} = 36.1$, $p < 0.0001$).

Comparison of model simulation results with field data

Soil temperature at depth -1m was significantly different between the two investigated consecutive winters (2005-06 vs. 2006-07: $t_{22} = 8.85$, $p < 0.0001$, Figure 7.4a). Soil temperature was $2.4 \pm 0.5^\circ\text{C}$ higher in the winter of 2006-07 than in 2005-06 according to estimated mean difference at correspondent time points. Difference in body weight at emergence was not significant between the normal ($203 \pm 5.5\text{ g}$) and warm ($220 \pm 8.4\text{ g}$) winter ($F_{1,84} = 0.9$, $p = 0.34$, Figure 7.4b). This fact was similar for both genders ($F_{1,84} = 0.01$, $p = 0.84$), nevertheless, males emerged $34 \pm 15.4\text{ g}$ heavier than females, regardless of winter soil temperature ($F_{1,84} = 16.4$, $p < 0.001$).

Simulated body mass data at emergence time was significantly affected by winter soil temperature ($F_{1,4999} = 6.9$, $p < 0.01$, Figure 7.4b). Body mass at emergence was $5 \pm 2.1\text{ g}$ higher in the normal winter than in the warm winter (2005-06: 163 ± 1.5 , 2006-07: 157 ± 2.1). Estimated standard deviation of inter-individual variance by mixed effects model process was 30.1 g and 7.2 g for field data and for simulation results, respectively.

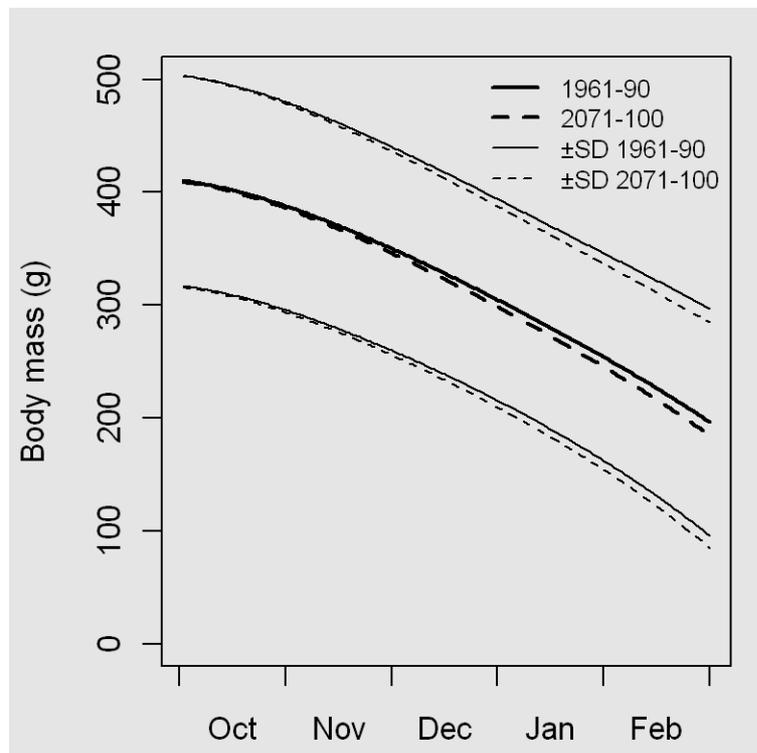


Figure 7.3 Predicted effect of climate change on body mass change during hibernation. Difference in body mass at emergence ($\sim 12\text{g}$) was found to be significant between the projected (2071-100) and control (1961-90) period ($p < 0.0001$). Differences among scenarios are negligible ($p = 0.77$). Results of 1000 iterations were pooled and the 29 year long periods were depicted as one hibernation period under KNMI's scenario.

DISCUSSION

Eco-physiological models are based on structural models including ecologically relevant parameters (Humphries et al. 2002; Landry-Cuerrier et al. 2008). However, a major criticism of eco-physiological modeling is that modeling results are isolated and probably only perform well in silico (Davis et al. 1998). Reliability of modeling results for the real world are in question, especially if they do not consider inter- and intra-individual differences. Our proposed method to answer this criticism was to imply non-linear mixed effects modeling in eco-physiology similarly to pharmacology where it has been successfully applied (Pillai et al. 2005). We used non-linear mixed effects (NLME) and population stochastic modeling technique (PSM) to determine population parameters of partial differential equations relating to the energetics of hibernation and their inter- and intra individual variations (Mortensen et al. 2007; Pinheiro and Bates 2002). Both population parameters and the magnitude of inter-and intra-individual variations were obtained using these methods (Appendix, Table 1 and 2). Estimated inter-individual variability is 10%, which confirms and justifies the inclusion of inter-individual variability, improving the capability of the model to predict hibernation energetics under changing environmental temperature.

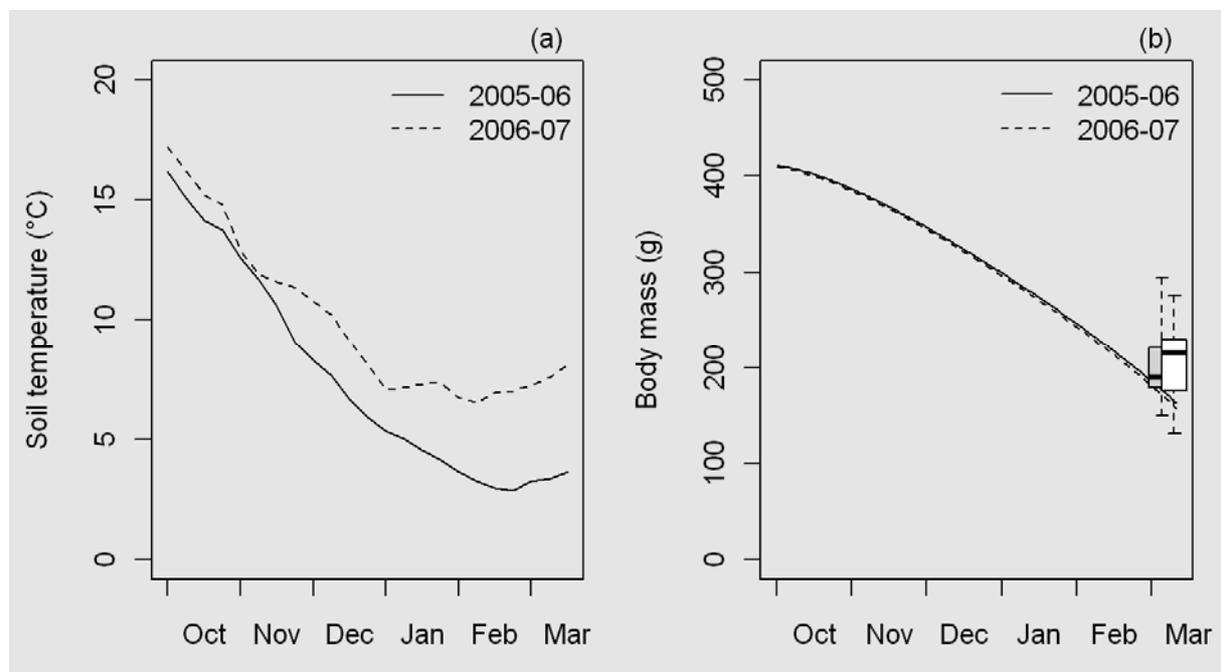


Figure 7.4 Graphical comparison of simulation and field study results of two consecutive years. Weekly soil temperature profiles during hibernation period from winter of 2005-06 was significantly lower than from winter of 2006-07 ($p < 0.0001$, subfigure a). Mean body mass-time profiles of 5000 simulation result (lines) with emergence body mass on field in winter of 2005-06 (grey box) and 2006-07 (empty box) are plotted (b; median, quartiles, confidence limits and outliers are represented by box plots).

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Model evaluation was carried out with visual predictive check as well as with the formal method, the posterior predictive check (Yano et al. 2001). Both methods confirm the reliability of the proposed partial differential equations and corresponding parameters. Accordingly, the chosen structural and random effect models are able to describe body mass change under various temperature conditions over hibernation (Figure 7.1). However, we have to point out that hibernation is convenient for modeling, because the entire process takes place in an isolated environment (~1m under ground) where the appearance of additive random effects is rare. Additionally, the simplicity of hibernation energetics facilitates the development of an eco-physiological model with relatively few parameters. Describing a more complex system, for example migration which is obviously exposed to many more factors, needs more care in the selection of regarded - disregarded factors (Ákos et al. 2008).

Model prediction on future climate and its species specific aspects

As some authors have pointed out, only a minority of organisms will not be affected by climate change (Parmesan and Yohe 2003). Nearly all aspects of ecological organization, geographical distribution, community structures, phenology and life history have been affected (McCarty 2001). The climatic effects on plants affect related animal species. Change of distribution and phenology of the plant coverage causes shifts in geographical distribution or seasonal activity of species in a community (Kausrud et al. 2008; Scholze et al. 2006). However, climate change also has a direct effect on hibernating animals. For example, a change in the phenology of the active season (*Marmota flaviventris*), and a shift in the species distribution area (*Myotis lucifugus*) have been reported due to the effect of climate change on hibernators (Humphries et al. 2002; Inouye et al. 2000). Presumably, the majority of these effects on hibernators are expressed through the sensitive energetics of hibernation. Animals in hibernation can save a considerable amount of energy because in the torpor state, no thermoregulation is required over a wide range of ambient temperatures and a fall in body temperature can lower the energy expenditure (Geiser 2004). Hibernation comprises two alternating states. The torpor state when animals are hypothermic lasting for approximately 2-20 days, and the arousal state when animals interrupt their low temperature torpid states by short normothermic state (~24 h, (Körtner and Geiser 2000).

The fall in energy demand allows some hibernating mammals to survive the hibernation period exclusively on body fat stored before they entered hibernation (Humphries et al. 2003). Additionally, relationship between environmental temperature and the energy requirement of hibernation is close and proven by many studies (Buck and Barnes 2000; Wang 1979a). Our

results also indicate that the effect of temperature is significant, but less pronounced on body mass change than would be expected. According to a study by Buck and Barnes, the predicted ~ 4 °C increase in year average soil temperature would cause a 12-20 % decrease in emergence body mass (Buck and Barnes 2000). However our results show an approximate 6-7 % decrease in body mass at emergence from the same change in soil temperature. This departure between results probably comes from the effect of temperature on the timing of arousals. This is the effect that primarily determines the overall energetics of hibernation, not the effect of temperature on energy consumption during torpor state. Energy consumption is about ten times higher in arousal than in torpor during a given time unit (Wang 1979a). One day in arousal consumes the approximate energy saved in ten days of torpor. In this sense, environmental temperature influences the overall energy consumption during hibernation via the timing of arousals, rather than via its relationship to the rate of torpor metabolism (Buck and Barnes 2000; Németh et al. 2009).

Nevertheless, a 6-7 % effect, which may seem negligible on a certain timescale, may have serious effects in a longer time period. The energetics of hibernation determines body weight at emergence, which is in close relationship with the further fate of the animals regardless of their gender and age. In many species, the timing of primiparity, maturity and competitive skills of the males, as well as the reproductive output of the females, are determined by body weight (Barnes 1984; Dobson and Michener 1995; Millesi et al. 1998; Millesi et al. 1999a; Neuhaus et al. 2004). The effects of these processes can be additive, which can appear as a decreased population growth rate on a long term scale.

Modeling prediction and field data

The lack of reliable long term experimental data renders it more difficult to evaluate predictions about the effect of climate change on animals. A possible solution is the use of data from unusual global events such as extraordinarily warm/cold years, eclipses, or volcano eruptions (Gu et al. 2003; Morton and Sherman 1978; Spoelstra et al. 2000). Similarly, to evaluate the reliability of our model, we modeled hibernation during an average and an extraordinarily warm winter. The model predicted emergence body weights were compared to data measured in the field. Although it is difficult to formally compare these results, predicted results of the model at emergence time is in the range of measured field data (Figure 7.4b). Considering the difference between model assumptions and the real value in the field, this result possesses acceptable error. Nevertheless, neither the results of the prediction, nor field data show any significant effect of a warm winter on BM_{em} . Considering the difference

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between model results and field data, the generalization of the results of simple physiological models should be interpreted carefully because of the many disregarded side effects of the studied factors. Warm winters may have a negative effect on emergence body mass, but it also causes elongated active periods and elevated primary production, which may have an opposite effect on BM_{em} (McCarty 2001; Walther et al. 2002).

Implications for conservation strategies

Hibernation might have appeared along with the first representatives of euthermic vertebrates (Grigg et al. 2004). There have been numerous global changes, including climate changes, during these approximately 60 million years (Barrett 2003). Considering the past events in the evolutionary history of hibernating species may help us to discover the consequences of the present climate change on hibernating species. The squirrel family is a relatively young group (first fossils are app. 34 million years old), but probably one of the groups which have the highest number of hibernators among its members (Sciuridae; (Barclay et al. 2001). There have been many global changes during these approximately 30 million years as well. Climate changes and tectonic movement frequently occurred during the evolution of the sciurid family. However, the major waves of these events coincide with the radiations of species rather than their extinctions (Mercer and Roth 2003). It seems probable that dispersion, moving across ecological corridors, played an important role in those past radiations and should play an important role in the present as well (Thomas et al. 2004).

In conclusion, global warming will affect the life of hibernating species and the energetics of hibernation, although other changes in relation to climate change (e.g.: phenology of season, human landscape use) will probably have a comparable effect on the survival and reproductive output of hibernating animals.

ACKNOWLEDGEMENTS

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APPENDIX

Modeling body mass change

The intra- and inter-individual differences in body mass change are described with the following stochastic differential equations:

$$(eq.1) \quad \frac{\partial BM^i}{\partial t} = \frac{EE^i}{37 \text{ kJ/g}} + e_t^i$$

$$(eq.2) \quad EE_t \equiv -\left(E_{t-1} + \frac{\partial E}{\partial t}\right)$$

$$(eq.3) \quad \partial E^i = \begin{bmatrix} \partial E_T^i & \text{if animal is in torpor} \\ \partial E_A^i & \text{if animal is in arousal} \end{bmatrix} dt + \sigma_{EE} d\omega_t$$

$$(eq.4) \quad \frac{\partial E_T^i}{\partial t} = BM_t^i \cdot \alpha_1^i - MR_T^i + T_a \cdot \alpha_2^i$$

$$(eq.5) \quad \frac{\partial E_A^i}{\partial t} = BM_t^i \cdot \alpha_1^i + MR_A^i - T_a \cdot \alpha_3^i$$

Body mass change (eq. 1) can be calculated using energy expenditure and the body mass-energy conversion constant. Energy expenditure at a certain time can be determined as it is defined in eq. 2. Change in energy expenditure is state dependent (eq.3). State dependency is detailed in eq. 4 for torpor and in eq. 5 for arousal state.

The measured body mass is assumed to incorporate a Gaussian white noise measurement error (eq. 1), $e_t^i \in N(0, S(EE^i))$ with S defining the covariance matrix for the observation noise, σ_{EE} defines the intra-individual variation of energy expenditure and ω_t is a standard Brownian motion (eq. 3). The notation using dx/dt cannot be used since the quotient $d\omega/dt$ is ill defined due to the changing values of ω (see: Mortensen et al. 2007; Mortensen and Klim 2008).

Inter-individual variation is included in the model through the initial (immersion) body mass $BM_{t=0}^i$ and the estimated state dependent metabolic rate constants (MR_T^i , MR_A^i) and coefficients of body mass (α_1^i) and ambient temperature (α_2^i , α_3^i). These individual parameters can be described as a sample from a population in the following way:

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$$\begin{aligned}
 BM_{t=0}^i &= BM_{t=0} \cdot \exp(\eta_1^i) \\
 \alpha_1^i &= \alpha_1 \cdot \exp(\eta_2^i) \\
 \alpha_2^i &= \alpha_2 \cdot \exp(\eta_3^i) \\
 \alpha_3^i &= \alpha_3 \cdot \exp(\eta_4^i) \\
 MR_T^i &= MR_T \cdot \exp(\eta_5^i) \\
 MR_A^i &= MR_A \cdot \exp(\eta_6^i)
 \end{aligned}$$

where η_k^i s are multivariate random effect parameters for the i^{th} individual, taken from a Gaussian distribution with zero mean and estimable covariance matrix (Ω).

The results of modeling are presented as the maximum likelihood estimations (MLE) of model parameters. The uncertainty of the parameter estimation was evaluated by calculating 95% Wald confidence intervals. The model assumptions were tested using QQ-plots and density plots of normalized residuals.

Modeling torpor – arousal cycles

Suppose that x_{ij} is an index variable of arousal event on a given day. Then our model describes the measured arousal-torpor cycle data in the following manner for the j^{th} day of i^{th} individual:

$$\begin{aligned}
 x_{ij} &\sim \text{Binom}(n_{ij}, p_{ij}) \\
 p_{ij} &= (A \cdot \exp(A_i) \cdot \cos(-j/\tau) + B \cdot \exp(B_i)) \\
 A &= a_0 + a_1 \cdot T_a \\
 B &= b_0 + b_1 \cdot T_a
 \end{aligned}$$

Where $n_{ij} = 1$ (number of days), p_{ij} is the probability of appearance of arousal on the j^{th} day during hibernation of the i^{th} individual. $A \cdot \exp(A_i)$ is the amplitude of the allowed probability range and $B \cdot \exp(B_i)$ determines the smallest probability, where A and B can both be decomposed into an intercept (a_0 and b_0) and an ambient temperature dependent part; and A_i and B_i are the individual random parts of A and B respectively, taken from a multinomial Gaussian distribution:

$$(A_i, B_i) \sim N([0,0], \Sigma) \quad \text{with} \quad \Sigma = \begin{pmatrix} \sigma_A^2 & \sigma_{AB} \\ \sigma_{AB} & \sigma_B^2 \end{pmatrix}$$

Soil temperature analysis

The change of soil temperature (T_{soil}) in time (t) was expressed as:

$$T_{soil,t} = T_{mean} + T_{amp} \cdot \cos(2\pi / 360 \cdot (t - t_{shift}))$$

where T_{mean} represents the mean year-round temperature, T_{amp} is the range of maximum deviation from T_{mean} (amplitude) and t_{shift} is a phase-delay (the time of first temperature peak). To reduce the number of parameters the length of periods were assumed to be 360 days long to meet the data structure of the PRUDENCE project.

Modeling Results

Population stochastic model included eight structural parameters. Each parameter was significantly different from zero, thus contributed to the meaningful description of the body mass change over hibernation (Table A.7.1).

Table A.7.1		
Tabulated ML estimations of model parameters		
	MLE	95% confidence limits
α_1	0.001	0.00028 - 0.00172
α_2	0.00005	0.00002 - 0.00008
α_3	0.001	0.00015 - 0.00185
MR_T	0.5	0.423 - 0.577
MR_A	2	1.47 - 2.53
$BM_{t=0}$	200	175 – 225
σ_{EE}	1	0.294 - 1.706
S	3	1.05 - 4.95

The effect of temperature on the appearance of arousals was significant on both temperature dependent parameters, which basically influence the probability of arousal

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occurrences (α_l , β_l). Two parameters, σ_a^2 and σ_{ab} were not estimated because of computational difficulties. These non-estimated parameters were not implemented into the further simulations.

Table A.7.2

Maximum likelihood parameter estimation of non-linear mixed effect modeling for the appearance of arousals on three different ambient temperatures (0, 5 and 9°C)

	Estimate	SE	DF	t	P
α_0	0.2	0.05033	13	3.97	0.0016
α_1	0.01	0.004614	13	2.17	0.0494
β_0	0.25	0.04402	13	5.68	<.0001
β_1	0.01	0.003073	13	3.25	0.0063
τ	25.0	1.7814	13	28.07	<.0001
σ_a^2	-	-	-	-	-
σ_b^2	0.01	0.003968	13	2.52	0.0256
σ_{ab}	-	-	-	-	-

-: Two parameters were not estimated because of computational difficulties

General discussion

This thesis describes a series of investigations related to physiological, behavioral and ecological aspects of hibernation in the European ground squirrel. The laboratory work on the timing mechanisms of torpor bouts and periodic euthermia was concerned with three related areas. One experiment was performed to test the “water economy” hypothesis (Chapter 2, Bintz 1982; Thomas and Geiser 1997). This hypothesis is based on the observation that the amount of lost water, via evaporation or urination, progressively increases in the course of torpor bouts. The second hypothesis which concerned the role of external (ambient temperature) and internal (circannual rhythm) factors in the timing of arousals was presented in Chapter 3. Early findings on the mechanisms that govern the timing of periodic euthermia showed that temperature and the time of season greatly affect the appearance of arousals (Pengelley et al. 1978; Pengelley and Fisher 1961; Pengelley and Fisher 1963; Twente and Twente 1965; Twente and Twente 1968). In Chapter 3, we proposed that interaction of these factors is as important as the factors per se. As it was found in Chapter 3, the pattern of arousals basically affects body mass loss during hibernation as was expected on the basis of hibernation metabolism research (Buck and Barnes 2000; Song et al. 1997; Wang 1979b). Going further, we hypothesized that the actual body mass also has an effect on the timing of arousals and in fact, timing of euthermic periods and body mass represent a feed back loop (Chapter 4). This third hypothesis was based on the observations that the body mass of hibernating animals rigidly traces a circannually changing set point of body mass (Mrosovsky and Fisher 1970; Mrosovsky and Sherry 1980).

The middle part of this thesis focused on the relationship between behavior and hibernation. In Chapter 5, we investigated nest building behavior and its effect on the energetics of hibernation in terms of body mass. As behavior before the onset of hibernation has an effect on hibernation, hibernation preceding the reproductive season also has an effect on mating behavior of ground squirrels as was found in the field study described in Chapter 6.

Finally, in Chapter 7 we investigated the effect of thermal environment on the energetics of hibernation using newly introduced modeling techniques as well as results and data of the experiment described in Chapter 3. Based on the results of this modeling we assessed the possible effects of climate change on hibernation energetics and its ecological relevance.

Heterothermy and euthermy during hibernation

Torpor effectively reduces the energy requirement of hibernation (Geiser 2004). The most elaborate use of torpor ensures the highest energy savings. Although torpid state can in some cases be extremely extended (up to one month: Körtner and Geiser 2000a), no species are able to sustain torpor state during the entire hibernation. In mammals there is limitation in the use of torpor related to body mass. Small rodents and insectivores (< 5 g) are limited by a body size which determines their lipid (fat) storing capacity (Geiser and Ruf 1995). The limit of their theoretical capacity in fat storing is far below the required amount of fat that should be stored for a successful hibernation (Geiser 2004). At the opposite end, large mammals (> 5 kg) are limited in their capacity to rewarm from low body temperature levels (Geiser and Ruf 1995). Large body size does not allow these animals to rewarm themselves to euthermic from a torpid body temperature (frequently < 10°C) in a timely manner, it rather prevents them in this process (Geiser 2004). Although, hypometabolism is a common and well regulated physiological response of endothermic organisms in energetically challenging situations (Arnold et al. 2004), reducing endogenous heat production to the level where cold adaptation is required to avoid damage by hypothermia is restricted to hibernators and daily heterotherms (Carey et al. 2003). In hibernators, torpor is regularly interrupted by periodic euthermy (Körtner and Geiser 2000a). The physiological limitations that cause periodic euthermy belong to the primary interests of hibernation research.

Since torpor metabolic rate basically depends on actual body temperature (Geiser 1988), the energetic gains depend on the temperature tolerance of the tissues of the hibernating animals. Hibernating animals are adapted to low and in some cases to sub-zero (-2.9°C, *Spermophilus parryii*: Barnes 1989), core body temperature conditions. However hibernators are able to tolerate an extremely low body temperature, they have to return to euthermy from time to time.

Homeostatic regulatory processes are continuous during torpor. Adaptive responsiveness of hibernators in torpid conditions have been observed in metabolism (Carey et al. 2003), thermoregulation (Florant and Heller 1977), heart rate (Lyman et al. 1982) and also to physical stimuli (Twente and Twente 1968). But many physiological processes are substantially suppressed or entirely ceased. Some torpor related problems occur and have to be repaired in euthermic state. Periodicity of euthermic states indicates some kind of underlying regulation. The energetic consequence of these periods is obvious (Chapter 3) which suggests some vital function of periodic euthermy.

Box 3

Hibernation: before fiction and beyond science

In a work that focuses on hibernation related studies, it is difficult to avoid the issue of human hibernation. When discussing human hibernation, one has to make sure that thinking about the subject does not become a journey into the realm of science fiction. However, since humans did not evolve into hibernators, the idea of making them hibernate has some strikingly characteristic features of science fiction.

Hypothermia in humans is pathological and has serious consequences. When a human is exposed to low environmental temperatures, body temperature begins to fall and hypothermia sets in. The homeostatic mechanism of shivering fails when the body temperature reaches 30-32 °C, the heart fibrillates at about 28 °C and ventilation stops at about 23 °C (McCullough and Arora 2004). Nevertheless, using mild hypothermia in clinical practice is not unusual, for example in the treatment of acute perinatal asphyxia in newborns (Shankaran et al. 2005).

In the 1960's, hibernation was induced in ground squirrels by perfusing them with the plasma of hibernating animals (Dawe and Spurrier 1969). Upon discovery of this encouraging result, many researchers began an enthusiastic search for the elusive protein factor (hibernation induction trigger: HIT) present in the blood of hibernators, which has been suggested as being responsible for the hibernation cascade, starting possibly at the nuclear level. HIT has never been completely characterized and there are also contradictory results as to its efficacy.

Marco Biggiogera and his team reviewed the possibilities in human hibernation based on recent facts for the request of European Space Agency in 2003 (Biggiogera et al. 2003). In this document the authors consider the aspects of the adaptations of hibernating animals and make comparisons between these and human physiology. They nominate six crucial areas of physiology involved in the possible induction, maintenance, control and termination of human hypometabolism. These are the regulation of gene expression, resetting the body temperature set point, ischemic preconditioning and protection from freezing, modification of metabolism and modulation of cell nuclear activity. Finally, the authors call our attention to the fact that even if the related difficulties were resolved, separate or synchronized perturbations in these mechanisms entail unpredictable and serious risks for subjects.

Regulatory processes in periodic euthermy

Most theories on the physiological function of periodic euthermy have focused on a specific physiological problem, transiently accumulating during torpor and in need of regular recovery during euthermy (Willis 1982b). The investigation of the role of water in hibernation began with an interesting hypothesis. Bintz came forward with an extraordinary proposal, suggesting that imbalance in water economy might be the cause of hibernation (Bintz 1982). As Bintz put forward, water balance through the winter is not possible on cached food because of its insufficient water content, and although water balance from stored fat catabolism may be possible in euthermy, insufficient fat is stored for survival over the winter if animals maintain euthermy. Hibernation allows reduced fat use and decreased water vapor pressure between the animal and its environment.

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Thomas and Geiser turned Bintz's hypothesis inside out and suggested that animals in fact recover the water that was lost during torpor via evaporative water loss in euthermic periods (Thomas and Geiser 1997).

We tested whether water loss is involved in the regulatory mechanism of periodic euthermia (Chapter 2). Elevated water loss, via increased urine production, was caused by using a diuretic drug. The prediction from the Thomas and Geiser (1997) hypothesis was that torpor bouts would be shorter, because diuresis would increase the rate of water loss, therefore the water deficit would rise sooner. In accordance with this prediction torpor bout duration changed as a result of the diuretic treatment. Arousals occurred sooner under diuretic treatment in comparison with the period when animals were only treated with vehicle control. Additionally, vehicle control per se significantly extended the length of torpor bouts. This suggests that water loss is involved in the mechanism that regulates the alternation of torpor and periodic euthermia.

Using diuretics to elevate water loss proved to be a successful resolution to uncouple environmental temperature and water loss (Thomas and Cloutier 1992; Thomas and Geiser 1997). The main concern with using environmental temperature to explain water loss via evaporation is its own effect on torpor bout length. Elevating environmental temperature shortens the time lag between two subsequent euthermic periods in many hibernating species (Chapter 3, Geiser and Kenagy 1988; Pengelley and Fisher 1963). However, correlation between the environmental (soil) temperature and the length of torpor bouts is also affected by innate circannual rhythms as is shown by studies using radio transmitting temperature probes during natural hibernation (Hut et al. 2002; Wang 1979b; Young 1990) and proven by experiment under constant environmental conditions (Pengelley and Fisher 1963; Zivadinovic et al. 2005). In Chapter 3 we tested the effects of both factors (innate timer and temperature) together. Beyond the expected results, we discovered an unexpected interaction between these two factors. Torpor bouts were lengthened by a drop in environmental temperature and the circannual rhythm still existed, as was expected from former experiments, within the usual range of soil temperature during hibernation in nature. However, when the temperature was shifted toward the extremity of the natural temperature range, the natural cycle of gradual torpor length change (Körtner and Geiser 2000a; Willis 1982b) seemed to disappear. Proposed explanation for this phenomenon was based on the effect of low temperature on the functioning of the central nervous system, particularly on parts related to biological rhythms.

In general, mammalian pacemakers in the central nervous system are considered to be temperature compensated (Pittendrigh and Daan 1976). This is in marked contrast to the high

temperature sensitivity of most biological processes that have Q_{10} s between 2 and 3. Although many chronobiologists assumed that changes in suprachiasmatic nucleus temperature of only a few degrees are insufficient to alter timekeeping functions, Ruby and Heller have proven that temperature affects the firing rate of pacemaker cells in mammals (Ruby and Heller 1996). Comparing hibernating and non-hibernating mammals, adaptation to cold in hibernating mammals results in markedly lower sensitivity of neurons to low temperature and very effective temperature compensation mechanisms (Ruby and Heller 1996). This result implied that timing mechanisms in hibernating animals might well be temperature compensated even under low environmental temperatures. Although authors argue that molecular clocks may function at low body temperature, some evidence opposes this assumption. Hibernating European hamsters (*Cricetus cricetus*) show strong circadian timing in the onset of torpor but not in the timing of the arousals (Wassmer and Wollnik 1997). Deeper analysis of this phenomenon revealed that the molecular circadian clock indeed stops during deep hibernation (Revel et al. 2007). This fact explains the findings on hibernating hamsters and might be in connection with our results. As low ambient temperature “turned off” the seasonal interval timer for torpor bout length, the lengths of torpor bouts were determined only by the low ambient temperature, hence torpor bouts did not shorten toward the end of hibernation.

Although this deficiency in the function of the seasonal interval timer indicates that animals had already reached the physiologically tolerable side of the hibernation temperature range, it was not reflected in the body mass of the ground squirrels (Geiser 2004). Increased body mass loss along with the onset of extra heat generation to avoid freezing damage (Song et al. 1997) did not occur, even ground squirrels without shortened torpor bouts lost less body mass during their hibernation than animals kept on higher ambient temperature (Chapter 3).

As body mass change during hibernation is determined by the number and extension of arousal episodes, actual body mass affects the timing of arousals independently of other factors, as it was tested in Chapter 4. This property of the rhythm organization confirms the complexity of the regulation of torpor-arousal cycles during hibernation. Surplus energy allows hibernating animals to display presumably advantageous, but highly costly arousal more frequently. This response to modified energy availability was observed both among fat storing (Mrosovsky and Barnes 1974) and food caching (French 2000) hibernators. The amount of surplus energy can obviously only be determined by using a reference point. This hypothetical reference might be the body weight set point proposed by Mrosovsky and Fisher (1970), that shows year round cyclic changing presumably in all hibernating animals (Davis 1976). The body mass in the course of hibernation shows a gradual lowering even if the animals are forced

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to being in euthermia and food is provided *ad libitum*. This transient lack of appetite was defined by the authors as an example of animal anorexia (Mrosovsky and Sherry 1980).

One of the most conspicuous properties of arousal timing is that as easy as it is to reduce torpor bout length (physical disturbance: Twente and Twente 1968), it is just as difficult to increase it. As far as we know, five treatments have been able to shorten torpor bout length: lesions in thermoregulatory brain structures (Ruby et al. 1996), saline treatment (Chapter 2), low ambient temperature (Chapter 3) (Geiser and Kenagy 1988), providing food with polyunsaturated fatty acid (Geiser and Kenagy 1987) and experimentally increased body weight (Chapter 4) (Mrosovsky and Barnes 1974). But considering all of the above mentioned hypotheses, none of them are able to explain the gradual lengthening of torpor bout length in the course of hibernation, because each of them explains the timing of arousals by the accumulation or depletion of a certain metabolic agent (Körtner and Geiser 2000a; Willis 1982b). Therefore, these approaches cannot deal with the seasonal changing of torpor bout lengths during hibernation. The reconciliation of this contradiction will be the next step in the mystery of periodic arousals.

All of the above proposals assume that arousals are counterproductive and should be avoided whenever possible. French (French 1985) questioned this approach by arguing that, with the strong selection pressure that should act against arousals, they should occur much less frequently than they do. Because large hibernators, which can carry larger fat reserves, arouse more often than small hibernators, which are energetically more challenged, he proposed that hibernators arouse as often as is energetically possible, and that they favor euthermia over torpor. French's original proposal was an attempt to open a new perspective in this question. Unfortunately, it was not considered a satisfactory theory by the mainstream of hibernation researchers and was neglected in further studies (Körtner and Geiser 2000a).

Behavioral aspects and ecological consequences of hibernation

The costs and benefits of a particular behavior have been weighed against each other through natural selection. Natural selection will result in a behavioral decision that maximizes the individual rate of gene propagation. The possibilities for gene propagation are set by an animal's individual physiological, behavioral and environmental limitation on reproductive success. For understanding the function of a behavior that is not directly linked to reproduction, the long term consequences of the specific behavior on reproductive success need to be assessed. Low reproductive success of ground squirrels has been associated with low body mass after hibernation (Barnes and York 1990; Millesi et al. 1998; Millesi et al. 1999a; Millesi

et al. 1999b). Relationship between body weight and survival is also evident among ground dwelling sciurid rodent species (Morton and Sherman 1978; Murie and Boag 1984). Thus any factor affecting body weight during hibernation or after the hibernation season acts basically on the animal's fitness.

Nesting behavior is adaptive and highly effective in decreasing energy expenditure of endothermic animals during resting periods (Bult and Lynch 1996; Bult and Lynch 1997). Although this effect is not as obvious in the case of hibernation as it is in constant euthermy, in Chapter 5 we demonstrated the advantageous effect of a well insulated nest on overall energy expenditure during hibernation. The significance of the nest is not limited to decreasing energy expenditure under unfavorable thermal conditions. The presence of the nest in the 1 m deep burrows is continuous (field endoscopic observation by Csongor Gedeon). Nest material collection behavior is observable on the surface throughout the entire active period. However up to this point, existing data have only shown that the structure of the nest influences the insulation (Gedeon et al. submitted), but the nest probably has some other function as well, because insulation is less pronounced during the summer period. Such a function can be the regulation of humidity (Thomas and Geiser 1997) or as a parasite repellent (Gwinner and Berger 2005) and may even play a role as a temporary food source. Nevertheless, the major role of the nest is undoubtedly related to thermal benefits and its relationship to energy savings. Animals constructing better insulated nests lose less body weight, thus acquiring advantage over animals with poorly constructed nests in survival or in the subsequent reproductive season.

Hibernation, especially for juveniles, is not as critical as the active period in terms of survival (Millesi et al. 1999b). The body weight of these animals can infrequently fall below the lower critical mass in nature, which might lead to insufficient warming up abilities and finally to death (Geiser 2004). The decreased body weight influences the reproductive abilities of the animals, rather than their survival, regardless of their gender.

Although some hormonal and cytological changes occur during the last third of the hibernation period in male ground squirrels, long lasting euthermy is required to complete gonadal maturation (Barnes et al. 1986). Our research and other studies have shown that male ground squirrels and closely related species are reproductively mature when first trapped in spring (Chapter 6, Michener 1998; Millesi et al. 1998; Millesi et al. 1999b), these findings confirm that males are euthermic for several weeks before they emerge from their hibernacula in the spring (Barnes et al. 1988). This period of gonadal development requires a considerable amount of stored energy, therefore only males in good condition are able to become

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reproductively active (Barnes 1984). This reproductive development dependence on energy stores may be related to the high energetic costs associated with breeding in males (Chapter 6, Millesi et al. 1998). When males emerge from hibernation in the spring, food availability is low, therefore compensation of body mass loss is difficult. Considering all of the above, the tight relationship between emergence timing, maturation and body mass in male ground squirrels is not surprising.

Since the active period is shorter for females than it is for males (Millesi et al. 1999b), reproductive decisions are much more rigidly controlled and influenced by the annual body mass cycle and emergence timing. Litter size increases with posthibernation body mass for yearlings, but not for older mothers, at the expense of producing slightly smaller young. In older females, on the other hand, emergence body mass influences offspring mass, whereas litter size is affected by oestrus date (Huber et al. 1999; Millesi et al. 1999a). Females with higher reproductive output and higher investment are unable to compensate for these costs before hibernation. Consequences for these individuals could therefore be lower over-winter survival or a delayed oestrus in the following season (Millesi et al. 1999a).

Low reproductive success of European ground squirrels is associated with low body mass after hibernation (Huber et al. 1999; Millesi et al. 1998; Millesi et al. 1999a). Body mass variation prior to hibernation is mainly due to variation in body fat (Dark et al. 1989). Body mass after hibernation will primarily reflect the loss of fat consumed during hibernation. In contrast to some hibernating species (Humphries et al. 2003), European ground squirrels have never been observed to store food, either in field or in laboratory. This suggests that the change in body mass over hibernation is directly caused by the energy expenditure of hibernation.

Energy expenditure during hibernation was calculated by combining data on body mass loss and temporal patterns of torpor and euthermic periods under different temperature conditions (Chapter 7). Variables such as ambient temperature and body mass have a substantial effect on energy expenditure (Buck and Barnes 2000; McNab 1978; McNab 2008). Ambient temperature also plays an important role in the appearance of periodic arousals (Chapter 3), which, in turn, also affect energy expenditure and thus body mass.

In some former studies the authors used estimates of energy expenditure during torpor and arousal episodes to calculate energy expenditure over hibernation (Strijkstra et al. 1999; Wang 1979b). Based on the timing of torpor bouts over the entire hibernation season of animals in the field, assessed by telemetry, and laboratory data on energy expenditure, estimates of energy expenditure were calculated (Wang 1979b). The observed relationship between environmental conditions and the timing and energy expenditure of both torpor bouts

GENERAL DISCUSSION

and periodic euthermy were combined in an integrative deterministic calculation model for energy expenditure over hibernation by Strijkstra (Strijkstra et al. 1999). The novel elements in the author's work compared to Wang's approach were the nonlinear modeling of energy expenditure and that energy expenditure over hibernation was subsequently used to estimate the consequences of hibernation for post hibernation body mass.

Further development of this approach was done in the present thesis (Chapter 7). The essence of this development was the usage of computer intensive population modeling methods, widely used in pharmacokinetics (Mortensen et al. 2007; Mortensen and Klim 2008; Pinheiro and Bates 2002; Pinheiro et al. 2008), to resolve an eco-physiological problem. Modeling of the body mass change over hibernation is composed of two parts. Metabolism description was used to estimate energy expenditure as a latent variable using stochastic modeling by partial differential equations and the main input variables were environmental temperature and the temporal pattern of hibernation (torpor-arousal cycles). Hibernation pattern modeling was used to estimate the temporal pattern of hibernation, the main input variables were the time of year and environmental temperature.

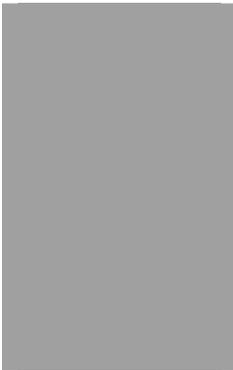
The model was used to predict the consequences of future climate change on body mass change over hibernation, and eventually on post hibernation body mass. We found no significant difference between the predictions of the three climate scenarios used. Based on each of the three climate scenarios, the predicted effect of climate change on body mass change could be judged biologically insignificant ($< 15\text{g}$), but further considerations are needed in this question. A 15 g difference in population emergence body mass between two consecutive years entails different consequences than a 15 g difference in population body mass over decades. Individuals with lower body mass are probably able to compensate for this before the next hibernation, but under permanently unfavorable conditions the body mass cycle of whole populations can drift downward with all of its collateral consequences described above.

Nevertheless, hibernating animals have experienced many global changes during their sixty million year history in the past (Grigg et al. 2004; McNab 1978). Detailed analysis of these events revealed that these periods were accompanied by intensive diversification instead of widespread extinctions (Mercer and Roth 2003). Outcomes of past events can only be partially applied to the present situation, considering the different circumstances.

A brief description of the collapse of a Northern Idaho ground squirrel colony helps outline these differences (*Spermophilus brunneus brunneus*, Sherman and Runge 2002). The concerned population was tallied yearly from 1987 to 1999, during which time it declined from 272 to 10 (!) animals. The authors believed that the ultimate cause of the population's collapse

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was inadequacy of food resources, particularly seeds, due to the drying of the habitat and changes in plant species composition, probably the result of fire suppression and grazing. Animals did not respond adaptively to impending food-plant failure by reducing litter sizes or curtailing reproduction in order to fatten early. The consequence may have been the increased overwinter mortality. No “rescue” by immigration occurred, probably because ground squirrels seldom disperse long distances and also because inappropriate landscape management allowed bush species to encroach on inhabited meadows, shrinking them and closing dispersal routes.



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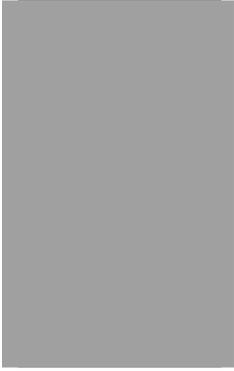
Összefoglaló

Disszertációm, a hibernáció élettani, viselkedési és ökológiai aspektusaival foglalkozik laboratóriumi kísérletek, terepi vizsgálatok és számítógépes modellezés segítségével.

A dolgozatban vizsgáltam; azokat a külső és belső tényezőket, amelyek potenciálisan befolyásolják a hibernáció alatt periodikusan megjelenő eutermikus szakaszok (arousalok) időzítését; a hibernáció és viselkedés kölcsönös kapcsolatát; valamint a hibernáló életmód ökológiai következményeit. Vizsgálataimat, a közép-európában endemikus elterjedésű, Közönséges ürgén (*Spermophilus citellus*) végeztem, 2002 és 2008 között. Ezek során kimutattam;

- laboratóriumi körülmények között, a periodikusan megjelenő arousalok időzítésére a vízháztartás kísérletes manipulációjának hatását
- hogy a külső hőmérséklet és a benső óra saját hatásán túl e kettő interakciója, is jelentősen befolyásolja az arousalok időzítését
- hogy amiképpen az arousalok megjelenése befolyásolja a hibernáció során a testtömeg-veszteség dinamikáját, úgy a testtömeg is visszahat ezen arousalok időzítésére
- a hibernációt megelőző időszakban készített fészkek minősége, elsősorban szigetelőképességén keresztül hat a hibernáció alatti testtömeg változásra
- terepi vizsgálataimban a testtömeg, a hibernáció befejezésének időzítése és a szaporodási viselkedés közötti kapcsolatot, valamint ezek költségét a hímek estében
- végül számítógépes modellezéssel leírtam az ürgék tömegváltozását különböző hőmérsékleti viszonyok között, majd ezek alapján közép-hosszútávon megbecsültem egy a klímaváltozás hatását a hibernáció alatti tömegváltozásra.

Munkám során az arousalok időzítésének mechanizmusában több külső és belső faktort is sikerült azonosítani, alátámasztva hogy e jelenség mögött számos, akár egymástól is független tényező játszik szerepet. A hibernáló állatok viselkedése hibernációs periódus előtt és után szoros, mondhatni ok-okozati kapcsolatban van. E szoros kapcsolat mögött a hibernáló életmód és az abból fakadó kényszereket sejthetjük. Míg a hibernáció nyilvánvalóan adaptív folyamatként értelmezendő, ugyanakkor egyes fajok estében a változó környezet következtében akár evolúciós zsákutca is lehet.



Summary

This thesis describes a series of investigations between 2002 and 2008, related to the physiological, behavioral and ecological aspects of hibernation in the European ground squirrel (*Spermophilus citellus*). The laboratory work dealt with the timing mechanisms of torpor bouts and periodic euthermy. Laboratory and field studies focused on the relationship between behavior and hibernation. Finally, we assessed the possible effects of climate change on hibernation energetics and its ecological relevance.

We demonstrated:

- the role of water economy in the timing of periodic euthermy
- beyond the effect of ambient temperature and the internal timing mechanism the significant effect of their interaction on the timing of periodic euthermy
- the effect of the actual body mass on the timing of periodic euthermy, similar to the effect of periodic euthermy, on body mass loss during hibernation
- the effect of the nest constructed before the onset of hibernation on body mass loss during the hibernation
- the relationships among body mass, emergence timing and mating behavior of male ground squirrels and the consequences of different mating behavior strategies in a field study
- the possible effect of climate change on body mass during hibernation using computational modeling

We pointed out the existence of several inner and outer factors that affect the timing of periodic euthermy. These results confirm the view which suggests that there are many, perhaps independent, contributing factors in the timing of periodic euthermy. The behavior of hibernating animals before and after hibernation is in a close, possibly causal, relationship. This tight relationship may come from the constraints of the hibernating life style. On one hand, hibernation is undoubtedly the result of a successful adaptation to seasonally harsh environmental conditions, on the other hand, if these conditions change, this adaptation can be an evolutionary dead end in certain species.

Köszönetnyilvánítás/Acknowledgement

Nehéz megírni egy köszönetnyilvánítást, hiszen olyan sokaknak kellene kiférni az első sorba,

és mégis, mindenképp szeretném kifejezni hálámat azoknak az ürgéknek, akikkel a munkánk során volt szerencsém foglalkozni. A velük folytatott munka segítette megérteni, hogy minden adat mögött egy lélegző teremtmény van. A legnagyobb áldozatot ők hozták. Nélkülük a dolgozatom nem készülhetett volna el. Köszönöm nekik.

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Let me change to English, however I am so poor in this language, too. Any in this thesis seems to be written in English is not my merit. My friends, Celeste and her husband Péter Pongrácz have invested enormous amounts of time and effort to improve the English of this thesis. Her suggestions, advice and gmail chats helped and encouraged me to believe that I am able to be a „doc”. Discussions, jokes and fun with Péter were so important for me during hard days in the field or in the house behind the sandy dunes. Thank you so very much!

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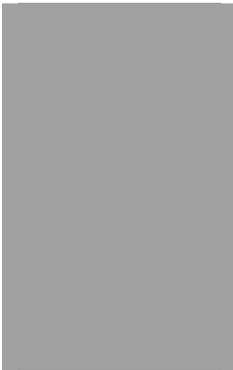
Köszönöm továbbá Magyar Gabinak és Zeöld Orsinak szakmai és emberi segítségüket is. Azokban a helyzetekben, mikor szándékosan ingoványos talajra tévedtem, sokszor ők rántottak vissza a szilárd talajra. Hálásan gondolok Kapás Margóra, aki lehetővé tette és támogatta, hogy munkám mellett befejezhessem a dolgozatot.

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Végül, minden balsejtelmem ellenére, amely önismeretre utal, csak leírhattam ezt a sort. Ehhez türelemre és kitartásra volt szükség, de nem részemről; az érdem egy embert illet. Ezt a munkát neki ajánlom,

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List of Publications

Articles

István Németh, Viktor Nyitrai, András Németh and Vilmos Altbäcker (2009): Diuretic treatment affects the length of torpor bouts in hibernating European ground squirrels (*Spermophilus citellus*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 180: 457-464

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