Faculty of Applied Ecology and Agricultural Sciences

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PhD-thesis

Ecophysiology of Brown Bears
Basic physiology and effects of hibernation, pregnancy, body mass, and capture

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Preface

My adventures in Norway began in 2009 with a 1-year fellowship from the American-Norwegian Fulbright Foundation and American-Scandinavian Foundation. I started off in Tromsø at the Norwegian School of Veterinary Science, Section for Arctic Veterinary Medicine. There I had excellent mentors and colleagues including Morten Tryland, Carlos das Neves, and Ingebjørg Nymo.

I was extremely fortunate with the timing of my arrival in central Scandinavia. In 2010, Sven Brunberg agreed to let me join the very first winter captures of bears, Stéphane Blanc, who became my co-supervisor, had just ordered the first temperature loggers and Ole Fröbert brought the first pair of heart monitoring devices. In 2011, Tim Laske, from Medtronic, joined and contributed not only all of our heart rate loggers, but also valuable insight and expertise from his years working with black bears. This, together with the enthusiasm of Jon M. Arnemo, who became my main supervisor, was the background and inspiration for my PhD. Jon welcomed me into his home, family and research projects.

I could not have a more enthusiastic and encouraging group of supervisors! Jon M. Arnemo, my main supervisor, always said that the most important job of the supervisor was to help the PhD student keep focused. Well, all of them have done exactly the opposite. Every time I had an off-topic idea, they said “go for it!” By believing in me, regardless of how over-ambitious ideas my ideas were, they forced me to take responsibility, to be realistic, and to make decisions and plans. I thank Stéphane Blanc for always believing in me and the numerous discussions on physiology, even though I didn’t always understand and Navinder Singh for his consistent discovery of statistical solutions, patience for redoing analysis with my many “discoveries” and for helping me to become more independent in my own analytical abilities.

The modern European PhD is very different than the monographs of earlier times. I am grateful that I had the opportunity to do my PhD in this way, including the compilation of manuscripts, building of collaborations and teamwork. Being so many PhD students and researchers in the Scandinavian Brown Bear Project has created a number of opportunities and an excellent support network. I am especially grateful to the PhD students whose work has overlapped with mine, including Veronica Sahlén, Andrea Fribe, Inge Revsbech and Berolla Sahdo. Veronica and Andrea introduced me to the brown bear project and Inge and Berolla broadened the scope of the work. Their initiative and collaboration, both in writing and in the field has been a highlight of this PhD. Many others including Jon S, Ole-Gunnar, Andrés, Andreas, Jonas, Fabrice, Isabelle, Johan, Marcus, Sam, Shane, Gro, Anne, Richard, and Nina have contributed with good companionship, insightful discussions, humor and some very good adventures (from the forests of Tackåsen, mountains of Poland and Greece, open landscapes like Denmark and Utah and even cities like Strasbourg). The excellent colleagues from other projects (Skandulv, Scandlynx, wolverine, beaver, moose, roe deer, Japanese black bear, etc) are too numerous to describe, but have also contributed greatly.

I also thank the veterinarians and vet students who have been part of our team. Their enthusiasm and energy has been priceless. Some have become my closest friends. Andrea Miller, Marianne Lian and Susanne Küker have
been present for some of the most difficult times during the PhD and have consistently been there to listen and advise, both personally and professionally. Although fieldwork can be demanding and the days can be long, the eagerness, dedication and positivity of Nikolaus Huber, Krista Jones, Anne Randi Græsli, Monica Bando, Martine Angel, Åsa Fahlman, Núria Fandos Esteruelas, Solveig Cecilie Minsaas and Johanna Painer, made it enjoyable and rewarding. Other veterinarians including Sven Björck, Kimberlee Beckmen, Peregrine Wolff, Sari Wedul and Sophie Rossi have shared their expertise and allowed me to join some fun fieldwork. All have contributed greatly to my development as a wildlife veterinarian, mentor and colleague.

Working with such a variety of field teams has been invaluable, and I have benefited from the combination of learning from some of the world’s most experienced field technicians (Sven Brunberg, Peter Segerström, Eric Andersson, Per Alqvist, Åke Nordström) and working as a team and making decisions together with some closer to my own age (Fredrik Stenbacka, Thomas Strømseth, Einar Segerström, David Alqvist). Together with the veterinarians and excellent pilots (especially Ulf Grinde, Mattias Eriksson, Fredrik Lundkvist and the late Åke Pettersson), the fieldwork was both effective and social.

The environment at Campus Evenstad has been a perfect place for a PhD. The academic culture is welcoming and creativity is encouraged. I am grateful to everyone here, including friends, neighbors, our excellent librarians and helpful administration.

The confidence and encouragement from my family has always been inspirational. My sister, Kelsi, has given me enormous love and support. My parents have been supportive in every endeavor, even as a small child, they encouraged me to work to pursue my own ideas... even when it meant that I, as a 16-year-old, saved money from my after-school job at the local vet clinic to buy a fourth dog (all four stayed with my parents when I left for college). When I decided, only one month ahead of time, to take a half-year to study in Tasmania, my dad responded, “I am coming too!” My Grandmother Verla told everyone she knew how much she hoped I got the Fulbright fellowship to Norway.

In preparation of the thesis and manuscripts, I have to thank my supervisors, coauthors, Jon Swenson and my partner, Boris Fuchs for many hours spent revising and helping. And especially to Boris for countless dinners and lots of love and support while I was working, and our dogs, Nala and Dasha who tolerated a lot of boring days at the office.

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Summary

Northern mammals show a host of behavioral, ecological, and physiological strategies for survival in harsh climates. Bears are unique in that they hibernate six months of the year without eating, drinking, urinating, or defecating, and the females give birth in their dens. Although there are numerous previous studies on the physiology and ecology of hibernation in bears, many gaps in our knowledge remain. Also, most physiological studies have been conducted in laboratory conditions, independently from the bear’s natural ecology.

This thesis begins by assessing the impacts of capturing brown bears in their winter den. These captures and the deployment of biologging devices then allowed for addressing fundamental questions about the basic biology of the bear’s annual cycle.

In blood samples taken in winter, analyzed together with similar sized bears in spring and summer, we found significant shifts in hematological and biochemical in winter (February-March) compared to in spring and summer (April-July), reflecting the lowered metabolic, renal and hepatic activity and shift to a lipid-based metabolism during hibernation. The lowered levels of leukocytes during hibernation were, when compared across species, explained by the decline in body temperature ($T_b$), suggesting that $T_b$ is the main driver of immune function regulation during hibernation. As a previous study has shown that metabolic rate and oxygen consumption are independent from lowered $T_b$, we investigated changes in O$_2$ binding affinity of red blood cells from hibernating bears, which consistently showed higher O$_2$ affinity than their summer counterparts. This likely maintains a relatively constant tissue oxygen tension during hibernation.

Over five years, we used biologgers for $T_b$ heart rate, heart rate variability (as proxy for autonomic nervous system levels) and activity to assess the timing and duration of pregnancy, the interplay between physiological and ecological drivers of den entry and den exit, and effects of body mass on hibernation depth and duration. The gestation period was 56 days (mean), with pregnant bears having higher $T_b$ during the gestation and lactation periods. Nonpregnant bears entered the den when the snow arrived and when the ambient temperature neared 0°C. Activity, heart rate and $T_b$ started to drop slowly several weeks before den entry. Denning appeared to be tightly coupled with metabolic suppression. During arousal, $T_b$ unexpectedly rose two months before den exit and was driven by ambient temperature ($T_A$), independently of autonomic nervous system activity which only became active three weeks before den exit. The difference between $T_b$ and $T_A$ decreased gradually. Although the sympathetic nervous system began to restore euthermic metabolism three weeks before den exit, it was not until $T_A$ reached the bear’s lower critical temperature that bears exited the den. We further evaluated $T_b$
throughout the year in 34 bears and found consistently lower $T_b$ in sequentially smaller bears in winter (Jan-Mar) with the opposite pattern in summer. The use of heart rate and $T_b$ data has allowed us to fill in important knowledge gaps in the basic ecology and physiology of free-ranging brown bears while also providing a solid foundation for exploring further details on conservation, management, and implicates of climate variability on bear biology.
Sammendrag

Pattedyr i nordområdene har ulike strategier for å overleve i et barskt klima. Brunbjørnen er unik ved at den hibernerer i seks måneder, uten å spise, drikke, urinere eller ha avføring og ved at binner føder unger i hiet midt på vinteren. Til tross for at det er gjort mange studier på hibernerende bjørner, mangler vi kunnskaper om fysiologiske og økologiske forhold. Dette skyldes i hovedsak at det meste av forskningen har blitt utført på bjørner i fangenskap, under forhold som er svært forskjellige fra artens naturlige levevilkår.

Denne avhandlingen starter med å beskrive bedøvelse av bjørner som ligger i hiet. Denne fangsten og instrumentering med fysiologiske sensorer gjorde det mulig å studere grunnleggende spørsmål rundt bjørnens biologi og årssyklus.

Blodprøver tatt på vinteren, våren og sommeren viste at bjørner reduserer stoffskiftet og nyre- og leveraktiviteten når de hibernerer sammenlignet med den aktive perioden. Om vinteren er stoffskiftet basert på fettforbrenning. Hibernerende bjørner har en lavere konsentrasjon av hvite blodlegemer. Denne reguleringen av immunfunksjonen, er styrt av reduksjonen i kroppstemperatur (Tb). Mens en tidligere undersøkelse fant at reduksjonen i stoffskiftet og forbruket av oksygen (O₂) hos hibernerende bjørner er uavhengig av nedsatt Tb, dokumenterer denne avhandlingen at O₂-affiniteten til røde blodlegemer er høyere om vinteren enn om sommeren. Dette bidrar sannsynligvis til å opprettholde en relativ konstant O₂-metning i vevene under hibernering.

Fysiologiske sensorer for kroppstemperatur og hjertaktivitet ble brukt over en femårsperiode for å undersøke effektiv drektighetslengde hos binner, samspillet mellom fysiologiske og naturgitte forhold som påvirker starten og slutten på hiperioden, hvordan kroppsvekten påvirker dybde og lengde av hiberneringen og fysiologiske effekter av fangst og bedøvelse.

Den gjennomsnittlige drektighetstiden var 56 dager og drektige binner hadde en høyere Tb under den effektive fosterveksten og laktasjonen enn andre bjørner. Ikke-drektige bjørner gikk i hiet når snøen kom og når lufttemperaturen (Tₐ) nærmet seg 0°C. Hiperioden er tilsynelatende nøye knyttet til en reduksjon i stoffskiftet. To måneder før bjørnene kom ut av hiet, begynte Tb uventet å stige, tilsynelatende påvirket av Tₐ og uavhengig av aktiviteten i det autonome nervesystemet, og differansen mellom Tb og Tₐ avtok gradvis. Selv om det sympatiske nervesystemet startet å gjenopprette normal metabolisme tre uker tidligere, forlot ikke bjørnene hiet før Tₐ nådde bjørnens nedre kritiske temperatur. Tb gjennom året for 35 bjørner viste at små bjørner hadde lavere Tb om vinteren sammenlignet med store bjørner mens forholdet var omvendt på sommeren.
Bruk av data for Tb og hjerteaktivitet har skaffet ny og viktig kunnskap om basal økologi og fysiologi hos villevende brunbjørner. Det har også dannet et solid grunnlag for å studere flere forhold av betydning for bevaring og forvaltning av denne arten, inkludert mulige konsekvenser av klimaendringer.
List of papers

Methodological issues


Basic biology


Ecophysiology of the bear


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Introduction

What is Ecophysiology?

“Ecophysiology is the study of how the environment, both physical and biological, interacts with the physiology of an organism. It includes the effects of climate and nutrients on physiological processes in both plants and animals, and has a particular focus on how physiological processes scale with organism size.” (Anonymous 2015)

Although the study of ecophysiology began in the late 1800’s and accelerated after WWII, it has been the more recent improvements in biologging techniques that have made the study of the ecophysiology of free-ranging animals feasible. These advances have allowed scientists to branch into new areas of study, including location, behavior, and interactions between animals and their external environment. Biologgers allows field ecologists to address scientific questions about ecology, physiology, and behavior that previously were only studied in the laboratory. These technologies have provided vast amounts of new knowledge about cryptic animals with large home ranges and have resulted in unexpected findings.

The Brown Bear as a Model

Within mammals, torpor is most commonly observed in endotherms under 10 kilograms. It is characterized by a hypometabolic state of fasting and inactivity and is a strategy applied at all latitudes from arctic to tropical regions (Wang and Lee 2010, Geiser 2013) and in members of at least seven mammalian orders (Monotremata, Marsupialia, Insectivora, Chiroptera, Rodentia, Carnivora and Primates) (Lyman 1982). The use of torpor in mammals (heterothermic endotherms) differs from ectotherms because body temperature ($T_b$) is regulated by a proportional increase in metabolic rate (Boyer and Barnes 1999) and return to euthermia can be achieved using internal heat generation through both shivering and non-shivering thermogenesis. Torpor is divided into two groups, based on the timing and duration of torpor: seasonal and non-seasonal torpor. Seasonal torpor can include both summer and winter. Here we focus on winter torpor, specifically, hibernation, which is defined as torpor bouts lasting consecutive days to several weeks without foraging (Ruf and Geiser 2015).
Examples of groups exhibiting hibernation are bats, chipmunks, dormice, hedgehogs, marmots and ground squirrels (Wang and Lee 2010, Geiser 2013). These animals show two clearly distinct annual physiological states. During the nonhibernating phase, the body mass of an adult animal has a relatively narrow range. Exposure to cold (0-5°C) results in increased heat production and heat conservation, similar to that of other nonhibernating species, to maintain $T_b$. Long-term exposure to cold leads to hypothermia and death. As winter approaches, the animal’s body mass increases drastically (up to 50%) within a short period of time, due to hyperphagia and fat deposition. Exposure to cold (0-5°C) typically results in hibernation. The timing of the annual active and hibernating cycle is determined complex and species-specific processes commonly based on an endogenous circannual clock (Helm et al. 2013). The duration of hibernation varies from weeks to months with the metabolic rate, on average, reduced to 5% of the basal requirements (Geiser and Ruf 1995). However, in some small species, the metabolic rate can decrease to below 1%, (Song et al. 1997). How this reduction is achieved remains controversial. In some species, such as the pigmy possum (*Cercartetus nanus*), the drop in metabolic rate precedes the drop in $T_b$ suggesting a regulated suppression of metabolism, rather than a passive adaptation to cold (Song et al. 1997). Interestingly, this does not apply to Ursids. They have a higher body temperature during hibernation (around 30-34°C) and do not urinate or defecate (Folk et al. 1974). They also do not need frequent periods of arousal from hibernation as seen in small hibernators (Hissa 1997).

Hibernating brown bears (*Ursus arctos*) undergo drastic changes in all aspects of their biology and physiology during the 5-7 months of winter dormancy (Nelson et al. 1973). Although bear physiology (primarily of American black bears, *Ursus americanus*) has been studied for decades, many aspects of their basic biology remain a mystery including the triggers for starting and ending hibernation in free-ranging animals.

Other well established aspects of bear hibernation physiology include decreases in body temperature ($T_b$), (Hissa 1997), heart rate (HR), respiratory rate, and metabolism (Folk et al. 1974, Tøien et al. 2011, Tøien et al. 2015) during hibernation and the increase in fat storage prior to hibernation (Hilderbrand et al. 1999). Due to these changes, physiology varies dramatically throughout the year, with hematological and biochemical variables illustrating many of the organ systems involved.
Capture of Hibernating Bears—Previous Studies

Previous studies on the capture of bears during hibernation included limited reports of immobilization of American and Asiatic black bears (Ursus thibetanus) during winter (Tinker et al. 1998, Harlow et al. 2002, Powell 2005, Asano et al. 2007).

The brown bear’s cryptic and shy nature makes them challenging to capture. Den sites are selected away from human activity (Linnell et al. 2000). These sites are usually selected before the denning period (Friebe et al. 2001). After the den is selected, bears are still very sensitive to human disturbance. In one study, bears exposed to human activity within 200 m abandoned their dens, moving an average of 5.1 km and up to 30 km before selecting a new den (Swenson et al. 1997). Den abandonment rates of bears in Sweden average 22%, but most abandonments occur during early denning (before 15 December) (Sahlen et al. 2015).

For chemical immobilization, ketamine has been used in combination with alpha-2 agonists at doses ranging from 1.5–17.1 mg/kg in both brown bears and American black bears (Addison and Kolenosky 1979, Jalanka and Roeken 1990, Seryodkin et al. 2003), 4.4 mg/kg in Asiatic black bears (Seryodkin et al. 2003), and 2.0–7.2 mg/kg in brown bears (Jalanka and Roeken 1990). American black bears are commonly captured during hibernation and, when approached quietly, can be localized without flushing them and immobilized with a blow dart, jab stick, or dart gun (Harlow et al. 2002, Powell 2005). In previous studies, brown bears had only been anesthetized during winter in captive situations. Some studies have used summertime doses or even higher doses for immobilization in winter (Tinker et al. 1998, Harlow et al. 2002, Powell 2005). However, one study of nonhibernating brown bears concluded that the ideal dose for oral carfentanil was 12.7 µg/kg in the summer and 7.6 µg/kg in the winter (60% of summer) (Mortenson and Bechert 2001) and a study of Asiatic black bears used 50% of nonhibernating doses during hibernation (Asano et al. 2007).

Methodological Issues—Evaluation

In addition to developing an appropriate immobilization protocol, it was essential to evaluate the consequences of captures using this protocol. The first phase of this evaluation was physiology during the capture event itself and evaluation of post-capture movements. Abandonment of dens by brown bears as a result of non-research human disturbance has previously been documented in Scandinavia (Swenson et al. 1997, Sahlen et al. 2015), and den abandonment was therefore considered a possible
response to our captures. The second phase was the evaluation of whether captures disrupted the hibernation period as a whole.

Arterial blood gases and acid-base status are useful for evaluating the immediate effects of capture methods, however, during hibernation, American black bears reduce oxygen consumption by 75% (Tøien et al. 2011). We did not know how oxygen consumption in bears was affected by anesthesia or what the optimal PaO₂ levels were during anesthesia of hibernating bears. Regardless, acidosis and hypoxemia could have undesirable yet undetectable effects, including organ dysfunction (Gutierrez 2006) and damage to the brain (Siesjö 1988), liver (Wang et al. 1997), and kidneys (Evans et al. 2011). In addition to ethical issues, these effects could potentially affect the quality of results for both short- and long-term studies.

In addition to possible detrimental effects from the capture itself (in terms of blood-gasses), we also considered the possibility of alterations in Tb and HR during the middle of hibernation to be potentially detrimental and costly. This is because arousal from hibernation is known to be energetically expensive for small hibernators, with the metabolic rate reaching several times the basal metabolic rate (Karpovich et al. 2009). In arctic ground squirrels (Urocitellus parryii), arousal episodes are the most energetically costly component of hibernation, accounting for the majority of energetic costs during the hibernation period (Karpovich et al. 2009). Therefore assessing whether the captures caused an “arousal” was an important part of the assessment of the impact of the captures.

Developing a safe and efficient capture protocol for wintertime and establishing the consequences for bear ecology and physiology were fundamental to address further questions on the basic biology and ecophysiology of brown bears.

**Basic Biology of Hibernating Bears**

A host of cell preservation strategies for muscle, bone, and the circulatory and innate immune systems accompany the period of inactivity during winter. Hibernating animals tolerate extremes in organ perfusion, oxygen saturation, immobilization, Tₜₜ, and calorie intake, which in combination would be lethal to humans. Bears are different from other hibernators in that their body temperature is downregulated less dramatically (from 37°C in summer to 33-30°C in winter) than in small hibernators, which can drop to near hibernaculum temperatures (Hissa et al. 1994, Ortmann and Heldmaier 2000), in some cases even dropping below freezing (Barnes 1989). O₂ consumption rates are downregulated by 75% in the bear (Tøien et al. 2011), in stark contrast to the Q₁₀-induced depression of metabolic rate in
small hibernators. Most hibernators do not experience hypoxia despite decreased ventilation and HR. For example, arctic ground squirrels even have a normal arterial O₂ tension (PaO₂) (Drew et al. 2004).

In most hibernating mammals, hibernation is composed of a series of torpor phases interrupted by euthermic arousal bouts. During torpor, metabolism is severely depressed, but hibernation also involves the inhibition of thermogenesis, leading to a considerable decrease in Tₘ. The induction of hibernation begins with lowering the metabolic rate, followed by hypothermia as the Tₘ drifts downward. Various degrees of cold torpor have been observed over a phylogenetically wide range of mammals with the most dramatic changes found in the Arctic ground squirrel, which exhibits Tₘ as low as -2.9°C (Barnes 1989). Bears do not show euthermic arousal bouts during winter but do exhibit shallow multiday cycles (1.6–7.3 days) in Tₘ (Tøien et al. 2015). Nevertheless, bears exhibit an active aerobic metabolic depression and a mass-specific metabolic rate, similar to that of smaller hibernators (Tøien et al. 2011). They are therefore recognized as true hibernators, even though they do not show the dramatic drop in Tₘ and arousals typical of small hibernators (Tøien et al. 2015). Whether and how the blood O₂ transport of bears adapts to a decreased O₂ supply to tissues during hibernation is, however, still unknown. Earlier studies have found that blood O₂ affinity increases markedly during hibernation in small hibernating mammals (Christoforides and Hedley-Whyte 1969).

A striking finding characteristic of hibernating mammals is reduced circulating leukocytes, and it is not known if this is a result of hibernation or a byproduct of the lowered body temperature (Bouma et al. 2010, Bouma et al. 2011). Reduced circulating leukocytes have also been noted in bears, with a 23% drop in hibernating captive Asiatic black bears compared to nonhibernating captive bears (Asano et al. 2007). In some species, Syrian hamsters (Mesocricetus auratus), and Djungarian hamsters (Phodopus campbelli), Tₘ during hibernation controls leukopenia (Bouma et al. 2011), however, the relationship between white blood cell counts and body temperature in the brown bear is unknown.

Nevertheless, there is also a critical need for understanding the interplay between internal state including these variables and external factors such as climate variability. With the knowledge of the physiological and environmental cues and drivers of bear behavior, we would better understand how well the bear’s phenotypic plasticity can respond to future changes.

**Ecophysiology in Brown Bears**

Although robust models have been developed to predict the effects of climate variability on animal population dynamics, scientists now recognize the necessity of incorporating physiological and behavioral data into models based on mechanistic, trait, and demographic data. Indeed, it is now
accepted that phenotypic plasticity can modify model predictions (Kearney and Porter 2009, Bradshaw and Holzapfel 2010) because it is the individual-centered physiological responses that govern the link between environmental change and individual performance. So far, most studies have investigated how environmental changes alter the phenology, morphological traits, and population dynamics of various species (Ozgul et al. 2010, Lane et al. 2012). However, the mechanisms underlying these responses remain largely unknown, owing to a paucity of long-term physiological data collected from free-ranging animals, particularly in conjunction with behavioral and environmental data. In light of these shortfalls, the extent to which phenology is based on physiological time-keeping mechanisms, such as in hibernation, has been widely overlooked.

In hibernating mammals, hibernation patterns are important determinants of survival (Turbill et al. 2011). In fact, studies of small hibernators suggest that climate variability might affect hibernation patterns and survival, as in the case of the yellow-bellied marmot (Marmota flaviventris) exiting the burrows much earlier due to warming spring temperatures in spite of a consistent length of snow cover (Inouye et al. 2000). Although energetics can be used as a simple predictor of a climate change associated with northward expansion of the distribution of species, including the little brown bat (Myotis lucifugus) (Humphries et al. 2002), climate variability can result in decreased fitness for other species, as a consequence of the decoupling of environmental cues and actual food availability (Lane et al. 2012). Mismatches between thermal and photoperiod cues can pose a major challenge for hibernators (Bradshaw and Holzapfel 2010). Therefore, the phenology and interdependency of physiological, behavioral, and ecological events bracketing the hibernation period are likely to provide important insight into individual plasticity to environmental challenges.

Data on hibernating bears are mainly derived from captive studies. The $T_b$ of captive American black bears appears to parallel decreases in ambient temperature ($T_A$) in autumn until a threshold of 31.8°C is reached (Craighead et al. 1976). In a study using HR monitors on three captive grizzly bears (also $U. arctos$), decreases in HR during winter were not observed until the removal of light, food, and water (Folk et al. 1976). When these bears were disturbed, fed, watered, and moved to different enclosures, their HRs increased again for several weeks (Folk et al. 1976). However, the artificial conditions, including composition and availability of food and visual, auditory, and olfactory stimuli, may have affected the timing of den entry and exit; thus, such studies may not necessarily give a true representation of what happens in the wild.

Data on free-ranging bears are scarce and suffer from the lack of environmental and phenological covariates to physiology and, more importantly, of methods to determine den entry and exit accurately.
In brown bears, the approximate timing of den entry and exit were reported to correlate with food availability (Servheen and Klaver 1983, Ciarniello et al. 2005), latitude, life history, and environmental factors, such as date of first snowfall, photoperiod and temperature (Friebe et al. 2001, Manchi and Swenson 2005). In fact, $T_a$ possibly provides a physiological cue for entering hibernation and, with food availability (Manchi and Swenson 2005), could explain why bears hibernate longer at high latitudes (Manchi and Swenson 2005). However, there are no studies on the relative importance and interdependence of these factors, nor the actual sequence of events that trigger den entry and exit (Friebe et al. 2001, Manchi and Swenson 2005).
Objectives of the Thesis

Overall Objectives

- to contribute to our knowledge of basic hibernation physiology in the brown bear, the role of $T_b$ in the processes of hibernation on both a small scale (blood chemistry and hematology) and a broader scale (the interplay between environment, hibernation, body size, $T_b$ and HR) and to evaluate the impact of potential stressors including capture

Methodological Issues

Develop a capture protocol for hibernating brown bears and evaluate its immediate effects

- to develop an efficient capture and anesthesia protocol for hibernating free-ranging brown bears, to assess arterial oxygenation to determine if supplemental oxygen should be administered and to evaluate the disturbance that the captures caused to the bears (Paper 1)

Evaluate longer term effects of capture of hibernating brown bears

- to describe the disturbance that the captures caused to the bears, determine if they returned to hibernation again, and document the length of time they were affected by altered behavior and physiology (Papers 1 and 2)
- to determine if the capture-caused disturbance could be considered an “arousal,” using definitions from small hibernators (Paper 2)

Basic Biology of Brown Bears

Investigate seasonal changes in hematology and serum biochemistry

- to describe seasonal changes in hematological and biochemical variables (Paper 3)
- to compare immune cell counts in peripheral blood during hibernation and the active period in summer (Paper 4)
- to describe seasonal changes in oxygen affinity and levels of the red cell hemoglobin-cofactor 2,3-diphosphoglycerate (Paper 5)

Ecophysiology

Describe the environmental, behavioral, and physiological factors contributing to den entry and den exit (Paper 6).

- to examine the interplay between ecological, behavioral, and physiological time-keeping mechanisms involved in the hibernation processes of free-ranging brown bears
• to identify and apply statistical techniques to estimate den entry and exit dates and assess causation between biotic and abiotic time-series variables

Use biologging techniques to describe the gestation of brown bears (Paper 7)

• to document dates of implantation, parturition, and the gestation by comparing the body temperature of pregnant females before, during, and after the gestation period and also to the body temperature of nonpregnant females

• to determine if activity data (recorded in GPS collars) and body temperature data (recorded in implanted temperature loggers) would yield the same dates of implantation or parturition

• to determine which factors influence the timing of gestation including age, litter size, primiparity, environmental conditions during the season before hibernation, and the date of the start of hibernation affect the timing of parturition

Evaluate the effect of body size on hibernation phenology (Paper 8)

• to describe the annual temperature cycle of brown bears and to determine if there is a seasonal pattern regarding relationships between body temperature and body mass
Materials and methods

Study area

The study area was located in south-central Sweden (Figure 1: 61°N, 15°E), in the northern boreal forest zone in Dalarna and Gävleborg counties. The terrain is hilly, with altitudes ranging from 200 m in the southeast to 1,000 m in the west, but is mostly (>90%) below timberline, which is at ~750 m (Dahle and Swenson 2003). Snow cover usually lasts from the end of October until late April and mean daily temperatures range from −7°C in January to 15°C in July (Swedish Meteorological and Hydrological Institute).

Study Population

The Swedish brown bear population was estimated to be 3,298 individuals (2,968-3,667; 95% confidence intervals) in 2008 (Kindberg et al. 2011). The denning period in the study area is from October until May, and its duration varies depending on individual demographics. The timing of den entry is influenced by sex, reproductive status, and environmental conditions, as well as age and body size (Friebe et al. 2001, Manchi and Swenson 2005). Pregnant females spend on average 196 days in the dens, about one month longer than nonpregnant bears (Friebe et al. 2001, Manchi and Swenson 2005). Pregnant females enter their dens first and leave their dens latest (Friebe et al. 2001).

Figure 1. The brown bears included in this thesis were from the core of the Southern study area of the Scandinavian Brown Bear Project (black box).
Monitoring and Devices

Brown bears were captured by darting from a helicopter from April to June 2010-2015 (Fahlman et al. 2011, Arnemo et al. 2012). The bears were fitted with collars, which included a global positioning system (GPS), dual-axis motion sensors to monitor activity (Friebe et al. 2014), very high frequency (VHF) transmitters, and a global system for mobile communications (GSM) modem (Vectronic Aerospace GmbH, Berlin, Germany). The devices recorded GPS positions every 30 minutes. The offspring of marked females were followed from birth; otherwise, age was determined by counting the annuli of a cross-section of the premolar roots (Harshyne et al. 1998). The Ethical Committee on Animal Experiments, Uppsala, Sweden (application numbers C47/9 and C7/12) and the Swedish Environmental Protection Agency approved all captures and procedures.

To conduct the studies in this thesis, we used several physiological sensors and loggers. For $T_b$, we used the Star-Oddi Centi loggers (DST Centi, Star-Oddi, Gardabaer, Iceland, 46 x15 mm; 19 g). With a memory capacity of 175,000 temperatures, the data loggers could record $T_b$ every 3 min for up to 1 year with an accuracy of $\pm 0.1^\circ C$ (Star-Oddi 2011). For cardiac monitoring, we used the Medtronic Reveal DX and XT (Medtronic Inc., Minneapolis, Minnesota, USA; 8 mm x 19 mm x 62 mm; 15 g). The Medtronic Reveal devices reported daytime mean HR (08:00–20:00) and nighttime mean HR (0:00–04:00) and contained ECG and acceleration sensors (Laske et al. 2011). It determined heart rate variability (HRV) by calculating 5-minute medians of ventricular intervals in milliseconds during sinus rhythm and computing the standard deviation of those medians over each 24 hour period (SDANN). The accelerometer recorded how many minutes of each day the bears were active.

We sterilized all implants with ethylene oxide gas (Anaproleve AN74i 60L, Andersen Europe, Kortrijk, Belgium) and programed temperature loggers to record $T_b$ at intervals ranging from 1 to 30 min, depending on other ongoing studies. Each temperature logger was individually calibrated from the manufacturer for 41 set points over the range 5°C to 45°C with a guaranteed accuracy of $\pm 0.1^\circ C$ for the full temperature range one-year post calibration. The equipment used for the calibration of the loggers, as stated on the calibration certificate from the manufacturer, is a Hart 7012 temperature bath, and the reference measurements are conducted with a Hart 1504 thermometer and a Hart 5610-9 thermistor probe with combined absolute accuracy better than $\pm 0.010^\circ C$. Each set point measurement was taken when the temperature was stable within 0.001°C. We surgically implanted temperature loggers into the abdomen (Arnemo et al. 2012). In some cases, temperature loggers were surgically removed and replaced in conjunction with a change of collar (at intervals of 1-2 years). We used insertable cardiac monitors (Medtronics Reveal DX and XT). We surgically implanted them peristernally on the left side.
between the muscle and subcutaneous fat and closed the incision using 2-0 monofilament glycomer (Biosyn Corporation, Carlsbad, California, USA).

We obtained $T_A$ and snow depth data for all of Sweden (620 weather stations) from the Swedish Meteorological and Hydrological Institute (SE-601 76 Norrköping, Sweden). These data were interpolated to a 1-km scale, which resulted in a daily map of $T_A$ and snow depth for the entire country. We extracted the local temperature at each bear location from these maps. Photoperiod was defined as the time between sunrise and sunset and was calculated for the same latitude (61.6) using the R-package Geosphere (Hijmans et al. 2012).

**Study Animals**

Bears were selected for the various studies based on several criteria including size, age, pregnancy status, and whether or not they were captured in winter. Papers 1, 2, 3, and 4 focused on the bears captured in both winter and summer (with Paper 2 including a control group of similar-sized bears that had not been captured). Papers 6 excluded bears captured in winter and those which were pregnant. Paper 7 included only pregnant females and a control group of similar-sized nonpregnant females. Paper 8 contained all available bears when possible but excluded pregnant bears from the months where the pregnancy was expected to have an effect and captured bears from February and March.

**Winter Captures**

Winter captures were carried out in February-March 2010-2015 with the same bears recaptured in June-July. We selected 29 individual subadult (2-4 years old) brown bears (seven were caught in multiple years, giving a total of 36 winter captures). Paper 1 includes the first 13 pairs of winter and summer captures (2010 and 2011). We chose subadult bears because larger animals were considered to pose greater risk to the capture team. Snow depth ranged from 70–120 cm with temperatures ranging from $-15^\circ$C to $+3^\circ$C.

Bears were located using GPS and VHF radio collars/implants. The dens were 500 m to 20 km from plowed roads, so when necessary we used snowmobiles to transport equipment and the field team to within 200-800 m of the den. Then field personnel used skis to approach and locate the den entrance. After the snow had been removed, a metal grate was placed over the opening. Two field personnel held the grate (with assistance from others when necessary) while anesthetic agents were administered by remote darting through the grate using a flashlight and CO$_2$ powered rifle (Dan-Inject®, Børkop, Denmark) fired from 0.3–3.5 meters distance. Darts were 3 ml with a 2.0×30 mm barbed needle with side-ports (Dan-Inject®). The bears were anesthetized with a total dose of 0.6–2.5 mg of medetomidine.
(Domitor® 1 mg/ml or Zalopine®, 10 mg/ml, Orion Pharma Animal Health, Turku, Finland) and 31–188 mg tiletamine-zolazepam (Zoletil®, 500 mg/vial, Virbac, Carros, France). In four bears, 75–100 mg ketamine (Narketan 10®, 100 mg/ml, Chassot, Dublin, Ireland) was hand-injected before handling and for the remaining 32 immobilizations; 37–113 mg of ketamine was included in the dart. We administered a second dart with a full dose if the bear was still mobile after 10 minutes.

Once anesthetized, we took each of the bears out of the den and placed them on an insulated blanket. We measured rectal temperature, HR, and respiratory rate in all bears. We were unable to obtain pulse oximetry readings with a veterinary sensor clip placed on the tongue, lip, ears, or vulva from the first four bears during February, so we abandoned this for the remaining bears. Blood samples from the femoral artery were collected anaerobically in preheparinized syringes from ten bears at 15–25 and 65–75 minutes from darting. The samples were immediately analyzed with a portable analyzer (iSTAT 1® Portable Clinical Analyzer, Abbott Laboratories, Abbott Park IL, 60064-6048, USA). Blood gas samples and pH were corrected to rectal temperature. Intranasal oxygen was provided from a portable oxygen cylinder to eight bears via a nasal line inserted 10 cm into one nostril with an oxygen flow rate of 0.5–2.0 liters per minute after the first arterial sample was collected. From 2012, we used only 0.5 liter per minute (the lowest possible setting) and took more limited sampling for monitoring, calibrating the oxygen flow rate and for other studies requiring blood gas results.

Blood samples from the jugular vein were collected as reported previously (Græsli et al. 2014) within 60 minutes of immobilization using a vacutainer system (BD Vacutainer®, BD Diagnostics, Preanalytical Systems, Franklin Lakes, NJ, USA). Blood for hematological analysis was collected in 4 mL tubes, with EDTA (ethylenediaminetetraacetic acid) as anticoagulant (Vacuette®, Greiner Bio-One International AG, Kremsmünster, Austria). The blood samples were kept cool from sampling until analysis at the Clinical Chemistry Laboratory at the Swedish University of Agricultural Sciences, Uppsala, Sweden. The time from sampling to analysis was within two days. Blood for biochemistry was collected in 9 mL tubes with gel and clot activating factor (Vacuette®, Greiner Bio-One International AG). The samples were kept at room temperature for 1-2 hours to ensure complete clotting and then centrifuged to separate the serum. The serum was stored in 2 mL cryogenic vials (Nalgene, Nalge Company, Rochester, NY, USA) and kept at -20°C until shipment to the Central Laboratory, Norwegian School of Veterinary Science, Oslo, Norway. The samples were kept cool during shipment.

After sampling, we placed the bears back into the dens and antagonized the effects of medetomidine with atipamezole (Antisedan®, 5 mg/ml, Orion Pharma Animal Health, Turku, Finland)
given intramuscularly at 5 mg per mg of medetomidine. We covered the entrance with branches and snow, and the bears were left to recover undisturbed.

**Laboratory Analysis (Papers 3, 4 and 5)**

Blood gasses, hematology, and chemistry values were evaluated in the field for evaluation of captures using an iSTAT portable analyzer (iSTAT 1® Portable Clinical Analyzer, Abbott Laboratories, Abbott Park IL, USA) and in the laboratory for evaluation of seasonal changes in hematology and chemistry. Several laboratories were used, including the Central Laboratory, Norwegian School of Veterinary Science, Oslo, Norway (Chemistry, Paper 3) and the Clinical Chemistry Laboratory at the Swedish University of Agricultural Sciences, Uppsala, Sweden (Hematology, Paper 3). These were selected so that the results would be comparable to a previous study in the same population (Græsli et al. 2014). The more in-depth analysis of white blood cell counts (Paper 4) was done at the Clinical Chemistry Laboratory at Örebro University Hospital, Sweden. The laboratory analysis of the hemolysate and hemoglobin component (Paper 5) was performed in Aarhus, Denmark. Briefly, the samples were evaluated for the presence of different isoforms of hemoglobin. Then O₂ binding curves were measured for red blood cell lysates, and these were further tested for cooperativity and O₂ affinity. Red cell hemoglobin-cofactor 2,3-diphosphoglycerate (DPG) levels were also measured. Further experiments were performed on purified hemoglobin with added DPG at the summer and winter levels to evaluate whether the lower DPG content was the cause of changes in the hemoglobin-O₂ equilibrium curve during hibernation.

**Statistical Analysis**

**Chemistry and Hematology (Papers 3, 4 and 5)**

Normality of data sets for chemistry and hematology was assessed using the Shapiro-Wilk test. Those with an approximate normal distribution were not transformed, whereas non-normal variables were transformed to normality with a Box-Cox transformation. In the analysis of differences between seasons, eight bears had been resampled in the same season during multiple years. In these cases, one sample from each bear in each season was selected randomly using a random number generator. Seasons were defined as winter (February and March), spring (April and May) and summer (June and July).
We performed comparisons between seasons and sex on the transformed variables using analysis of variance. A significant $F$-test was followed by a simultaneous comparison of paired means using the Tukey-Kramer test. After calculating the 95% confidence intervals in the significantly different seasons, the transformed data were reconverted to the original data scale and corrections were made for any added values. For variables for which significant differences were not detected, sex and season cohorts were grouped together for presentation in Paper 3.

Mixed linear models with time as the repeated measure (hibernation vs. active), and bears and year of capture as random effects were used to test for effects of hibernation on innate immune cells. Further analyses were performed by adjusting for sex, age, and body masses to check for possible confounding factors on cell counts. The relationship between leukopenia (expressed as a percentage of active period levels) and the drop in $T_b$ during hibernation was checked by simple regression analysis. Two-sided $p$-values are reported, and significance was set to $<0.05$.

For the analysis of hemoglobin and its components, paired $t$-tests were used for the comparison between summer and winter. Comparisons in that study were statistically significant with $P \leq 0.001$. In one case, $P_{50}$ of hemolysates at summer 37 and 30°C was non-normally distributed, so the Wilcoxon signed rank test was used.

**GPS Data Analysis (Paper 1 and 6)**

To determine the time and distance moved after capture, we used the movement data recorded from GPS collars. Activity data collected using accelerometers fitted within the GPS collars was used to determine when the bear had become inactive, so the last inactive measurement was considered the end of the period of use for a den or bed site. Denning sites were identified by clusters of positions (defined as 6 GPS positions within 50 m). Clusters were divided into dens and beds by follow-up visits during May and June. Arrival at a den or bed was the first GPS location within the cluster. Bed or den use was defined to have begun at the time of the first inactive measurement after arrival.

To determine den entry and exit with greater precision than in Paper 1, we used behavioral changepoint analysis (BCPA, see Paper 6) on the GPS data. This method sweeps through changes in the magnitudes of animal movement speeds and changes in direction, to detect points of behavioral change (Gurarie *et al.* 2009). While this method proved reliable and useful, we were able to compare the results of this analysis to $T_b$ (bears exited the dens at mean $+S.E. 36.7\pm0.15^\circ C$) and concluded that $T_b$ can be used as an efficient means of determining the end of the denning period – this was used in subsequent analysis (Papers 2 and 7).
**Body Temperature, Heart Rate and the SDANN Index**

For Papers 4 and 5, rectal temperature was collected using an accu-temp digital thermometer (Jahpron Medical Int., Bodø, Norway), with a manufacturer-reported accuracy of ±0.1ºC. In Paper 4, this was used in a simple regression analysis to compare white blood cell counts and T_b between species from earlier studies. In subsequent papers, T_b was collected using Star-Oddi loggers as described in “Monitoring and Devices.”

As a measure of autonomic nervous system activity, we used the SDANN (standard deviation of all the five-minute NN interval means). We used this index as an indirect measure of sympathovagal balance, or the dynamic interaction between the acceleratory sympathetic and deceleratory parasympathetic nervous system’s input (Maros et al. 2008). Low HRV is indicative of increased sympathetic or decreased parasympathetic tone and increased HRV indicates the reverse. HR is highly affected by movement, whereas HRV is less so, making it a better indicator of psychological stress, especially in combination with HR.

To identify changes in the slope of T_b and other variables (T_a, HR, photoperiod, and activity), we used GAMMS and determined the periods in which each was increasing or decreasing by computing the first derivatives of the fitted trends.

We then aligned both abiotic and physiological factors to the dates of den entry and exit, separated the den entry and exit periods, and set the entry/exit dates as time zero. We then aligned all data on time zero and determined the dates of significant increases or decreases in each parameter by fitting GAMMs. We superimposed the dates of significant changes on yearly average environmental variables (i.e. T_a, snow depth, and photoperiod) as a proxy of the microclimate experienced by the bear. This enabled us to determine the sequence of environmental and physiological events that were associated with den entry and den exit.

To identify the causal relationships between the monitored environmental and physiological variables, we used a convergent cross mapping approach devised to detect causal relationships between pairs of variables represented by time series (Clark et al. 2015). We used this method to test for causal relationships between T_a, T_b, HR, and SDANN. We tested specific relationships during critical periods of interest.

To determine the length of time bears had abnormal T_b and HR after capture (Paper 2), we used changepoint analysis (with binary segmentation algorithm) (Killick et al. 2014) to identify the significant points of change during hibernation, including den capture and return to the hibernation curve (which was already in the a warming phase). The period from den capture to return to hibernation was called
the “disturbance period.” Summary statistics were done for the disturbance period and the week before and after for both variables for each bear.

To quantify the magnitude of the disturbance, we made a two-step analysis. We first calculated the under-the-hibernation curve for $T_b$ for each bear (captured and not captured) and then subtracted this from the area given by the highest daily average $T_b$ measured during the hibernation period (39.3°C)(Figure 2). We used the area between this two curves (ABC) as an energy saving index. Using linear mixed effect regression models (lme) we analyzed how capture and body mass related to this energy saving index with bear ID as random effect. (Paper 2). This analysis was repeated in Paper 8 to further evaluate the effect of body size, by adding additional bears (45 years of data from 34 bears) of higher body mass and grouping the bears into three size groups, with “small” defined as 30-60 kg (N=19, 11 captured in winter), “medium” as 60-120 kg (N=19, 1 captured in winter), “large” as 120-240kg (N=6). Pregnant bears were included in the descriptive statistics for June-Sept, but for the models they were removed from the categories “medium” (N=7).

Figure 2. Illustration of the area between the curves (ABC) used as an index of energy saving. The method takes the area between highest daily average $T_b$ measured during the hibernation period (dashed line, 39.3 °C) and the measured body temperature curve (solid line, $T_b$) example (a), captured bear with body mass 58 kg and (b) an undisturbed bear with body mass 57 kg.
In both Papers 2 and 8, we used the day of the year that the daily mean $T_b$ rose above 36.7°C as the den exit date, as this $T_b$ is associated with den exit in brown bears (Paper 6). In Paper 2, we used a GLM model to test for effects of capture and body mass on the end of the hibernation period and in Paper 8 this analysis was repeated with additional bears to evaluate further the effect of body mass. In Paper 8, we further evaluated the effect of body mass on den entry, In Paper 8, we further evaluated the effect of body mass on den entry (date when $T_b$ remains below 36.5°C, Paper 6), den exit, and denning duration. Again, we used lme models with to test for effects body mass on the dates of den entry and exit and on the duration hibernation period including Bear ID as a random effect.

To further evaluate the effect of body mass, we used $T_b$ as the response variable and the three size groups as predictors in lme models for each month, again with Bear ID as a random effect. Tukey contrasts were then used to compare means and p-values between the size groups in each month.
Results and Discussion

Methodological Issues

Development of a Winter Capture Protocol for Brown Bears (Paper 1)

When the bears were located in their dens, they were alert and attempted to escape, often digging at the opposite end of the den. In 2010-2011 (Paper 1), two bears that were denning under rocks were darted while fleeing, running 40 and 200 meters. In the other 11 captures, the darting and induction were uneventful, except for one case where the drugs in the dart froze, and the bear required a second dart, and another instance where the bear managed to escape around the grate after darting. During 2012-2015, an additional 23 captures were carried out, most of these went smoothly, although one more bear under a rock den was darted while fleeing (running 200 m before being immobilized), two bears fled the dens before the capture team had reached the area, and one fled when a field worker was scouting the area to plan the capture. Two two-year-old sibling bears denned together and were immobilized together. Although the immobilization went smoothly, they moved to separate dens afterward. One bear broke two incisors while trying to escape from the den.

Drugs and doses are presented in Table 1 and Paper 1 (2010-2011). The alert behavior we observed is contrary to that reported for American black bears, which can be approached even when denning in open nests (Powell 2005). We achieved the best quality of anesthesia during winter for the bears darted with the lowest doses of medetomidine, zolazepam-tiletamine, combined with ketamine in the dart to speed up induction, and to deepen anesthesia without depressing respiration or prolonging recovery. With the addition of ketamine in winter, ground darting and anesthesia of hibernating bears was possible with 25% of the doses of medetomidine and tiletamine-zolazepam used for the same bears in summer.
Table 1. Body mass, age (years), and drug doses (mg) used for anesthesia of brown bears during winter and summer. Individuals captured in 2010 and 2011 are included in Paper 1. Missed darts are not included. “D” after medetomidine dose indicate the two individuals that received dexmedetomidine instead of medetomidine during summer. They received 1.25 mg dexmedetomidine, theoretically equivalent to 2.5 mg medetomidine. Four bears were not recaptured in summer, § indicates killed by adult males, # drowned during the capture, and ¤ escaped from the capture team without being darted. Two bears* stopped breathing during induction, were given manual respiratory support and recovered.

<table>
<thead>
<tr>
<th>Bear</th>
<th>Year</th>
<th>ID</th>
<th>Weight (Kg)</th>
<th>Tiletamine-zolazepam</th>
<th>Medetomidine</th>
<th>Ketamine</th>
<th>Induction time (min)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>Summer</td>
<td>Winter</td>
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<td>1</td>
<td>63 250</td>
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<td>2.5 5.0</td>
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<td>2 1</td>
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<td>2.5 5.0</td>
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<td>1.3 5.0</td>
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<td>1 1</td>
<td>63 250</td>
<td>1.3 5.0</td>
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</table>

**Physiological Evaluation at Time of Capture (Paper 1)**

During the physiologic evaluation at the capture, we found that bears were hypothermic, bradycardic, and had alterations in pulmonary gas exchange and acid-base status. The hypoxemia was readily corrected with intranasal oxygen supplementation.

Although combinations with medetomidine and zolazepam have been given at high doses to captive bears without apnea or cardiac arrest during the active season, one bear became apneic. This individual (a 2-year old male, 27 kg) received the highest dose (per kg) of all bears in the study (0.05 mg/kg medetomidine, 2.5 mg/kg zolazepam-tiletamine, and 3 mg/kg ketamine). The bear was...
unresponsive to 50 mg doxapram administered intravenously and was intubated and ventilated manually (with additional supplemental oxygen). This bear received what was ultimately determined to be four times the recommended dose, which illustrates that the therapeutic index for this combination during winter captures is lower than during the active period, where bears reportedly tolerate up to 11 times the standard doses used without major respiratory or cardiac depression (Arnemo et al. 2003).

Hypoxemia was recorded before oxygen supplementation in seven of ten bears in winter (PaO$_2$ 30–74 mmHg) and two of ten bears in summer (PaO$_2$ 66–69 mmHg). A second arterial sample was collected from nine bears in winter, receiving 0, 1 and 2 liters per minute of oxygen, and ten bears in summer, receiving 0.5 or 1 liter per minute. In the second winter sample, the seven bears receiving 1 liter per minute had PaO$_2$ levels of 100–387 mmHg and the bear receiving 2 liters per minute had a PaO$_2$ of 301 mmHg. On the second summer sample (while receiving either 0.5 or 1 liter per minute O$_2$), bears had PaO$_2$ levels of 89–180. In winter, eight of the bears evaluated had initial pH values of less than 7.25 (marked acidemia) and the other two had mild acidemia (7.25–7.35). In summer, three of ten bears had marked acidemia (7.12–7.25), five had mild acidemia (7.25–7.34), and two were 7.35 (within the normal range).

Despite the lower drug doses used in winter, seven of nine bears captured in winter developed mild to marked hypercapnia, whereas only two of ten bears developed mild hypercapnia during capture in June. Mild hypercapnia can be beneficial, because it causes a shift in the oxygen-hemoglobin dissociation curve to the right, increasing the unloading of oxygen at tissues, enhancing oxygen delivery, and carrying capacity (Johnson and Morais 2006). This expected effect of hypercapnia is in contrast to the finding of a left-shift later discussed in Paper 5. This difference likely illustrates that the hypercapnia seen here is a result of physiologic alterations during anesthesia, rather than related to hibernation per se. Despite the potential benefits of hypercapnia, monitoring hypercapnia was important, as severe hypercapnia can cause tachyarrhythmia, hemodynamic instability, and coma (Johnson and Morais 2006). The acidosis (decreased pH) seen in winter, was mainly attributed to the hypercapnia (respiratory acidemia), whereas in summer, the bears more commonly developed lactic acidemia, which can be attributed to the muscle activity while fleeing the helicopter. All tested bears had higher glucose, hematocrit, and hemoglobin during winter than during summer, consistent with Paper 3. As the intranasal oxygen supplementation at a flow rate of 1 L/min increased the PaO$_2$ to 100-387 mmHg (target 80-120 mmHg) in the seven bears receiving this flow rate, evaluation of 0.5 L/min was conducted after publication of Paper 1. All 12 bears weighing 27 to 56 kg had PaO$_2$ levels above 80 mmHg 15 minutes after oxygen supplementation.
**Behavioral Disturbance (Paper 1)**

Bears left their original dens following capture on 32 of 36 occasions. Four of the 35 instances of capture from which we obtained GPS and activity data remained at the original den. The first 13 captures were included in Paper 1 and were included in more detailed analysis than the subsequent captures. One of the 13 bears included in Paper 1 remained at the original den until spring, one did not have a GPS collar and eleven left their dens, (mean ± standard deviation) 3.2±3.6 (range 0.5–10.5) days after capture. They used 1.9±0.9 intermediate resting sites during 6.2±7.8 days before entering a new permanent den. The eleven new permanent dens were located 730±589 m from the original dens. A subsample of bears from the same area which not captured in the dens had an abandonment rate of 26% (20/76, 2004-2011), but the majority occurred in October/November and only 3 (4%) abandoned their dens during January-March (4%). Den-captured bears were significantly more likely to leave their dens compared to marked, but uncaptured bears during the same period ($\chi^2_{(1, N=70)}=\text{59.1}, p<.0005$). However, the abandonment rates of den-captured bears were highly significant, even when comparing with overall abandonment rates ($\chi^2_{(1, N=70)}=\text{20.5}, p<.0005$) or when excluding those occasions where bears moved between dens early in the season ($\chi^2_{(1, N=56)}=\text{45.2}, p<.0005$). Den emergence occurred from 5 April until 23 April in 2010, and for six of seven bears, 19 April until 22 April in 2011, similar to other bears in the study area in the respective years.

Captures in rock dens (N=5, two of these are reported in Paper 1) proved to be more complex than other types of den captures. Three of five bears approached while in rock dens escaped from their dens and were darted outside the dens. These were the only bears that escaped from their dens before darting. One additional bear escaped after darting and ran 200 m before being immobilized. All of the bears originally denning in rock dens changed dens and chose new rock dens. Although the difficulties with capture at rock dens would suggest that pre-assessment of the den site before capture would be useful, there was also one instance where the bear left the den during the pre-assessment and could not be included in winter captures. Therefore, den type and surrounding terrain should be considered when planning captures, although not at the cost of disturbing the bear.

Den abandonment for captured bears was dramatically higher than the 4% typically changing dens during mid-winter. Thus, the process of captures likely was energetically costly, especially for the bears taking longer to find new dens (Swenson et al. 1997, Linnell et al. 2000). Once out of the dens, most of the bears made a couple of attempts before locating a den that they settled into for the rest of the denning period. Anthill and soil dens were the most common den types for the original dens, whereas rock dens, nest dens, and beds were the most common types of the second permanent dens. The
difference between a bed and a nest den is the amount of material used in its construction. The choice of second den type likely reflected availability and the terrain around the den sites, especially when considering the deep snow cover and frozen ground.

**Physiological Disturbance (Paper 2)**

The changepoint analysis showed that the mean duration of the body temperature disruption of the normal hibernation pattern following capture was 16.1±6.9 days. For HR, the disturbance period lasted for 20.9±6.8 days. The week before disturbance, bears had a mean±SD, min-max temperature of (33.6±0.8°C, 30.6°C-35.3°C) and daytime HR of (15±3, 10-28 beats/min). During the disturbance period itself, the mean temperature was (36.0±1.3°C, 32.0-39.3°C) and HR (32±9, 13-64 beats/min). The week afterward the body temperature was 34.6±0.9°C (32.3-36.8°C) and HR was 22±5, 15-47 beats/min.

Den capture of hibernating brown bears caused increased HR, $T_b$, and activity levels for several weeks postcapture. $T_b$ and HR did not return to their precapture levels, but rather to a later phase in the normal arousal process. However, the overall effects of the capture on the magnitude and duration of hibernation were undetectable in terms of the overall body temperature profiles during hibernation. Although the body temperature returned to the hibernation curve faster than the daily mean HRs, both were affected. Arousal from hibernation is known to be energetically costly, with metabolic rate reaching several times the basal metabolic rate. In arctic ground squirrels, arousal episodes are the most energetically costly component of hibernation, accounting for the majority of energetic costs during the hibernation period (Karpovich et al. 2009). In small hibernators, arousal is defined as a period of euthermia (Karpovich et al. 2009). In this study the bears reached 36.28±1.31°C, consistent with this definition of arousal, and it can be hypothesized that this episode has similarly high energetic costs. Unlike ground squirrels (Karpovich et al. 2009), the bears in our study did not return to their pre-arousal body temperatures (Figure 3) but $T_b$ and HR curves followed the patterns of the undisturbed bears. In contrast to arousals in small hibernators, the bears did not have a sustained period of active-level HRs, which in the small hibernators can be up to 500 beats per minute (Milsom et al. 1999).
Figure 3. Comparison of physiological variables in captured (blue line) and undisturbed (red line) hibernating brown bears in Sweden. (a) Mean daily body temperature ($T_b$) of captured (N=11) and undisturbed (N=11) bears and (b) mean daytime heart rates (HR) of captured (N=7) and undisturbed (N=11) bears. The solid lines show the daily means for individual captured bears with standard errors as shaded areas; the dotted vertical line shows the average beginning and end of the disturbance period.
Methological Issues – Summary

We developed an effective and quite safe capture and anesthesia protocol for hibernating brown bears. During anesthesia, most bears were hypoxemic, but this was easily treated or prevented with supplemental oxygen. We found, as expected, that the brown bears were extremely sensitive to disturbance, with the majority changing dens after capture. The arousal caused by capture during hibernation appears to be energetically costly, but we could not detect changes in depth and duration of hibernation due to a capture-caused period of physiological disruption lasting 2-4 weeks.

Basic Biology of Seasonal Changes

Chemistry and Hematology (Paper 3, 4 and 5)

Seasonal changes were found in eight of twelve hematological variables and in all biochemical variables, except creatinine kinase, calcium, potassium, α₁-globulin, γ-globulins, lipase, and cortisol. Hemoglobin, hematocrit, albumin, β-hydroxybutyrate, creatinine, and triglycerides were significantly higher during winter compared to spring, and significantly higher during spring compared to summer. The values of the serum enzymes alanine transaminase, aspartate transaminase, alkaline phosphatase, and lactate dehydrogenase, were significantly higher during summer than during spring, and the values during spring were significantly higher than during winter. The levels of red blood cells, eosinophils, lymphocytes, total protein, and cholesterol were significantly higher during winter compared to spring and summer, whereas the values of mean corpuscular volume, total white blood cell count, segmented neutrophils, amylase, γ-glutamyl transeptidase, and bile acids were significantly lower during winter compared to spring and summer.

The changes found here represent the many aspects of altered physiology seen during winter. The increased hemoglobin, red blood cells, hematocrit, total protein, and albumin can be attributed to hypovolemia from the lack of liquid intake. The shift from carbohydrate and protein metabolism to fat metabolism results in decreased urea production and reduced demand for the enzymes important for protein breakdown (alanine transaminase, aspartate transaminase, lactate dehydrogenase, γ-glutamyl transeptidase, glutamate dehydrogenase, and amylase). Fat catabolism also resulted in increased blood lipids, triglycerides, cholesterol, and free fatty acids and fasting resulted in increased β-HBA, lower bile acids, enzyme (lower in 5 out of 9 enzymes tested), and urea levels. The general depression of metabolic rate is also represented by the decreased organ function, including decreased kidney (increased creatinine and magnesium), liver (increased ALP), and pancreas (decreased amylase) function and suppression of the innate immune system in winter (neutropenia).
In Paper 4, we did an additional evaluation of leukocytes. Peripheral total blood leukocyte counts, neutrophils, and monocytes were significantly lower during hibernation than during the active period, as reported above. These findings are consistent with results from small hibernators (Bouma et al. 2010). In contrast to Paper 3, where % lymphocytes was much higher in winter, Paper 4 used total lymphocyte counts; there was no change in total lymphocyte counts between the hibernation and active periods. This indicates that the increase in % lymphocytes was due mainly to decreases in the other types of white blood cells. Using body temperature taken at the captures in spring and during summer and comparing with $T_b$ and leukocyte counts from 10 other studies on 9 hibernating species suggested that $T_b$ explained the decrease in immune cells and an important driver of immune function regulation during hibernation.

Our data from brown bears are consistent with reports from other species that the immune system is suppressed during hibernation, likely resulting in altered and reduced defense mechanisms (Bouma et al. 2010), as seen in fungal infections in hibernating bats (Cryan et al. 2010). In small hibernators, it is hypothesized that one reason hibernators have periodic arousals is to reactivate the immune system to combat any pathogens entering during this period of reduced immune function (Prendergast et al. 2002).

For the evaluation of hemoglobin structure and function, we found a single hemoglobin component, indicating a consistent pathway for the synthesis of hemoglobin. For the oxygen binding curves at 30°C and 37°C, there was less temperature sensitivity in the oxygen affinity than that seen in other vertebrates. In winter, hemolysates showed lower cooperativity and higher oxygen affinity than in summer. The increased oxygen affinity seen in winter corresponded to a significant decrease in hemoglobin-cofactor 2,3-diphosphoglycerate (DPG) during hibernation to approximately half of the summer value. The left shift in hemoglobin-O$_2$ equilibrium (Figure 4) was attributed to the low DPG content by experiments performed on purified hemoglobin, where DPG had been added to match summer and winter levels (Paper 5). We could use the low levels of plasma lactate (from Paper 1) to conclude that glycolysis was not upregulated during hibernation and that metabolism was primarily aerobic. The increased hemoglobin-Oxygen affinity and decrease in cooperativity, as a result of the
decreased red cell DPG, might be a crucial mechanism for maintaining consistent tissue oxygen tension during hibernation. These results are in spite of the increased CO2 found in bears during winter (Paper 1), which, according to the Bohr Effect, should shift the hemoglobin-oxygen saturation curve to the right (Bohr et al. 1904), thereby indicating that these mechanisms override the effects of hypercapnia.

**Basic Biology Summary**

We describe seasonal changes in eight of twelve hematological variables, and in all biochemical variables except creatinine kinase, calcium, potassium, $\alpha_2$-globulin, $\gamma$-globulins, lipase, and cortisol. Most of these changes illustrate the bear’s altered physiology during winter. We were able to link the change in leukocyte counts with $T_b$ changes, showing that the immune system changes are $T_b$-dependent. The seasonal changes in oxygen affinity and red cell hemoglobin-cofactor 2,3-diphosphoglycerate were independent of $T_b$, but allow for maintenance of tissue oxygenation in the face of lower respiratory rates and lower blood oxygen levels during winter.
The behavioral changepoint analysis allowed for the determination of den entry and exit dates using the GPS data. We found that the bears entered their dens during October and November (median 30 October) and exited from 21 March to 6 May (median 6 April). The high variability seen between years could be explained by variation in \( T_A \) between years (Table S2), with the warmer years associated with later entry and shorter hibernation (winter 2010-11 hibernation, mean±S.D: 175.3±22.4 days versus 151.2±15.3 days for winter 2011-12; t-value= 6.78, p=0.03). The order of the drop of the variables is presented in Figure 5, with \( T_b \) falling 13 days before entry, 25 days before the activity drop, and 24 days before the HR drop. Bears entered the den when the snow arrived and when \( T_A \) reached 0°C. HRV, taken as a proxy of sympathetic nervous system activity, dropped dramatically once the bears entered the dens. This drop indirectly suggests that denning is tightly coupled to metabolic suppression. During arousal, the unexpected early rise in \( T_b \) (two months before den exit) was driven by \( T_A \), but was independent of HRV. The difference between \( T_b \) and \( T_A \) decreased gradually, suggesting that bears were not thermoconforming (adapting to the surrounding environment to avoid thermoregulation). HRV increased only three weeks before exit, likely indicating activation of the sympathetic nervous system to restore euthermic metabolism. Bears later exited the den when \( T_A \) reached their presumed upper critical temperature. Therefore, we found that hibernation was initiated primarily by environmental cues and terminated by physiological constraints.

Figure 5. Sequence of environmental and physiological events in the entry and exit into hibernation (From Paper 6).
**Gestation (Paper 7)**

Six adult female bears became pregnant after instrumentation with temperature loggers. Using the spike in $T_b$ as the primary indicator for the start of pregnancy, the mean date of implantation was 1 December ($\text{SD} = 12$), the mean date of parturition was 26 January ($\text{SD} = 12$), and the mean duration of the gestation period was $56\pm2$ days. The $T_b$ of pregnant bears rose to euthermia for the gestation period and dropped after parturition. We found that parturition could also be detected from activity recordings; however start and length of gestation was less accurate with activity recordings.

$T_b$ clearly indicated dates of implantation and parturition (Figure 6) with the gestation period calculated to be between 54 and 59 days long. $T_b$ was significantly higher during the gestation period compared with pre-gestation, lactation, and hibernation in nonpregnant adult females and there were no multiday cycles, as observed in nonpregnant hibernating bears (Tøien *et al.* 2015). The mean daily $T_b$ during gestation stayed above $35.9^\circ\text{C}$.

The temperature dropped at the end of gestation, clearly showing parturition. However, the brown bears, in contrast to American and Asiatic black bears (Tøien *et al.* 2011, Shimozuru *et al.* 2013), did not return to the level of nonpregnant bears, but rather had a slightly higher $T_b$ during lactation, consistent with a previous report in brown bears (Hissa 1997). Metabolic activity during lactation likely requires or results in higher $T_b$ levels.

Analysis of activity data produced similar results for determining the date of parturition and detecting pregnancy, however determining the date of implantation was not possible with activity data alone, because the timing of implantation varied $17\pm5$ days between $T_b$ and activity recordings. In some cases activity rose before implantation, which could be due to hormonal changes before implantation, or just the general, previous finding that activity is higher in early hibernation (Friebe *et al.* 2013). However, activity data can still be used to pinpoint implantation by subtracting 56 days from the parturition date calculated by the activity.

![Figure 6](image_url)

**Figure 6.** Body temperature ($^\circ\text{C}$) for pregnant (N=6, colored lines) and the mean body temperature and standard deviation of nonpregnant bears (N=9, black dots).
The dramatic range in parturition dates between individuals (43 days) showed a surprising amount of flexibility. A similar range has been reported for free-ranging American black bears (over 53 days for 150 litters (Bridges et al. 2011)). We did not find a relationship between the date of den entry and date of parturition, nor was there a significant effect of age. One study on captive brown bears found that larger females give birth earlier in winter (Robbins et al. 2012). Although we did not have predenning body mass, we used an environmental condition index and assumed females were heavier when environmental conditions were favorable. We found the opposite of that seen in captive bears – parturition occurred later when conditions were better. During the years with favorable environmental conditions, the pregnant females began hibernating earlier, rather than using energy reserves for early parturition and lactation, which would have maximized offspring mass at den emergence. During the years with poorer environmental conditions, there was a shorter period of hibernation before implantation. Early hibernation can be a strategy for predator avoidance, as seen in small mammals during good years (Bieber et al. 2014). The autumn is a high disturbance period for brown bears, with bear and moose hunting bringing people into the forests, so it is possible that this strategy of prioritizing early denning results in better survival.

Body Mass and Hibernation (Paper 8)

Size group was a significant predictor for Tb during most of the year; all size groups had significantly different means during November, December, January, February, July, and August. In November, December, January, and February, the smallest bears were warmest, with the reverse pattern in summer (Figure 8a & b). During the spring and fall, there was overlap between some groups, as the pattern reversed itself (Table 1 in Paper 8). According to the lme model used to test for the effect of body mass on the area under the Tb curve the effect of body mass (t= -5.48, effect size= -2.10) significantly affected the index of energy savings (p<0.01). The area between the curves (ABC) is plotted by body size in (Figure 7). We found no detectable effect of body mass on den entrance date (t=1.81, effect size= 0.07, p=0.11), however we found a significant effect of body mass on den exit date (t=-4.9536, effect size=-0.15, p<0.01) and denning duration (t=-4.19, effect size=-0.22, p=0<0.01).
We found that body mass played a significant role in some of the phenological aspects of hibernation; the smallest bears hibernated longest, coming out of the dens last. However, body size did not have an effect on den entrance date; in addition to the non-significant p-value, the low t-statistic also indicates indications that a relationship is unlikely. This is consistent with our previous finding that den entrance timing is dependent primarily on environmental cues (Paper 6). The small bears were warmest in summer and had the lowest $T_b$ in winter. This more dramatic difference between summer and winter can likely be attributed to the fact that smaller animals have a lower surface-to-volume ratio, resulting in more thermal conductance and poorer heat conservation. This result is consistent with the ABC, energy savings index; the smallest bears also had a consistently higher ABC, likely because they needed to save the most energy. The observation that the smallest bears came out of the dens last may be due to either, 1) that their thermal neutral zone is at a higher temperature than the larger bears, so they wait for a higher ambient temperature to come out in spring or 2) they have lower fat reserves a) are dependent on hibernating longer to conserve energy food availability increases.

Figure 7. Boxplot of area between the body temperature curves (ABC, an index of energy savings; a higher ABC shows lower energy savings) for brown bears in Sweden, compared between body mass size groups. “Small” (S) was defined as 30-60 kg (N=19), “medium” (M) as 60-120 kg (N=12) and “large” (L) as 120-240 (N=6). Pregnant bears (N=6) were excluded from the ABC analysis.
Figure 8. a). Pooled mean daily body temperatures applying a LOESS smoother to body mass (Size) of brown bears in Sweden centred on the hibernation period (a), and (b) centred on the active period. “Small” (blue) was defined as 30-60 kg (N=7), “medium” (green) as 60-120 kg (N=6) and “large” (pink) as 120-240 (N=6). Pregnant (N=6) and captured bears (N=11) were excluded from both graphs.
An alternative interpretation is that the larger bears did not have as deep or long hibernation, likely because of the greater energetic cost of warming up. The smallest bears probably need to save more energy than the largest bears and can warm up again at lower costs. For the larger bears, the benefit of hibernating at lower $T_b$ is probably offset by the increased cost of warming up a large body, and they also have more substantial energy reserves making the energy-sparing from deep hibernation less important. The larger bears can exit their dens earlier in the spring, as they have more fat stores in the spring. Thus, they can afford to search for uncommon, but rare, protein-rich food, such as ungulate carrion or weakened moose ($Alces alces$) (Dahle and Swenson 2003, Stenset et al. 2016), and can better withstand harsh weather if it occurs.

The large dataset available here allowed for comparisons across multiple size groups in free-ranging bears, where the relationship between environment and physiology was not manipulated. We conclude that the smallest bears hibernate most deeply and longest, likely because smaller animals have a lower surface-to-volume ratio, resulting in higher thermal conductance (Tøien et al. 2015), poorer heat conservation and greater need for energy savings due to their smaller fat reserves.

**Ecophysiology Summary**

To our knowledge, we have built the first chronology of both ecological and physiological events from before the start to the end of hibernation in the field, examining the interplay between environmental, behavioral, and physiological time-keeping mechanisms and applying statistical techniques to determine den entry and exit dates and to assess causation between factors.

We further used biologging techniques to describe the pregnancy of the brown bear, including implantation, parturition, and gestation, by comparing body temperature in pregnant and nonpregnant females and determined that activity data can be used to establish parturition date, and to give an estimate for the implantation date based on the gestation length. During years with better environmental conditions, pregnant females began hibernating earlier, but did not implant earlier. During years with poor conditions, there was a shorter period of hibernation before implantation.

We found that the differences in mass between individual brown bears had a dramatic effect on depth of hibernation, with the smallest bears hibernating at $T_b$, approximately 1.5°C lower than the largest bears. We found consistent relationships between body size and $T_b$ during December-March (with smallest bears coldest) and from July-September (with smallest bears warmest). During the other months, there was overlap between the groups, as they transitioned between these two patterns.
Conclusion

Biologging techniques are becoming an integral part of wildlife research and management. Here we report results from five years of biologging experience, including temperature and heart rate loggers in free-ranging brown bears. We used these loggers to assess the timing and duration of pregnancy, impacts of capture on hibernation, the interplay between physiological and ecological drivers of den entry and den exit, and effects of body mass and latitude on hibernation depth and duration. Den entry was driven by environmental conditions, specifically ambient temperature. HRV, regarded as a proxy of sympathetic nervous system activity, dropped dramatically once the bear entered the den, indirectly suggesting that denning might be tightly coupled with metabolic suppression. During arousal, the unexpected early rise in $T_b$ (two months before den exit) was driven by ambient temperature, $T_a$, but was independent of HRV. The difference between $T_b$ and $T_a$ decreased gradually, suggesting that bears were not thermoconforming. HRV increased only three weeks before exit, indicating that late activation of the sympathetic nervous system likely restored euthermic metabolism. Interestingly, it was not until $T_a$ reached the presumed lower critical temperature that bears exited the den. Hibernating bears took 15-20 days from capture before their $T_b$ and HR returned to the hibernation curve, but capture did not influence the length or magnitude of hibernation. We evaluated mean temperatures throughout the year in 34 bears and found that smaller bears consistently had higher $T_b$ in summer and lowered in winter (Dec-March). The use of heart rate and body temperature data have allowed us to fill in knowledge gaps about the basic ecology of free-ranging brown bears, while also providing a solid foundation for exposing further details of bear biology.

This thesis makes contributions to our knowledge of basic hibernation physiology in the brown bear, the role of body temperature in the processes of hibernation at both a small scale (blood chemistry and hematology) and a broader scale (the interplay between environment, hibernation, body size, body temperature and HR). It also applies these technologies to evaluate the impact of capture.
References


Barnes B. 1989. Freeze avoidance in a mammal: body temperatures below 0 degree C in an Arctic hibernator. Science 244: 1593-1595. 10.1126/science.2740905


Bradshaw WE, CM Holzapfel. 2010. Light, time, and the physiology of biotic response to rapid climate change in animals. Annual Review of Physiology 72: 147-166. 10.1146/annurev-physiol-021909-135837


Clark AT, H Ye, F Isbell, ER Deyle, J Cowles, GD Tilman, G Sugihara. 2015. Spatial convergent cross mapping to detect causal relationships from short time series. Ecology 96: 1174-1181. 10.1890/14-1479.1


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Inouye DW, B Barr, KB Armitage, BD Inouye. 2000. Climate change is affecting altitudinal migrants and hibernating species. Proceedings of the National Academy of Sciences of the United States of America 97: 1630-1633. 10.1073/pnas.97.4.1630


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Paper I
Capture, Anesthesia, and Disturbance of Free-Ranging Brown Bears (Ursus arctos) during Hibernation

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Abstract

We conducted thirteen immobilizations of previously collared hibernating two- to four-year-old brown bears (Ursus arctos) weighing 21–66 kg in central Sweden in winter 2010 and 2011 for comparative physiology research. Here we report, for the first time, an effective protocol for the capture and anesthesia of free-ranging brown bears during hibernation and an assessment of the disturbance the captures caused. Bears were darted in anthill, soil, or uprooted tree dens on eleven occasions, but two bears in rock dens fled and were darted outside the den. We used medetomidine at 0.02–0.06 mg/kg and zolazepam-tiletamine at 0.9–2.8 mg/kg for anesthesia. In addition, ketamine at 1.5 mg/kg was hand-injected intramuscularly in four bears and in six it was included in the dart at 1.1–3.0 mg/kg. Once anesthetized, bears were removed from the dens. In nine, arterial blood samples were analyzed immediately with a portable blood gas analyzer. We corrected hypoxemia in seven bears (PaO2 57–74 mmHg) with supplemental oxygen. We placed the bears back into the dens and antagonized the effect of medetomidine with atipamezole. Capturing bears in the den significantly increased the risk of den abandonment. One of twelve collared bears that were captured remained at the original den until spring, and eleven, left their dens (mean ± standard deviation) 3.2±3.6 (range 0.5–10.5) days after capture. They used 1.9±0.9 intermediate resting sites, during 6.2±7.8 days before entering a new permanent den. The eleven new permanent dens were located 730±589 m from the original dens. We documented that it was feasible and safe to capture hibernating brown bears, although they behaved differently than black bears. When doing so, researchers should use 25% of the doses used for helicopter darting during the active period and should consider increased energetic costs associated with den abandonment.

Introduction

Growing interest in hibernation physiology requires development of safe and effective field techniques for immobilizing hibernating bears with the least possible risk to both researchers and bears. Free-ranging brown bears (Ursus arctos) in Sweden hibernate six to seven months each year and with fewer disruptions than the three months for brown bears in captivity at the same latitude [1]. Due to a longer hibernation and different physiology [2,3], free-ranging bears are likely to be a better model for human medical research regarding cardiovascular disease, space medicine, bed-ridden patients, and obesity than captive bears. When a Scandinavian brown bear goes into hibernation in the fall it has typically gained 40% in weight most of which is stored fat. For the next half year the bear lies still and plasma cholesterol levels rise to an average of 12 mmol/L [4]. However, when the bear emerges from the den in spring it has remained free from vascular thrombosis, atherosclerosis [5] and heart failure [6] despite these quite dramatic risk factors. Although there are many research projects that can utilize samples from hibernating bears [7,8], research on capture, anesthesia, and disturbance is important to ensure the welfare of the research animals, safety of the capture personnel, and to evaluate the ethics of such research. Evaluation of disturbance and impact of research on free-ranging animals is becoming more valued [9,10]. We developed this capture protocol for hibernating brown bears based on limited reports of immobilization of American and Asiatic black bears (Ursus americanus and U. thibetanus) during winter [11,12,13], immobilization of captive brown bears in wintertime [14], springtime brown bear immobilization protocols in the same study areas [15,16], and knowledge of denning ecology [17,18,19] and hibernation physiology [3,20].
Brown bears select their den sites prior to hibernating [18], typically at least 1–2 km from human activity [17]. Human activity closer than this, particularly closer than 200 m, can cause bears to abandon their dens [17]. Brown bears that abandoned their dens in our study area moved on average 5.1 km before finding a new den, with 56% moving 2 kilometers or less [21]. In a study where 14 denning female American black bears with cubs were captured, none abandoned their dens [22]. However, den abandonment by brown bears as a result of non-research human disturbance has been documented in Scandinavia [21], and den abandonment was therefore considered a possible response to our captures.

In Scandinavia, free-ranging brown bears are immobilized during their active period with a combination of medetomidine and tiletamine-zolazepam, with atipamezole used for antagonism of the effects of medetomidine [15]. In April captures, subadults were given a mean ± SD dose of 0.08±0.02 mg/kg medetomidine combined with 4.1±1.3 mg/kg tiletamine-zolazepam [16]. Recent studies showing hypoxemia correctable with intranasal oxygen resulted in the addition of oxygen supplementation for all bears during spring and summer captures [16,23]. During hibernation, American black bears reduce oxygen consumption by 75% [24], but we do not know how oxygen consumption in bears is affected by anesthesia or what the optimal PaO2 levels are during anesthesia of hibernating bears. Ketamine has been used in combination with alpha-2 agonists at doses ranging from 1.5–17.1 mg/kg in American black bears [25,26,27], 4.4 mg/kg in Asiatic black bears [27], and 2.0–7.2 mg/kg ketamine in brown bears [25,27]. American black bears (Ursus americanus) are commonly captured during hibernation and when approached quietly, can be localized without disturbing or flushing them and immobilized with a blow dart, jab stick or dart gun [12,13].

In previous studies, brown bears have only been anesthetized during winter in captive situations. One study of non-hibernating brown bears concluded that the ideal dose for oral carfentanil was 12.7 μg/kg in the summer and 7.6 μg/kg in the winter (60% of summer dose) [14]. Another study mentions, but does not describe, the anesthesia of four captive brown bears with tiletamine-zolazepam during hibernation [6]. In that study, 2 mg/kg tiletamine-zolazepam was used during hibernation and 5 mg/kg during the summer months (personal communication, Nelson, 12/2009).

Our objectives were to develop an effective capture and anesthesia protocol for hibernating free-ranging brown bears, to evaluate arterial oxygenation in order to determine if supplemental oxygen should be administered and to evaluate the disturbance that the captures caused to the bears. Our hypothesis was that a low-dose combination of medetomidine and zolazepam-tiletamine would be effective for capture and anesthesia of hibernating brown bears, and that these captures would cause the bears to abandon their dens.

Materials and Methods
All captures were approved by the Swedish Ethical Committee on Animal Research (application numbers C212/9 and C47/9) and the Swedish Environmental Protection Agency. Fieldwork was carried out in Dalarna, Sweden during February–March (winter) and in June (summer) 2010 and 2011. We selected six female and six male hibernating brown bears, two to four years old, previously fitted with global positioning system (GPS) collars and very high frequency (VHF) abdominal implants. One female was anesthetized during both years. We only anesthetized subadults to reduce the chance of encountering females with cubs in the dens and to avoid older animals, considered to pose greater risk to the capture team. Snow depth ranged from 80–120 cm with temperatures ranging from –15°C to +1°C.

We located bears using GPS and VHF radio collars/implants (Figure S1 and S2). All dens were between 5 and 20 km from plowed roads, so we used snowmobiles to transport equipment and the field team to within 200–800 m of the den. Once we had located the den entrance and removed the snow (Figure S3), a metal grate was placed over the entrance. Two field personnel held the grate over the entrance using their own body weight and were assisted by up to three more people if necessary to keep the bear in the den. Anesthetic agents were administered by remote darting through the grate (Figure S4) using a flashlight and CO2 powered rifle (Dan-Inject®, Berkop, Denmark) fired from 0.3–3.5 meters distance. Darts were 3 ml with a 2.0×30 mm barbed needle (Dan-Inject®). The bears were anesthetized with a total dose of 0.6–2.5 mg of medetomidine (Domitor® 1 mg/ml, and Zalopine®, 10 mg/ml, Orion Pharma Animal Health, Turku, Finland) and 31–125 mg tiletamine-zolazepam (Zoletil®, 500 mg/vial, Virbac, Carros, France). A second dart with a full dose was administered if the bear was mobile after 10 minutes. In four bears, 75–100 mg ketamine (Narketan 10®, 100 mg/ml, Chassot, Dublin, Ireland) was hand-injected before handling and for six immobilizations; 37–75 mg of ketamine was included in the initial dart.

Once anesthetized, we took each of the bears out of the den (Figure S5) and placed them on an insulated blanket. We measured temperature, heart rate, and respiratory rate in all bears. We were unable to obtain pulse oximetry readings with a veterinary sensor clip placed on the tongue, lip, ears, or vulva were from the first four bears during February, so we abandoned this for the remaining bears. Blood samples from the femoral artery were collected aero-statically in pre-heparinized syringes from ten bears at 15–25 and 65–75 minutes from darting. The samples were immediately analyzed in a portable analyzer (iSTAT 1® Portable Clinical Analyzer, Abbott Laboratories, Abbott Park IL, 60064-6048, USA) with the bear captured both years only sampled during the second year. Blood gas samples and pH were corrected to rectal temperature. Intranasal oxygen was provided from a portable oxygen cylinder to eight bears via a nasal line inserted 10 cm into one nostril with an oxygen flow rate of 0.5–2.0 liters per minute after the first arterial sample was collected.

After sampling, we placed the bears back into the dens and antagonized the effects of medetomidine with atipamezole (Antisedan®, 5 mg/ml, Orion Pharma Animal Health, Turku, Finland) given intramuscularly at 5 mg per mg of medetomidine. We covered the entrance with branches and snow and the bears were left to recover undisturbed.

In June we recaptured bears by darting from a helicopter as previously described [16]. Ten bears were captured with 5 mg medetomidine combined with 250 mg zolazepam-tiletamine and one was darted twice for a total of 10 mg medetomidine and 500 mg zolazepam-tiletamine. Two smaller bears (22 and 28 kg) were immobilized with 2.5 mg medetomidine and 125 mg zolazepam-tiletamine. Sampling was conducted as described for February bears, except that a narrower time range was selected for each arterial sample (20–30 minutes and 60–65 minutes from darting).

Hypoxemia was defined as mild (PaO2 60–80 mmHg), marked (PaO2 40–60 mmHg), or severe (PaO2 <40 mmHg). Acidemia was defined as a pH <7.35, and acidemia was considered marked if pH <7.25. Hypocapnia was defined as a PaCD02c <35 mmHg and hypercapnia was defined as mild (PaCO2c 45–60 mmHg) or marked (PaCO2c >60 mmHg). A paired two-
tailed t-test was used to compare the first and second sample at both winter and summer captures, and between winter and summer for both the first and second samples. Bears not receiving oxygen were excluded from comparisons that included a second sample for the variables with direct relation to oxygen (PaO₂, PaCO₂, SaO₂, HCO₃ and pH).

**Disturbance Data Analysis**

Twelve of the thirteen winter-captured bears were fitted with GPS Plus and GPS Plus Pro collars with GSM lateral modems (Vectronic Aerospace GmbH, Berlin, Germany), which allowed collection of GPS and activity data. The GPS collars also had dual-axis motion sensors and VHF transmitters. We programmed the collars to register GPS-position data every ten minutes from the date of capture until at least four days after capture. The collars registered only one GPS-position per day (at noon) until 31 March, and from 1 April reverted to the standard programming of one GPS-position per 30 minutes. GPS position data were stored in the collar and sent to a base station in packages of seven positions per text message, via the GSM (Global System for Mobile Communications) network. We retrieved collars during captures in June and downloaded GPS data in order to obtain any data not sent via text messaging.

The collars recorded activity data at 5-minute intervals, based on the average of 4–8 measurements per second for five minutes immediately preceding the time of recording. Activity level was measured in two orthogonal directions, yielding two numeric activity values ranging from 0–255. The average of these two values indicated whether a bear is active (≥50) or passive (<50) [28]. Activity data were not sent via mobile network text messages, but were stored in the collar and downloaded after we retrieved the collar.

GPS data documented the time and distance of movements following immobilization. We defined a cluster of positions (hereafter called a cluster) as the equivalent of six GPS positions within 50 m, with a 30-minute position interval. We divided clusters into dens and beds, i.e. outside dens, based on follow-up visits to the sites during May and June. The activity data identified activity changes associated with movements. We considered a bear to have remained at a den or bed (a temporarily used above-ground site) until the time of the last inactive measurement before movement. We defined arrival at a bed or den as the first GPS location within the cluster, and we considered bed or den use to have begun at the time of the first inactive measurement following arrival.

We considered a new permanent den as the location where the bear remained for the majority of the remaining denning period. We defined resumed inactivity at the new permanent den as the first inactive measurement during five consecutive days where less than 5% of the daily activity measurements were active. We defined den emergence as the time of the last GPS position within 50 meters of the den. Data for all variables are presented as mean ± standard deviation (range). We used a subsample of marked bears in the study area that were not captured in the den, for which activity data, GPS data and den location data were available for 76 denning events in 2004–2011. We conducted a chi-squared test of association with Yates’ Correction for Continuity to compare the den abandonment rate of bears captured in the den with that of bears that were not. We have no information on other non-research related human disturbance around the dens, and thus could not compare the effects of different types of human disturbance on den abandonment rates.

**Results**

In 2010, two of the bears were in rock dens at the time of capture. On the other capture occasions, bears were denned in anihill (6), soil (4), or uprooted tree (1) dens. All of the sites used between original dens and new permanent dens were beds (7) or nest dens (3). The difference between a bed and a nest den is the amount of material used in its construction. Dens used as new permanent dens were rock (4), bed (3), nest (2), uprooted tree (1), anihill (1) and soil (1) dens.

**Ground Darting and Adequate Anesthesia of Hibernating Bears was Possible with 25% of the doses of Medetomidine and Tiletamine-zolazepam Used for the same Bears in Summertime**

We documented hypothermia, bradycardia and mild to marked alterations in pulmonary gas exchange and acid-base status. Intranasal oxygen supplementation markedly improved arterial oxygenation.

During winter captures, all bears moved as far as possible from the entrance into the den when capture personnel entered it. Two bears in dens under large rocks escaped using alternate exits. Due to difficulties in carrying out captures in rock dens, bears in rock dens were not captured in 2011. One was darted in the den and both were darted as they left the dens, running 40 and 200 meters respectively, before recumbency. On the remaining eleven occasions, bears were in soil or anihill dens. In these, the captures went smoothly, except for one instance where the drug in the dart froze and the bear required a second dart, and a second case where the bear was darted in the den and managed to escape around the grate.

Induction time was 16±8 (6–26) minutes. Doses were 0.03±0.01 (0.02–0.05) mg/kg medetomidine, 1.7±0.7 (0.9–2.8) mg/kg zolazepam-tiletamine in all bears. In four bears, ketamine at 1.5 mg/kg was hand-injected and in six it was included in the dart at 1.1–3.0 mg/kg (Table 1). During summer captures, doses for bears darted once were 0.10±0.03 (0.07–0.11) mg/kg medetomidine and 4.7±0.6 (4.3–5.7) mg/kg tiletamine-zolazepam. Induction time in the eleven bears darted once was 2±1 minutes. The two bears darted multiple times received a total dose of 0.13 mg/kg medetomidine and 6.5 mg/kg tiletamine-zolazepam, and 0.18 mg/kg medetomidine and 6.8 mg/kg tiletamine-zolazepam, respectively.

The bear darted with the highest dose in winter (0.05 mg/kg medetomidine, 2.5 mg/kg zolazepam-tiletamine and 3 mg/kg ketamine), a 2-year old, 27 kg male, was apneic on removal from the den at 12 minutes after darting. The apnea did not respond to 50 mg doxapram (Dopram®, Wyeth Lederle, Wyeth-Ayerst International Inc., Philadelphia, PA, USA) given intravenously and the bear was therefore intubated and ventilated manually with a bag valve mask (Ambu-bag®, Ambu Ltd. Cambridgeshire, United Kingdom). This bear was supplemented with oxygen-enriched air by connecting the oxygen tube to the bag valve mask. We did not take an arterial blood sample until after manual ventilation with oxygen enriched air began, so this bear was excluded from the blood-gas data presented in Table 2. The bear did not resume spontaneous breathing until after atipamezole was given at 2 hours and 24 minutes after darting.

**Physiological evaluation.** Heart rate, respiratory rate, and body temperature for winter and summer are presented in Table 2. Paired analysis of arterial blood samples was performed in the same ten bears in winter and in summer (Table 2). Due to cartridge errors, some variables were not available for the second sample of one bear during winter and for the first sample of one
Table 1. Body mass, age (years) and drug doses (mg) used for anesthesia of brown bears during winter and summer.

<table>
<thead>
<tr>
<th>Bear</th>
<th>Weight (Kg)</th>
<th>Tiletamine-zolazepam Winter</th>
<th>Medetomidine Winter</th>
<th>Ketamine Winter</th>
<th>Induction time (minutes from darting) Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (3)**1</td>
<td>NR</td>
<td>62.5</td>
<td>1.25</td>
<td>N/A</td>
<td>16</td>
</tr>
<tr>
<td>Male^2 (2)</td>
<td>54</td>
<td>62.5</td>
<td>2.25</td>
<td>N/A</td>
<td>26</td>
</tr>
<tr>
<td>Female (3)^3</td>
<td>45</td>
<td>62.5</td>
<td>2.25</td>
<td>N/A</td>
<td>42</td>
</tr>
<tr>
<td>Female (3)^4</td>
<td>55</td>
<td>62.5</td>
<td>2.5</td>
<td>75</td>
<td>13</td>
</tr>
<tr>
<td>Female (3)^4</td>
<td>51</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>13</td>
</tr>
<tr>
<td>Female (3)^4</td>
<td>53</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>13</td>
</tr>
<tr>
<td>Male (3)^3</td>
<td>66</td>
<td>125</td>
<td>2.5</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>Female (3)^4</td>
<td>57</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>10</td>
</tr>
<tr>
<td>Male (3)^3</td>
<td>58</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>12</td>
</tr>
<tr>
<td>Female (2)^2</td>
<td>21</td>
<td>62.5</td>
<td>0.63</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>Male (4)^2</td>
<td>59</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>7</td>
</tr>
<tr>
<td>Male (2)^2</td>
<td>25</td>
<td>62.5</td>
<td>1.25</td>
<td>75</td>
<td>12</td>
</tr>
<tr>
<td>Female (2)^2</td>
<td>35</td>
<td>62.5</td>
<td>0.63</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>mean</td>
<td>48±14</td>
<td>77±35</td>
<td>1.5±1.0</td>
<td>70±19</td>
<td>16±10</td>
</tr>
</tbody>
</table>

*Denotes the bears that had the best quality of anesthesia. For bears requiring several darts to be anesthetized in summer, the dose presented is the total dose and the induction time is not included in the mean.

^Escaped from rock dens, darted while running.

*Induction not observed (ran 200 meters), not included in the mean.

Captured in 2010.

Captured in 2011.

doi:10.1371/journal.pone.0040520.t001

Capture of Hibernating Free-Ranging Brown Bears

A bear during summer. Hypoxemia was recorded in arterial samples before oxygen supplementation in seven of ten bears in winter (PaO2 30–74 mmHg) and two of ten bears in summer (PaO2 66–69 mmHg). A second arterial sample was collected from nine bears in winter receiving 0.5 or 1 liter per minute of oxygen and ten bears in summer receiving 0.5 or 1 liter per minute. On the second winter sample, the seven bears receiving 1 liter per minute had PaO2 levels of 89–180 (Table 3). In winter, eight of the bears had PaO2 of 301 mmHg. On the second summer sample (while receiving either 0.5 or 1 liter per minute O2), bears had PaO2 30–74 mmHg and two of ten bears in summer (PaO2 66–74 mmHg). A second arterial sample was collected from nine bears. In winter, eight of the bears had PaO2 of 301 mmHg. On the second summer sample (while receiving either 0.5 or 1 liter per minute O2), bears had PaO2 30–74 mmHg and two of ten bears in summer (PaO2 66–74 mmHg).

During winter captures, hypercapnia was initially recorded in five of ten sampled bears and in the second measurement in seven of nine bears. In summer, one of nine bears had hypercapnia on initial sampling. Hypocapnia was recorded in anesthetized bears both during winter and summer. All bears that were tested had higher glucose, hematocrit and hemoglobin during winter than both during winter and summer. All bears that were tested had higher glucose, hematocrit and hemoglobin during winter than during summer (table 2).

Behavioral consequences. Bears left their original dens following capture on twelve of thirteen occasions (summarized in Table 4). One of the twelve bears from which we obtained GPS and activity data remained at the original den. The 11 bears remained at their dens for 3.2±3.6 days before leaving, and spent 6.2±2.8 days before resuming inactivity at a new permanent den. On five occasions, bears moved directly from the original den to the new permanent den, spending 2.2±1.1 hours before locating and settling into the new den. On the remaining occasions, bears used 1.8±0.5 beds for 12.4±7.0 days before locating and settling into the new permanent den. One bear did however move from the original den to a new den within 1.5 hours. It remained at this den for 17.5 days, left and stayed at a bed for 2.0 days, before resuming inactivity at a new den for 22 days. From the activity pattern and duration, we consider the bear to have resumed inactivity at both new den sites. The data from this bear’s denning was therefore only included in calculations of time spent at the original den (i.e. the den where it was captured).

Non-den captured bears had a den abandonment rate of 26% (n = 20), but the majority occurred in October/November and only 3 abandoned their dens during January-March (4%). Den captured bears were significantly more likely to abandon their dens compared to marked, non-captured bears during the same time period (χ² (1, N = 76) = 59.1, p < .0005). However, the abandonment rates of den-captured bears were highly significant even when comparing with overall abandonment rates (χ² (1, N = 76) = 20.5, p < .0005) or when excluding those occasions where bears moved dens early in the season (χ² (1, N = 56) = 45.2, p < .0005).

Den emergence occurred from 5 April until 23 April in 2010, and for six of seven bears, 19 April until 22 April in 2011, similar to other bears in the study area in the respective years. One bear in 2011 emerged on 5 May, which was somewhat later than most other bears in the study area. The straight-line distance between the original and (final) new permanent dens was 730±589 m (225–2123 m, Table 4).

Discussion

The capture technique with ground-darting of hibernating brown bears in dens was successful. All bears were alert, frightened, with three escaping from their dens and darted while running. This is in contrary to black bears, which can even be snuck up on when denning in open nests [13]. The best quality of
anesthesia during winter was achieved in the bears darted with the lowest doses of medetomidine, zolazepam-tiletamine combined with ketamine in the dart to deepen anesthesia without depressing respiration or prolonging recovery. The ketamine was added after experiencing depressed respiration at higher doses of medetomidine-zolazepam-tiletamine and a shallow plane of anesthesia at the low doses. Ground darting and anesthesia of hibernating bears was possible with 25% of the doses used in summer.

The bear that became apneic during winter captures was darted with medetomidine-zolazepam-tiletamine at 50% of the mean dose given in summertime combined with 75 mg ketamine. The apnea may be attributed to the dose of medetomidine, which can depress respiration [29]. Although medetomidine tiletamine-zolazepam combinations have a wide safety margin during anesthesia of brown bears in springtime [15], the therapeutic range may be narrower in hibernating bears.

<table>
<thead>
<tr>
<th>Table 2. Physiological variables and blood gas results from seven brown bears anesthetized during winter and summer 2010 and 2011.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time from darting</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Heart ratea,b,c,d</td>
</tr>
<tr>
<td>Respiratory rateb</td>
</tr>
<tr>
<td>Rectal Tempab,d</td>
</tr>
<tr>
<td>Lactatec,d</td>
</tr>
<tr>
<td>PaO2a,c,d</td>
</tr>
<tr>
<td>SaO2d</td>
</tr>
<tr>
<td>pHd</td>
</tr>
<tr>
<td>PaCO2a,b</td>
</tr>
<tr>
<td>BUNa</td>
</tr>
<tr>
<td>Glucosea,c,d</td>
</tr>
<tr>
<td>Hcta,b,c</td>
</tr>
<tr>
<td>HCO3a,b,d</td>
</tr>
</tbody>
</table>

Variables corrected to rectal temperature are marked with an *. Statistically significant differences using a paired two-tailed t-test are denoted by:

- aBetween winter and summer sample 1,
- bwinter and summer sample 2.
- cwinter sample 1 and 2 and d. summer sample 1 and 2.

The bear that became apneic during winter captures was darted with medetomidine-zolazepam-tiletamine at 50% of the mean dose given in summertime combined with 75 mg ketamine. The apnea may be attributed to the dose of medetomidine, which can depress respiration [29]. Although medetomidine tiletamine-zolazepam combinations have a wide safety margin during anesthesia of brown bears in springtime [15], the therapeutic range may be narrower in hibernating bears.

<table>
<thead>
<tr>
<th>Table 3. Partial pressure of oxygen (PaO2) before (Pre-O2) and during oxygen supplementation in individual bears anesthetized winter and summer captures.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter PaO2 (mmHg)</td>
</tr>
<tr>
<td>Kg Pre-O2</td>
</tr>
<tr>
<td>Male (3)§</td>
</tr>
<tr>
<td>Male (2)</td>
</tr>
<tr>
<td>Female (3)</td>
</tr>
<tr>
<td>Female (3)*</td>
</tr>
<tr>
<td>Male (3)#</td>
</tr>
<tr>
<td>Female (3)</td>
</tr>
<tr>
<td>Male (3)</td>
</tr>
<tr>
<td>Female (2)</td>
</tr>
<tr>
<td>Male (4)</td>
</tr>
<tr>
<td>Male (2)</td>
</tr>
<tr>
<td>Female (2)</td>
</tr>
</tbody>
</table>

*denotes the results of the only bear not given oxygen that was sampled during the second sampling interval.
doi:10.1371/journal.pone.0040520.t003
Table 4. Movements of twelve GPS-collared brown bears after capture during winter 2010 and 2011 in central Sweden.

<table>
<thead>
<tr>
<th>Sex (years of age)</th>
<th>Den type (original)</th>
<th>Days at original den</th>
<th>Times moved</th>
<th>Intermediate beds</th>
<th>Days before resumed inactivity at new permanent den</th>
<th>Distance between original and new dens (m)</th>
<th>Den emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (3)</td>
<td>Soil</td>
<td>2.1</td>
<td>2</td>
<td>1</td>
<td>16.8</td>
<td>320</td>
<td>4/22/10</td>
</tr>
<tr>
<td>Female (3)</td>
<td>Soil</td>
<td>0.5</td>
<td>3</td>
<td>2</td>
<td>1.5</td>
<td>775</td>
<td>4/22/10</td>
</tr>
<tr>
<td>Female (3)</td>
<td>Anthill</td>
<td>1.6</td>
<td>3</td>
<td>2</td>
<td>17.4</td>
<td>363</td>
<td>4/23/10</td>
</tr>
<tr>
<td>Male (3)</td>
<td>Rock</td>
<td>10.4</td>
<td>1</td>
<td>0</td>
<td>1 hour</td>
<td>225</td>
<td>4/15/10</td>
</tr>
<tr>
<td>Female (3)*</td>
<td>Rock</td>
<td>1.9</td>
<td>1</td>
<td>0</td>
<td>2 hour</td>
<td>342</td>
<td>4/14/10</td>
</tr>
<tr>
<td>Male (2)</td>
<td>Anthill</td>
<td>0.8</td>
<td>3</td>
<td>2</td>
<td>15.3</td>
<td>264</td>
<td>4/5/10</td>
</tr>
<tr>
<td>Female (4)*</td>
<td>Soil</td>
<td>3.2</td>
<td>2**</td>
<td>1**</td>
<td>**</td>
<td>1013**</td>
<td>4/20/11</td>
</tr>
<tr>
<td>Female (3)</td>
<td>Soil</td>
<td>1.7</td>
<td>1</td>
<td>0</td>
<td>2 hour</td>
<td>1419</td>
<td>4/21/11</td>
</tr>
<tr>
<td>Male (3)</td>
<td>Anthill</td>
<td>1.7</td>
<td>3</td>
<td>2</td>
<td>10.3</td>
<td>534</td>
<td>4/19/11</td>
</tr>
<tr>
<td>Male (2)</td>
<td>Uprooted tree</td>
<td>Did not move</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>4/19/11</td>
</tr>
<tr>
<td>Male (2)</td>
<td>Anthill</td>
<td>10.5</td>
<td>1</td>
<td>0</td>
<td>2 hour</td>
<td>647</td>
<td>4/22/11</td>
</tr>
<tr>
<td>Female (2)</td>
<td>Soil</td>
<td>1.3</td>
<td>1</td>
<td>0</td>
<td>4 hour</td>
<td>2123</td>
<td>5/5/11</td>
</tr>
</tbody>
</table>

Individual information is presented from the twelve GPS-collared bears including days spent at the original den before moving, number of times moved before entering a permanent den, hours spent until resuming hibernation at their permanent den and date of den emergence. Grey denotes bears in rock dens, *denotes the individual captured twice. **Moved directly to new permanent den, but relocated to yet another new permanent den, staying at an uncovered bed site for 49 hours in between. Distance was calculated to the final new permanent den.

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The hypoxemia recorded during nine of 19 anesthesia events in both winter and summer indicates that bears in both capture situations may benefit from oxygen supplementation. Since intranasal oxygen at a flow rate of 1 L/min increased the PaO₂ to 100–387 mmHg in the seven bears that were supplemented with this flow rate during wintertime, evaluation of lower flow rates during hibernation is warranted. The failure of pulse oximetry to work during hibernation captures was likely due to the low body temperatures and the vasoconstrictive effects of medetomidine [30].

Hypercapnia during anesthesia is commonly caused by drug-induced hypventilation (respiratory center depression) [31]. Despite the lower drug doses used in winter than in summer, seven of nine bears captured in winter developed mild to marked hypercapnea, whereas only two of ten bears developed mild hypercapnea during capture in June. Mild hypercapnia can be beneficial, because it causes a shift in the oxygen-hemoglobin dissociation curve, increasing the unloading of oxygen at tissues, enhancing oxygen delivery, and carrying capacity [31]. On the contrary, severe hypercapnia can cause tachyarrhythmia, hemodynamic instability, and coma [31]. The higher hematocrit and hemoglobin values recorded in winter than in summer were most likely due to dehydration from not eating or drinking during hibernation.

The decreased pH recorded during anesthesia of hibernating brown bears was mainly due to increased values of PaCO₂ (respiratory acidemia), whereas in summer, bears more commonly developed lactic acidemia (Table 2). As previously documented, brown bears anesthetized by darting from a helicopter develop higher lactate levels than brown bears anesthetized in captivity [16]. In the present study, the lower lactate levels of most hibernating bears during anesthesia were in the same range as reported in captive bears [16], indicating less physical exertion than capture by darting from a helicopter. However, one bear that escaped from its den and ran approximately 300 meters in deep snow before being anesthetized developed lactate levels up to 7.5 mmol/L. Bradycardia and hypothermia were recorded during anesthesia of hibernating bears, consistent with previous studies on denning physiology [24].

The two captures in rock dens were more complicated than captures in other types of dens because both bears in rock dens escaped from their dens and were darted outside. When planning for den captures, den type and surrounding terrain must be considered.

Bears left their dens following the disturbance associated with entering the den and capture on 12 of 13 occasions (Table S1), compared to only 4% den abandonment during the equivalent time of year or 26% overall in non-den captured bears in this study area. The 26% overall den abandonment rate is higher than documented in a previous study, which may be attributed to the higher resolution of GPS/Activity data compared to VHF telemetry data [21]. As in the present study, most of the non-capture related abandonments occurred early in the denning period (November/December), and were mostly attributed to non-research related human disturbance [21,32]. The lower abandonment rates further into the denning period agrees with findings from an earlier study [21]. As all den captures occurred during late February/early March, the lower rate provides the most relevant comparison. Den abandonments in our den-captured bears are likely to have conferred an energetic cost to the bears, particularly for those bears that used a couple of attempts before successfully locating a den that they used for the rest of the denning period [17,21]. Although den emergence dates were similar to other bears in the study area and the bears appeared to be in good physical condition when recaptured in June, we recommend that researchers consider the effects of den abandonment when planning to immobilize hibernating brown bears. In an earlier study in our study area, 68% of the presumed pregnant females that abandoned their dens emerged from their new dens without cubs, compared to 6% who did not abandon their den [21]. Cub mortality following den abandonment due to human disturbance has also been documented in American black bears [33]. Thus, we conclude that immobilization of hibernating females that are suspected to be pregnant may be especially intrusive, even if they are immobilized prior to giving birth.

Once out of the dens, most of the bears made a couple of attempts before locating a den that they settled into for the rest of the denning period. Anthill and soil dens were the most common den types for the original dens, whereas rock dens, nest dens and beds were the most common types of the second permanent dens. The choice of second den type likely reflected availability and the terrain around the den sites, especially when considering the deep snow cover (approximately 70 cm).

Conclusions

This paper describes the only documented method for capture of brown bears during hibernation. Bears were stable with consistent physiological variables under anesthesia and exhibited hypoxemia that was correctable by low doses of supplemental oxygen. They showed much greater sensitivity to the disturbance of the captures than that caused to black bears in North America with similar capture methods. The doses presented here should result in an appropriate level of anesthesia if the size of the bear can be correctly predicted. This study presents a capture method for sub-adult Scandinavian brown bears and cannot be extrapolated to other age-categories or species of bears that may not have the same behavioral responses to capture.

Supporting Information

Figure S1 Radiotracking using VHF radiocollars/implants to find the location of the denning bear. (TIF)

Figure S2 Locating a bear denning underneath a rock den using VHF radio tracking. (TIF)

Figure S3 Snow is removed and a metal grate is held ready to cover the den entrance. (TIF)

Figure S4 Darting through the metal grate placed over the den entrance. On ten of thirteen occasions, bears were in anthill or earth dens such as this one. (TIF)

Figure S5 After removal from the dens, bears were placed on an insulated blanket and physiological monitoring was performed. (TIF)

Table S1 Original, Intermediate and permanent den sites for each of the captured bears. (XLSX)

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Author Contributions
Conceived and designed the experiments: JMA ALE SB OF JES. Performed the experiments: ALE VS SB OG AF KM. Analyzed the data: ALE VS JMA AF OGS JES. Contributed reagents/materials/analysis tools: SB JMA. Wrote the paper: ALE VS.

References
Paper II
Physiological reactions to capture in hibernating brown bears

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Abstract

Human disturbance can affect animal life history and even population dynamics. However, the consequences of disturbance are difficult to measure. Hibernation is a process of major physiological changes, involving conservation of energy during a resource-depleted time of year. Hibernating animals are therefore highly vulnerable to disturbance. During the winters of 2011-2015, we captured 16 subadult brown bears (*Ursus arctos*) and recorded their body temperatures (N=11) and heart rates (N=7) before, during, and after capture using biologgers. We estimated the time for body temperature (Tb) and heart rate (HR) to normalize after the capture event. We then evaluated the effect of the captures on pattern, depth of hibernation and the day of den emergence by comparing captured bears to the Tb (N=11) and HR (N=11) of undisturbed subadult bears. Both Tb and HR increased during the capture and returned to the hibernation curve after 15-20 days, but capture did not influence the overall length or depth of hibernation. We show that, although bears required two to three weeks to return to “normal” after winter captures and metabolic costs were likely high, there was no detectable effect on the overall depth or duration of the hibernation period.

**Keywords:** chemical immobilization, ecophysiology, research ethics, *Ursus arctos*. 
Introduction

Assessing potential negative effects of wildlife capture is an ethical imperative. For hibernating animals, disturbance during hibernation could have dramatically different implications than disturbance during the active period. In many species, the metabolic savings afforded by hibernation are critical to survival. Disturbances during this period may have consequences on behaviour, habitat use, body condition, foraging opportunities, and juvenile survival [1]. In small mammals such as bats, arousal during hibernation is energetically costly and frequent arousals are thought to decrease winter survival, with body mass lost during hibernation correlating with body temperature and number of arousals [2, 3]. This suggests that animals with greater fat reserves may be able to better tolerate disturbance and capture during hibernation. We previously reported that capture of hibernating brown bears (*Ursus arctos*) resulted in den abandonment in 12 (92%) of 13 captures, compared to 22% overall den abandonment rate in the study area [4]. Although we do not know of other reports of capture of denning brown bears, a study reporting the capture of 14 hibernating female American black bears (*Ursus americanus*) with cubs found that none abandoned their dens [5]. Based on these reports, it appears that the Scandinavian brown bear may be more sensitive to winter disturbance than the American black bear. Here we used biologgers to document how winter captures affected hibernation patterns, and hibernation depth, and timing of emergence from the hibernaculum in brown bears. We hypothesized that, after controlling for potential size-related effects, this disturbance would result in disruptions to the pattern of hibernation, decrease its overall depth and duration.
Methods

We carried out captures of 16 biologger-outfitted solitary sub-adult brown bears (2-4 years old, 28-72kg) in Dalarna County, Sweden during February and March 2011-2015. Bears included in this study had been captured by darting from a helicopter in April-May the previous year [6]. They were fitted with global positioning system (GPS) collars (Vectronics Aerospace GmbH, Berlin, Germany) and very high frequency (VHF) abdominal implants (Telonics Inc., Mesa, AZ, USA). For 11 captures, bears had previously implanted abdominal body temperature ($T_b$) loggers (DST Centi, Star-Oddi, Gardabaer, Iceland); two of these and five additional bears had previously implanted heart monitors (Reveal® XT, Medtronic Inc., MN, USA), which recorded day and nighttime heart rates (HR) and activity. The captures were carried out as previously described [7] and detailed here (supplementary material). Data on $T_b$ (N=11, 43-100 kg) and HR (N=11, 55-76 kg) of undisturbed bears of the same age in the study area were used as a control group. We accounted for repeated inclusion of several individuals in models by using bear ID as a random effect.

To describe the capture-induced effect to the hibernation pattern, the length of the period between capture and return to hibernation (disturbance period) was determined using a binary segmentation changepoint analysis [8] for daily means of both $T_b$ and daytime HR (detailed description in supplementary material). The disturbance period was then compared by summary statistics to the week before capture (pre-disturbance period) and the week after return to hibernation (post-disturbance period).

We made a two-step analysis to quantify the differences in overall depth of hibernation in the disturbed bears using an energy-savings index. We first calculated this index as the area between curves (ABC) from the maximum daily mean $T_b$ and the measured $T_b$ curve during hibernation (start date defined by when the body temperature dropped below 36.5 and end date when it rose above 36.7, Paper 6), i.e. the greater the ABC, the deeper and and/or longer
the hibernation period (Figure S2). The ABC was calculated for each bear (11 captured, 49 ± 13 kg body mass; 11 undisturbed, 65 ± 18 kg). Secondly, we compared this index of energy savings for both captured and undisturbed bears using linear mixed-effects model (lmer) [9], with ABC as the response variable, captured or not and body mass as explanatory variables, and bear ID as a random effect.

We then compared dates of the end of hibernation between captured versus undisturbed bears using the day of the year that the daily mean $T_b$ rose above 36.7°C, as this body temperature is associated with den exit in brown bears in the study area (Paper 6). Again, we used a lmer model to test for effects of capture and body mass on the end date of hibernation period. All analysis were done using statistical extensions available in R 3.2.0 [10].

Results

The pattern of hibernation between captured and undisturbed bears is visually different for mean daily $T_b$ and HR (Figure 1). The closest change points for $T_b$ between the point of disturbance and return to normal yielded a mean duration of the disturbance period of 16.1±6.9 days (mean±SD). For HR, the disturbance period lasted 20.9±6.8 days (Figure 1). From the GPS positions, we saw that only two of 15 bears remained at the den site (Table S2). These had 10 and 11 day disturbance periods based on $T_b$.

Using the periods defined individually for each variable, bears had on average 2.6°C higher $T_b$ and HR increased from 15 beats per minute (bpm) to 31 bpm during the disturbance period compared to before disturbance. $T_b$ and HR did not return to their pre-capture levels, but rather to a later phase in the rising process (examples in Figure S2), returning to a similar pattern as those of the uncaptured bears (Figure 1). In the post-disturbance period, the captured bears had mean $T_b$ and HR 1.0°C and 7 bpm higher than pre-disturbance (Table 1).
For the energy savings index representing overall depth of hibernation, we found no detectable effect of capture ($t= -0.23$, effect size $=8.89$, $p=0.82$). The effect of body mass ($t=-3.41$, effect size $=-6.32$) was statistically significant ($p<0.01$) (Figure 2) with heavier bears saving more energy regardless of whether or not they were captured (Table S1). We also found no detectable effect of capture on date of emergence from hibernation ($t=0.76$, effect size$= 2.67$ $p= 0.45$). However, the effect of body mass was statistically significant with heavier bears exiting earlier ($t=-5.04$, effect size$= -0.53$, $p<0.01$).

**Discussion**

As hypothesized, capturing hibernating brown bears in their dens disrupted their hibernation pattern, causing increased HR and $T_b$ levels for up to four weeks post-capture. A striking finding of our study was that instead of returning to their pre-capture values $T_b$ and HR values of the disturbed bears returned to the pattern shown in undisturbed bears (Figure 1). This suggests that, although hibernation $T_b$ was disrupted for 16 days, it did not affect the internal clock regulating hibernation phenology. A similar type of internal set point was observed when an energetic challenge was induced by fasting in 13-lined ground squirrels (*Citellus tridecemlineatus*) during the annual fattening cycle [11].

$T_b$ returned to the hibernation curve (similar to the control bears) faster than the daily mean HR. This is in contrast to a study showing that capture had extremely short-term effects on the HR and activity levels of American black bears, which usually remain in the same dens after capture [12]. In our study, the only bears remaining in the original den had shorter than average disturbance periods (10 and 11 days) based on $T_b$. Nevertheless, we detected neither effects of capture on the energy-savings index (ABC) nor the duration of hibernation in terms of $T_b$ profiles.
Arousal from hibernation is energetically costly, with metabolic rate reaching several times the basal metabolic rate [13]. One study on edible dormice (*Glis glis*) found that fatter animals aroused more frequently, but had a similar length of hibernation and concluded that surplus energy was used to allow shallower hibernation with more frequent arousal rather than shorter hibernation [14]. The same study also documented that the body mass lost during hibernation correlated with $T_b$ and number of arousals, concluding that the heavier animals could afford to minimize torpor. This also suggests that animals with greater fat reserves may be able to better tolerate disturbance and capture during the hibernation period and is consistent with reports of adult male brown bears hibernating shorter than other demographic groups[15]. In Arctic ground squirrels (*Spermophilus parryi*), arousal episodes are energetically the most costly component of hibernation, accounting for the majority of energetic costs during the hibernation period [13]. In small hibernators, arousal is defined as a period of euthermia [13]. In our study, bears sustained a daily mean $T_b$ of 36.0°C during the disturbance period with maximum daily means reaching 39.3°C, consistent with this definition of arousal. Thus, although we did not find significant differences in overall energy savings, the disturbance likely had high additional energetic costs. Unlike the ground squirrels, the bears in our study did not return to their pre-arousal $T_b$ (figure 2). In contrast to arousals in small hibernators, the bears we studied did not have a sustained period of active-level heart rates [16].

Much of the variation in duration and depth of hibernation among individuals was attributed to body mass. Such an importance of body mass on the depth of hibernation is well known for small species [17]. Our findings imply that, although den captures have energetic costs during arousal and the subsequent period of euthermia, there were no measurable impacts of capture on hibernation phenology.
Ethics

All captures were approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (application #s C47/9, C7/12, C18/15, C212/9, and C268/12) and the Swedish Environmental Protection Agency.

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References


Figure 1. Comparison of physiological variables in captured (blue line) and undisturbed (red line) hibernating brown bears in Sweden. (a) Mean daily body temperature ($T_b$) of captured ($N=11$) and undisturbed ($N=11$) bears and (b) mean daytime heart rates (HR) of captured ($N=7$) and undisturbed ($N=11$) bears. The solid lines show the daily means for individual captured bears with standard errors as shaded areas; the dotted vertical line shows the average beginning and end of the disturbance period.
**Figure 2.** Area between the body temperature curves (ABC, an index of energy savings; a higher ABC shows greater energy savings) for brown bears in Sweden that were captured during hibernation, compared with a sample of undisturbed bears, plotted by body mass.

**Table 1.** Descriptive statistics for daytime mean heart rate and daily mean body temperature using the periods defined by changepoint analysis on the data for each variable. Period 1 is the week before the capture, Period 2 starts with the day of capture and goes until daily heart rate or daily mean body temperature reached the hibernation curve again, and Period 3 is the week following Period 2. The last four columns show the result of a linear mixed model distinguishing the three periods for the heart rate and body temperature patterns.

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Supplementary Material

Methods

Capture

Bears were located using previously deployed GPS and VHF radio collars/implants. Dens were located between 300m and 20 km from plowed roads, so when necessary, snow mobiles were used to transport the field team to the den area. We used skis or snowshoes for the last 200-800 m. Once the den was located, a metal grate was placed over the entrance and the bear was darted in the den through the grate using a flashlight and a CO₂-powered rifle (Dan-Inject®, Børkop, Denmark) fired from 0.3–3.5 m. Darts were 3 ml with a 2.0×30 mm barbed needle (Dan-Inject®). The bears were anesthetized with medetomidine (Domitor® 1 mg/ml, and Zalopine®, 10 mg/ml, Orion Pharma Animal Health, Turku, Finland), tiletamine-zolazepam (Zoletil®, 500 mg/vial, Virbac, Carros, France and ketamine (Narketan 10®, 100 mg/ml, Chassot, Dublin, Ireland). Doses are presented in supplemental table 2. Bears not asleep after 15 minutes were given a second dart with the same or half-dose, depending on their initial reaction to the drugs. Once immobilized, we took each of the bears out of the den and placed them on an insulated blanket for monitoring and sampling. Fat, muscle and blood samples were collected and echocardiography was performed for other studies. Afterwards, bears were placed into the dens and the effects of medetomidine were antagonized with atipamezole (Antisedan®, 5 mg/ml, Orion Pharma Animal Health, Turku, Finland) intramuscularly at 5 mg per mg of medetomidine. The dens were covered with branches and snow and bears were left to recover undisturbed.

Changepoint analysis

This method detects multiple change points in a time series using a pruned exact linear time (PELT) algorithm which has increased accuracy over binary segmentation and uses a dynamic programming technique to identify an optimized cost function and the maximum number of segments a time series can be split into [18]. We used the ‘cpt.meanvar’ function in package ‘changepoint’ in R. We used the ’Normal’ as the test statistic and set the penalty value as 0.
Figure S1. Area (grey) between measured mean daily body temperature (solid line) and the highest measured daily mean body temperature (39.3°C, dashed line), used as an energy saving index (ABC). Examples of a) a captured bear with body mass 58 kg and, b) an undisturbed bear with body mass 57 kg.
Figure S2. Plots of body temperature of four of the captured bears in this study. The green highlight shows the day of capture.
<table>
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<th>Variable</th>
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</table>

| mg/kg     | 1.6±0.8  | 0.02±0.01 | 1.4±0.4 |
| Mean      | 45±13    | 1.1±0.4   | 71±40   | 1.1±0.4 | 63±27 | 11±9 |

1 Denotes the bears that did not change dens after capture
2 Denotes one bear that required manual ventilation after respiratory arrest during anaesthesia
3 Denotes one bear that was killed and eaten by another bear after den emergence in spring
Paper III
Seasonal variation in haematological and biochemical variables in free-ranging subadult brown bears (*Ursus arctos*) in Sweden

Anne Randi Græsli1,2, Alina L. Evans1*, Åsa Fahlman3, Mads F. Bertelsen2, Stéphane Blanc4,5 and Jon M. Arnemo1,6

**Abstract**

**Background:** Free-ranging brown bears exhibit highly contrasting physiological states throughout the year. They hibernate 6 months of the year, experiencing a decrease in body temperature, heart rate, respiratory rate and metabolism. An increase in food consumption and the resulting weight gain (mostly through fat storage) prior to hibernation are also part of the brown bear’s annual cycle. Due to these physiological changes, haematological and biochemical variables vary dramatically throughout the year. Seasonal changes in 12 haematological and 34 biochemical variables were evaluated in blood samples collected from 40 free-ranging subadult brown bears (22 females, 18 males) immobilised in Sweden in winter (February-March), spring (April-May), and summer (June).

**Results:** Higher levels of haemoglobin, haematocrit and red blood cell count, and a lower white blood cell count and mean cell volume was found during hibernation than in spring and summer. Lower values of the enzymes; aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GD) and amylase, and increased values of β-hydroxybutyrate (B-HBA) and blood lipids; triglycerides, cholesterol and free fatty acids, were present during hibernation compared to spring and summer.

**Conclusions:** This study documents significant shifts in haematological and biochemical variables in samples collected from brown bears anaesthetised in winter (February-March) compared to in spring and summer (April-June), reflecting the lowered metabolic, renal and hepatic activity during hibernation. Lower values of enzymes and higher values of blood lipids during hibernation, likely reflect a lipid-based metabolism.

**Keywords:** Brown bear, *Ursus arctos*, Haematology, Biochemistry, Seasonality

**Background**

Hibernation is an important feature of the biology of the brown bear (*Ursus arctos*). Brown bears spend about half of the year in a den and survives extreme winter conditions without eating. Brown bears in Sweden enter their dens in October-November and emerge during March-April. The precise mechanisms determining den entry and exit are still unknown [1, 2]. Prior to hibernation, the bears increase their food consumption (primarily of berries) and store adipose tissue [3, 4]. During hibernation, their body weight is reduced by 20-40 %, depending on sex and age, and metabolism decreases with up to 75 % [5, 6], becoming mainly lipid based [7]. However, this decrease in metabolism is associated with only a moderate drop in body temperature. Body temperatures down to 32 °C during immobilisation in winter (February-March), have been documented in brown bears in Scandinavia [8]. Compared to resting heart rate in the active season, hibernating brown bears are bradycardic [3, 9]. A 35 % decrease in

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heart rate and a 69 % decrease in stroke volume during hibernation, compared to during summer, have been reported in subadult brown bears anaesthetised with a drug combination including medetomidine [9].

Blood variables are important in understanding the impact of disease on both an individual and a population level and to assess the health of individual animals [10]. Seasonal influences on blood variables of brown bears can also aid in understanding hibernation physiology. Several reports have demonstrated seasonal variations for selected haematological variables in brown bears [11–15]. This however, is the first multi-season report of changes in blood variables of hibernating free-ranging brown bears. The objective of this study was to describe seasonal changes in haematological and biochemical variables for free-ranging subadult brown bears in Sweden. Only subadult bears were included in this study, because female adult bears may have newborn cubs during hibernation and because it may be risky for the personnel to wake up male adult bears from hibernation. Variation between age categories have earlier been studied in brown bears [11, 14–16], because of these results we don’t expect a difference in subadult and adult bears, which means that the data could infer to adult bears.

Methods

Study site and animals
This study included 40 free-ranging subadult (2-4 years old) brown bears (22 females, 18 males), which were immobilised in the period of 2006 through 2013. Mean age at the time of immobilisation for females and males were 2.9 and 2.7 years old, respectively. Season was assigned to three categories according to the date the bear was immobilised; winter (immobilised from the 4th of February to the 2nd of March), spring (immobilised from April 12th to May 3rd) and summer (immobilised between the 8th of May and the 23rd of June). The grouping was based on the seasonal changes in the bears’ life during the year, with winter being the hibernation period, spring the transition period and summer the active period. There was at least one month gap between each season group.

Blood samples from bears captured during spring (April-May) were included in a previous study [16]. The study area was in the county of Dalarna, Sweden, approximately 61°N, 15°E. Blood samples were collected during immobilisation for GPS collaring for ecological studies within the Scandinavian Brown Bear Research Project. All bears appeared clinically healthy as determined by clinical examination during immobilisation. All sampling was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (application numbers: C 7/12, C 47/9 and C 59/6).

During spring and summer (April–June), bears were immobilised by remote darting from a helicopter using medetomidine-zolazepam-tiletamine, as previously described [17]. In February and early March, bears were immobilised in their winter dens [8]. Subadult bears, previously fitted with global positioning system (GPS) collars and very high frequency (VHF) abdominal implants, were selected and localised by radio tracking. Field workers transported the equipment with snowmobiles within 200-800 m of the den, and thereafter approached the den on skis. After removing the snow, a metal grate was placed over the entrance, and the bear was darted with medetomidine-zolazepam-tiletamine-ketamine [8].

Sample collection and transport
Blood was sampled as reported previously [16] within 60 min of immobilisation from the jugular vein using a vacutainer system (BD Vacutainer®, BD Diagnostics, Preanalytical Systems, Franklin Lakes, NJ, USA). Blood for haematological analyses was collected in 4 mL tubes, with EDTA (ethylenediaminetetraacetic acid) as anticoagulant (Vacuette®, Greiner Bio-One International AG, Kremsmünster, Austria). The blood samples were kept cool from sampling until analysis at the Clinical Chemistry Laboratory at the Swedish University of Agricultural Sciences, Uppsala, Sweden. The time from sampling to analysis was within two days.

Blood for biochemistry was collected in 9 mL tubes with gel and clot activating factor (Vacuette®, Greiner Bio-One International AG). The samples were kept at room temperature for 1-2 h to ensure complete clotting, and then centrifuged at 1500 g for 10 min to separate the serum. The serum was stored in 2 mL cryogenic vials (Nalgene, Nalge Company, Rochester, NY, USA) and kept at -20 °C until shipment to the Central Laboratory, Norwegian School of Veterinary Science, Oslo, Norway. The samples were kept cool during shipment.

Laboratory analyses

The haematological analyses and complete blood cell counts were carried out upon arrival at the laboratory. For haematological analyses in 2006 and 2007, a Cell-Dyn 3500 Hematology Analyser (Abbott Laboratories, Abbott Park, Illinois, USA) was used. In 2008, the Cell-Dyn 3500 was replaced by an ADVIA®2120 Hematology System (Bayer HealthCare, Diagnostics Division, Tarrytown, NY, USA). Cell differentiation was carried out by manual examination of a blood smear, by the same technician at the laboratory.

The haematology profile included red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin, hematocrit, mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC).
In addition, a white blood cell differential count (percentage of total) for band shaped neutrophils, segmented neutrophils, eosinophils, basophils, lymphocytes and monocytes was included.

All clinical chemistry analyses, except cortisol analysis, were carried out using an ADVIA®1650 Chemistry System (Siemens Healthcare, Tarrytown, NY, USA) from 2006 to October 2011. Thereafter it was replaced with an ADVIA®1800 Chemistry System (Siemens Healthcare). The methods were the same for both analysers. Upon arrival at the Central Laboratory, samples were kept at -80 °C until analysis. Cortisol was measured with an Immulite system (Diagnostic Products Corporation, Los Angeles, CA, USA). The protein fractions were measured by Paragon CZE TM 2000 Capillary Electrophoresis System (Beckman Coulter, Brea, California, USA) from 2006 to September 2008, thereafter it was replaced with a Capillarys TM 2 (Sebia®, Evry, France).

The biochemical profile included aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GD), creatinine kinase (CK), amylase, lipase, lactate dehydrogenase (LD), albumin, α1-globulin, α2-globulin, β-globulin, γ-globulin, albumin-globulin ratio, total protein, β-hydroxybutyrate (β-HBA), bile acid, total bilirubin, cholesterol, creatinine, free fatty acids (FFA), glucose, triglycerides, urea, uric acid, calcium, chloride, iron, magnesium, inorganic phosphorus, potassium, sodium, and cortisol. Not all analyses were performed on each bear.

Statistical analysis
JMP®10.0.0 statistical software (SAS Institute, Cary, North Carolina, USA), was used to perform statistical analysis, and a *p*-value of <0.05 was considered significant. Eight bears were resampled in the same season during multiple years; in these cases, one sample from each bear in each season was randomly selected using a random number generator. The population was characterised based on sex (male and female) and the season during which the blood was collected.

The distributions of the haematological and biochemical variables were tested for normality. Those with an approximate Normal distribution were kept on the direct scale, whereas non-normal variables were transformed to normality with a Box-Cox transformation.

| Table 1 Seasonal changes in haematological variables for subadult brown bears (Ursus arctos) presented as 95 % confidence interval |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Unit            | λ              | 2.5 - 97.5 interval | Sex | Season | n   | Range | *p*-value       |
|                  |                 |                |                  |     |        |     |       |                 |
| Haemoglobin g/L  | 2               | 141-213        | F + M            | W   | W      | 11  | 187–215 | <0.0001         |
|                  |                 |                |                   |     | Sp     | 19  | 153–187 |                 |
|                  |                 |                |                   |     | Su     | 15  | 134–183 |                 |
| RBC ×10¹²/L      | 0.8             | 6.1–9.5        | F + M            | W   | W      | 11  | 8.4–10.0 | <0.0001         |
|                  |                 |                |                   |     | Sp + Su| 34  | 6.2–8.0  |                 |
| Haematocrit L/L  | –0.2            | 0.40–0.63      | F + M            | W   | W      | 11  | 0.54–0.66 | <0.0001         |
|                  |                 |                |                   |     | Sp     | 19  | 0.46–0.55 |                 |
|                  |                 |                |                   |     | Su     | 15  | 0.42–0.53 |                 |
| MCV fL           | –0.6            | 60–75          | F + M            | W   | W      | 11  | 58–71    | 0.0038          |
|                  |                 |                |                   |     | Sp + Su| 34  | 62–74    |                 |
| MCHC g/L         | 2               | 289–385        | F + M            | W + Sp + Su| 45  | 318–388 |                 |
| WBC ×10⁹/L       | 0               | 2.7–13.2       | F + M            | W   | W      | 11  | 3.1–12.7 | 0.0208          |
|                  |                 |                |                   |     | Sp + Su| 34  | 3.9–18.3 |                 |
| Segm.neutroph. % | 2               | 41.3–89.5      | F + M            | W   | W      | 11  | 31.4–70.8 | <0.0001         |
|                  |                 |                |                   |     | Sp + Su| 34  | 50.7–87.9 |                 |
| Eosinophils %    | 0.4             | 0.0–21.4       | F + M            | W   | W      | 11  | 1.2–18.4 | 0.0091          |
|                  |                 |                |                   |     | Sp + Su| 34  | 0.0–10.7 |                 |
| Lymphocytes %    | 0.6             | 2.6–44.3       | F + M            | W   | W      | 11  | 15.1–45.4 | 0.0004          |
|                  |                 |                |                   |     | Sp + Su| 34  | 3.1–34.9 |                 |
| Monocytes %      | 0.4             | 1.0–21.4       | F + M            | W + Sp + Su| 45  | 0.6–14.6 |                 |

aRBC = red blood cell count; MCV = mean corpuscular volume; MCHC = mean corpuscular haemoglobin concentration; WBC = white blood cell count; Segm.neutroph. = segmented neutrophils

* = the transformation value from the Box-Cox transformation

2.5 and 97.5 percentiles on untransformed data

F = female; M = male. *F = winter (February-March); Sp = spring (April-May); Su = summer (June)

*p*-values are given for significantly different seasons groups
The formula for the Box-Cox transformation, where \(\bar{y}\) is the geometric mean [18];

\[
y'_{\lambda} = \frac{y^{\lambda} - 1}{\lambda \bar{y}^{\lambda - 1}} \text{ for } \lambda \neq 0, \quad \text{and} \quad y'_{\lambda} = \bar{y} \cdot \log(y) \text{ for } \lambda = 0
\]

When >90% of the values for a given variable was 0, transformation was considered non-sensible, therefore the reference values for these variables were presented as 90th, 97.5th and 100th percentiles. For variables including observed values of 0, a factor of 0.05 or 0.5 was added to all values before transformation.

Comparisons between sex and seasons were performed on the transformed variables using analysis of variance. A significant \(F\)-test was followed by a simultaneous comparison of paired means using the Tukey-Kramer test. After calculating the 95% confidence intervals in the significantly different seasons, the transformed data were reconverted to the original data scale and corrections were made for any added values. For variables for which significant differences were not detected, sex and season cohorts were grouped together for presentation. Data is presented as 95% confidence intervals.

**Results**

Results for haematological and biochemical variables for subadult bears in different seasons are presented in Tables 1, 2, 3, 4 and 5. The \(\lambda\)-values from the Box-Cox transformations are presented.

For the variables band-shaped neutrophils and basophils >90% of the values were 0, they were presented as 90th, 97.5th and 100th percentiles. The percentiles for band-shaped neutrophils and basophils were 2.5; 11.3 and 12.4 and 0.0; 1.3 and 1.4, respectively.

Seasonal changes were found in eight out of twelve haematological variables and in all biochemical variables except creatinine kinase, calcium, potassium, \(\alpha_1\)-globulin, \(\gamma\)-globulins, lipase and cortisol. Haemoglobin, haematocrit, albumin, \(\beta\)-hydroxybutyrate, creatinine and triglycerides

<p>| Table 2: Seasonal changes in serum enzymes for subadult brown bears (Ursus arctos) presented as 95% confidence interval |
|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>(\lambda)</th>
<th>2.5 - 97.5 interval</th>
<th>Sex</th>
<th>Season</th>
<th>(n)</th>
<th>Range</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>U/L</td>
<td>−1.2</td>
<td>43–317</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>38–95</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ALT</td>
<td>U/L</td>
<td>−1</td>
<td>11–63</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>10–20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AP</td>
<td>U/L</td>
<td>−0.4</td>
<td>13–214</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>12–35</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GGT</td>
<td>U/L</td>
<td>−0.4</td>
<td>4–98</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>4–16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GD</td>
<td>U/L</td>
<td>−0.2</td>
<td>2–24</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>2–7</td>
<td>0.009</td>
</tr>
<tr>
<td>CK</td>
<td>U/L</td>
<td>−0.6</td>
<td>56–843</td>
<td>F + M</td>
<td>W + Sp</td>
<td>60</td>
<td>61–522</td>
<td>0.0001</td>
</tr>
<tr>
<td>Amylase</td>
<td>U/L</td>
<td>−0.4</td>
<td>26–207</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>23–86</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lipase</td>
<td>U/L</td>
<td>−1.2</td>
<td>12–150</td>
<td>F</td>
<td>W + Sp</td>
<td>33</td>
<td>15–197</td>
<td>0.00185</td>
</tr>
<tr>
<td>LD</td>
<td>U/L</td>
<td>0.6</td>
<td>379–1021</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>363–753</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(\mathbf{a}\)AST = aspartate aminotransferase; ALT = alanine transaminase; AP = alkaline phosphatase; GGT = \(\gamma\)-glutamyl transpeptidase; GD = glutamate dehydrogenase; CK = creatinine kinase; LD = lactate dehydrogenase

\(\mathbf{b}\)\(\lambda\) = the transformation value from the Box-Cox transformation

\(\mathbf{c}\)2.5 and 97.5 percentiles on untransformed data

\(\mathbf{d}\)F = female; M = male

\(\mathbf{e}\)W = winter (February-March); Sp = spring (April-May); Su = summer (June)

\(\mathbf{f}\)\(p\)-values are given for significantly different seasons groups: S = \(p\)-value for sex

\(\mathbf{g}\)Non-estimable 95% confidence interval, the range is given as lowest-highest measured value
were significantly higher during winter compared to spring, and significantly higher during spring compared to summer. For the serum enzymes alanine transaminase, aspartate transaminase, alkaline phosphatase and lactate dehydrogenase the values during summer were significantly higher than during spring, and the values during spring were significantly higher than during winter. The values of red blood cell count, eosinophils, lymphocytes, total protein and cholesterol were significantly higher during winter compared to spring and summer, while the values of mean corpuscular volume, white blood cell count, segmented neutrophils, amylase, γ-glutamyl transpeptidase and bile acids were significantly lower during winter compared to spring and summer.

Discussion

Uniquely, this study documents seasonal differences in blood variables in free-ranging brown bears. Bears were captured in their natural habitat and, when hibernating, inside their dens. Previous studies have used limited numbers of captive bears or non-hibernating bears. The seasonal changes in blood variables demonstrated here reflect the drastic physiological changes that bears undergo during the year. From the hibernation period in winter with decreased metabolism and reduced renal, liver and pancreas function, to the active state during summer where bears increase their food consumption to prepare for hibernation.

During the capture event there are several factors that potentially can affect the blood variables; both the immobilising drugs, and the capture method chosen to enable darting may affect the animals’ physiology [17, 19]. In the present study the bears in April-June were immobilised from a helicopter using a combination medetomidine-zolazepam-tiletamine. In February-March, when hibernating, ketamine was added to the drug combination, and the bears were immobilised inside the den. High amounts of haemoglobin, red blood cells and haematocrit in winter have also been reported in other bear studies [11, 12, 20–22] and, as accompanied by higher total protein and albumin, can likely be attributed to dehydration and haemoconcentration. Higher albumin levels during hibernation compared to the other two seasons, and a nearly constant level of total globulins throughout the three seasons, gave a rise in the albumin:globulin ratio during winter. The lower mean cell volume in winter compared to in spring and summer, has also been reported in black bears [23] and is possibly related to iron status. Indeed, iron levels found in this study were significantly lower during winter than in summer and spring.

Several reports, including this present study, have shown lower white blood cell counts in bears during

<table>
<thead>
<tr>
<th>Table 3 Seasonal changes in serum proteins for subadult brown bears (Ursus arctos) presented as 95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>α₁-globulin</td>
</tr>
<tr>
<td>a₂-globulin</td>
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<tr>
<td></td>
</tr>
<tr>
<td>β-globulin</td>
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<td></td>
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<tr>
<td>γ-globulins</td>
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<tr>
<td>A:G</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

*³A:G = Albuminglobulin ratio
²λ= the transformation value from the Box-Cox transformation
²2.5 and 97.5 percentiles on untransformed data
²F = female; M = male
²W = winter (February-March); Sp = spring (April-May); Su = summer (June)
²p-values are given for significantly different seasons groups; S = p-value for sex
hibernation [12, 13, 20, 24]. One previous study in brown bears has reported lymphocytosis during winter [12]. Neutropenia during winter has been previously reported for brown bears [24]. This may, in addition to the decrease in total white blood cell count, be a consequence of a suppressed innate immune system during hibernation [24]. Lower enzyme levels during winter, as demonstrated in this study, have been reported in black bears [23, 25, 26]. Long-term fasting involves three phases based on changes in body tissue utilization as energy sources [27]. Fasting in bears was found to follow phase II [25] with increased fat catabolism associated with use of ketone bodies as substitute substrates for glucose. This inhibits the gluconeic pathway and reduces the membrane transport of glucose, and as a result of this, the demand for protein breakdown in the body is decreased [25, 27].

Alanine transaminase, aspartate transaminase, lactate dehydrogenase, γ-glutamyl transpeptidase, glutamate dehydrogenase and amylase are all enzymes that catalyse reactions involved in amino acid deamination, anaerobic glycolysis, glutathione reaction, conversion of glutamate to 2-oxoglutarate and hydrolysis of starches [28]. The decrease in protein breakdown and inhibition of the gluconeic pathway during hibernation may explain the significantly lower enzyme levels during winter. The lower values in 5 out of 9 enzymes during spring compared to summer, may reflect decreased bone turnover.

Creatinine and urea are good indicators of renal function, with high values indicating impaired function [28]. During hibernation the glomerular filtration rate (GFR) in bears decreases by about 70 % [29]. As a result, a rise in creatinine and magnesium concentrations in the

### Table 4: Seasonal changes in serum metabolites for subadult brown bears (Ursus arctos) presented as 95 % confidence interval

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>λ</th>
<th>2.5 - 97.5 interval</th>
<th>Sex</th>
<th>Season</th>
<th>n</th>
<th>Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-HBA</td>
<td>mmol/L</td>
<td>0.2</td>
<td>0.0–1.2</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>0.5–1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp</td>
<td>27</td>
<td>0.0–0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>0.0–0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp + Su</td>
<td>45</td>
<td>6–36</td>
<td></td>
</tr>
<tr>
<td>Bile acids</td>
<td>μmol/L</td>
<td>–1</td>
<td>4–52</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>4–9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp + Su</td>
<td>45</td>
<td>6–36</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (total)</td>
<td>μmol/L</td>
<td>0.2</td>
<td>0–4</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>0–4</td>
<td>0.0131</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>0–2</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mmol/L</td>
<td>1</td>
<td>4.3–12.6</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>8.7–12.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp + Su</td>
<td>45</td>
<td>4.0–11.4</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>μmol/L</td>
<td>0.2</td>
<td>72–303</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>166–322</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp</td>
<td>27</td>
<td>105–196</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>52–152</td>
<td></td>
</tr>
<tr>
<td>FFA</td>
<td>mmol/L</td>
<td>0.4</td>
<td>0.1–1.1</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>0.2–1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>0.1–0.8</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/L</td>
<td>0.8</td>
<td>2.6–10.6</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>4.0–10.2</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>1.9–9.2</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>mmol/L</td>
<td>0.4</td>
<td>1.8–7.2</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>3.4–7.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp</td>
<td>27</td>
<td>2.1–5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>1.7–4.1</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>0</td>
<td>0.5–26.3</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>1.0–12.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp</td>
<td>27</td>
<td>0.3–9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Su</td>
<td>18</td>
<td>0.6–32.4</td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>μmol/L</td>
<td>–0.6</td>
<td>48–270</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>42–146</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sp + Su</td>
<td>45</td>
<td>68–215</td>
<td></td>
</tr>
</tbody>
</table>

*β-HBA = β-hydroxybutyrate; FFA = free fatty acids

λ = the transformation value from the Box-Cox transformation

2.5 and 97.5 percentiles on untransformed data

F = female; M = male

W = winter (February-March); Sp = spring (April-May); Su = summer (June)

p-values are given for significantly different seasons groups

Non-estimable 95 % confidence interval, the range is given as lowest-highest measured value
blood is expected, and as far as creatinine goes, this has been previously documented in hibernating brown and black bears [12, 23, 30]. An increase in urea concentration is also expected, because of the decreased GFR, however, this study along with several previous reports [12, 23, 31] demonstrated a decrease. In a study on starvation of black bears during summer, bears became both dehydrated and azotemic [31]. The reason for decreased urea concentrations during hibernation is that the urea formed during catabolism of proteins is hydrolysed in the intestinal tract, and the nitrogen formed re-enters the protein synthetic pathways. This process is faster than the synthesis of urea [31, 32]. Hibernating bears are mainly metabolising fat, and their urea production decreases partly due to the shift from protein to fat metabolism. It has also been shown that the ability of bears to recycle urea during hibernation is not associated with den entry and exit, which means that it can occur before denning and some days after the bear has left the den [33]. This can explain the decreased urea values during spring compared to winter in this study, since the bears were captured shortly after leaving the den.

Increased values of blood lipids; triglycerides, cholesterol and free fatty acids, during hibernation found in this study, have been reported earlier in bears [22, 23, 25, 26, 34–36]. Catabolism of fat supplies the energy for metabolism during hibernation and the increase of blood lipids during hibernation, reflects lipolysis from adipose tissue [26, 37]. Bile acids are released during digestion, this leads to a postprandial increase in concentration of bile acids in the blood. The lower values of bile acids found in the present study, during winter, are consistent with fasting [28].

β-hydroxybutyrate (β-HBA) was also significantly higher in the winter compared to the spring and summer; as previously reported for denning bears [26, 32]. Even though β-HBA levels rise during hibernation, they are not high enough to result in ketosis [32]. In a study of changes in hepatic gene expression during hibernation, results indicated that the prevention of ketosis in hibernating bears is achieved by a rapid consumption of ketone bodies by peripheral tissue and not by a reduction of ketogenesis [26]. This process results in glucose sparing, likely an advantage for the fasting bear.

The changes reported here represent the many aspects of altered physiology seen during winter. The increased haemoglobin, red blood cells, haematocrit, total protein and albumin can be attributed to hypovolemia from the lack of liquid intake. The shift from carbohydrate and protein metabolism to fat metabolism results in decreased urea production and reduced demand for the enzymes important for protein breakdown (alanine transaminase, aspartate transaminase, lactate dehydrogenase, γ-glutamyl transpeptidase, glutamate dehydrogenase and amylase). Fat catabolism also resulted in increased blood lipids, triglycerides, cholesterol and free fatty acids and fasting resulted in increased β-HBA, lower bile acids, enzyme and urea levels. The general depression of metabolic rate is also represented by the decreased organ function including decreased kidney (increased creatinine and magnesium), liver (increased ALP), and pancreas (decreased amylase).

### Table 5 Seasonal changes in serum minerals and cortisol for subadult brown bears (*Ursus arctos*) presented as 95 % confidence interval

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>λ²</th>
<th>2.5 - 97.5 Interval³</th>
<th>Sex¹</th>
<th>Season¹</th>
<th>n</th>
<th>Range</th>
<th>p-value⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>mmol/L</td>
<td>2</td>
<td>1.9–2.6</td>
<td>F + M</td>
<td>W + Sp + Su</td>
<td>60</td>
<td>1.9–26</td>
<td>0.0006</td>
</tr>
<tr>
<td>Chloride</td>
<td>mmol/L</td>
<td>2</td>
<td>92–103</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>92–102</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12–105</td>
<td>Su</td>
<td></td>
<td>18</td>
<td>92–105</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>μmol/L</td>
<td>0.2</td>
<td>12–48</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>12–26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15–45</td>
<td>Sp + Su</td>
<td></td>
<td>45</td>
<td>15–45</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>mmol/L</td>
<td>1.6</td>
<td>0.71–1.25</td>
<td>F + M</td>
<td>W</td>
<td>15</td>
<td>0.98–1.30</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15–45</td>
<td>Sp + Su</td>
<td></td>
<td>45</td>
<td>0.67–1.08</td>
<td></td>
</tr>
<tr>
<td>Phosphorous (inorganic)</td>
<td>mmol/L</td>
<td>1.2</td>
<td>0.8–2.4</td>
<td>F + M</td>
<td>W + Sp</td>
<td>42</td>
<td>0.8–2.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15–45</td>
<td>Su</td>
<td></td>
<td>18</td>
<td>1.2–2.8</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/L</td>
<td>–1.8</td>
<td>3.4–5.6</td>
<td>F + M</td>
<td>W + Sp + Su</td>
<td>60</td>
<td>3.5–5.5</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>2</td>
<td>130–143</td>
<td>F + M</td>
<td>W + Su</td>
<td>33</td>
<td>133–142</td>
<td>0.0003</td>
</tr>
<tr>
<td>Cortisol</td>
<td>nmol/L</td>
<td>0.6</td>
<td>29–734</td>
<td>F + M</td>
<td>W + Sp + Su</td>
<td>57</td>
<td>25–668</td>
<td></td>
</tr>
</tbody>
</table>

²: λ = the transformation value from the Box-Cox transformation
³: 2.5 and 97.5 percentiles on untransformed data
⁴: F = female; M = male
⁵: W = winter (February-March); Sp = spring (April-May); Su = summer (June)
⁶: p-values are given for significantly different seasons groups
function and suppression of the innate immune system in winter (neutropenia).

Conclusion
Significant seasonal differences in hematological and biochemical variables were documented in free-ranging subadult bears. The changes show the shifts in activity of the liver, kidneys, pancreas and overall metabolic processes that characterizes long term fasting and hypometabolism.

Competing interests
The authors have declared that no competing interests exist.

Author contributions
ARG analyzed the data and drafted the manuscript. JMA and ÅF initiated the study and carried out collection of blood samples. ALE and SB conceived of the seasonal approach to data analysis and continued sample collection. MFB assisted with study design and drafting of the manuscript. All authors participated in writing the manuscript and approved the final version.

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References
34. Nelson RA, Wahner HW, Jones JD, Ellefson RD, Zollman PE. Metabolism of 
35. Matula GJ, Lindzey JS, Rothenbacher H. Sex, age, and seasonal 
differences in the blood profile of black bears captured in 
36. Storm GL, Arne GL, Matula GJ, Nelson RA. Blood-chemistry of 
black bears from Pennsylvania during winter dormancy. 
Paper IV
Body Temperature during Hibernation Is Highly Correlated with a Decrease in Circulating Innate Immune Cells in the Brown Bear (Ursus arctos): A Common Feature among Hibernators?

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Abstract

Background: Hibernation involves periods of severely depressed metabolism (torpor) and decreases in body temperature (Tb). Small arctic mammals (<5kg), in which Tb generally drop drastically, display leukopenia during hibernation. This raised the question of whether the decreased leukocyte counts in mammalian hibernators is due to torpor per se or is secondary to low Tb. The present study examined immune cell counts in brown bears (Ursus arctos), where torpor is only associated with shallow decreases in Tb. The results were compared across hibernator species for which immune and Tb data were available.

Methods and Results: The white blood cell counts were determined by flow cytometry in 13 bears captured in the field both during summer and winter over 2 years time. Tb dropped from 39.6±0.8 to 33.5±1.1°C during hibernation. Blood neutrophils and monocytes were lower during hibernation than during the active period (47%, p=0.001; 43%, p=0.039, respectively), whereas no change in lymphocyte counts was detected (p=0.599). Further, combining our data and those from 10 studies on 9 hibernating species suggested that the decline in Tb explained the decrease in innate immune cells (R²=0.83, p<0.0001).

Conclusions: Bears have fewer innate immune cells in circulation during hibernation, which may represent a suppressed innate immune system. Across species comparison suggests that, both in small and large hibernators, Tb is the main driver of immune function regulation during winter dormancy. The lack of a difference in lymphocyte counts in this context requires further investigations.

Key words: Brown bear, Ursus arctos, Hibernation, Innate immunity, Leukocytes, Torpor.

Introduction

The state of lowered metabolism during hibernation in mammals is a showcase of cell preservation strategies for muscle, bone, and the circulatory and innate immune systems. Hibernating mammals tolerate extremes in organ perfusion, oxygen saturation, temperature, immobilization, and calorie intake -
In most hibernating mammals, torpor phases are interrupted by euthermic arousal phases (reviewed in: (1)). During torpor, metabolism is severely depressed but hibernation also involves the inhibition of thermogenesis, leading to a considerable decrease in body temperature (Tb). The induction of torpor begins with lowering of the metabolic rate, followed by hypothermia as the Tb drifts downward (reviewed in: (1-3)). Various degrees of cold torpor have been observed over a phylogenetically wide range of mammals and is most drastic in bats and rodents. The champion of all torpid mammals is the hibernating arctic ground squirrel (*Urocitellus parryii*), previously called *Spermophilus parryii*, which exhibits body temperatures as low as -2.9°C (4).

In a recent paper by Bouma et al. (5), the current knowledge of the immune system of hibernating mammals was reviewed. Although there are few data from this field, one of the most striking phenomena is the reduced number of circulating leukocytes found in all hibernating mammals studies so far. The studies reviewed by Bouma et al. (5), finding leukopenia in hibernating mammals, were all conducted on small species, including the European hamster (*Cricetus cricetus*), European hedgehog (*Erinaceus europaeus*), European ground squirrel (*Spermophilus citellus*), arctic ground squirrel (*Urocitellus parryii*), and the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*, previously called *Spermophilus tridecemlineatus*); all species in which Tb drops drastically during hibernation. The authors (5) raised the question of whether the reduced number of circulating leukocytes in mammalian hibernators is due to torpor *per se* or secondarily to the low Tb. Most recently, the same authors showed that, at least in small species (Syrian and Djungarian hamsters), Tb during hibernation indeed controls leukopenia (6).

The brown bear (*Ursus arctos*) is an exception among hibernators. The definition of hibernation as a temporary physiological state, characterized by a controlled lowering of the metabolic rate, as well as a dramatic drop in Tb (1), does not strictly apply to the brown bear. Instead, the bear has been classified as a hibernator based on the length of the torpid period (5-7 months in Scandinavia) (7-9). However, in a recent study on black bears (*Ursus americanus*), it was shown that bears are true hibernators; involving processes of both metabolic and thermal regulation (10). Whereas the bear's body temperature remains relatively stable during hibernation with a less dramatic drop in comparison to other hibernators (from 37°C in summer to 33-30°C in winter) (10, 11), the absolute metabolic rate in the bear is substantially reduced by 75%, and it takes several weeks after emergence for metabolic rate to return to normal levels (10). The slight Tb lowering in hibernating black bears is uncoupled from metabolic suppression (10), and, most recently, it was demonstrated that the expression of a number of genes involved in regulating the metabolism is adjusted during winter hibernation (12). By this, the bear offers a unique opportunity to further elucidate whether immune depression during hibernation is primarily caused by a lowered Tb or might be intrinsic to torpor *per se*.

In the present study, we examined immune cell counts in peripheral blood of 13 free-ranging Scandinavian brown bears during hibernation and again during the active period in summer. The results were compared to data from the literature available on different species on immune function during hibernation.

### Materials & Methods

#### Study subjects

Blood samples were collected from 13 free-ranging brown bears, (7 females, 6 males; age 2 (n=4), 3 (n=8) and 4 (n=1) years old), during hibernation (February/March 2010 or 2011) and again from the same bears during their active period in summer (June 2010 or 2011). The bears were immobilized in the den during February/March with combination of tiletamine-zolazepam, medetomidine and ketamine and from a helicopter during June by darting with a combination of tiletamine-zolazepam and medetomidine (13, 14). Blood was collected from the jugular vein, as described previously (14, 15). An accu-temp digital thermometer (Jahpron Medical Int., Bodo, Norway), with a manufacturer reported accuracy of ±0.1°C, was used. The temperatures reported is the temperature taken immediately on capture. All bears were clinically examined by a wildlife veterinarian, and all animals were apparently healthy with no signs of infection. None of the bears were pregnant, and since bears do not have menstruation or any other bleeding during the ovulatory cycle, we did not exclude female bears. The study was approved by the Swedish Ethical Committee on animal research (C212/9). All procedures described were in compliance with Swedish laws and regulations.

#### Routine blood cell count

White blood cell (WBC) count and the number of different leukocytes were determined at the accredited Clinical Chemical Laboratory at Örebro Univer-
sity Hospital, Sweden, by a fully automated hematology analyzer (XE-5000, Sysmex Corporation, Kobe, Japan). Blood cells were differentiated by the instruments using standardized “WBC differential analysis settings” by applying a combination of forward scatter, side scatter and fluorescence of nucleic acid material using highly specific polymethine dyes. Blood smears were prepared at the field site and stored. The leukocyte differential counts were confirmed manually by the same lab technician for all samples, with a microscope at the Haematology and Chemistry Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Comparative approach

A Web of Science search was performed to find studies on hibernators in which both white blood cell counts and Tb data were available. This search identified ten studies, including 9 different species (6, 16-22); European hamster (*Cricetus cricetus*), Arctic ground squirrel (*Urocitellus parryii*), woodchuck (*Marmota monax*), 13-lined ground squirrels (*Ictidomys tridecemlineatus*), Syrian hamster (*Mesocricetus auratus*), European ground squirrel (*Spermophilus citellus*), Northern leopard frog (*Rana pipiens*), painted turtle (*Chrysemys picta*) and grass snake (*Natrix natrix*). For the later study, no data on Tb was given in the reference (22). As the grass snake is an ectotherm, we made a search on meteorological websites. The average temperature of grass snakes, corresponding to the month the study was conducted, was assumed as Tb. Including the present study, 10 species were included in the comparative analysis.

Statistical analysis

Normality of data sets was assessed using the Shapiro-Wilk test. The effects of hibernation on innate immune cells were analyzed by using mixed linear models with time as the repeated measure (hibernation vs. active), and bears and year of capture as random effects. Further analyses were performed by adjusting on sex, age and body mass to check for possible confounding factors on cell counts. The relationship between leukopenia (expressed as a percentage of the active period) and the drop in Tb during hibernation was checked by simple regression analysis. Two-sided *p*-values are reported and significance was set to <0.05 (SPPS version 16).

Results & Discussion

Blood samples were collected from all 13 bears during hibernation and again in summer. During winter, the rectal temperature, recorded with a digital thermometer, was 33.5 ± 1.1 (32.2-35.4) °C and during summer 39.6 ± 0.8 (38.8-40.9) °C (Figure 1a). Rectal temperatures seen during summer captures were typical for bears immobilized during the active period (23). WBC count and the presence of different types of leukocytes were investigated. Bears had significantly lower counts of peripheral blood leukocytes during hibernation compared to the active period (4.5 ± 1.1 vs. 7.4 ± 2.4 x10⁹/L, *p* = 0.001; Figure 1b). These data are in agreement with a previous study showing lower leukocyte counts in captive brown bears during denning compared to non-hibernating animals (11). In addition, we found that the bears had significantly fewer neutrophils (3.0 ± 0.7 vs. 5.7 ± 2.4 x10⁹/L, *p* = 0.001; Figure 1c) and monocytes (0.35 ± 0.08 vs. 0.61 ± 0.4, *p* = 0.039; Figure 1d) during hibernation. These findings are consistent with the results for small hibernating animals (5). On the other hand, we could not detect any change in lymphocyte counts between hibernation and active periods (1.1 ± 0.4 vs. 1.1 ± 0.4 x10⁹/L, *p* = 0.599; data not shown). In one of the bears, WBC was 21.9 x10⁹/L during hibernation, which is 4-5 times higher compared to the other bears (Figure 1). Neutrophils, monocytes and lymphocytes were slightly elevated (4.3, 1.1 and 2.0 x 10⁹/L, respectively) compared to the other bears (Figure 1). However, the number of eosinophils was extremely high in this bear compared to the other bears (14.9 vs. 0.22 ± 0.11 x10⁹/L). C-reactive protein (CRP) was not elevated (0.085 vs. 0.046 ± 0.04 mg/L for the other bears). The leukocyte count of this bear was similar to the other bears when examined in the summer. Still, based on the haematology analyses, this bear was excluded from the study.

Our data support the view that the innate immune system is suppressed during hibernation. As discussed by Bouma et al. (5), this could affect the defence towards infections, as illustrated by the massive death of hibernating bats caused by a fungus (24) that has an optimal growth temperature of 12.5-15.8 °C but grows well at 3-6 °C (25). Interestingly, it has been suggested that one reason why animals arouse periodically from torpor is to reactivate a dormant immune system to combat pathogens that enter the body during the torpor bouts (26). Although arousals in bears show very different patterns as compared to small-bodied hibernators (10), further investigation is required to find out whether or not hibernating bears exhibit increased cell counts upon arousal from torpor.
In contrast to the extreme decrease in peripheral blood lymphocytes in small hibernating animals (5, 6), our data suggest that cells of the adaptive immune system are not affected in bears during hibernation. However, given that many acquired immune responses are stimulated by innate cells, there may still be an effect on the functioning of this immunological aspect. During arousal bouts in small hibernating animals, there is a rapid (within a few hours) reappearance of retained lymphocytes probably from the gut and spleen to the blood (6, 27, 28). Hibernating black bears do not show spontaneous arousals to normothermic levels of Tb (10), as do small hibernators. Instead, they change position on a daily basis and display multiday cycles of Tb between 30-36°C (10). Still, bears can be aroused relatively easily, and one could speculate that the short awaken period before we anesthetized the bears could have influenced the number of lymphocytes. However, this period of time was very short (approx. 15 min), and, in addition, the number of circulation lymphocytes during arousal found in small hibernators is still only half of the number found in active animals during summer (29), suggesting that this was not a major factor influencing our results. Another explanation of depressed neutrophils and monocytes during torpor could be that longer-lived lymphocytes would remain in circulation longer than the short-lived neutrophils. On the other hand, this explanation requires a decreased rate of granulopoiesis or a shorter life span of granulocytes. If bear leukocytes are sequestered in peripheral tissue during hibernation as found in small hibernators (6, 27, 28), this phenomenon might explain why monocytes are depressed with torpor due to their ability to leave the circulation. Also, further studies should investigate the hibernating effect of lymphocytes in sub-groups to see if there are different patterns between, for instance, B- and T-lymphocytes, and if this could explain the numbers of lymphocytes found in hibernating vs. active bears. Although the length of time and body temperature history during hibernation could play a role in determining the leukocyte profile at the particular time of sampling, this is unlikely based on work done in ground squirrels (19) that found that the numbers of leukocytes decreased by 90% within 24 hours of torpor and remained unchanged during the remainder of the torpor-period. Although this indicates that the length of time hibernating prior to sampling may not have an impact on the results, further studies including the temperature history (using biologging techniques) would be necessary to determine if these results in ground squirrels apply to the brown bear.
Our study thus supports the concept that hibernation has significant effects on the immune system. Whether or not the hibernation-induced leukopenia is a general phenomenon of hibernation, independently of body mass, resulting from a direct body temperature effect, is an important question from an evolutionary point of view. In small hibernators, the number of circulating white blood cells drops dramatically when the body temperature decreases to 5˚C, and it was thus recently argued that the hibernation induced immune depression is a temperature-dependent mechanism (6). Even more recently, metabolic suppression in bears during hibernation was suggested to be independent of Tb (10); indicating that processes of hibernation may occur in a temperature-independent matter. The direct corollary is to question whether the hibernation-induced leukopenia is independent of Tb when body size is large or whether the temperature effect is a general feature of hibernation. Clearly, this would bring lights on many questions of the above discussion. The between-species-comparison we performed shows that, across taxa, the depression of the immune function appears indeed as a direct function of the temperature reached during torpor (Figure 2). The intercept is slightly off zero, indicating that we cannot rule out the contribution of other mechanisms. However, given the strength of the relationship with Tb explaining 83% of the relationship, it is likely that any alternative mechanisms are of modest importance, but further studies on large animals, such as different bear species, are needed. Taken together, our results suggest that the drop in Tb, reached during the torpor bout, is the main drive of the process of leukopenia observed during hibernation and this may be a general mechanism in hibernators. We think that similar approaches are needed to investigate the relationship between metabolic depression and hypothermia. Indeed, we find it hard to explain how such an independency is possible given the first law of thermodynamics. We rather think that further studies are clearly needed to look at regional/organ body temperature regulations.

Figure 2. Relationship between leukopenia and the drop in Tb during hibernation across heterothermic species. European hamster (Cricetus cricetus; (16)), Arctic ground squirrel (Urocitellus parryii; (17), woodchuck (Marmota monax; (18), 13-lined ground squirrels 1 (Citellus tricemlineatus; (18), 13-lined ground squirrels 2 (Ictidomys tridecemlineatus; (38), 13-lined ground squirrels 3 (Ictidomys tridecemlineatus; (39), Syrian hamster (Mesocricetus auratus; 6), European ground squirrel (Spermophilus citellus; (19), Northern leopard frog (Rana pipiens; (20), painted turtle (Chrysemys picta; (21), and grass snake (Natrix natrix; (22) . The bear data are from the present study.
In conclusion, the brown bear offers novel information on the innate immune system during hibernation. The present study supports the hypothesis that the reduced number of white blood cells during hibernation is a temperature-dependent phenomenon conserved across evolution. Future studies are needed to understand the mechanisms regulating the innate immune system during hibernation. We hypothesize that the reduced number of neutrophils during hibernation is associated with increased survival. One explanation could be that the suppression in immune function, as a function of body temperature, is proportional to the decrease in virulence of any pathogens present in the hibernator. Whether this is due to energy benefits of a reduced immune function (6) or other mechanisms must be determined in future studies.

In perspective, our findings of a hibernation-associated drop in white blood cells in brown bears could inspire research in human pathophysiological states and increase the understanding of hibernation physiology in general and aspects of ecological immunology. High numbers of neutrophils are associated with disorders, including ischemic heart disease (30), complications of metabolic syndrome (31) and hypertension (32). Patients with coronary artery disease display dysregulated neutrophils (33, 34), and activated neutrophils play a role in morbid obesity (35), rheumatoid arthritis (36) and bronchial asthma (37). If the regulatory mechanisms behind white blood cell lowering in bears could be worked out, this might have therapeutic potential. Understanding the mechanisms of specific physiological alterations involved in hibernation is of relevance for pharmacologically induced suspended animation - a therapeutic option in patients with acute severe cerebral or cardiovascular disease (6).

**Author Contributions**

**Study design:** Sahdo, Evans, Arnemo, Fröbert, Särndahl.

**Acquisition of data:** Sahdo, Evans, Blanc, Fröbert.

**Analysis and Interpretation of data:** Sahdo, Evans, Arnemo, Blanc, Fröbert, Särndahl.

**Preparation of manuscript:** Sahdo, Evans, Arnemo, Blanc, Fröbert, Särndahl.

All authors were involved in revising the manuscript for important intellectual content and approved the final version.

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**Statement of Responsibility:** The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Abbreviations**

Tb: body temperature; WBC: white blood cells.

**Competing Interests**

The authors have declared that no competing interest exists.

**References**


Paper V
Decrease in the red cell cofactor 2,3-diphosphoglycerate increases hemoglobin oxygen affinity in the hibernating brown bear *Ursus arctos*

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Revbesch IG, Malte H, Fröbert O, Evans A, Blanc S, Josefsson J, Fago A. Decrease in the red cell cofactor 2,3-diphosphoglycerate increases hemoglobin oxygen affinity in the hibernating brown bear *Ursus arctos*. Am J Physiol Regul Integr Comp Physiol 304: R43–R49, 2013. First published November 21, 2012; doi:10.1152/ajpregu.00440.2012.—During winter hibernation, brown bears (*Ursus arctos*) reduce basal *O*₂ consumption rate to ~25% compared with the active state, while body temperature decreases moderately (to ~30°C), suggesting a temperature-independent component in their metabolic depression. To establish whether changes in *O*₂ consumption during hibernation correlate with changes in blood *O*₂ affinity, we took blood samples from the same six individuals of hibernating and nonhibernating free-ranging brown bears during winter and summer, respectively. A single hemoglobin (Hb) component was detected in all samples, indicating no switch in Hb synthesis. O₂ binding curves measured on red blood cell lysates at 30°C and 37°C showed a less temperature-sensitive O₂ affinity than in other vertebrates. Furthermore, hemolysates from hibernating bears consistently showed lower cooperativity and higher O₂ affinity than their summer counterparts, regardless of the temperature. We found that this increase in O₂ affinity was associated with a significant decrease in the red cell Hb-cofactor 2,3-diphosphoglycerate (DPG) during hibernation to approximately half of the summer value. Experiments performed on purified Hb, to which DPG had been added to match summer and winter levels, confirmed that the low DPG content was the cause of the left shift in the Hb-O₂ equilibrium curve during hibernation. Levels of plasma lactate indicated that glycolysis is not upregulated during hibernation and that metabolism is essentially aerobic. Calculations show that the increase in Hb-O₂ affinity and decrease in cooperativity resulting from decreased red cell DPG may be crucial in maintaining a fairly constant tissue oxygen tension during hibernation in vivo.

metabolic suppression; body temperature; oxygen binding curves; heat of oxygenation; hibernation

A DISTINCT TRAIT OF MAMMALLIAN hibernation is the highly controlled decrease in body temperature and metabolic rate. A hibernating brown bear (*Ursus arctos*) routinely spends 5–7 mo per year in continuous dormancy with no food or water intake, no urination, and no defeation (19, 36). During this time, bears appear to be resistant to loss of muscle mass, strength, or bone density (18, 24, 29, 43, 14, 44). During hibernation body temperature is downregulated only slightly, fluctuating from ~37°C to a minimum of 30°C, as found in brown and in black bears (*Ursus americanus*) (17, 26, 27, 36, 43), whereas *O*₂ consumption rate is downregulated by 75% (43). In comparison, in most smaller hibernators, such as ground squirrels and marmots, body temperature drops dramatically to values close to ambient temperatures (32, 37, 48), with a consequent strong Q₁₀~10~ induced depression of metabolic rate (where Q₁₀ is the rate coefficient for a 10°C change in temperature). In spite of substantial downregulation of ventilation and heart rate, most hibernators likely experience only slight or no hypoxia and in some ground squirrels arterial *O*₂ tension (P*O*₂) is normal during torpor (16). As opposed to smaller hibernators that exhibit periods of spontaneous arousals back to normothermic temperature and metabolic rate (33, 31), bears do not arouse to normothermic temperature during winter but have shallow multiday cyclic (1.6–7.3 days) fluctuations in body temperature (43). Nevertheless, as demonstrated in a recent study (43), bears exhibit a strong active aerobic metabolic depression and a weight-specific metabolic rate similar to that of smaller hibernators (20, 25, 43), and for this reason, they are now recognized as true hibernators, even though they do not show the dramatic drop in body temperature and arousals typical of small hibernators (43). Whether and how the blood *O*₂ transport of bears adapts to a decreased *O*₂ supply to tissues during hibernation is however, still unknown.

Earlier studies have found that in small hibernating mammals, blood *O*₂ affinity increases markedly during hibernation (4, 9, 32). Potentially, blood *O*₂ affinity can be affected in two ways: by structural changes in the blood *O*₂ carrier hemoglobin (Hb) that lead to changes in the protein sensitivity to allosteric cofactors and temperature, or by changes in the concentration of allosteric cofactors inside the red blood cell. Such allosteric cofactors include organic phosphates that bind to the central cavity of the Hb tetramer and decrease *O*₂ affinity by shifting the allosteric T-R equilibrium between the low-affinity (T) and the high-affinity (R) protein conformation toward the T state. In most mammalian Hbs, the main anionic cofactors are the organic phosphate 2,3-diphosphoglycerate (DPG) and Cl⁻, that in bear Hb have a large synergistic effect in regulating *O*₂ binding (11). DPG binds allosterically to the low-affinity T-state conformation of mammalian Hbs and, thereby, decreases Hb-O₂ affinity (5). An increase in erythrocytic DPG decreases Hb-O₂ affinity, and, conversely, a decrease in DPG increases Hb-O₂ affinity.

An increase in blood *O*₂ affinity has been observed in several small hibernating animals during dormancy (4, 34), but has not yet been measured in bears. Although the molecular mechanisms for a hibernation-induced increase in blood *O*₂ affinity have not been much investigated, reductions in DPG levels
have been found to be involved in the hedgehog (28) and possibly in some hibernating rodents (23, 32).

Here, we report O2 binding curves of red blood cell hemolysates and purified Hb from free-ranging radio-collared brown bears during summer activity and winter hibernation. Curves were measured at temperatures close to the lowest measured body temperature of hibernating bears and the normothermic temperature of nonhibernating bears, 30°C and 37°C, respectively (26, 43), to take into account the effect of temperature on Hb oxygenation. We also examined Hb multiplicity, concentration levels of the allosteric cofactor DPG present in the red cells, and of plasma lactate to evaluate possible differences in glycolytic activity for hibernating and nonhibernating bears.

**MATERIALS AND METHODS**

**Blood sample collection and preparation.** Samples of blood were taken from the same six free-ranging 2- to 3-yr-old Eurasian brown bears, Ursus arctos, three females and three males captured during winter hibernation (February: females 35, 57, and 59 kg; males 21, 25 and 58 kg) and summer (June: females, 28, 72, and 47 kg; males, 27, 51, and 22 kg) in Dalarna county, Sweden, as described previously (17). The bears were immobilized by darting in the den during February 2011 and again by darting from a helicopter during June. Bears were anesthetized as described in detail in a previous study (17). Briefly, in winter, a mixture of tiletamine-zolazepam (1.1 mg/kg, except 2.5 mg/kg in one male bear, 25 kg), medetomidine (0.03 mg/kg) and ketamine (1.3 mg/kg, except 3 mg/kg in one male bear, 25 kg) was used, and in summer, a mixture of tiletamine-zolazepam (4.7 mg/kg) and medetomidine (0.09 mg/kg) was used. Doses were based on body mass and time of year (due to differences in expected metabolism), as previously reported (17). Blood samples were taken within ~20 min from darting. All animal handling and sampling was carried out under approval of the Swedish Ethical Committee on animal research (C212/9). The performed procedure was in compliance with Swedish laws and regulations. In the field, blood samples (~1 ml) were taken from the jugular vein of anesthetized animals into syringes containing 50 μl of 200 mM EDTA as anticoagulant. At the time of sampling, rectal temperature of the bears was 33.8 ± 1.5°C during winter sampling and 39.1 ± 1.4°C during summer sampling. Plasma pH was unchanged (7.25 ± 0.09 for winter bears and 7.25 ± 0.06 for summer bears), and slightly acidic, probably due to anesthesia, as previously reported (17). Blood samples were centrifuged with a portable centrifuge in Eppendorf tubes immediately after collection to separate plasma and red blood cells (RBCs). Plasma and RBCs from each individual were immediately frozen on dry ice and shipped to Aarhus University, Denmark.

In the laboratory, samples were processed individually. Aliquots of frozen RBCs were added to 0.2 M HEPES buffer (pH 7.40) at a 1:1 volume ratio, and cell debris was removed by centrifugation (7,000 g, 5 min, 4°C). Levels of oxidized (met) Hb were negligible in all samples, as judged form the absorbance ratios at 577 and 541 nm (A577/A541 > 1).

**DPG, hemoglobin, and chloride.** DPG concentration was assessed spectrophotometrically in all individual hemolysates using the DPG assay kit (Roche Diagnostics, Mannheim, Germany; cat no. 10 148 334 001), where concentration of DPG in the reaction assay is stoichiometrically coupled to the decrease of NADH to NAD+ that was followed at 340 nm (extinction coefficient 6.22 mM-1 cm-1) using a Uvikon 923 B double-beam UV/Vis spectrophotometer (Kontron Instruments, Milan, Italy) in 1-cm cuvettes. Deproteinization with 0.6 M perchloric acid and neutralization of the supernatant using 25 M K2CO3 was carried out with one tenth of the prescribed volumes. In the protocol used, 100 μl of hemolysate was added to 500 μl ice-cold 0.6 M perchloric acid, mixed and centrifuged (2000 g, 10 min), 400 μl of the clear supernatant was then neutralized with 50 μl of ice-cold 2.5 M K2CO3, and left on ice for ~70 min. Samples were centrifuged again to spin down precipitate (2,000 g, 5 min, 4°C), and 100 μl of supernatant was used for the spectrophotometric assay following the instructions provided by the manufacturer. One blank served as reference for every 4–6 samples. The reaction was completed after 25 min, whereafter the final absorbance did not change noticeably. The method was validated in control experiments made with fresh human blood and frozen RBCs. These experiments yielded DPG values for human blood (4.25 ± 0.54 mmol/l RBC) equivalent to those reported by the kit manufacturer (4.83 ± 0.15 mmol/l RBC).

To determine the DPG to tetrameric Hb ratios in individual samples, Hb concentration was measured using Drabkin’s method (15, 42). Absorbance was read at 540 nm using a Uvikon 923 B Double Beam UV/Vis spectrophotometer (Kontron instruments; Milan, Italy) and the hemoglobin concentration determined from the cyanmethemoglobin extinction coefficient at 540 nm of 10.99 mM-1 cm-1 (heme basis) (49).

**Hemoglobin multiplicity.** To evaluate Hb multiplicity, individual winter and summer hemolysates were analyzed on an isoelectric focusing (IEF; pH range 3–9) polyacrylamide gels (Phastigel GE Healthcare Biosciences AB, Upplands Väsby, Sweden). A heme concentration of 200 μM in the samples diluted in milliQ water yielded visible red bands. Adult human hemolysate was used for comparison.

**Oxygen binding measurements of hemolysates.** O2 equilibrium curves were determined at constant temperatures of 30 and 37°C (± 0.2°C). These temperatures were chosen as they are near to the lowest body temperature measured in hibernating bears and the normal temperature in summer active state, respectively (26, 36, 43). Curves were determined using a modified diffusion chamber, as described previously (40, 45) at a heme concentration of 1 mM in 0.1 M HEPES buffer, pH 7.4. A pH of 7.4 was chosen, as it is close to normal mammalian blood pH. Two HEPES buffer stock solutions (1 M) differing slightly in pH were used to achieve the same final pH of 7.4 of the sample at the two chosen temperatures. For each sample, pH was measured using a Radiometer BMS2 Mk2 microelectrode assembly coupled to a Radiometer PHM64 pH meter. In each experiment, a thin layer (~0.01 mm) of sample was equilibrated with humidified gases of varying O2 tensions supplied by two cascaded Wösthoff (Bochum, Germany) gas-mixing pumps mixing pure (99.998%) N2 and air. Changes in absorbance at 436 nm upon stepwise increases in PO2 within the chamber were monitored to determine changes in O2 saturation as a function of changes in PO2. Zero and 100% O2 saturation were obtained from equilibration with pure N2 and O2, respectively. For each equilibration step, absorbance was obtained using the in-house made data acquisition software Spectrosampler (available on request). The O2 partial pressure required to achieve 50% saturation of the Hb (P50) and cooperativity coefficient at 50% saturation (n50) were calculated from the zero intercept and slope, respectively, of Hill plots, log[Y/(1-Y)] vs. log[PO2], where Y is fractional saturation. Hill plots were based on at least 4 saturation steps between 0.3 and 0.7.

The temperature dependence of O2 binding expressed as the apparent heat of oxygenation (kcal/mol, 1 kcal = 4.184 kJ/mol) was calculated by the van’t Hoff equation:

\[
\Delta H = -4.57 \left[ \frac{1}{T_1} - \frac{1}{T_2} \right] \times \Delta \log P_{50}/1,000 \text{ kcal/mol, where } T_1 \text{ and } T_2 \text{ are the absolute temperatures (Kelvin) and } \Delta \log P_{50} \text{ is the corresponding difference in logP}_{50} \text{ at the two temperatures. The } \Delta H \text{ values presented have been corrected for heat of O2 in solution (~3.0 kcal/mol (1)).}
\]

**Oxygen binding measurements of purified hemoglobin.** Hb was purified from three winter and three summer individual hemolysates. Bear Hb was stripped from DPG by gel filtration by passing the individual hemolysates (~<1 ml sample) through a Sephadex G-25M, PD-10 column (GE Healthcare, New York, NY) equilibrated with 10 mM HEPES, pH 7.6, at 4°C. To facilitate removal of DPG, NaCl was added to the hemolysate samples to a final concentration of 0.2 M before loading on column. Stripped Hb samples were then concentrated by ultrafiltration at 4°C using Amicon ultra 0.5 ml 10 K
(Millipore) spin tubes. O2 binding curves for all Hb samples were measured at 30°C and 37°C, as described above (0.1 M HEPES, pH 7.4, 1 mM heme) in the presence of added DPG and Cl\(^-\). For each Hb sample, summer or winter, DPG was added to obtain the same Hb tetramer to DPG ratio as that measured in the (unstripped) hemolysates. Chloride (Cl\(^-\)) was added to the same final concentration as that measured in the hemolysates (10 mM) using 1 M NaCl. Cl\(^-\) concentration was measured using a Sherwood MKII model 926S chloride analyzer (Sherwood Scientific), requiring 5 μl per assessment (measurements were done in duplicates).

**Plasma lactate concentration.** To assess for changes in anaerobic metabolism, concentration of lactate was determined enzymatically in the plasma of winter and summer blood samples, following a previously described method (38). This method utilizes the stoichiometric conversion of NAD\(^+\) and lactate to NADH and pyruvate by lactate dehydrogenase (LDH), reaction 1 in the scheme below. The reaction is kept shifted to the right by the constant removal of pyruvate, which reacts with glutamate in the presence of glutamic pyruvate transaminase (GPT), reaction 2, as shown below. Production of NADH (equivalent to the lactate present) is quantified from the increase in absorbance at 340 nm using the extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\).

\[
\text{LDH} \quad \text{Lactate} + \text{NAD}^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + H^+ \quad (I)
\]

\[
\text{GPT} \quad \text{Pyruvate + glutamate} \rightarrow \text{alanine} + \alpha - \text{keto glutamate} \quad (2)
\]

The original method of Passonneau and Lowry was slightly modified. Thawed plasma (35–50 μl) was used directly for the assay (1 ml total volume) in 1-cm cuvettes. Final concentrations in the cuvette were: 50 mM 2-amino-2-methylpropanol buffer (ICN Biomedicals, Santa Ana, CA), pH 9.9; 50 mM glutamate (l-glutamic acid monosodium salt; ICN Biomedicals), pH 9.8; 3 mM NAD\(^+\) (NAD\(^+\) free acid, grade II, 98%; Roche Diagnostics); 100 μg/ml LDH (from bovine heart, type XVII; Sigma-Aldrich), 100 μg/ml GPT (Roche Diagnostics). Glutamate and NAD\(^+\) solutions were prepared each day and kept on ice until used. Absorbance of samples was recorded immediately after addition of GPT and again after 30-min incubation at room temperature until absorbance was stable. A blank (containing milliQ water instead of plasma) was run in parallel every 1–4 samples. A standard curve was constructed from 0.1 to 2 mM lactate measured at 30°C was significantly higher (i.e., P50 was significantly lower) than that of summer hemolysates measured at 37°C and 30°C (Fig. 2B) was found to be significant by the Wilcoxon signed rank test, with P = 0.031.

**RESULTS**

There was no indication of variation in Hb isoforms between summer and winter blood samples, as determined by IEF gels (Fig. 1). Bears expressed a single Hb with an isoelectric point similar to that of human HbA.

Oxygen binding curves of summer and winter hemolysates measured at 30°C and 37°C, and the corresponding changes in P50 are shown in Fig. 2. When measured at the respective physiological temperatures, O2 affinity of winter hemolysates measured at 30°C was significantly higher (i.e., P50 was significantly lower) than that of summer hemolysates measured at 37°C (Fig. 2, Table 1). Although a significant effect of temperature on P50 values was observed (Fig. 2B), the temperature change alone was not enough to provide the observed shift in the O2 equilibrium curve (Fig. 2A), and P50 values of winter and summer samples measured at identical temperatures were significantly different (Fig. 2B), suggesting changes in a soluble red cell allosteric cofactor. The cooperativity coefficient n50 was also significantly lower in winter compared with summer (Table 1), regardless of the temperature of measurement, indicating a change in the overall allosteric equilibrium between T and R state. The heat of oxygenation (ΔH) was similar in summer and

![Table 1. O2 affinity (P50), cooperativity coefficients (n50) measured at 30°C and 37°C and derived heat of oxygenation (ΔH) in brown bear hemolysates during winter and summer](https://www.ajpregu.org)

<table>
<thead>
<tr>
<th></th>
<th>Summer 37°C</th>
<th>Summer 30°C</th>
<th>Winter 37°C</th>
<th>Winter 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>P50, torr</td>
<td>15.4 ± 0.6</td>
<td>10.0 ± 0.4</td>
<td>11.4 ± 0.8*</td>
<td>7.3 ± 0.6*</td>
</tr>
<tr>
<td>n50</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>1.9 ± 0.2*</td>
<td>1.7 ± 0.2*</td>
</tr>
<tr>
<td>ΔH, kcal/mol</td>
<td>-8.5 ± 0.8</td>
<td>-8.5 ± 0.8</td>
<td>-8.7 ± 1.5</td>
<td>-8.7 ± 1.5</td>
</tr>
</tbody>
</table>

*Significant differences (P < 0.001) from summer values (means ± SD); n = 6.

\[C_{O_2} = \beta_{O_2} \cdot P_{O_2} + 4C_{Hb} \cdot \frac{P_{O_2}^n}{P_{O_2}^{n50}} + P_{O_2}^{n50} \quad (4)\]

where n is Hill’s cooperativity coefficient (n50) and P50 is the half-saturation partial pressure and B0 is the physical solubility of O2 (8). We used n50 and P50 values measured here at 37°C and 30°C for summer and winter samples, respectively (see Table 1 under RESULTS). Cao2 was first calculated from Eq. 4 assuming an arterial PaO2 of 100 torr and an intracellular tetrameric Hb concentration of 5 mM and using reported values of hemocrit (Hct) of 45 and 56.8% for brown bears measured during summer and winter, respectively (2). O2 consumption rates (VO2) of 0.30 and 0.069 ml O2·g\(^{-1}\)·h\(^{-1}\) and heart rates of 55 bpm and 14.4 bpm were taken from previously published values (43) for summer and winter black bears, respectively. Cardiac output was calculated from the heart rate using a stroke volume of 0.06 liter as expected for a 60-kg bear (i.e., the mean weight of the bears used in the study of 43), as described by Schmidt-Nielsen (39). After obtaining CaO2, the Cvo2 value for summer active and hibernating bears was obtained by Eq. 3, and was then used in Eq. 4 to obtain Pvo2 values. Calculations were performed by iteration using a Mathematica script (Wolfram Research, Champaign, IL).

**Statistics.** Values are presented as mean ± SD. Paired t-test was used for the statistical comparison between summer and winter individual samples. Comparisons were statistically significant with P ≤ 0.001. One comparison of P50 of hemolysates at summer 37 and 30°C (Fig. 2B) was found to be significant by the Wilcoxon signed rank test, with P = 0.031.
winter samples, with values of $-8.5 \pm 0.8$ kcal/mol and $-8.7 \pm 1.5$ kcal/mol, respectively.

The red cell hemolysate DPG concentration was significantly lower in winter compared with summer hemolysates (Table 2). O2 equilibria are largely affected by DPG to Hb tetramer ratios rather than by DPG concentrations alone. In the winter samples this ratio was $1:1$, whereas in the summer samples it increased significantly to $\sim 2:1$. Plasma lactate did not vary significantly between winter and summer (Table 2).

To evaluate whether the observed left shift in the O2 equilibrium curve found for the hemolysate of hibernating bears (Fig. 2) was due to a decrease in DPG, we removed endogenous DPG from the hemolysate from three bears, added exogenous DPG to the same DPG:Hb tetramer ratio as in the untreated hemolysate (i.e., 2:1 for summer samples and 1:1 for winter samples) and measured O2 equilibria at the two temperatures. Cl was added to the same final concentration as measured in the untreated hemolysates (10 mM Cl). The $P_{50}$ and $n_{50}$ values (means $\pm$ SD, $n = 3$) obtained in these samples were for winter bears $6.6 \pm 0.5$ torr, and $1.8 \pm 0.04$ (30°C), $9.5 \pm 0.9$ torr and $1.7 \pm 0.02$ (37°C), respectively. For summer bears, the same parameters were $9.2 \pm 0.2$ torr and $2.0 \pm 0.18$ (30°C), $12.6 \pm 0.2$ torr and $2.0 \pm 0.09$ (37°C), respectively.

As shown in Fig. 3, the change in $P_{50}$ between summer and winter samples obtained with purified Hb added to DPG was not significantly different from that obtained with the untreated RBC lysates, at both temperatures (Fig. 3), demonstrating that the change in DPG concentration was responsible for the left-shifted O2 equilibrium curves of hibernating bears reported in Fig. 2.

When assuming similar heart rates and O2 consumption as in undisturbed hibernating and active black bears (43), the O2 tension of mixed venous blood (that approximates that existing in tissues) in hibernating and active brown bears can be
predicted from the Fick equation when knowing values for $P_{50}$, cooperativity coefficients $n_{50}$ and blood Hb concentration, as described in detail under MATERIALS AND METHODS. Figure 4 shows the predicted $O_2$ equilibrium curves for winter and summer animals along with the estimated arterial and venous $O_2$ saturation and respective $P_O_2$ values. In the calculations, $P_{50}$ and $n_{50}$ values were those measured at the physiological temperatures of $37^\circ C$ and $30^\circ C$ for summer and winter samples, respectively (Table 1). As evident from Fig. 4, winter $O_2$ content of the blood is considerably elevated because of the increase in Hct during hibernation (2). If the $O_2$ binding curve had remained unchanged during winter (dotted line, Fig. 4), venous $P_O_2$ would have been substantially elevated ($\sim 21.8$ torr). However, a left-shift in the Hb-$O_2$ binding curve may avoid this situation and maintain $P_{O_2}$ of hibernating brown bears relatively unchanged (summer $\sim 14.8$ and winter $\sim 11.5$ torr) (Fig. 4).

**DISCUSSION**

In this study of free-ranging brown bears, we found a marked left-shift of the $O_2$ equilibrium curve during hibernation, which was associated with an increase in the Hb-$O_2$ affinity and a decrease in cooperativity. We demonstrated that the differences between hibernation and active state can be explained by a decrease in the red cell cofactor DPG, a major allosteric cofactor of Hb, whereas the reduction in body temperature during hibernation alone cannot account for the observed shift in Hb oxygenation. In concert with decreased ventilation and heart rate, a left-shifted $O_2$ equilibrium curve would maintain the necessary $O_2$ supply to tissues under conditions of a prolonged depressed $O_2$ consumption rate, as during hibernation. Such long-term effects are typically mediated by changes in the levels of organic phosphates, from high-altitude mammals (46) to hypoxic fish (47). A similar decrease in DPG levels as found here has been demonstrated in other hibernating mammals, such as the 13-lined ground squirrel, the golden-mantled ground squirrel, the woodchuck, and the hedgehog (7, 23, 28). However, only in the hedgehog has the DPG decrease been demonstrated to be directly coupled with the increase in blood $O_2$ affinity during hibernation (28).

By contrast, high $O_2$ consumption rates, as during intense muscle exercise, are typically associated with a transient right shift of the $O_2$ binding curve, as, for example, due to the Bohr effect. In this study, we found that during hibernation, a depressed $O_2$ consumption rate was not compensated for by increased glycolysis, as plasma lactate levels did not increase in winter samples, in agreement with previous measurements (17). Levels of plasma lactate found in winter ($3.05 \pm 1.32$ mM) were comparable to normal human values (22, 30, 41). The higher variation of plasma lactate concentration in the summer samples may reflect a more variable activity of the individual bears at the time of sampling (Table 2). This further confirms that energy metabolism in hibernating bears is essentially aerobic, as also indicated by the use of fatty acids as the primary energy fuel (35, 36).

When in the presence of anionic allosteric effectors, the Hb from brown bear, similar to that from other polar and cold-tolerant mammals, shows a reduced temperature sensitivity (i.e., a less negative $\Delta H$) compared with other mammals, including humans (3, 11, 12, 21), a feature that has been attributed to the presence of an additional allosteric Cl$^-$ binding site on the $\beta$ subunit between residues Lys88 and Lys76$\beta$ (11, 12). Limited effect of temperature on Hb-$O_2$ binding facilitates $O_2$ delivery to poorly insulated body parts, including cold extremities in contact with ice, a feature that has been interpreted as an energy-saving adaptive mechanism (10, 13). In bears, the comparatively small decrease in body temperature during hibernation would then cause a slight (albeit significant, Fig. 2B) effect on the $O_2$ binding curve, as shown here by us and earlier by others (11). The calculated heat of oxygenation ($\Delta H$) values ($-8.5 \pm 0.8$ summer and $-8.7 \pm 1.5$ kcal/mol winter) were consistent with previous $\Delta H$ data reported for bear Hb, with values ranging from $-7$ to $-8.5$ kcal/mol (6, 11).

Temperature sensitivity of $O_2$ binding to Hb was overall similar in summer and winter hemolysates, indicating that a fall in body temperature alone is necessary but not sufficient for the decrease in $P_{50}$ of hibernating bear hemolysates. We found that a significant part of the observed shift of the $O_2$ equilibrium curve in hibernating bears is due to a decrease in red blood cell DPG levels (Figs. 2 and 3). No indication of switch in Hb expression with synthesis of new isoHb components with a higher $O_2$ affinity was found in the brown bears (Fig. 1).

By binding to a specific site (including His2$\beta$, Lys82$\beta$, and His143$\beta$ in bear Hbs) in the central cavity of the T-state conformation, DPG is a potent allosteric cofactor of brown bear Hb acting synergistically with Cl$^-$ ions (11). The overall effect of a DPG decrease is an increase in the Hb-$O_2$ affinity and a decrease in cooperativity. These effects are explained by a destabilized T state due to a decrease in the DPG to Hb tetramer ratio, with the consequent shift of the T-R equilibrium toward the high-affinity R state. We demonstrated that DPG is the allosteric cofactor responsible for the affinity changes, as these were reproducible when purified Hb and DPG were mixed in exactly the same ratios as found in summer and winter samples. In addition, higher DPG to Hb
titer expressed as rate of O₂ consumption are to a certain extent
been presented that changes in body temperature and metabolic
confirm the results here obtained on diluted hemolysates.
be crucial for defending internal PO₂ during the hibernation
Hct (2) (Fig. 4). In the case of an unaltered O₂ binding curve,
increases to their use. Interestingly, P₅₀ values calculated from the
induced elevation, particularly in the heart rate, poses limita-
physiological data from hibernating and active brown bears
indicates that such remodeling may play a role in regulating
levels, whereas their basal metabolic rate seems to remain
dens show a rapid recovery of their body temperature to normal
Blood O₂ affinity and decrease in cooperativity of O₂ binding may be
of this metabolic remodeling. In this case, regulation of glycolytic enzymes upstream of DPG production may be crucial for maintaining mixed venous PO₂ at a
physiologically reasonable level in spite of the strong elevation of Hct (2) (Fig. 4). In the case of an unaltered O₂ binding curve, the high Hct would have elevated winter P₅₀ substantially (Fig. 4) and cause a potentially detrimental increase in dissolved O₂. The same conclusion is reached when applying physiological data from hibernating and active brown bears under anesthesia (17), although the sedative and disturbance-induced elevation, particularly in the heart rate, poses limitations to their use. Interestingly, P₅₀ values calculated from the whole blood saturation data of anesthetized winter and summer bears (19.7 and 32.1 torr, respectively) (17), also show a similar left-shifted O₂ binding curve during hibernation and confirm the results here obtained on diluted hemolysates.

In black bears during and after hibernation, evidence has been presented that changes in body temperature and metabolic rate expressed as rate of O₂ consumption are to a certain extent independent from each other (43). Bears emerging from their dens show a rapid recovery of their body temperature to normal levels, whereas their basal metabolic rate seems to remain suppressed and recovers only slowly. These findings indicate a strong temperature-independent remodeling of their metabolism during hibernation. Our results in brown bears further indicate that such remodeling may play a role in regulating Hb-O₂ binding during hibernation.

Perspectives and Significance

The physiology of brown bear hibernation is inherently intriguing as bears seem less dependent on reductions in body temperature to aid metabolic regulation compared with other (smaller) hibernators, but rather may rely more heavily on active aerobic metabolic suppression.

Here, we found indications that a side product of glycolysis, DPG, is substantially downregulated during hibernation, in effect increasing blood O₂ affinity substantially. The described changes in O₂ affinity and cooperativity of Hb-O₂ binding may be crucial for defending internal Po₂ during the hibernation period. Clearly, further studies will be needed to identify the mechanisms involved in metabolic suppression in hibernating bears and to establish whether the observed decrease in erythrocyte DPG, besides its role in adapting the blood O₂ equilibrium curve, is part of this metabolic remodeling. In this case, regulation of glycolytic enzymes upstream of DPG production by pH, temperature, or other factors could be involved. Such findings could be of relevance to identify pharmacological manipulation of energy metabolism that would preserve tissue integrity in critically ill human patients. Further studies on hibernators such as the brown bear may reveal details of how the substantial metabolic regulation is achieved.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


Paper VI
Drivers of hibernation in the brown bear

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Abstract

Background: Hibernation has been a key area of research for several decades, essentially in small mammals in the laboratory, yet we know very little about what triggers or ends it in the wild. Do climatic factors, an internal biological clock, or physiological processes dominate? Using state-of-the-art tracking and monitoring technology on fourteen free-ranging brown bears over three winters, we recorded movement, heart rate (HR), heart rate variability (HRV), body temperature (Tb), physical activity, ambient temperature (TA), and snow depth to identify the drivers of the start and end of hibernation. We used behavioral change point analyses to estimate the start and end of hibernation and convergent cross mapping to identify the causal interactions between the ecological and physiological variables over time.

Results: To our knowledge, we have built the first chronology of both ecological and physiological events from before the start to the end of hibernation in the field. Activity, HR, and Tb started to drop slowly several weeks before den entry. Bears entered the den when snow arrived and when ambient temperature reached 0 °C. HRV, taken as a proxy of sympathetic nervous system activity, dropped dramatically once the bear entered the den. This indirectly suggests that denning is tightly coupled to metabolic suppression. During arousal, the unexpected early rise in Tb (two months before den exit) was driven by TA but was independent of HRV. The difference between Tb and TA decreased gradually suggesting that bears were not thermoconforming. HRV increased only three weeks before exit, indicating that late activation of the sympathetic nervous system likely finalized restoration of euthermic metabolism. Interestingly, it was not until TA reached the presumed lower critical temperature, likely indicating that the bears were seeking thermoneutrality, that they exited the den.

Conclusions: We conclude that brown bear hibernation was initiated primarily by environmental cues, but terminated by physiological cues.

Keywords: Body temperature, Denning ecology, Metabolic inhibition, Physiological ecology, Thermoregulation

Background

Hibernating mammals are good models for investigating the relationship between physiology, behavior, and environment, as hibernation patterns are important determinants of survival [1]. Studies of small hibernators suggest that climate variability might affect hibernation patterns and survival, as seen in the yellow-bellied marmot (Marmota flaviventris) exiting the burrows much earlier due to warming spring temperatures in spite of a consistent duration of snow cover [2]. Moreover, whereas energetics has been used as a predictor of a climate-change-associated northward expansion for certain species, including the little brown bat (Myotis lucifugus) [3], increased climate variability has been shown to decrease fitness in Columbian ground squirrels (Urocitellus columbianus), due to decoupling of environmental cues and food availability [4]. Mismatches between thermal and photoperiod cues pose a major challenge for hibernators [5]. Therefore, the phenology, interdependency, and chronology of physiological, behavioral, and ecological events, bracketing the hibernation period and their causative relationships, could provide important insights into individual plasticity to environmental challenges and predict whether climactic changes will cause mismatches between behavior, physiology, and food availability.

Many studies have focused on how environmental changes alter the phenology, morphological traits, and...
population dynamics of various species [4, 6], but have failed to account for the fact that phenotypic plasticity can modify model predictions [5, 7]. Indeed, because individual-centered physiological responses govern the link between environmental change and individual performance, scientists now recognize the necessity of incorporating physiological, behavioral, and demographic data into combined models [8]. However, this approach remains a challenge, due to the paucity of long-term physiological data collected on free-ranging animals, particularly in conjunction with behavioral and environmental data.

Much of the existing knowledge on the interplay between environment, physiology, and behavior in timing of denning comes from studies on small hibernating mammals; studies of free-ranging hibernating bears, the only large hibernators, are scarce. Yet owing to their low surface-to-volume ratios, bears experience different energetic challenges, specifically slower cooling rates and an inability to rely on dropping body temperature for metabolic rate reduction, as do small mammals [9].

Collecting data from free-ranging bears represents additional challenges. First, assessing hibernation patterns in response to climate variability requires long-term monitoring of environmental and phenological covariates to compare with physiology and behavior. Second, evaluation of causative relationships between environmental cues and physiological variables are required to assess drivers of behavior. Third, methods are required to accurately determine hibernation duration.

We sought to overcome these three challenges by developing novel applications of both behavioral change point analysis, BCPA [10], to accurately estimate den entry and exit dates, and convergent cross-mapping (CCM) [11], to assess causation between given biotic and abiotic time-series variables. CCM works on the premise that the Pearson correlation (\( \rho \)) coefficient increases significantly with increasing length (L) of the period of association. We applied these methods to a unique longitudinal data set collected from 14 free-ranging Scandinavian brown bears (\( U. arctos \)) over a period of three years, to examine the interplay between ecological, behavioral, and physiological time-keeping mechanisms involved in the hibernation processes of free-ranging brown bears.

**Methods**

**Study area**

The study area encompassed about 21,000 km\(^2\) in south-central Sweden (61°N, 15°E, Additional file 1: Figure S1). The topography in this region is rolling hills, with <10 % above 750 m above sea level. The area is forested and dominated by Scots pine (\( P. sylvestris \) L.) and Norway spruce (\( P. abies \) H. Karst) [12]. The area is heavily used by hunters with dogs, during both the moose (\( A. alces \)) hunting season (September–October) and the bear hunting season (21 August to 15 October or until the quota is filled). This hunting period overlaps with the predenning period [13].

**Data collection**

Fourteen bears (8 males, 6 females, 2–8 years old, 30–233 kg) were captured by darting from a helicopter from April to June 2010, 2011, and 2012 [14, 15]. The bears were fitted with collars, which included a global positioning system (GPS), dual-axis motion sensors to monitor activity, described in detail previously [16], very high frequency (VHF) transmitters, and a Global System for Mobile modem (Vectronic-aerospace, Berlin, Germany). GPS positions were recorded every 30 min. The offspring of marked females were followed from birth; otherwise, age was determined by counting the annuli of a cross-section of the premolar roots [17]. We excluded six females that became pregnant during the study (also excluded from above total; these have been reported elsewhere [16]).

All implants were sterilized with ethylene oxide gas (Anaprolene AN74i 60 L, Andersen Europe, Kortrijk, Belgium). We programed temperature loggers (DST Centi, Star Oddi, Gardabaer, Iceland, 46 x15 mm; 19 g) to record body temperature (\( T_b \)) at intervals ranging from 1 to 30 min, depending on other ongoing studies. With a memory capacity of 175,000 temperatures, the data loggers could record \( T_b \) every 3 min for up to 1 year [18]. Each temperature logger was individually calibrated by the manufacturer for 41 set points over the range 5 °C to 45 °C with a guaranteed accuracy of ±0.1 °C for the full temperature range one year post calibration. The equipment used for the calibration of the loggers, as stated on the calibration certificate from the manufacturer, is a Hart 7012 temperature bath and the reference measurements are conducted with a Hart 1504 thermometer and a Hart 5610–9 thermistor probe with combined absolute accuracy better than ±0.010 °C. Each set point measurement was taken when the temperature was stable within 0.001 °C. We surgically implanted temperature loggers into the abdomen, as previously described [14]. In some cases, temperature loggers were surgically removed and replaced in conjunction with a change of collar (at intervals of 1–2 years).

We used insertable cardiac monitors (Reveal DX and XT; Medtronic Inc., Minneapolis, Minnesota, USA; 8 mm x 19 mm x 62 mm; 15 g). We surgically implanted them peripherally on the left side between the muscle and subcutaneous fat and closed the incision using 2–0 monofilament glycomer (Biosyn Corporation, Carlsbad, California, USA). The device reported daytime mean heart rate (HR) (08:00–20:00) and nighttime mean HR (00:00–04:00) and contained ECG and acceleration sensors, as described previously [19].
The device determined heart rate variability (HRV) by calculating 5-min medians of ventricular intervals in milliseconds during sinus rhythm and computing the standard deviation of those medians over each 24-h period (SDANN: standard deviation of all the five-minutes NN interval means; the term "NN" was used in place of RR, to emphasize that the processed beats were "normal" beats, which means that extra systolic beats were not included).

We obtained ambient temperature (TA) and snow depth data for all of Sweden (620 weather stations) from the Swedish Meteorological and Hydrological Institute (SE-601 76 Norrköping, Sweden). This data were interpolated to a 1-km scale, which resulted in a daily map of TA and snow depth for the entire country. From these maps, we extracted the local temperature at each bear location. Photoperiod was defined as the time between sunrise and sunset and was calculated for the same latitude (61° 6′ N) using the R-package Geosphere [20].

### Den entry and exit dates

We estimated den entry and exit dates using BCPA on the GPS data. This method sweeps through changes in the magnitudes of animal movement speeds and changes in direction, to detect points of speed and directionality change [10]. Because we were interested in the dates on which bears entered and exited the dens, we calculated mean daily location, which we used to estimate the velocities and changes in direction of the study animals at the daily scale. The method we used first computed the velocity (V) and changes in direction (Ψ) from the data and then decomposed these results into orthogonal components of persistence velocity \( V_p(t) \) and directional change \( V_d(t) \) defined as:

\[
V_p(T_i) = V(T_i)\cos(\Psi(T_i)) \quad V_d(T_i) = V(T_i)\sin(\Psi(T_i))
\]

where \( V_p \) is the tendency and magnitude of a movement to persist in a given direction, and \( V_d \) is the tendency of movement to head in a perpendicular direction in a given time interval. Thus, we could estimate mean velocity (\( \mu \)), variation (\( \sigma \)), and directional persistence (\( \rho \)). \( \rho \) is the first-order autocorrelation (also called the autocorrelation coefficient) at a measured time lag one. A more detailed description has been reported previously [10].

This method identifies change points by the simultaneous changes in \( \mu, \sigma \), and \( \rho \). We considered a bear to have entered the den in the autumn on the date that the values of these parameters became 0, and we considered the bear to have left the den on the date that the values became positive in spring (Additional file 1: Figure S4). The advantage of this method is that it provides easily obtainable and analyzable parameters, which contain more information than simple estimates of speed and turning angles, and controls for differences in the distance traveled by animals.

Thus, the speed and turning velocities were comparable across individuals. We applied this method to each individual bear to identify the den entry and exit dates. We then overlaid the GPS position of the bear on this specifically identified date with the GPS location of the den to confirm if the bear was at the site. Some bears entered or exited the dens several times before the final entry or exit, as detected by the BCPA.

After estimating the den entry and exit dates, we aggregated (using means) the activity, \( T_b \), and HR data sets at the daily scales, to compare to movement data and to infer simultaneous daily changes in these variables and their values on the days of den entry and exit. We constructed plots for each bear showing \( T_b \), HR, activity, displacement (change in GPS positions), and \( T_A \).

### Generalized additive mixed models (GAMMs)

We used GAMMs to identify the days of increases and decreases in \( T_b \) and other variables. We modeled these changes for all recorded variables (\( T_A, T_b \), HR, photoperiod, and activity) using GAMMs in the mgcv package in R [21].

For the variables measured at the individual animal level for multiple years (\( T_b \), HR, HRV, and activity), we fitted the GAMMs with a spline of ‘Day of the year/Julian day’ as a fixed effect and ‘animal-year ID’ as a random effect. For variables measured at the daily level, but for multiple years (\( T_A \), snow depth, and photoperiod), we fitted the GAMMs with the spline of ‘Julian day’ as a fixed effect and ‘year’ as a random effect. We used GAMMs to fit trends to the variables, because all of the variables showed nonlinearity (increasing and decreasing at different times of the year). We also explored the autocorrelation function (ACF) and partial autocorrelation function (PACF) for the variables to account for temporal autocorrelation and decided to use autocorrelation moving average correlation structures (corARMA) [21], because these autoregressive (AR) models fit the data better (tested using ANOVAs). After fitting the models, we determined the periods in which the variables were significantly increasing or decreasing. We determined these periods by computing the first derivatives of the fitted trends (GAMMs above). We used a method of finite differences, where we calculated the values of the fitted trend at a grid of point over the entire data set. We then swept through the grid by one point and recalculated the values of the trend at the new locations. The differences between the two sets of values provided a slope of the trend at that particular point, and the trend was calculated at 365 points. We then overlaid the estimated dates and periods of increase and decrease over the fitted models.

### Relationship between physiology and the external environment

To identify the predictors of changes in \( T_b \), HR, and activity, we again used GAMMs. We aggregated all of the
variables measured at the individual animal level (\(T_b\), HR, and activity) to a mean daily value for that variable across individuals. We then merged all the variables (\(T_A\), \(T_b\), HR, snow depth, photoperiod, and activity) into a data set at a daily scale for multiple years. We then created three sets of GAMMs using snow depth, \(T_A\), and photoperiod as explanatory variables to predict changes in \(T_b\), HR, and activity and used year as a random effect. We again used the corARMA structure to account for temporal autocorrelation for both sets of models. To test for which ecological variable drove the changes in physiological variables during entry and exit, we split the year into two halves and ran the same GAMMs, as described above.

To determine the contribution of abiotic and physiological factors to dates of den entry and exit, we separated den entry and exit periods and set the entry/exit dates as time zero. We then aligned all data on time zero and determined the dates of significant increases or decreases in each parameter by fitting GAMMs (Fig. 2). We superimposed the dates of significant changes on yearly average environmental variables (i.e. \(T_A\), snow depth, and photoperiod) as a proxy of the den climate. This enabled us to determine the sequence of environmental and physiological events that were associated with den entry and den exit.

Causal relationships

To identify the causal relationships between the monitored environmental and physiological variables, we used a convergent cross-mapping approach devised to detect causal relationships between pairs of processes represented by time series [11]. We used this approach to test for causal relationships between \(T_A\), \(T_b\), HR, and SDANN. We tested specific relationships during critical periods of interest (Table 1). The periods were based on patterns observed during data exploration. Note that we used GAMMS to determine correlates of den entry and exit, whereas CCM was used to determine causation between pairs of variables.

A causal relationship was detected when the Pearson correlation coefficient \(\rho\) was significantly greater than zero for a large library length (defined as \(L\)), and \(\rho\) increased significantly with increasing \(L\). The method was comprised of three steps; selecting an embedding dimension value that should correspond to a high predictability for a time step into the future. The predictive power should drop as the length of the prediction time step increases. Bootstrapping was then used to increase the precision of \(\rho\), the number of iterations were increased in order to reduce Monte Carlo stochasticity, and iterated until mean and S.D. stabilized [11].

Results

The longitudinal design of the study resulted in a combined total of 38 years of bear data (data summary, Fig. 1, example individual Additional file 1: Figure S2, change points for individual variables Fig. 2 and Additional file 1: Figure S3). According to the BCPA (Additional file 1: Figure S4), bears entered the den during the months of October and November (median date 30 October) and exited from 21 March to 6 May (median date 6 April).

Denning behavior was highly variable among individuals, but none of the bears changed dens during the study period. Both entry and exit date variability could be explained by \(T_A\) variation between years (Additional file 1: Table S1), with the warmer winter associated with later entry and shorter hibernation (winter 2010–11 hibernation, mean ± SD: 175.3 ± 22.4 days versus 151.2 ± 15.3 days for winter 2011–12; t-value=6.78, \(p=0.03\)).

In order to determine the ecophysiological triggers of denning, we corrected for the between-individual variation

<table>
<thead>
<tr>
<th>Period</th>
<th>Description</th>
<th>Relationship tested</th>
<th>(P)-value (\rho)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period 1</td>
<td>From 25 days before den entry (the day activity starts to drop) until den entry</td>
<td>SDANN causes HR</td>
<td>0.1</td>
</tr>
<tr>
<td>Period 2</td>
<td>30 days following the date of den entry</td>
<td>SDANN causes (T_b)</td>
<td>0.05</td>
</tr>
<tr>
<td>Period 2</td>
<td>30 days following the date of den entry</td>
<td>(T_A) causes (T_b)</td>
<td>0.1</td>
</tr>
<tr>
<td>Period 3</td>
<td>Between 63 to 25 days before the date of den exit</td>
<td>(T_A) causes (T_b)</td>
<td>0.06</td>
</tr>
<tr>
<td>Period 3</td>
<td>Between 63 to 25 days before the date of den exit</td>
<td>SDANN causes (T_b)</td>
<td>0.9</td>
</tr>
<tr>
<td>Period 3</td>
<td>Between 63 to 25 days before the date of den exit</td>
<td>(T_b) drives HR</td>
<td>0.07</td>
</tr>
<tr>
<td>Period 4</td>
<td>From 25 days before den exit to the den exit date</td>
<td>SDANN causes (T_b)</td>
<td>0.01</td>
</tr>
<tr>
<td>Period 4</td>
<td>From 25 days before den exit to the den exit date</td>
<td>SDANN causes HR</td>
<td>0.03</td>
</tr>
<tr>
<td>Period 5</td>
<td>From 25 days before den exit to the den exit date</td>
<td>(T_A) causes (T_b)</td>
<td>0.3</td>
</tr>
<tr>
<td>Period 5</td>
<td>From 10 days before den exit to the den exit date</td>
<td>Activity causes (T_b)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 1 Cross Convergent Mapping (11) analyses of the causation between different ecophysiological variables. Standard deviation of all the five-minutes normal heart beat interval means (SDANN), heart rate (HR), ambient temperature (\(T_A\)), and body temperature (\(T_b\)). The library length of the association \(L\) was set to 100 for all analyses and \(\rho\) is the range of Pearson correlation coefficients for the tested relationship during the defined period (Column 1).
Fig. 1 Average of the daily mean values for ambient temperature (a) bear body temperature (b), heart rate (c) and activity level in accelerometry units (d) for 14 individual free-ranging brown bears in central Sweden collected over 3 years. The X-axis indicates the time of year. Green vertical bars indicate the den entry and exit periods. The width of the green bars denotes the range of den entry and exit dates across all individuals. Trend lines were calculated using GAMMs.
Fig. 2 (See legend on next page.)
in denning behavior by aligning all data to individual den entry and exit dates. We then referenced den entry and exit dates as zero time points for all measured parameters. Mean and standard error of the physiological variables at den entry and exit are presented in Additional file 1: Table S1. We observed that \( T_b \) (mean ± S.E.; 37.2 ± 1.6 °C) started to drop on average 13 days prior to den entry (Fig. 2) and activity and HR decreased 25 and 24 days before den entry, respectively. We used the SDANN index as an indirect measure of sympa-thovagal balance [22]. We observed that SDANN was not related to HR in a way that could be explained by the models used in this study (SDANN drives HR, \( p=0.1 \), Table 1). SDANN declined only five days before den entry (Fig. 2). It is difficult to assess the contributions of the sympathetic and parasympathetic nervous systems to the overall autonomic balance during entry into hibernation for bears and we cannot exclude that alterations in autonomic nervous system activity may be driving processes that we would not be able to detect by assessing SDANN alone. However, the drop in SDANN observed in this study likely reflected metabolic depression due either to increased parasympathetic activity or decreased sympathetic activity (or both).

Den entry occurred when \( T_A \) was 1.03 ± 0.95 °C (mean ± S.E.) and when snow had started to settle (Fig. 2 and Additional file 1: Figure S3, Additional file 1: Table S1). Activity stabilized nine days after den entry, but it took 20 days for HR and SDANN to reach a plateau. \( T_b \) stabilized at 33.8 ± 2.1 °C during the first 30 days after den entry and causation analysis by CCM showed that SDANN was a driver for changes in \( T_b \) (SDANN drives \( T_b \), \( p < 0.05 \), Table 1) to a larger degree than \( T_A \) (\( T_A \) drives \( T_b \), \( p < 0.1 \), Table 1).

Environmental drivers of physiology

On an annual scale, the only recorded environmental parameter that was significantly correlated with \( T_b \) was the average daily \( T_A \) (\( F=14.76, p < 0.001 \), Additional file 1: Table S2). Neither average daily snow depth nor photoperiod significantly influenced \( T_b \) or HR (Additional file 1: Table S2). The average daily activity level was positively affected by snow depth and \( T_A \) (interaction of snow depth and \( T_A \), \( F=2.63, p < 0.001 \)) (Additional file 1: Table S3).

During the den entry period, \( T_A \) and its interaction with snow depth were significantly correlated with \( T_b \). In contrast, during the exit period, \( T_A \), snow depth, and their interactions were not associated with \( T_b \) (Additional file 1: Table S3). None of the environmental variables were significantly correlated with HR for either den entry or exit (Additional file 1: Table S2 and S3). The difference between \( T_b \) and \( T_A \) gradually decreased during the first period (Additional file 1: Figure S5).

\( T_b \) was the first physiological parameter to change during arousal from hibernation. \( T_b \) started to rise gradually as early as 2 months prior to den exit (mean ± S.E. from 33.2 ± 0.8 °C at 63 days before exit). HR started rising a month later and was followed by SDANN (20 days before exit) and activity (10 days before exit). We observed two patterns of thermoregulation during arousal from hibernation. From the rise of \( T_b \) to the rise of the SDANN (the period beginning 63 days and ending 25 days before den exit), causation analyses revealed that \( T_A \) influenced \( T_b \) (\( p=0.06, \) Table 1, Fig. 3) and \( T_b \) influenced HR (\( p=0.07 \)). During that period, SDANN did not influence \( T_b \) (\( p=0.98 \)). The second period of hibernation arousal was observed when SDANN started to rise (25 days prior to exit). At this time SDANN caused a rise in both \( T_b \) (\( p < 0.01 \)) and HR (\( p=0.03 \)). During this period, \( T_b \) was no longer associated with \( T_A \) (\( p=0.3, \) Fig. 3). Activity increased 10 days prior to den exit; its contribution to the return to euthermia was confirmed by the CCM, finding that it caused a rise in \( T_b \) (\( p=0.05 \)). Den exit occurred when \( T_b \) had almost reached euthermia (mean ± S.E. 36.7 ± 0.15 °C) and \( T_A \) was 3.7 ± 1.3 °C. It took 10 and 15 days, respectively, for the bears to stabilize their \( T_b \) and SDANN after den exit. It took another month before HR and activity had stabilized. Fig. 3 combines these results and summarizes the different environmental and physiological drivers of brown bear denning in this population.

Discussion

During the den entry period, \( T_b \) and activity level appeared to be influenced by environmental factors, such as \( T_A \) and snow depth. \( T_A \) declined before activity levels and both parameters preceded the decline in \( T_b \) and the first snow event (HR and the SDANN both declined later than these variables). The den emergence process began with increases in \( T_A \), \( T_b \), HR, SDANN, and activity level, in that order, apparently independently of snow depth. The observation that SDANN increased only 13 days after the increase in HR (i.e. a gradual shift in autonomous nervous system balance) suggested that the
first increase in HR was adaptive thermoregulation (through a temperature-dependent increase in metabolic activity). Although this is well described in small hibernators and even in humans during induced therapeutic hypothermia, this observation would require further investigations.

The BCPA greatly increased our ability to estimate den entry and exit dates (Additional file 1: Figure S4). A simple evaluation of GPS positions would not have sufficed, because the transition period between hibernating and active states was drawn out, and some bears regularly or periodically returned to the denning area after emergence. We observed significant differences in the timing of den entry and exit between years, with the latest den entry dates during the warmer years. As climate change projections predict warming wintertime temperatures for the [23], shorter hibernation periods can be expected. For example, the decline in $T_b$ before den entry was significantly correlated with $T_A$, whereas HR was independent of the predictor variables we tested, except for day of the year. This suggests that environmental variability can affect behavioral and physiological aspects of hibernation independently in the bear.

Our results regarding timing of den entry and exit were consistent with a previous study on this population [24]; den entry and den exit dates occurred within the same ranges and males and older animals entered the dens later and exited earlier than females and younger animals [25]. Although a study on brown bears in Alaska documented a correlation between den emergence and the timing of snow melt [26], the bears in our study area had emerged from their dens either before or during snow melt (Fig. 3). Moreover, during emergence, there was no apparent impact of snow depth or photoperiod on $T_b$ or HR; however, activity level was affected by both snow depth and photoperiod. This result indicated that,
although snow depth was associated with a change in the behavior of the bears (e.g. den entry, consistent with the previous report [25]), it was not associated with any physiological change measured in this study. However, we found a difference in den entry from year to year, with bears entering the dens earlier in a colder year.

Drivers of den entry

Although $T_b$ was correlated with several factors during den entry (Additional file 1: Table S2), convergent cross mapping revealed the greatest causation due to SDANN, followed by $T_A$ (Table 1). SDANN and HR were closely related at this stage of hibernation, likely because activity was low, eliminating activity’s confounding effect on HR. SDANN declined steeply just before den entry. Although the SDANN is not the best proxy to assess autonomic nervous system balance, we were unfortunately not able to use another index due to the duration of the experiment and the storage capacity required for a full ECG to be stored. Therefore, it is difficult to determine if changes in SDANN should be attributed to changes in the parasympathetic or sympathetic nervous system or both. Previous studies [27] have shown that hibernating bears have an enhanced respiratory sinus arrhythmia, indicating increased parasympathetic nervous system activity. Based on the literature on small hibernating mammals (e.g. [28, 29]), the observed decrease in SDANN likely indicated that a predominant parasympathetic tone drives metabolic suppression and decreases in most physiological functions. In small hibernators, cooling is achieved by both metabolic depression and passive body cooling. Our data are in the line with the previously reported observation that large hibernators initially rely on metabolic depression to achieve depressed metabolic rate during hibernation [9, 30] to a larger extent than passive body cooling, although both mechanisms occur. The early rise in $T_b$ in the bear is in contrast to most hibernators, with likely two involved mechanisms that cannot be dissociated but likely happen sequentially: $Q_{10}$ effect due to slow warming with concomitant ANS activity and final warming from a massive SNA burst.

Both SDANN and HR stabilized 20 days after den entry, but it took an additional 10 days for $T_b$ to stabilize, probably because of the bear’s large body mass and decreased heat exchange in the den. This is similar in sequence to that found in woodchucks (Marmota monax), where the decrease in metabolic rate occurred in 6 h, whereas the drop in $T_b$ continued for 12 h [31], and in golden hamsters (Mesocricetus auratus), with metabolic rate decreasing for 3 h and $T_b$ dropping for 8 h [32, 33]. However, the mechanisms of metabolic rate reduction are thought to differ between large and small hibernators [9]. Large hibernators, such as bears, are expected to rely to a greater extent on active metabolic suppression to reduce their metabolism, due to their larger body size, compared to small hibernating species, which benefit more from the $Q_{10}$ effect in torpor. Even small marsupials (<20 g) actively suppress their metabolism during torpor [9]. Using active metabolic suppression, bears are able to reach metabolic rates (despite having a $T_b$ above 30 °C) as low as small hibernators in deep (<5 °C $T_b$) torpor [34].

Including SDANN in our study proved to be particularly valuable. In contrast to previous approaches [33], HR was not used to infer metabolic rate, because, this parameter is confounded by activity and stress [33] which are expected prior to hibernation with the combination of both the hunting season and den entry behavior [13]. However, activity affects SDANN to a much lesser extent [22]. By including SDANN, we avoided the confounding effect of activity on HR and had indirect access to information on the sympathovagal regulation of metabolism. One study in dogs found that HRV did not differ between slow movements, lying, sitting, or standing, but did change when a favorite toy was presented [22]. In stressful situations, dogs had consistently increased HR and decreased HRV [35]. Therefore, whereas HRV may often be connected to HR, it is more independent of movement-induced changes. Variability in HR can be caused by changes in thermoregulation, circadian rhythms, respiration, blood pressure, and both physiological and psychological stressors and can be used to evaluate the state of balance between the sympathetic and parasympathetic nervous systems [36], but does not give an overall level of either system’s activity.

$T_b$ in captive brown bears was reported to decline gradually over 5 weeks from the date that food and water were removed [37]. Our finding that changes in $T_b$ began long before changes in HR suggested that previous studies focusing on captive bears with an artificially defined end of the food/water season might not represent the actual sequence of events in the wild. In our study, SDANN declined steeply just before den entry. This change was probably associated with the enhanced respiratory sinus arrhythmia previously reported to occur in hibernating bears [27]. Based on the literature on small hibernating mammals, the observed decrease in SDANN suggested that a massive parasympathetic tone, likely with a reduction in sympathetic activity, drives metabolic suppression and decreases in most physiological functions. The role of SNS in thermogenesis and cardiovascular control has been the topic of a number of experiments starting more than 60 years ago [38]. In studies of small hibernators, the initial fall in HR during entry into hibernation is due to parasympathetic activation and the exit due to SNS activation [39]. Treatment with atropine, an inhibitor of parasympathetic pathways, prevented 13-lined ground
squirrels (*Citellus tridecemlineatus*) from entering hibernation [40]. Our results are in line with those from previous studies and suggest that increased parasympathetic activation plays a key role in the reduction in HR at den entry in bears as well, but does not rule out potential decreases in sympathetic nervous system activity.

**Drivers of den exit**

Although den exit was not correlated with either T_A or photoperiod, the bears exited the dens at T_A of 3.7° ± 1.3 °C. A bear den is not an adiabatic shell, however, the inside air temperature could easily rise, depending on the type of den (ant hill, under rocks or nests [41]). The fairly narrow range of T_b between bears on the day of exit (36.7 ± 0.15 °C, Additional file 1: Table S1) suggested that the bears exited when they reached a specific set point. At T_A of > 0 °C, water also could start draining into the den, causing the bear to become uncomfortable and leave the den. American black bears (*Ursus americanus*) in artificial dens have a mean lower critical T_A of 5 °C, below which the bears’ thermal conductance increased [42]. This suggests that the bears’ cue to exit the den was that they became too warm when the temperatures rose in springtime or that they were seeking more optimal temperatures outside the dens.

That T_A does not drive T_b during the phase before exit (period 5, Table 1) might be due to the adaptive thermoregulation that occurred over several months, making the T_A immediately around the day of exit less important. It could also be that the den temperature was more relevant, as the bears exited when T_A reached approximately 3.7 ± 1.3 °C (Additional file 1: Figure S1), nearing the lower critical temperature for established for black bears and polar bear (*Ursus maritimus*) cubs [42, 43], possibly because the den temperature was above thermoneutrality.

T_b started rising 2 months prior to exit, whereas HR rose a month later, and was followed by SDANN (20 days prior to exit) and activity (10 days prior to exit). During the first part of arousal, the causation analysis revealed that T_A caused T_b and T_b caused HR. There was no causal relationship between SDANN and T_b, but a different trend emerged 20 days before exit.

The gradually decreasing difference between T_b and T_A during the first period (Additional file 1: Figure S5), suggests that bears were thermoregulating at a lower thermoregulatory set point during hibernation. This is consistent with recent findings from captive bears showing a negative relationship between den temperatures and hibernating metabolic rates [42]. Then, SDANN started rising and may have caused T_b and HR to rise, likely via an increase in sympathetic nervous system activity, a decrease in parasympathetic nervous system activity, or a combination. At this stage T_b lost its causal association with T_A although T_b-T_A remained stable during the second period, suggesting that euthermic metabolism was reestablished later by active thermogenesis, likely involving the sympathetic nervous system. The exact roles of the sympathetic and parasympathetic nervous systems in this process can only be assessed by direct measurements of sympathetic and parasympathetic nervous system activity in free-ranging conditions, which would be difficult to conduct.

This second phase of den exit was driven by SDANN, with SDANN driving T_b (p < 0.01). When SDANN began to rise, the thermoregulatory pattern shifted. This could indicate transitioning out of hibernation i.e., sympathetic nervous system activation combined with potentially a more profound change in metabolic state [42]. Activity increased from 10 days before exit, showing a causative relationship with the increase in T_b (activity drives T_b, p=0.05). Den exit occurred when T_b was almost at euthermia (mean 36.7 °C), nearing the lower critical temperature for bears [42]. T_b and SDANN stabilized quickly (within two weeks after den exit), but HR and activity took longer, indicating that the bears took longer to return to their original activity levels.

Although shivering may play a role in active thermogenesis, it occurs at the end of arousal in the species studied to date, excluding tropical hibernators [44]. Increased T_b allows restoration of enzyme functioning through a Q_{10} effect and contributes to restoration of muscle function. Early on, the processes start with SNS activation of the vascular system to increase body temperature and heart rate [44]. The role of SNS in thermogenesis in addition to vascular control has also been the topic of numerous investigations starting from the early studies of Lyman. Studies on American black bear in the laboratory show a role for shivering at the end of arousal [34], although our results show that it was less important in free-living conditions.

A recent study on captive American black bears found that metabolic rate was related to den temperature and showed that larger bears showed more variation in length of T_b cycles [42]. During experimental manipulations of den temperature, they found no direct relationship between den temperature and T_b, although the time between peaks in T_b became longer at higher den temperatures. The authors suggested, based on a single bear that increased its T_b to 35.9 °C when the den warmed to 10 °C, that the bear may have inhibited heat dissipation mechanisms. It is not clear whether this was merely an effect of being inside an isolated den or was a physiological phenomenon. In addition, they found that the lower critical temperature varied from 1° to 10 °C, from which they concluded that the smaller bears partially compensated for higher thermal conductance with increased metabolic rate. Interestingly, they found no relationship between T_A and den temperatures. Although it
was not possible to measure the den temperatures in our study, we would expect a correlation with \( T_A \), because the bears in our study were not in adiabatic shells; they were under rocks or tree roots or in anthills, with oxygen exchange varying from a small ventilation hole to large openings under rocks. We found, however, that \( T_A \) drives \( T_b \) during the first phase, and the differential between \( T_b \) and \( T_A \) decreased until the point in the spring when HRV rose. Although [42] found a negative relationship between \( T_A \) and metabolic rate, we conclude that this is more likely an adaptive thermoregulation allowing maintenance and slowly rising \( T_b \) at a minimal cost, simultaneously with the increasing \( T_A \).

In a previous study, the HR in captive black bears was reported to decline gradually over five weeks from the date that food and water were removed [45]. Our finding that changes in \( T_b \) began long before changes in HR suggests that studies on captive bears with an artificially defined end of the food/water season might not represent the actual sequence of events in the wild. Our results would have been enhanced considerably had we succeeded at measuring den temperature. Bears in this population are very susceptible to disturbance in winter [46], repeatedly changing dens after captures or capture attempts, so putting temperature loggers inside the den was not realistic.

Our novel results and the methods adapted for this analysis could impact our general understanding of how climate change influences other ecophysiological and behavioral adaptations. In this study, we demonstrate mechanisms for the entry and exit into hibernation by the brown bear in Sweden that have implications for both bear population monitoring and management. These results highlight some of the differences between the bear and small hibernators, reinforcing the importance of not generalizing results from small hibernators to bears.

This work is an example of how different types of datasets can be combined to provide coherent ecophysiological timeseries with potential applications for other ecophysiological and adaptation studies beyond hibernation. Other time-series datasets that could be analyzed in a similar way include phenological and reproduction data on different organisms that are commercially important (crops) or used as indicator species for habitat/ ecosystem quality measures (e.g. birds, butterflies). The results from such analyses would provide management strategies and production optimization, while minimizing ecosystem-level impacts. Besides conservation practices, our study demonstrates the importance of several physiological and behavioral characteristics that are important for studies of adaptation, in this case to winter conditions and to climate change, in the context of selection pressures for matching the start and end of hibernation with resource availability.

**Conclusions**

We demonstrate that changes in brown bear \( T_b \) during den entry were driven by environmental factors, particularly \( T_A \). This indicates that a warming climate could result in later den entry. Thus, although many studies have shown that den entry and exit are related to food availability, climate change also appears to be an important factor affecting the timing of the life events of the brown bear and could pose a threat through the mismatch of important physiological cues. Consequences would be a shortening of the bears’ hibernation period and potentially prolonging the den-entry period, which has been shown to be the highest risk period for bear caused injuries to humans [13]. This should be anticipated by wildlife management agencies in areas where there is a large overlap between humans and bears.

Further, this study suggests that Scandinavian brown bears terminated their hibernation due to physiological cues. Although body temperature started to rise slowly very early in the hibernation period, it was only few weeks before exit that we observed activation of the sympathetic nervous system to restore euthermic metabolism. Body temperatures were close to euthermia when ambient temperature reached 0 °C and bears exited the dens when ambient temperature was close to the lower critical temperature. Hibernation in brown bears seems to be initiated based on environmental cues and terminated due to physiological cues.

**Ethics**

All captures were approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (application #C47/9 and C7/12) and the Swedish Environmental Protection Agency.

**Additional file**

Additional file 1: Supplemental Figures and Tables. (DOCX 10538 kb)

**Abbreviations**

HR: heart rate; HRV: heart rate variability; SDANN: standard deviation of all the five-minute NN interval means; \( T_A \): ambient temperature; \( T_b \): body temperature.

**Competing interests**

Timothy Laske is an employee of Medtronic Inc.

**Authors’ contributions**

ALE, SB, OF, JMA, JES initiated the study and designed the experiments. ALE, AF, SB, OF, JMA, TGL contributed during fieldwork and data collection. Equipment was provided by SB, TGL, OF, JMA. Data management was done by NJS and ALE. Data analysis and preparation of fig. was done primarily by NJS, with contributions from ALE and SB. ALE, NS and SB drafted the manuscript, JES and JMA critically revised the manuscript, and all authors participated in revisions and approved the final manuscript.
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References

Supplemental Figures

Figure S1. Map of the GPS positions from the studied bears in central Sweden, illustrating that they all occurred at similar latitudes.
Figure S2. Dataset for an individual brown bear in central Sweden, demonstrating the simultaneous changes in daily mean activity, movement, heart rate, and body temperature. Vertical bars indicate the den entry and exit dates, respectively. W1019 Den Entry Date- 2011-11-13; W1019 Den Exit Date- 2012-03-26.
**Figure S3.** Smoothed annual variation in ambient temperature (°C), photoperiod, and snow depth (cm) on the study area in central Sweden and body temperature (°C), heart rate (bpm), and activity (AU) of the brown bears; all values were estimated using generalized additive mixed models. Green vertical bars show the median date of den entry and exit. Annotated numbers denote the Julian day. Blue numbers represent the dates of den entry and exit, and red numbers denote the date when an increase began and ended (change points). Dotted vertical bars demarcate the periods of increase and decrease in the variables, with blue overlaid curves showing the increase phase and red showing the decrease phase based on the fitted model. Gray dots show daily means for the individuals.
Figure S4. Estimation of the den entry date for two brown bears in Sweden using the GPS data for changes in velocity. The day the change in velocity became zero was considered to be the den entry date and the day the change was no longer zero, the den exit. Each dot is a bear position; the color of the dot represents the autocorrelation value ($\rho$). X axis shows the date, $\mu$ is the orthogonal velocity, and $\sigma$ is the variance (Gurarie et al. 2009). Vertical bars represent the periods with significant change in behavior. The location of the last and first yellow vertical bar was selected as the entry and exit date.
Figure S5. Difference between mean body temperature °C and ambient temperature of brown bears in central Sweden during the days leading up to den exit, using the dataset aligned by den exit date (day 0).

**Tables**

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**Table S1:** Mean and standard error values of the eco-physiological variables during the den entry and exit of brown bears in central Sweden.
Table S2. Results of the annual generalized additive mixed models for body temperature, heart rate, and activity for brown bears in central Sweden. Values in blue show statistically significant variables with p values <0.01. Body temperature, heart rate and activity were included as response variables, and snow depth, day length, ambient temperature, and the interaction between ambient temperature and snow depth were included as predictor variables.

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Table S3. Results of the half-year generalized additive mixed models for body temperature, heart rate, and activity of brown bears in central Sweden. Parameter values are presented along with their standard errors. Values in blue show statistically significant variables with p values <0.01.

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### Activity
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### Heart rate
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#### Half Year 2

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Table S4. Correlation matrix of the monitored variables to identify the drivers of den entry and exit of brown bears in central Sweden.

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<th>Mean Heart Rate</th>
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<td>0.77</td>
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Paper VII
Factors Affecting Date of Implantation, Parturition, and Den Entry Estimated from Activity and Body Temperature in Free-Ranging Brown Bears

Andrea Friebe1*, Alina L. Evans2, Jon M. Arnemo3,9, Stéphane Blanc4, Sven Brunberg5, Günther Fleissner1, Jon E. Swenson5,6, Andreas Zedrosser7,8

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Abstract

Knowledge of factors influencing the timing of reproduction is important for animal conservation and management. Brown bears (Ursus arctos) are able to vary the birth date of their cubs in response to their fat stores, but little information is available about the timing of implantation and parturition in free-ranging brown bears. Body temperature and activity of pregnant brown bears is higher during the gestation period than during the rest of hibernation and drops at parturition. We compared mean daily body temperature and activity levels of pregnant and nonpregnant females during preimplantation, gestation, and lactation. Additionally we tested whether age, litter size, primiparity, and environmental conditions, and the start of hibernation influence the timing of parturition. The mean date of implantation was 1 December (SD = 12), the mean date of parturition was 26 January (SD = 12), and the mean duration of the gestation period was 56 days (SD = 2). The body temperature of pregnant females was higher during the gestation and lactation periods than that of nonpregnant bears. The body temperature of pregnant females decreased during the gestation period. Activity recordings were also used to determine the date of parturition. The parturition dates calculated with activity and body temperature data did not differ significantly and were the same in 50% of the females. Older females started hibernation earlier. The start of hibernation was earlier during years with favorable environmental conditions. Dates of parturition were later during years with good environmental conditions which was unexpected. We suggest that free-ranging pregnant brown bears in areas with high levels of human activities at the beginning of the denning period, as in our study area, might prioritize investing energy in early denning than in early parturition during years with favorable environmental conditions, as a strategy to prevent disturbances caused by human.


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Data Availability: The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Please contact the project leader of the Scandinavian Brown Bear Research project: jon.swiftson@nmbu.no concerning data availability.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Embryonic diapause, a widespread strategy to ensure and optimize successful reproduction, is common in plants, insects, fish, birds, and mammals [1,2]. Diapause and delayed implantation involve several independently controlled steps and many of the biological processes are still poorly understood [3]. Bears are the only mammals with delayed implantation, gestation, parturition, and lactation during hibernation, when they do not eat, drink, urinate, or defecate for several months. During this period they survive solely on their stored energy resources [4–6].

Gestation in ursids lasts approximately 60 days [7–9]. This short period limits the energetic costs of reproduction by truncating embryonic development, which in turn reduces the size of offspring and thus the initial costs of lactation [8,10]. The gestation period of bears has been estimated mainly with macroscopic and histological investigations of the ovaries and uteri of hunter-killed females or with blood serum analysis in captive and free-ranging bears [7,8,11]. Quest [9] determined a...
54-56-day gestation period in captive brown bears using ultrasonic examination.

Examinations of the reproductive organs of free-ranging and captive brown (Ursus arctos) and American black bears (U. americanus) indicate that implantation occurs in late November to early December, and parturition occurs in late January to early February [11–16]. Studies of serum plasma progesterone concentrations of pregnant and nonpregnant female American and Asiatic black bears (Ursus thibetanus) and brown bears gave similar results [17–20]. The time of parturition has also been determined for American black bears by listening for vocalizations of cubs at the den sites [21,22].

Many aspects of the reproductive biology of ursids are still poorly understood, such as reproductive cycles, hormone and estrous cycling, and factors that trigger implantation and birth. Most of these studies have been carried out in captivity [8,23,24] and little information is available about the timing of implantation and parturition in free-ranging bears. The reproduction biology of ursids is controlled by a complex timing system, in which the chronological sequence is determined by seasonality [8,25]. Although photoperiod is an important regulator of the reproductive cycle, the mating season and the duration of embryonic diapauses vary among ursid species and individuals [8,25,26]. The mating season of most bear species occurs in spring or early summer and lasts approximately 2-2.5 months. Fertilized eggs undergo diapause at the blastocyst stage for 4-5 months until delayed implantation occurs [11,17,19,20]. The duration of embryonic diapauses varies, because the time of implantation and birth is uncoupled from the mating season [8,10]. Cubs in a litter are normally born at the same date independently of the dates of estrus and mating [8,10,12]. Split parturition has been observed in a captive brown bear, but has not been documented in the wild [27].

Several studies of bears have shown a strong correlation between a females’ body condition in fall and their reproductive success. Well-nourished females have larger litter sizes and shorter litter intervals [28–33]. A minimum amount of body mass and fat content (19% in brown bears) prior to hibernation is necessary for reproduction [34–38]. Thus, brown bears are able to vary both the birth date and growth rate of their cubs in response to their fat stores, which means that females in superior condition give birth earlier and lactate longer and produce more and higher quality milk in the den than females in poorer condition. This also accelerates cub growth relative to females in poorer condition [37,39]. Knowledge about the timing of reproductive events is therefore important for conservation and management.

Our first aim was to document, for the first time, the dates of implantation, parturition, and the gestation period of free-ranging brown bears. Embryo development requires euthermia and the implantation, parturition, and the gestation period of free-ranging bears is therefore important for conservation and management.

Our second aim was to determine which factors influence the timing of gestation. We tested whether age, litter size, primiparity, environmental conditions during season before hibernation, or the date of the start of hibernation influence the timing of parturition. In addition, we evaluated whether age, primiparity, environmental conditions, or weather conditions in autumn influence the start of hibernation.

Methods

Study area

The study area was located in the northern boreal forest zone in Dalarna and Gävleborg counties, south-central Sweden (~61°N, 15°E). The area is hilly, with altitudes ranging from 200 m in the southeast to 1,000 m in the west, but are mostly (>90%) below timberline, which is at ~750 m [45]. Snow cover usually lasts from the end of October until late April, and mean daily temperatures range from ~7°C in January to 15°C in July (Swedish Meteorological and Hydrological Institute). The bear population density is ~30/1000 km² [46,47]. The denning period in the study area is from October until May, and its duration varies due to reproductive status. Pregnant females spend an average 196 days in den, about one month longer than nonpregnant bears in the study area [48,49]. Timing of den entry is influenced by sex, reproductive status, and environmental conditions (e.g. first snowfall), as well as age and/or body size [48,49]. Pregnant females enter their dens first and leave their dens latest [48].

Capture, sensors, and the bears

We captured bears in spring after they left their dens. For detailed capture and marking procedures, see Arnemo et al. [50]. The permission to capture and instrument bears was granted by the Swedish Environmental Protection Agency (permit Dnr 412-7327-09 Nq) and the Ethical Committee on Animal Experiments in Uppsala (approval C47/9). Every bear was equipped with a dual-axis motion sensor mounted on a GPS-GSM collar (Vectorcan Aerospace GmbH, Berlin). This sensor measures true acceleration six to eight times per second in two orthogonal directions. The acceleration values were accumulated and averaged for each direction for a recording interval of 5 minutes, resulting in average acceleration values ranging from 0 to 255 for each axis. These averaged acceleration values were stored in the neck collar with the associated date and time until they were downloaded as a text file via Link Manager (Vectorcan Aerospace GmbH, Berlin). We implanted abdominal temperature data loggers (DST Genti, Star Oddi, Iceland), programmed to record body temperature every 30 minutes (see Arnemo et al. [50] for further details on the implantation procedures). These temperature data were stored in the logger’s internal memory with a real-time clock reference for each measurement. After recapturing the bears, we recovered the temperature loggers and uploaded the body temperature data with SeaStar software and the Communication Box (Star Oddi, Iceland), which served as a wireless interface between the logger and a PC.

Only females with verified reproductive status in a given year were included in the data set. Pregnant females were defined as solitary-hibernating females that had been observed with cubs of the year (hereafter referred to as cubs) after den emergence in spring, or which had been captured shortly after den emergence and showed signs of lactation and that cubs had used the nipples collars and body temperature data (recorded in implanted temperature loggers) would yield the same dates of implantation or parturition.
(to exclude cases of pseudopregnancy). Females were defined as nonpregnant when they had emerged from the den without cubs and showed no signs of lactation when captured. We defined the hibernation period as 1 November–31 March and calculated the mean daily body temperature and mean daily activity during this hibernation period for all females, based on the methods described by Friebe et al. [44]. It is common that bears abandoned their first dens (~22% of the cases), mainly as a result of human disturbance [51–53]. Two of our bears changed dens at the end of October and entered new dens in early November. For those bears we chose the second den entry as the start of hibernation.

Definition of the gestation period

Body temperature data. The body temperature T(b) of pregnant females bears is on average higher and more stable during the period of gestation than that of nonpregnant females [40,41]. After parturition, T(b) drops to the level of nonpregnant bears [41,42]. We defined the hibernation period as 1 November until 31 March [48,49] and calculated the mean body temperature during hibernation for each individual. The date of implantation was defined as the first day in November/December when an individual’s mean daily body temperature exceeded the same individual’s mean temperature during hibernation. Occasional high body temperature recordings, apparently caused by external factors, e.g. disturbances during hibernation, were excluded from the data set [40,41,54]. We defined the date of parturition as the first day in January/February when an individual’s mean daily body temperature declined below the individual’s mean temperature during hibernation. The gestation period was defined as the time interval between the dates of implantation and parturition.

Activity data. Bears are inactive ~98% of the time during hibernation, but they periodically make small movements [41,44,55,56]. Therefore, only a few position movements may have a large impact on the mean daily activity level. Robbins et al. [57] observed that pregnant captive brown bears did not stand up during the first 3 weeks postpartum. However, Friebe et al. [44] observed that some females have this low activity level for a shorter time after parturition. We therefore defined the date of parturition as the first day when the individual’s mean activity level decreased below the same individual’s mean hibernation activity level for at least 2 weeks. In central Sweden, 22% of the brown bears change winter dens, most often early in the denning period, when human hunting activities are still high [53]. Activity levels during hibernation are also lowest during midwinter [44,55]. For these reasons, occasional high activity peaks often occur early in hibernation, when implantation also occurs. To minimize the effect of high activity peaks, we used the moving averages (5th order) of the mean daily activity levels when defining the dates of implantation. The first day when this moving average exceeded the individual’s mean hibernation activity level for at least the next 2 weeks was defined as the day of implantation.

We used the dates of implantation and parturition calculated with body temperature data to compare the recorded activity and body temperature data during the gestation period with that obtained from two other periods: 14 days before gestation (preimplantation period) and 14 days after parturition (lactation period). We used relatively short periods of 14 days, because we wanted to compare data collected only during the hibernation period. Implantation may occur some weeks after the start of hibernation, and the time in den during lactation may be short for females that give birth to cubs very late. The body temperature of hibernating nonpregnant American and Asian black bears show multiday cycles, whereas pregnant females remain normothermic during gestation [41,42]. We compared the mean body temperature and also the daily variation in body temperature during the preimplantation, gestation, and lactation periods for pregnant and nonpregnant bears. For nonpregnant bears, we used the mean date of implantation and parturition determined from pregnant bears with body temperature recordings to define the periods of preimplantation, gestation, and lactation. Activity levels of pregnant and nonpregnant bears has been compared in a previous study [44].

Factors influencing date of birth and start of hibernation

Maternal body condition prior to denning influences reproductive success in bears [39]. Because we did not capture bears in autumn or winter, we had no information about the maternal body mass or fat content in autumn, nor information about cub growth. Instead, we calculated a yearling condition index for each year, which reflects the combined effect of environmental factors on the bear’s condition. The environmental condition index had been used in former studies as a proxy for food conditions [58]. We regressed the spring yearling body mass of 307 yearlings as a function of maternal size, litter size, population density, and sex, variables that are known to influence yearling mass independently of environmental conditions. The standardized residual values from this regression were averaged for each year and used as the environmental condition index for the previous year, when the yearlings had been cubs [58]. We then tested whether the environmental condition index, age, primiparity, litter size, and the start of hibernation influenced the date of parturition.

Harsh climate and weather conditions may trigger the start of hibernation and prolong the duration of denning [59–61]. Additionally it has been reported that black and brown bears in excellent condition start hibernation earlier [28,61,62]. We created individual activity indices by summing the acceleration values on the orthogonal axes (0–510) for each 5-minute interval. A bear was considered to be physically active when its activity index was higher than 22.9 [63]. The start of hibernation was defined as the first day in autumn when activity dropped below 1 hour per day (defined as fewer than 12 activity recordings with values on the orthogonal axes (0–510) for each 5-minute interval).

Harsh climate and weather conditions may trigger the start of hibernation and prolong the duration of denning [59–61].

Figure 1. Mean daily body temperature (T(b)) of pregnant (N=6) and nonpregnant (N=9) hibernating female brown bears in Sweden, during 2010–2013. The solid lines show the mean daily T(b) of 6 individual pregnant females, the dotted line shows the mean daily T(b) of 9 nonpregnant females, including the daily SE (gray bars). The T(b) decreased throughout gestation (Estimate = −0.002, SE<0.001, P<0.001).

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### Table 1. Gestation periods of 6 pregnant female brown bears in Sweden, during 2010–2013, calculated from body temperature (T(b)) and activity data.

<table>
<thead>
<tr>
<th>Bear Id-Year-Age</th>
<th>Date Implantation</th>
<th>Difference (days)</th>
<th>Date Parturition</th>
<th>Difference (days)</th>
<th>Gestation (days)</th>
<th>Difference (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W0820-12-6</td>
<td>30 Nov T(b)</td>
<td>56</td>
<td>25 Jan</td>
<td>54</td>
<td>56</td>
<td>−2</td>
</tr>
<tr>
<td></td>
<td>activity 01 Dec</td>
<td>1</td>
<td>24 Jan</td>
<td>1</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>W0605-11-7</td>
<td>19 Nov T(b)</td>
<td>54</td>
<td>12 Jan</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>activity 11 Nov</td>
<td>−8</td>
<td>12 Jan</td>
<td>0</td>
<td>62</td>
<td>8</td>
</tr>
<tr>
<td>W0703-11-6</td>
<td>25 Nov T(b)</td>
<td>56</td>
<td>20 Jan</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>activity 12 Nov</td>
<td>−13</td>
<td>20 Jan</td>
<td>0</td>
<td>69</td>
<td>13</td>
</tr>
<tr>
<td>W0610-11-7</td>
<td>18 Nov T(b)</td>
<td>54</td>
<td>10 Feb</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>activity 01 Dec</td>
<td>−17</td>
<td>09 Feb</td>
<td>−1</td>
<td>70</td>
<td>16</td>
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<td>W0610-12-8</td>
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<td>59</td>
<td>8 Feb</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>activity 02 Dec</td>
<td>−9</td>
<td>8 Feb</td>
<td>0</td>
<td>68</td>
<td>9</td>
</tr>
<tr>
<td>W0720-11-12</td>
<td>21 Nov T(b)</td>
<td>56</td>
<td>16 Jan</td>
<td>56</td>
<td></td>
<td></td>
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<td></td>
<td>activity 22 Nov</td>
<td>1</td>
<td>18 Jan</td>
<td>2</td>
<td>57</td>
<td>1</td>
</tr>
<tr>
<td>Total mean</td>
<td>T(b) 01 Dec</td>
<td>55.8</td>
<td>26 Jan</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total median</td>
<td>T(b) 28 Nov</td>
<td>56</td>
<td>23 Jan</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SD</td>
<td>T(b) 11.6</td>
<td>1.8</td>
<td>12.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Range</td>
<td>T(b) 29</td>
<td>5</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>activity 24 Nov</td>
<td>63.3</td>
<td>26 Jan</td>
<td>63.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total median</td>
<td>activity 27 Nov</td>
<td>65</td>
<td>23 Jan</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SD</td>
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<td>6.7</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total Range</td>
<td>activity 21</td>
<td>16</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0101410.t001
Data analysis

We tested for relationships between mean daily activity and mean daily body temperature during hibernation with a general linear mixed model with normal distribution and with individual identity as a random factor. A second-order polynomial term for mean daily body temperature was included into this analysis to account for nonlinear effects. We used paired-samples t-tests to compare the dates of implantation and parturition and the gestation period between the estimates based on activity and body temperature data. Activity and body temperature data during the preimplantation, gestation, and lactation periods were compared with paired Wilcoxon signed rank tests. To evaluate the effect of the day of gestation on body temperature, we used a general linear mixed model with a normal distribution and with individual identity as a random factor. A second-order polynomial term for day of gestation was included into this analysis to account for nonlinear effects. Mann Whitney U tests (MWU) were used to compare body temperatures during preimplantation, gestation, and lactation periods between pregnant and nonpregnant females.

We evaluated the factors affecting the date of parturition with a general linear mixed model with a normal distribution and assessed the effects of the following factors: age, primiparity (as binomial variable, with no = 0; yes = 1), litter size, date of hibernation start, and the environmental condition index. Because some mothers contributed several litters to our datasets during their lifetime, we included individual identity as a random effect to account for nonindependence. Year was not included as a random effect, because the environmental condition index was included as a fixed variable to describe the different environmental conditions among years. We used a backward procedure to select the best models, based on P values with a significance level of \( \alpha = 0.05 \), starting with a full model of all covariates and relevant second-order interactions.

We used a linear mixed model with a normal distribution to evaluate the effects of age, primiparity, and environmental condition indices on the start of hibernation, with individual identity as random effect. Ambient temperature in October was excluded from the model, because of collinearity with the environmental condition index (Pearson’s r: \(-0.745, P<0.001\)).

![Figure 2. Mean body temperature (T(b)) (A) and mean activity (B) during preimplantation, gestation, and lactation periods for pregnant females brown bears (N = 6) in Sweden, during 2010–2013. We calculated the dates of implantation and parturition with body temperature data. Preimplantation was defined as the 14-day period before implantation occurred and lactation was defined as the 14-day period after parturition. Extreme outliers are plotted as asterisks. In figure B, the highest activity values in all periods originate from the same pregnant female. doi:10.1371/journal.pone.0101410.g002](image)

![Figure 3. Daily variation in body temperature (T(b)) during preimplantation, gestation, and lactation for pregnant (N = 6) and nonpregnant (N = 9) female brown bears in Sweden during 2010–2013. For nonpregnant bears, we used the mean date of implantation and parturition determined from pregnant bears with body temperature recordings to define the periods of preimplantation, gestation, and lactation. The box indicates the median, 25, and 75% percentiles; the whiskers show the minimum and maximum observed values that are not statistically outliers. The extreme outlier is plotted as an asterisk. doi:10.1371/journal.pone.0101410.g003](image)
evaluate the effects of mean ambient temperature in October on the start of hibernation, with individual identity as a random effect. A linear mixed model with a normal distribution was also used to evaluate the effects of age and environmental condition index on the duration of hibernation prior to parturition. Residuals from all final models were inspected visually to ensure that the assumptions of constancy of variance and normality of errors were met. All statistical tests were carried out in SPSS (PASW Statistics 21).

Results

We compared body temperature data from 9 hibernation events from 8 nonpregnant adult females during 2010–2013, and body temperature and activity data from 6 hibernation events from 4 pregnant females, during 2010–2013.

Body temperature

The body temperature of pregnant females increased in November/December and remained high until January/February (Fig. 1). Based on body temperature, the estimated mean date of implantation was 1 December ± 12 days (median: 28 November, range: 29 days, from 19 November – 18 December). The mean date of parturition was 26 January ± 12 days (median: 23 January, range: 29 days, from 12 January – 10 February). The mean duration of the gestation period was 56 ± 2 days (median: 56 days, range: 54 – 59 days) (Table 1). Mean body temperature during the preimplantation, gestation, and lactation periods was 34.07 ± 0.42°C (median: 34.00°C), 37.11 ± 0.04°C (median: 37.11°C), and 34.64 ± 0.32°C (median: 34.71°C), respectively. We excluded the preimplantation period of one pregnant female that had shifted den 3 days before implantation from the analysis. Mean body temperature was significantly higher during the gestation period than during both the preimplantation and the lactation periods (paired sample Wilcoxon test: preimplantation vs gestation: Z = 2.02, P = 0.043; gestation vs lactation: Z = 2.20, P = 0.028) (Fig. 2A). We found no significant difference in body temperature between the preimplantation and lactation periods of pregnant females (Wilcoxon signed rank test: Z = -2.15, P = 0.080; Fig. 2A). The body temperature of pregnant females decreased during the gestation period (Estimate = -0.002, SE < 0.001, P < 0.001; Fig. 1). The mean body temperature of nonpregnant bears during the time period corresponding to the preimplantation period of pregnant females was 33.85 ± 0.54°C.

Figure 4. Example of the mean daily activity and mean daily body temperature (T(b)) recordings for a hibernating pregnant female brown bear in Sweden. The horizontal gray and the black dotted lines show the mean individual body temperature and activity during hibernation, respectively, which were used to calculate the dates of implantation and parturition.
doi:10.1371/journal.pone.0101410.g004

Figure 5. The effect of age and environmental conditions on the start of hibernation (A), date of parturition (B) and on the days of hibernation prior parturition (C) for 46 hibernating pregnant female brown bear in Sweden. The environmental condition index was significant related to the start of hibernation, date of parturition and on days of hibernation prior parturition. Age was significantly related only to the start of hibernation.
doi:10.1371/journal.pone.0101410.g005
Activity data

The activity and body temperature data of 6 pregnant females showed similar patterns (Fig. 4). Mean daily activity and body temperature were positively related during the defined hibernation period (estimate = 0.205, SE = 0.021, t = 9.865, P < 0.001). The implantation dates we estimated based on activity data differed up to 17 (±5) days from the implantation dates we estimated based on body temperature, but the means were only barely statistically equal (paired-sample t-test: t = 2.512, df = 5, P = 0.054). The calculated parturition dates differed only by a maximum of 2 days, which was not statistically significant (paired-sample T-test: t = 0.000, df = 5, P = 1.000). The calculated gestation periods based on activity data were significantly longer than those based on body temperature data (paired-sample T-test: t = -2.667, df = 5, P = 0.045; Table 1). Mean activity during preimplantation, gestation, and lactation periods was 0.39±0.3 (median: 0.33, 1.0±1.08 (median: 0.72), and 0.29±0.27 (median: 0.27), respectively. Activity during the gestation period was significantly higher than during both the preimplantation and the lactation periods (paired-sample Wilcoxon test: preimplantation vs. gestation: Z = 2.02, P = 0.043; gestation vs. lactation: Z = -2.20, P = 0.028; Fig. 2B). Activity during the lactation period was significantly lower than during the preimplantation (paired-sample Wilcoxon test: Z = 2.02, P = 0.043; Fig. 2B).

We used a data set of 46 hibernation events from 30 females with only activity data to investigate which factors influence the date of parturition and the start of hibernation. The mean age of the females during these 46 hibernation events was 9.0±4.0 years (range: 16, from 3 – 19 years). Eleven females were primiparous, 33 were multiparous, and in 2 cases the previous reproductive status could not be classified. Mean litter size after hibernation was 9.0 (median: 8.0, range: 6–11). The mean start of hibernation was 18 October (SD: 9, median: 16 October, range: 34 days, from 2 October – 5 November). The mean duration of denning prior to parturition was 93 days (SD: 13, median: 9, range: 57 days, from 66 – 123 days).

The activity and body temperature data of 6 pregnant females showed similar patterns (Fig. 4). Mean daily activity and body temperature were positively related during the defined hibernation period (estimate = 0.205, SE = 0.021, t = 9.865, P < 0.001). The implantation dates we estimated based on activity data differed up to 17 (±5) days from the implantation dates we estimated based on body temperature, but the means were only barely statistically equal (paired-sample t-test: t = 2.512, df = 5, P = 0.054). The calculated parturition dates differed only by a maximum of 2 days, which was not statistically significant (paired-sample T-test: t = 0.000, df = 5, P = 1.000). The calculated gestation periods based on activity data were significantly longer than those based on body temperature data (paired-sample T-test: t = -2.667, df = 5, P = 0.045; Table 1). Mean activity during preimplantation, gestation, and lactation periods was 0.39±0.3 (median: 0.33, 1.0±1.08 (median: 0.72), and 0.29±0.27 (median: 0.27), respectively. Activity during the gestation period was significantly higher than during both the preimplantation and the lactation periods (paired-sample Wilcoxon test: preimplantation vs. gestation: Z = 2.02, P = 0.043; gestation vs. lactation: Z = -2.20, P = 0.028; Fig. 2B). Activity during the lactation period was significantly lower than during the preimplantation (paired-sample Wilcoxon test: Z = 2.02, P = 0.043; Fig. 2B).

Discussion

Body temperature

This is the first time the timing of gestation has been documented in free-ranging brown bears. The body temperature data clearly identified the dates of implantation and parturition. The calculated gestation periods ranged between 54 – 59 days and were similar to early reports for black and brown bears in other studies. Body temperature averaged higher during the gestation period compared to the preimplantation and lactation periods for pregnant females and compared to the body temperature of nonpregnant females. Besides the energetic costs of lactation, the maintenance of a high body temperature during gestation may be an additional reason why pregnant females loose more body mass during hibernation than nonpregnant bears [38]. The mean daily body temperature during gestation varied very little compared to the periods before and after the gestation and compared to the body temperature of nonpregnant females. Multiday cycles of body temperature have been documented for nonpregnant hibernating bears [41,64]. We did not observe this in pregnant females during the gestation period. Instead, the mean daily body temperature was stable and did not fall below 35.9°C, as also observed in one pregnant American black bear [41]. Fetal development might be intolerant of high variations in body temperature. Raised hormone levels during pregnancy could be another reason for the low variation of body temperature during gestation [7].

We also observed that the body temperature of pregnant females decreased during the course of gestation. Studies have shown that the maximum serum progesterone level of pregnant brown bears occurs approximately 60 days before parturition and decreases during gestation [7]. The decrease in body temperature during gestation that we observed might be caused by changes in progesterone or other hormone levels. A drop in body temperature at parturition has been reported previously for American and Asiatic black bears and brown bears;
in both species of black bears, the body temperature decreased to the level of nonpregnant bears after parturition [41,42]. However, our results for brown bears showed that the body temperature during lactation did not fall as low as that of nonlactating bears, as also reported by Hisa [40] for brown bears. Our data showed that body temperature during the preimplantation period did not differ significantly from that during lactation for pregnant females. However, nonpregnant females had lower body temperature levels than pregnant females during the lactation period. Body temperature is probably lowest during midwinter, as it is for activity [40,44]. Metabolic activity during lactation might require or result in higher body temperature levels.

Activity
Parturition dates estimated using activity and body temperature data differed by only one or two days and were the same for 50% of the females. Thus, we consider that either activity data or body temperature can be used to determine dates of parturition. However, because of the high variation in mean daily activity during the early hibernation period, it was more difficult to estimate the dates of implantation. We used the moving 5th-order average, because in some cases, activity did not reach the mean hibernation level for more than a few days before implantation, in other cases activity rose before the implantation calculated from the body temperature. Raised activity during this period could be caused by hormonal changes prior implantation, or because activity is in general higher during the beginning of the hibernation period than during midwinter [44,55]. Our calculated dates of implantation varied 17±5 days between body temperature and activity recordings. We can therefore not recommend using activity recordings to determine the date of implantation. However, because the gestation period was stable, showed little variation and lasted on average 56 days, we recommend estimating the date of implantation using activity data by subtracting 56 days from the calculated date of parturition.

Factors that influenced the date of parturition
Parturition dates ranged over a period of 43 days, which showed a high flexibility in the timing of gestation. Whereas dates of parturition have not been recorded in wild-living brown bears before, Bridges et al. [65] documented that parturition dates of 150 litters of wild-living American black bears ranged over 53 days from late December to mid-February (39 days excluding an outlier). Robbins et al. [39] reported only a 17-day range of parturition in January for a smaller sample of 13 captive brown bear births, perhaps due to similar conditions between bears in captivity.

Because the date of denning did not correlate with the date of parturition, we suggest that other factors than the start of denning trigger implantation. Age had no significant effect on the timing of parturition, as there was only a tendency for older females to give birth earlier. Bridges et al. [65] observed later parturition in pregnant female American black bears < 5 years old. However, in our study, only 2 females were < 5 years. A larger dataset of young pregnant females might be necessary to document an effect of age on the date of parturition.

Studies on captive brown bears have shown that larger females give birth earlier during winter than smaller females [39]. However, in our study, favorable environmental conditions correlated with late parturition. Although we had no information about the females’ body mass prior to denning, we expected that food availability was the most important factor affecting the environmental condition index [58] and that the females were heavier when the environmental conditions had been favorable.

With this reasoning, our results differed from those found in captive bears [39]. It is possible that free ranging females might budget their energy resources differently than captive females [66,67].

Timing of the start of hibernation
The start of hibernation varied 34 days, with a mean start of 18 October, similar to previous studies in our study area [48,53]. Good environmental conditions were highly significantly correlated with an earlier start of hibernation. Early start of hibernation has been observed as a strategy for extremely well nourished female bears [61]. Limited fat-storing capacity can be a reason for early start of hibernation during years with good environmental conditions [36].

Similar to other studies, low temperatures in October, and high age were factors that initiated an early start of hibernation for pregnant females [60,61]. Bears in colder climates hibernate longer [49]. The temperature in October also correlated negatively with the environmental condition index. In our study area, bears mainly forage on berries in autumn [68]. In late autumn when food availability decreases, the trade-off between energy expenditure and energy consumption might diminish [56]. Older females may have experienced that an early start of hibernation had a positive impact on the energy balance and started to hibernate earlier than younger inexperienced pregnant females. Schooley et al. [69] suggested that pregnant American black bears den after they have stored sufficient fat reserves for winter survival and reproduction in order to avoid being active during periods when food become less abundant.

Pregnant free-ranging bears must cope with more challenging environmental factors than bears in captivity, such as limited food availability, harsh weather conditions, disturbances by humans, or hunting activities. They must gauge the energy costs and benefits of an early denning start. In central Sweden 68% of the presumed pregnant females that had abandoned their dens emerged from their new dens without cubs and 22% of the first dens were abandoned, primarily due to human disturbance [52]. Previous studies have shown that disturbance during hyperphagia and during hibernation period have a negative effect on the bears’ fitness and reproductive success [31,35,51,52,70]. Pregnant females are not protected from hunting, however, they play a crucial role in population growth and start to hibernate earliest [48,71–73]. In our study 47% of the pregnant females started hibernation before the 15 October, the last day hunting is permitted if the quota has not been filled. Therefore, an early start of hibernation could also be a strategy to avoid disturbance and loss of energy during the hunting season. Restricted use of their home range, combined with reduced movements, are known strategies of female brown bears with cubs of the year to avoid male bear encounters during mating season [45,74]. Several studies have shown that bears try to avoid human disturbance during hibernation, e.g., by selecting den sites far from roads or in concealed and rugged terrain [75–80]. Additionally, pregnant females choose better concealed den types, like anthill, soil, and rock dens, than male bears, which often hibernate in open nest dens [81]. Also, previous studies on free-ranging female brown bears in central Sweden have shown that females select predetermined places for denning by visiting their den areas on average more than once a month during season [48]. Male brown bears in the same study area have higher abandonment rates when they had not visited their den sites previously [33]. In our study, during years with good environmental conditions, pregnant females began hibernating earlier rather than using energy reserves for early parturition and lactation, which would have
maximized offspring weight at den emergence. During years with bad environmental conditions, the duration of hibernation prior parturition also was shorter. Further research is necessary to determine whether early denning combined with tactically wise denning strategies help pregnant females avoid disturbance. Early start of hibernation has been hypothesized as a strategy for predator avoidance in small mammals [82]. In this regard, it would be important to compare the timing of hibernation and parturition in our hunted population living in a human-dominated landscape with brown bear populations living in areas with low human activities during autumn. In addition, more information about the relationship between female body condition prior to hibernation and the timing of gestation is needed for wild-living bears.

Author Contributions

Conceived and designed the experiments: AF AE JMA S. Brumberg S. Blanc GF AZ JS. Performed the experiments: AF AE JMA S. Brumberg S. Blanc. Analyzed the data: AF AZ JS S. Blanc. Contributed reagents/materials/analysis tools: AF AZ JS S. Brumberg JA S. Blanc. Contributed to the writing of the manuscript: AF JS AZ.

References

Paper VIII
Body size determines depth and length of hibernation in free-ranging brown bears

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Abstract

During hibernation, animals undergo major reductions in body temperature and metabolic rate. The amplitude of metabolic rate reduction in hibernators is dependent on body size; small hibernators have a high metabolic rate when active, resulting in drastic reductions to reach the very low metabolic rate of most hibernators. The lower magnitude of temperature fluctuations during hibernation in bears, compared to other hibernators, is thought to be related to body size, although detailed studies on intra-specific variations in body temperature are lacking. Here we document, with 45 years of data from 34 bears, relationships between body mass and body temperature, hibernation duration, den exit date, and in an energy savings index, with the smallest bears having lower body temperatures, hibernating longer, exiting later and saving more energy than large bears. These relationships were consistent across a range of masses from 30 to 233 kg. However, in July-September, the smallest bears had higher temperatures. Our results demonstrate that basic thermodynamics most likely regulate the cooling process of the bear as they enter the dens and lower the metabolic rate, with smaller bears which have higher thermal conductance, having lower body temperatures.

Keywords: bears, hibernation, thermal conductance, thermoregulation
**Introduction**

An age-old question in hibernation biology is how a large mammal, such as the bear, can hibernate and moreover, how a bear’s large size affects its status as a hibernator. Most studies on small mammals focus on the effects of body condition and energy reserves on hibernation and have concluded that fatter individuals have a higher mean minimum body temperature and arouse more often and for longer periods than leaner animals (Bieber *et al.* 2014). However, few hibernators have such a wide range of body mass as the bear, with spring body mass in Scandinavian brown bears (*Ursus arctos*) ranging from 8–44 kg at one year of age (Painer *et al.* 2012) to 62 to 241 kg as adults (Fahlman *et al.* 2011). This is considerably different than in the small hibernators. So, a fundamental question is how does body mass affect the pattern of hibernation in bears?

Body mass correlates with total fat stores in woodchucks (*Marmota monax*) (Snyder *et al.* 1961), and individuals with higher body mass have been shown to spend less time in torpor and more in euthermia (Zervanos *et al.* 2014). In a study comparing torpor bouts across a range of mammalian hibernator species, hibernation duration increased with latitude, but was independent of body mass (Lovegrove 2012). The same study found that minimum body temperature increased with increasing body masses, although no such relationship has been found for mammals during euthermia (Lovegrove 2012). Minimum metabolic rate increases with increasing body mass (Geiser 2004). Decreased metabolic rate as the primary mechanism for energy saving during hibernation and torpor has evolved to balance a variety of costs (Munro *et al.* 2005). Costs include increased risk of predation, reduced protein synthesis, sleep deprivation (Larkin and Heller 1996, Boyles *et al.* 2007) and attenuated immune responses (Humphries *et al.* 2003). Earlier it was thought that, across species, torpor bout duration decreased with increased body mass (French 1985), but later studies (Geiser and Ruf 1995, Malan 2010, Ruf and Geiser 2015) have not found evidence of this. Overall hibernation
length, however, has been connected to body size with larger species hibernating longer (French 1985). Body size determines the amplitude of metabolic rate reduction between active and hibernating periods; small hibernators have a higher metabolic rate when active, resulting in drastic reductions to reach the low metabolic rates of most hibernators (Heldmaier et al. 2004). The amplitude of rate reduction in larger hibernators, like bears, is much less (Toien et al. 2011). The lowered body temperatures, characteristic of hibernation, have often been considered to be one of the critical indicators of metabolic rate (Watts et al. 1981, Németh et al. 2009). The lower magnitude of temperature fluctuations during hibernation in bears, compared to other hibernators, is thought to be related to the bears’ large body size (Watts et al. 1981, Heldmaier et al. 2004, Heldmaier 2011).

Body mass affects thermodynamics and one study in American black bears (Ursus americanus) found that larger bears had lower thermal conductance (Toien et al. 2015) and that total body thermal conductance decreased at low den temperature. It also found that although smaller bears had higher total body conductance, their lower critical temperatures were not significantly higher than that of larger bears. The hibernation-optimization hypothesis (Boyles et al. 2007) suggests that hibernators that can afford it spend less time in torpor and more time at euthermic body temperatures in order to reduce the negative effects of cooling. In this regard, larger bears with more fat reserves would be expected to hibernate less.

Although several studies have been conducted on hibernating black or brown bears in captivity (Hissa et al. 1994, Toien et al. 2011), few have had enough individuals to evaluate the effect of body mass. Here we report the effects of large-scale mass differences on an intra-species scale throughout the bear’s annual cycle.
Methods

A total of 34 sub-adult and adult bears (1-13 years old), in south-central Sweden were captured by darting from a helicopter as previously described (Arnemo et al. 2012). They were fitted with global positioning system (GPS) collars (Vectronics Aerospace GmbH, Berlin, Germany), very high frequency (VHF) abdominal implants (Telonics Inc., Mesa, AZ, USA) and with implanted intra-peritoneal temperature loggers (DST Centi, Star Oddi, Gardaber, Iceland). Bears had body temperature (Tb) recorded at intervals ranging from 1 to 15 minutes for 1-3 years, giving a total of 45 years of data. Twelve bears were captured during winter, and for these bears, the data for February and March were excluded. The majority of the other bears were captured in April, so the data from April were influenced by the capture event. Six bears became pregnant during the study and were excluded from the models because their body temperature has been described elsewhere (Friebe et al. 2014).

Using daily mean body temperatures, we first investigated descriptive statistics (mean, median, minimum, and maximum) of Tb. We grouped the bears by size based on their weight in the winter/spring following the Tb measurements for some analyses. “Small” was defined as 30-60 kg (N=19, 11 captured in winter), “medium” as 60-120 kg (N=19, 7 of which pregnant, 1 captured in winter), “large” as 120-240 (N=6). For descriptive statistics, pregnant bears were removed from the categories “medium” (N=7) and from October through May and den captured bears were removed from February and March.

To determine which times of the year Tb was related to body size, we created linear mixed effect models (lme, (Pinheiro et al. 2013)) , including mean daily body temperature as the response variable, bear size category as a predictor and bear ID as a random effect. We then compared Tb between size groups during each month with Tukey contrasts.

We made a two-step analysis to quantify the differences in overall depth of hibernation among size groups. We first calculated this as the area between curves (ABC) using 39.3°C
(T\textsubscript{b} of the maximum daily mean measured during hibernation) and the measured T\textsubscript{b} curve during the entire hibernation, i.e. the greater the ABC, the deeper and and/or longer the hibernation period (Figure S1). Secondly, we tested for effect of body mass using linear mixed-effect models (lme), with ABC as the response variable, body mass and a binary term for den capture as a fixed effect and bear ID and den capture as random factors. Following a determination of the random structure the fixed variables were selected by backward selection.

To determine the length of the hibernation period, we used the day that the daily mean T\textsubscript{b} decreased below 36.5°C in the autumn and increased above 36.7°C in the spring. These temperatures have previously been reported to be associated with den entry (36.47± 0.14°C) and exit (36.70±0.15°C) (Paper 6). Again, we used lme, with the same structure and selected as above, to test for effects of body mass on the date of den entry and exit and on the duration of the hibernation period. All analysis were done using statistical extensions available in R 3.2.0 (R Development Core Team 2014).

**Results**

The groups had significantly different and sequentially lower T\textsubscript{b} as body size decreased from November through February. The opposite pattern was seen from July through August with March-June and September-October having overlap between groups (Table 1 and Figure 1). According to the size group models, size grouping was a significant predictor of T\textsubscript{b} during most of the year; all size groups had significantly different mean T\textsubscript{b} during December, January, February, July, August, and September.

Models with the ID and the binary term for den capture as random effects did not converge. The difference between the models with ID as a random factor and no random factor were not significant for all three response variables (LL<0.3, p>0.58). To avoid potential violation of independence, the ID remained as random factor in all of the models.
Whether bears were captured in the den or not, was not significant and had the highest p-value in any of the models and was therefore dropped. There was a significant effect of body mass (t= -5.48, effect size= -2.10) on the ABC, the index of energy savings (Figure 2). We found no detectable effect of body mass on den entry date (t=1.81, effect size= 0.07, p=0.11), but we found a significant effect of body mass on den exit date (t=-4.95, effect size=-0.15, p<0.01) with smaller bears emerging last. The denning duration was significantly longer (t=-4.19, effect size=-0.22, p=0<0.01) for smaller bears.

Discussion

We evaluated the effect of body mass on Tₘ of the brown bear throughout their annual cycle. We found that body mass played a significant role in some of the phenological aspects of hibernation; the smallest bears hibernated longest and exited their dens last. However, body mass did not have an effect on den entrance date, which is consistent with our previous finding that den entrance timing is dependent primarily by environmental cues (Paper 6).

The small bears were the warmest group in summer, but as they entered hibernation, their Tₘ dropped to below that of the other groups (Figure 1a). This can likely be attributed to the fact that smaller animals have a lower surface-to-volume ratio, resulting in higher thermal conductance (Tøien et al. 2015) and poorer heat conservation (Geiser 2004). This is consistent with interspecies comparisons where larger hibernators have slower cooling rates (Geiser 2004), confirming that differences can be attributed to body size. The smallest bears also had a consistently higher energy savings index, perhaps because they lacked the energy reserves to hibernate at a higher temperature or to compensate for the higher mass-specific metabolic rate reported in smaller American black bears (Tøien et al. 2015). The smallest bears may have exited their dens last, either because 1), their thermal neutral zone is higher than the larger bears (consistent with the trend noted in American black bears (Tøien et al. 2015)), so they wait for a higher ambient temperature to come out in spring or 2), they have lower fat
reserves and must hibernate longer to conserve energy until food availability increases. Although a study examining the lower critical temperature across black bear body masses of 40-120 kg did not find a significant effect of body mass (Tøien et al. 2015), the limited range of sizes and small sample size in that study may have precluded finding a statistically significant result.

An alternative interpretation is that the larger bears did not have as deep or long of a hibernation period, because of the greater energetic cost of warming up a large body mass, potentially undo benefits of a deep hibernation. The smallest bears likely need to save more energy than the largest bears and can warm up again at lower cost. Larger bears may be able to better afford to search for rare, protein-rich food, such as ungulate carrion or weakened moose (Alces alces) (Dahle and Swenson 2003, Stenset et al. 2016), and can better withstand harsh weather if it occurs.

The large dataset available here allowed for comparisons across multiple size groups in free-ranging bears, where the relationship between environment and physiology has not been manipulated. We conclude that the smallest bears hibernate most deeply and longest, likely from a combined effect of basic thermodynamics (high thermal conductance due to a low surface-to-body-mass ratio) and the higher need for energy savings and a lower cost of warming up a small body.

Ethics

All captures were approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (application #s C47/9, C7/12, C18/15, C212/9, and C268/12) and the Swedish Environmental Protection Agency.
Acknowledgments

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References


Figure 1. a). Pooled mean daily body temperatures applying a LOESS smoother to body mass (Size) of brown bears in Sweden centred on the hibernation period (a), and (b) centred on the active period. “Small” (blue) was defined as 30-60 kg (N=7), “medium” (green) as 60-120 kg (N=6) and “large” (pink) as 120-240 (N=6). Pregnant (N=6) and captured bears (N=11) were excluded from both graphs.
Figure 2. Boxplot of area between the body temperature curves (ABC, an index of energy savings; a higher ABC shows lower energy savings) for brown bears in Sweden, compared between body mass size groups. “Small” (S) was defined as 30-60 kg (N=19), “medium” (M) as 60-120 kg (N=12) and “large” (L) as 120-240 (N=6). Pregnant bears (N=6) were excluded from the ABC analysis.
### Supplementary Material

**Table S1.** Mean body temperatures (in °C) of brown bears in Sweden, by month. It is noted which bears are excluded from the means, either pregnant, both (pregnant and winter captured) or none. “Sig diff” indicates which group each is significantly different from in the Tukey contrasts from the linear mixed effect models (S, Small, M, Medium, L, Large).

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**Figure S1.** Area (grey) between measured mean daily body temperature (solid line) and the highest measured daily mean body temperature (39.3°C, dashed line), used as an energy saving index (ABC). Here, a) a bear with body mass 233 kg and b), a bear with body mass 57 kg.