Molecular mechanisms regulating oxygen transport and consumption in high altitude and hibernating mammals

PhD dissertation
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Zoophysiology, Department of Bioscience
Aarhus University
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Preface

This dissertation represents the partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) at the Faculty of Science and Technology, Aarhus University. The work described in this dissertation was carried out at the section of Zoophysiology, Department of Bioscience, Aarhus University under the supervision of Angela M. Fago. Part of this work, including field expeditions was conducted in collaboration with Jay F. Storz, University of Nebraska, Lincoln, and with the Scandinavian Brown Bear Research Project.

The overall aim of this project was to investigate molecular mechanisms of oxygen uptake, delivery and consumption in animals living at extremes, with focus on hemoglobin oxygen affinity changes and allosteric effectors as well as concentration changes and possible effects of the signaling molecules nitric oxide and hydrogen sulfide.

My first informed contact with hibernator blood was at a drenched cemetery in Lincoln Nebraska 2010. I had just flown over after spending half a year as an exchange student in Seattle, Washington, and literally walked from arrival hall to rainy cemetery and lie-in-wait 13-lined ground squirrel hunt armed with our bait of peanut butter and oats. Peanut butter in stages of dissolving everywhere (my pockets included) does leave a lasting impression in a European olfactory memory. During my studies of high and low altitude ground squirrels for what became Paper III, I became captivated by hibernators. When we got contacted by the Scandinavian Brown Bear Research Project, and I later was taken on as the blood specialist of the field team, I had to skip aside to spend some time just jumping up and down. Six years later, I am only the more fascinated by these animals, and still eager to learn and understand more.

Chapter I in this dissertation consists of a broad review-like introduction to molecular mechanisms controlling oxygen transport and consumption, discussing the work of this thesis in relation to the literature. The introduction finalizes by a conclusion and perspectives paragraph as well as future experiments, including two ongoing projects. Chapter II consists of four peer reviewed papers, published in international journals. I am first-author on three of these papers, having been the major contributor to all fases of the research, and contributed majorly to planning, data collection and writing of paper II. Paper III won the 2013 Outstanding Paper Prize in Journal of Experimental Biology.

Aarhus, January 2016

Inge G. Revsbech
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There are many people I wish to thank for being important parts of my past 6 years as a PhD-student on the 3+5 model, plus a maternal leave.

First and foremost, I wish to truly thank my supervisor Angela Fago, for believing and investing in the young yet bachelor student who stumbled upon her door those six years ago. Angela has been a great advisor and support and has shared my enthusiasm as well as helped me see the positive in the - at first hand - less than ideal experiment outcomes. She even took a turn with the bears when I was field work incapable, tackling of snowshoes being tricky due to advancing pregnancy. Angela also unusually quickly planned around a fast idea of an especially interesting conference mid-teaching, both of us departing within a few weeks. For all investments in me and my project, thank you.

I also wish to thank our collaborator Jay F. Storz at the University of Nebraska, Lincoln. During the first years of my PhD, it was much due to Jay and wonderful people of his lab, that I got a running start, including exciting early morning field work at 4000 meters. Our labs are in close collaboration and I have enjoyed inputs from visits, especially from Post. Doc. Joana Projecto-Garcia.

A large and heartfelt thanks goes to everyone in the Scandinavian Brown Bear Research Project. Especially to my “bear-boss” Ole Frøbert and his partner-in-crime, Johan Josefsson at Örebro University Hospital, Sweden, as well as to Alina Evans, Peter Godsk Jörgensen, Jon Arnemo, Jon Swenson and Sven Brunberg. It has been much through my involvement in this project that my passion for hibernators grew and flourished.

Thank you goes out to our good collaborators Christopher G. Kevil and Xinggui Shen at LSU heath Sciences Center, Shreveport Louisiana for being willing to ‘jump in the bear cave’ with us and for many good hydrogen sulfide discussions.

Also thank you to Tom Brittain and his kind lab for hosting me during two months at Auckland University, New Zealand.

There has been a whole bunch of people involved more or less directly in my work during the years at Zoophysiology. I wish to thank all of the staff at our department, all of them being ready with a helping hand in their field. A special thanks goes to Hans Malte, Tobias Wang and Hans Gesser for assisting with ideas for experimental planning. As well as to Nini Skovgaard and Nina Iversen for being part of planning and conducting myography experiments. Huge thanks goes to our lab-techs Elin and Kirsten, who are just wonderful people. Last but not least at all, a big thank you to our workshops and especially John Svane and Niels Skyberg for kind help with quickly fixing all kinds of things.

Next, I wish to thank all students at Zoophysiology. Without a good work-community, work gets less fun. Special thanks to former and present office mates, Frants Jensen, Cristian Bech Christensen, Christian Damsgaard, Amanda Bundgård, Lærke Reinholdt, Mikkel Thomsen and to my PhD colleague and friend Signe Helbo. Also thanks for shared teaching, courses and interests to Jonas, Anders, Anete and Catherine. And thank you to everyone I forgot.

Lastly, I thank my good friends and family. My exceptional friends Marie and Sunna and “Sis” Emma. My parents and my brother Lars for believing in and supporting me. Thank you to my patient husband Henrik who has aided me in stress and exhilaration, 2-hour mice-recaptures in university basements and feedback on countless talks and illustrations. Thank you for always being there to bring me back home, also in my head. And finally, thank you to our bandit son Mikkel for making every day a brighter place – e.g. by cheerfully uttering “fart!”
ENGLISH SUMMARY

The aim of this thesis is to broaden the knowledge of molecular mechanisms of adjustment in oxygen (O$_2$) uptake, conduction, delivery and consumption in mammals adapted to extreme conditions. For this end, I have worked with animals living at high altitude as an example of environmental hypoxia, and hibernating mammals, as an example of closely balanced internal low O$_2$. Studies have had two main focus points. Firstly, I have investigated variations in hemolysate and hemoglobin (Hb) O$_2$ affinity, working to pinpoint whether and how functional changes in intrinsic affinity or cofactor sensitivity of the Hb molecule compares to amino acid substitutions in the molecule, i.e., can be characterized as evolved genetic adaptation. Phenotypic acclimatization in Hb- O$_2$ affinity responses involves changes in cofactor to Hb tetramer ratio. Secondly, I have worked with (in a cardiovascular perspective) fine-tuners of delivery and consumption: the signaling molecules nitric oxide (NO) and hydrogen sulfide (H$_2$S) that are ubiquitously produced in mammalian tissues. These molecules and their potential effects seem particularly relevant to hibernators.

My dissertation consists of two chapters. Chapter I contains an introductory review to my field of study, and chapter II consists of four published, peer-reviewed papers.

The introductory review deals firstly with the background for animal adaptation and acclimatization to challenging conditions via changes in Hb-O$_2$ affinity. Elevated blood O$_2$ affinity is one of the repeatedly found adaptive traits in animals living at high altitude and in hibernating mammals during hibernation compared with the active state. Factors that affect O$_2$ affinity of Hb include temperature, H$^+/CO_2$ via the Bohr effect as well as Cl' and organic phosphates, in mammals mainly 2,3-diphosphoglycerate, DPG. I here show how my results fit into the current knowledge. Secondly, I review the current literature on cardiovascular effects of NO and H$_2$S and discuss their potential effects in mainly hibernating mammals.

SUMMARY OF IMPORTANT FINDINGS OF THE INCLUDED PAPERS TO BE FOUND AT THE END OF THIS DISSERTATION:


Paper I: Revsbech et al., 2013a. Paper I in this thesis concerns variations in the red blood cell cofactor DPG with hibernation in the brown bear and how this affects hemolysate and Hb O$_2$ affinity. Decreases in allosteric cofactor DPG has been observed in several small hibernators, but only one study in hedgehogs have found a correlation with the observed hibernation-induced increases in blood O$_2$ affinity. Bear hibernation is in some aspects physiologically different from that of smaller hibernators (warmer body temperature, no arousals). We found the DPG/Hb tetramer ratio to be reduced to about half of summer levels in hibernating brown bear, and the winter hemolysate to indeed show increased O$_2$ affinity when measured at physiologically relevant temperatures (37 vs 30°C). The difference in temperature was necessary but not sufficient to induce this change. We then demonstrated the change in DPG to be responsible by adding the measured amount of DPG to purified Hb from the individual bears. Modeling moreover showed that the
winter decrease in DPG (and thus changes in affinity $P_{50}$ and cooperativity $n_{50}$) related to a somewhat lower venous $PO_2$ than in summer. If not for these changes, winter venous $PO_2$ would have been appreciably higher than summer due to an increase in hematocrit, which would likely have led to high reactive oxygen species production.


Paper II: Tufts et al., 2013. Paper II also concerns variation in DPG in the red blood cells, here measured in high versus low altitude deer mouse populations. Elevations in erythrocyte DPG, hematocrit, Hb concentration, lower mean corpuscular Hb concentration and smaller red blood cells were found in a high altitude deer mouse population. By conducting a common garden experiment over six weeks (housing the animals at the same conditions at low altitude), these changes were reversed. Our group and collaborators have published on the genetic adaptation of deer mouse Hb to high altitude, involving low cofactor sensitivity. The reversal of these traits in the common garden experiment proves them to be acclimatization responses that are part of a remarkable phenotypic plasticity, that we provide evidence can also be found in a population in other aspects considered high altitude adapted.


Paper III: Revsbech et al., 2013b. The third paper to be published was actually the first study of my thesis, however data collection was conducted over several years. This project concerned a thorough comparison of six species of high and low altitude marmotine ground squirrel Hb function and sequence data. Globin sequencing by our collaborators showed highly unusual substitutions, according to the human HbA model expected to affect the Bohr effect of the ground squirrel Hbs. In particular the $\beta_{146}$His→Gln of the golden mantled ground squirrel was expected to be detrimental to the Bohr effect of this Hb. Functional analysis in our lab showed general high similar Hb-$O_2$ affinity for all species, with suppressed Cl$^-$ and DPG sensitivities, regardless of native altitude. Additionally the 13-lined and golden mantled ground squirrel Hbs had substantial Bohr effects, despite their substitutions. Taken together, these ground squirrels do not conform to the human HbA model of Hb function, but rather the mentioned substitutions have a different effect on the genetic background of these animals. Instead the collective number of surface histidines seems to decide the Bohr effect. In addition, the hibernation-induced decrease in DPG observed in ground squirrels may not have any appreciable direct allosteric effect, despite the fact that the DPG binding site was conserved. To our surprise and delight, this paper won the Outstanding Paper Prize 2013 in the Journal of Experimental Biology.

Paper IV: Revsbech et al., 2014. The fourth and final paper included in this thesis was our entrance into H$_2$S biology. In this paper we provide the, to our knowledge, first measurements of plasma and red blood cell H$_2$S metabolites in the hibernating versus awake state of a hibernating animal. We investigated levels and metabolites of both ubiquitous gaseous signaling molecules NO and H$_2$S that can potentially inhibit mitochondrial cytochrome c oxidase, and contribute to metabolic reduction during hibernation. H$_2$S can be divided into three major pools. I: acid-labile sulfur, containing mainly Fe-S clusters and persulfides that will be released under acidic conditions. II: bound sulfane sulfur pool containing mainly thiosulfate and polysulfides, converted into H$_2$S under reducing conditions. And III: free H$_2$S, at physiological pH mainly in the form of HS$^-$. To our surprise we did not find any increase in free H$_2$S, but rather a shift in the pools of H$_2$S. During winter hibernation we observed a decrease in bound sulfane sulfur, indicating that this pool in the low O$_2$ environment is utilized for H$_2$S production. The available cysteine, substrate for enzymatic H$_2$S production, can then be allocated for production of glutathione, a major antioxidant also found in high levels in red blood cells of hibernating bears. In contrast, changes in nitrite taken as a marker for NO production as well as SNO-modification of a key glycolytic enzyme, was not significantly different. We thus propose mainly H$_2$S, and likely not NO, to be involved in hibernation, as has also been indicated by its ability to induce a hibernation-like state in mice.

In conclusion the results of these studies shows that, when it comes to O$_2$ transport, phenotypic plasticity and in particular variations in red blood cell DPG levels plays large roles in acclimatizing animals that are in other aspects considered adapted. However, not all hibernators necessarily utilize falls in DPG during hibernation, and small hibernators may rely more on fall in body temperature to elevate blood O$_2$ affinity. Additionally, our studies indicate a role for H$_2$S during hibernation, possibly as part of the metabolic downregulation. Finally, results from this dissertation support the growing theory that not necessarily only a few amino acids are paramount to protein function, but rather their roles can be substituted by the cumulative action of other amino acid substitutions working on the specific genetic background.
**Dansk Resumé (Summary in Danish)**

Denne ph.d.-afhandlings formål er at øge vores viden om de molekylære mekanismer, som justerer ilts optag, transport, levering og forbrug i pattedyr, der er tilpasset ekstreme vilkår. Jeg har arbejdet med dyr, som er tilpasset til at leve i høje bjerger som eksempler på tilpasning til hypoxi i miljøet, samt dyr, der sover vintersøvn som eksempel på kontrolleringen af den hårfine balance af lavt ilt indeni et dyr, der skal spare på ”madpakken”. Mine studier har haft to primære fokuspunkter. Jeg har undersøgt variationer i hæmolysat (en opløsning af lyserede røde blodceller) og hæmoglobins (Hb) iltaffinitet. Jeg har arbejdet på at udpege hvilke og hvordan funktionelle ændringer i iboende ilt affinitet af Hb molekylet i sig selv eller Hbs sensitivitet overfor kofaktorer har rødder i aminosyre udskiftninger i molekylet, og derfor kan betragtes som en genotypisk nedarvet tilpasning. Fenotypisk tilpasning i Hb iltaffinitet er til gengæld styret af plastiske eller foranderlige ændringer, og involverer typisk ændringer i kofaktor til Hb tetramer ratioen. Mit andet fokuspunkt har været arbejde med signalmolekylerne nitrogenoxid (NO) og hydrogen sulfid (H₂S), der konstant produceres i pattedyrs væv. Disse molekyler kan i et cardiovasculært perspektiv fin-justere iltlevering og forbrug, hvilket især synes relevant for et dyr i vintersøvn.

Min afhandling består af to hovedkapitler. Kapitel I udgøres af et introducerende review til mit forskningsfelt, og kapitel II består af fire udgivne peer-reviewede artikler.

Det introducerende review omhandler i første del baggrunden for dyrs tilpasning gennem genetisk tilpasning og fænotypisk tilpasning via ændringer i Hb iltaffinitet. Øget iltaffinitet af blodet er en af de ofte fundne træk i dyr, som lever i højde samt i dvale tilstanden sammenlignet med den vågne, aktive tilstand. Faktorer som påvirker iltaffiniteten af Hb inkluderer temperatur, H⁺/CO₂ via Bohr effekten samt Cl⁻ og organiske fosfater, i pattedyr hovedsageligt 2,3-difosfoglycerat, DPG. Jeg diskuterer og sætter mine resultater i perspektiv til den forhåndenværende litteratur på området. Den anden del af introduktionen omhandler og diskuterer literaturen samt mine resultater omhandlende cardiovasculære niveauer af NO og H₂S og deres potentielle effekter især i dyr, som sover vintersøvn.

**Sammendrag af Vigtige Resultater i Mine Publicerede Artikler (Papers), som findes sidst i Afhandlingen:**


Paper I: Revsbech et al., 2013a. Paper I i denne afhandling omhandler variationer i de røde blodcellers Hb kofaktor DPG under vintersøvn i den brune bjørn, og hvordan dette påvirker hæmolysat og Hb iltaffinitet. Fald i den allosteriske kofaktor DPG har været set i adskillige mindre dyr, som sover vintersøvn, men en sammenhæng med den observerede stigning i blodets iltaffinitet er hidtil kun set hos pindsvin. Vintersøvn i brune bjørne er i nogle aspekter fysiologisk anderledes fra det, vi ser i små dyr (varmere kropstemperatur, ingen opvågninger). Vi fandt en halvering i forhold til niveauet om sommeren i DPG/Hb tetramer ratioen i
den hibernerende brune bjørn, samt en øget iltaffinitet i vinter hæmolysaterne når de blev målt ved fysiologisk relevante temperaturer (37 vs 30˚C). Forskellen i temperatur var nødvendig, men ikke tilstrækkelig til at forårsage ændringerne i iltaffiniteten. Vi demonstrerede ved et forsøg, hvor vi tilsatte den nøjagtige målte mængde DPG til oprenset Hb for hver bjørn, at ændringerne i DPG var årsagen til den yderligere ændring i iltaffiniteten. Yderligere viste matematisk modelering, at vinterens fald i de rød blodcellers DPG niveau (og derfor ændringer i iltaffinitet $P_{50}$ og cooperativitet $n_{50}$), var relateret til en lidt lavere venøs ilttension $PO_2$ om vinteren end om sommeren i bjørnens blod. Hvis de DPG-relaterede ændringer i iltbinding ikke var sket, ville den venøse $PO_2$, grundet en højere hæmatokrit i blodet om vinteren, omvendt have været en del højere end om sommeren. Høj venøs $PO_2$ ville have ført til skadelige niveauer af frit ilt. Altså bidrager faldet i DPG til en lavere venøs $PO_2$, hvilket bidrager til en lavere produktion af frie iltradikaler.


**Paper IV: Revsbech et al., 2014.** Den fjerde og sidste artikel inkluderet i denne afhandling var vores første bekendtskab med H$_2$S biologi. I denne artikel rapporterer vi, efter mit kendskab, de første målinger af plasma og røde blodcellers niveauer af frit H$_2$S og metabolitter i et hibernerende versus vågent stadie af et dyr, som sover vintersøvn. Vi undersøgte niveauer og metabolitter af begge de fysiologisk allosterdrevende gasser NO og H$_2$S, som potentielt kan inhibere mitokondriel cytocrom c oxidase og bidrage til metabolisk depression under vintersøvn. H$_2$S kan opdeles groft i tre store pools. I: Acid-labile sulfur, der består af mestendels Fe-S-samlinger og persulfider, og som frigøres under forsurede forhold. II: bundet sulfur, der hovedsagelig består af thiosulfat og polysulfider, og som frigøres til fri H$_2$S i et reducerende miljø. Og III: Fri H$_2$S, ved fysiologisk pH primært på formen HS$. Til vores overraskelse fandt vi ingen øgning i frit H$_2$S under hibernering, men i stedet et skift i H$_2$S pools. Under vintersøvnen så vi et fald i den bundne sulfur pool, hvilket indikerede, at denne pool i det lave iltmiljø bliver brugt til produktion af frit H$_2$S. Den tilgængelige cystein, som er substrat for enzymatisk H$_2$S produktion, kan så allokeres til produktionen af glutathion, en vigtig antioxidant som vi også fandt høje niveauer af i røde blodceller. Til gengæld fandt vi ingen signifikante ændringer i nitrit eller i SNO-modifikation af et nøgle-enzym i glycolysen, som blev brugt som markør for NO biotilgængelighed. Vi foreslår derfor, at H$_2$S er involveret i vintersøvn i brune bjørne, som også indikeret ved dets evne til at forårsage et stadie lig vintersøvn i mus.

I konklusion viser resultaterne fra mine ph.d.- studier, at fænotypisk plasticitet og særligt variation i røde blodcellers DPG niveauer i et iltransport perspektiv kan spille en væsentlig rolle i yderligere at tilpasse dyr til deres givne miljø. Til gengæld kan vi også konkludere, at ikke alle dyr som sover vintersøvn tilsyneladende har nytte af en direkte allosterisk effekt af faldet i DPG i de røde blodceller. I stedet må det store fald i kropstemperatur i mindre dyr være tilstrækkeligt til alene at øge Hbs iltaffinitet under vintersøvnen. Desuden indikerer mine studier at H$_2$S spiller en rolle under vintersøvn, muligvis som et led i den metaboliske nedjustering. Slutteligt støtter resultater fra denne afhandling den voksende teori, at ikke nødvendigvis kun få aminosyrer er afgørende for et proteins funktion, men at hele den genetiske baggrund og indbyrdes påvirkninger kan træde i stedet.

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CHAPTER I: INTRODUCTORY REVIEW
ABBREVIATIONS USED IN THIS INTRODUCTION:

Hb: hemoglobin; Mb: myoglobin; NO: nitric oxide; H₂S: hydrogen sulfide; CcOx: Cytochrome c oxidase, complex IV of the electron transport chain; NOS: nitric oxide synthase; CSE: cystathionine γ-lyase; CBS: cystathionine β-synthase; MST: 3-mercaptoppyruvate sulfurtransferase; PO₂: partial pressure of oxygen; P₅₀: oxygen tension at half-saturation; n₅₀: Hill’s cooperativity coefficient at half-saturation; Kₜ and Kᵣ: O₂ association equilibrium constants of Hb in the deoxygenated (tense T) state and oxygenated (relaxed R) states, respectively; DPG: 2,3-diphosphoglycerate; BMR: basic metabolic rate; Tₖ: body temperature. T: temperature. GSH: glutathione; GSSG: glutathione disulfide; NADPH: nicotineamide adenine dinucleotide-phosphate; NADH: nicotineamide adenine dinucleotide; FADH₂: flavin adenine dinucleotide; ROS: reactive oxygen species; GTP: guanosine triphosphate.
OXYGEN MEANS ENERGY – FOR SOME, A LITTLE CAN GO A LONG WAY

Mammals are as endotherms inherently slaves of a high energy demand, and thus a high oxygen (O₂) consumption most often requiring a high O₂ environment. Some mammalian species are, however, to some extent able to work around these constraints. Some mammals can cope well with low O₂ conditions, and some utilize periods of low O₂ consumption. Whether accomplished by more permanent genetic adaptation or by temporary plastic acclimatization responses, the means to go beyond the usual mammalian O₂-linked constraints are intriguing to unravel. During my thesis I have dealt with molecular scale physiological responses to the environmental setting of living at high altitude as well as the physiological mechanisms enabling an accurate control of O₂ inside a hibernating animal. The inherent appeal in this subject lay for me first in mere fascination of the physiology. Secondly my captivation has come to lie in understanding these responses and wishing to play part in the basic research that is paramount to potential human application. Findings could well be relevant for conditions where O₂ lack and reintroduction are central, as in ischemia-reperfusion injury of the heart and brain.

Most multicellular life utilizes O₂ as electron acceptor in the mitochondrial electron transport chain enabling ATP production. Because O₂ is of paramount importance for the production of energy, conditions limiting the O₂ supply to an animal is a challenging state. The animal in question must respond appropriately, or energy deficiency will lead to loss of membrane potentials over cell and mitochondrial membranes, ultimately resulting in cell death.

ORGANISMAL RESPONSES: ACCLIMATION, ACCLIMATIZATION AND ADAPTATION

A living being can cope with changes in its environment by responding. Responses over timespans of hours and longer are usually classified as one of three: Responding to a single changed variable such as temperature in an artificial or laboratory setting is termed acclimation. Responding to a changed natural environment involving multiple variables such as allocation to high altitude is termed acclimatization. Finally species living at e.g. high altitude often over multiple generations evolve changes in their genome that adapts the population to this environment, known as adaptation (Gatten et al., 1988; Giordano, 2013). Responses can happen on different physiological levels: Firstly, by optimizing the four linked mechanisms of O₂ transport: ventilation, pulmonary diffusion, circulation and tissue diffusion (Lenfant and Sullivan, 1971). Secondly, by reducing consumption; aerobic energy metabolism or shut-down of parts of the organism in order to conserve available O₂ for vital organs such as the brain. Metabolism and thus O₂ consumption is tightly controlled in a hibernator, but the details of this control are yet unclear. Thirdly, some species manage by upregulating glycolysis, which in most vertebrates (except from the crucian carp and freshwater turtles) takes place primarily during short term need, yielding potentially troublesome lactic acid. One of the highly repeated features observed across extensively divergent species adapted to hypoxia, is in
the first category: increased O$_2$ affinity of blood hemoglobin (Hb) (Bunn, 1980). High Hb-O$_2$ affinity may enhance pulmonary O$_2$ uptake in hypoxia whilst defending arterial O$_2$ saturation, thus maintaining an O$_2$ pressure gradient that drives the diffusion steps of O$_2$ transport from lung epithelia to mitochondria (Mairbäurl and Weber, 2012). Consequently, faced with hypoxia, increases in Hb-O$_2$ affinity can play an important part of coping responses (Mairbäurl, 1994; Turek et al., 1973; Turek et al., 1978a). Other adjustments are often hematocrit and capillary density alterations as well as lung volume increases (Arieli and Ar, 1979; Avivi et al., 2005; Monge and León-Velarde, 1991; Turek et al., 1978b). Employing an increase of Hb-O$_2$ affinity may be the case in both high altitude and hibernating mammals, although not necessarily achieved in the same manner, or for the same effects, as we shall see in this introduction.

In this introduction, I will discuss physiologic adaptations in O$_2$ transport and consumption found in animals living and coping with hypoxia. I have mainly worked with two general varieties of low O$_2$; high altitude environmental hypoxia along with the low fluctuating O$_2$ delivery and consumption of hibernators. The first half of the introduction deals with conditions of low O$_2$ and O$_2$ delivery, introducing the blood O$_2$ carrier Hb, and discussing the molecular adaptations in and around Hb in achieving sufficient O$_2$ delivery in hypoxic conditions. The second half focuses on control of O$_2$ consumption as ideally studied in a hibernating animal downregulating metabolism and thus O$_2$ utilization, compared with the same animal in its summer active state. The endogenous gaseous signaling molecules nitric oxide and hydrogen sulfide may be part of metabolic control, as they are both potential inhibitors of complex IV of the mitochondrial electron transport chain, along with other effects. The introduction is finalized by a conclusion and perspectives section, as well as future experiments where I will discuss future avenues of further understanding the mechanisms controlling O$_2$ delivery and consumption.

**THE ENVIRONMENT SETS THE BAR, OR THE BODY DOES - EITHER WAY, YOU HAVE TO COPE**

In high altitude life, the by far most important stressor is the direct effect of height: hypobaric hypoxia. The reduction in ambient $P$O$_2$ exposes all animals living at height to a degree of chronic environmental hypoxia. No short term solution is feasible. The native high altitude mammal generally follows one major strategy; increase in O$_2$ supply and transport capabilities, including blood O$_2$ affinity (Bunn, 1980).

Examples of utilizing short term adaptations are the hibernating animals switching from euthermic state to 5-7 months of drastically reduced metabolism every year. Hibernation is generally induced by periods of food limitations rather than alone by the cold environmental temperatures per se (Milsom and Jackson, 2011; Ortmann and Heldmaier, 2000). During bear hibernation energy is supplied by burning of fat (Robbins et al., 2012). Decrease in body temperature and resultant $Q_{10}$-effects on metabolic rate ($Q_{10}$ being the rate coefficient for a 10°C change in temperature) play a large but not sufficient role in sustaining metabolic
depression. During entrance and arousal, metabolism is the regulated variable, falling before body temperature in entrance as seen in the dormouse (Heldmaier et al., 2004) and in some remaining low for weeks at normal body temperature after arousal, as seen in black bears (Tøien et al., 2011). The extensive metabolic reduction entails a drastic reduction of O\textsubscript{2} consumption. During hibernation, basal metabolic rate (BMR) will drop substantially; metabolism is down-regulated to between 0.02 to 0.06 ml O\textsubscript{2} g\textsuperscript{-1} h\textsuperscript{-1} for all hibernators, regardless of body size and euthermic BMR (Heldmaier et al., 2004; Milsom and Jackson, 2011; Ortmann and Heldmaier, 2000; Tøien et al., 2015). For 33 species of small hibernating mammals the metabolic reduction is to around 1-10\% of BMR, with a mean of 4\% (Geiser and Ruf, 1995). In bears, the reduction is to 25\% of summer BMR (Tøien et al., 2011).

Of organismal O\textsubscript{2} consumption ~ 90\% is utilized directly as electron acceptor in the mitochondrial electron transport chain during ATP production. Both delivery to and consumption at the mitochondria are areas of potential downregulation in order to achieve the reduced metabolic demand needed to survive all winter. In addition hibernators are likely to experience mismatches in supply to consumption of O\textsubscript{2} especially at arousal from hibernation when body temperatures and metabolism return to euthermic states. Elevated O\textsubscript{2} affinities during hibernation could work to protect blood O\textsubscript{2} saturation during arousals and apneas of 30-60 min as seen in some smaller hibernators (Heldmaier et al., 2004) where PO\textsubscript{2}’s might become low.
All known hibernators except the bears and the Madagascar lemurs do not hibernate continuously during their hibernation period, but rather express torpor bouts of stable and low body temperature ($T_b$) and metabolism of up to 20 days, interrupted by 1-2 day arousals back to euthermic temperature, then start over, as in the alpine marmot, figure 1 (Heldmaier et al., 2004). This behavior is exceedingly expensive energetically, thus requiring an estimated 72% of all energy reserves expended during the hibernation season of a marmot (Heldmaier et al., 1993). Despite including costly arousals, energy expenditure during hibernation is reduced to ~15% of normothermic expenditure (Wang, 1979). It has been speculated that obtaining normal sleep as well as enabling protein production may be the background necessitating these costly returns to normothermia (Srere et al., 1992; Daan et al., 1991). Regardless, hibernators are likely to have evolved a suite of adaptations to handle inevitable mismatches in O$_2$ delivery to consumption resulting from this behavior.

In comparison the larger bears do not arouse between bouts of deep hibernation, but instead show multiday cyclic oscillations in body temperature (Tøien et al., 2011). The rises in $T_b$ are induced by shivering (Tøien et al., 2015), and thus metabolic rate also increases albeit very transiently during warming phases (figure 1). In black bears the cycles in $T_b$ seem to come at no metabolic cost and with an unknown effect besides defending a lower critical body temperature ~ 30°C (Tøien et al., 2015). Around 30°C also seems to be the critical temperature for the African fat-tailed dwarf lemur that only exhibits periodic arousals if not passively warmed (by the sun) to above 30°C on a daily basis (Dausmann et al., 2004; Dausmann et al., 2005). Thus also bears are likely to be adapted to handle sudden variations in O$_2$ consumption during hibernation, and especially in preparation for the final spring arousal.

**Figure 1. Continuous versus interrupted hibernation.**
Parts A shows traces from an Alpine marmot. Part B,C shows traces from a black bear in hibernation. MR is metabolic rate. $T_a$ ambient temperature. Part A from Heldmaier et al., 2004. Parts B,C modified from Tøien et al., 2011
HEMOGLOBIN - THE OXYGEN TRANSPORTER

Hemoglobin Hb is the blood O\textsubscript{2} carrier and thus Hb and the erythrocyte environment surrounding it are the main sites of molecular adaptation of O\textsubscript{2} transport. All vertebrate Hbs are composed of four heme-carrying globins, two α or α-like subunits, and two β- or β-like subunits. Each heme group contains a porphyrin ring with an iron atom in the ferrous state (Fe\textsuperscript{2+}) able to reversibly bind a single O\textsubscript{2} molecule. In the major adult human Hb, HbA, as well as most vertebrate Hbs the α-globin is 141 amino acid residues long and β-globin is 146 residues long. The structure of each globin subunit is characterized by its ‘globin-fold’ consisting of seven to eight α-helices (named A to H) linked by non-helical segments, with amino and carboxyl termini extensions (named NA and HC, respectively). During the oxygenation-deoxygenation cycle the Hb undergoes a change in quaternary structure as the two αβ dimers rotate from another by 15°, switching from the low affinity deoxy T (tense) state stabilized by salt bridges and hydrogen bonds, to the high affinity oxygenated R (relaxed) state, where some of these bonds are broken (Perutz, 1972; Perutz, 1989). The change in quaternary structure with oxygenation forms the background for Hb-O\textsubscript{2} transport to be modified by allosteric binding of cofactors stabilizing the T-state, as well as is the structural basis for cooperative binding of O\textsubscript{2}.

COOPERATIVITY OF OXYGEN BINDING TO HEMOGLOBIN

The tetrameric Hb exhibits cooperative binding of O\textsubscript{2}, giving rise to an S-shaped O\textsubscript{2} binding curve (figure 2B). How exactly the cooperative binding has roots in protein structure has been the gnawing bone of studies for decades. Two models have been promoted in particular: the first work by Pauling and later Koshlad to elucidate Hb cooperativity established the sequential model, arguing for the idea that each O\textsubscript{2} binding induces conformational change in the protein by direct heme-heme interaction (Koshland et al., 1966; Pauling, 1935). Later the allosteric model or the MWC was put forward (Monod et al., 1965), proposing that cooperativity arises from differences in quaternary structure of the low affinity T state and the high affinity R state. Thus rising O\textsubscript{2} tensions shifts the equilibrium from most in low affinity T-state to a higher population of R-state quaternary structure, but each heme O\textsubscript{2} binding is independent of the total number of O\textsubscript{2} bound in the tetramer. Perutz then elegantly demonstrated how much fitted together by describing how the T structure is stabilized by salt bridges and how O\textsubscript{2} binding to heme displaces iron causing helix movement, salt bridge breaks and release of protons, collectively causing destabilization of the tetramer at the sliding interface of αβ dimers, and shifting the quaternary equilibrium toward the R-state (Eaton et al., 1999; Perutz, 1970). The Perutz/allosteric model is the essential base of our understanding of Hb-O\textsubscript{2} binding today (Eaton et al., 1999). The often described heme-heme interactions essential to Hb cooperativity seems a relic of the pure sequential model, and is an imprecise way to describe the background for cooperativity. The details are not yet fully agreed upon, but Hb does certainly bind O\textsubscript{2} in a cooperative fashion that can be assessed by measuring the Hill or cooperativity coefficient, \(n\), the slope of a Hill-plot (figure 2C). Besides mere O\textsubscript{2}
affinity of a given Hb, the degree of cooperative binding logically would be another likely factor under evolutionary pressure in adaptations to low O₂ conditions. Cooperativity coefficient n was indeed found to significantly differ between hibernating and summer active bear hemolysates, by our modeling playing an important role in achieving a suitable venous PO₂ despite the high winter hematocrit as will be discussed in more detail later (Paper I: Revsbech et al., 2013a).

**OXYGEN AFFINITY ADJUSTMENTS**

Adjustments in Hb-O₂ affinity may be mediated through changes in the intrinsic interaction between heme groups in a dimer, affecting the inherent O₂ affinity. Affinity can also be affected by amino acid substitutions changing the sensitivity towards effectors that modify Hb-O₂ affinity, or simply though changes in the concentration of effectors inside the red blood cell (RBC). Allosteric effectors bind specific sites in the Hb, increase T-state stabilization and thus increase the O₂ tension required for half-saturation of the Hb, the $P_{50}$ as well as may affect cooperativity, n (figure 2B), (Nikinmaa, 2001; Weber and Fago, 2004). The major allosteric effectors for regulating Hb-O₂ affinity in mammals are protons and CO₂, both giving rise to the Bohr effect, and the anions 2,3-diphosphoglycerate (DPG) and chloride. Cl` ions in Human Hb are thought to bind at two sites: between residues $\alpha1$Val and $\alpha131$Ser as well as $\beta82$Lys and $\beta1$Val (O’Donnell et al., 1979; Riggs, 1988).
Box 2: Heat of Oxygenation and Hill Plots

As discussed in the text, oxygen binding to hemoglobin is affected by allosteric effectors H⁺, Cl⁻, organic phosphates P⁻ and then temperature T. The binding of O₂ is an exothermic reaction, whereas the dissociations of bound allosteric effectors are endothermic reactions, consuming heat (figure 2A). The resultant heat of oxygenation is thus diminished by the number of allosteric effectors bound (figure 2A). The apparent heat of oxygenation ΔH’, i.e. the enthalpy here depicted is calculated by use of the Van’t Hoff equation:

\[
\Delta H = 2.303R(\Delta \log P_{50}) / ((1/T_1)-(1/T_2))
\]

Where R is the gas constant (1.987 cal K⁻¹mol⁻¹), T₁ and T₂ are the absolute temperatures (K), and ΔlogP₅₀ is the corresponding difference in logP₅₀. ΔH’ values are then corrected for heat of O₂ in solution (-3.0 kcal mol⁻¹) (Antonini and Brunori, 1971). The slight endothermic component of the conformational shift T→R is constant and not considered here. Thus ΔH’ is depicting how sensitive Hb-O₂ binding will be to variations in temperature. Accordingly high temperature is amongst the factors causing a right shifting of the Hb-O₂ binding curve (figure 2B), causing the blood to require a higher PO₂ to get 50% saturated (P₅₀).

Hill plots are used for interpreting O₂-binding of a given Hb sample. figure 2C is a schematic of a Hill plot, log (oxyHb/deoxy Hb) or logY/(1-Y) vs. log PO₂, where Y is fractional saturation. An estimate of n₅₀, the cooperativity coefficient for Hb O₂ binding, is given by the slope of the hill plot at 50% saturation of the Hb, that is, when log (oxy/deoxy) is log1= 0 on the Y-axis. This value moreover corresponds to logP₅₀ on the x-axis. In Fig. 2D the upper and lower asymptotes depict effector-induced decrease in O₂ affinity of the T-state (equilibrium constant for T state, Kₜ) and less so that of the R-state (Kᵣ) resulting in increased cooperativity, n₅₀, the slope at line Y=0.

Figure 2. Details of oxygen binding
A: Heat forms part of the T-R equilibrium.
B: Allosteric effector right shifts the Hb-O₂ binding curve.
C: Schematic Hill plot depicting the cooperativity coefficient n₅₀, this slope is typically around 2.8 for human Hb.
D: Schematic Hill plots depicting the change in specifically Kᵣ with effector binding.

Figure redrawn and modified from Weber and Fago 2004.
**Affinity Adjustments: DPG**

The organic phosphate DPG is produced as a side product of red cell glycolysis. DPG has been shown to have a direct right-shifting effect on the Hb-O\(_2\) dissociation curve in humans (figure 2B), (Benesch and Benesch, 1967; Benesch and Benesch, 1969). The effect is believed mediated through asymmetric binding directly or through water molecules to seven positively charged sites in the central cavity between the two \(\beta\)-chains of the deoxy T-state Hb molecule (figure 3). Thus DPG binds with \(\beta2\)His, \(\beta82\)Lys (via different interactions) and \(\beta143\)His (weakly) of both \(\beta\) chains, and \(\beta1\)Val of one chain though water molecules, the other \(\beta1\)Val binds back to its own chain \(\beta78\)Leu (Richard et al., 1993). The major difference from a previous model being that DPG does not directly bind both \(\beta1\)Val as previously assumed (Arnone, 1972). The binding of DPG is inhibited in the R-state where the central cavity is narrowed (Lukin and Ho, 2004; Perutz, 1970).

As a nondiffusible polyanion, DPG furthermore indirectly affects intracellular proton concentration, affecting the Donnan equilibrium across the erythrocyte membrane. High intracellular DPG leads to an influx of \(H^+\), which via the Bohr effect (below) may affect Hb-O\(_2\) binding (Duhm, 1971). We suggest DPG affecting Hb O\(_2\) affinity through the Donnan equilibrium to be likely in ground squirrel Hbs that are largely insensitive to DPG, as will be discussed further (Paper III: Revsbech et al., 2013b).

**Affinity Adjustments: Temperature and Bohr Effect**

Temperature and pH/pCO\(_2\) are other central factors affecting O\(_2\) binding to Hb. O\(_2\) binding is an exothermic reaction, and dissociation of ligands is endothermic (figure 2A). Therefore a rise in temperature causes a fall in Hb-O\(_2\) affinity (see box). The alkaline Bohr effect takes place between pH ~ 6-9 covering the physiological range for a mammal, and consists of both direct CO\(_2\) binding as well as H\(^+\) binding to Hb. The Bohr effect below pH 6.3 is termed the acid Bohr effect (Imai, 1982), but is unlikely to be applicable in a mammalian physiological setting and therefore not considered further here. CO\(_2\) forms carbamates with the uncharged NA termini of both \(\alpha\)- and \(\beta\) chains, giving rise to the direct CO\(_2\) Bohr effect (Imai, 1982). The fixed acid Bohr effect arises when H\(^+\) binding reduces Hb-O\(_2\) affinity at low pH as in working muscles, and
H⁺ dissociation at respiratory surfaces increases affinity. The size of the Bohr effect for a given Hb can be evaluated by estimating the Bohr coefficient given the change in logP₅₀ upon a change in pH by \( \varphi = \frac{\delta \log P_{50}}{\delta pH} \).

In the blood a local drop in pH would promote unloading of O₂ at metabolically active tissues and loading of O₂ is facilitated when CO₂ and protons dissociate from Hb at the lung alveoli, the latter mostly being relevant in low O₂ environments. Early studies have favored the view that the Bohr effect generally for all vertebrate Hbs originates from a few key cationic groups that raise their pKa in the T→R transition as they form salt bridges with chloride, phosphates or carboxylates in the T state and are free in the R state (Perutz, 1983).

Especially histidines and in particular one, 146His of both β chains, has been and is considered responsible for approx. 40-60% of the fixed acid Bohr effect, (Berenbrink, 2006; Busch et al., 1991; Perutz, 1983; Shih et al., 1993) (figure 4A). According to this theory, at low pH the Bohr protons will primarily protonate α-amino termini and the C-terminal His (146His) of the β-chain thus stabilizing the deoxy T-state by formation of a salt bridge with Asp94 of the same β chain (Perutz, 1970; Perutz, 1983; Van Beek and De Bruin, 1980). The vicinity of the negatively-charged Asp raises the pKₐ of the β146His imidazole ring (Im) \( pK_a = -\log K_a = -\log \left( \frac{[m^-][H^+]}{[Hm^+]} \right) \) to a value substantially higher than in the oxy form, making H⁺ binding more likely in deoxy T state Hb.

Furthermore, the α-chain C-termini α141Arg (figure 4B) has been attributed to stabilize the Hb deoxy C-terminus by binding a Cl⁻ ion together with α1Val (Perutz, 1970). Oxygenation-induced conformational change breaks the ionic bond and induces deprotonation of the C-termini back to the NH₂-form. This residue thus has been considered to contribute to the Bohr effect in the presence of Cl⁻ (Lukin and Ho, 2004). Newer reports attributing the Bohr effect to almost exclusively histidine residues also estimates the β146His residue...
to be responsible of approximately 60% of the human HbA Bohr effect in the presence of 0.1M Cl⁻ (Berenbrink, 2006; Lukin and Ho, 2004).

The alkaline Bohr effect has historically largely been considered ascribable to a difference in pKₐ of specific amino acid residues, from high pKₐ (and high affinity for H⁺) in the unliganded deoxy T form to low pKₐ (and low affinity for H⁺) in the liganded R-state (Perutz, 1970; Perutz, 1983; Shih et al., 1993). Addition of anions will also affect the contribution of each residue involved, and the Bohr effect is sensitive to position and interactions of all hydrogen and anion binding sites in the molecule (Busch and Ho, 1990; Lukin and Ho, 2004; Sun et al., 1997). Collectively the presence or absence of substitutions at or near a few number of sites at first hand seems to determine Hb-O₂ affinity, aligning with the neutral theory of molecular evolution (Kimura, 1968; Perutz, 1983). However, as we shall also see in this thesis and Paper III, some species retain “normal” Bohr coefficients although lacking what is still considered key residues (Paper III: Revsbech et al., 2013b).

For all allosteric effects in Hb, it has for several species been shown that a palette of different substitutions at a far distance from the ligand binding sites can also modify function of Hb, whereby small contributions from many mutations may produce cumulative effects on function, again depending on the presence or absence of other mutations, a phenomenon linked to genetic epistasis (Natarajan et al., 2013; Natarajan et al., 2015; Tufts et al., 2015).

All in all, low in vivo levels or decreased Hb sensitivity of one or more of the mentioned effectors will cause a higher O₂ affinity of Hb, but will also limit the regulatory potential of this affinity. Readily reversed regulations of the erythrocyte concentrations of effectors, e.g. the transient rise in human red cell DPG upon translocation to high altitude favoring O₂ unloading (Lenfant et al., 1968), is considered acclimatization restrained by the phenotypic plasticity of the individual. On the other hand, regulations of intrinsic affinity or sensitivity towards cofactors through structural changes in the Hb, is considered genotypic adaptation. Either type of regulation may adapt an animal to its environment. Animals native to hypoxic environments like high altitude are often characterized by a strategy of structural changes in the Hb protein chains affecting O₂ affinity, thus making them genetically adapted to their environment (Bunn, 1980; Monge and León-Velarde, 1991; Weber, 2007).
HEMOGLOBIN – AMINO ACID SUBSTITUTIONS AND COFACTOR VARIATION AT PLAY

Adjustments in blood \( \text{O}_2 \) delivery with altitude is a product of organismal adaptations (e.g. higher capillary density, higher lung diffusive conductance) and cellular to molecular adaptation. Changes can be at the level of concentration differences of effectors affecting Hb-\( \text{O}_2 \) binding, as well as changes in the concentration, function and structure of the Hb molecule itself, affecting intrinsic \( \text{O}_2 \) affinity or efficacy of effectors. In low altitude human subjects translocated into moderate altitude below 2000m a rise in erythrocyte DPG is part of the response, largely because stimulated erythropoiesis with height yields a greater fraction of young erythrocytes in circulation. Young RBCs have a higher glycolytic rate and therefore a higher formation of DPG (Lenfant et al., 1968; Mairbäurl, 1994). The reduced Hb-\( \text{O}_2 \) affinity improves overall \( \text{O}_2 \) delivery despite a slight loss in arterial saturation (Mairbäurl, 1994; Turek et al., 1973; Weber, 2007).

At moderate altitude ventilatory compensated hypoxia leads to alkalosis and the decreased pH will via the Bohr effect push Hb \( \text{P}_{50} \) within normal sea level values, compensating for the effects of DPG. It is not before extreme altitudes that a right shifted \( \text{O}_2 \) binding curve becomes counterproductive and a higher Hb \( \text{O}_2 \) affinity favors \( \text{O}_2 \) delivery. The limit is theoretically above 5000 meters in man (Samaja et al., 1986), but will vary between species according to specific physiological limitations. High Hb-\( \text{O}_2 \) affinity compared to conspecifics is often observed in species adapted to high altitude, for mammals examples are the Andean *Chinchilla brevicaudata* resident in 3000-5000m (Ostojic et al., 2002) and in North America the deer mouse *Peromyscus maniculatus* resident at sealevel-4300m at least (Snyder, 1982; Storz et al., 2007). I will in the following consider first genetic adaptation to altitude and in the next paragraph give examples of phenotypic plasticity responses.

ENVIRONMENTALLY LOW OXYGEN SELECTS FOR HIGH HEMOGLOBIN-\( \text{O}_2 \) AFFINITY

In high altitude native animals, genotypic specialization in Hb-\( \text{O}_2 \) affinity is predominant. A classic example is the bar-headed goose that flies over the Himalayas at 9000 m and can tolerate extreme hypoxia. This tolerance is thought to be due mainly to one Hb substitution compared to the lowland graylag goose; \( \alpha_1 \text{119Pro} \rightarrow \text{Ala} \) that deletes a contact to \( \beta_1 \text{55} \) and thereby destabilizes the \( \alpha_1 \beta_1 \) interface T-structure of HbA (birds coexpress two Hbs, a major HbA and a minor HbD), giving rise to high \( \text{O}_2 \) affinity (Jessen et al., 1991; Liang et al., 2001; Perutz, 1983). In addition, two substitutions on the \( \alpha \) chain of HbD may affect binding of the avian organic phosphate, inositol pentaphosphate (McCracken et al., 2010), which could be expected to lead to an even higher \( \text{O}_2 \) affinity of the high affinity avian Hb D isoform. In a different part of the world, the Andean goose also exhibiting high Hb-\( \text{O}_2 \) affinity shows a substitution deleting the exact same contacts (\( \beta_5 \text{55Leu} \rightarrow \text{Ser} \)) (Jessen et al., 1991). However, it was later found that this last substitution is not unique to altitude adapted species (McCracken et al., 2010).
High altitude Andean camelids llama and guanacho have Hbs with lower DPG sensitivities compared to most mammals (Petschow et al., 1977; Scott et al., 1977). In the llama this is considered due to an β2His→Asn substitution that eradicates two out of the usual DPG contacts in the Hb tetramer yielding a threefold lower binding constant of DPG to llama Hb compared to camel Hb (Bauer et al., 1980). An array of mammals expresses multiple isoHbs. Alpaca are expressing 55% fetal Hb with a higher O₂ affinity (Reynafarje et al., 1975). Yaks have two major fetal Hb components and two to four major adult Hb components, that via different O₂ affinities extend the altitudinal range of the animals (Weber et al., 1988).

Deer mice are amongst the more intricate, expressing a complex Hb polymorphism with two α-globin genes and two β-globin genes (Storz et al., 2007; Storz et al., 2009). The Hb composition of highland mice yields slightly higher intrinsic O₂ affinities, that, in combination with lowered DPG sensitivity and lowered Cl⁻ sensitivities, results in an elevated blood O₂ affinity of high compared with low altitude mice (Storz et al., 2009; Storz et al., 2010). Deer mice do at the same time show curious elevations of erythrocyte DPG concentrations in high altitude populations compared with low altitude populations (Snyder, 1982), as was also later found by our group to be part of the phenotypic plasticity of these animals (Paper II: Tufts et al., 2013) and discussed in the following paragraph.

**TEMPORARY LOW OXYGEN LINKED WITH PLASTIC ADAPTATIONS IN Hb-O₂ AFFINITY**

High altitude adapted deer mice populations possess genetically adapted Hbs with higher O₂-affinities. Concurrently, high altitude mice have elevated erythrocyte DPG. High DPG would reduce Hb-O₂ affinity and thus seems counterintuitive in an adapted population, albeit the significance may be small due to the low DPG sensitivity of highland mice Hb. Interestingly, we found DPG levels of high altitude mice to be lowered upon 6 week translocation to low altitude (Paper II: Tufts et al., 2013). This implies that diverse DPG levels inside the RBCs are part of the phenotypic plasticity of deer mice, even further broadening their abilities to handle very different environments. One can speculate that highland mice may have a constant higher red cell turnover and that the elevated levels of DPG of highland mice red cells may be an unavoidable side-effect of the increased red cell glycolysis of young erythrocytes, as first proposed by Snyder (Snyder, 1982). High DPG levels are then further compensated by the evolution of a low sensitivity toward this allosteric effector and thus high altitude adapted deer mice retain high Hb-O₂ affinity. When translocated to low altitude, the mice acclimatize and DPG levels are no longer elevated (Paper II: Tufts et al., 2013). Or perhaps the elevated DPG has roots in population admixture (Storz et al., 2010), retaining it as an acclimatization response. Variations in organic phosphates to accommodate needs of increased O₂ affinity that are not permanent are also known in fish exposed to hypoxia (Lykkeboe and Weber, 1978; Weber and Lykkeboe, 1978). As well as in hibernating mammals (Burlington R.F. and Whitten, 1971; Harkness et al., 1974; Kramm et al., 1975; Maginniss and Milsom, 1994; Tempel and Musacchia, 1975) as we also found in the large hibernator, the brown bear (Paper I: Revsbech et al., 2013a)
Blood hematocrit and Hb concentration will also affect O$_2$ capacity, but have not consistently been measured to rise or fall with hibernation, perhaps due to sampling at very different periods of the hibernating state, as observed in woodchucks (Harkness et al., 1974). Elevated hematocrits have been observed in translocated humans (Monge and León-Velarde, 1991), in high altitude in the Russian wood mouse and in the deer mouse (Hock, 1964; Kalabuchov, 1937; Paper II: Tufts et al., 2013), decreasing again with low altitude adaptation (Reynafarje et al., 1975; Paper II: Tufts et al., 2013), thus classifying these changes as part of phenotypic plasticity. It is intriguing to see the exact same plastic acclimations as in humans persist as part of the phenotype of a high altitude adapted deer mouse population.

**Hibernators Utilize Temporary Phenotypic Plasticity in Oxygen Transport**

Hibernators downregulate metabolism to extremely reduced levels (see box 1), implying also a much reduced rate of breathing and heart beats, in bears breathing is reduced to 1-2 breaths/min and heart rate to ~14 beats per min from ~55 beats per min in summer resting state (Tøien et al., 2011). This implies, that even though the external environment has plenty of available O$_2$, the internal environment of the bear does not, and also must not (Paper I: Revsbech et al., 2013a). Hibernation is a temporary state, in many species further interrupted by arousals (see box 1), and thus adaptations in O$_2$ transport need to be reversible to optimize delivery in both the hibernating and the normothermic states. Species like the hibernating hedgehog, the 13-lined and the golden mantled ground squirrel, as well as our investigated brown bear have been shown to increase their blood O$_2$ affinities during hibernation compared to the euthermic state (Bartels et al., 1969; Clausen and Ersland, 1968; Maginniss and Milsom, 1994; Musacchia and Volkert, 1971; Paper I: Revsbech et al., 2013a).

Elevations in Hb-O$_2$ affinities are highly attributable to direct effects of fall in body temperature on the Hb-O$_2$ equilibrium curve, but a temperature independent component has also been proposed, namely DPG. In 13-lined and golden-mantled ground squirrels, woodchucks and golden hamsters, a clear reduction in DPG in the range 39-45% has been detected during hibernation (Burlington R.F. and Whitten, 1971; Harkness et al., 1974; Kramm et al., 1975; Maginniss and Milsom, 1994; Tempel and Musacchia, 1975) and some studies including work from our group on the brown bear (Paper I: Revsbech et al., 2013a) have proposed a direct coupling between decreased DPG and rise in Hb-O$_2$ affinity (Kramm et al., 1975; Paper I: Revsbech et al., 2013a). In contrast are our studies in smaller hibernators: In six marmotine ground squirrels investigated in Paper III, and perhaps general to rodent Hbs (Clementi et al., 2003; Runck et al., 2010; Storz et al., 2012), sensitivity of Hb to the anion DPG is generally low (Paper III: Revsbech et al., 2013b). A low DPG sensitivity makes the hibernation-induced observed DPG decreases across a large range of smaller hibernating rodents unlikely to have any substantial effect on Hb-O$_2$ affinity. Thus our work with small rodent hibernators does not support the hypothesis of a direct relationship between decreases in allosteric effector DPG and increases in Hb-O$_2$ affinity during hibernation in rodents (Burlington R.F. and Whitten, 1971; Maginniss and Milsom, 1994; Tempel and Musacchia, 1975).
PERMPTION OF THE DONNAN DISTRIBUTION OF IONS

Instead of a direct effect of decreases in red cell cofactor DPG on Hb-O₂ affinity in the marmotine ground squirrels investigated in Paper III, we suggest the possibility of an indirect relationship; namely that decreases in the anion DPG via perturbation of the Donnan equilibrium of ions across the erythrocyte membrane may still effect a decrease in \( P_{50} \) during hibernation. Fall in intraerythrocytic nondiffusable polyanion DPG will cause an equal decrease in intracellular H⁺ and thus a rise in pH. In Paper III we found a normal Bohr effect of the investigated Hbs, implying that the observed fall in DPG during hibernation may...
have this indirect effect on Hb-O₂ affinity, although the fall in Tₙ is likely to be the prominent effector (Paper III: Revsbech et al., 2013b). However, for the larger and warmer hibernator, the brown bear (see box 1), DPG also decreases during hibernation and this decrease together with the limited fall in Tₙ is in this hibernator sufficient to explain the observed elevation of bear O₂ affinity of hemolysate (Paper I: Revsbech et al., 2013a). Taken together, decrease in DPG works directly in some hibernators like hedgehogs and bears towards aiding a high Hb O₂ affinity (Kramm et al., 1975; Paper I: Revsbech et al., 2013a), but may in others, like grounds squirrels, marmots and woodchucks contribute only little or none (Harkness et al., 1974; Paper III: Revsbech et al., 2013b).

**THE WHOLE PICTURE VERSUS FEW IMPORTANT BINDING SITES**

In the above mentioned study our group compared amino acid substitutions with functional data and found highly unusual substitutions involving an expectedly detrimental substitution β146 His→Gln in the major Hb β chain of the golden mantled ground squirrel. β146 His is considered responsible for ~60% of the Bohr effect for all Hbs (Berenbrink, 2006). However, functional analysis showed a normal Bohr effect of this Hb. Likewise, the observed low DPG sensitivity coincided in all six species with contained cationic residues considered implicated in DPG binding in human Hb (Paper III: Revsbech et al., 2013b), namely β-chain 1Val, 2His, 82Lys and 143His (Richard et al., 1993). Conclusively, not all species seem to conform to the human model of Hb function, or to theory dictating only a few amino acid residues to be responsible for overall function. Instead, our study on ground squirrels amongst others (Natarajan et al., 2013; Natarajan et al., 2015; Paper III: Revsbech et al., 2013b; Tufts et al., 2015) suggest that the general genetic background and multiple substitutions rather than only a few key residues has a major impact on the Hb-O₂ affinity.
FINE-TUNED REGULATORS OF O₂ DELIVERY AND CONSUMPTION: INTRODUCTION TO NO AND H₂S

Oxygen arrives to tissues by bulk transport bound to Hb inside the RBCs, as described earlier in this introduction. Part of fine-tuning both O₂ delivery and consumption, amongst a cascade of other signaling effects, are the endogenously produced gaseous messengers hydrogen sulfide, H₂S, and nitric oxide or nitrogen monoxide, NO. Both NO and H₂S are ubiquitous cellular signaling molecules naturally synthesized and degraded in peripheral tissues by endogenous enzymes. NO and H₂S are now recognized as signaling molecules invoking physiological function. Together with carbon monoxide (CO), they are collectively referred to as gasotransmitters. Both NO and H₂S are highly toxic at high concentrations, but have signaling effects in lower concentrations. Their physiological functions involved in O₂ delivery and consumption range from control of blood vessel dilation (NO and H₂S in systemic arteries) or contraction (H₂S in pulmonary vessels) to cytoprotection and inhibition of respiration. Albeit normally produced in an O₂-dependent manner, all production is not lost at low O₂ conditions, as both NO and H₂S can be reconstructed from oxidated end products during particular conditions (Lundberg et al., 2008; Olson et al., 2013).

Reaction products of both NO and H₂S may modify proteins at reactive cysteine residues and radically alter function, causing major downstream effects (Paulsen and Carroll, 2013). Redox signaling through site-specific and reversible cysteine modifications enables an oxidant signal to be turned into an appropriate biological response within and between cells. Both H₂S and NO are proving increasingly interesting to study as they appear involved in multiple adaptations and responses to hypoxia. Both alleviate ischemia/reperfusion damage mainly through respiratory inhibition of cytochrome c oxidase (CcOx) in the electron transport chain of mitochondria. NO plays a key role in anoxia and hypoxia adaptations from anoxia in red-eared slider turtles and crucian carps in the natural overwintering habitat of frozen ponds (Jacobsen et al., 2012; Jensen et al., 2014; Sandvik et al., 2012) to high altitude adaptations in Tibetan highland human residents (Erzurum et al., 2007). H₂S has the ability to induce an artificial hibernation-like state (Blackstone et al., 2005), making it a molecule of special interest to study in natural hibernators. The current paragraph serves to introduce NO and H₂S as physiological players. In the following I will focus on mechanisms and occurrence of selected NO and H₂S mediated changes in physiology that could be relevant to mainly hibernators. Cardiocascular origins, fates and effects of the two molecules are summarized in figure 6 at the end of this paragraph. Some applications may also be relevant in high altitude adaptation as I will discuss under future research.
Nitric oxide – a short introduction

NO is a well-established gaseous signaling molecule with a palette of different effects on blood flow and metabolism. NO is a free radical and stabilizes its unpaired electron by binding other compounds with unpaired electrons. It also interacts with d-orbitals of transition metals of both non-heme and heme irons (Thomas et al., 2003). In particular, NO has a high affinity for ferrous heme. One effect is through binding to the heme of soluble guanylate cyclase in the endothelium causing an induction of a vasodilatory cascade (Moncada and Higgs, 1993). Another effect is transient inhibition of respiration by binding to cytochrome c oxidase (CcOx), complex IV in the mitochondrial electron transport chain (Cleeter et al., 1994). NO plays an important role in alleviating ischemia/reperfusion injury through metabolic inhibition and additionally, through binding to a reactive cysteine on complex I by S-nitrosation, suppresses reactive oxygen species (ROS) generation in the mitochondria (Chouchani et al., 2013). All effects will be discussed below.

NO production and lifetime

A family of NO synthesizing enzymes, NOS enzymes, generates NO endogenously by catalyzing an NADPH and O$_2$-dependent oxidation of L-arginine to L-citrulline and NO by the help of a number of cofactors (Alderton et al., 2001; Li and Poulos, 2005). The isoform endothelial NOS, eNOS was first found in the vascular endothelium, but since also discovered in epithelial cells, cardiac myocytes and neurons (Dudzinski and Michel, 2007). The form eNOS can be upregulated by sheer stress of vasculature blood flow, enabling close coupling of eNOS regulation to forces of flow (Balligand et al., 2009). Neuronal NOS, nNOS, is found in neurons, epithelial cells and skeletal muscle. Inducible NOS, iNOS, can generate the highest levels of NO in response to inflammatory stimuli in a great variety of tissues. All NOS isoforms are regulated in activity and location by Ca$^{2+}$ and calmodulin in addition to posttranslational modifications, cofactor availability and substrate levels (Kone et al., 2003).

NO has a half-life of 2 ms to 2 seconds in vasculature, in effect keeping NO signaling effects local (Thomas et al., 2001). NO is consumed by reaction with oxygenated Hb or Mb, to create nitrate, NO$_3^-$, (Moncada and Higgs, 1993; M. P. Doyle and J. W. Hoekstra, 1981). In reaction with freely dissolved O$_2$, NO is transformed into nitrite, NO$_2^-$. A major avenue of O$_2$–independent production of NO away from site-specific NOS is by reduction of the metabolite nitrite, NO$_2^-$, to NO (Lundberg et al., 2008). Heme proteins such as deoxygenated hemoglobin (Cosby et al., 2003) and myoglobin (Shiva et al., 2007a) can exhibit high nitrite reductase activity. This enables what was former considered a product alone (NO$_2^-$) to regenerate the active form NO under hypoxic conditions. Nitrate can additionally be converted to nitrite by one of two ways to uphold nitrite levels. One way is via the ubiquitous enzyme xanthine oxidoreductase, that also converts nitrite to NO, and is able to convert nitrate to nitrite although the latter reaction is much slower (Jansson et al., 2008). The other avenue is by a bacterial symbiosis with oral bacteria converting dietary and recycled nitrate excreted in the salivary glands to nitrite, later taken up in the gut (Lundberg et al., 2008). Dietary
supplementation with nitrite or nitrate thus seems to alleviate ischemia/reperfusion injury in various settings (Lundberg et al., 2009).

**NO PRODUCTS AND FUNCTIONS**

NO induces and regulates a broad range of biological functions, typically by binding either to ferrous iron in heme proteins creating iron nitrosyl compounds, FeNO, or by binding to protein thiol groups, S-nitrosation, SNO, or to aromatic rings, causing nitration (Hill et al., 2010).

The signaling action of NO depends strongly on the presence of other free radicals. Once reacted with superoxide, O\(_2^–\), NO forms peroxynitrite ONOO\(^–\), a nitrating and oxidating agent that may modify protein, lipids, and DNA, but no longer interact with ferrous heme (McAndrew et al., 1997). Some target specificity is achieved by location of O\(_2^–\) production, as superoxide is not freely diffusible across cell membranes (Hill et al., 2010). Targets are cysteine and methionine (S-containing amino acids) as well as their incorporated forms in protein thiols, with the latter reaction being much slower (Hill et al., 2010). Aromatic rings (amino acids tryptophan and tyrosine) are also prone to nitration (Hill et al., 2010). Nitration of proteins can also happen through other avenues. Nitrogen dioxide, NO\(_2\), and dinitrogen trioxide, N\(_2\)O\(_3\), are byproducts of the reaction of NO with O\(_2\), and may also cause nitration or S-nitrosation alone or in combination with ROS radicals (Hill et al., 2010). In hypoxia an avenue of N\(_2\)O\(_3\) production requires acidic conditions and high concentrations of nitrite, as nitrous acid, HNO\(_2\) is in equilibrium with N\(_2\)O\(_3\) in water (see Fago and Jensen, 2015). All protein modifications via NO are likely to be part of complex NO signaling.

In hypoxic conditions NO generated from nitrite reduction by deoxy hemoglobin will in vasculature bind soluble guanylate cyclase, causing an increase in local vasodilation and blood flow in accord with hypoxia as explained in detail further below (Moncada and Higgs, 1993). Nitrite is found in tenfold higher concentrations in intracellular compartments in the hypoxic heart of crucian carp, in goldfish and in the red-eared slider turtle, possibly by transfer from extracellular location or increased NOS expression (Hansen and Jensen, 2010; Jensen et al., 2014; Sandvik et al., 2012). In hypoxic organs, NO binding to ferrous heme iron in mitochondrial CcOx is competitive with O\(_2\) binding (Cleeter et al., 1994). Thus NO-induced metabolic inhibition as well as vasodilation is most prevalent in low O\(_2\) conditions as during ischemia, where local deoxy myoglobin will also supply NO generation from nitrite (Totzeck et al., 2012). During ischemia, Complex I and III of the respiration chain become highly reduced because of electron loading from reducing equivalents, causing a high potential for superoxide and other ROS production upon reoxygenation. NO has the ability to prevent reverse electron transport by keeping complex I in an inactive state due to SNO modification (Babot and Galkin, 2013). The reactant is likely N\(_2\)O\(_3\) that will react with a specific cysteine residue on complex I, which will be inhibited by the resulting S-nitrosation, thus blocking this main site of ROS generation when reperfusion takes place (Babot and Galkin, 2013; Chouchani et al., 2013; see Fago and Jensen, 2015). In low O\(_2\) conditions the available NO will largely stem from nitrite reduction, explaining why additional nitrate or nitrite supplementation decrease ROS production (Shiva et al., 2007b). Tissue
specific increases of FeNO and SNO compounds can be used to indicate areas of NO production from nitrite during hypoxia (Sandvik et al., 2012).

**HYDROGEN SULFIDE – A SHORT INTRODUCTION**

Hydrogen sulfide (H\textsubscript{2}S) is easily recognized by the rotten egg odor and is known as a potentially lethal acquaintance if inhaled in too large doses. However H\textsubscript{2}S has within the last two decades been recognized as a signaling molecule invoking physiological function akin to other gaseous species such as NO. H\textsubscript{2}S is endogenously synthesized and is a small molecule that easily diffuses across cell membranes (Mathai et al., 2009) with potential to be involved as a signaling agent in a plural of physiological processes. Possible H\textsubscript{2}S involvement has been documented ranging from vasocontrol to neuromodulation, cardio protection, metabolic inhibition and anti-inflammatory activities (Kimura, 2009). Research in possible involvement of H\textsubscript{2}S and its metabolites in physiology have literally exploded in the past two decades and resulted in a wide range of sulfide-signaling now known of in smaller or greater detail. Despite the great interest in H\textsubscript{2}S, uncertainty still exists of what form or forms of H\textsubscript{2}S exhibits biological activity. Under physiological conditions (37°C and pH 7.4) only 18.5% of free hydrogen sulfide exists as H\textsubscript{2}S, while the remainder dissociates to HS\textsuperscript{-} and H\textsuperscript{+} with trace amounts of S\textsuperscript{2-} (Dombkowski et al., 2004; Hughes et al., 2009). H\textsubscript{2}S, HS\textsuperscript{-}, or even products of H\textsubscript{2}S combining with proteins may be the active form or forms of H\textsubscript{2}S signaling. Or perhaps many forms have each their special reactions and activity. The field still refers to sulfide signaling as H\textsubscript{2}S signaling, and thus, so will this review.

**PRODUCTION AND POOLS OF SULFIDE**

At least three endogenous enzymes produce H\textsubscript{2}S. H\textsubscript{2}S is created from cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MST), from substrate L-cysteine and homocysteine as well as 3-mercaptopyruvate, in the case of MST. 3-mercaptoppyruvate is produced from cysteine and α-ketoglutarate by cysteine aminotransferase (Shibuya et al., 2008). In humans the enzymes are mostly site-specific in their distribution with CSE found predominantly in the smooth muscles of vasculature, MST in vasculature endothelial cells, RBCs and in mitochondria, and CBS in brain, liver and kidney (Calvert et al., 2009; Kabil et al., 2014) Kimura 2014, Szabó 2007, Vitvitsky et al 2015). Only the zinc-dependent protein MST is found in mitochondria as well as the cytosol of cells, whereas CSE and CBS are heme proteins with primary cytosolic location.

Hydrogen sulfide is constantly produced and oxidized at a tissue-specific rate, enabling an efficient control of H\textsubscript{2}S tissue levels (Vitvitsky et al., 2012a). The exponential decay of H\textsubscript{2}S with time is highest under aerobic conditions as it primarily occurs via a mitochondrial sulfide oxidation pathway involving an \textsubscript{O\textsubscript{2}} consuming step (Vitvitsky et al., 2012b). A mechanism for \textsubscript{O\textsubscript{2}}-dependent methemoglobin (MetHb) catalyzed H\textsubscript{2}S turnover in RBCs has moreover recently been found (Vitvitsky et al., 2015). MetHb is the state of Hb where the heme iron is oxidized to the ferric state, Fe\textsuperscript{3+} instead of normal ferrous Fe\textsuperscript{2+} state, rendering the Hb
non-functional for O₂ transport until reduced by methemoglobin reductase. The oxidation of H₂S by metHb seems also mostly O₂ dependent, but ironbound polysulfides (e.g. Feⁿ⁻⁰-S-S-S-H) may be created under low O₂ conditions (Vitvitsky et al., 2015). This finding indicates that other massively present heme proteins around the body, with myoglobin Mb as the likely best candidate, also may play a role in H₂S equilibrium and creation of reactive metabolites, much akin to deoxy Hb and Mb regenerating NO from nitrite. Estimated free H₂S half-life is between 2 and 10 minutes in bovine liver, kidney and brain homogenates (Vitvitsky et al., 2012a). Normal steady-state levels of free H₂S are debated as measurements depend highly on techniques that have only recently become precise enough. In the order of 14-17nM as found in whole tissue liver and brain of mice (Furne et al., 2008) up to a few micro molar (µM) as found by our group in bears seems within a realistic range of endogenous H₂S (Paper IV: Revsbech et al., 2014). All H₂S products can be divided into pools: (1) free sulfide, containing mainly freely dissolved H₂S and HS⁻; (2) Bound sulfane sulfur (BSS), the products of H₂S oxidation, containing thiosulfate S₂O₃²⁻ and polysulfides (e.g. RSSSR) that can be converted to H₂S under reducing conditions; (3) acid-labile sulfur, containing mainly Fe-S clusters and persulfides (e.g. RSSH), that are considered convertible into H₂S only under rare acidic conditions (Kolluru et al., 2013).

**Sulfur Binding to Protein Thiols and Heme Groups Can Alter Function**

H₂S can be stored as bound sulfane sulfur (BSS) on protein cysteine (thiol) residues. BSS is created by a yet not fully determined range of reactive sulfur species combining preferably with a previously oxidized thiol species, e.g. cysteine disulfide (R-SSR) or sulfenic acid (R-SOH) by the process of sulfuration (also known as S-sulfhydration) to form persulfides (RSSH or HSSH) and polysulfides (e.g. RSSSR) (Francoleon et al., 2011; Mustafa et al., 2009; Park et al., 2015). It should here be noted that the number of active or available cysteine thiols in selected proteins may be background for species differences. Any addition of cysteines yield sites for posttranslational modifications like S-nitrosation and sulfuration and can potentially affect function of proteins and alter enzymatic capability as part of redox signaling. This yields a great potential for downstream signaling. Hyper-activation is the result of sulfuration in cysteine, CysSSH as explained further below (Ida et al., 2014) as well as increased catalytic activity for glyceraldehyde-3-phosphate dehydrogenase GAPDH. Oppositely, inhibition following persulfidation has been observed for glucose 6-phosphate dehydrogenase and other erythrocyte enzymes (Valentine et al., 1987). NO-induced S-nitrosation of proteins and enzymes on the other hand often inactivates function, as seen for GAPDH-induced incorporation of hemes into proteins (Chakravarti et al., 2010).

Per- and polysulfides have recently been seen to elicit highly interesting reactions. In brain tissue a sizable proportion of the sulfur pool is in the bound state, which may serve to keep free H₂S low in order to avoid toxicity and to function as a readily released storage (Ishigami et al., 2009). Persulfides are now emerging as highly potent compounds themselves, and recently it was shown that CBS and CSE can generate substantial amounts of CysSSH when utilizing cystine (CysS-SCys) rather than singular cysteine (Ida et al., 2014). CysSSH appear to react with endogenous glutathione (GSH) forming GSSH persulfides and polysulfides with high occurrence as well as uniquely potent nucleophilic and antioxidant/reducing activities in mammals.
(Ida et al., 2014). Where parental H$_2$S is mainly involved in reduction of cysteine disulfide bonds (Kimura, 2015), Polysulfides (R-S$_n$-R) have also recently been shown to be potential signaling molecules acting by the addition of sulfur to cysteine (thiol) residues on target proteins, generating BSS by sulfuration (Kimura, 2015). It is likely that several species currently considered H$_2$S products have their distinct targets and reactive functions, with some likely overlapping and contributing to one another.

H$_2$S is primarily toxic owing to the fact that it binds to the binuclear Cu-heme iron complex in CcOx, causing reversible inhibition of the whole terminal complex (CcOx, complex IV) of the electron transport chain (Nicholls and Kim, 1982). Heme proteins exist in a great variety in cells and many of them could potentially bind H$_2$S. Proteins like myoglobin and Hb typically have a low affinity for H$_2$S binding in mammals (Nguyen et al., 1998), but could potentially still play a role due to their massive presence alone. To prove this point stands the recent finding that the low levels of metHb normally present in blood (1-3% of total Hb), are sufficient to bind and oxidize H$_2$S to thiosulfate and heme –iron bound polysulfates in RBCs, thus clearing H$_2$S from circulation (Vitvitsky et al., 2015). OxyHb is then speculated to be recreated by MetHb reductase releasing the ironbound polysulfides (Vitvitsky et al., 2015).
Figure 6. Overview of H₂S and NO origin, principal fates and effects with focus on the cardiovascular (CV) system. For references, see text. CcOx: cytochrome c oxidase, complex IV of the electron transport chain.
FINE- TUNED REGULATORS OF DELIVERY AND CONSUMPTION AT PLAY: NO AND H₂S

In this paragraph I will focus on mechanisms and occurrence of selected NO and H₂S mediated changes in physiology that could be relevant to mainly hibernators. As I have worked with these gaseous signaling molecules in potentially regulating delivery and consumption in hibernating animals (see Paper IV and ongoing projects), I will focus on the following effects: vasodilation, antioxidant effects and metabolic inhibition.

ON THE PERFUSION SIDE: NO AND H₂S AFFECT BLOOD VESSEL DIAMETER

NO primarily mediates vasodilation in larger conductance vessels and has been recognized as a major endothelium-derived relaxing factor (Nagao et al., 1992). Endothelium-derived hyperpolarizing factors also control vessel tone and are dominant in the smaller resistance blood vessels that generally determine blood pressure. H₂S has been shown to be a major relaxing factor of the latter kind (Mustafa et al., 2011; Yang et al., 2008). Via activation of three channels, the ATP-sensitive K⁺ as well as the intermediate and small conductance Ca²⁺ sensitive K⁺ channels, H₂S facilitates vasodilation in systemic arteries (Mustafa et al., 2011; Zhao et al., 2001). In case of the ATP-sensitive K⁺ channel, the activation is now known to be due to sulfuration or sulfhydration (addition of an –SH group) to a cysteine residue in the subunit region where ATP binding would normally reduce activity (Mustafa et al., 2011). NO works through a different avenue. In the vasculature, cholinergic stimulation (release acetylcholine) or physical shear stress in the vessel wall induces Ca²⁺ dependent activation of eNOS that lead to NO production. NO freely diffuse into nearby smooth muscle of the vessel wall to activate the heme-containing soluble guanylate cyclase, which by cGMP generation cause vasorelaxation (Moncada and Higgs, 1993; Waldman and Murad, 1988). Possibly, a synergistic effect of NO and H₂S in combination may also exist on systemic vascular smooth muscle relaxation (Coletta et al., 2012; Hosoki et al., 1997).

Localized vasorelaxation and resulting dilation of the vessel will increase blood flow to specific organs or tissues, unless countered by other stimuli. Differences in utilized pools, but not in total quantity, of endogenous H₂S metabolites have been observed by our group during summer active and winter hibernating states of brown bears, suggesting consumption of free H₂S during hibernation (Paper IV: Revsbech et al., 2014). During hibernation the sympathetic nervous system is responsible for keeping a marked vasoconstriction in peripheral blood vessels, Likely foremost to keep blood pressure at reasonable levels despite the decrease in heart rate (Lyman and O’Brien, 1963; Lyman et al., 1960; Toien et al., 2011) Also, diving mammals are capable of a similar response during breath-hold dives (Bron et al., 1966). The strong adrenergic tone seen in peripheral vessels of many hibernators and divers would most likely prevent any general vasodilatory effect of H₂S and NO. This would serve to uphold the peripheral vasoconstriction that
may, besides keeping a stable blood pressure, be part of a conserved adaptive trait aiding metabolic allocation to important organs during hibernation and breath-hold dives.

Nitrite in the circulation will surely be converted to NO in low O\textsubscript{2} environments because of a higher quantity of deoxy hemoglobin and myoglobin. It has been shown that sensitivity to NO donor-induced vasodilation is unaltered in hibernation compared to euthermic state (Karoon et al., 1998). Given the high peripheral vasoconstriction, NO-induced dilation of large conducting vessels would further enable these to contain most of the cardiac output (Fago and Jensen, 2015). It is still plausible, that some degree of e.g. critical organ (heart, brain) microcirculation regulation in the hibernating animal is regulated to avoid hypoxia by compounds such as NO and H\textsubscript{2}S during hibernation. Even more so as mammalian RBCs may be able to regulate their own H\textsubscript{2}S levels, as just discovered in human RBCs (Vitvitsky et al., 2015). Besides the bear, differentiating endogenous H\textsubscript{2}S production in a natural hibernator has been reported only in one other species: In lung tissue of the Syrian hamster torpor increased CBS expression and thus H\textsubscript{2}S production twofold with H\textsubscript{2}S playing a role in hibernation linked lung remodeling (Talaei et al., 2011a; Talaei et al., 2012).

Opposite to the relaxation response in systemic arteries, H\textsubscript{2}S in pulmonary arteries of most mammals can enhance hypoxic vasoconstriction (Olson et al., 2006; Olson et al., 2013). Hypoxic vasoconstriction may in the lungs of a hibernator serve to optimize lung efficiency even more than recognized in non-hibernators. On one hand arterial O\textsubscript{2} saturation is kept high as only parts of the lung still containing high P\textsubscript{O}2 is perfused. On the other hand, when not inflated at the normal frequency, lung segments may be restricted in blood flow and thus less energy is consumed in an organ that is not working at normal capacity during deep hibernation: the Syrian hamster breathes 5-7 times per minute in hibernation versus 84± 20 times per minute in awake state (Rubin et al., 1978; Talaei et al., 2011a). The golden-mantled ground squirrel will be in apnea for about 17 minutes between breathing bouts in deep hibernation (Milsom and Jackson, 2011). Yet again, in some divers H\textsubscript{2}S can induce hypoxic vasodilation (Olson et al., 2010).

**THE BALANCE OF TISSUE OXYGEN CONSUMPTION: POSSIBLE INVOLVEMENT OF H\textsubscript{2}S AND NO**

Hibernators go through an extensive metabolic reduction entailing a drastic reduction of O\textsubscript{2} consumption. Of organismal O\textsubscript{2} consumption 90% is utilized directly as electron acceptor in the mitochondrial electron transport chain during ATP production. Of particular interest is therefore the ability of both H\textsubscript{2}S and NO to reversibly inhibit mitochondrial CcOx of the electron transport chain, impeding mitochondrial O\textsubscript{2} consumption and energy production (Cleeter et al., 1994; Collman et al., 2009; Cooper and Brown, 2008; Hill et al., 1984). H\textsubscript{2}S of ~80ppm given to mice induce a hibernation-like state with reversible, dose-dependent and temperature independent reductions in O\textsubscript{2} consumption, ventilation, CO\textsubscript{2} production and body temperature (Blackstone and Roth, 2005). This ability showcases H\textsubscript{2}S as a possible key element in substantial metabolic reduction. All pools of H\textsubscript{2}S (free sulfide, bound sulfane sulfur and the acid labile pool
(see figure 6) are relevant to assess when evaluating the status of sulfide signaling. In our studies on brown bears, we did not, as first expected, see an increase in concentration of free or total sulfide when evaluated in plasma and RBCs, but rather, we saw a constant concentration but a shift in composition; a shift in the pools (Paper IV: Revsbech et al., 2014). During hibernation, remodeling of H$_2$S metabolism was visible in blood plasma as a significantly reduced quantity of free sulfide and BSS, and changes in red cells approached significance as compared to the summer active state. Erythrocytes showed higher CSE activity in winter. From that data we propose that the plasma pool of BSS is utilized during hibernation to generate free H$_2$S in the RBCs, which then may diffuse to nearby tissues. At low tissue O$_2$ levels as are potentially the case in hibernation (see below), the generated H$_2$S may aid metabolic depression by inhibiting mitochondrial O$_2$ consumption (Paper IV: Revsbech et al. 2014). Our study highlights the importance of evaluating all forms of sulfide, especially under varying metabolic demands that may induce low O$_2$ conditions and/or changes in acidity that could affect the bound pools of H$_2$S. In contrast we did not detect any significant differences in nitrite of hibernating versus awake brown bears, suggesting NO metabolism is less involved (Paper IV: Revsbech et al., 2014).

The strong metabolic suppression of hibernating bears implies a reduced O$_2$ consumption. This is balanced by small O$_2$ supply from blood with a higher affinity (Paper I: Revsbech et al., 2013a). A steady state of reduced supply and consumption invoking a maintained tissue PO$_2$ is theoretically possible. However, it is also logical that a lower but sufficient O$_2$ tension in the tissues could be the outcome, as is the case at least in brain tissue of the hibernating arctic ground squirrel (Ma and Wu, 2008). This latter situation is additionally supported in the brown bear by our finding of sulfide recycling that is favored by local hypoxia (Paper IV: Revsbech et al., 2014), as well as a lower arterial PO$_2$ measured in hibernating, anesthetized bears (Evans et al., 2012) and in hibernating 13-lined ground squirrel (Musacchia and Volkert, 1971). Further, we have modeled an estimate on venous P$_{O2}$ in the hibernating bear and found a slightly lower value than summer venous PO$_2$ (Paper I: Revsbech et al., 2013a). Our modeling assumed the same arterial PO$_2$ in summer and winter bears, and thus our results only understate the result that hibernating bears likely operate at a lower internal PO$_2$, but still sufficient to sustain aerobic metabolism.

The reduced PO$_2$ of the blood required for full saturation following a markedly higher Hb-O$_2$ affinity in the hibernating brown bear (Paper I: Revsbech et al., 2013a) will result in a smaller PO$_2$ diffusion gradient from blood to tissues. This may perhaps aid in achieving or maintaining the new metabolic state directly by limiting O$_2$ delivery for oxidative phosphorylation, whilst still enabling it where it is needed. The creation of free H$_2$S from the bound sulfane sulfur pool has until now only been observed in neurons (Ishigami et al., 2009) as well as in isolated arteries in strong hypoxia (PO$_2$ <5mmHg) and deoxygenated homogenized tissues (Olson et al., 2013). In the hibernating bear we propose the same mechanism (Paper IV: Revsbech et al., 2014), but likely occurring at only slight hypoxia in line with our hypothesis of a reduced PO$_2$ diffusion gradient (Paper I: Revsbech et al., 2013a).
There are currently to my knowledge no reports of NO involved in inducing hibernation-like states, and thus far levels of red cell and plasma nitrite in brown bears have not revealed any significant differences between summer and winter states (Paper IV: Revsbech et al., 2014). Instead, slightly higher circulating nitrite levels, possibly due to elevated NOS activity, seem to be a mark of hypoxia adapted ectotherms (Hansen and Jensen, 2010; Jensen, 2009; Sandvik et al., 2012), as well as in at least one diving mammal (Soegaard et al., 2012) and as mentioned earlier even in humans living in high altitude hypoxia in Tibet (Beall et al., 2012; Erzurum et al., 2007). It is still conceivable that NO could play some role in hibernation.

**Cytoprotective and Antioxidant Effects of H$_2$S and NO**

Despite the low O$_2$ intake and transport levels, hibernators are not continuously hypoxic, but remain aerobic at a low steady state. However the steady state is in small hibernators interrupted by repeated arousals, and in bears by cyclic oscillations in T$_b$ and metabolism (see box 1). Thus mismatches in O$_2$ delivery to consumption are expected. It is likely that the electron transport chain of the inner mitochondrial membrane is reduced during the low O$_2$ flux of hibernation. A sudden surplus of available O$_2$, largest at the resumed euthermic breathing and heart rate at arousal, is therefore expected to yield a pulse of reactive oxygen species, ROS. In particular organs that drastically change their activity level, like the heart, must be under stress. Yet, a hibernating bear is fully able to engage in fight or flight at any disturbance. Clearly hibernators have adapted to handle all parts of hibernation and arousal well, and an efficient antioxidant system must be involved.

The major antioxidant effect of NO lies in the inhibition of oxidant production as the production of ROS can be inhibited by S-nitrosation of complex I of the electron transport chain, a major site of ROS generation. In hibernating Syrian hamsters, upregulation of H$_2$S production seems to protect against hypothermia-induced cell apoptosis, ROS formation and acidosis (Talaei et al., 2011b; Talaei et al., 2012). Cytoprotective and antioxidant effects have been attributed to varying concentrations of H$_2$S and its products or downstream signaling. H$_2$S protects neurons against ischemia-reperfusion-injury and oxidative stress primarily by recovering levels of the major intracellular antioxidant glutathione (Kimura and Kimura, 2004; Kimura et al., 2009). Excessive neurotransmitter glutamate buildup following ischemic events suppresses cystine import into cells where cystine reduction yields cysteine, the substrate for the production of major endogenous antioxidant glutathione (GSH). This is the background for oxidative glutamate toxicity (see Kimura and Kimura, 2004). H$_2$S increases the activity of a rate-limiting enzyme in thiol-containing GSH production ($\gamma$-glutamylcysteine synthetase) as well as reduces the cysteine/glutamate antiporter inhibition by glutamate and thus increase cystine import (Kimura and Kimura, 2004). More importantly, H$_2$S directly reduces cystine to cysteine in the extracellular space and a more efficient transporter imports the cysteine, contributing dominantly to GSH production (Kimura et al., 2009). Together, these two mechanisms ensure a markedly higher GSH production in the presence of H$_2$S.
Additionally, H₂S facilitates GSH redistribution to mitochondria where GSH can be in the first line of defense to scavenge produced ROS (Kimura et al., 2009). As a reducing substance H₂S may also exhibit direct ROS scavenging in the mitochondria, however this direct role is minor compared to that of GSH (Kimura et al., 2009). Although these roles of H₂S in antioxidant defense has been established for neurons, similar mechanisms could be envisaged to take place in other cells as GSH is a ubiquitous antioxidant and H₂S readily crosses cell membranes (Cuevasanta et al., 2012; Riahi and Rowley, 2014).

GSH reaction with ROS yields oxidized GSSG, which needs to be reduced back by NADPH-dependent GSH reductase, or actively exported from the cell (Raftos et al., 2010). Thus enough reducing power needs to be present to reduce GSSG to GSH during mismatches in oxygen supply to consumption and resultant ROS generation. During brown bear hibernation our group found indications that erythrocyte glycolysis is downregulated substantially, as the side product of glycolysis, DPG was reduced about 50% (Paper I: Revsbech et al., 2013a). Thus both aerobic respiration in surrounding tissues as well as red cell glycolysis are operating at reduced rates during hibernation, therefore also yielding less NADPH. However, ROS generation must be temporally restricted due to very transient increases in metabolic rate, especially in bears (see box 1). It is therefore not unlikely that a hibernator may possess enough glucose to generate sufficient additional NADPH by the pentose phosphate pathway, or possibly by other avenues like NADP-dependent isocitrate dehydrogenase (Jo et al., 2001).

In the hibernating brown bear we did indeed see a large increase in RBC total GSH (Paper IV: Revsbech et al., 2014). Using correlations, we found the high RBC GSH to correlate closely with plasma and RBC cysteine availability, as also evidenced in humans where GSH synthesis of RBC is rate limited by plasma and RBC cysteine (Raftos et al., 2010). Furthermore, it is interesting to note that in bear RBCs, the high GSH occurs concurrently with what we hypothesize is H₂S formation from thiosulfate as evidenced by a depleted BSS pool during hibernation. Our hypothesis is that the hibernator in this way allocates the majority of available cysteine to fuel GSH production, whilst still upholding a sufficient H₂S production by regeneration from oxidation products (Paper IV: Revsbech et al., 2014).

**Metabolic inhibition: not a ligand banding battle**

Depressed mitochondrial function can be induced by moderate to high levels of H₂S (see below). Reversibly inhibiting mitochondrial CcOx implies reducing metabolic demand for O₂ and therefore reducing metabolism as a whole. Inhalation of ~80ppm of H₂S has induced reversible suspended animation in mice involving all the hallmarks of natural hibernation; reductions in O₂ consumption and CO₂ production and therefore in metabolic rate as well as reductions in lung ventilation and body temperature (Blackstone et al., 2005). The ability of H₂S to induce a form of suspended animation or hibernation-like state has been attributed to organismal mitochondrial inhibition (Blackstone and Roth, 2007; Blackstone et al., 2005; Cooper and Brown, 2008; Li et al., 2012).
H₂S-induced protection against ischemia-reperfusion injury in myocardia associated with preserved mitochondrial structure, function and inhibition of respiration has been identified (Chen et al., 2007; Elrod et al., 2007). Blockade of electron transport as well as a degree of mitochondrial uncoupling decreases oxidative phosphorylation and causes reduced accumulation of reducing equivalents in the electron transport chain (Chen et al., 2007). Most publications agree that the binding of H₂S to CcOx heme is uncompetitive with that of O₂, in effect meaning O₂ concentration does not affect binding directly (Cooper and Brown, 2008). Affinity of binding is weak with half-inhibition of respiration rate, Kᵢ of 0.2-12.5µM (Collman et al., 2009; Cooper and Brown, 2008). In comparison NO binding is competitive with O₂ binding with an Kᵢ of reduced CcOx for NO of about 0.2 nM in total absence of O₂, 60nM NO at 30 µM O₂ (typical O₂ concentration of tissues) up to 270nM at 145µM O₂ (typical O₂ concentration of arterial blood) (Brown and Cooper, 1994; Cooper and Brown, 2008). The two ligands are not in direct competition as they do not inhibit the same redox state of the enzyme; NO inhibits the reduced state (as well as interacts with the oxidized state at Cu₈, see below) and sulfide binds to the oxidized states of the binuclear centre (Cooper and Brown, 2008). Mitochondrial inhibition by H₂S and NO is thus favored in low O₂ conditions, precisely as would be the case in hibernators (Revsbech et al., 2013a). Low O₂ conditions are also the conditions where thiosulfate may work as a pool to regenerate H₂S (Olson et al., 2013), as data suggested to happen in bears (Paper IV: Revsbech et al., 2014).

**SUBSTRATE OR INHIBITOR – WHY NOT BOTH?**

Due to the weak binding of H₂S to CcOx, the response is highly concentration-dependent: Only moderate to high H₂S concentrations (>10-50µM, dependent on tissue and species) will inhibit CcOx O₂ consumption (Collman et al., 2009; Goubern et al., 2007; Nicholls and Kim, 1982; Yong and Searcy, 2001) On the other hand, low H₂S concentrations will function as a reducing agent and therefore as a substrate, donating electrons to the electron transport chain and quite oppositely stimulate O₂ consumption (Goubern et al., 2007; Yong and Searcy, 2001). H₂S can reduce both the oxidized CcOx active site as well as reduce cytochrome c, the electron carrier normally reduced by electrons from the electron transport chain, originally donated from food metabolism derived NADH or FADH₂ at complex I and II (Collman et al., 2009).

Recently, Módís et al (2013) working in mammalian mitochondria found that low 1µM H₂S stimulated mitochondrial function synergistically with succinate (yielding FADH₂ through Krebs cycle), however no stimulation of mitochondrial electron flow was seen when succinate was absent (Módís et al., 2013). Thus at low levels H₂S can be a source of electrons and an inorganic energy substrate in a mammalian cell, but seemingly only so in the presence of an active Krebs cycle. At higher levels however, H₂S can indeed inhibit mitochondrial respiration.

A similar situation to that of H₂S is at play for NO, as NO noncompetitively binds the copper (Cu₈) of the heme:copper binuclear center of oxidized CcOx, reducing the enzyme whilst oxidizing NO to nitrite. Thus NO is acting like a substrate and an inhibitor simultaneously (Cooper, 2002; Torres et al., 2000). Oxidized Cu₈ does not bind O₂ and NO binding to the Cu₈ is thought to happen mainly in settings of low electron flux.
(Cooper, 2002). Biased as I am, I cannot help but speculate here: NO-induced inhibition of CcOx at CuB could be a potential avenue of mitochondrial respiration reduction in a fully aerobic environment with low electron flux due to reduced metabolism. Could NO be both a substrate contributing to electron delivery and a regulator of metabolism and ROS production in hibernators? It would require the affinity of CuB for NO to be high enough compared with the reaction of NO and O2 to produce nitrate, and currently the precise affinity is unknown (see Cooper, 2002). Despite our recent inconclusive findings with regard to changes in nitrite levels during hibernation (Paper IV: Revsbech et al., 2014), NO and metabolites still hold potential for the hibernation story.

**Nitrate reductase activity increases with hemoglobin oxygen affinity**

It seems a vertebrate trend that higher O2 affinity of Hb is combined with a higher nitrite reductase activity of the deoxy (R) form in hypoxia, as the R state of Hb has the higher nitrite reductase activity over the T-state (Fago and Jensen, 2015; Jensen, 2009). We know that DPG levels fall drastically during hibernation in several hibernating species, including our investigated brown bears (Kramm et al., 1975; Maginniss and Milsom, 1994; Paper I: Revsbech et al., 2013a), as well as is the case for ATP or GTP in teleost fish exposed to hypoxia (Jensen, 2004). This high O2 affinity of Hb translates directly into increased nitrite reductase activity due to the shift of the T-R equilibrium towards the R-state as the T state is less stabilized by phosphates (Paper I: Revsbech et al., 2013a). Thus a slightly higher capacity for nitrite reductase activity in a hypoxic milieu can potentially be at play in blood of hibernators, enabling local vasodilation and perhaps release of NO to aid CcOx inhibition as well as plug ROS production from complex I. Whilst NO may inhibit respiration, it does not decrease contractility of heart muscle in some ectotherms; the hypoxic muscle has been reported to contract equally well or even with a up to 60% higher force per O2 consumed, as seen in the hypoxia tolerant turtle *Trachemys scripta* (Imbrogno et al., 2014; Misfeldt et al., 2009). The effect can be attributed to increased mitochondrial coupling as the leak current of H+ over the inner mitochondrial membrane is diminished, yielding more ATP per O2 consumed, thus increasing muscle efficiency (Larsen et al., 2011). In mild hypoxia (9% O2) trout Mb is more efficient as a nitrite reductase than hypoxia-tolerant high affinity goldfish Mb, as a larger part is deoxygenated at any given PO2 (Pepersen et al., 2010). Nitrite-derived NO may be crucial in keeping the heart of a hypoxia-adapted animal pumping even in anoxia (Stecyk et al., 2004), and the mechanism behind may well apply for hibernators where energy stores, and thus the reducing equivalents creating the H+ gradient in the first place, must be stretched throughout the winter. Also of interest is the ability of known hypoxia-tolerant ectotherms to translocate nitrite from plasma and extracellular stores into tenfold higher concentrations in intracellular compartments in the hypoxic heart of crucian carp as well as in goldfish and the red-eared slider turtle (Hansen and Jensen, 2010; Jensen et al., 2014; Sandvik et al., 2012). We do not yet know if any part or how these adaptations to hypoxia seen in the exothermic specialists, may also apply for the more energy demanding mammalian hibernators faced with a similar, albeit less extreme task of coping with low O2.
CONCLUSION AND PERSPECTIVES

HYDROGEN SULFIDE AND NITRIC OXIDE: NEWCOMERS IN HIBERNATION SCIENCE

In this thesis I report the first work on a whole body scale measurement of the signaling molecules hydrogen sulfide and nitric oxide in a hibernating animal like the brown bear. Until papers included in this thesis were published, endogenous H$_2$S in natural hibernation had only been reported for lung tissue of Syrian hamsters (Talaei et al., 2011a; Talaei et al., 2012). A very recent paper (Dugbartey et al., 2015) reports plasma free H$_2$S levels to go up as well as increased renal expression of CBS, CSE and 3-MST under an artificially induced hibernation-like state using 5'-Adenosine monophosphate (5'-AMP) in Syrian hamsters. However, injection of the non-specific inhibitor of H$_2$S production, amino-oxyacetic acid, did not affect induction of the torpor-like state albeit did hinder H$_2$S production, leading to the conclusion that H$_2$S is not necessary for artificial 5’-AMP-induced hibernation (Dugbartey et al., 2015). However, our group did find changes in H$_2$S during natural hibernation in the brown bear (Paper IV: Revsbech et al., 2014).

In the blood of hibernating brown bears we did not see increases in total H$_2$S as first expected, but instead a fine-tuned remodeling of H$_2$S metabolism, recycling oxidation products and thus conserving amino acid cysteine for other purposes. We propose that hibernators prioritize the available free Cys for the production of antioxidant glutathione, GSH, found in high levels in winter erythrocytes (Paper IV: Revsbech et al., 2014). High antioxidant defenses are especially important during arousal induced mismatches in delivery and consumption. Blood H$_2$S is conveniently distributed throughout the body and thus ready to effect local systemic vasodilation where needed in important organs as the heart and the brain, and to aid metabolic suppression in tissues by inhibiting CcOx of the electron transport chain. We remain to see evidence of NO and H$_2$S affecting key glycolytic enzyme activities during hibernation, but SNO-GAPDH levels as well as other enzyme activities could be relevant to reevaluate in smaller hibernators. Clearly more studies are needed to evaluate the possible involvement of H$_2$S in natural hibernation. Perhaps studies can even contribute towards future incuduction of a state of reduced metabolism in organs for transplants or in wider perspectives, in humans after serious trauma.

CONSEQUENCES OF 2,3-DIPHOSPHOGLYCERATE VARIATIONS FROM SMALL TO LARGE MAMMALS

This thesis also reports the first comparison and elucidation of mechanisms behind O$_2$ transport in hibernating versus euthermic states of the large hibernator the brown bear. As I hope made clear during this thesis, bear hibernation is not a direct expansion of hibernation in small mammals, but involves a larger degree of active metabolic reduction due to an only slight reduction in body temperature $T_b$ as well as a more continuous mode of hibernation. Nevertheless, as reported before in small hibernators, we found erythrocytes to about half their DPG content during hibernation (Paper I: Revsbech et al., 2013a). We proved the decrease in DPG to via a direct allosteric action affect Hb-O$_2$ affinity. In concord with the decrease in $T_b$, the DPG
decrease thus yields a substantially higher hemolysate O$_2$ affinity during winter compared with summer state. In contrast, we found that six small rodent hibernators likely have no noteworthy effect of the decreases in DPG (Paper III: Revsbech et al., 2013b), but probably rely more on their strong decrease on T$_b$ to effect a rise in Hb-O$_2$ affinity (see box 1). As decreases in DPG will also affect the Donnan distribution of anions across the erythrocyte membranes, an indirect effect on Hb-O$_2$ affinity may take place through the Bohr effect (Paper III: Revsbech et al., 2013b).

Additionally, decreases in DPG likely will affect the cooperativity of Hb-O$_2$ binding, $n$, through less stabilization of the reduced affinity of only the T state and thus a less steep Hill plot (see box 2) around 50% saturation. We modeled this DPG reduction-induced decrease in $n_{50}$ to likely be of substantial significance in vivo in hibernating bears, as the reduction in cooperativity in consort with the elevated Hb-O$_2$ affinity yielded a sensible venous $P_{O_2}$ despite the high winter hematocrit (Paper I: Revsbech et al., 2013a). The substantially higher venous $P_{O_2}$ if not for these changes in $n$ and $P_{50}$, would lead to untimely O$_2$ delivery to tissues and major ROS production (see Box 3). Also high altitude deer mice engage variations in DPG in their adaptation to altitude. However, unlike elevated Hb-O$_2$ affinity and reduced sensitivity to cofactors in high altitude deer mice, the elevations in DPG, hematocrit and other factors are readily reversed with translocation to low altitude. This indicates the plastic changes as part of a perhaps necessary impressive phenotypic plasticity of these mice (Paper II: Tufts et al., 2013). The phenotypic plasticity of deer mice can play part also in high altitude adaptation, although normally categorized as an acclimatization response.

In this thesis we provide support for the growing theory (Berenbrink, 2006; Natarajan et al., 2013; Natarajan et al., 2015; Tufts et al., 2015) that not necessarily only specific amino acid residues are important for protein function, but rather that the entire genetic background and multiple substitutions far away from the active sites yields the protein phenotype (Paper III: Revsbech et al., 2013b). In Paper III we found the Bohr effect unaffected by an expectedly detrimental amino acid substitution $\beta$146 His$\rightarrow$Gln, at the same time as sensitivity towards DPG was low in spite of the entire DPG-binding site being retained (Paper III: Revsbech et al., 2013b). Additional work from our group on pika Hb reports how substitution sequence or chronology on a genetic background has a major impact on protein function (Tufts et al., 2015); my own contribution to this study was minor although I am a co-author, and the paper not included here as the deeper genetics are outside the scope of this thesis.

**INTERACTIONS OF MECHANISMS REGULATING OXYGEN TRANSPORT AND CONSUMPTION**

For the ease of maintaining a logical overview, this thesis has largely been structured into discussing modifications in oxygen transport involving Hb-O$_2$ affinity adjustments via phenotypic adaptation or via genotypic adaptation, separated from adjustments in oxygen delivery and consumption involving the signaling molecules H$_2$S and NO. In the integrated physiology of a high altitude animal or a hibernator, modifications and interactions in transport and consumption obviously may occur. As described earlier in this thesis, the high O$_2$ affinity of Hb during hibernation will increase the nitrite reductase activity of Hb during
hibernation, potentially enabling higher NO production at low O\textsubscript{2}. Changes between summer and winter nitrite, taken as a marker of NO-metabolism, was not statistically significant in our study (Paper IV: Revsbech et al., 2014), but with P=0.043, they likely will be in a different study comparing fewer parameters. I here speculate further: Given that sufficient NO is not converted to nitrite in the meeting with free O\textsubscript{2}, or with localized production, NO-induced inhibition of CcOx at Cu\textsubscript{B} (see paragraph on substrate or inhibitor) could be a potential avenue of sustaining reduced mitochondrial respiration. And it would be possible in a fully aerobic environment with low electron flux due to otherwise actively reduced metabolism. This speculation would be difficult to test, but surely NO has interesting effects, also in a hibernator perspective. For H\textsubscript{2}S, it has been recently shown that RBCs are able not only to produce H\textsubscript{2}S enzymatically, but also to oxidize it to thiosulfate by reaction with ferric hemoglobin, also known as MetHb (Vitvitsky et al., 2015). This opens up the avenue of all heme complexes in their ferric form to potentially play some role in H\textsubscript{2}S homeostasis. In addition, on the vascular side a synergistic effect of NO and H\textsubscript{2}S in combination may also exist on systemic vascular smooth muscle relaxation (Coletta et al., 2012; Hosoki et al., 1997).

Production of DPG inside the erythrocyte happens as a side product of glycolysis, and is thus directly proportional to the level of glycolysis. In the context of hibernation, a downregulation of RBC glycolysis, and thus of DPG production as found in the brown bear (~50%) fit in well (Paper I: Revsbech et al., 2013a). However, as for the general reduction in metabolism, it is interesting to search for the cause, although one must appreciate that the search may be a question of who came first. Mammalian erythrocytes possess no mitochondria, but NO and H\textsubscript{2}S may still affect cell turnover by S-nitrosation and/or sulfuration of key enzymes, likely upstream of DPG in glycolysis. The enzyme Glyceraldehyde 3-phosphate dehydrogenase, GAPDH, catalyzing the sixth step of glycolysis is known to be S-nitrosated (Chakravarti et al., 2010). GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, the precursor to DPG. We therefore set out to test the hypothesis of whether the decrease in DPG in winter could be linked with a hibernation induced increase in GAPDH-SNO. In Paper IV we investigated activity and by biotin switch S-nitrosation of GAPDH, but found no significant changes using the applied, admittedly strict (P<0.002), statistical method (Paper IV: Revsbech et al., 2014). Applying an altered setup with less tested parameters and more stable base levels of in-house kept animals may well provide a different outcome.

The molecular mechanisms of an endotherm body at low metabolism and limited O\textsubscript{2} supply may give us new knowledge of physiology at extremes and, if we can learn just parts of what aids metabolic suppression in a hibernator, perhaps even the tools to prolong the window of opportunity for large operations as well as organ transplants.
ONGOING AND FUTURE RESEARCH

ONGOING: MUSCLE AND ADIPOSE TISSUE HYDROGEN SULFIDE LEVELS OF HIBERNATING BEARS

In a previous study, we found seasonal changes in the plasma levels of selected H\textsubscript{2}S metabolites, suggesting that hydrogen sulfide, H\textsubscript{2}S, may be one of the temperature independent factors implicated in the metabolic depression of hibernating brown bears (Paper IV: Revsbech et al., 2014). Specifically, our data analysis indicated remodeling of H\textsubscript{2}S metabolism during hibernation and that the plasma pool of BSS may be utilized during hibernation to generate free H\textsubscript{2}S in the RBCs. We proposed that H\textsubscript{2}S may then freely diffuse to perfused tissues, where at the low O\textsubscript{2} levels of hibernating tissues (Paper I: Revsbech et al., 2013a) it may aid metabolic depression by inhibiting mitochondrial O\textsubscript{2} consumption. The question then arises as to whether changes in H\textsubscript{2}S plasma metabolites during hibernation are reflected by changes of the same or other metabolites in other body parts. As muscle and adipose tissue are dominant tissues of a hibernating bear, and in addition relatively simple to sample with minimal risk to the bear, we set out to investigate H\textsubscript{2}S metabolites in these tissues of hibernating versus summer active bears.

A few organs are considered producers of the majority of endogenous H\textsubscript{2}S: Liver and kidney produce the highest levels in most species (Kabil et al., 2011). In the rat, levels of H\textsubscript{2}S in adipose tissue are within the range of other major H\textsubscript{2}S producing organs as kidney and liver (Feng et al., 2009). In humans skeletal muscle levels of H\textsubscript{2}S producing enzymes CSE and CBS are comparable to that found in liver and kidney, however expression levels of H\textsubscript{2}S generating enzymes are highly species specific (Chen et al., 2010; Du et al., 2013; Veeranki and Tyagi, 2015). Collectively, both adipose tissue as well as skeletal muscle in hibernating versus summer active brown bears may or may not have high expression patterns of H\textsubscript{2}S generating enzymes, and thus until now unknown H\textsubscript{2}S metabolite profiles.

SKELETAL MUSCLE

Several studies have found skeletal muscle mass and morphology to be preserved during hibernation in ground squirrel following specific changes in the mammalian target of raptamyosin (mTOR) pathway, mTOR being a key kinase that upregulates muscle protein synthesis and limits fasting-induced proteolysis and muscle degradation (Andres-Mateos et al., 2013; Nowell et al., 2010; Wu and Storey, 2012). However muscle maintenance is not necessarily static, but rather hibernation can be associated with an initial slight skeletal muscle loss, stabilization and then recovery and return to summer protein synthesis levels in late hibernation, prior to emergence, at least in the 13-lined ground squirrel (Hindle et al., 2015). In hibernating bears lean body mass seems preserved due to low turnover and recycling of urea into amino acids as well as de novo protein synthesis of selected proteins only (Nelson, 1980; Nelson et al., 1973; Stenvinkel et al., 2013).
ADIPOSE TISSUE
During bear hibernation energy is supplied by burning of fat (Robbins et al., 2012), a process that – albeit at lower rates – still requires O$_2$. Hibernating bears do not accumulate lactate and are not hypoxic (Paper I: Revsbech et al., 2013a). Adipose tissue is therefore metabolically active during hibernation. Adipose tissue may also act as an insulin-sensitive organ and regulate blood glucose (Feng et al., 2009). Endogenous H$_2$S production by the enzyme CSE has been shown to inhibit glucose uptake in rat adipose tissue (Feng et al., 2009), suggesting that H$_2$S may be a novel insulin resistance regulator of adipose tissue (Feng et al., 2009).

Skeletal muscle and adipose tissue are both relevant to hibernation. Skeletal muscle is the largest organ in terms of weight and would then contribute the most to the whole animal metabolic depression, especially during inactivity, while adipose tissue is essential due to its role in maintaining energy metabolism during hibernation. Therefore in principle H$_2$S metabolites could differ between the two organs and between seasons, aligning with different winter and summer metabolic activities and requirements.

Our aim in this project is to better understand the physiological role of H$_2$S in natural hibernation and to this end we have quantified composition of H$_2$S metabolites in skeletal muscle and adipose tissue compared to that of plasma and RBCs in winter hibernating and summer active wild brown bears. This study included free-ranging subadult brown bears (2-4 years old), 7 in winter (February 2014, 4 females, 3 males), of which 5 were recaptured in summer (June 2014, 3 females, 2 males). We are additionally in the process of obtaining measurements for CSE enzyme activity in adipose tissue and skeletal muscle during winter hibernation and summer active state of these bears.

The results of this study are in the analysis and writing process, while awaiting more data.

ONGOING: NITRIC OXIDE MEDIATED REGULATION OF ARTERIAL FLOW IN A HIGH ALTITUDE ADAPTED ANIMAL

NO causes vasodilation and thereby increased local perfusion trough activation of smooth muscle guanylyl cyclase producing cyclic GMP. Additionally, an O$_2$-independent formation of NO can happen by nitrite reduction by deoxyHb, yielding NO in hypoxia independently of the endothelium.

Our collaborators have observed genetic differences in Hb-O$_2$ affinity between high-and low altitude deer mice (Storz et al., 2007; Storz et al., 2009; Storz et al., 2010). We now wish to expand this knowledge on divergent O$_2$ delivery by uncovering possible differences in the function of blood perfusion regulation between high and low altitude adapted mice. It has before been observed that major differences in NO metabolites and localized blood flow can exist between populations adapted to high and low altitude as the study by Erzurum et al., comparing Tibetans (4,200m) and low-altitude American residents (206m) (Beall et
al., 2012; Erzurum et al., 2007). We aim to integrate this knowledge into understanding possible variation and feedback in blood pressure response to acute hypoxia exposure in the same deer mice cohort.

Microscale blood pressure control studies have been performed by wire myography on mesenteric arteries. Mesenteric arterial NO-mediated perfusion regulation responses were evaluated using noradrenaline as contractant, acetylcholine (Ach) as an invoker of endogenous NO-production, sodium nitroprusside (SNP) as an exogenous NO-donor, and N-nitro-L-arginine methyl ester hydrochloride (L-NAME) as a blocker of endogenously produced NO, respectively. Representative traces from performed wire myography experiments on first order mesenteric arteries are shown below; the top line was incubated 20 min with L-NAME (10^{-5}M, as negative controls) before the ACh protocol was performed. Experiments have been performed on six mice from each cohort and data are to be evaluated.

Figure 7. Representative traces of relaxation responses from a low- and high altitude mouse 1st order mesenteric arteries. Arteries (2 per mouse) were precontracted with noradrenaline and relaxation in response to NO, produced endogenously by the endothelium eNOS by addition of Ach was evaluated. Intervals were of 2 minutes between each addition to the chamber. The top line of each mouse was incubated for 20 min with L-NAME (10^{-5}M) prior to additions to attempt blocking endogenously produced NO. These mice seemed not to respond as expected to L-NAME, however the function of eNOS seemed necessary to archive relaxation.
Preliminary data analysis gives the picture that high altitude mice seem to be more transient NO generators than low altitude mice, as they show recontraction during each 2 min period between chamber additions. On the other hand low altitude mice show more relaxation with endogenous produced NO. In both cases L-NAME did not exclude NO production, however loss of eNOS function seemed to delay full relaxation. For the cumulative SNP addition, both high and low altitude mice show continuous relaxation with increasing concentration (traces not shown). Data awaits further analysis.

The aim of this experiment is to evaluate possible differences in the regulation of blood pressure and perfusion between high and low altitude populations of deer mice, in response to hypoxia and to NO. To gain this insight we are collaborating closely with Tobias Wang and Post Doc Nini Skovgaard from the Zoophysiology department at Aarhus University as well as Nina Kerting Iversen, PhD from the same department and currently Post Doc at Department of Clinical Medicine - Center for Functionally Integrative Neuroscience, Aarhus University, for the experiments performed in Aarhus. Externally we are collaborating with Jay Storz, University of Nebraska.

**Future: Hydrogen Sulfide Metabolites Measurement Method Setup Using Unisense Microsensors**

The major obstacle to studying H\(_2\)S in physiology is to obtain correct measurements. As also discussed in this thesis, H\(_2\)S and derived metabolites have been measured using several different methods, and it is only recently that expected *in vivo* concentration ranges have been agreed on. As recently recognized, and also evident from Paper IV (Paper IV: Revsbech et al., 2014), it is of great importance to evaluate all pools of H\(_2\)S (free sulfide, bound sulfane-sulfur and acid-labile sulfane sulfur). However, the only applicable method so far consists of a series of complicated and equipment-heavy procedures using the monobromobimane method (Kolluru et al., 2013). H\(_2\)S is a volatile gas with a half-life of approximately 2-10 minutes in a physiological environment (Vitvitsky et al., 2012a). It is also light sensitive and best measured under anaerobic conditions (Kolluru et al., 2013).

In this project we aspire to set up a simple new measuring method of H\(_2\)S and metabolites applying a Unisense microsensor as well as a gas tight MicroRespiration chamber from the same company in which we can conduct experiments under controlled conditions. The Unisense H\(_2\)S sensor is an amperometric sensor with an internal reference, a sensing and a guard anode. Both anodes are polarized against an internal reference. H\(_2\)S from the environment penetrates the membrane at the sensor tip according to the external partial pressure. Inside the tip is an alkaline electrolyte, where formed HS\(^-\) ions are immediately oxidized by ferricyanide, producing sulfur and ferrocyanide. The signal is then generated indirectly by the re-oxidation of ferrocyanide at the anode (Jeroschewski et al., 1996). The internal guard electrode contributes to the low
zero-current. As is the case for use of other very small microsensors, the Unisense \( \text{H}_2\text{S} \) microsensor will cause a minute, nonsignificant consumption of \( \text{H}_2\text{S} \) in the sample.

Our aim is to evaluate the free \( \text{H}_2\text{S} \) created from the major bound pools, I. by adding a reducing agent, e.g. Dithiothreitol, DTT, to reduce the bound sulfane sulfur pool to free \( \text{H}_2\text{S} \), II. by adding an acidic buffer (e.g. tested with 10mM Hepes, 0.5 mM ethylenediaminetetraacetic acid (EDTA) pH 3.4 at 20°C) to the sample, thereby releasing the acid-labile pool. Obviously, constraints of volume and concentrations to obtain full liberation of the bound pools will occur, and will be solved as we go. During a three day company workshop at Unisense I have tested a coarse version of this method using human blood and was successful in obtaining a signal. From that I can conclude that a microsensor built to high sensitivity is likely sufficiently sensitive for mammalian measurements (1-5\( \mu \text{M} \) \( \text{H}_2\text{S} \)) (Revsbech et al., 2014; Shen et al., 2012). Free sulfide \( \text{H}_2\text{S} \) in the blood samples before manipulation may, however, be too low to quantify alone. If measurable, the free \( \text{H}_2\text{S} \) will depend on the equilibrium constant at the given temperature as the majority of \( \text{H}_2\text{S} \) at pH >7 will be in \( \text{HS}^- \) form (Dombkowski et al., 2004; Hughes et al., 2009), and only \( \text{H}_2\text{S} \) will cross the micro sensor tip membrane. The total free sulfide can then be back calculated from the given \( \text{pK}_a \) at that temperature. The \( \text{pK}_a \) of sulfide is also susceptible to high salinity, however we will not work at salinities much higher than physiological solution (0.9% NaCl) and thus the given error will be of no great consequence for the measurement.

Once running we plan to transport the setup to places where it will be possible to work with fresh blood samples. Samples can be taken from hibernators, and if possible, with modifications of the method also on tissue homogenate. This method is best suited for immediate measurement of samples, and thus sample processing for storage will also be an area of investigation, as no method has yet been routinely reported.

**FUTURE: NITRITE AND HYDROGEN SULFIDE METABOLITES AND GLYCOLYTIC ENZYME MODIFICATIONS IN A SMALL HIBERNATOR**

Some of the limitations in applying wild brown bears would certainly be alleviated in studies on small hibernators kept in controlled animal facilities and used to some degree of handling, hopefully limiting the individual variations that we see in wild-caught, and in summer helicopter-hunted bears. Small hibernators have more stable states of reduction in metabolism, whereas multiday oscillations in \( \text{T}_b \) and metabolism may have blurred results taken from hibernating bears. Until now we have worked with bears following the argument of natural hibernation in a natural environment and a large animal, from which repeated and sizeable sampling is (relatively) straightforward. It has also been hypothesized that bear physiology is more akin to human physiology than that of rodents, which particularly holds true for weight specific metabolism. On the other hand, however, especially when it comes to volatile gasotransmitters, the concentration changes needed in a smaller hibernator to downregulate a much higher weight specific metabolism (Schmidt-Nielsen,
1984), are likely more pronounced. In the future, it would certainly be interesting to look at H$_2$S and NO metabolites, their distribution and possible enzyme modifications in organs like heart, liver and kidney taken from small hibernating animals during deep hibernation. Hibernation does not entail hypoxia per se, but hypoxia coping mechanisms could be envisaged to play a substantial role in protecting crucial organs like the heart and the brain at inevitable mismatches in O$_2$ supply and consumption with animal arousal from hibernation. This is specifically the case in the smaller hibernators where ~ 6 months of hibernation is interrupted by arousals to normal temperature and metabolism about once every or every second week (see box 1).

Our group is in contact with Kelly Drew at the University of Alaska Fairbanks working with arctic ground squirrels, *Spermophilus parryii*. We further aim to establish contacts at the 15$^{th}$ international Hibernation symposium, to be held August 2016 in Las Vegas. We hope to conduct measurements of H$_2$S and derived pools in house using the above pilot method, but we will verify this method in collaboration with the Kevil lab, LSU Health Sciences Center Shreveport, Louisiana. Nitrite measurements will be conducted in house or in collaboration with Frank Jensen, University of Southern Denmark.
REFERENCES


CHAPTER II: PAPERS
Paper I

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

Revsbech 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*

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A DISTINCT TRAIT OF MAMMALIAN 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 defecation (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 bear 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 O₂ 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-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 O₂ tension (P_{O₂}) 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 O₂ transport of bears adapts to a decreased O₂ supply to tissues during hibernation is however, still unknown.

Earlier studies have found that in small hibernating mammals, blood O₂ affinity increases markedly during hibernation (4, 9, 32). Potentially, blood O₂ affinity can be affected in two ways: by structural changes in the blood O₂ 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 O₂ 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 O₂ 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.

In blood O₂ 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 O₂ 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-year-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 tetratole-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 tetratole-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 ± 0.9°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 from 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 assy is stoichiometrically coupled to the decrease of NADH to NAD+ that was followed at 340 nm (extinction coefficient 6.22 mmol/l 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 2.5 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 (2,000 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 cyaanmethemoglobin extinction coefficient of 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 (Phastgel GE Healthcare Biosciences AB, Uppsala Sweden). A heme concentration of 200 µM in the samples diluted in distilled 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 assembled 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 such equilibration step, absorbance was obtained using the in-house made data acquisition software Spectrosonar (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: ΔH = −4.57 [T1/T1/(T1 − T2)] × ΔlogP50/1,000 kcal/mol, where T1 and T2 are the absolute temperatures (Kelvin) and ΔlogP50 is the corresponding difference in logP50 at the two temperatures. The ΔH 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
HEMOGLOBIN OXYGEN AFFINITY IN HIBERNATING BROWN BEAR

R45

\[ C_{O_2} = \beta_{O_2} \cdot P_{O_2} + 4C_{Hb} \frac{p_{O_2}^n}{p_{O_2} + P_{50}^n} \]  

where \( n \) is Hill’s cooperativity coefficient (\( n_{SO} \)) and \( P_{50} \) is the half-saturation partial pressure and \( \beta_{O_2} \) is the physical solubility of \( O_2 \). We used \( n_{SO} \) and \( P_{50} \) values measured here at 37°C and 30°C for summer and winter samples, respectively (see Table 1 under RESULTS). \( C_{O_2} \) was first calculated from Eq. 4 assuming an arterial \( P_{O_2} \) of 100 torr and an intracellular tetrameric \( Hb \) concentration of 5 mM and using reported values of hematocrit (Hct) of 45 and 56.8% for brown bears measured during summer and winter, respectively (2). \( O_2 \) consumption rates (\( VO_2 \)) of 0.30 and 0.069 ml \( O_2 \cdot g^{-1} \cdot h^{-1} \) 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 \( C_{O_2} \), the \( C_{O_2} \) value for summer active and hibernating bears was obtained by Eq. 3, and was then used in Eq. 4 to obtain \( P_{O_2} \) 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 \( P \leq 0.001 \). One comparison of \( P_{SO} \) of hemolysates at summer 37 and 30°C (Fig. 2B) was found to be significant by the Wilcoxon signed rank test, with \( P = 0.031 \).

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 \( P_{50} \) are shown in Fig. 2. When measured at the respective physiological temperatures, \( O_2 \) affinity of winter hemolysates measured at 30°C was significantly higher (i.e., \( P_{50} \) was significantly lower) than that of summer hemolysates measured at 37°C (Fig. 2, Table 1). Although a significant effect of temperature on \( P_{50} \) values was observed (Fig. 2B), the temperature change alone was not enough to provide the observed shift in the \( O_2 \) equilibrium curve (Fig. 2A), and \( P_{50} \) 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 \( n_{SO} \) 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 (\( \Delta H \)) was similar in summer and winter samples.

Table 1. \( O_2 \) affinity (\( P_{50} \)), cooperativity coefficients (\( n_{SO} \)) measured at 30°C and 37°C and derived heat of oxygenation (\( \Delta H \)) in brown bear hemolysates during winter and summer

<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>( P_{50} ) 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>( n_{SO} )</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>( \Delta H ), 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); \( n = 6 \).
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). O$_2$ 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 O$_2$ 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 O$_2$ equilibria at the two temperatures. Cl$^-$ was added to the same final concentration as measured in the untreated hemolysates (10 mM Cl$^-\text{Cl}$). 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 O$_2$ equilibrium curves of hibernating bears reported in Fig. 2.

When assuming similar heart rates and O$_2$ consumption as in undisturbed hibernating and active black bears (43), the O$_2$ tension of mixed venous blood (that approximates that existing in tissues) in hibernating and active brown bears can be

<table>
<thead>
<tr>
<th></th>
<th>RBC DPG, mM</th>
<th>[DPG]/[Hb+]</th>
<th>Plasma Lactate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>4.41 $\pm$ 0.50</td>
<td>2.12 $\pm$ 0.39</td>
<td>5.81 $\pm$ 2.77</td>
</tr>
<tr>
<td>Winter</td>
<td>2.37 $\pm$ 0.39*</td>
<td>0.99 $\pm$ 0.21*</td>
<td>3.05 $\pm$ 1.32</td>
</tr>
</tbody>
</table>

*Significant differences ($P < 0.001$) from summer values (means $\pm$ SD); $n = 6$. RBC, red blood cells; DPG, 2,3-diphosphoglycerate.
predicted from the Fick equation when knowing values for P50, cooperativity coefficients n50 and blood Hb concentration, as described in detail under MATERIALS AND METHODS. Figure 4 shows the predicted O2 equilibrium curves for winter and summer animals along with the estimated arterial and venous O2 saturation and respective P02 values. In the calculations, P50 and n50 values were those measured at the physiological temperatures of 37°C and 30°C for summer and winter samples, respectively (Table 1). As evident from Fig. 4, winter O2 temperatures of 37°C and 30°C for summer and winter samples (Table 1). As evident from Fig. 4, winter O2 content of the blood is considerably elevated because of the increase in Hct occurring during hibernation (2). If the O2 binding curve had remained unchanged during winter (dotted line, Fig. 4), venous P02 would have been substantially elevated (~21.8 torr). However, a left-shift in the Hb-O2 binding curve may avoid this situation and maintain PvO2 of hibernating brown bears relatively unchanged (summer ~14.8 and winter ~11.5 torr) (Fig. 4).

**DISCUSSION**

In this study of free-ranging brown bears, we found a marked left-shift of the O2 equilibrium curve during hibernation, which was associated with an increase in the Hb-O2 affinity and a decrease in cooperativity. We demonstrated that the differences between hibernation and active state can be attributed to the presence of an additional allosteric Cl- binding site on the β subunit between residues Lys88β and Lys76β (11, 12). Limited effect of temperature on Hb-O2 binding facilitates O2 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 O2 binding curve, as shown here by us and earlier by others (11). The calculated heat of oxygenation (∆H) values (~8.5 ± 0.8 summer and ~8.7 ± 1.5 kcal/mol winter) were consistent with previous ∆H data reported for bear Hb, with values ranging from ~7 to ~8.5 kcal/mol (6, 11). Temperature sensitivity of O2 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 P50 of hibernating bear hemolysates. We found that a significant part of the observed shift of the O2 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 O2 affinity was found in the brown bears (Fig. 1).

By binding to a specific site (including His2β, Lys82β, and His143β 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-O2 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
tetramer ratios in summer bears (~2:1) than in humans (~1:1) may indicate some differences in the regulation of DPG as a red blood cell metabolite.

Calculations show that the hibernation-associated elevation in blood O2 affinity and decrease in cooperativity of O2 binding 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 PVo2 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.

ACKNOWLEDGMENTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


Paper II

Phenotypic plasticity in blood-oxygen transport in highland and lowland deer mice

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**RESEARCH ARTICLE**

Phenotypic plasticity in blood–oxygen transport in highland and lowland deer mice

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**SUMMARY**

In vertebrates living at high altitude, arterial hypoxemia may be ameliorated by reversible changes in the oxygen-carrying capacity of the blood (regulated by erythropoiesis) and/or changes in blood–oxygen affinity (regulated by allosteric effectors of hemoglobin function). These hematological traits often differ between taxa that are native to different elevational zones, but it is often unknown whether the observed physiological differences reflect fixed, genetically based differences or environmentally induced acclimatization responses (phenotypic plasticity). Here, we report measurements of hematological traits related to blood–O₂ transport in populations of deer mice (Peromyscus maniculatus) that are native to high- and low-altitude environments. We conducted a common-garden breeding experiment to assess whether altitude-related physiological differences were attributable to developmental plasticity and/or physiological plasticity during adulthood. Under conditions prevailing in their native habitats, high-altitude deer mice from the Rocky Mountains exhibited a number of pronounced hematological differences attributable to developmental plasticity and/or physiological plasticity during adulthood. However, these differences disappeared after 6 weeks of acclimation to normoxia at low altitude. The measured traits were also indistinguishable between the F₁ progeny of highland and lowland mice, indicating that there were no persistent differences in phenotype that could be attributed to developmental plasticity. These results indicate that the naturally occurring hematological differences between highland and lowland mice are environmentally induced and are largely attributable to physiological plasticity during adulthood.

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Key words: physiological plasticity, high altitude, hemoglobin, hematocrit, hypoxia, Peromyscus maniculatus, red blood cell.

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**INTRODUCTION**

In air-breathing vertebrates living at high altitude, the reduced O₂ tension of inspired air requires compensatory physiological adjustments to ensure an adequate tissue O₂ supply. Some of these adjustments involve reversible changes in O₂ transport by red blood cells (RBCs), which is a function of the O₂-carrying capacity of the blood (regulated by erythropoiesis) and blood–O₂ affinity [regulated by allosteric effectors of hemoglobin (Hb) function]. Mammals that are native to high-altitude environments are typically characterized by a suite of hematological and vascular traits that include an elevated blood–O₂ affinity, a normal or slightly increased hematocrit (Hct), and increased muscle capillary density (Hall et al., 1936; Chiodi, 1962; Bullard et al., 1966; Bullard, 1972; Monge and León-Velarde, 1991; Weber, 1995; Weber, 2007; Storz et al., 2010b; Mairbäurl and Weber, 2012). In comparisons between species or conspecific populations that are native to different elevational zones, common-garden and/or reciprocal-transplant experiments are required to assess whether observed physiological differences reflect fixed, genetically based differences or environmentally induced acclimatization responses (phenotypic plasticity). In the latter case, the acclimatization response may involve irreversible changes in phenotype (reflecting developmental plasticity) and/or reversible changes during adulthood (physiological plasticity or ‘phenotypic flexibility’) (Piersma and Drent, 2003). Regardless of the ontogenetic stage at which the plasticity is manifest, it is generally an open question as to whether a given change in phenotype represents an adaptive acclimatization response that has evolved under the influence of natural selection (Kingsolver and Huey, 1998; Huey et al., 1999; Woods and Harrison, 2002; Ghalambor et al., 2007).

If physiological differences between highland and lowland natives are attributable to adaptive phenotypic plasticity, then it prompts the question of why traits that are characteristic of hypoxia-tolerant high-altitude species are often not congruent with the typical acclimatization response to hypoxia in lowland species (Monge and León-Velarde, 1991; Storz et al., 2010b). Lowland mammals that are not genetically adapted to environmental hypoxia typically respond to chronic O₂ deprivation with an increased erythropoietic activity (resulting in correlated increases in Hb concentration and Hct) and a decreased blood–O₂ affinity [mediated by an increased RBC concentration of 2,3-diphosphoglycerate (DPG), an allosteric effector that reduces Hb–O₂ affinity] (Lenfant et al., 1968; Baumann et al., 1971; Duhm and Gerlach, 1971; Mairbäurl et al., 1993; Quatrini et al., 1993). DPG directly reduces Hb–O₂ affinity by preferentially binding and stabilizing deoxygenated Hb, thereby shifting the allosteric equilibrium in favor of the low-affinity ‘T-...
state’ quaternary structure. The hypoxia-induced increase in the molar DPG/Hb ratio also indirectly reduces Hb–O2 affinity by altering the Donnan equilibrium across the RBC membrane, as the increased intracellular concentration of non-diffusible anions leads to an associated influx of hydrogen ions, thereby enhancing the Bohr effect (Duhm, 1971; Samaja and Winslow, 1979).

Within limits, an increased Hb concentration may enhance tissue O2 delivery under hypoxia because the associated increase in arterial O2 content can compensate for a reduced O2 saturation. However, results of several empirical and theoretical studies suggest that increasing the Hb concentration of partially saturated blood is not an ideal long-term solution to the problem of chronic hypoxia because the associated increase in blood viscosity produces an elevated peripheral vascular resistance that can compromise cardiac output (Guyton and Richardson, 1961; Bullard, 1972; McGrath and Weil, 1978; Winslow and Monge, 1987; Monge and León-Velarde, 1991; Connes et al., 2006; Schuler et al., 2010; Storz, 2010). Studies of humans at high altitude have suggested that the optimal Hb concentration at rest and at exercise may actually be quite close to the typical sea level value (Winslow, 1988; Villafuerte et al., 2004), or perhaps only slightly higher (Reeves and León-Velarde, 2004), and it is well documented that excessive polycythemia is a causal factor in the development of chronic mountain sickness (Winslow et al., 1985; Winslow and Monge, 1987; Monge and León-Velarde, 1991; Rivera-Ch et al., 2007).

The adaptive significance of hypoxia-induced reductions in blood–O2 affinity depends on the severity of hypoxia as well as the physiological attributes. Under conditions of severe hypoxia, theoretical and experimental results indicate that an increase in RBC DPG concentration and the concomitant decrease in Hb–O2 affinity will generally have detrimental effects on tissue oxygenation because the reduced arterial O2 saturation more than offsets any benefit of increased O2 unloading in the peripheral circulation (Turek et al., 1973; Eaton et al., 1974; Bencowitz et al., 1982; Willford et al., 1982).

Some of the best opportunities for examining plasticity in traits associated with hypoxia tolerance are provided by studies of population-level variation in species that are distributed across steep altitudinal gradients. One such species is the deer mouse (Peromyscus maniculatus), which has the broadest altitudinal distribution of any North American mammal (Hock, 1964). As deer mice are continuously distributed from sea level to elevations above 4300 m, this species has proven to be an exemplary subject for research on mechanisms of adaptation and acclimatization to high-altitude hypoxia. An extensive body of work has documented that deer mice native to high altitude have elevated blood–O2 affinities relative to lowland conspecifics, and these differences are largely attributable to allelic differences in Hb–O2 affinity (Snyder, 1982; Snyder et al., 1982; Snyder, 1985; Chappell and Snyder, 1984; Chappell et al., 1988; Storz, 2007; Storz et al., 2009; Storz et al., 2010a). Additional evidence that allelic variation in Hb function contributes to local adaptation is provided by tests of whole-organism physiological performance involving wild-derived strains that express different Hb variants (Chappell and Snyder, 1984; Chappell et al., 1988; Hayes and Chappell, 1990) and population genetic analyses of variation in the underlying globin genes (Snyder et al., 1988; Storz, 2007; Storz et al., 2007; Storz and Kelly, 2008; Storz et al., 2007; Storz et al., 2009; Storz et al., 2012a). In light of this evidence for adaptive, genetically based differences in Hb–O2 affinity between deer mouse populations that are native to different elevational zones, it is also of interest to assess the role of phenotypic plasticity in modulating aspects of blood–O2 transport. Here, we report measurements of hematological traits related to blood–O2 transport capacity in populations of deer mice that are native to high- and low-altitude environments. The main objectives were (i) to characterize altitude-related differences in hematological traits, and (ii) to assess what fraction of the observed trait differences are attributable to phenotypic plasticity.

MATERIALS AND METHODS

Animals

In July–August of 2010 and 2011, we collected a total of 118 deer mice, P. maniculatus (Wagner 1845), from a high-altitude locality in the Southern Rocky Mountains and a low-altitude locality in the Great Plains, 770 km to the East. We collected 58 highland mice from the summit of Mount Evans (Clear Creek County, CO, USA; 39°35′18″N, 105°38′38″W, elevation 4350 m above sea level) and 60 lowland mice from the prairie grassland of eastern Nebraska (Nine Mile Prairie, Lancaster County, NE, USA; 40°52′12″N, 96°48′20″3″W, elevation 430 m above sea level). All mice were captured using Sherman live traps baited with peanut butter and oats. We drew approximately 200 μl of blood from the maxillary vein of each mouse using a 5 mm Goldenrod lancet (MEDpoint Inc., Mineola, NY, USA).

A subset of 46 mice (24 highland and 22 lowland) were transferred to a common-garden environment at the Animal Research Facility at the University of Nebraska (elevation 300 m) where they were allowed to acclimate for 6 weeks. All mice were maintained at a constant temperature (25°C) and on a standard light:dark cycle (12h:12h) for the duration of the experiment. During the 6-week acclimation period all mice were offered a standard diet ad libitum (Harlan Teklad Rodent Chow, Indianapolis, IN, USA). After the acclimation period, we again drew blood from each mouse as described above, for repeat measurements of the same hematological traits. We also conducted crosses between wild-caught mice from the same collection locality and we reared the resultant F1 progeny at the University of Nebraska and the University of Illinois under the same common-garden conditions as described above. A total of 71 F1 mice (N=48 and 23 descendants of highland and lowland nates, respectively) were phenotyped after they reached a minimum age of 66 days. All experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Nebraska (IACUC no. 522) and the University of Illinois (IACUC no. 10244).

Measurement of hematological traits

We measured Hb concentration in whole blood using a HemoCue Hb 201+ analyzer, following the manufacturer’s protocol (HemoCue AB, Angelholm, Sweden). We measured Hct as the volume of packed RBCs relative to total blood volume in a heparinized capillary tube that was spun at 13,600 g for 5 min in a ZPcrot centrifuge (IW Scientific Inc., Lawrenceville, GA, USA). We also calculated mean cell Hb concentration, MCHC (=Hb concentration×100/Hct). To measure RBC size, we used a Zeiss Axiosplan 2 imaging microscope (Carl Zeiss, Gottingen, Germany) to measure the diameter of 10 RBCs per sample.

RBC DPG concentrations were determined spectrophotometrically using a DPG kit, following the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN, USA), with the exception that we used sample volumes of 100 μl whole blood rather than 1 ml. We drew blood from a subset of 21 mice (N=10 and 11 highland and lowland mice, respectively). Blood was collected in chilled heparinized capillary tubes and was
immediately deproteinized with 500 μl of cold 0.6 mol l⁻¹ perchloric acid (HClO₄). Samples were centrifuged at 5000 r.p.m. for 10 min, and 400 μl of clear supernatant was then removed and neutralized with 50 μl of 2.5 mol l⁻¹ potassium carbonate (K₂CO₃). Samples were kept on ice for at least 60 min and were then centrifuged again at 5000 r.p.m. for 10 min. The supernatant was removed and immediately frozen in liquid nitrogen prior to the spectrophotometric analysis. The concentration of DPG in whole blood was estimated from the coupled reduction of NADH to NAD⁺ in the reaction assay at 340 nm (UVIKON 923 B Double Beam UV/VIS Spectrophotometer, Kontron Instruments, Milan, Italy). The reaction was completed after 25 min and absorbance did not change noticeably in later readings. Negative controls were run with each analysis and positive controls were performed with human blood.

**Statistical analysis**

For measures of Hb concentration in wild-caught mice (phenotyped at the site of capture) and for measures of all hematological traits in the F₁ progeny of wild-caught/lab-acclimated mice, we tested for altitude-related differences in phenotype using standard t-tests. For the subset of mice that were included in the common-garden acclimation experiment, we compared pre- and post-acclimation trait values using a repeated measures two-way ANOVA with native altitude (high versus low) and time (pre- versus post-acclimation) included as independent variables. In the analysis of trait variation in the common-garden F₁ mice, we controlled for variation in individual age (66–454 days) by performing an ANCOVA with native altitude as an independent variable and age as a covariate. We did not detect any significant differences between the sexes for any of the hematological traits, so data from males and females were pooled in all analyses. Similarly, we did not detect significant trait variation among sibships within each set of F₁ mice from high- and low-altitude, so data from all families were pooled. Kolmogorov–Smirnov (K–S) tests did not reveal any significant deviations from normality in any of the trait-specific data sets. All statistical analyses were conducted using the SAS software package (SAS Institute Inc., 2009) or VassarStats online statistical calculator (vassarstats.net).

**RESULTS**

Under the conditions prevailing in their natural habitats, the highland *Peromyscus maniculatus* exhibited a significantly higher Hb concentration than the lowland *Peromyscus maniculatus* [mean ± 1 s.d., 2.83±0.28 versus 2.33±0.32 mmol l⁻¹, respectively (Hb molecular mass 64.45 kDa); \(t_{116}=9.079, P<0.0001\); Fig. 1]. However, after 6 weeks of acclimation to normoxia in the common-garden laboratory environment, the mean Hb concentration of the highland mice dropped by 8.8% to 2.54±0.21 mmol l⁻¹ and was statistically indistinguishable from that of the lab-acclimated lowland mice (2.55±0.20 mmol l⁻¹; Table 1, Fig. 2A). The F₁ progeny of highland and lowland *Peromyscus maniculatus* also had Hb concentrations that were statistically indistinguishable (2.33±0.21 versus 2.29±0.24 mmol l⁻¹, respectively; \(t_{63}=0.739, P=0.489\)). Similar to the repeated-measures analysis of Hb concentration (Fig. 2A), an analysis of pre- and post-acclimation measures of Hct, MCHC, RBC size and RBC DPG concentration for the same subset of highland and lowland mice revealed similar degrees of plasticity (Fig. 2B–E). Highland deer mice exhibited a significantly higher Hct relative to lowland mice (59.67±4.83 versus 48.94±4.82%, respectively; Table 1, Fig. 2A), but after 6 weeks of acclimation to normoxia, the average Hct of the highland mice dropped by 17% such that post-acclimation values of the highland and lowland mice were statistically indistinguishable (49.77±3.35 versus 49.96±3.78%, respectively; Fig. 2B). Relative to the lowland mice, the highland mice had a lower MCHC (4.74±0.28 versus 5.05±0.45 mmol l⁻¹, respectively) and a smaller average RBC size (5.38±0.12 versus 5.72±0.41 μm, respectively), but these differences disappeared after acclimation to normoxia (Fig. 2C,D). The highland mice exhibited a significantly higher RBC DPG concentration (2.16±0.44 versus 1.47±0.39 mmol l⁻¹; Table 1, Fig. 2E), but as with MCHC and RBC size, this difference disappeared after acclimation to normoxia. For each of the hematological traits, the ‘in situ’ measurements of wild-caught mice were consistent with data from previous studies of *Peromyscus* mice (Fig. 3).

Similar to the case with Hb concentration, the F₁ progeny of highland and lowland parents did not exhibit significant differences in Hct (49.20±3.21 versus 50.00±4.26%; \(t_{50}=0.733, P=0.528\)), MCHC (4.81±0.34 versus 4.84±0.44 mmol l⁻¹; \(t_{50}=-0.228, P=0.841\)),

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Tests were carried out using a repeated-measures, two-way ANOVA. Hb, hemoglobin; Hct, hematocrit; MCHC, mean corpuscular Hb concentration; RBC, red blood cell; DPG, 2,3-diphosphoglycerate.
RBC size (5.57±0.28 versus 5.45±0.21 µm; \(t_{23}=1.380, P=0.126\)) or DPG/Hb ratio (2.45±0.51 versus 2.24±0.61; \(t_{23}=0.858, P=0.458\)). These results indicate that the naturally occurring hematological differences between highland and lowland mice are environmentally induced and are largely attributable to physiological plasticity during adulthood.

**DISCUSSION**

Under conditions prevailing in their native habitats, highland deer mice from the summit of Mount Evans exhibited a number of pronounced hematological differences relative to lowland conspecifics from the Great Plains. In comparison with lowland mice, the highland mice exhibited higher Hb concentrations, higher Hcts, higher DPG/Hb ratios, lower MCHC values and smaller RBCs. However, these differences disappeared after 6 weeks of acclimation to normoxia at low altitude (Fig. 2). The measured traits were also indistinguishable between the F1 progeny of highland and lowland conspecifics that have no known evolutionary history of residence at high altitude (Monge and León-Velarde, 1991). The elevated Hb concentrations and Hcts observed in the highland deer mice are consistent with results of previous studies of *P. maniculatus* (Gough and Kilgore, 1964; Hock, 1964; Sealander, 1964; Dunaway and Lewis, 1965; Thompson et al., 1966; Sawin, 1970; Snyder, 1982; Snyder et al., 1982; Wyckoff and Frase, 1990; Hammond et al., 1999; Hammond et al., 2001) (supplementary material Table S1; Fig. 3). By contrast, most rodent species that are native to high altitudes appear to have Hb concentrations and Hcts that are substantially lower than those of hypoxia-acclimated laboratory rats or house mice (Hall et al., 1936; Chiodi, 1962; Morrison et al., 1963a; Morrison et al., 1963b; Bullard et al., 1966) and hematological traits in the majority of high-altitude Andean rodents studied by Morrison and colleagues remained unaltered after acclimation to normoxic conditions at sea level (Morrison et al., 1963a; Morrison et al., 1963b).

In humans, highlanders that are indigenous to the Tibetan and Ethiopian plateaus exhibit low Hb concentrations relative to Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010). The high Hb concentrations of Andean highlanders that are resident at comparable altitudes (Beall and Reischsman, 1984; Winslow et al., 1989; Beall et al., 1990; Beall et al., 1998; Beall et al., 2002; Garruto et al., 2003; Wu et al., 2005; Beall, 2006; Beall, 2007; Beall et al., 2010; Simonson et al., 2010).
The elevated Hb concentrations of highland deer mice mirror the acclimatization response to hypoxia in lowland species (e.g. Baumann et al., 1971; Duhn and Gerlach, 1971), suggesting that the highland deer mice have a hematological profile that is more similar to that of Andean humans than to that of Tibetans or Ethiopians. However, additional experiments are required to assess whether highland and lowland mice differ in their acclimation responses to hypoxia, and it is an open question as to whether the elevated Hb concentration of high-altitude deer mice represents an example of adaptive or maladaptive plasticity.

In addition to the potentially adverse effects of increased blood viscosity on cardiac output, an increased Hct can also diminish plasma transport of free fatty acids and other metabolic fuels (McClelland, 2004). This is because the fuel transport capacity of blood plasma is determined by the product of plasma flow and fuel concentration, and plasma flow is an inverse function of Hct. In deer mice living at high altitude, the effects of elevated Hct on plasma fuel transport may be especially significant because deer mice rely heavily on fatty acid oxidation to fuel shivering thermogenesis (Cheviron et al., 2012).

The elevated RBC DPG concentrations observed in highland deer mice are mostly consistent with previous reports. Snyder measured RBC DPG concentrations in deer mice sampled from localities ranging from 2590 to 4340 m in the White Mountains of eastern California (Snyder, 1982). Although the DPG/Hb ratio was somewhat reduced in the high-altitude sample of mice from 4340 m, there was no monotonic altitudinal trend, as DPG/Hb ratios were highest in mice from intermediate elevations. Similar to the results reported by Snyder (Snyder, 1982), our common-garden experiment revealed a reversal in relative DPG concentrations such that the highland mice actually exhibited a slightly lower baseline DPG/Hb ratio after acclimation to normoxia (Fig. 2E). If the reduced baseline DPG/Hb ratio and elevated Hb–O2 affinity of highland deer mice are construed as adaptive mechanisms for maintaining an elevated blood–O2 affinity under hypoxia, then the hypoxia-induced increase in RBC DPG would seem to be counterproductive (Storz et al., 2010b).

In considering this seemingly maladaptive acclimatization response, Snyder suggested that physiological constraints of RBC energy metabolism may prevent evolutionary modifications of intracellular DPG levels as the formation of DPG is stimulated by overall glycolytic activity (Snyder, 1982). However, given that the optimal blood–O2 affinity is a non-linear function of ambient $P_{O_2}$ (Turek et al., 1973; Eaton et al., 1974; Bencowitz et al., 1982; Willford et al., 1982), empirical performance curves are needed to evaluate whether hypoxia-induced reductions in the DPG/Hb ratio are advantageous or disadvantageous at a given altitude. As changes in the DPG/Hb ratio also affect intracellular pH and CI– concentration, and as the adult Hbs of deer mice and other muroid rodents have ligand affinities that are more strongly modulated by CI– ions than by DPG (Storz et al., 2009; Storz et al., 2010a; Storz et al., 2012b; Runck et al., 2010), the net effects on blood–O2 affinity are difficult to predict. Moreover, if deer mice have a hyperventilatory response to hypoxia, as in other mammals, the resultant increase in plasma pH (respiratory hypocapnia) may effectively counterbalance the inhibitory effects of DPG on Hb–O2 affinity (Mairbäurl and Weber, 2012).

In contrast to the hematological changes that are typically associated with the acclimatization response to hypoxia in lowland mammals, genetically based changes in Hb structure that increase intrinsic O2 affinity or that suppress sensitivity to allosteric effectors are generally thought to make more important contributions to hypoxia tolerance in species that are high-altitude natives (Bunn, 1980; Monge and León-Velarde, 1991; Storz, 2007; Weber, 1995; Weber, 2007; Storz and Moriyama, 2008; Storz et al., 2010b; Mairbäurl and Weber, 2012). High-altitude deer mice seem to defy Monge and León-Velarde’s proposed dichotomy between the hypoxia response strategies of highland and lowland natives (Monge and León-Velarde, 1991), as they possess a set of hematological traits that appear to fit both profiles. On the one hand, highland deer mice exhibit elevated Hb concentrations, Hcts and RBC DPG concentrations relative to lowland mice (Hock, 1964; Savin, 1970; Snyder, 1982; Snyder et al., 1982), characteristics that seem to fit the profile of a hypoxia-acclimated lowland species. On the other hand, highland deer mice possess structurally distinct Hbs with slightly elevated O2 affinity (Storz et al., 2009; Storz et al., 2010a), which fits the profile of a genetically adapted, hypoxia-tolerant highland species.

In the case of highly labile hematological traits like Hb concentration, Hct and RBC DPG concentration that are responsive to minute changes in arterial $P_{O_2}$ and acid–base balance, it is probably often the case that altitude-related trait differences between species or between conspecific populations are purely attributable to physiological plasticity, reflecting hypoxia-induced regulatory changes in erythropoietic activity and RBC metabolism. The high degree of plasticity that we have documented for hematological traits in deer mice highlights the importance of using common-garden experiments to assess whether physiological differences between species or conspecific populations represent reversible, constitutively expressed traits or irreversible, fixed differences.


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Paper III

Hemoglobin function and allosteric regulation in semi-fossorial rodents (family Sciuridae) with different altitudinal ranges

Hemoglobin function and allosteric regulation in semi-fossorial rodents (family Sciuridae) with different altitudinal ranges

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SUMMARY

Semi-fossorial ground squirrels face challenges to respiratory gas transport associated with the chronic hypoxia and hypercapnia of underground burrows, and such challenges are compounded in species that are native to high altitude. During hibernation, such species must also contend with vicissitudes of blood gas concentrations and plasma pH caused by episodic breathing. Here, we report an analysis of hemoglobin (Hb) function in six species of marmotine ground squirrels with different altitudinal distributions. Regardless of their native altitude, all species have high Hb–O₂ affinities, mainly due to suppressed sensitivities to allosteric effectors [2,3-diphosphoglycerate (DPG) and chloride ions]. This suppressed anion sensitivity is surprising given that all canonical anion-binding sites are conserved. Two sciurid species, the golden-mantled and thirteen-lined ground squirrel, have Hb–O₂ affinities that are characterized by high pH sensitivity and low thermal sensitivity relative to the Hbs of humans and other mammals. The pronounced Bohr effect is surprising in light of highly unusual amino acid substitutions at the C-termini that are known to abolish the Bohr effect in human HbA. Taken together, the high O₂ affinity of sciurid Hbs suggests an enhanced capacity for pulmonary O₂ loading under hypoxic and hypcapnic conditions, while the large Bohr effect should help to ensure efficient O₂ unloading in tissue capillaries. In spite of the relatively low thermal sensitivities of the sciurid Hbs, our results indicate that the effect of hypothermia on Hb oxygenation is the main factor contributing to the increased blood–O₂ affinity in hibernating ground squirrels.

Key words: adaptation, allostery, globin, hibernation, hypoxia.

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INTRODUCTION

Fossorial and semi-fossorial mammals face challenges to respiratory gas transport associated with the chronic hypoxia and hypercapnia of underground burrows (Boggs et al., 1984). At high altitude, the hypoxic conditions of underground burrows are further compounded by the reduced partial pressure of atmospheric O₂ (P₀₂). In mountain ranges of western North America, burrow-dwelling rodents in the squirrel family (Sciuridae) are common denizens of the alpine and subalpine zones, most notably the golden-mantled ground squirrel (Callospermophilus lateralis) and the yellow-bellied marmot (Marmota flaviventris), which occur at the highest possible elevations in the contiguous United States (Armstrong, 1972; Chappell and Dlugosz, 2009). In addition to the hypoxic challenges associated with life underground and life at high altitude, many sciurid rodents face additional gas transport challenges associated with depressed ventilation and episodic breathing during winter hibernation (Milsom, 1992; Milsom and Jackson, 2011; Webb and Milsom, 1994). In hibernating ground squirrels, prolonged periods of apnea can result in wide fluctuations in arterial P₀₂ and plasma pH (Maginniss and Milsom, 1994; Malan et al., 1973; Musacchia and Volkert, 1971).

In mammals adapted to life underground or life at high altitude, an elevated blood–O₂ affinity can enhance pulmonary O₂ loading to compensate for the reduced P₀₂ of inspired air (Eaton, 1974; Eaton et al., 1974; Turek et al., 1973). Likewise, in hibernating mammals, a left-shifted blood–O₂ equilibrium curve can help safeguard arterial O₂ saturation during apneic periods and prevent unfavorable O₂ release to tissues (Maginniss and Milsom, 1994; Milsom and Jackson, 2011; Revsbech et al., 2013). An increase in blood–O₂ affinity can be achieved by changes in the intrinsic O₂ affinity of hemoglobin (Hb), changes in the sensitivity of Hb to allosteric effectors and/or changes in the concentrations of allosteric effectors within the erythrocyte. In mammalian red cells, the main organic phosphate effector, 2,3-diphosphoglycerate (DPG), decreases Hb–O₂ affinity by binding stereochemically at specific positively charged residues in the central cavity of deoxygenated Hb: β₁Val, β₂His, β₈₂Lys and β₁₄₃His (Arnone, 1972). The resultant stabilization of deoxy (T-state) Hb shifts the allosteric equilibrium between the low-affinity T-state and the high-affinity R-state in favor of the low-affinity quaternary structure (Perutz, 1970). Other factors that modulate Hb–O₂ affinity are Cl– ions (Chiancone et al., 1972) and temperature (Antonini and Brunori, 1971), as well as protons and CO₂, which also bind preferentially to deoxy-Hb and promote O₂ unloading in the tissue capillaries via the Bohr effect (Perutz, 1983; Weber and Fago, 2004). Increases in blood–O₂ affinity during winter hibernation have been documented in a number of mammals, and such changes are generally attributable to reductions in red cell DPG, increases in pH and/or decreased body temperature (Burlington and Whitten, 1971; Maginniss and Milsom, 1994; Musacchia and Volkert, 1971; Revsbech et al., 2013; Tempel and Musacchia, 1975). To assess the relative efficacy of different mechanisms for increasing blood–O₂ affinity during
hibernation, experiments on purified Hbs are required to evaluate the independent and joint effects of DPG, pH and temperature on Hb–O2 affinity under controlled conditions.

Here, we report an examination of the structural and functional properties of Hb in six species of marmotine ground squirrels (subfamily Xerinae, tribe Marmotini): golden-mantled ground squirrel (*C. lateralis*) (Say 1823), yellow-bellied marmot (*M. flaviventris*) (Audubon and Bachman 1841), hoary marmot (*M. caligata*) (Eschscholtz 1829), thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (Mitchell 1821), Uinta chipmunk (*Tamias umbrinus*) (Allen 1890) and least chipmunk (*T. minimus*) (Allen 1890) and least chipmunk (*T. minimus*) (Allen 1890). All six species are semi-fossorial, winter hibernators that are native to North America. These six species inhabit a range of different elevations (Fig. 1). For example, the yellow-bellied marmot is a strictly montane species that mainly occurs at elevations of 2000–4300 m in the Rocky Mountains, the Sierra Nevada, and other mountain ranges in western North America (Armstrong, 1972). By contrast, the closely related hoary marmot is mostly restricted to elevations <2500 m in Alaska and western Canada (Braun et al., 2011). The golden-mantled ground squirrel, Uinta chipmunk and least chipmunk are most common in the montane and subalpine zones, whereas the thirteen-lined ground squirrel is mainly restricted to low-elevation grassland (Fig. 1) (Armstrong, 1972; Streubel and Fitzgerald, 1978).

The objectives of this study were (i) to test the hypothesis that the elevated blood–O2 affinities of burrow-dwelling, hibernating ground squirrels are attributable to genetically based changes in Hb function (i.e., an increase in intrinsic Hb–O2 affinity and/or a decrease in sensitivity to allosteric effectors), (ii) to evaluate the efficacy of different allosteric effectors in regulating Hb–O2 affinity, and (iii) to test whether interspecific variation in Hb–O2 affinity is associated with native altitude.

**MATERIALS AND METHODS**

**Sampling**

We collected blood samples from wild-caught specimens of each species in July–August 2010 and 2011. Golden-mantled ground squirrels (*N* = 6) were collected from two localities in the Front Range of the southern Rocky Mountains: Boulder County, CO, USA (40°01′45″N, 105°50′77″W; 3103 m above sea level) and Grand County, CO, USA (38°84′37″N, 105°75′12″W; 2397 m). Yellow-bellied marmots (*N* = 5) were collected from near the summit of Mount Evans, Clear Creek County, CO, USA (39°15′24″N, 106°10′54″W; 4300 m), and hoary marmots (*N* = 6) were collected from two localities in central Alaska, viz., Eagle Summit, Yukon-Koyukuk County, AK, USA (65°29′8″N, 145°24′0″W; 1119 m) and Wickersham Dome, Fairbanks North Star County, AK, USA (65°12′40″N, 148°3′37″W; 915 m). Uinta chipmunks (*N* = 2) and least chipmunks (*N* = 6) were collected from Park County, CO, USA (39°34′71″N, 105°63′19″W; 2013 m) in addition to the same Grand County locality mentioned above. Thirteen-lined ground squirrels (*N* = 2) were live-trapped in Lancaster County, NE, USA (40°52′12″N, 96°48′20″W; 430 m).

Animals were handled in accordance with guidelines approved by the University of Nebraska Institutional Animal Care and Use Committee (IACUC no. 07-07-030D and 519) and the National Institutes of Health (NIH publication no. 78-23). The thirteen-lined ground squirrels were anesthetized using isoflurane and blood was drawn from the femoral vein. Blood was collected in cryotubes, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen blood samples were used as a source of Hb for experimental studies and reticulocytes served as a source of globin mRNA for cDNA sequencing. The animals were released at the site of capture after they had regained consciousness. In the remaining species, blood was collected *via* cardiac puncture immediately after each animal was killed and the blood samples were processed and stored as described above. Additionally, bone marrow was collected from the femurs of each animal as a source of globin mRNA. Bone marrow was preserved in RNA later (Qiagen, Valencia, CA, USA) and stored at −80°C prior to RNA extraction. With the exception of the thirteen-lined ground squirrels, all animals were prepared as vouchered museum specimens and were deposited in the vertebrate collections of the Denver Museum of Nature and Science (Denver, CO, USA; catalog numbers 11959–11962, 11967–11969, 11979–11983, 11989–11990), the University of Alaska Museum of the North (Fairbanks, AK, USA; catalog numbers AF71385, AF71391–AF71395), and the University of Nebraska State Museum (Lincoln, NE, USA; catalog numbers NP1203–NP1207).

**Globin sequencing**

After annotating the α- and β-globin genes from the genome assembly of the thirteen-lined ground squirrel and other rodents (Hoffmann et al., 2008a; Hoffmann et al., 2008b; Opazo et al., 2008), we designed locus-specific primers for 5′ and 3′ RACE (rapid amplification of cDNA ends; Invitrogen Life Technologies, Carlsbad, CA, USA) to obtain cDNA sequence for the 5′ and 3′ untranslated regions (UTRs) of each adult-expressed globin gene. After designing paralog-specific PCR primers with annealing sites in the 5′ and 3′ UTRs, complete cDNAs were synthesized for each gene by reverse-transcriptase PCR (RT-PCR) using the OneStep
RT-PCR kit (Qiagen). We performed RT-PCR on RNA template to amplify α- and β-globin cDNAs, which were then cloned and sequenced. Total RNA was extracted from reticulocytes or hematopoietic bone marrow cells using the RNaseq Plus Mini Kit (Qiagen), and cDNA was generated from 1 μg RNA by reverse transcription. For RT-PCR, we used the SuperScript III Platinum One-Step RT-PCR system with Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were as follows: one cycle at 50°C for 10 min, followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 2 min, and a final extension cycle at 68°C for 7 min. We then cloned gel-purified RT-PCR products into pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). For each individual ground squirrel specimen, we sequenced a total of 20 clones containing products of paralog-specific RT-PCR (10 cloned α-globin cDNAs and 10 cloned β-globin cDNAs per individual). Thus, full-length inserts representing cDNAs of the adult-expressed squirrel specimen, we sequenced a total of 20 clones containing products of paralog-specific RT-PCR (10 cloned α-globin cDNAs and 10 cloned β-globin cDNAs per individual). Thus, full-length inserts representing cDNAs of the adult-expressed α- and β-globin genes were sequenced at 10× coverage. PCR and RT-PCR primers for all species are available upon request. All DNA sequences were deposited in GenBank under the accession numbers KF010591–KF010606, KF153033–KF153050.

Analysis of Hb isoform composition

Hemolysates were prepared by adding a 3- to 5-fold volume of ice-cold 10 mmolL−1 Hepes pH 7.8 to frozen blood. The red supernatant was cleared by centrifugation and stripped from endogenous organic phosphates using a mixed-bed resin (MB-1, AG501-X8, Bio-Rad, Hercules, CA, USA) (Storz et al., 2012). The Hb isoform (isoHb) composition of hemolysates from each specimen was analyzed by isoelectric focusing (IEF, pH5–8) using polyacrylamide gels (Phastgel, GE Healthcare Biosciences AB, Uppsala, Sweden). After separation of isoHbs by means of IEF, bands excised from each gel were digested with trypsin, and the resultant peptides were identified by means of tandem mass spectrometry (MS/MS). The peak lists of the MS/MS data were generated by Distiller (Matrix Science, London, UK) using the charge state recognition and de-isotoping with default parameters for quadrupole time-of-flight data. Database searches of the resultant MS/MS spectra were performed using Mascot v1.9.0 (Matrix Science). Specifically, peptide mass fingerprints derived from the MS/MS analysis were used to query a customized database of α- and β-globin amino acid sequences (based on conceptually translated DNA sequences) from each of the six sciurid species included in the study, including the full complement of pre- and post-natally expressed globin genes from the thirteen-lined ground squirrel genome assembly. The following search parameters were used: no restriction on protein molecular weight or isoelectric point, and methionine oxidation allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses. We identified all significant protein hits that matched more than one peptide with P<0.05.

O2 equilibrium measurements

For all species, O2 equilibria were measured on 3 μl samples of purified hemolysate (0.2–0.3 mmolL−1 heme, 0.1 mmolL−1 Hepes) at pH 7.4 and 37°C using a thermostatted thin-layer chamber technique (Sick and Gersonde, 1969; Weber, 1992). Curves were measured for each sample in the absence (stripped hemolysate) and presence of DPG (DPG/Hb tetramer ratio=2.0), Cl− ions (0.1 mmolL−1 KCl) and of both anionic effectors as described previously (Storz et al., 2010a; Storz et al., 2012). As a measure of Hb–O2 affinity the P50 for 50% heme saturation (P50) and the corresponding cooperativity coefficient (n50) were calculated by linear regression (r2≥0.99) from the zero intercept and slope, respectively, of Hill plots {log[Y/(1−Y)] versus log P50, where Y is the fractional saturation} fitted to at least four saturation steps between 20 and 80%.

The sensitivity of Hb–O2 affinity to changes in pH (Bohr effect) and temperature were examined in the golden-mantled ground squirrel and in the thirteen-lined ground squirrel. These species were chosen to compare the functional consequences of unusual amino acid substitutions (see Results) found in the β-chain Hbs of the golden-mantled ground squirrel (a highland native) and the thirteen-lined ground squirrel (a lowland native). For the golden-mantled ground squirrel Hb, O2-binding curves were also measured in duplicate in the absence (stripped) and presence of DPG as described above at three different pH values (range 7.0–8.0, 37°C) (to assess the Bohr effect) and three different temperatures (range 23–37°C, pH 7.4; to measure the temperature effect). For thirteen-lined squirrels, only one of the two individual hemolysates was suitable for functional studies (N=1) and, because of limited sample availability, measures of pH and temperature sensitivity for the stripped Hbs of this species were based on two pH values (7.0 and 7.4) and two temperatures (23 and 37°C). The Bohr effect was quantified as φ=ΔlogP50/ΔpH (Bohr factor) by linear regression (r2≥0.99 for golden-mantled ground squirrel Hb) of the logP50 versus pH plot. The temperature dependence of O2 binding, expressed as the apparent heat of oxygenation (AH, kcal mol−1; 1 kcal=4.184 kJ, was derived by linear regression (r2≥0.99 for golden-mantled ground squirrel Hb) of the van’t Hoff plot ΔH=2.303R(ΔlogP50)/ Δ(1/T), where R is the gas constant (1.987 cal K−1 mol−1) and T is the absolute temperature (K). The ΔH values here reported were corrected for heat of O2 in solution (−3.0 kcal mol−1) (Antonini and Brunori, 1971).

RESULTS

The IEF analysis revealed that each of the marmotine ground squirrels expressed one to three isoHbs, with one major isoHb (isoelectric point=7.2–7.4) that typically accounted for 50–65% of the total (Fig. 2). Combined results of the MS/MS analysis and cDNA sequence analysis revealed that isoHb heterogeneity is attributable to the expression of two to three structurally distinct β-globin chains (except for the Uinta chipmunk, which co-expresses two distinct α-chain isoforms) (Fig. 3). Multiple alignments of α- and β-globin amino acid sequences from each of the study species (Fig. 3) revealed unusual substitutions at highly conserved residue positions. Specifically, the golden-mantled ground squirrel, thirteen-lined ground squirrel, Uinta chipmunk and least chipmunk share an unusual substitution at the C-terminus of the α-chain, α141Arg→Cys, and the major β-chain of the golden-mantled ground squirrel has an unusual substitution at the C-terminus, β146His→Gln (Fig. 3). Both C-terminus residues are known to contribute to the pH sensitivity of Hb–O2 binding (the Bohr effect) in human Hb (Perutz, 1970; Weber and Fago, 2004; Perutz, 1983; Berenbrink, 2006; Fang et al., 1999; Lukin and Ho, 2004). The golden-mantled ground squirrel also has a highly unusual substitution at another highly conserved β-chain position, β55Met→Ile (Fig. 3). In each species, the α- and β-chain tryptic peptides of the major isoHb yielded highly significant matches to the translated cDNA sequences from the same individual specimens. The MS/MS analysis confirmed the presence of the unusual C-terminal residues mentioned above. The MS/MS analysis did not reveal the presence of any major subunit isoforms that were not already characterized at the DNA level.
The experimental measures of Hb function performed on the hemolysates under physiologically relevant conditions revealed that the six sciurid species have overall similar Hb–O2 affinities (i.e. \( P_{50} \) values) in the presence and absence of allosteric effectors (Table 1). Sensitivities to DPG and Cl\(^-\) ions, as indexed by the difference in \( \log P_{50} \) values for stripped samples in the presence both effectors, were in the range 0.13–0.35, with the exception of thirteen-lined squirrel Hb from a single individual, which showed a greater sensitivity of 0.45 (Table 1). Under similar experimental conditions, the measured anion sensitivity of human HbA was reported as \( \Delta \log P_{50(\text{DPG+KCl})} - \log P_{50} \approx 0.4 \) (Weber, 1992). Thus, Hbs of the marmotine ground squirrels have generally low anion sensitivities relative to human HbA (see also Table 1). The Hill coefficients \( (n_0) \) were in the range 1.8–2.9 under all conditions, indicating cooperative O2 binding and intact homotropic interactions (Table 1).

Measures of the pH sensitivity of Hb–O2 affinity revealed substantial Bohr effects for the Hbs of the golden-mantled and thirteen-lined ground squirrels, with Bohr factors \( (\alpha) \) of 0.57–0.70 and 0.56 (Weber, 1992). The small changes in the Bohr factor observed upon addition of DPG \( (\alpha) \) were similar to that of the thirteen-lined ground squirrel Hb. The small changes in the Bohr factor observed upon addition of DPG \( (\alpha) \) are consistent with the suppressed anion sensitivity of Hb–O2 affinity in these species (Table 1). Because the Bohr factor represents the number of additional protons bound per O2 molecule (i.e. the number of additional protons released upon oxygenation if the Bohr factor is negative), these values indicate the presence of two to four O2-linked proton-binding sites per Hb tetramer in the T-state. For comparison, under similar experimental conditions, the Bohr coefficient for stripped human HbA has been reported to be \(-0.56\) (Weber, 1992).

The temperature sensitivity of stripped Hb oxygenation at pH 7.4 (data not shown), expressed as the heat of oxygenation \( (\Delta H, \text{i.e. enthalpy}) \), was \(-6.8\text{kcal mol}^{-1}\) in the golden-mantled ground squirrel Hb and \(-3.3\text{kcal mol}^{-1}\) in the thirteen-lined ground squirrel Hb, whereas it is \(-14.3\text{kcal mol}^{-1}\) in human HbA (Weber, 1992). In the golden-mantled ground squirrel, the enthalpy of Hb oxygenation decreased in magnitude to \(-4.4\text{kcal mol}^{-1}\) in the presence of DPG, revealing the endothermic contribution of oxygenation-linked DPG dissociation. Representative O2-binding curves for golden-mantled ground squirrel Hb measured at different pH values and temperatures are shown in Fig. 4.

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**Table 1.** Sensitivities to DPG and Cl\(^-\) ions, as indexed by the difference in \( \log P_{50} \) values for stripped samples in the presence of both effectors, were in the range 0.13–0.35, with the exception of thirteen-lined squirrel Hb from a single individual, which showed a greater sensitivity of 0.45 (Table 1). Under similar experimental conditions, the measured anion sensitivity of human HbA was reported as \( \Delta \log P_{50(\text{DPG+KCl})} - \log P_{50} \approx 0.4 \) (Weber, 1992). Thus, Hbs of the marmotine ground squirrels have generally low anion sensitivities relative to human HbA (see also Table 1). The Hill coefficients \( (n_0) \) were in the range 1.8–2.9 under all conditions, indicating cooperative O2 binding and intact homotropic interactions (Table 1).

**Table 2.** Representative O2-binding curves for golden-mantled ground squirrel Hb measured at different pH values and temperatures are shown in Fig. 4.
Although Hb–O₂ affinities are often increased in mammals that are native to high altitude (Storz, 2007; Storz et al., 2010b; Weber, 2007), we found that the yellow-bellied marmot (a highland species) and hoary marmot (a predominantly lowland species) have Hbs with very similar O₂ affinities (Table 1). For the six sciurid species that we examined, linear regressions revealed a suggestive but non-significant positive relationship between Hb–O₂ affinity and native elevational range limit, 

\[ \Delta \log P_{50} = 0.13 \pm 0.04 \ \text{KCl–stripped} \]
\[ \Delta \log P_{50} = 0.15 \pm 0.04 \ \text{DPG–stripped} \]
\[ \Delta \log P_{50} = 0.16 \pm 0.04 \ \text{(K+DPG)–stripped} \]

Table 1. Mean Hb–O₂ affinities, cofactor sensitivities and cooperativity coefficients of purified hemolysates from six sciurid species

<table>
<thead>
<tr>
<th>Species</th>
<th>Golden-mantled ground squirrel</th>
<th>Hoary marmot</th>
<th>Yellow-bellied marmot</th>
<th>Thirteen-lined ground squirrel</th>
<th>Least chipmunk</th>
<th>Uinta chipmunk</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(N=6)</td>
<td>(N=4)</td>
<td>(N=4)</td>
<td>(N=1)</td>
<td>(N=6)</td>
<td>(N=2)</td>
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<tr>
<td><strong>P₅₀ (Torr)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Stripped</td>
<td>7.1±1.4</td>
<td>5.2±0.3</td>
<td>6.0±0.4</td>
<td>7.9</td>
<td>5.2±0.3</td>
<td>6.0±0.4</td>
</tr>
<tr>
<td>KCl</td>
<td>9.2±0.8</td>
<td>10.8±0.9</td>
<td>12.±1.5</td>
<td>11.1</td>
<td>8.7±0.4</td>
<td>11.2±1.8</td>
</tr>
<tr>
<td>DPG</td>
<td>9.8±0.9</td>
<td>12.2±0.8</td>
<td>10.7±0.5</td>
<td>11.2</td>
<td>9.0±0.5</td>
<td>10.1±2.3</td>
</tr>
<tr>
<td>KCl+DPG</td>
<td>9.8±0.9</td>
<td>11.7±0.8</td>
<td>11.9±1.4</td>
<td>13.1</td>
<td>9.3±0.6</td>
<td>11.3±2.2</td>
</tr>
<tr>
<td><strong>KCl–stripped</strong></td>
<td>0.13±0.04</td>
<td>0.32±0.02</td>
<td>0.31±0.03</td>
<td>0.38</td>
<td>0.10±0.02</td>
<td>0.18±0.07</td>
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<tr>
<td><strong>DPG–stripped</strong></td>
<td>0.15±0.04</td>
<td>0.37±0.01</td>
<td>0.25±0.04</td>
<td>0.39</td>
<td>0.12±0.02</td>
<td>0.13±0.10</td>
</tr>
<tr>
<td><strong>(K+DPG)–stripped</strong></td>
<td>0.16±0.04</td>
<td>0.35±0.01</td>
<td>0.30±0.03</td>
<td>0.45</td>
<td>0.13±0.03</td>
<td>0.18±0.08</td>
</tr>
</tbody>
</table>

Data are means (± s.d.) from golden-mantled ground squirrel (Callospermophilus lateralis), hoary marmot (M. caligata), yellow-bellied marmot (Marmota flaviventris), thirteen-lined ground squirrel (Ictidomys tridecimlineatus), least chipmunk (T. minimus) and Uinta chipmunk (Tamias umbrinus) Curves were measured at 0.2–0.3 mmol l⁻¹ heme in 0.1 mol l⁻¹ Hepes buffer at pH 7.4, 37°C, in the absence (stripped) and presence of added KCl (0.1 mol l⁻¹) and/or 2,3-diphosphoglycerate (DPG; DPG/Hb tetramer ratio, 2.0), as indicated. The sample size in parentheses (N) refers to the number of individuals analysed per species.

P₅₀, Hb–O₂ affinity (where 1 Torr=133 Pa); ΔlogP₅₀(x-stripped), cofactor-induced change in logP₅₀ (where x is the cofactor); n₅₀, cooperativity coefficient.

The elevated Hb–O₂ affinities of the marmotine ground squirrels are attributable to a generalized suppression of anion sensitivity combined with a high intrinsic Hb–O₂ affinity (i.e. of the stripped Hb). In conjunction with results from other recent studies (Clementi et al., 2003; Natarajan et al., 2013; Storz et al., 2009; Storz et al., 2010a; Storz et al., 2012; Weber, 1992), the data summarized in Table 1 indicate that rodent Hbs are generally far less responsive to DPG than the Hbs of humans and many other mammals.

In hibernating golden-mantled ground squirrels and thirteen-lined ground squirrels, red cell DPG concentrations are reduced by nearly 50% compared with levels in non-hibernating individuals of the same species (Burlington and Whitten, 1971; Maginniss and Milsom, 1994). However, as the Hbs of these two species have such low anion sensitivities, the dramatic reductions in intracellular DPG may not have important direct effects on the regulation of Hb–O₂ affinity during hibernation. Instead, the reduced DPG concentration may indirectly affect Hb–O₂ affinity by altering the Donnan equilibrium of protons across the red cell membrane, as the reduced intracellular concentration of non-diffusible anions produces a corresponding reduction in proton concentration (Duhm, 1971). The resulting increase in intracellular pH at a given plasma pH would then increase Hb–O₂ affinity via the pronounced Bohr effect of the scurid Hbs (Fig. 4).

The elevated Hb–O₂ affinities of the marmotine ground squirrels should enhance pulmonary O₂ loading under hypoxia, but the trade-off is a reduced P$_{O_2}$ gradient for O₂ diffusion from capillary blood to the perfused tissue. As suggested previously (Maginniss and Milsom, 1994), the strong Bohr effect should compensate for the high Hb–O₂ affinity and promote O₂ unloading in tissue capillary beds (where tissue pH is decreased) while simultaneously contributing to O₂ loading at the pulmonary surfaces (where pH is increased). The large Bohr effect in the Hbs of marmotine ground squirrels may also play a role in pulmonary CO$_2$ excretion, where Bohr protons liberated upon Hb oxygenation drive the formation of gaseous CO$_2$ from plasma bicarbonate. Thus, a pronounced Bohr effect could be beneficial for tissue O$_2$ delivery as well as pulmonary CO$_2$ excretion not only at high altitudes but also in hypercapnic burrows and during hibernation when CO$_2$ accumulates during prolonged apneic periods (Heldmaier et al., 2004; Milsom and...
Hemoglobin in semi-fossorial rodents

Fig. 4. Representative O2-binding curves for golden-mantled ground squirrel Hb measured (A) at 37°C and pH 7.0, 7.4 and 8.0 in the presence of 2.3-diphosphoglycerate (DPG; DPG/Hb tetramer ratio 2:0) and 0.1 mol l⁻¹ KCl, and (B) at pH 7.4 and 37, 30 and 23°C in the presence of DPG (DPG/Hb tetramer ratio 2:0) and 0.1 mol l⁻¹ KCl. Fitting of data according to the sigmoidal Hill equation \([\frac{1}{1+P_{50}^{(\text{Hb})}(P_{50}^{(\text{Hb})}+P_{50}^{(\text{KCl})})]}\] is shown by solid lines. The same O2-binding curve measured at pH 7.4 and 37°C in the presence of DPG and KCl (filled circles, thick line) is shown in both panels to illustrate the effects of changes in pH (A) or temperature (B).
mammals (Hall, 1965; Hall, 1966; Jelkmann et al., 1981; Lechner, 1976; Campbell et al., 2010). Comparisons among the six species that we examined revealed a suggestive (but non-significant) positive relationship between Hb–O₂ affinity and native altitude. However, the yellow-bellied marmot and hoary marmot exhibited no appreciable difference in Hb–O₂ affinity in spite of their dramatically different elevational ranges. As ground squirrels are adapted to the respiratory gas transport challenges associated with semi-fossorial habits and winter hibernation, it may be that their ability to survive and function at high altitude does not require any additional modifications of Hb function. Consistent with this hypothesis, there are no appreciable differences in blood–O₂ affinity between highland and lowland populations of burrowing mole rats (Cryptomys hottentotus mahali) (Broekman et al., 2006). Similarly, pocket gophers of the genus Thomomys have elevated blood–O₂ affinities relative to non-fossorial rodents, but there are no apparent differences in Hb function between closely related pocket gopher species that are native to different elevational zones (Lechner, 1976).

CONCLUSION

The Hbs of marmotine ground squirrels are surprisingly insensitive to the modulating effects of DPG and Cl– ions, but are characterized by large Bohr coefficients and low thermal sensitivities relative to the Hbs of humans and other mammals. The suppressed anion sensitivity of the sciurid Hbs investigated here is unexpected in light of the fact that they retain all the canonical organic phosphate- and Cl–-binding sites in the central cavity, and the pronounced Bohr effect of gold-nanted ground squirrel Hb is surprising in light of two highly unusual substitutions, α141Arg→Cy5 and β146His→Gln, that are known to abolish the Bohr effect in human HbA. The elevated blood–O₂ affinity of ground squirrels suggests enhanced capacities for pulmonary O₂ loading under hypoxic and hypercapnic conditions, while the large Bohr effect should help to ensure efficient O₂ unloading in tissue capillaries.

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AUTHOR CONTRIBUTIONS

I.G.R. and D.M.T. contributed equally to this work. The research was conceived and designed by I.G.R., D.M.T., J.F.S. and A.F.; I.G.R., D.M.T., J.P.-G., H.M., R.E.W., J.F.S. and A.F. executed and interpreted the results of experiments; I.G.R., J.F.S. and A.F. drafted the manuscript; all authors edited, revised and approved the manuscript.

COMPETING INTERESTS

No competing interests declared.

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Paper IV

Hydrogen sulfide and nitric oxide metabolites in the blood of free-ranging brown bears and their potential roles in hibernation


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Original Contribution

Hydrogen sulfide and nitric oxide metabolites in the blood of free-ranging brown bears and their potential roles in hibernation

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During winter hibernation, brown bears (Ursus arctos) lie in dens for half a year without eating while their basal metabolism is largely suppressed. To understand the underlying mechanisms of metabolic depression in hibernation, we measured type and content of blood metabolites of two ubiquitous inhibitors of mitochondrial respiration, hydrogen sulfide (H2S) and nitric oxide (NO), in winter-hibernating and summer-active free-ranging Scandinavian brown bears. We found that levels of sulfide metabolites were overall similar in summer-active and hibernating bears but their composition in the plasma differed significantly, with a decrease in bound sulfane sulfur in hibernation. High levels of unbound free sulfane correlated with high levels of cysteine (Cys) and with low levels of bound sulfane sulfur, indicating that during hibernation H2S, in addition to being formed enzymatically from the substrate Cys, may also be regenerated from its oxidation products, including thiosulfate and polysulfides. In the absence of any dietary intake, this shift in the mode of H2S synthesis would help preserve free Cys for synthesis of glutathione (GSH), a major antioxidant found at high levels in the red blood cells of hibernating bears. In contrast, circulating nitrite and erythrocytic S-nitrosation of glyceraldehyde-3-phosphate dehydrogenase, taken as markers of NO metabolism, did not change appreciably. Our findings reveal that remodeling of H2S metabolism and enhanced intracellular GSH levels are hallmarks of the aerobic metabolic suppression of hibernating bears.

Abbreviations: CSE, cystathionine γ-lyase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RBC, red blood cell; DTPA, diethylenetriaminepentaacetic acid; SNO, S-nitrosothiol; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; BSS, bound sulfane sulfur; SBDF, 4-fluoro-7-sulfonylbenzofurazan; HPLC, high-performance liquid chromatography
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The remarkable ability of bears and other mammalian species to hibernate has remained poorly understood in terms of the underlying mechanisms. After some early attempts to identify a circulating “trigger” molecule in the blood from hibernators [8,9], a first clue to understanding the key to metabolic depression in hibernation came from experiments [10] showing that mice inhaling ~80 ppm hydrogen sulfide (sulfane, H₂S) underwent drastic but fully reversible reductions in metabolic rate, body temperature, lung ventilation, O₂ consumption, and CO₂ production. The dramatic changes observed, albeit artificial, were strikingly similar to those of natural hibernators. This hypometabolic effect has been ascribed to the known ability of H₂S to reversibly inhibit mitochondrial cytochrome c oxidase when present at low levels [10–12]. However, it was not known whether levels of H₂S and of its physiological in vivo metabolites in fact change in natural hibernators such as bears.

Suppression of O₂ consumption in hibernation necessarily originates from the mitochondria, where ~90% of whole-animal O₂ consumption takes place [13]. In principle, other signaling molecules capable of reversible inhibition of cytochrome c oxidase could also be involved in the metabolic suppression of hibernators. One such molecule is nitric oxide (nitrogen monoxide, NO). H₂S and NO are ubiquitous signaling molecules synthesized by naturally occurring enzymes (including cystathionine γ-lyase for H₂S and nitric oxide synthases for NO) with profound physiological effects on mitochondrial respiration, blood pressure regulation, and cytoprotection [14,15]. Because of their reactivity, both these signaling molecules generate in vivo a broad range of oxidative products, each with distinctive biological activities. The complex in vivo effects of NO and its products, in particular nitrite and S-nitrosothiols (SNOs; formed when Cys thiol is modified by NO), are known in good detail [16] because highly sensitive (e.g. chemiluminescence and biotin switch) methods have been available for some time for the detection of their low-nanomolar in vivo levels [17,18]. These methods have revealed important roles for circulating nitrite as a storage pool of NO, from where NO can be regenerated during hypoxia and contribute to vasodilation and cytoprotection [16,20,21], and for S-nitrosation as a site-specific redox-dependent protein modification in mammals [17] and in ectotherm vertebrates [22–24]. In contrast, the biological roles of H₂S and its metabolites in vivo have remained more elusive owing to technical limitations for their detection [25,26] and new methods are being currently developed to obtain reliable measures of physiological levels of H₂S and related compounds [14,27].

As fluctuations in respiratory rates are associated with oxidative stress, physiological metabolic suppression is tightly linked with antioxidant capacity. Hibernating bears most likely possess enhanced tolerance against oxidative stress and regenerative capacity as known for other animals capable of prolonged metabolic suppression [2,28]. Enhanced oxidative stress typically occurs whenever mitochondrial activity varies independent of available O₂ and potentially damaging reactive oxygen species (ROS) are generated as a product [29]. The ubiquitous tripeptide glutathione (GSH) is a key element in the thiol-dependent cellular defense against ROS and redox imbalance. For instance, ectotherms experiencing seasonal periods of prolonged hypoxia and severe oxidative stress at arousal are known to possess much higher levels of GSH compared to their hypoxia-intolerant counterparts [28].

In this study, we report measurements of a large number (23 in total) of blood parameters taken from winter-hibernating and summer-active free-ranging brown bears (the same individuals in winter and summer), with the intent to identify which parameters could be involved in hibernation. Specifically, we examined the circulating levels of major H₂S and NO metabolites; the activity of the enzyme cystathionine γ-lyase (CSE), an important enzyme catalyzing the production of H₂S from l-Cys in the circulation; and the levels of free l-Cys and GSH and other thiols. We have also investigated levels, activity, and S-nitrosation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme known to undergo S-nitrosation [30], as a marker of targeted S-nitrosation-dependent control of energy metabolism and potentially involved in the reduction of downstream 2,3-diphosphoglycerate in RBCs during hibernation [6]. All of the investigated parameters were subjected to a stringent statistical analysis to test for significant differences and mutual correlations between hibernating and summer active individual bears. The findings of this exploratory study unveil distinct potential roles of H₂S and NO-dependent signaling in physiological metabolic suppression.

Materials and methods

Animals and blood samples

Animal handling and sampling were approved by the Swedish Ethics Committee on Animal Research (C212/9) and the Swedish Environmental Protection Agency. Blood samples were collected from seven 3- to 5-year-old (two males and five females) free-ranging anesthetized Eurasian brown bears (Ursus arctos) in Dalarna and Gävleborgs Counties, Sweden. The bears were previously equipped with global-positioning system collars as well as radio transmitters for tracking. Bears were immobilized by darting in the den during winter (February 2013) and the same bears again by darting from a helicopter during summer (June 2013). Anesthetics used in winter were a mixture of tiletamine–zolazepam (1.1 mg/kg), medetomidine (0.03 mg/kg), and ketamine (1.3 mg/kg) and in summer a mixture of tiletamine–zolazepam (4.7 mg/kg) and medetomidine (0.09 mg/kg) [7]. The medetomidine was antagonized with 5 mg antisedan for each milligram of medetomidine after the procedures were finished and after placing the bears back into the dens in winter [6,7]. Blood was taken from the jugular vein using heparinized vacuum tubes and immediately centrifuged in the field (4 min, 9000g) to separate plasma from RBCs. For each individual, RBC aliquots were immediately frozen in dry ice for later measurement of GAPDH activity or treated before freezing as described below under H₂S products and NO products. All processing and freezing of blood samples were done in the field within 10 min of blood sample collection. Samples were protected from light during processing. All chemicals were from Sigma–Aldrich unless otherwise stated.

H₂S products

Biochemical forms of H₂S were measured using the HPLC monobromobimane (MBB) assay as previously reported [27,31]. Aliquots of RBCs and plasma from individual bears were immediately diluted 1:5 in rubber cap-sealed anaerobic Eppendorf vials containing previously degassed 100 mM Tris–HCl buffer, pH 9.5, 0.1 mM diethylenetriamine pentaacetic acid (DTPA) and frozen in dry ice for later measurement of H₂S products, total GSH, cysteine, and homocysteine concentrations [27,31,32]. Additional RBC and plasma samples were frozen without further treatment immediately after centrifugation for measurement of CSE activity. All samples were stored in liquid N₂ and analyzed within 2 weeks of collection [33].

Measurement of total GSH, Cys, and homocysteine

Total GSH, Cys, and homocysteine were measured after thiol reduction and derivatization with 4-fluoro-7-sulfobenzofurazan (SBD-F) as described [32,34]. Briefly, samples were reduced by
incubation with 30 mM tris(2-carboxyethyl)phosphine hydrochloride at room temperature for 30 min. Trichloroacetic acid (100 mg/ml) was added to precipitate proteins. After centrifugation for 10 min, the supernatants were derivatized with 90 mM SBD-F and the fluorescent thiol derivatives were separated on a C18 column by reverse-phase high-performance liquid chromatography and detected by fluorescence (extinction 385 nm, emission 515 nm).

CSE activity

CSE activity was measured as previously reported [35]. Briefly, tissue lysates were mixed with 2 mM cystathionine, 0.25 mM pyridoxal 5′-phosphate in 100 mM Tris–HCl buffer and incubated for 60 min at 37 °C. Trichloroacetic acid (10%) was added to the reaction mixture and spun down. The supernatant was then mixed with 1% ninhydrin reagent and incubated for 5 min in a boiling-water bath. The solution was then cooled on ice for 2 min and the absorbance measured at 455 nm using a Smart Spect Plus spectrophotometer (Bio-Rad). CSE activity was expressed as nanomoles of cystathionine consumed per milligram of total protein per hour of incubation.

NO products

Aliquots of RBCs from individual bears were diluted 1:5 with a SNO/nitrite-stabilizing solution containing 4 mM ferricyanide (K3FeIII(CN)6), 10 mM N-ethylmaleimide, 0.1 mM DTPA [18] and frozen in dry ice. Thawed hemolysate and plasma were vortexed and centrifuged (2 min, 16,000g, 4 °C), and supernatants were immediately measured. NO metabolites were measured by reductive chemiluminescence using a Sievers (Boulder, CO, USA) nitric oxide analyzer (Model 280i) and previously described procedures [18,36]. Levels of SNO, iron–nitrosyl, and N-nitrosyl compounds were below the detection limit (approximately 10 nM at the volume and dilution used). Nitrite was subsequently determined on samples deproteinized with ice-cold ethanol (1:1). Nitrite peaks were integrated with Origin software (OriginLab Corp., Northampton, MA, USA).

Biotin switch

RBC samples were stored at −80 °C in stabilization solution containing 10 mM N-ethylmaleimide. The biotin switch assay was performed as described [19,37] and pull-downs from Neutravidin resin (Pierce, Rockford, IL, USA) were probed for the presence of GAPDH by Western blotting. Total GAPDH was tested in RBC samples before processing samples for biotin switch.

GAPDH activity

GAPDH activity was assessed at 25 °C in the absence and presence of 0.3 mM dithiothreitol by monitoring the time-dependent decrease in NADH absorbance at 340 nm (extinction coefficient 6.22 mM−1 cm−1) following the Sigma enzymatic assay protocol (EC 1.2.1.12, Sigma–Aldrich) [37]. The assay proceeds in two steps, catalyzed by 3-phosphoglyceric phosphokinase (step 1) and GAPDH (step 2):

3-phosphoglyceric acid + ATP → 1,3-DPG + ADP,

1,3-DPG + NADH → glyceraldehyde-3P + NAD+ + P1. (1)

Reactions were completed after 150 s. For each sample, the GAPDH activity reported is expressed as units per milligram of total hemoglobin, measured at 540 and 576 nm using known extinction coefficients [38].

Statistical analysis

Statistical differences between plasma and RBC parameters of the same seven individual winter-hibernating and summer-active bears were assessed by parametric paired t test with a significance level set at P < 0.002 to account for multiple tests. Because of the high number of parameters measured on the same samples, a low P value of <0.002 (~0.05/23) is required to validate that differences between parameters measured in hibernating and summer-active bears are significant. Nonparametric paired Wilcoxon signed rank tests produced similar results (significance level P < 0.016). To search for relationships between pairs of individual parameters measured in winter-hibernating and summer-active bears, pairwise Pearson correlations were calculated. The most interesting pairs were determined based on the strength (r > 0.7 or r < −0.7) and significance (P < 0.05) of the correlation. Statistical analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC, USA).

Results

We used the recently developed MBB method for the detection of physiological levels of various H2S metabolites [31] in plasma and RBCs isolated from the same individual hibernating and summer-active brown bears. H2S metabolites can be divided into (1) acid-labile sulfur, which mainly contains Fe–S clusters and persulfides and is converted into H2S under acidic conditions; (2) bound sulfane sulfur (BSS), which contains thiosulfate and polysulfides and is converted into H2S under reducing conditions; and (3) free sulfide, containing mainly freely dissolved H2S and HS − [14]. We found that in winter-hibernating bears, plasma contained significantly (P < 0.002) lower BSS and free sulfide and significantly (P < 0.002) higher acid-labile sulfane than in summer-active bears (Fig. 1A; Supplementary Tables S1 and S2). Marked changes were also found when measuring thiols. Levels of plasma Cys (the most abundant plasma thiol) and total GSH decreased significantly (P < 0.002) during hibernation (Fig. 1B, Supplementary Tables S1 and S2). Less pronounced changes were found in other plasma parameters, such as an increase in CSE activity (P=0.033) (Fig. 1C) and a decrease in total sulfane sulfur (P=0.0035) and nitrite (P=0.043) (Supplementary Tables S1 and S2), all approaching significance. Conversely, in RBCs, all parameters examined remained relatively constant with the exception of total GSH, which increased significantly (P < 0.002) during hibernation (Figs. 2A and B; Supplementary Tables S3 and S4). CSE activity was higher in RBCs than in plasma and possibly increased in hibernating bears (Fig. 2C; P=0.05). RBC nitrite also increased during hibernation, albeit not significantly (P=0.05; Supplementary Tables S3 and S4). Despite the level of total SNO compounds being below the detection limit, S-nitrosated GAPDH was detected in RBCs by the biotin switch method (Fig. 3A), but its levels did not change significantly during hibernation and showed a large individual variation (Fig. 3B, Supplementary Tables S3 and S4).

We then analyzed all pairwise Pearson correlations for plasma and RBC variables (Supplementary Figs. S1 and S2) within individual hibernating and summer-active bears to search for significant patterns (r higher than 0.7 for direct correlations and less than −0.7 for inverse correlations). Selected correlations are shown in Fig. 4. In hibernating bears plasma, BSS, and free sulfide were inversely correlated (r = −0.77; Fig. 4A), whereas Cys was positively correlated with GSH
(r=0.89; Fig. 4B) and free sulfide (r=0.87; Fig. 4C). Within RBCs, GSH was positively correlated with Cys (r=0.82) in hibernating bears (Fig. 4D). None of these parameters were significantly correlated in summer (Figs. 4A–D). These correlations suggest that during hibernation plasma Cys availability is important for the generation of free sulfide and GSH (as also in RBCs) and that plasma BSS is used as a source of free sulfide. Furthermore, nitrite and SNO–GAPDH levels were not correlated (Supplementary Fig. S2), suggesting a nitrite-independent mechanism for S-nitrosation in this enzyme. Other correlations, albeit significant, are not clearly interpretable in terms of seasonal patterns of their variations. We note, however, that a strong correlation between two parameters indicates that they are equivalent in what they are measuring. For example, in both hibernating and summer-active bears, total and bound sulfane sulfur were tightly correlated (r=0.91 in plasma and 0.94 and 0.96 in RBCs; Supplementary Figs. S1 and S2), meaning that the amount of variation in one variable is largely due to the other variable (as given by r²). In other words, in these samples measuring total sulfane sulfur is largely equivalent to measuring bound sulfane sulfur.

**Discussion**

How brown bears and other mammalian hibernators are capable of drastically reducing their metabolic rate for long periods of time while still preserving organ integrity is largely unknown. A major finding of this study is that hibernation in free-ranging brown bears is associated with highly significant changes in plasma H₂S metabolites and enhanced intracellular GSH levels.

**The possible origin of H₂S in hibernation**

Overall, total sulfide did not change significantly in either plasma (Fig. 1A) or RBCs (Fig. 2A) upon hibernation, indicating that the balance between H₂S generation and consumption is largely the same. Plasma values of ~5 μM total sulfide in bears are about the same as those found in mice (~4.5–4.8 μM) [31]. These results indicate that it is not a general increase in H₂S levels that is associated with hibernation, but rather a shift in the way it is produced and consumed. Consistent with this interpretation, the
The relative composition of H₂S metabolites changed markedly in plasma (Fig. 1A) but not in RBCs (Fig. 2A), with more sulfide present in the plasma as the acid-labile fraction and less as free sulfide or BSS (Fig. 1A). The significant decrease in the plasma BSS pool (Fig. 1A) and the negative correlation between BSS and free sulfide found in hibernating bears (Fig. 4A) are interesting, as these results suggest that H₂S is generated at the expense of the BSS pool, whereas in summer bears, there is no obvious correlation between these two parameters. Polysulfides and thiosulfate (S₂O₃⁻) are major products of H₂S oxidation contained in the BSS.
fraction [14,25,39] that can be recycled back to H$_2$S under reducing conditions [14,25], and enzymes catalyzing the conversion of thiolsulfate to H$_2$S, including a ubiquitous GSH-dependent thiolsulfate reductase [25] and mitochondrial rhodanese and 3-mercaptopyruvate sulfur transferase [40], have been identified. A recent study [40] has reported H$_2$S formation from thiolsulfate and various reducing agents in tissue homogenates, indicating a biological role for thiolsulfate in its reduction to H$_2$S. Although future studies will be needed to identify the BSS source of H$_2$S in hibernating bears, the regeneration of H$_2$S from one or more of its oxidative products would be of particular physiological importance for the hibernating bear, as it would help preserve levels of Cys for protein and GSH synthesis during hibernation. This strategy would also contribute to preservation of body nitrogen stores and sustain protein synthesis despite the absence of dietary intake of amino acids [9,41].

Fig. 5 shows a plausible model for H$_2$S origin and fate consistent with our findings. In the blood of summer-active bears, H$_2$S generated in RBCs from the CSE-catalyzed conversion of Cys freely diffuses out into plasma and is rapidly metabolized to generate thiolsulfate and other oxidized products [14,39]. Because of its propensity to become oxidized [42,43], at normal O$_2$ levels most H$_2$S generated would be inactivated before reaching cytochrome c oxidase in the mitochondria of perfused tissues. During hibernation, part of the plasma BSS pool is transferred to the RBC, where it is converted to H$_2$S, a reaction that is favored by reduced GSH [25]. The low arterial O$_2$ tension [7] and high hemoglobin O$_2$ affinity [6] in hibernating brown bears indicate conditions of low O$_2$, under which H$_2$S would be able to diffuse into nearby cells and contribute to suppressing mitochondrial respiration. It can be envisaged that similar O$_2$-linked processes could also take place in cells and tissues other than blood.

**Generation of H$_2$S from Cys**

Although the BSS may function as an alternative source of H$_2$S, the positive correlation between plasma free sulfide and Cys (Fig. 4C) indicates that H$_2$S is still produced by erythrocytic CSE, possibly even functioning at higher rates (Fig. 2C), whereas the lower CSE activity in plasma (Fig. 1C) would reflect a release from hepatocytes and endothelium [44]. Other enzymes, including cystathionine β-synthase, 3-mercaptopyruvate sulfurtransferase, and cysteine aminotransferase, may also synthesize H$_2$S from Cys in various tissues and cellular compartments [45], and CSE may even translocate to mitochondria and improve ATP production in vascular muscle cells [46], a process that may well occur in vivo in hibernating animals. Taken together, these results indicate that plasma may contain circulating available pools of BSS and Cys for uptake into RBCs and tissues where they can be used in H$_2$S synthesis and for mitochondrial function during hibernation.

**Effects of H$_2$S on mitochondrial respiration**

Several studies have consistently reported lower mitochondrial respiration rates in several hibernating ground squirrel species, especially in the liver [47,48] and in the skeletal muscle in 13-lined ground squirrels [49]. In this species, the suppressed O$_2$ consumption was not due to phosphorylation of respiratory complexes [50], thus supporting that a soluble factor, such as H$_2$S, could be involved. Interestingly, mitochondrial respiration in cardiac muscle and brain was not depressed in hibernating ground squirrels [51], suggesting that during winter hibernation energy resources are preferentially allocated to these two vital organs.

H$_2$S is a weak reversible inhibitor of the ferrous heme of cytochrome c oxidase in mitochondria, with estimated affinity constants in the physiological micromolar range (0.2–12.5 μM) [11,12]. In binding to cytochrome c oxidase with low affinity, it is readily displaced by stronger ligands, such as O$_2$. Inhalation of ~80 ppm gaseous H$_2$S that induced suspended animation in mice [10] corresponds to ~1 μM H$_2$S [39], levels that are compatible with those found in this study (1.08 and 2.00 μM free sulfide for winter-hibernating and summer-active bears, respectively; Supplementary Table S1). The results of this study extend the original conclusion that H$_2$S is involved in hibernation [10] by showing that in hibernating brown bears H$_2$S may in part originate from plasma BSS.

**Effects of H$_2$S on the circulation**

In addition to inhibiting mitochondrial respiration, H$_2$S also has marked effects on the circulation, by acting as a hypoxic vasodilator in the pulmonary and systemic circulation,
A major finding of this study is the large increase in erythrocytic total GSH found in hibernating bears (Fig. 2B). Total GSH was highly dependent on the availability of Cys in plasma and RBCs (Figs. 4B and D) and, consistent with these results, the rate of synthesis of GSH is rate limited by the levels of Cys present in human plasma and RBCs, which Cys enters through a Na+-dependent transporter [57]. Thiol-containing GSH is an essential component of the defense against oxidative stress in that it reacts with ROS to generate oxidized GSSG, which is then reduced back to GSH via NADPH-dependent GSH reductase or actively exported from the erythrocyte [57]. Although relying on aerobic metabolism, hibernating animals undergo periodic oscillations in their metabolic rates, with inevitable mismatches between local O2 supply and consumption and resulting generation of potentially damaging ROS [4,28]. Although we could not measure oxidized vs reduced GSH because of the time delay in collection and analysis of samples from the free-ranging bears, a large pool of total GSH available in RBCs (Fig. 2B) and probably in other tissues during the hibernation period would help limit periodic oxidative damage. We note that reduction of any oxidized GSSG back to GSH predicts that sufficient NADPH is available as a reducing agent, whereby glucose reserves (not the primary energy fuel during hibernation) might be diverted away from glycolysis to fuel the NADPH-generating pentose–phosphate pathway. In this process, reversible inactivation of phosphofructokinase, a key enzyme of glycolysis, mediated by low temperature and pH may well play a role in hibernating bears as found in a small hibernating rodent [58]. This would also explain our earlier finding of a substantial reduction in RBC 2,3-diphosphoglycerate, a side product of glycolysis, during hibernation in brown bears [6].

HS and NO: a comparison between two signaling molecules

Perhaps surprisingly, we did not find significant changes in the major NO metabolite nitrite in plasma or RBCs nor in S-nitrosation levels or activity of erythrocytic GAPDH, although we cannot rule out that some of the changes (for example plasma nitrite with \(P=0.043\); Supplementary Table S2) may become statistically significant in separate studies with a lower number of parameters investigated (where \(P < 0.05\) instead of \(P < 0.002\) is sufficient). Other parameters that were not investigated here may also reveal a role for NO in the control of hibernation, such as changes in NOS activity, nitrate, or targeted S-nitrosation of key proteins or enzymes in blood or other tissues. Previous studies have shown that H2S and NO share some important characteristics: they both originate enzymatically from amino acids (L-Cys and L-Arg, respectively) [59,60], can be regenerated from their respective oxidative products [40,61], and interact in the control of vasodilation [62] and in cytoprotection [63]. However, results from this and previous investigations suggest that these two signaling molecules may operate in different physiological and pathological contexts [64]. Under hypoxia, the enzymatic rate of NO synthesis from L-Arg decreases (as O2 is a cosubstrate), whereas conversion of nitrite to NO increases, a process also favored by acidic conditions. Such conditions are present during acute exercise and heart ischemia in mammals or even during prolonged acclimation to extreme hypoxic and anoxic conditions as achieved by some fish and turtles [28]. Accordingly, in these hypoxia-tolerant ectotherms, levels of plasma nitrite are constitutively higher than in mammals and hypoxia-intolerant species, and plasma nitrite is shifted to tissues and used for NO synthesis during hypoxia and anoxia [22,36,65]. Conversely, mammalian hibernators use stored fat as the major energy fuel to sustain a hypometabolic state in which little O2 is consumed and supplied, without becoming hypoxic. As a result, blood pH and lactate remain relatively stable under hibernation in brown bears [67]. Although the role of NO in mammalian hibernation is less clear than that of H2S, we speculate that H2S-dependent inhibition may prevail in aerobic metabolic suppression, as it occurs in hibernating bears, whereas NO-dependent inhibition may be dominant in hypoxic or even anoxic metabolic suppression, as it occurs in a few ectotherm species, such as crucian carp [22] and turtle [23,65], that overwinter in a total lack of O2. These complementary abilities of H2S and NO to induce controlled and reversible hypometabolic states and to protect cells and organs against O2 deprivation would have far-reaching consequences in biology and medicine.

Conclusion

In summary, our study is the first to show that in a hibernating species in its natural environment, hibernation is associated with (1) a significant remodeling of H2S metabolism consistent with generation of H2S from both BSS and Cys and (2) a large increase in the intracellular GSH pool available. Although the role of NO in hibernation remains to be conclusively established, these findings highlight the emerging importance of sulfane metabolism in metabolic depression and antioxidant defense and provide a rare snapshot into the physiological processes underlying the fascinating phenomenon of mammalian hibernation.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.05.025.


