On the effect of physiological acidosis in hibernating rodents

by

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Abstract

Metabolic rate suppression during hibernation requires an orchestrated global reduction in metabolism while still matching O\textsubscript{2} supply and demand. Acidosis has been suggested as a mechanism for suppressing metabolic rate in hibernation, although the mechanism behind this process has not been thoroughly investigated. Findings from my thesis indicate that hemoglobin of both the facultative hibernator (Syrian hamster) and the obligate hibernator (13-lined ground squirrel) exhibit a reduced temperature sensitivity and increased Bohr effect relative to the non-hibernating rat, ultimately enhancing O\textsubscript{2} offloading at the tissues. I also found that in the obligate hibernator, due to metabolic state-dependent changes in intracellular buffering constituents, the ionisation ratio of intracellular imidazole (\(\alpha_{\text{im}}\)) did not vary between euthermia and hibernation. In the facultative hibernator, \(\alpha_{\text{im}}\) remained constant regardless of hibernation state. These findings contradict previous speculation that acidosis may suppress metabolism in quiescent tissues such as the brain and skeletal muscle during hibernation.

Furthermore, mitochondrial respiration increased at low pH despite the reduced activity of electron transport system (ETS) enzymes at low pH. This suggests that, despite ETS enzyme activity being reduced at low pH, acidosis may reverse the inhibitory mechanisms that suppress mitochondrial respiration during steady-state hibernation, leading to the observed increase in mitochondrial respiration at low pH. Lastly, acidosis decreased cellular ATPase activity in the hibernating species but not in the rat. In the obligate hibernator, this inhibitory effect of acidosis on cellular ATPase activity was present regardless of hibernation state and assay temperature (37 – 10 °C). In the facultative hibernator, the effect of acidosis on ATPase activity was only present during euthermia and at warm temperatures.

Taken together, these findings indicate that acidosis does not occur in quiescent tissues of hibernators or lower mitochondrial O\textsubscript{2} consumption. Instead, it may serve a role in matching O\textsubscript{2} supply to the high O\textsubscript{2} demand during arousal by increasing O\textsubscript{2} offloading by hemoglobin. Collectively, these data provide novel insight into the role of acidosis during hibernation that were previously uncharacterised.
Unlike most mammals, hibernating animals show incredible metabolic flexibility as they can lower their metabolism by more than 90%. The physiological mechanisms that allow for this flexibility in hibernators are unclear. One suspected mechanism has been the change in physiological pH as animals enter hibernation. I found that tissue pH was not lower during hibernation in the two species of rodents I studied (Syrian hamsters and 13-lined ground squirrels). Furthermore, although low pH decreased enzyme activity in the two species of hibernators (which may reduce ATP consumption during hibernation), low pH in fact increased O$_2$ usage in mitochondria. This contradicted the hypothesis that low pH may be a mechanism to lower metabolism during hibernation. I found that low pH increased O$_2$ unloading from the blood to the tissues as animals arouse from hibernation when O$_2$ demand rises with increasing metabolism. Collectively, my thesis expands our understanding of how changes in physiological pH may affect metabolism during hibernation.
A version of Chapter 1 has been published as part of a larger invited review as: Kim, A.B., and Milsom, W. K. (2019). pH regulation in hibernation: implications for ventilatory and metabolic control. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology. 237, 110536. I wrote the manuscript with editorial guidance from Dr. Milsom.

Chapters 2 to 4 have been written as separate manuscripts to be submitted to peer-reviewed journals. I conceived the study and designed the experiments of all my data chapters (Chapters 2-5) with guidance from Dr. Milsom and Dr. Richards.

Chapter 2 has been submitted as: Kim, A. B., Morrison, P. R., Richards, J. G., and Milsom, W. K. (2022). Opposing temperature and pH sensitivity increases hemoglobin oxygen offloading in hibernating rodents. I carried out all experiments and analysed the data with training and help from Dr. Phillip Morrison. I also prepared the manuscript with editorial guidance from Dr. Milsom and Dr. Richards. I carried out all experiments, data analysis and manuscript preparations for Chapter 3, with editorial input from Dr. Milsom and Dr. Richards. I also carried out all the experiments and data analysis for Chapter 4, with help from Reece Long in collecting the enzymatic activity data. I wrote the manuscript with editorial input from Dr. Milsom and Dr. Richards. Lastly, I carried out the experiments of Chapter 5 with assistance from Daniel Lee with hepatocyte isolation and cell culture media preparation. I analysed all the data and wrote the chapter with editorial input from Dr. Milsom and Dr. Richards.

Ground squirrels used in this thesis were trapped with the approval of Manitoba Conservation and Water Stewardship, under the wildlife scientific permit WB15027. All experimental procedures in this thesis conformed to the guidelines of the Canadian Council on Animal Care and were approved by the UBC Committee on Animal Care (under protocol A21-0006).
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<th>Definition</th>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CI, CII…</td>
<td>Complex I, II…of the ETS</td>
</tr>
<tr>
<td>eEF-2</td>
<td>Eukaryotic elongation factor-2</td>
</tr>
<tr>
<td>eIF-2</td>
<td>Eukaryotic initiation factor-2</td>
</tr>
<tr>
<td>ETS</td>
<td>Electron transport system</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors and protein tyrosine kinase 2</td>
</tr>
<tr>
<td>IBE</td>
<td>Interbout euthermia</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OEC</td>
<td>Oxygen equilibrium curve</td>
</tr>
<tr>
<td>P50</td>
<td>Partial pressure at which 50% of the blood is saturated with O2</td>
</tr>
<tr>
<td>pH_e</td>
<td>pH of extracellular fluid</td>
</tr>
<tr>
<td>pH_i</td>
<td>pH of intracellular fluid</td>
</tr>
<tr>
<td>pH nw</td>
<td>pH of neutral water</td>
</tr>
<tr>
<td>P_i</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
</tr>
<tr>
<td>Pyk2</td>
<td>Protein tyrosine kinase 2</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RFID</td>
<td>Radio-frequency identification</td>
</tr>
<tr>
<td>T_b</td>
<td>Body temperature</td>
</tr>
<tr>
<td>TMR</td>
<td>Torpid metabolic rate</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>α-stat</td>
<td>Alpha-stat regulation (maintaining the ionisation ratio of αim constant)</td>
</tr>
<tr>
<td>αim</td>
<td>Ionisation ratio of intracellular imidazole</td>
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Chapter 1: General introduction

1.1 Heterothermy

The vast majority of mammals are homeothermic endotherms that maintain a constant body temperature of around 37 °C (T_b) with endogenously generated heat. However, homeothermy is energetically costly, as metabolic heat production requires constant and high rates of fuel catabolism and O_2 consumption. To reduce energetic demands, several mammalian species exhibit varying degrees of heterothermy, which for the purpose of this thesis, is defined as a significant (> 5 °C) reduction in body temperature below resting T_b and a concomitant reduction in metabolic rate (Cooper & Geiser, 2007). The term heterotherm is used to refer to animals capable of endogenously regulating variable T_b. Specifically, when pertaining to endothermic species capable of entering physiological states of inactivity, reduced T_b, and metabolic rate, the term temporal heterotherm is used (c.f. regional heterothermy). The metabolic rate depression associated with heterothermy is considered by most to be a continuum ranging from a relatively small decrease in metabolic rate during sleep (10-15% decrease from resting metabolic rate) to shallow torpor lasting less than 24 hours (daily torpor), as well as prolonged periods (several days) of up to 90% decrease in metabolic rate in hibernation (Zepelin & Rechtschaffen, 1974; Berger, 1993; van Breukelen & Martin, 2015).

At the onset of entrance into hibernation, a rapid reduction in heart rate, ventilation, and metabolic rate occurs before any significant decrease in T_b is observed. Following the initial active decrease in metabolic rate, T_b subsequently falls in a steady and controlled manner, to match the gradually decreasing thermoregulatory set point in the pre-optic anterior hypothalamus. As T_b continues to fall, passive thermal effects further lower metabolic rate in addition to the active mechanisms (Carey et al., 2003; Heldmaier et al., 2004; Heller & Colliver, 1974; Millsom & Jackson, 2011; Staples, 2014). Thus, torpid metabolic rate (TMR) is the result of the interaction between initial active metabolic suppression at the onset of entrance into hibernation and passive metabolic suppression caused by declining T_b.
Throughout the hibernation season, hibernators periodically elevate metabolic rate and $T_b$ back to typical homeothermic levels for 24 – 48 hours resulting in periods of interbout euthermia (IBE), for reasons that are yet unclear (for a comprehensive review of IBE, see Ruf et al., 2022)

### 1.1.2 Obligate vs facultative hibernation

Hibernation can be categorised as obligate or facultative hibernation. Both obligate and facultative hibernators exhibit similar torpor patterns in which several days of torpor bouts are interrupted by brief periods of IBE. Facultative hibernators typically exhibit shorter torpor bouts (1 - 6 days) than obligate hibernators (8 – 11 days), although torpor bout duration is shortened at warmer ambient temperature and dependent on the fasting duration (Geiser & Kenagy, 1988; Lehmer et al., 2001; MacCannell & Staples, 2021; Talaei et al., 2011). Despite the similar torpor patterns, the manner in which animals prepare for and enter the hibernation period distinguishes the two types of hibernators.

Obligate hibernation is a circannual cycle that occurs in the absence of environmental cues such as cold temperatures, short photoperiods, or food deprivation (Geiser, 2020; MacCannell & Staples, 2021). Obligate hibernation typically occurs between autumn to spring (November – April in the Northern Hemisphere), although the precise seasonality of obligate hibernation is known to vary with species, sex, and age (Geiser, 2020). Extensive physiological remodelling occurs in the months prior to the hibernation season, as well as between torpor and IBE. This has been referred to as a “two-switch model”, as one switch in metabolic phenotype occurs between seasons (summer and winter), while the second switch occurs between states (hibernation and IBE). For example, during the summer, lipogenic capacity is higher in many species of obligate hibernators to increase white adipose tissue mass, while genes associated with lipolysis are upregulated during the winter as most obligate hibernators typically fast for the duration of the hibernation season (Mostafa et al., 1993; Serkova et al., 2007; Wang et al., 1997). This switch from increasing fat storage in the summer, to fat catabolism in the winter exemplifies the first switch of the two-switch model. During the winter, the second switch occurs within a matter of hours as an animal cycles in
and out of hibernation and IBE. For example, the transcriptomic profiles of the cerebral cortex of the 13-lined ground squirrels (an obligate hibernator) exhibit marked changes between hibernation and IBE, in order to restore neuronal function during IBE (Schwartz et al., 2013). As a result, obligate hibernators are often broadly characterised into three distinct phenotypes: summer, hibernation, and IBE. In contrast, entrance into facultative hibernation is non-seasonal and can occur at any time of the year. They are typically induced by prolonged exposure to cold ambient temperatures, short daylight duration, and low food availability. Although there is comparatively less research on metabolic remodelling in facultative hibernators, studies using Syrian hamsters (a facultative hibernator) have reported decreases in body mass and body temperature, as well as changes in lung tissue composition to avoid cold-induced organ damage, and increase in mitochondrial content in white adipose tissue during several weeks of pre-hibernation cold acclimatisation (Chayama et al., 2016; Talaei et al., 2011). Therefore, while the two-switch model is most commonly applied to obligate hibernators, two switches in metabolic phenotype also appear to occur in facultative hibernators as well. One switch in metabolic phenotype occurs during the pre-hibernation acclimatisation period in facultative hibernators, with the second switch occurring between hibernation and periods of IBE. As such, while the first switch in metabolic phenotype in obligate hibernators occurs in sync with a circannual rhythm in the absence of cold or shorter photoperiod exposure, the first switch in facultative hibernators appears to require prolonged cold induced constitutive metabolic changes that eventually lead to hibernation.

With the exception of a few studies that compare the metabolite and proteomic profiles of facultative and obligate hibernators (Frank et al., 1998; Harlow & Frank, 2001), very few studies have investigated the differences or similarities in the mechanisms that may be associated with reducing the metabolic rate in these two types of hibernators. As acidosis of the blood has been shown to occur in species of both facultative and obligate hibernators, it has been suggested as a mechanistic candidate for active metabolic rate suppression in hibernators for several decades (Kreienbühl et al., 1976; Lyman & Hastings, 1951). To my knowledge, no study has compared and contrasted the physiological effect of this acidosis on metabolism in the two types of hibernators.
1.2 Acid-base balance with changing body temperature

During early entrance into hibernation, the ventilation rate decreases faster than the metabolic rate, which leads to CO$_2$ retention, and therefore a decrease in the ratio of ventilatory CO$_2$ elimination over ventilatory O$_2$ uptake (respiratory exchange ratio) (Bickler, 1984a; Elvert & Heldmaier, 2000, 2005; Nestler, 1990b; Snapp & Heller, 1981; Sprenger & Milsom, 2022). This CO$_2$ retention occurs rapidly and the CO$_2$ is then retained throughout the bout of hibernation (Sprenger & Milsom, 2022). Due to the retention of CO$_2$, the blood undergoes a relative acidosis during hibernation (Malan et al., 1985; Milsom & Jackson, 2011). The term relative acidosis is used in this case as measured blood/extracellular pH (pH$_e$) stays relatively constant, and in some cases increases slightly, between euthermia and steady state hibernation (Table 1.1), which overall represents an acidosis due to the thermal effects on neutral pH (Figure 1; pH$_{nw}$). Water dissociates into H$^+$ and OH$^-$ to a lesser degree when the temperature decreases, as the dissociation of H$_2$O is an endothermic reaction. As a result, pH$_{nw}$ increases with decreasing temperature ($\Delta$ pH$_{nw}$/\$\Delta$t temperature: 0.018), but this does not represent alkalinisation of water, as less of both products of water dissociation (namely H$^+$ and OH$^-$) are formed. Thus, pOH is still identical to pH at lowered temperatures and the solution is still neutral. Therefore, maintaining the same pH$_e$ at 37$^{\circ}$C and 10$^{\circ}$C means that the degree of alkalinity relative to pH$_{nw}$ is reduced at 10$^{\circ}$C, resulting in relative acidosis. The rapid initial CO$_2$ accumulation has been estimated to account for up to 95% of the reduction in the relative alkalinity of blood to pH$_{nw}$ in hibernation (Nestler, 1990b; Snapp & Heller, 1981). Maintaining a constant pH with changing body temperature (referred to as pH-stat) and the resulting relative acidosis has long been suggested as a possible mechanism for lowering metabolic rate in hibernation (Dubois, 1896). Acidosis is known to reduce whole animal O$_2$ consumption rate without a change in body temperature, as well as decrease shivering and non-shivering thermogenesis by reducing the firing rate of preoptic anterior hypothalamic neurons that are involved in T$_b$ regulation (Bharma & Milsom, 1993; Kuhnen et al., 1987; Schaefer & Wünnenberg, 1976). Upon arousal from hibernation, hyperventilation precedes the increase in O$_2$ consumption, leading to CO$_2$ unloading (Bickler, 1984a; Elvert & Heldmaier, 2000, 2005; Nestler, 1990b; Snapp & Heller, 1981; Sprenger & Milsom, 2022). In the European hamster, up to 63% of the increased CO$_2$
content, that accrued during entrance may be eliminated during this early hyperventilation upon arousal (Malan et al., 1988). Thus, the rapid onset and reversibility of acidosis makes it a suitable mechanistic candidate for rapidly suppressing metabolic rate during entrance into hibernation, and reversing this suppression in a matter of hours upon arousal. As attractive as acidosis is as a mechanistic candidate for metabolic rate suppression during hibernation, whether this correlation reflects causation, particularly at the cellular level, requires further investigation.

1.2.1 α-stat hypothesis

Upon observing that not all tissues of the hibernating European hamster maintained a constant pH with changing body temperature, as is the case in blood, Malan et al. (1985) applied the α-stat hypothesis to hibernators. The α-stat hypothesis was conceived by Reeves (1972) based on several studies that reported that ectothermic vertebrates increased pH_\text{e} to maintain the same degree of relative alkalinity to pH_\text{nw} at all T_b. Reeves (1972) suggested that maintaining the same degree of relative alkalinity serves to maintain the ionisation ratio of intracellular imidazole (\(\alpha_{\text{im}}\)), which acts as an important physiological buffer. The pK of the imidazole ring on histidine is close to pH_\text{nw} at 37°C (6.8) and increases with decreasing temperature with an almost identical slope to that of pH_\text{nw}. In other words, the unprotonated imidazole ring (capable of accepting H\(^+\)) and the protonated form (capable of donating H\(^+\)) are relatively equal at physiological pH, making histidine an important buffer for ameliorating acid-base disturbances. By maintaining the ratio of the imidazole ring that is in the unprotonated form (i.e., maintaining \(\alpha_{\text{im}}\)) as temperature falls, the net charge on all proteins (enzymes, receptors, transporters, etc.) is kept constant ensuring that they can function optimally despite temperature changes. For example, the active site of lactate dehydrogenase requires protonated histidine residues to bind its substrate pyruvate. Regulating \(\alpha_{\text{im}}\) in vitro has been shown to conserve the substrate binding affinity (K\(_m\)) of lactate dehydrogenase with decreasing temperature (Yancey & Somero, 1978). Whether hibernators exhibit the same pattern of intracellular acid-base regulation as ectothermic species, and how this may affect biochemical pathways in different tissues is relatively understudied, although hibernators vary T_b as much as, or more than many ectothermic species.
1.3 Thesis overview

The overarching objective of this thesis is to determine whether acidosis contributes to the suppression of metabolic rate in both facultative and obligate hibernators. Although the occurrence of acidosis in the blood has been well documented in a wide range of hibernating species, very few studies have examined whether it may affect blood function, such as O$_2$ transport. Furthermore, the effect of extracellular acidosis on intracellular acid-base state, and how this might affect cellular processes in tissues of hibernators, have not been well investigated. Intracellular acidosis is known to affect vital cellular processes including (but not limited to) metabolic pathways such as glycolysis and oxidative phosphorylation, cell volume regulation, and protein synthesis (Putnam, 2012). Therefore, acidosis may also ultimately decrease energy supply and/or demand, and contribute to metabolic rate suppression during hibernation. However, our understanding of the cellular effects of acidosis during hibernation is still limited.

In Chapter 2 of my thesis, I test the effects of acidosis on O$_2$ transport in the blood, and how it may vary with temperature (Chapter 2). I then examine the temperature, seasonal and metabolic state effects on intracellular pH (pH$_i$) to elucidate whether intracellular acidosis occurs in tissues during hibernation (Chapter 3). Lastly, I investigate the effect of pH and temperature on O$_2$ consumption in the mitochondria (Chapter 4), and on ATP-consuming intracellular processes (Chapter 5). Throughout my thesis, I carry out a three species comparison between a non-hibernator (Rattus norvegicus; Sprague Dawley rat), facultative hibernator (Mesocricetus auratus; Syrian hamster), and obligate hibernator (Ictidomys tridecemlineatus; 13-lined ground squirrel) to understand whether observed pH effects are 1) present in all rodent species, or only in hibernating species, and 2) present all year round in hibernators, or whether these effects were acquired after the pre-hibernation remodelling period. For the remainder of this chapter, I provide background for the four data chapters that follow. Lastly, in Chapter 6, I synthesise the results from my data chapters and elaborate on future directions that may advance our understanding on the effect of acidosis during hibernation.
1.4 Blood O₂ transport in hibernation

The binding of O₂ to hemoglobin is cooperative, giving rise to a sigmoid shaped curve termed an O₂ equilibrium curve (OEC) which describes the association between blood O₂ saturation and O₂ partial pressure. A decrease in temperature causes a left shift in the OEC, which reflects a higher O₂ binding affinity with a lower $P_{50}$ (the partial pressure at which hemoglobin is 50% saturated with O₂). The overall enthalpy of the oxygenation process is exothermic (i.e., heat is released when O₂ molecules bind to the heme group). Conversely, the deoxygenation process is endothermic, requiring heat for hemoglobin to release O₂. As Le Chatelier’s principle states, low temperatures would cause the equilibrium reaction to favour the exothermic reaction, in which hemoglobin is oxygenated, thereby increasing hemoglobin’s affinity for O₂ at lowered temperatures. The HbO₂ affinity decreases in the presence of allosteric regulators such as organophosphates, CO₂, Cl⁻ and H⁺. The way in which H⁺/CO₂ affects the O₂ binding affinity of hemoglobin is known as the Bohr effect. CO₂ influences the blood O₂ carrying capacity directly as hemoglobin molecules bind CO₂, as well as indirectly by altering pH through the CO₂ hydration reaction. More specifically, in the presence of H⁺, the side chain of histidine 146 becomes protonated, which subsequently forms an ionic salt bridge with the carboxyl group of aspartate 94. This stabilises hemoglobin in the T-state, ultimately decreasing hemoglobin’s affinity for O₂.

The change in metabolic rate, $T_b$, and acid-base balance in hibernation poses an interesting conundrum for HbO₂ binding kinetics. As an animal enters hibernation, $T_b$ is drastically lowered which increases HbO₂ binding affinity and decreases O₂ offloading at the peripheries. However, as hibernators are known to experience relative acidosis of the blood during entrance, this decreases HbO₂ binding affinity and increases O₂ offloading at the tissues during a time when O₂ demand is decreasing. Upon arousal, metabolic O₂ demand begins to increase rapidly before a significant increase in $T_b$ occurs. The high HbO₂ binding affinity at low $T_b$ poses a challenge for HbO₂ offloading which must also increase to match the O₂ demand upon arousal. The increased binding affinity requires a reduced partial pressure at the tissues for O₂ unloading, which reduces the diffusion gradient for the movement of O₂ from plasma into surrounding cells.

HbO₂ offloading during arousal may be further hindered by the fact that most hibernators are
semi-fossorial animals. Semi-fossorial animals typically exhibit a higher HbO₂ binding affinity to increase O₂ loading at the lungs in potentially hypoxic burrows. The P₅₀ of hemoglobin in euthermic summer golden-mantled ground squirrels is around 18 mm Hg, which is approximately half that of normothermic rats that are of comparable size (Maginniss & Milsom, 1994; Woodson et al., 1973). Thus, hibernating species exhibit a left shifted OEC even during euthermia compared to that of non-hibernators. During hibernation, HbO₂ increases even further as organophosphate concentrations have been repeatedly shown to decrease during hibernation, thereby decreasing the P₅₀ (Burlington & Whitten, 1971; Maginniss & Milsom, 1994; Revsbech et al., 2013). A higher HbO₂ binding affinity is typical of hypoxia tolerant species, including semi-fossorial animals such as hibernators, and has been suggested to be beneficial in underground burrows (Revsbech et al., 2013, Revsbech & Fago, 2017).

To mitigate the negative effects of high HbO₂ binding affinity on O₂ unloading, it has been suggested that hibernators benefit from a smaller thermal effect on the HbO₂ binding affinity (Revsbech et al., 2013, Revsbech & Fago, 2017). The enthalpy associated with oxygenation in hibernators is lower than in humans (i.e., less heat is required to oxygenate the hemoglobin of hibernators, and therefore ΔH is less negative) (Revsbech & Fago, 2017). Thus, the temperature coefficient (Δlog P₅₀/ ΔT °C) of whole blood of golden-mantled ground squirrels (0.014; Maginniss and Milsom, 1994) and hedgehogs (0.017; Clausen & Ersland, 1968) is significantly less than that of humans (0.025; Zwart et al., 1984) and when the temperature is lowered, human blood exhibits a greater increase in hemoglobin’s O₂ binding affinity, in comparison to euthermic hibernators. Furthermore, there appears to be no effect of hibernation on temperature sensitivity in the golden-mantled ground squirrel, as the temperature coefficient is the same in euthermia and during hibernation (Maginniss & Milsom, 1994). As a result, low temperature is expected to induce a smaller increase in hemoglobin O₂ binding affinity in hibernators and this has been suggested to be an adaptation to facilitate O₂ offloading at the cold peripheries (Coletta et al., 1992; di Prisco et al., 1991; Revsbech et al., 2013).

The Bohr coefficient of the euthermic golden-mantled ground squirrel is higher (-0.6; Maginniss & Milsom, 1994; Revsbech et al., 2013) than that of rats (-0.47; Turek et al., 1978) and humans (-0.5; Astrup et al., 1965). This larger Bohr coefficient has been suggested
to facilitate O\(_2\) offloading at the tissues in hypercapnic and hypoxic burrow conditions, typical of hibernating species (Revsbech et al., 2013). While the Bohr coefficient is reported to be unaffected by temperature in human blood (-0.46 to -0.51 between 13 and 43 °C; Reeves, 1980), Maginniss and Milsom (1994) reported a decreased Bohr coefficient from -0.6 to -0.4, when the temperature was lowered, in both euthermic and hibernating golden-mantled ground squirrels. This indicates that as body temperature is lowered, pH enhances O\(_2\) offloading to a lesser extent in this species. However, a comprehensive study investigating the effects of temperature, pH, and hibernation state on the HbO\(_2\) binding kinetics of species other than the golden-mantled ground squirrel is limited.

In Chapter 2, I examine the effects of pH and temperature on HbO\(_2\) binding affinity in hibernators to elucidate how O\(_2\) transport may be affected by the opposing effects of low T\(_b\) and relative acidosis of the blood during hibernation. I hypothesised that the hibernating species will exhibit lower temperature sensitivity and greater Bohr effect to offset the higher HbO\(_2\) binding affinity in order to counter the deleterious effects of low body temperature on O\(_2\) offloading at the peripheries, particularly during arousal from hibernation when metabolic demands increase well before T\(_b\) does.

1.5 Intracellular acid-base balance in hibernation

The α-stat hypothesis was first applied to hibernators by Malan et al. (1985) based on the observation that the pH\(_i\) of some organs increased more than others during hibernation. In hibernating European hamsters, the temperature coefficient of pH (ΔpH/Δtemperature) was lowest in the blood plasma and brain (-0.0016 and -0.005, respectively), intermediate in striated muscle (-0.010) and highest in heart and liver (both -0.013) (Malan et al., 1985). Thus, the pH\(_i\) of the liver and heart change in an α-stat like fashion despite the fact the pH\(_e\) remains relatively constant with temperature changes (pH-stat). The brain, on the other hand, behaves similarly to blood plasma while skeletal muscle exhibits neither a clear α-stat or pH-stat pattern, with only a moderate increase in pH\(_i\) at low temperatures (Malan et al., 1985). This observation led to the hypothesis that acidosis was selectively inhibiting quiescent tissues, such as the brain (as neuronal activity is markedly attenuated during torpor; Krilowicz et al., 1988) and skeletal
muscle (as movement ceases during torpor) while following α-stat allowed the liver and heart to remain functional in hibernation (Malan et al., 1985). The liver continues to be the main sources of glucose and ketone bodies required by the central nervous system even during hibernation (Burlington & Klain, 1967; Krilowicz, 1985). While lipid catabolism is the main source of energy during fasting as in hibernation, the resulting free fatty acids are unable to cross the blood-brain barrier to be utilized as a metabolic fuel in the central nervous system (Carey et al., 2003). The heart also remains functional during hibernation, although the heart rate decreases to less than 20% of euthermic values (Harris & Milsom, 1995; Lyman, 1958). Therefore, α-stat regulation of liver and heart pH\(_i\) has been hypothesised to maintain the activity of proteins in these organs during hibernation. The brain and skeletal muscle of the European hamster have also been shown to maintain constant pH\(_i\) during hibernation, similar to the plasma (Malan et al., 1985), supporting the hypothesis that this may be adaptive in suppressing the metabolic rate of quiescent tissues that do not play any functional role during hibernation. In agreement, the activity of phosphofructokinase (PFK) isolated from skeletal muscle of active California ground squirrels (Otospermophilus beecheyi) is inhibited by a 0.3 – 0.4 unit decrease in pH. This suggests that acidosis may control glycolytic flux in the skeletal muscle of hibernators, although whether this inhibition contributes to significant suppression of metabolic rate is contested (Hand & Somero, 1983; MacDonald & Storey, 2001). Whether this selective acidosis of quiescent tissues occurs in other species of hibernators requires further investigation.

In Chapter 3 of my thesis, I tested the hypothesis that acidosis selectively occurs in quiescent tissues (such as the skeletal muscle and brain) as a means to decrease metabolic rate during hibernation. Based on the findings of Malan et al. (1985), I predicted that the quiescent tissues will maintain a relatively constant pH as temperature is lowered, thereby exhibiting relative acidosis at low temperatures. On the other hand, I predicted that the pH\(_i\) of tissues that remain functional during hibernation (such as the heart and liver) will increase with decreasing temperature to maintain α\(_{im}\) constant at all temperatures.

1.6 Mitochondrial O\(_2\) consumption in hibernation

As the ultimate consumers of cellular O\(_2\) that are responsible for up to 90% of whole animal O\(_2\)
consumption, the role of the mitochondria in hibernation has been extensively studied (Rolfe and Brown, 1997; Staples et al., 2022). The mitochondrial electron transport system (ETS) oxidises substrates to undergo a series of redox reactions with O$_2$ acting as the final electron acceptor. In this process, complexes I, III, and IV (CI, CIII, and CIV, respectively) move protons from the mitochondrial matrix to the intermembrane space. This proton motive force is utilised by F$_{O}F_1$ ATPase (complex V; CV) to phosphorylate ADP, thereby coupling electron transport to chemiosmosis of the proton gradient to generate adenosine triphosphate (ATP). Because the hydration of O$_2$ by CIV represents the last reaction of the ETS, mitochondrial O$_2$ consumption is often used as a metric to assess ETS function. Much of our understanding of the role of mitochondria during hibernation comes from the measurement of respiration in isolated mitochondria from animals in different states of hibernation.

Suppression of mitochondrial respiration during hibernation compared to IBE appears to be tissue-specific, with no suppression in the brain cortex, but a modest suppression (~30%) in skeletal and cardiac muscle (Brown et al., 2012; Brown & Staples, 2014; Gallagher & Staples, 2013; Muleme et al., 2006). Respiration in isolated mitochondria from the liver has been consistently shown to be significantly (~70%) lower in hibernating animals in comparison to IBE animals when assayed at warm temperatures (25 °C - 37 °C) (Brown et al., 2012; Martin et al., 1999; Staples et al., 2022). However, the difference in respiration rate between hibernation and active states becomes less apparent when the temperature is lowered to 10 °C (Armstrong & Staples, 2010; Chung et al., 2011; Muleme et al., 2006). This finding is consistent with the idea that active metabolic rate suppression is most critical at high $T_b$, while passive thermal effects are sufficient to suppress metabolism once body temperature decreases further. This active mechanism of suppression does not involve decreasing the mitochondrial density of the liver (approximated by citrate synthase quantity) or the oxidative phosphorylation capacity (no change in ETS enzyme quantity) (Mathers et al., 2017; Mathers & Staples, 2015). As mitochondrial respiration in the liver exhibits the greatest degree of suppression during hibernation compared to the brain, heart, and skeletal muscle, it has been extensively studied as the best candidate for identifying other regulatory mechanisms (Brown et al., 2012; Mathers & Staples, 2015, 2019; Muleme et al., 2006).
Mitochondrial respiration is rapidly inhibited during entrance into hibernation, as respiration rate reaches torpid levels within 2 hours. On the other hand, reversing the suppression is more gradual as respiration rates are restored to IBE levels around 10 hours after the initiation of arousal (Staples, 2014). This implies that the mechanism behind the suppression of mitochondrial respiration rate is temperature dependent, in which the initiation of suppression acts quickly at 37 °C, but the reversal is slower due to the colder body temperature (~10 °C) at the onset of arousal. As a result, post-translational modification (PTM) of ETS complexes has been the subject of recent studies. PTMs are facilitated by regulatory enzymes (such as protein kinases and acetyltransferases) acting on existing proteins. As with all enzymatic reactions, PTMs are temperature-sensitive and rapid-acting as existing proteins can be readily modified. In agreement, increased phosphorylation of CII during IBE has been implicated in controlling flux through the ETS in 13-lined ground squirrels (Mathers & Staples, 2019). In addition to PTMs, Jensen et al. (2021) recently reported that hydrogen sulfide (H₂S) is a potent inhibitor of liver mitochondrial respiration in hibernating 13-lined ground squirrels, but not in summer or IBE animals. Therefore, the suppression of mitochondrial respiration during hibernation appears to be mediated by more than one mechanism.

As mitochondrial function is intimately tied to pH at many levels, acidosis may be another potential mechanism that lowers mitochondrial function during hibernation. Oxidative phosphorylation for ATP synthesis is coupled to the chemiosmotic proton gradient between the intermembrane space and matrix. Changes in extramitochondrial pH are known to influence the proton gradient, as the outer mitochondrial membrane is permeable to small molecules such as protons (Porcelli et al., 2005; Lemeshko, 2006). Furthermore, the proton motive force that drives ATP synthesis is generated by the activity of ETS complexes which, as with all protein function, is sensitive to changes in pH. Furthermore, the movement of key substrates (such as pyruvate and glutamate) into the mitochondria via transmembrane transporters requires proton symport. Therefore, given the aforementioned role of the mitochondria in lowering metabolic rate during hibernation, and the complex association between pH and mitochondrial function, it is conceivable that acidosis may play a regulatory role on mitochondrial O₂ consumption during hibernation.
In Chapter 4, I hypothesised that acidosis inhibits metabolic rate during entrance into hibernation by lowering mitochondrial O$_2$ consumption. I predicted that the suppressive effects of acidosis on mitochondrial respiration will be greatest at high temperatures, as active metabolic rate suppression is most important during early entrance when $T_b$ has not yet significantly decreased.

### 1.7 Cellular ATP consumption in hibernation

When metabolic substrates and O$_2$ are abundant, ATP consuming processes indirectly control ATP synthesis. Intracellular sensors of AMP/ATP or ADP/ATP ratio downregulate ATP synthesis when these ratios are low to preserve metabolic substrates as ATP is in excess. During times of metabolic substrate or O$_2$ limitation, ATP supply constraints ATP consumption. States of controlled metabolic rate suppression, such as in hibernation, results from the orchestrated reduction in both ATP synthesis and consumption (Staples & Buck, 2009). As a result, the ATP content (i.e., the net result of ATP synthesis – ATP consumption) of the liver in golden-mantled ground squirrels does not differ between summer and IBE animals (Staples, 1995). This indicates that energy metabolism remains balanced in hibernation, as ATP synthesis and consumption are downregulated to similar degrees.

Whether one mechanism (such as acidosis) can inhibit both ATP synthesis and ATP consumption to the same magnitude to maintain energy balance during hibernation remains unclear. It is conceivable that the inhibitory role of acidosis on whole animal metabolic rate may be paralleled in the ATP synthesis pathways, the ATP consuming processes or both. The mechanisms reducing ATP synthesis at the mitochondrial level have been extensively studied, while the mechanisms controlling the reduction in ATP consumption during hibernation are relatively less studied. In chapter 5 of my thesis, I focus on the effect of acidosis on protein synthesis and ion homeostasis, as these two pathways have been regarded as the two biggest consumers of intracellular ATP (Buttgereit & Brand, 1995; Wieser & Krumschnabel, 2001).

#### 1.7.1 Protein synthesis

De novo protein synthesis is an essential but energetically consuming process that is responsible for up to 12 – 25% of the total cell O$_2$ consumption rate. Protein synthesis incurs a high
metabolic cost, as it is estimated to consume up to 70% of the intracellular ATP that is utilised for anabolic processes (Pontes et al., 2015). Therefore, in circumstances where conservation of metabolic fuel is required, protein synthesis is downregulated in both in vitro cell cultures as well as in whole animals (Buttgereit & Brand, 1995; Hand & Hardewig, 1996). It should be noted here, however, that although protein synthesis technically involves transcription and translation, for the brevity of this introduction only translation will be discussed in the context of protein synthesis, as is commonly done in the literature (Carter & Houlihan, 2001).

Protein synthesis consists of three phases: initiation, elongation and termination. The elongation stage of translation is of particular interest in the bioenergetic context as it is the most ATP consuming stage of translation (Chetverin & Spirin, 1982). Translation of messenger RNA (mRNA) into a polypeptide chain begins with the initiation phase when mRNA binds to the ribosomal complex. Subsequently, during the elongation phase, charged tRNA molecules carrying amino acids bind to the ribosomal complex, and extend the polypeptide chain. ATP is utilised during the process of tRNA charging, which requires ATP to attach the amino acid to the complimentary tRNA molecule. Furthermore, during the elongation phase, ATP is indirectly consumed for the production of GTP, which is required for the movement of the ribosome along the mRNA to the next codon. Lastly, translation is terminated at the stop codon of the mRNA through the binding of polypeptide chain release factors.

The overall rate of protein synthesis is controlled by several regulatory factors associated with each phase of translation, which are beyond the scope of this thesis (for a comprehensive review on regulatory control of translation, refer to Hershey et al., 2012). In this section of my thesis, I focus on the inhibition of the elongation phase, as ATP consumption is directly and indirectly involved with this phase of translation. Furthermore, previous studies have demonstrated a downregulation of the elongation phase of translation in hibernating 13-lined ground squirrels (Frerichs et al., 1998) although the mechanistic cause behind this downregulation remains to be elucidated. As with most biological reactions, the passive effects of low $T_b$ in hibernation decrease overall protein turnover and thus the need to carry out protein synthesis (van Breukelen & Martin, 2001; Velickovska et al., 2005). However, there is evidence to suggest that protein synthesis in the brain and liver are actively downregulated during hibernation by post-
translational phosphorylation of regulatory proteins involved in the initiation (eukaryotic initiation factor 2; eIF2) and elongation (eukaryotic elongation factor 2; eEF-2) phases of translation (Chen et al., 2001; Frerichs et al., 1998). In particular, eEF-2 activity in vitro is known to decrease at low pH (6.6), as the activity of eEF-2 kinase (eEF-2K) increases (Dorovkov et al., 2002). Increased eEF-2K activity at low pH results in increased eEF-2 phosphorylation, and subsequent inhibition of protein synthesis. Therefore, it is plausible that acidosis contributes to metabolic rate suppression in hibernation by decreasing rates of protein synthesis through the inhibition of eEF-2 activity.

1.7.2 Na\(^+\)-K\(^+\) ATPase

Maintaining cellular ion homeostasis is another major energy consuming process that is responsible for around 20% of cellular O\(_2\) consumption (Buttgereit and Brand, 1995). Cells need to maintain low intracellular Na\(^+\) and Ca\(^+\), but high K\(^+\) relative to the extracellular fluid to regulate cell resting membrane potential, cell volume, and signal transduction (Hoffmann et al., 2009). To avoid dissipation of these cellular ion gradients, which would lead to cell death, ATP-driven transmembrane ion transporters move ions against their concentration gradients. Hibernators are faced with a conundrum, as ion homeostasis must continue even during hibernation to maintain cellular integrity, yet cellular ion homeostasis is energetically costly. Furthermore, as with any enzyme catalysed reaction, the active movement of these ions is temperature dependent. Therefore, the drastic change in T\(_b\) experienced during hibernation poses a challenge for the maintenance of intracellular ion homeostasis. To this end, hibernating species exhibit lower passive movement of ions across the plasma membrane to maintain ion gradients (Dorovkov et al., 2002; Kimzey & Willis, 1971; Marjanovic & Willis, 1992; Wang et al., 2002; Zhou & Willis, 1989).

By reducing passive ion fluxes, hibernators can reduce the need for active transport of ions across the membrane and thereby reduce ATP demand during times of metabolic rate suppression. Na\(^+\)-K\(^+\) ATPase alone consumes 5 – 40% of cellular ATP, as it transports 3 Na\(^+\) ions out of the cell in exchange for 2 K\(^+\) moved into the cell for every ATP molecule utilised (Buttgereit & Brand, 1995). Na\(^+\)-K\(^+\) ATPase activity in the liver of golden-mantled ground
squirrels is suppressed by 50% compared to eutherian animals when assayed at 25 °C (MacDonald & Storey, 1999). Post-translational phosphorylation has been suggested as a putative mechanism for lowering Na\(^{+}\)-K\(^{+}\) ATPase activity, although additional mechanisms of active suppression of other cellular ATPases during hibernation may exist and remain yet to be elucidated. As rates of enzymatic reactions are also pH-dependent, it is conceivable that low pH may have an inhibitory role on ATP consumption in hibernation by lowering cellular ATPase activity. Previous studies have reported optimal Na\(^{+}\)-K\(^{+}\) ATPase activity at around pH 7.5 as Na\(^{+}\) selectivity at the cytoplasmic side decreases with lowering pH (Cornelius et al., 2018; Salonikidis et al., 2000). Whether hibernators exhibit the same decrease in Na\(^{+}\)-K\(^{+}\) ATPase activity with decreasing pH, and how changes in temperature and hibernation state may influence the effect of pH on Na\(^{+}\)-K\(^{+}\) ATPase activity remains to be characterised. Plasma membrane Ca\(^{2+}\) ATPase (PMCA) activity is another significant (~10%) contributor to basal cellular respiration, as it consumes ATP to maintain a low cytosolic Ca\(^{2+}\) concentration. As an intracellular secondary messenger, fluctuations in Ca\(^{2+}\) concentration is essential for signal transduction. To date, with the exception of Na\(^{+}\)-K\(^{+}\) ATPase, no study has examined the downregulation of other (or total) cellular ATPases during hibernation.

In Chapter 5, I predicted that acidosis inhibits ATP consumption during hibernation by increasing the phosphorylation of eEF-2 to decrease protein synthesis, and by reducing cellular ATPase activity in the liver. I further hypothesised that because active suppression of ATP demand is more important when Tb is high during early entrance into hibernation, the inhibition of these ATP-consuming processes will be greatest at low pH and high temperature.
Table 1.1. Blood pH and PCO$_2$ data compiled from literature.

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<tr>
<th>Species</th>
<th>Temperature (°C)</th>
<th>PCO$_2$</th>
<th>pH</th>
<th>Reference</th>
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<td>47.7</td>
<td>7.4</td>
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</tr>
<tr>
<td></td>
<td>6</td>
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<td>7.44</td>
<td></td>
</tr>
<tr>
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<tr>
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<td>7.38</td>
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2.1 Introduction

Mammalian hibernation is characterized by remarkable reductions in metabolic rate and body temperature. Even during such times of suppressed metabolism, however, O\textsubscript{2} transport must still be maintained (albeit at a lower rate) to sustain beta oxidation of fatty acids (Bailey & Davis, 1965). Hibernators also experience rapid changes in O\textsubscript{2} demand as they cycle between states of hibernation with associated metabolic suppression and interbout arousal where whole animal metabolic rate and body temperature return to euthermic levels. To meet these changing O\textsubscript{2} demands, O\textsubscript{2} transport is regulated in part, by modulating the binding affinity of the intracellular O\textsubscript{2} carrying protein, hemoglobin (Hb).

Hibernators are faced with an interesting physiological conundrum as two key regulators of HbO\textsubscript{2} binding affinity change rapidly between hibernation and interbout euthermia. Temperature is a key regulator of HbO\textsubscript{2} binding, with low temperatures increasing HbO\textsubscript{2} binding affinity as the binding of O\textsubscript{2} is an exothermic reaction which is favoured at low temperatures (Weber & Campbell, 2011). Dramatic decreases in body temperature to within 1-2 °C of winter ambient temperatures during hibernation can induce a marked increase in HbO\textsubscript{2} affinity. Such an increase in HbO\textsubscript{2} affinity reduces the PO\textsubscript{2} at which O\textsubscript{2} is unloaded at the tissues, decreasing the diffusion gradient for O\textsubscript{2} from the blood into the tissues.

However, the drastic reduction in body temperature in hibernation is accompanied by a dramatic fall in O\textsubscript{2} demand as metabolic rate is reduced by more than 90% in hibernation (Staples, 2014). Therefore, the reduced O\textsubscript{2} offloading at the tissues due to increased HbO\textsubscript{2} binding affinity in the blood is balanced by a low O\textsubscript{2} demand during hibernation. On the other hand, during arousal from hibernation, a rapid reversal of the metabolic rate suppression occurs and the O\textsubscript{2} demand for thermogenesis increases before body temperature changes. While the subsequent rapid increase in body temperature will promote O\textsubscript{2} offloading by decreasing HbO\textsubscript{2} binding affinity in the blood, the kinetics of this process in different body tissues are not well established. Secondly, H\textsuperscript{+} is an allosteric regulator that reduces O\textsubscript{2}
The pH-sensitivity of HbO₂ binding affinity, termed the Bohr effect, occurs as protonation of specific histidine residues stabilises the low affinity (T) conformation of the hemoglobin tetramer. The Bohr effect is well studied in mammalian hemoglobin and is observed in nearly all species examined to date, although its magnitude varies widely across species (Riggs, 1960; Zhang et al., 2006). The blood pH of several hibernating species is reported to remain similar in eutherma and hibernation (Kreienbühl et al., 1976). However, this in fact represents a relative acidosis of the blood as the body temperature of euthermic animals (37 °C) and hibernating animals (~10 °C) affects the degree of relative alkalinity to neutral pH of water (pH₆w). As pH₆w increases with decreasing temperature, the dissociation of water into constituent ions (OH⁻ and H⁺) also decreases. As pH is a measure of [H⁺], pH₆w increases as temperature decreases, although by definition, the solution is still neutral because pOH and pH are equal. Thus, the relative degree of alkalinity of blood relative to pH₆w decreases with decreasing body temperature in hibernators. This phenomenon in which measured absolute pH value does not change with temperature is termed pH-stat regulation, and this relative acidosis may enhance O₂ unloading from hemoglobin. It has been suggested that this relative acidosis (as the degree of relative alkalinity to pH₆w is decreased when pH 7.4 is maintained while pH₆w increases with lowered temperatures) may contribute to the metabolic suppression during torpor (Malan et al., 1985). Furthermore, during arousal, the rapid increase in metabolic rate will lead to increased CO₂ production and acidosis during thermogenesis, which may also contribute to O₂ unloading.

The interactive effects of relative acidosis and low temperature on HbO₂ binding and unloading will depend on the magnitude of the Bohr effect and temperature sensitivity of HbO₂ binding in the blood, each of which may be species specific. An increased sensitivity to lowered body temperature along with a reduced Bohr effect would reduce offloading of O₂ at the tissues during entrance into hibernation possibly contributing to the reduction in O₂ consumption during torpor, while a reduced temperature sensitivity and enhanced Bohr effect would serve to enhance the O₂ offloading that is critically important at the onset of arousal when metabolic demand begins to rapidly increase while body temperature is still low. To better understand the quandaries of HbO₂ binding kinetics, I constructed oxygen equilibrium
curves (OECs) using whole blood of two semi-fossorial rodents capable of hibernation, the 13-lined ground squirrel and the golden-Syrian hamster, as well as the Sprague Dawley Rat, a rodent that does not undergo any form of torpor. I hypothesise that the hamster and ground squirrel will exhibit a lower temperature sensitivity and greater Bohr effect to offset the higher HbO₂ binding affinity of whole blood that is intrinsic to hibernators, in order to facilitate oxygen offloading that is essential during arousal.

2.2 Materials and Methods

2.2.1 Animals

All procedures were conducted under protocol A21-0006, approved by The University of British Columbia (UBC) Animal Care Committee (ACC) in compliance with the standards of the Canadian Council on Animal Care (CCAC). 13-lined ground squirrels were captured and transported from Carman, Manitoba, Canada, and housed at an animal care facility at UBC. During the active season (May-October; 230g–316g), all animals were fed ad libitum dog food (IAMS small chunk dog food) and water, with apples and cereal as occasional supplements. No food or water was provided during the hibernation period. All animals were kept in a temperature-controlled chamber (20 ± 2 °C) on a photoperiod that matched the daily photoperiod in Vancouver, Canada during the active period and at (5±2 °C) in 24 h dark during the hibernation period (November-April). Winter animals (150–270g) were previously intraperitoneally implanted with RFID temperature chips (IPTT-300; 1 mm × 3 mm, < 0.5 g) and scanned with a DAS-8017 reader (Biomedic Data Systems, DE, USA) at the time of sampling (see section 2.2.2), as outlined in Sprenger and Milsom (2022). Adult Sprague-Dawley rats (256g–568g) and Syrian hamsters (80g–130g) were acquired from commercial breeders (Envigo and/or Charles Rivers). Rats and hamsters were fed ad libitum rodent chow (Purina LabDiet 5001) and water, with cereal as occasional supplements. Rats and hamsters were maintained at 20 ± 2 °C and sampled throughout the year.
2.2.2 Blood sampling

All animals were euthanised by isoflurane overdose followed by exsanguination through cardiac puncture. Between 3 – 10 ml of blood were collected using heparinised 23-gauge needles and stored in heparinised vacutainers at 4 °C. All experiments were carried out within four days of blood collection. Rats (n = 8), euthermic 13-lined ground squirrels (n = 6) and euthermic golden-Syrian hamsters (n = 9) were sampled during summer months (May-August) at 37 °C. Hibernating 13-lined ground squirrels (n = 6) were sampled during the hibernation season (November-April) after a minimum of 48 hours in hibernation and with body temperatures between 5 – 10 °C. Body temperature was determined using previously implanted RFID temperature loggers without disrupting the animal. Animals were confirmed to be in torpor for at least 48 hours by daily monitoring and if wood chips placed on the hibernating animals remained undisturbed. Interbout euthermic animals (n = 6) were sampled by agitating animals in hibernation at least 12 hours prior to sampling, and only when the animal was fully active and had returned to ~37 °C body temperature at the time of sampling.

2.2.3 $O_2$ equilibrium curves (OECs)

OECs were constructed at three temperatures (10, 25, and 37 °C) and at three CO$_2$ tensions (~15, 35 and 50 mm Hg). These temperatures and CO$_2$ levels were chosen to alter blood temperature and pH within a physiologically relevant range (Musacchia and Volkert, 1971). OECs were generated using a custom microplate-based tonometry cell as described by Lilly et al., 2013. Briefly, microcuvettes were formed by sandwiching ~3 μL of whole blood between two sheets of low-density polyethylene secured with plastic O-rings on an aluminium ring, which were then placed in a tonometry cell designed to fit into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, USA). The inside of the tonometry cell was isolated from external gases (i.e., atmospheric air), and precise gas mixes were introduced through a gas inlet. Optical density was measured every 30 seconds at 436 nm (near the peak absorption for deoxygenated Hb) and at 390 nm (near the isosbestic point where optical density is independent of HbO$_2$ saturation). Initially, samples were
deoxygenated with pure N\textsubscript{2} for at least 45 minutes, until the optical density at 436 nm had plateaued, which was assumed to indicate full Hb deoxygenation. Subsequently, HbO\textsubscript{2} saturation was increased with nine stepwise increments of PO\textsubscript{2} (from 0 – 190 mm Hg, balanced with N\textsubscript{2}). Full HbO\textsubscript{2} saturation was assumed after the final increment to a PO\textsubscript{2} of 190 mm Hg. Gases (O\textsubscript{2}, CO\textsubscript{2} and N\textsubscript{2}) were mixed with three mass flow controllers (Alicat Scientific Inc., Tuscon AZ, USA) to desired levels, which generated a total flow rate of ~500 ml/min. The difference in optical density (ΔOD) between 390 and 436 nm was determined for each equilibration step and the fractional HbO\textsubscript{2} saturation at each step was determined as the change in ΔOD from full deoxygenation relative to the change in ΔOD between full deoxygenation (pure N\textsubscript{2}) and full oxygenation (PO\textsubscript{2} of 190 mm Hg / 25% O\textsubscript{2}):

\[ \text{HbO}_2 \text{ Saturation} = 100 \times \frac{[(\text{OD}_{430nm} - \text{OD}_{390nm})_{\text{sample}} - (\text{OD}_{430nm} - \text{OD}_{390nm})_{\text{fully deoxygenated}}]}{[(\text{OD}_{430nm} - \text{OD}_{390nm})_{\text{fully oxygenated}} - (\text{OD}_{430nm} - \text{OD}_{390nm})_{\text{fully deoxygenated}}]} \]

In order to estimate the corresponding pH associated with the aforementioned measurement of OECs in the microcuvettes, pH was measured in the same blood samples using a combination microelectrode (Hach Co., CO, USA). Approximately 400 μL of each blood sample was equilibrated for 1 hour with either ~15, 35 or 50 mm Hg CO\textsubscript{2} and 160 mm Hg O\textsubscript{2} (balanced with N\textsubscript{2} and humified at the experimental temperature), in a rotating glass tonometer thermostated to either 10, 25, or 37 °C.

2.2.4 Data analysis

An OEC was constructed for each blood sample using a best-fit non-linear regression curve that was then used to predict the PO\textsubscript{2} at ten saturation levels (10-95%). Subsequently, the estimated PO\textsubscript{2} of each saturation for each animal was plotted against the measured whole blood pH (Bohr plots). PO\textsubscript{2} for each saturation at fixed pH levels was interpolated from the linear regression equation of the Bohr plots. These interpolated values were then used to construct species-specific OEC at 10, 25, and 37 °C, and defined pH levels. Bohr coefficients (\( \phi \pm \text{CI} \)) were determined as the slopes of the linear regression. It should be noted that due to insufficient blood volume, Bohr coefficients for each individual animal could not be determined at each temperature. Therefore, the Bohr plots were constructed by fitting linear
models at each temperature, using pooled data where some individuals were represented by more than one data point (at different pH levels).

Typical mammalian arterial pH is known to be tightly regulated around pH 7.4 (Fregosi & Dempsey, 1984; Stringer et al., 1992), which is 0.6 pH units more alkaline than the pH of neutrality (pH$_{nw}$) at 37 °C. In accordance with pH-stat regulation, I calculated P$_{50}$ values at pH 7.4 at each of these temperatures. In addition, the P$_{50}$ values at pH 7.6 at 25 °C and pH 7.87 at 10 °C, were also estimated as these pH values are 0.6 pH units more alkaline to the pH$_{nw}$ at each respective temperature and correspond to α-stat regulation. P$_{50}$ values across species were compared using a 2-way ANOVA, followed by a Tukey’s multiple comparison test across species within each temperature. All statistical analyses and model fitting were performed in R v 3.5.2. (R Core Team, 2017) and Graphpad Prism v8 (CA, USA).

Hemoglobin concentrations ([Hb]) used in calculating the O$_2$ carrying capacity of the blood ([Hb] x 1.34 ml O$_2$/ g Hb) were provided by the commercial suppliers for rats (Charles River Laboratories, MA, USA) and hamsters (Envigo, IN, USA), while [Hb] for ground squirrels were used from Cooper et al. (2016). Comparison of the volume of O$_2$ offloaded at 10 °C at pH 7.4 or 7.87 was calculated by multiplying the O$_2$ carrying capacity by 100 - % saturation (i.e., offloaded %) that results in a PO$_2$ of 6.3 mm Hg (the reported PO$_2$ of mixed venous blood in hibernating 13-lined ground squirrels; Musacchia and Volkert, 1971).

2.3 Results

2.3.1 Effect of temperature on HbO$_2$ binding affinity

Reduction of blood temperature significantly increased HbO$_2$ affinity (p < 0.0001) in all groups (Fig. 2.1, Table 2.1). When temperature was lowered, P$_{50}$ decreased the most in the rat, as indicated by the highest temperature coefficient (ΔlogP$_{50}$/ΔT; Table 2.2). HbO$_2$ affinity also differed across species and state (p = 0.012), although there was no significant interaction between temperature and species or state. Of the hibernating species groups, only the hamster and hibernating squirrel had a lower P$_{50}$ (22 mm Hg ± 1.4 and 25 mm Hg ± 1.2, respectively) compared to the rat at 37 °C and pH 7.4 (Figure 2.1, Table 2.1). As temperature
was lowered to 25 °C and 10 °C (still at pH 7.4), there was no significant effect of species or state on HbO₂ affinity, with $P_{50}$ ranging between 19-21 mm Hg, and 9-11 mm Hg, at 25 °C and 10 °C respectively (Figure 2.1, Table 2.1). Hibernation state did not have an effect on HbO₂ affinity as the difference in $P_{50}$ was not statistically significant between summer active, hibernating or interbout euthermic animals at any assay temperature.

2.3.2 Effect of pH on HbO₂ binding affinity

In all species and hibernation states, $P_{50}$ values estimated at the pH corresponding to $\alpha$-stat at each respective temperature (i.e., 0.6 pH units more alkaline than that of pH$_{aw}$ at that temperature) were lower than $P_{50}$ values estimated at pH 7.4 (Figure 2.1, Table 2.1). The rat had the smallest Bohr coefficient at all temperatures examined. At 25 °C, the greatest Bohr effect was seen in the blood of hibernating squirrels, and therefore the $P_{50}$ was reduced by 5 mm Hg at pH 7.6 ($\alpha$-stat pH at 25 °C) (Figure 2.1, Table 2.1). At 10 °C, the euthermic hamster had the largest Bohr effect, with a $P_{50}$ reduced by 5 mm Hg. Temperature and pH effects on Hill’s cooperativity coefficient ($n_{50}$) was negligible, with coefficients ranging between 2-3 in all species/states, indicating high cooperativity in all conditions.

Table 2.3 presents calculated amounts of O₂ that would be unloaded from arterial to venous blood at a venous PO₂ equivalent to that of hibernating 13-lined ground squirrels (6.3 mm Hg as reported by Musacchia and Volkert, 1971). This calculation was carried out with the assumption that arterial blood was 100% saturated, and calculations were made using the OECs and Bohr coefficients for the different groups (Table 2.1). Based on this I estimate that 7.4 to 11.9 ml of O₂ would be unloaded from each dL of blood at pH 7.87 ($\alpha$-stat regulation) at 10 °C in the different groups. More O₂ would be unloaded from blood following pH-stat regulation as the volume of O₂ offloaded at pH 7.4 ranges from 15.1 to 18.4 ml O₂ per dL of blood. The % increase in the volume of O₂ offloaded when following pH-stat (as compared to following $\alpha$-stat) was lowest in rats (37%) and highest in hamsters (134%). By following pH-stat, the volume of O₂ offloaded for the 13-lined ground squirrels increases by 108, 88, and 54% in summer, hibernating and IBE animals, respectively.
2.4 Discussion

To investigate the potential interactive influence of acidosis and low temperature on O\textsubscript{2} transport during hibernation, I constructed OECs using whole blood from two species of hibernators and the non-hibernating rat. The findings of this study support the hypothesis that the HbO\textsubscript{2} binding kinetics of hibernators facilitates O\textsubscript{2} offloading which may be crucial for meeting high O\textsubscript{2} demands during arousal. This reflects a lower temperature sensitivity of HbO\textsubscript{2} binding combined with higher Bohr coefficients in the species capable of hibernation.

High HbO\textsubscript{2} affinity has long been regarded as an adaptive trait in species inhabiting environments with low oxygen availability (Clementi et al., 2003; Maginniss & Milsom, 1994; Revsbech & Fago, 2017). The dens of semi- or fully fossorial animals are considered to be hypoxic by many, although very few direct measurements of den PO\textsubscript{2} exist (Kühnen, 1986). Nevertheless, it has been suggested that high HbO\textsubscript{2} affinity may be an adaptive trait in hibernators to facilitate O\textsubscript{2} loading in burrow conditions with low O\textsubscript{2} availability (Clementi et al. 2003). In agreement, hibernators such as the golden-mantled ground squirrel have also been reported to have a much higher HbO\textsubscript{2} binding affinity than predicted by the allometric equation for mammalian P\textsubscript{50} based on body size (Predicted P\textsubscript{50} = 37.5 mm Hg; Measured P\textsubscript{50}=18 mm Hg; Boggs et al., 1984; Maginniss & Milsom, 1994). However, I found that while the P\textsubscript{50} of blood from hamsters and hibernating 13-lined ground squirrels at 37 °C was significantly lower than that of the rat, summer euthermic 13-lined ground squirrel blood did not have a significantly lower P\textsubscript{50} (29 mm Hg) compared to the P\textsubscript{50} of the rat at the same temperature (31 mm Hg). The P\textsubscript{50} of the rat found in this study is consistent with previously published literature values for this species (Cartheuser, 1993; Woodson et al., 1973). Given that the 13-lined ground squirrel and the golden-mantled ground squirrel belong in the same Sciuridae family, exhibit similar degrees of fossoriality, body size, and hibernation patterns the high P\textsubscript{50} in the summer euthermic 13-lined ground squirrels was surprising but suggests that the higher HbO\textsubscript{2} affinity of hibernating animals may only be a seasonal phenomenon in some species. It also suggests that a higher HbO\textsubscript{2} affinity may not be a universal trait exhibited by all hibernating and/or fossorial species of mammals as previously suggested.
In addition, I found that $P_{50}$ at 37 °C in the 13-lined ground squirrel was modestly lower (~3 mm Hg) in the two winter metabolic states, compared to active summer animals. This is similar to the findings of Maginnis and Milsom (1993) in which hibernating golden mantled ground squirrels exhibited a $P_{50}$ that was ~3 mm Hg lower than summer active animals at 37 °C and pH 7.49. This decrease in $P_{50}$, albeit small, has been attributed to a lower 2,3-DPG concentration in golden-mantled ground squirrels during the winter. Similarly, Burlington and Whitten (1971) reported a significant decrease in red blood cell 2,3-DPG during hibernation in 13-lined ground squirrels.

As blood temperature was decreased, I observed almost no differences in $P_{50}$ between species. At 10 °C, the sigmoidal curves become increasingly steep, and while a small decrease in PO$_2$ results in significant O$_2$ offloading, the diffusion gradient for O$_2$ from the blood to the tissue, and ultimately to the mitochondria becomes small. Hibernators experience a drastically lowered metabolic rate at such low temperatures. Whether this is because O$_2$ becomes limiting and subsequently contributes to metabolic suppression in hibernation is unclear. While the $P_{50}$ of the different species and metabolic states were similar at low temperatures, this does reflect a reduced temperature sensitivity of the blood oxygen binding affinity in hibernators. If their temperature sensitivity was similar to that of the rat, their HbO$_2$ affinity would increase even further as temperature decreased, and the tissue O$_2$ diffusion gradient at the tissues would be even more reduced. Therefore, a reduced temperature sensitivity of the HbO$_2$ affinity may be a mechanism to prevent O$_2$ offloading from becoming limiting. Furthermore, it may also prevent O$_2$ from becoming limiting at the onset of arousal when metabolic O$_2$ demand begins to increases rapidly while body temperature is still low. Similar properties of Hb binding kinetics have been demonstrated in fish that have adapted to functional hypoxemia, such as that in trout, which exhibit a high degree of cooperativity and pH sensitivity to favour unloading of O$_2$ to meet the high metabolic demand during exercise (Eddy et al., 1977; Wells, 2009).

To offset the increased HbO$_2$ binding affinity at low temperatures, a large Bohr effect would also contribute to O$_2$ unloading at the tissues at the onset of arousal. Sympathetic activation of brown adipose tissue leads to intense thermogenesis both requiring increased O$_2$ delivery
as well as increased CO₂ production (Kitao & Hashimoto, 2012; Smith, 1964). This would produce a local acidosis that would lead to a right shift in the OEC and enhance O₂ unloading. My findings that the HbO₂ binding affinity at 10 °C of the two hibernating species is more pH sensitive than that of the rat are consistent with this. It should be noted that the Bohr effects in blood of hibernators examined at 37 °C in this study were not appreciably greater than those reported in previous findings in other non-hibernating mammals (mice φ=-0.96; Riggs 1960, humans φ = -0.48; mouse deer φ = -0.48; Snyder & Weathers, 1977). Nonetheless, due to the increased Bohr coefficient at 10 °C, the hibernating ground squirrel can be predicted to offload up to 86% more oxygen for a 0.47 unit decrease in pH (a right shift of the OEC) at a PO₂ of 6.3 (reported venous PO₂ of hibernating 13-lined ground squirrels; Musacchia & Volkert, 1971), in comparison to the rat (37%) at the same PO₂ and pH change (Table 2.3). The increase in O₂ offloading in the hamster was even greater (134%). Nonetheless, I emphasise that I do not consider these values to be absolute values representative of in vivo extraction rates, but rather a theoretical consideration of the consequences of the differences in Bohr effect, given that all else (including tissue diffusive capacity and cardiac output) is equal across the species.

Albeit at low rates, oxygen delivery must continue during hibernation, and thus hemoglobin must continue to function even at body temperatures as low as – 2.9 °C (Barnes, 1989). The α-stat hypothesis suggested that maintaining pHₐ with the same degree of relative alkalinity as at 37 °C (0.6 pH units above pHₐw) with decreasing body temperature, would be of physiological advantage as protein function would be maintained. In all species and hibernation states examined in this study, however, I demonstrate that following α-stat regulation instead of maintaining pHₐ at 7.4 in whole blood (pH-stat) would approximately halve the P₅₀ at 10 °C. This would be deleterious for the diffusion gradient between O₂ from blood to tissues. To offload the same volume of O₂ from hemoglobin with a relatively more left shifted OEC at higher pH (following α-stat regulation of the blood at 10 °C), the PO₂ of blood would have to fall drastically.

Taken together, the reduced temperature sensitivity and increased Bohr coefficient in hibernators suggest that the kinetics of HbO₂ binding in hibernators is beneficial for
enhancing O₂ offloading during arousal. Given the low metabolic rate in hibernation, whether a left shifted OEC at low temperatures also reduces the diffusion gradient for O₂ leading to an O₂ supply limitation that contributes to the reduced metabolic rate in hibernation, as suggested by others (Malan et al. 1985), requires further investigation.
Figure 2.1. Whole blood oxygen equilibrium curves (OEC) for rat, hamster and ground squirrel at 37 °C (● - pH 7.4), 25 °C (○ - pH 7.4; ▲ - pH 7.6), and 10 °C (● - pH 7.4; ▲ - pH 7.87). Dashed lines for 25 °C and 10 °C indicate OEC at pH at which 0.6 pH units of alkalinity relative to pH_{nw} is maintained (i.e., α-stat pH).
Table 2.1. Whole blood oxygen equilibria parameters for rats, hamster and ground squirrels. Whole blood \( P_{50} \) is reported at pH 7.4 and at respective \( \alpha \)-stat pH for 25 °C and 10 °C. Interspecies differences in \( P_{50} \) were only observed at 37 °C using a two-way ANOVA, followed by a Tukeys post-hoc analysis. Significant differences of \( P_{50} \) at 37 °C is denoted by different letters. Bohr coefficients (\( \Phi \)) were determined as the slopes of \( \Delta \log P_{50}/\Delta \text{pH} \). Hill cooperativity coefficients (\( n_{50} \)) were determined at \( P_{50} \) using the Hill equation.

<table>
<thead>
<tr>
<th></th>
<th>10 °C</th>
<th>25 °C</th>
<th>37 °C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH-stat (7.4)</td>
<td>95% CI</td>
<td>( \alpha )-stat (7.6)</td>
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<tr>
<td><strong>Rat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_{50} ) (mm Hg)</td>
<td>10.3</td>
<td>9.5 to 11.2</td>
<td>8.6</td>
</tr>
<tr>
<td>( n_{50} )</td>
<td>2.54</td>
<td>2.53 to 2.55</td>
<td>2.62</td>
</tr>
<tr>
<td>( \Phi )</td>
<td>-0.17 (-0.44 to -0.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hamster</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_{50} ) (mm Hg)</td>
<td>10.0</td>
<td>8.5 to 11.6</td>
<td>4.9</td>
</tr>
<tr>
<td>( n_{50} )</td>
<td>3.15</td>
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<tr>
<td>( \Phi )</td>
<td>-0.65 (-0.99 to -0.31)</td>
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<tr>
<td><strong>Summer squirrel</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>( P_{50} ) (mm Hg)</td>
<td>11.0</td>
<td>10.0 to 12.1</td>
<td>5.5</td>
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<tr>
<td>( n_{50} )</td>
<td>2.63</td>
<td>2.61 to 2.65</td>
<td>3.31</td>
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<tr>
<td>( \Phi )</td>
<td>-0.64 (-0.87 to -0.41)</td>
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<td><strong>Hibernating squirrel</strong></td>
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<td>( P_{50} ) (mm Hg)</td>
<td>10.6</td>
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<tr>
<td><strong>IVE squirrel</strong></td>
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</tr>
<tr>
<td>( P_{50} ) (mm Hg)</td>
<td>9.9</td>
<td>8.5 to 10.3</td>
<td>7.3</td>
</tr>
<tr>
<td>( n_{50} )</td>
<td>2.92</td>
<td>2.90 to 2.94</td>
<td>2.76</td>
</tr>
<tr>
<td>( \Phi )</td>
<td>-0.22 (-0.40 to -0.04)</td>
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Table 2.2. Temperature coefficients (ΔlogP_{50}/ΔT) of whole blood for rats, hamster and ground squirrels.

<table>
<thead>
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<th>Temperature coefficient</th>
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<td>Rat</td>
<td>0.0182</td>
</tr>
<tr>
<td>Euthermic hamster</td>
<td>0.0127</td>
</tr>
<tr>
<td>Summer euthermic squirrel</td>
<td>0.0156</td>
</tr>
<tr>
<td>Hibernating squirrel</td>
<td>0.0132</td>
</tr>
<tr>
<td>IBE squirrel</td>
<td>0.0171</td>
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Table 2.3. Calculated volume of O₂ offloaded at PO₂ of 6.3 mm Hg (reported venous PO₂ of hibernating 13-lined ground squirrels; Musacchia and Volkert, 1971) with changes in pH at 10 °C. Hemoglobin concentrations obtained from: ¹Charles River Laboratories, MA, USA; ²Envigo, IN, USA; ³Cooper et al. (2016).

<table>
<thead>
<tr>
<th></th>
<th>[Hb] (g/dL)</th>
<th>O₂ carrying capacity (ml O₂/dL)</th>
<th>mL O₂ offloaded at pH 7.4 (pH-stat)</th>
<th>mL O₂ offloaded at pH 7.87 (α-stat)</th>
<th>% Increase in ml O₂ relative to pH 7.87</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>15.6¹</td>
<td>20.90</td>
<td>16.2</td>
<td>11.9</td>
<td>37</td>
</tr>
<tr>
<td>Euthermic hamster</td>
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<td>22.78</td>
<td>18.4</td>
<td>7.9</td>
<td>134</td>
</tr>
<tr>
<td>Summer euthermic squirrel</td>
<td>14.1³</td>
<td>18.89</td>
<td>15.3</td>
<td>7.4</td>
<td>108</td>
</tr>
<tr>
<td>Hibernating squirrel</td>
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<td>19.43</td>
<td>15.9</td>
<td>8.5</td>
<td>86</td>
</tr>
<tr>
<td>IBE squirrel</td>
<td>14.9³</td>
<td>19.97</td>
<td>15.1</td>
<td>9.8</td>
<td>54</td>
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</table>
3.1 Introduction

During times of food scarcity, caloric intake cannot sustain continued high metabolic rates to maintain homeothermy in endothermic vertebrates. To survive these periods, many taxa have evolved the capacity to abandon homeothermy and enter a state of dormancy with reversible suppression of metabolic rate, body temperature and activity (Geiser, 2004). Of the many diverse animals that lower metabolic rate in challenging environments, hibernators experience one of the most drastic reductions in metabolism (to less than 10% of resting metabolic rate) and body temperature (from 37 °C to within 1-2 °C of winter ambient temperatures) (Geiser, 2004; Lyman, 1948; Staples, 2016). Even though body temperature is well known to influence acid-base balance, the changes that accompany metabolic suppression and falls in body temperature have received relatively little attention in hibernators, compared to ectothermic vertebrates and clinical research on hypothermia (Howell et al., 1970).

Many ectotherms increase blood pH (pH_e) and intracellular pH (pH_i) as body temperature decreases to maintain the same degree of relative alkalinity to the pH of neutrality (pH_{nw}; where pH and pOH are identical). pH_{nw} increases with decreasing temperature (0.017 units/°C between 37 and 3 °C) as the autoionisation of water into H^+ and OH^- decreases. However, this increase in pH_{nw} does not represent alkalinisation of water, as both products of water dissociation continue to exist in equal ratio. Imidazole serves as an important intracellular buffer since the pK of imidazole (pK 6.7 at 37 °C) is relatively close to pH_i which is typically between 6.5 - 7.5, although pH_i varies greatly depending on the species, body temperature, metabolic activity, and tissue type (Somero, 1985; Putnam, 2012). When the pK of a molecule is equal to the pH of a solution, the molecule is able to accept/donate protons, i.e., act as a buffer. The pK of imidazole increases by, on average, 0.018 units/°C with decreasing temperature. As the physiological pH of ectotherms increases with decreasing body temperature in parallel to the pK of imidazole, Reeves et al. (1972)
hypothesised that by regulating pH in this fashion, protein function would be maintained with varying body temperatures since the ionisation ratio of their major intracellular buffer would be kept constant. This hypothesis was referred to as the $\alpha$-stat hypothesis, with $\alpha_{im}$ referring to the ionisation ratio of histidine’s imidazole ring ($\alpha_{im} = [\text{imidazole}] / ([\text{protonated imidazole}] + [\text{imidazole}])$. At any given temperature, intracellular $\alpha_{im}$ varies with pH$_i$, increasing as pH$_i$ increases. In accordance with the $\alpha$-stat hypothesis, the $\Delta$pH$_i$/$\Delta$temperature of tissues of many ectotherms from fish to iguanas mirrors that of pH$_{nw}$ and the $\Delta$pK$_i$/$\Delta$temperature of imidazole (Bickler, 1982; Bock et al., 2001; Malan et al., 1976).

Conversely, deviation from $\alpha$-stat implies protein function is impaired as temperature falls. Maintaining the same pH with changing temperature is termed ‘pH-stat’ regulation which represents a relative acidosis at low body temperature in which the degree of alkalinity of tissues relative to pH$_{nw}$ is reduced. However, these acid-base strategies ($\alpha$-stat and pH-stat) have almost exclusively been applied to ectotherms such as reptiles and fish (Cameron & Kormanik, 1982; Howell et al., 1970; Malan et al., 1976), although the theory is not necessarily confined to animals that regulate body temperature based on ambient temperature. Rather, it applies to any animal that varies body temperature widely enough that acid-base state may be altered as a result, including heterotherms.

To date, only three papers (Malan et al., 1985; McArthur et al., 1990; Nestler, 1990a) have investigated pH$_i$ in mammalian species capable of torpor, even though hibernators vary body temperature as much as, if not more drastically, than many ectothermic species. The liver and heart of hibernating European hamsters were reported to have a higher pH$_i$ when collected and assayed at 10 °C, compared to those of interbout euthermic (IBE) animals collected and assayed at 37 °C ($\Delta$pH$_i$/$\Delta$temperature: -0.013). As a result, $\alpha_{im}$ was similar between hibernation and IBE (i.e., $\alpha$-stat), and this was hypothesised to maintain protein function for cardiac contractility and gluconeogenesis during hibernation (Malan et al., 1985). On the other hand, the increase in pH$_i$ was less ($\Delta$pH$_i$/$\Delta$temperature: -0.005) and $\alpha_{im}$ was reduced in the brain between hibernation and IBE, which was suggested to inhibit neuronal function and lower the temperature set point of the hypothalamus in heterotherms (Malan et al., 1985; Wright & Boulant, 2007; Wünnenberg & Baltruschat, 1982). This was further corroborated by the findings of Nestler (1990), who also reported similar results in liver and heart and the
greatest reduction in $\alpha_{im}$ between daily torpor and euthermia in the brain of deer mice *(Peromyscus maniculatus)*. However, by comparing pH$_i$ measured in tissues taken from animals in hibernation/torpor and euthermia to determine $\Delta$pH/$\Delta$temperature, previous studies were unable to elucidate whether the observed changes were due to the effects of temperature *per se*, or due to changes in intracellular buffering associated with changes in metabolic state and/or of hibernation season.

The Syrian hamster (*Mesocricetus auratus*) is a facultative hibernator that can be induced into torpor at any time of the year when acclimatised to cold temperatures, short photoperiod and reduced food availability (Chayama et al., 2016; Harlow & Menkens., 1986; Lehmer et al., 2006). In contrast, an obligate hibernator such as the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) displays distinct seasonality in its metabolic phenotype, with hibernation only occurring during the winter even in captivity in the absence of any environmental cues (MacCannell & Staples, 2021). Several previous studies have reported extensive physiological remodelling in the brain, skeletal muscle, white adipose tissue and lungs (Chayama et al., 2016, 2019; Nowell et al., 2011; Schwartz et al., 2013) during a pre-hibernation acclimatisation period that precedes hibernation bouts in facultative hibernators and during the fall in obligate hibernators. Whether changes in intracellular buffering constituents occur with the pre-hibernation remodelling (seasonal effects) or between metabolic states within the same season (metabolic state effects) have not yet been reported. In this study, I further investigate the temperature effects on pH$_i$ by comparing state and seasonal changes in pH$_i$ regulation in these two different types of hibernators. I hypothesised that a non-hibernating species, such as the rat, would not follow $\alpha$-stat in any organ, since the ability to maintain protein function with changing body temperature is only necessary in species that routinely change body temperature. I also make intraspecific comparisons between seasons and metabolic states in the facultative hibernator, the Syrian hamster, and the obligate hibernator, the 13-lined ground squirrel, to elucidate whether the observed traits are part of the seasonal and/or metabolic state remodelling that occurs prior to entering hibernation, or whether they are intrinsic traits of hibernating species. Based on the findings of Malan et al. (1985) and Nestler (1990), I hypothesised that the heart and liver would exhibit $\Delta$pH/ $\Delta$temperature values similar to those predicted by the $\alpha$-stat hypothesis (-0.018 pH/ °C) and that this is an intrinsic trait of hibernating species, as the heart and liver remain
functional in all metabolic states and seasons. I further hypothesized based on these previous studies that tissues that are quiescent in hibernation, such as the brain, would exhibit ΔpH/Δtemperature values closer to pH-stat regulation, contributing to metabolic suppression.

3.2 Materials and Methods

3.2.1 Experimental animals and tissue collection

All procedures were conducted under protocol A21-0006, approved by The University of British Columbia (UBC) Animal Care Committee (ACC) in compliance with the standards of the Canadian Council on Animal Care (CCAC). Adult Sprague-Dawley rats (256g–568g), and Syrian hamsters (80g–130g) were acquired from commercial breeders (Envigo and/or Charles Rivers) and housed at 20 ± 2 °C with ad libitum rodent chow (Purina LabDiet 5001) and water. Surplus 13-lined ground squirrels from a previous study by Sprenger and Milsom (2022) were used in this study. Wild caught squirrels were transported to an animal care facility at UBC, Vancouver from Carman, MB, Canada, under the wildlife scientific permit WB15027. Euthermic summer squirrels were supplied with ad libitum IAMS small chunk dog food and water.

Summer euthermic ground squirrels (230g–316g) were sampled during the active season (May–October). Winter animals (150–270g) were previously intraperitoneally implanted with RFID temperature chips (IPTT-300; 1 mm × 3 mm, < 0.5 g) and scanned with a DAS-8017 reader (Biomedic Data Systems, DE, USA) as described by Sprenger and Milsom (2022). Hibernating 13-lined ground squirrels were sampled during the hibernation season (at 5±2 °C) in 24 h dark during November-April) after a minimum of 48 hours in hibernation and with body temperatures between 5 – 10 °C. The RFID temperature loggers were scanned without disrupting the animal. Animals were confirmed to be in hibernation for at least 48 hours by daily monitoring to determine whether wood chips placed on the hibernating animals remained in place. No food or water was provided during the hibernation period. Interbout euthermic animals were sampled after agitating animals to arouse from hibernation at least 12 hours prior to sampling, and only when the animal was fully active and had returned to ~37 °C body temperature at the time of sampling.
Rats and euthermic hamsters were sampled throughout the year. As facultative heterotherms, hamsters can be induced into torpor at any time of the year following acclimatisation to short photoperiod and cold ambient temperatures (Chayama et al., 2016). Hamsters were acclimatised to 8:16 light:dark at 20 °C for 8 weeks, followed by 23 hours of darkness at 5 °C, with access to food and water, until torpor was observed. Between the hours of 1100-1200 every day, daily welfare checks were carried out under dim room light. As food restriction alone has been reported to increase induction into torpor in this species (Trefna et al., 2017), hamsters were supplied a small daily ration of food (3-5 pellets) that was mostly hoarded rather than eaten. Animals were considered to be in torpor when breathing frequency was markedly reduced but still consistent for 5 minutes (approximately 3-5 breaths/minute) and wood chips placed on the hibernating animals remained in place for 24 hours. Animals that did not enter torpor after 12 weeks were removed from the environmental chamber. It should be noted here that only around 15% of hamsters entered hibernation. Difficulty in inducing hibernation in commercially bred Syrian hamsters has been previously reported by Trefna et al. (2017).

3.2.2 Tissue collection

All animals were euthanised by isoflurane overdose. The brain (cerebrum and cerebellum), brainstem, liver, heart and skeletal muscle were collected within two minutes of the last breath and were freeze clamped in liquid nitrogen. The quadriceps muscles from the hindlimbs were collected for skeletal muscle samples, and the four muscles comprising the quadriceps (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis) were collected in an indiscriminatory manner. All tissue samples were stored at -80 °C for less than a month before use.

3.2.3 pH measurement

pHt was measured using the metabolic inhibitor tissue homogenate method, first described by Pörtner et al. (1990). Tissues were powdered under liquid nitrogen and subsequently suspended in 1:5 tissue to metabolic inhibitor ratio (150 mM potassium fluoride; 6 mM nitrilotriacetic acid, pH 7.4). Typically, every tissue of each individual was divided into three
40 mg samples of tissue (with the exception of the brainstem, ~10mg each), and suspended in 200 μl of metabolic inhibitor. The suspension was vortexed and equilibrated to either 37, 25 or 10 °C for 30 minutes before the pH$_i$ of the supernatant was measured with a combination pH electrode (Mettler Toledo; calibrated at the measurement temperature). The dissociation ratio of histidine’s imidazole ring ($\alpha_{im}$) was calculated for each individual as: $1/(1+10^{pK_{imidazole}−pH})$. $\alpha_{im}$ was calculated at 37 °C for euthermic hamsters, summer and winter euthermic squirrels, and at 10 °C for the torpid hamster and hibernating squirrel (i.e., at physiologically relevant body temperatures).

3.2.4 Statistical analysis

A two-way ANOVA, followed by a Tukey’s post hoc test (p<0.05) was carried out between the pH$_i$ of each tissue at each temperature to assess species-specific differences in euthermic animals. A separate two-way ANOVA and Tukey’s post hoc test were carried out within each tissue and temperature to test for differences across hibernation states in the hamster and ground squirrel. A linear regression model was used to determine $\Delta$pH$_i$/Δtemperature of each tissue. Regression slopes in the range of -0.010 to -0.020 were considered to be consistent with $\alpha$-stat regulation (see Discussion for justification). Finally, to test state-induced differences in $\alpha_{im}$, a two-way ANOVA was carried out for each tissue in Syrian hamsters (between two states, euthermia and torpor). I also carried out a two-way ANOVA for each tissue in the 13-lined ground squirrel to compare the three states (summer euthermia, hibernation, and interbout euthermia), followed by a Tukey’s multiple comparison test). All statistical analysis was carried out on Graphpad Prism v8 (CA, USA).

3.3 Results

I compared pH$_i$ in five different tissues from euthermic animals in each of the three species at 37 °C. pH$_i$ did not differ in any organ in the three species except for the skeletal muscle, which had a lower pH$_i$ in the summer euthermic squirrel compared to the rat (Figure 3.1; Tukey’s post hoc p = 0.0032).
All tissues of the rat and summer euthermic squirrel exhibited slopes of the relationship between pH$_i$ and body temperature lesser than that required for α-stat (Rat: -0.0047 – -0.0077 pH units/ °C; Summer euthermic squirrel: -0.0036 – -0.0079 pH units/ °C; Figure 3.1). In the euthermic hamster, however, all tissues except the skeletal muscle (-0.0044 pH/ °C) had slopes close to, or consistent with α-stat (-0.011 – -0.016 pH/ °C).

In euthermic hamsters, temperature had a significant effect on pH$_i$ in all tissues, except skeletal muscle (Figure 3.2). Temperature had a suggestive (but statistically insignificant) effect on pH$_i$ of skeletal muscle (p = 0.055), and the ΔpH/Δtemperature value for skeletal muscle (-0.0044 pH/ °C) was furthest from that of α-stat. Entering torpor did not affect pH$_i$ in any tissue in the hamster. There was no interaction effect between temperature and torpor state of the hamster. In all tissues examined, there was no difference in α$_{im}$ between euthermia and hibernation (Figure 3.3).

In 13-lined ground squirrels, both temperature and hibernation state had a significant effect on pH$_i$, with significant interactions between temperature and state in all tissues (Figure 3.4). In the summer and interbout euthermia animals, ΔpH/Δtemperature did not follow α-stat regulation (summer euthermic squirrels: -0.0036 – -0.0079 pH/ °C; IBE squirrels: -0.0051 – 0.0038 pH/ °C). However, in the hibernating squirrels, ΔpH/Δtemperature closely followed α-stat in all tissues, ranging from -0.0147 – 0.0183 pH/ °C. When compared at a common temperature, the hibernating animals had a higher pH$_i$ in all tissues relative to the summer animals at all three assay temperatures. pH$_i$ did not differ between the two winter states at 37 °C but diverged to be significantly higher in the hibernating animals when the assay temperature was lowered.

With the exception of the brainstem and heart, α$_{im}$ increased in all tissues in both hibernation and IBE, when compared to summer animals (Figure 3.5). α$_{im}$ of the brainstem was maintained in all three states, whereas the hearts of IBE squirrels exhibited a higher degree of protonation (higher α$_{im}$) in IBE compared to summer euthermia and hibernation.

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3.4 Discussion

The changes in pH\textsubscript{i} that accompany changing body temperature have been implicated in regulating tissue specific metabolic rate in hibernators for several decades (Malan et al., 1985; Nestor, 1990). It has been hypothesized that maintaining the same pH\textsubscript{i} in quiescent tissues (such as brain and skeletal muscle) at low body temperature results in a relative acidosis that changes the ionisation ratio of histidine, and thereby decrease protein function in these tissues during hibernation, contributing to metabolic suppression. To test the hypothesis that quiescent tissues would exhibit acidosis to inhibit metabolic rate, this study used three species of rodents that exhibit different degrees of heterothermy to tease out the changes in pH\textsubscript{i} that occur with changes in temperature, hibernation state and season. I determined the $\Delta$pH/$\Delta$temperature of five different tissues in each species/hibernation state to elucidate whether the temperature induced changes in intracellular pH paralleled $\Delta$pK/$\Delta$temperature of imidazole. I also calculated the $\alpha_{im}$ of each state at their physiologically relevant body temperatures (37 °C for all active states, and 10 °C for hibernation), to determine whether the ionisation ratio of histidine was maintained constant as animals transitioned from the active state to hibernation, and whether this was a tissue specific phenomenon in the heart and liver as suggested by Malan et al. (1985).

With the exception of skeletal muscle, I found that the pH\textsubscript{i} in each tissue did not differ between rats, euthermic hamsters and summer euthermic squirrels when compared at their resting body temperature (37 °C) within their thermal neutral zone (Figure 3.1). This is perhaps not surprising, given that the function of each tissue at 37 °C in resting animals would be the same across the three species during euthermia, and therefore the pH\textsubscript{i} at which intracellular processes occur would be similar if not the same across species. The pH\textsubscript{i} of skeletal muscle, however, is known to vary significantly between species, time of sampling since intense exercise, and the fibre composition of the skeletal muscle sampled (Mannion et al., 1995). Skeletal muscle pH\textsubscript{i} as low as 5.99 in human muscle to values as high as 7.33 in mice have been reported (Carter et al., 1967; Westerblad & Allen, 1992). Therefore, I interpret the lower pH\textsubscript{i} in skeletal muscle of summer euthermic 13-lined ground squirrels in
the present study to be an interspecies difference, but not a significant deviation from the range of pH values in the literature.

There were more notable differences in the pH\textsubscript{i} of individual tissues in rats, eutheremic hamsters and summer eutheremic squirrels when temperature was lowered \textit{in vitro}, owing to differences in the magnitudes of the temperature effect on pH\textsubscript{i} across the three species (Figure 3.1). The increases in pH\textsubscript{i} with decreasing temperature in the different tissues of the rats and summer eutheremic ground squirrels were not significant. The increase in pH\textsubscript{i} with decreasing temperature ($\Delta$PH/ $\Delta$temperature), however, was consistently highest in euthermic hamsters in all tissues, except skeletal muscle where temperature had a suggestive (but statistically insignificant) effect on pH\textsubscript{i} of skeletal muscle in the hamsters. I hypothesised that varying pH\textsubscript{i} to maintain $\alpha$\textsubscript{im} constant at all body temperatures is an intrinsic characteristic of heterotherms and that it would not be present in a non-hibernating species. The data suggest that while this is the case for the facultative hibernator, the hamster that can enter hibernation all year round, it is not for the obligate hibernator, the 13-lined ground squirrel that enters hibernation seasonally. The tissues of the rat and the summer euthermic ground squirrel did not exhibit $\Delta$PH/$\Delta$temperature consistent with the $\alpha$-stat hypothesis, and as a result would exhibit different $\alpha$\textsubscript{im} at different temperatures.

It is well established that extensive seasonal metabolic remodelling occurs in 13-lined ground squirrels as they approach the hibernation season (winter). Despite both the summer euthermic and IBE animals being in metabolically active states, there was a significant seasonal increase in the pH\textsubscript{i} of tissues from summer to winter in animals at 37 °C body temperature (Figure 3.4). Furthermore, there was a consistent trend in the $\alpha$\textsubscript{im} of 13-lined ground squirrels. $\alpha$\textsubscript{im} of every tissue was lower in the summer euthermic animals (0.47 – 0.54) compared to that of animals in hibernation (0.58 – 0.66) or IBE (0.61 – 0.70), although the differences in $\alpha$\textsubscript{im} between the three states were not statistically significant in the brainstem, and not significant between summer and hibernation in the heart (Figure 3.5). The reason behind the lack of statistical significance is unclear. Nonetheless, the lower $\alpha$\textsubscript{im} in summer animals compared to the other two states is the result of pH\textsubscript{i} in all tissues of summer animals being the same or lower than the pK of imidazole. This results in ~50% of the
intracellular imidazole being unprotonated at the pH$_i$ of summer animals. Conversely, a pH$_i$ higher than the pK of imidazole at any given temperature, as is the case in hibernation and IBE, means a greater proportion of imidazole residues are unprotonated resulting in a greater $\alpha_{im}$ value. The lower pH$_i$ and lower $\alpha_{im}$ of summer euthermic ground squirrels compared to winter animals may be the result of seasonal differences in intracellular buffer constituents and the regulatory set point of pH$_i$ in winter animals, but the functional significance and precise mechanistic cause remain unclear. To elucidate this, experiments using live cells or whole animals with intact regulatory mechanisms (such as the activity of plasma membrane ion transporters, and systemic control of ventilation), will be necessary. I also report metabolic state dependent changes in $\Delta$ pH/ $\Delta$ temperature in the same season (i.e., between hibernation and IBE) in this species, as tissues taken from the squirrels during IBE showed no significant difference in $\Delta$ pH/ $\Delta$ temperature compared to summer euthermic animals. In all tissues taken from hibernating squirrels, however, the $\Delta$ pH/ $\Delta$ temperature was significantly higher and consistent with predictions based on the $\alpha$-stat hypothesis.

The lack of a significant effect of temperature on the pH$_i$ of the different tissues in the rat and both summer and winter euthermic squirrels point to phosphate as a major buffer group in these animals. Intracellular buffering is predominantly due to inorganic phosphate and imidazole related compounds as their pK values coincide closely to physiological pH (Castellini & Somero, 1981). The dissociation of phosphoric acid has a low enthalpy (does not require a large amount of energy to dissociate phosphoric acid into phosphate), and as a result, pH$_i$ changes very little with decreasing temperature (low $\Delta$ pH/ $\Delta$ temperature). However, it is unlikely that any single chemical group such as phosphate is solely responsible for maintaining intracellular buffering capacity in any cell, and significant species differences have been noted. Previous studies have shown species-specific differences in the buffering groups present in mammalian muscle. While the contribution of inorganic phosphate to total muscle buffering capacity is reported to be relatively constant between mammalian species (20 – 27% in pig, ox and whales), histidine proportions appear to vary widely across species, depending on the species’ glycolytic capacity (Castellini & Somero, 1981; Abe, 2000; Dolan et al., 2019). In some, such as thoroughbred horses and greyhounds, histidine related compounds contribute 25 – 31% to the total buffering capacity.
of skeletal muscle although the contribution was less than 10% in human skeletal muscle (Sahlin and Henriksson, 1984). The significant effect of temperature on the pH of the different tissues in the hamster and the hibernating ground squirrel suggests that histidine related compounds also contribute to the intracellular buffering capacity of these tissues. Further investigation on the composition of intracellular buffers is necessary to identify the mechanistic cause of the interspecific/state differences in ΔpH/Δtemperature.

The data suggest that there may be significant differences between facultative and obligate hibernators. The data were obtained from only one species employing each of three different thermoregulatory strategies, but the consistently high values of ΔpH/Δtemperature of all tissues in both the euthermic and hibernating hamsters implies that intracellular buffering in facultative hibernators retains the activity of functional proteins with changing temperature throughout the year. In the obligate hibernator, however, there appears to be a rapid change in intracellular buffer constituents between states, with ΔpH/Δtemperature being consistent with the α-stat hypothesis only during the hibernation state. The time course of this change during both entrance and arousal from hibernation bouts remains unknown.

The α-stat hypothesis suggests that increasing pH with decreasing body temperature in parallel to the changing pK of imidazole retains the functionality of proteins. Whether such changes in pH substantially affect functionality of proteins, however, has not been widely investigated. The pH optima of acetyl CoA carboxylase, fatty acid synthetase, NADH- and succinate- cytochrome C reductase, and NADH ferricyanide reductase isolated from rainbow trout all appear to increase with lowered assay temperature (Hazel et al., 1978). Such changes mirror the temperature dependent change in intracellular and extracellular pH observed in trout at different body temperatures, indicating enzyme functionality is dependent on maintaining $a_{im}$ constant. Also, phosphofructokinase (PFK) isolated from skeletal muscle of hibernating California ground squirrels has been shown to decrease activity with a 0.3-0.4 pH unit decrease (Hand & Somero, 1983). Such acidification is known to convert the active tetrameric form of PFK to the inactive dimeric form in vitro. The findings of Hand and Somero (1983) have been accepted by many as functional evidence supporting the hypothesis that acidosis may be a mechanism of metabolic rate suppression in
hibernation (Bickler, 1984; Malan et al., 1985; Nestler, 1990; Yacoe, 1983). In contrast, MacDonald and Storey (2001) report that physiologically relevant intracellular protein and urea concentrations mitigate the effects of acidosis and low temperature on PFK activity previously reported by Hand and Somero (1983). Therefore, whether acidosis can have a substantial regulatory role in reducing the rate of glycolysis in hibernation is still unclear and requires investigation in vivo.

In both hibernating hamsters and ground squirrels, I found all tissues to have $\Delta p\text{H}/\Delta \text{temperature}$ similar to the $\Delta pK/\Delta \text{temperature}$ of imidazole. The results are contrary to the findings of Malan et al. (1985) and Nestler (1990) in two respects. Firstly, the two aforementioned studies report that both European hamsters during hibernation and deer mice during daily torpor exhibit $\Delta p\text{H}/\Delta \text{temperature}$ near zero in the brain, indicating a relative acidosis during torpor/hibernation (Malan et al., 1985; Nestler, 1990). In this study, $\Delta p\text{H}/\Delta \text{temperature}$ for brain and brainstem were -0.0147 and -0.0161 respectively and $\alpha_{\text{im}}$ remained the same between hibernation and interbout euthermia. I do not currently have an explanation for the difference in $\Delta p\text{H}/\Delta \text{temperature}$ for brain between studies. Malan et al. (1985) sampled the cerebral cortex (outermost tissue layer of the cerebrum) while Nestler (1990) sampled the cerebral hemispheres (the two halves comprising the cerebrum), whereas I sampled the cerebrum and cerebellum. Thus, the difference in area of the brain sampled may in part explain the differences in $\Delta p\text{H}/\Delta \text{temperature}$ between this study and the aforementioned studies. The cerebellum has been shown to have a higher $pH_i$ ($7.04 \pm 0.03$) compared to the cerebrum ($6.99 \pm 0.02$) in humans (Buchli et al., 1994). However, there is limited data on how the $pH_i$ of these different parts of the brain change with decreasing temperature.

Brain acidosis was suggested to lower the thermoregulatory set point of the preoptic anterior hypothalamus during hibernation by both Malan et al. (1985) and Nestler (1990). The results suggest that this is not a universal phenomenon in all heterotherms. Furthermore, while respiratory acidosis has been shown to inhibit thermogenesis in whole animals (Jennings, 1979; Kuhnen et al., 1987; Schaefer & Wünnenberg, 1976; Wünnenberg & Baltruschat, 1982), the mechanism behind $pH$ induced lowering of the thermoregulatory set point is not
clear. Both hypercapnic and isocapnic acidosis have been shown to downregulate the firing of temperature sensitive neurons of the preoptic anterior hypothalamus in hamsters and rats (Matsumura et al., 1987; Wright & Boulant, 2007). Inhibition of these neurons during acidosis was hypothesised by these researchers to disrupt normal heat loss mechanisms and cause hyperthermia, not a reduction in body temperature (Dean, 2007; Wright & Boulant, 2007).

Secondly, Malan et al. (1985) also report a relative intracellular acidosis in striated muscle during hibernation which they also suggested contributes to metabolic depression. In this study, I did not observe relative acidosis in the skeletal muscle of either hibernating hamsters or ground squirrels. The difference between the study of Malan et al. (1985) and both this study and that of Nestler (1990), however, is more a matter of interpretation. Malan et al. (1985) found that the $\Delta p\text{H}/\Delta \text{temperature}$ for skeletal muscle was only -0.010 while that for heart and liver were each -0.013. Their comparison of $\Delta p\text{H}/\Delta \text{temperature}$ of tissues to the $\Delta p\text{K}/\Delta \text{temperature}$ of imidazole, however, overlooked the complexity of in situ biochemical and physical properties of proteins. The $\Delta p\text{K}/\Delta \text{temperature}$ value commonly attributed to imidazole (-0.018) is not entirely accurate in situ, as the value varies depending on the molecular interactions between neighbouring amino acids within proteins (Burton, 1986; Cameron, 1984; Nattie, 1990). For example, $\Delta p\text{K}/\Delta \text{temperature}$ values of in situ imidazole residues within proteins, such as ribonuclease, range from -0.010 to -0.020 (Burton, 1986; Roberts et al., 1969). Thus, the suggestion by Malan et al. (1985) that the skeletal muscle with a $\Delta p\text{H}/\Delta \text{temperature}$ value of -0.010 undergoes acidosis while the heart and liver with a $\Delta p\text{H}/\Delta \text{temperature}$ value of -0.013 do not, based on the $\Delta p\text{H}/\Delta \text{temperature}$ of -0.018 commonly provided for imidazole residues alone, disproportionately emphasises the regulatory role of acid-base balance on maintaining heart and liver function during hibernation. Nestler (1990) also found that skeletal muscle (like all tissues other than brain) in deer mice exhibited changes in $\alpha_m$ similar to that of the heart and liver.

The results contribute to previous work on the acid-base physiology of heterotherms. The data show that the $\Delta p\text{H}/\Delta \text{temperature}$ of hibernators is similar to that of ectotherms and mirror the $\Delta p\text{K}/\Delta \text{temperature}$ of imidazole. However, there are discrepancies with the
findings of Malan et al. (1985) and Nestler (1990) that differences in methodology do not explain. The DMO method (developed by Waddell and Butler, 1959) was used in the aforementioned earlier studies, while the metabolic inhibitor tissue homogenisation method was used in this study. Both techniques have been shown to produce similar results (Pörtner et al., 1990). However, both methods suffer from the shortcoming that PCO$_2$ tensions must be overlooked as the assays are not conducted in a closed system. The data suggest that there may be significant differences in intracellular buffering constituents between species capable of entering hibernation at any time of the year (facultative hibernator) and those that enter seasonal hibernation (obligate hibernator). Given the restricted data set (only one species employing each of three different thermoregulatory strategies) the conclusions of this study must be taken with caution but suggest that intracellular buffering in facultative hibernators retains the activity of proteins with changing temperature throughout the year while that in obligate hibernators changes rapidly between states. Both cases produce changes in pH$_i$ consistent with $\alpha$-stat predictions with changing temperature during hibernation and suggest that the proposed inhibitory role of relative acidosis in quiescent tissues may not be a widespread trait in all hibernating species.
Figure 3.1. Intracellular pH (pH$_i$) of tissues of the rat (○), euthermic hamster (●), and summer euthermic ground squirrel (●). All tissues were collected at 37 °C but measured at indicated temperatures in vitro. $P$ values for two-way ANOVA are indicated on each panel. Different letters denote statistical significance between species within each temperature. ΔpH/Δtemperature indicated on the left. Data are mean ± SEM.
Figure 3.2. Intracellular pH comparison between the euthermic hamster (○) and hibernating hamster (▲). P values for two-way ANOVA are indicated on each panel. No statistically significant differences were observed between states within each temperature. ΔpH/Δtemperature indicated on the left. Data are mean ± SEM. Data for euthermic hamsters are also shown in Figure 3.1.
Figure 3.3. Calculated dissociation ratio of histidine imidazole ($\alpha_{im}$) in hamster tissues in two states (euthermia, n=5; torpor, n=4). No statistically significant differences were observed between states within each tissue. Data are mean ± SEM.
Figure 3.4. Intracellular pH comparison between the summer euthermic squirrel (○), hibernating squirrel (Δ), and interbout euthermic squirrel (□). P values for two-way ANOVA are indicated on each panel. Different letters denote statistical significance between hibernation states within each temperature. ΔpH/Δtemperature indicated on the left. Data are mean ± SEM. Data for euthermic ground squirrels are also shown in Figure 3.1.
Figure 3.5. Calculated dissociation ratio of histidine imidazole ($\alpha_{im}$) in squirrel tissues in three states (summer euthermia, n=6; hibernation, n=4; interbout euthermia n =6). Letters denote statistically significant differences between states within each tissue. Data are mean ± SEM.
Chapter 4 - Temperature, pH and seasonal effects on mitochondrial respiration in hibernating rodents

4.1 Introduction

The vast majority of mammals are homeothermic endotherms that maintain body temperature constant at 37 °C by endogenously generating heat. Some mammals, however, are capable of heterothermy as they enter torpor to reduce the energetic demands associated with endothermy. Torpor is accompanied by carefully regulated reductions in MR and body temperature (Tb). It may last for a part of the day (daily torpor), or for days to weeks (hibernation) (Geiser, 2020). In facultative hibernators, such as the Syrian hamster, hibernation can be triggered by prolonged exposure (several weeks) to cold ambient temperatures (around 5-10 °C) and short daylengths. In obligate hibernators, the induction of torpor is governed by an intrinsic circannual rhythm regardless of photoperiod, food availability or ambient temperature. During the pre-hibernation acclimatisation period that precedes hibernation in facultative hibernators and during the fall in obligate hibernators, extensive metabolic remodelling occurs that enables temporal heterothermy and energy savings of up to 88% (Chayama et al., 2016; Nowell et al., 2011; Talaei et al., 2011, 2012). Both facultative and obligate hibernation can exhibit a marked reduction in metabolic rate (by up to ~90%) and lowering of body temperature (often to within 1-2 °C of ambient temperatures), although the depth of hibernation varies widely depending on the species, body size, sex, and age of the animal (Geiser & Ruf, 1995). It is well established that passive thermal effects do not entirely account for the decrease in metabolic rate in hibernators, as metabolic rate declines to torpid metabolic rates (TMR) within 8-9 hours (Lyman, 1958; Staples, 2014), with body temperature subsequently reaching near ambient levels more gradually (10-24 hours since the onset of entrance) (Boyer & Barnes, 1999; Carey et al., 2003). Thus, metabolic rate suppression in hibernation is the result of interactive effects of active mechanisms that induce suppression at high body temperature, alongside the passive effects of cooling body temperature as entrance progresses. These periods of hibernation (3-25 days) are interrupted by rapid arousals (2-3 hours) where metabolic rate and body
temperature return to, and remain at high levels for 12-24 hours (interbout euthermia; IBE), before re-entrance into hibernation. The mechanisms that underlie the progressive fall in metabolic rate and the establishment of the TMR have been an active area of research. The current literature points to TMR being the result of an orchestrated downregulation in both energy (ATP) supply and demand (Staples & Buck, 2009).

Acidosis has been suggested for several decades to lower metabolic rate in hibernation (Malan et al., 1985). This idea was rooted in the findings of several studies that reported CO₂ retention during entrance into hibernation and a relative acidosis of the blood during hibernation (Bickler, 1984a; Malan, 1988; Malan et al., 1985; Nestler, 1990b; Snapp & Heller, 1981; Sprenger & Milsom, 2022). Indeed, artificially lowering blood pH (pHₑ) has been shown to decrease whole animal metabolic rate by ~10% per 0.1 unit decrease in pH in the golden-mantled ground squirrel (Callospermophilus lateralis; Bharma & Milsom, 1993). While there are comparatively fewer reports of changes in intracellular pH (pHᵢ) during hibernation, the brain and skeletal muscle of European hamsters have been reported to undergo acidosis, which has been suggested to induce quiescence in these tissues during hibernation (Malan, 1985). While a mechanistic basis for an acidosis induced metabolic suppression has not yet been identified, as the ultimate consumers of oxygen and predominant supplier of cellular ATP, it seems plausible that the suppression of whole animal oxygen consumption may be regulated at the mitochondrial level. Despite being the main site of cellular oxygen consumption, the effects of pH on mitochondrial respiration during hibernation, and how this effect may vary depending on temperature and hibernation state have not yet been explored.

It is well established that suppression of respiration in isolated mitochondria from thermogenic and non-thermogenic tissues mirror the metabolic rate suppression seen at the whole-animal level (Martin et al., 1999; Staples et al., 2022). In 13-lined ground squirrels, the phosphorylating respiration rate (state 3) of isolated liver mitochondria is significantly lower in tissue from hibernating animals than in IBE, but only when assessed at warm temperatures (37 and 25 °C; Brown et al., 2012). This suggests that, whatever the mechanism may be, it only contributes to the active suppression of metabolic rate at high body
temperature in animals entering hibernation. At lower body temperatures, passive thermal $Q_{10}$ effects may replace the need for active mechanisms, including the possible inhibitory effect of acidosis, to achieve TMR. Thus, if low pH contributes to the suppression of metabolic processes, it likely does so in the early phase of entrance into hibernation while body temperature is still high.

Changes in pH influence enzyme structure, and therefore function, by changing the ionisation state of amino acid side chains. For example, acidosis has been shown to decrease the affinity of pyruvate dehydrogenase for its substrate (pyruvate) and phosphofructokinase activity in vitro (Blier & Guderley, 1993; Hand & Somero, 1983; Ui, 1966). The inhibitory effect of low pH on key metabolic enzymes may decrease downstream substrate availability and thereby indirectly affect mitochondrial oxygen consumption. Previous studies have consistently reported reduced substrate oxidation in states of metabolic rate suppression, in estivating snails, frogs and squirrels (Bishop et al., 2002; Staples et al., 2022). However, studies of the direct effects of acidosis on the electron transport system (ETS) have been equivocal. Activity of complex II (CII) and state 3 respiration has been shown to decrease at low pH in mice liver mitochondria (Milliken & Brookes, 2020), but pH has no effect on state 3 respiration in rat liver mitochondria (Tobin et al., 1972) while low pH increases the proton motive force of the intermembrane space and state 3 respiration in skeletal muscle of Crucian carp (Moyes et al., 1988). Thus, how acidosis influences flux through the ETS, and whether it plays a regulatory role in metabolic rate suppression is still unclear.

The 13-lined ground squirrel (*Ictidomys tridecemlineatus*) is an obligate hibernator that has been extensively studied in hibernation research for their predictable hibernation pattern and remarkable ability to suppress metabolic rate. Most of our understanding of changes in mitochondrial bioenergetics in hibernation comes from comparisons of the 13-lined ground squirrel during hibernation and IBE and has provided valuable insight into the rapid and reversible changes that occur in the mitochondria (within several hours) (Mathers et al., 2017; Staples et al., 2022). However, there is still much unknown regarding the seasonality of metabolic phenotypes in obligate hibernators. Extensive metabolic remodelling during the fall results in a distinct winter state in which animals in IBE differ in body composition,
metabolomic, genomic and proteomic profile in comparison to summer active animals, despite both states being active (Epperson et al., 2011; Staples, 2016). Furthermore, while facultative hibernators such as Syrian hamsters can be induced to enter hibernation at any time of the year, there is limited research on the physiological remodelling that occurs in the pre-hibernation acclimatization period (Chayama et al., 2016, 2019; Talaei et al., 2011). By comparing the obligate hibernator in three distinct metabolic states in different seasons, and the facultative hibernator during euthermia, before the pre-hibernation acclimatization period, I can identify whether the observed effects of pH on mitochondrial bioenergetics are intrinsically present in a species capable of hibernation, or whether these traits become apparent after pre-hibernation remodelling.

In this study, I hypothesised that if acidosis plays a role in inducing metabolic rate suppression during entrance into hibernation, lower extramitochondrial pH would reduce mitochondrial respiration at warmer temperatures by either direct effects on the function of ETS proteins or by passively increasing the proton motive force. I further hypothesised that the effect of pH on mitochondrial respiration would be associated with entering hibernation, and therefore absent in the non-hibernating rat and before pre-hibernation remodelling in the two hibernating species. To test this hypothesis, I compared the effect of pH and temperature on aspects of mitochondrial respiration and ETS complex activity between the non-hibernating Sprague-Dawley rat, the facultative hibernator (Syrian hamster) during euthermia, and the 13-lined ground squirrel in three metabolic states (summer euthermia, hibernation, and interbout euthermia). I examined two different types of hibernators in this study to elucidate whether the purported pH effects on mitochondrial respiration may be a shared mechanism of suppressing metabolic rate in both obligate and facultative hibernators.

4.2 Material and methods

4.2.1 Animals

All experiments were conducted under protocol A21-0006, which was approved by the UBC Animal Care Committee (ACC) in compliance with the Canadian Council on Animal Care (CCAC). Only females of each species were used to account for sexual dimorphism in
oxidative capacity that has been previously reported in rats (Valle et al., 2007). Adult rats (256g–568g) and hamsters ((80g–130g) were acquired from commercial breeders (Envigo and/or Charles Rivers) and housed at 20 ± 2 °C all year round. Captive-born adult 13-lined ground squirrels were used in this study. These animals were born in captivity and raised until adulthood (6+ months) under a 12:12 light:dark cycle. Ground squirrels were divided into three groups based on hibernation states: summer euthermic, hibernation, and interbout euthermic. All summer squirrels (230g–316g) were kept in a temperature-controlled chamber (20 ± 2 °C) on a photoperiod that matched the daily photoperiod in Vancouver, BC, Canada during the active period (May – October). Summer euthermic animals were fully active and sampled in July. During November-April, all squirrels were housed at 5 ± 2 °C and 24 hours in the dark. Winter animals (150–270g) were previously intraperitoneally implanted with RFID temperature chips (IPTT-300; 1 mm × 3 mm, < 0.5 g) and scanned with a DAS-8017 reader (Biomedic Data Systems, DE, USA) as described by Sprenger and Milsom (2022). Body temperature was scanned at the time of sampling, and animals were considered to be in hibernation when body temperature was 1-2 °C above environmental chamber temperature. Only animals that were confirmed to be in hibernation for 48 hours were sampled. This was done by placing woodchips on the back of the animal to verify lack of movement for 48 hours. Interbout euthermic animals were sampled by gently agitating animals in hibernation and sampled after at least 12 hours when the animal was fully active with a body temperature around 37 °C.

4.2.2 Mitochondrial isolation

All animals were euthanised by an isoflurane overdose. The liver was excised and rinsed in a buffer consisting of 250 mM sucrose, 5 mM TRIZMA, 1 mM EGTA (STE buffer) at pH 7.4 at 25 °C and immediately placed on ice. The liver was then diced using a razor blade on a petri dish placed on ice. Subsequently, the diced liver was homogenized in 25 mL STE using a dounce homogeniser. The homogenate was centrifuged at 1,000 g for 10 min at 4 °C. The supernatant was then filtered through four layers of cheesecloth and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was discarded and the remaining pellet was resuspended in 25 ml of STE. This was repeated twice more and the final pellet was resuspended in 1 ml
of STE and placed on ice until assayed. Aliquots (~300 μL) of the mitochondrial suspension were immediately frozen at -80 °C for complex activity assays. Protein content of the final mitochondrial suspension was determined using Bradford’s reagent (Bio-Rad), using BSA standards. The mitochondrial suspension was used within one hour of isolation.

4.2.3 Mitochondrial respiration

Oxygen consumption of isolated mitochondria was measured using high resolution respirometers (O2k-MiPNetAnalyzer, Oroboros, Austria). Oxygen electrodes were calibrated to air saturated mitochondrial respiration buffer 05 (MiR05; 0.5 mM EGTA, 3 mM MgCl₂, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM Sucrose). Before experimentation, MiR05 pH was adjusted to one of pH 7.0, 7.4 or 7.8 using 5 mM KOH at one of three temperatures (10, 25 and 37°C). Due to the time limited viability of isolated mitochondria, mitochondrial suspension from each animal was only assayed at one temperature, but at the three different pH levels in three separate oxygraph chambers simultaneously (n = 5–7 animals at each temperature).

Approximately 0.1 mg of mitochondrial protein was added to 2 ml of temperature equilibrated and oxygen saturated MiR05 at 25 and 37 °C. Due to the low respiration rate at 10°C, a greater amount of mitochondrial protein (0.4 mg) was used to improve signal:noise ratio. Due to the low respiration rate, substrate depletion was not of concern when using more mitochondria and therefore the following concentrations of substrate were kept consistent throughout the three assay temperatures. State 2 respiration through CI was obtained by adding 10 mM glutamate + 2 mM malate, and 1 mM ADP was subsequently added to stimulate state 3 respiration through CI. State 3 respiration through CII was achieved with the addition of 0.5 μM rotenone to inhibit CI and 10 mM succinate. Leak (state 4) respiration was induced by adding 5 nM oligomycin. Subsequently, carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP; 0.5 μM) was incrementally added until respiration was fully uncoupled from ATP synthesis. Any preparations with more than a 25% increase in respiration after the addition of cytochrome C (10 μM) were considered to have compromised outer membrane integrity and discarded (Toleikis et al.,
Lastly, 2.5 μM antimycin A was added to correct for non-mitochondrial oxygen consumption.

4.2.4 ETS complex activity assay

Frozen mitochondrial pellet aliquots were thawed on ice and centrifuged at 15,000g at 4 °C for 10 minutes. The pellet was then resuspended in hypotonic medium (25 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, pH 7.4) to yield a protein concentration of 5 mg/ml. The same individuals were used for assays across the three temperatures and pH (n = 6 – 10 animals).

CI activity was measured by adding 5 μl mitochondrial suspension (25 μg mitochondrial protein) to 250 μl of assay medium containing 25 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, 2 μg/ml antimycin A, 100 μM DCPIP, 65 μM ubiquinone$_2$, 2.5 mg/ml BSA, and 0.2 mM NADH (adjusted to pH 7.0, 7.4, or 7.8). The assay was carried out at 10, 25, and 37 °C. Absorbance values for the oxidation of DCPIP were collected for 15 minutes at 600 nm. All samples were run in triplicates, along with triplicates of rotenone containing assay medium to control for non-specific DCPIP consumption. CII activity was measured following the same procedure as for CI with the exception of NADH, which was substituted for 20 mM of succinate to fuel CII, and 2 μg/ml of rotenone to inhibit CI. Complex V (CV) activity was measured by adding 5 μl mitochondrial suspension (25 μg mitochondrial protein) to assay medium (25 mM KH$_2$PO$_4$, 5 mM MgCl$_2$, 100 mM KCl, 2.5 mg/ml BSA, 5 mM ATP, 2 mM PEP, 0.2 mM NADH, 3 U/ml lactate dehydrogenase, 3 U/ml pyruvate kinase; adjusted to pH 7.0, 7.4, or 7.8). The assay was carried out at 10, 25 and 37 °C. Absorbance values were collected for 15 minutes at 340 nm. All samples were run in triplicates, along with triplicates of oligomycin containing assay medium to control for non-specific NADH consumption.

4.2.5 Statistical analysis

All data were analysed in Graphpad Prism v8 (CA, USA). A repeated measures two-way ANOVA or mixed effects model (in cases where some individual animals were missing values) were used to test the effects of pH and temperature on maximal complex activity and
mitochondrial respiration rates. A Tukey’s multiple comparison test was subsequently carried out to identify means of maximal activity or respiration rates within each temperature that were significantly different from another. P-values below 0.05 were considered significant differences and all data are displayed as the mean ± standard error of the mean. Q_{10} values for state 3 respiration and for complex activity at each pH were calculated using:

\[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{\frac{10}{T_2-T_1}} \]

where R2 and R1 refer to the rate of reaction, and T2 and T1 refer to the temperature at which R2 and R1 occurred, respectively. For state 3 respiration, Q_{10} values were calculated using mean state 3 respiration rates for each temperature (at each pH) as different individuals were used at each temperature. Individual Q_{10} values were calculated for complex activities and reported as the mean ± SEM.

### 4.3 Results

#### 4.3.1 Sprague-Dawley rat (non-hibernator)

Temperature had a significant effect on state 3 respiration with glutamate + malate and succinate (p < 0.0001; Fig. 4.1). The greatest reduction in state 3 respiration rate was observed between 25 and 10 °C for both glutamate + malate (Q_{10} = 2.3 – 2.7) and succinate fueled respiration (Q_{10} = 2.3 – 2.6; Supplementary Table 4.1). pH did not affect glutamate + malate or succinate fueled state 3 respiration at any temperature in the rat.

Leak respiration and FCCP induced maximal respiration were reduced with decreasing temperature (p < 0.0001; Figure 4.2). pH had a near-significant effect on leak respiration (p = 0.0526), with decreasing pH lowering leak respiration. pH significantly reduced maximal respiratory capacity (p = 0.0116) at 25 °C at pH 7.0, compared to pH 7.8.

Decreasing temperature reduced CI, CII and CV activity in the rat (p < 0.0001; Fig. 4.3, Supplementary Figure 4.1). However, changing pH only affected CV activity (p=0.0027), with lower pH decreasing CV activity at 25 °C and 10 °C.
4.3.2 Syrian hamster (facultative hibernator)

Temperature had a significant effect on state 3 respiration with glutamate + malate (p = 0.0003), and succinate (p < 0.0001; Fig. 4.1). The greatest reduction in state 3 respiration rate was observed when temperature was lowered from 25 °C to 10 °C for both glutamate + malate \( (Q_{10} = 1.7 - 2.6) \) and succinate fueled respiration \( (Q_{10} = 1.8 - 2.4) \); Supplementary Table 4.1). Changing pH did not have an effect on glutamate + malate fueled state 3 respiration but lowering pH increased state 3 respiration with succinate (p = 0.0256) at 37 °C and 25 °C.

Leak respiration and FCCP induced maximal respiration decreased at lower temperatures (p < 0.0001; Fig.4.2). However, changing pH did not affect leak respiration or maximal respiratory capacity.

Decreasing temperature lowered CI, CII and CV activity in the euthermic hamster (Fig. 4.3, Supplementary Fig. 4.1). Lowering pH did not affect CI activity but did decrease CII activity at all assay temperatures (p < 0.0001) with a significant interaction effect with temperature (p=0.0022). CV activity was also decreased at lower pH (p < 0.0001) but only at 37 °C, with an interaction effect between pH and temperature (p = 0.0001).

4.3.3 13-lined ground squirrel (obligate hibernator)

In all metabolic states of the ground squirrel, temperature had a significant effect on glutamate + malate and succinate fueled state 3 respiration (Fig. 4.1; Supplementary Table 4.1 and 2). Decreasing temperature from 25°C to 10 °C had the greatest Q_{10} effect on reducing state 3 respiration with either substrate in all three metabolic states (Supplementary Table 4.1). State 3 respiration with glutamate + malate was only affected by pH during hibernation (p < 0.0001) with a significant interaction effect between pH and temperature (p = 0.0052) as lowering pH increased respiration rate at 25 °C. In summer euthermic animals, decreasing pH significantly increased succinate fueled state 3 respiration (p = 0.0259) but post-hoc analysis did not reveal specific differences between the pH treatments at any assay temperature (Fig. 4.1) However, decreasing pH significantly increased succinate fueled state
3 respiration during hibernation (p <0.0001) at all three assay temperatures. The effect of pH varied with temperature in the hibernating squirrels, as there was a significant interaction effect between pH and temperature (p<0.0001). There was also a significant increase in succinate fueled state 3 respiration in IBE animals with decreasing pH (p = 0.0047), but only at 37 °C (p = 0.0008).

Decreasing temperature significantly decreased leak respiration in all three metabolic states (p<0.0001 in all three states). There was no effect of pH on leak respiration during the two active states. However, lower pH increased leak respiration (p = 0.0121) at 25 °C in hibernating animals. FCCP induced maximal respiration rate was significantly reduced as temperature decreased in all three metabolic states (p<0.0001 in all three states). pH had no effect on maximal respiratory capacity in the two active states of the ground squirrel. Lower pH significantly increased maximal respiratory capacity during hibernation (p=0.0012), with a significant interaction effect between temperature and pH (p<0.0001).

Lowering temperature decreased CI, CII and CV activity in all states of the ground squirrel (p < 0.0001; Fig. 4.3; Supplementary Fig. 4.1). Lowering pH only decreased CI activity in the two winter states (p < 0.0001 in both states). CII activity was significantly decreased by lower pH in all three metabolic states (summer: p < 0.0001; hibernation: p < 0.0001; IBE: p=0.0009). CV activity was inhibited by lower pH in the two active states (summer: p < 0.0001; IBE: p = 0.0005), but not in hibernation, although the pH effects were only marginally insignificant (p=0.0646). The effect of pH on complex activity was temperature dependent in all metabolic states, with the notable exception of CI activity in summer euthermic animals (although only marginally insignificant; p = 0.0560) and CII activity in IBE animals.

4.4 Discussion

4.4.1 pH effects

The findings of Chapter 3 indicated that euthermic animals may exhibit relative acidosis in all tissues, as pH increased very little with decreasing body temperature. Although acidosis was not present during steady state hibernation, it is plausible that during early stages of
entrance into hibernation (i.e. as animals transition from euthermia to hibernation), acidosis may contribute to metabolic rate suppression. Therefore, in this chapter of my thesis, I tested the hypothesis that acidosis is involved in active metabolic rate suppression during the early stages of entrance into hibernation when body temperature is still relatively high. This hypothesis led me to predict that low pH would have a greater inhibitory effect on mitochondrial respiration at warmer temperatures than at lower temperatures. The findings of this chapter did not support this hypothesis as low pH increased state 3 respiration in liver mitochondria of hibernators at all assay temperatures (37, 25, 10 °C; Figure 4.1), despite decreased ETS complex activity at lower pH (Figure 4.3). In particular, CII activity was suppressed at lower pH at all assay temperatures, in both species of hibernators in all metabolic states, while state 3 respiration through CII increased at lower pH. This stimulatory effect of pH on state 3 respiration rate was unique to the hibernators and not seen in the rat at any assay temperature, with CI and CII activity also being largely unaffected by pH in the rat. While CV activity decreased at low pH, this did not contribute to a decreased state 3 respiration either through CI (glutamate + malate) or CII (succinate) in the rat.

The findings in the rat are consistent with that of Tobin et al. (1972), in which rat liver mitochondria did not increase state 3 respiration and did not exhibit changes in P/O ratio with changes in extramitochondrial pH (pH_{em}) between 5.9 – 8.0. This indicates that lower pH does not increase ATP production for the same amount of oxygen consumed in isolated liver mitochondria of the rat. In contrast, a decreased pH_{em} has been shown to increase state 3 respiration in isolated mitochondria of Crucian carp skeletal muscle and rat brain (Moyes et al., 1988; Selivanov et al., 2008). This was accompanied by an increase in the pH gradient between the intermembrane space and matrix, and the membrane potential. Although the proton motive force was not quantified in this study, I expected that a low pH induced increase in state 3 respiration would be accompanied by an increased proton motive force as shown in the aforementioned studies. As lower pH stimulated state 3 respiration in the hibernating species, while state 4 respiration did not noticeably increase, this implies that the proton motive force is coupled to potentially increased ATP production. This is contrary to the hypothesis put forward by Malan et al. (1985) that acidosis may be a mechanism of mitochondrial metabolic rate suppression in hibernators. Furthermore, my findings also
contradict the idea that suppressing mitochondrial oxygen consumption by acidosis is an adaptive trait in species tolerant of hypoxia (such as hibernators). As acidosis often accompanies hypoxia, limiting oxygen consumption during times of environmental oxygen deprivation would be beneficial (Ivanina & Sokolova, 2013). Hibernators are known to have a higher hypoxia tolerance in comparison to non-hibernators (such as rats), which has often been suggested to correlate with their semi-fossorial nature (Drew et al., 2004; Dzal & Milsom, 2019). Thus, the biological significance behind increased oxygen consumption and purported increased ATP production at low pH in hibernating species is unclear.

In contrast to the stimulatory effect of acidosis on state 3 respiration, I found that CI, CII and CV activities were decreased at lower pH in the hibernating species. The decrease in ETS complex activity at lower pH may be due to the pH sensitive nature of intermolecular forces that ultimately affect substrate-enzyme binding affinity, as well as the stability of the protein structure. Similarly, acidosis has been shown to lower phosphofructokinase activity in California ground squirrels (Otospermophilus beecheyi), a species also capable of hibernation (Halperin et al., 1969; Hand & Somero, 1983). Therefore, it appears that although low pH decreases activity of key metabolic enzymes involved in glycolysis and oxidative phosphorylation, and may inhibit activity of these enzymes in hibernation, a compensatory mechanism exists in the intact mitochondria that increases state 3 respiration at lower pH.

How this inhibitory effect of lower pH is compensated for to increase respiration at low pH remains unclear. It is possible that substrate transport may increase at lower pH as the transport of key monocarboxylate substrates into the matrix requires a proton symport (e.g. pyruvate). Furthermore, the mitochondrial dicarboxylate transporter moves malate and succinate in exchange for phosphate, and phosphate in turn is transported into the matrix through a phosphate/proton symporter, thereby indirectly connecting dicarboxylate transport and pH. If proton concentration (i.e., pH) was a limiting factor in transporting metabolites into the matrix, the observed increase in oxygen consumption rate may be explained by increased substrate transport into the mitochondrial matrix with more proton availability at lower pH. Previous studies have demonstrated that succinate transport at pH 7.2 does not
inhibit mitochondrial respiration unless concentrations are below \( \sim 4\text{mM} \) (Quagliariello & Palmieri, 1968; Staples & Brown, 2008). While it is conceivable that the lower proton concentration at high pH may decrease succinate transport even when the substrate is present at saturating concentrations, I am unaware of any studies that investigate the pH dependent nature of substrate transport in hibernators. Furthermore, previous studies have identified NADH production, as well as ADP and \( P_i \) availability, and CIV activity as the rate limiting factors for oxidative phosphorylation in intact cells (From et al., 1990; Villani and Attardi, 2000; Kadenbach, 2021). Therefore, whether the purported acidosis induced limitation on succinate transport plays any regulatory role on reducing oxidative phosphorylation during hibernation is unclear. Taken together, the stimulatory effect of lower pH on mitochondrial respiration in hibernators does not appear to be a direct effect on ETS protein function, but through a different mechanism that requires further investigation.

4.4.2 Temperature effects

In this study, thermal effects on succinate fueled state 3 respiration between 37 °C and 10 °C in the Syrian hamster mirrored that of whole animal metabolic rate (Figure 4.1). The \( Q_{10} \) values of state 3 respiration (1.8 – 2.0, depending on the pH) and CII activity (1.9 – 2.0) between 37 °C and 10 °C were similar to that of whole animal metabolic rate (2.4) across this temperature range (Geiser, 2004; Lyman, 1948). However, I observed discrepancies between \( Q_{10} \) values at the mitochondrial level and whole animal metabolic rate in the 13-lined ground squirrels. \( Q_{10} \) values for succinate fueled state 3 respiration between 37 – 10 °C in hibernating animals were 2.3 – 2.4 and 2.1 – 2.3 for CII activity between 37 °C and 10. The \( Q_{10} \) values here were in good alignment with the findings of Brown et al. (2012), which were between 2 – 3 for succinate fueled state 3 respiration between 37 °C and 10 °C in the liver. In contrast, the \( Q_{10} \) value of whole animal metabolic rate was 5.0 over a similar temperature range in the same species (Sprenger & Milsom, 2022). This indicates whole animal metabolic rate suppression is highly temperature sensitive, beyond what is expected for a typical biological reaction, and is not due to thermal effects at the ETS level.
Furthermore, I found that $Q_{10}$ values for state 3 respiration between 37 °C – 25 °C were generally less than 2-3, and were smaller than those between 25 °C and 10 °C, in all three species, regardless of assay pH, hibernation state or substrate type. The smaller $Q_{10}$ values between 37 °C and 25 °C indicate a less temperature sensitive reaction, which indicates that suppression of state 3 respiration between these temperatures is active. This is in agreement with the notion that active metabolic rate suppression in hibernators is greatest when body temperature is high, while passive thermal effects predominate as body temperature falls (Milsom & Jackson, 2011; Staples, 2014). While it is conceivable that mitochondrial respiration with high thermal sensitivity at warmer temperatures may be beneficial in reducing metabolic rate during the early phase of entrance into hibernation alongside active mechanisms, my findings do not support this idea.

### 4.4.3 Effect of hibernation state

To further identify whether the observed effects of pH and temperature were seasonally plastic and associated with metabolic state, I compared data from the facultative hibernator and the obligate hibernator in different metabolic states. In the hamster, the stimulatory effect of low pH on respiration and inhibitory effect on complex activity were present during euthermia. This suggests that the pH and temperature effects in this species are intrinsically present, and are not the result of the metabolic remodelling that occurs during the pre-hibernation acclimatization period. However, in order to better understand any metabolic state dependency of pH and temperature, intraspecific comparison with tissue taken from hamsters in hibernation would be necessary. I was unable to determine whether the observed traits were present in tissues from hamsters during hibernation as hamsters did not enter hibernation in my hands. Although the Syrian hamster is a species capable of hibernation in the wild and certain laboratory colonies have been used in hibernation research, it appears that prolonged commercial breeding may have selected against the hibernating phenotype in some strains (Trefna et al., 2017).

In the obligate hibernator, the effect of pH on state 3 mitochondrial respiration through CI was influenced by metabolic state (i.e., hibernation), rather than season. There was a
significant effect of pH on state 3 respiration through CI during hibernation, although post-hoc analysis reveals the stimulatory effect of acidosis was only prevalent at the intermediate temperature (25 °C). The biological significance or biochemical mechanism behind this observation only at 25 °C is unclear and requires further investigation. Nonetheless, the pH effects on respiration were again not mirrored at the individual complex level, as pH effects on CI activity appear to be seasonally plastic, rather than being caused by a rapidly reversible mechanism associated with metabolic state. CI activity was unaffected by pH during the summer but decreased at lower pH in both the winter states of the 13-lined ground squirrel (during hibernation and IBE). Seasonal changes in the mitochondria are well documented in hibernators as the mitochondrial phosphoproteome, phospholipid composition, and ETS supercomplex formation are known to change between summer and winter (Ballinger et al., 2016; Chung et al., 2013; Heim et al., 2017; Hutchinson et al., 2022). However, to my knowledge I am unaware of any studies that report CI specific changes between summer and winter that could explain the suppressive effect of acidosis on CI activity in winter animals but not in the summer.

The increase in respiration with decreasing pH acting through CII was persistent at 37, 25 and 10 °C in hibernation. This was not observed during summer euthermia where pH had only a weak effect on respiration (with no significant pairwise comparison at any temperature), or in IBE where pH only increased respiration at 37 °C at the lowest pH. The effects of pH and temperature on CII activity were of particular interest as previous studies have identified CII as the predominant regulatory site of oxidative phosphorylation in arctic ground squirrels and 13-lined ground squirrels, without a change in the quantity of the enzyme (Barger et al., 2003; Brown et al., 2012; Chung et al., 2011; Fedotcheva et al., 1985; Mathers et al., 2017).

The stimulatory effect of lower pH on respiration through CII was not mirrored at the individual complex level. To begin with, decreasing pH led to a decrease in CII activity. Furthermore, the pH effect on CII activity was not seasonal or metabolic state dependent, but constantly present in every metabolic state. The data suggest that while lower pH did have an inhibitory effect of CII activity, and therefore could be a mechanism of decreasing substrate oxidation in hibernators, there is an unknown compensatory mechanism that increases
mitochondrial oxygen consumption at lower pH. This mechanism allows 13-lined ground squirrels to maintain a constant level of respiration with decreasing pH in the euthermic states (summer and IBE) and increase respiration during hibernation.

Taken together, my data did not support the hypothesis that acidosis is a mechanism of suppressing metabolic rate in hibernators by reducing mitochondrial respiration rate. Contrary to my prediction, the data suggest that acidosis in fact increases respiration at lower pH and that this is not caused by the increase in individual complex activity at lower pH. The results suggest the existence of a compensatory mechanism which increases respiration at low pH and mitigates the inhibitory effect of pH on ETS enzyme activity. Thus, at least at the level of the ETS, changes in pH do not contribute to metabolic rate suppression in hibernating animals.
Figure 4.1. ADP stimulated respiration in isolated liver mitochondria with 10 mM glutamate + 2 mM malate or 10 mM succinate. O$_2$ consumption rates (nmol O$_2$/min/mg protein) were measured at 10 °C, 25 °C, 37 °C, and pH 7.0 ( ), 7.4 ( ), and 7.8 ( ). P-values for the statistical models are indicated on each panel. Statistical significances between pH at each temperature as determined by Tukey’s post-hoc test are indicated (****=p<0.0001; ***=p<0.001; **=p<0.01; *=p<0.05).
Figure 4.2. Leak respiration in isolated liver mitochondria (5 nM oligomycin), and FCCP induced uncoupled respiration (0.5 – 2.5 µM FCCP). O₂ consumption rates (nmol O₂/min/mg protein) were measured at 10 °C, 25 °C, 37 °C, and pH 7.0 ( ), 7.4 ( ), and 7.8 ( ). P values for the statistical models are indicated on each panel. Statistical significances between pH at each temperature as determined by Tukey’s post-hoc test are indicated (**** = p<0.0001; *** = p<0.001; ** = p<0.01; * = p<0.05).
Figure 4.3. Complex I, II, and V activity in isolated liver mitochondria measured at 10 °C, 25 °C, 37 °C, and pH 7.0 ( ), 7.4 ( ), and 7.8 ( ). P values for the statistical models are indicated on each panel. Statistical significances between pH at each temperature as determined by Tukey’s post-hoc test are indicated (****=p<0.0001; ***=p<0.001; **=p<0.01; *=p<0.05).
Chapter 5 - Temperature, pH and seasonal effects on eEF-2 phosphorylation and ATPase activity in hibernating rodents

5.1 Introduction

By reducing metabolic rate to <10% of basal metabolic rate and body temperatures close to ambient winter temperatures, hibernators are able to lower energy expenditure during times of low ambient temperature and food availability (Geiser, 2004). In hibernation, the reduction in metabolic rate is achieved through the regulated reduction in both adenosine triphosphate (ATP) synthesis and ATP consumption (MacDonald & Storey, 1998; Staples et al., 2022). The precise biochemical mechanisms that underly the downregulation of ATP turnover during hibernation are still active areas of research. There is currently a wealth of literature that identify several downregulated cellular processes such as cell division, apoptosis, and biosynthesis which ultimately lower metabolic demand in hibernation (Fleck & Carey, 2005; Wu & Storey, 2012).

Protein synthesis and ion homeostasis have been estimated to be responsible for 12 – 20% and 20 – 40% of total cellular respiration rate, respectively (Buttgereit and Brand, 1995). It is conceivable that these two highly ATP demanding processes are most sensitive to changes in ATP supply, as ATP supply and demand are inextricably connected. There is evidence to suggest that ATP demand controls ATP supply, while evidence for the converse (where ATP supply controls ATP demand) also exists (Nath, 2019). The cellular ratio of ATP to its hydrolysed states (adenosine diphosphate, ADP; and adenosine monophosphate AMP) serves as an important regulator of enzymes that control metabolic pathways (e.g. AMP kinase; Hardie & Hawley, 2001; Ramaiah et al., 1964). On the other hand, reduction in O$_2$ consumption (which ultimately leads to reduced ATP synthesis) has also been shown to decrease ATP consumption rates, thereby suggesting that ATP supply controls demand (Buttgereit and Brand, 1995). In cultured rat thrombocytes, protein synthesis and intracellular ion (Na$^+$, K$^+$ and Ca$^{2+}$) regulation were two of the most sensitive cellular processes to changes in ATP supply, with a 60% and 45% reduction in these processes, respectively, when a modest (30%) reduction in cellular O$_2$ consumption was induced (Buttgereit and
As mitochondrial oxidative phosphorylation capacity is significantly reduced in hibernation (see Chapter 4), it is possible that ATP consuming processes will also be reduced in parallel to this decrease in ATP supply.

For several decades, the blood of hibernators has been consistently reported to be relatively acidic, which has led to the hypothesis that acidosis may be involved in metabolic rate suppression (Kreienbühl et al., 1976; Malan et al., 1985). This hypothesis was supported by the findings of Bharma and Milsom (1993) as lowering blood pH decreased whole animal metabolic rate in golden mantled ground squirrels (Callospermophilus lateralis) by ~10% per 0.1 unit decrease in pH. The suggested mechanisms behind acidosis induced reduction in metabolic rate during hibernation ranges from lowering the thermoregulatory set point in the brain (Malan et al., 1985), lowering thermogenesis in the brown adipose tissue by inhibiting ADP binding (Malan and Mioskowski, 1988), and inhibiting PFK activity in skeletal muscle (Hand and Somero, 1982). If acidosis was a mechanism of reducing metabolic rate during hibernation, it is conceivable that this would occur through the downregulation of the two most ATP consuming cellular processes; protein synthesis and ion homeostasis.

Firstly, as the rate of mRNA translation is regulated by the activation or inactivation of initiation or elongation factors through post-translational modifications, acidosis may decrease the activity of these regulatory proteins (Frerichs et al., 1998). Of these regulatory proteins, eukaryotic elongation factor (eEF-2) is a protein essential in the elongation process as it recruits transfer RNAs (tRNA) carrying amino acids to the ribosome, for the elongation of the growing peptide chain. eEF-2 is regulated by the highly-specific eEF-2 kinase (eEF-2K) which phosphorylates and inhibits eEF-2 activity. Increased activity of eEF-2K and a concomitant increase in phosphorylated eEF-2 (phospho-eEF2) were reported in 13-lined ground squirrels during hibernation, in comparison to animals in IBE (Chen et al., 2001). Although low pH (6.6 – 6.8) is known to increase eEF-2K activity and decrease protein synthesis in mice, whether acidosis could be responsible for the increase in eEF-2 phosphorylation in hibernating animals has not yet been elucidated (Dorokov et al., 2002).
Secondly, the maintenance of transmembrane ion gradients is vital for signal transduction, cell volume regulation as well as secondary metabolite (e.g., glucose) transport. Homeostatic ion regulation is the second most ATP consuming intracellular process, as active transmembrane transporter proteins hydrolyse ATP to maintain low intracellular Na\(^+\) and Ca\(^+\), and high K\(^+\). In particular, 10 - 20\% of hepatocyte respiration has been attributed to Na\(^+\)-K\(^+\) ATPase alone (Nobes et al., 1989; Staples, 1995). Limited data in the literature suggests that Na\(^+\)-K\(^+\) ATPase activity is downregulated by \(~50\%\) in hibernating golden mantled ground squirrels in comparison to IBE through increased phosphorylation (MacDonald and Storey, 1999). However, whether total ATPase activity (including, but not limited to Na\(^+\)-K\(^+\) ATPase activity) is reduced in hibernation, and whether acidosis contributes to this reduction, has not yet been investigated. On the other hand, the purported acidosis during hibernation may stimulate the activity of intracellular pH regulating transporters that regulate cytosolic pH. For example, the Na\(^+\)-H\(^+\) exchanger is a secondary active pH regulator that is activated during acidosis and accounts for 70\% of intracellular pH regulation in hepatocytes (Henderson et al., 1987; Renner et al., 1989; Wadsworth & van Rossum, 1994). As pH regulation by cotransporters utilises the Na\(^+\) gradient established by the Na\(^+\)-K\(^+\) ATPase, the potential stimulation of these cotransporters during acidosis may increase the ATP demand which would be unfavourable during hibernation. Furthermore, Staples (1995) reported no hibernation state associated reduction in Na\(^+\)-K\(^+\) ATPase activity at 37 °C and pH 7.4 in hepatocytes from golden-mantled ground squirrels. On the other hand, MacDonald and Storey (1999) reported a 50\% reduction in Na\(^+\)-K\(^+\) ATPase activity in liver homogenates of hibernating golden-mantled ground squirrels at 25 °C and pH 7.4 when compared to euthermic counterparts. Whether the discrepancy in these studies stems from the reversal of metabolic rate suppression at warmer (37 °C) assay temperatures requires further investigation.

While our understanding of the mechanisms that govern metabolic rate suppression in obligate hibernators continues to grow, very little research examines whether these mechanisms are shared between obligate and facultative hibernators. The Syrian hamster is a facultative hibernator that can be induced into hibernation at any time of the year (without a circannual rhythm) after several weeks in pre-hibernation acclimatisation conditions of cold
ambient temperatures, low daylight length and reduced food availability. There is limited research to indicate that metabolic remodelling occurs during this pre-hibernation acclimatisation period, although how ATP consuming cellular pathways are downregulated in facultative hibernation is not well studied (Chayama et al., 2016, 2019; Talaei et al., 2012). On the other hand, the 13-lined ground squirrel is an obligate hibernator that routinely enters hibernation in the winter months. These animals have provided valuable insight into the rapidly reversible mechanisms that facilitate the cycling of metabolic rate between hibernation and IBE within a matter of hours. Their metabolic phenotype has been well characterised throughout the seasons, with several studies describing a summer active state that gradually remodels into the winter metabolic phenotype that can cycle between hibernation and IBE. By comparing a non-hibernating species (the Sprague-Dawley rat), a facultative hibernator during euthermia and hibernation, and an obligate hibernator in three metabolic states (summer euthermia, hibernation and interbout euthermia), I aimed to elucidate whether the effects of acidosis on ATP consuming cellular processes are intrinsically present in species capable of hibernation, or whether seasonal metabolic remodelling is required for these pH effects to manifest.

To test the hypothesis that acidosis inhibits protein synthesis and ATPase activity during hibernation, I compared the effect of pH on the phosphorylation state of eEF-2 in hepatocytes and total activity of ATPases in liver homogenates of two species of hibernators to the non-hibernating rat. As low pH has been shown to increase activity of eEF-2K at low pH in rabbit reticulocytes and mice liver, I predicted that incubation at low pH would increase phosphorylation state of eEF-2 in hibernators (Dorokov et al., 2002; Xie et al., 2015). I predicted that acidosis would increase the phosphorylation of eEF-2 in the non-hibernating rat and two hibernating species alike, and that perhaps this may not be a unique trait in hibernating species per se, but that acidosis could still serve as a regulatory mechanism to lower ATP consumption during hibernation. I also predicted that ATPase activity in the liver would be lower in hibernating animals compared to euthermic animals, especially at lower pH and temperature. I predicted that the pH effect on ATPase activity would be exhibited in both facultative and obligate hibernating species but absent in the non-hibernating rat.
5.2 Methods

5.2.1 Animals

All procedures were conducted under protocol A21-0006, approved by the UBC Animal Care Committee (ACC) and were in compliance with the standards set out by the Canadian Council on Animal Care (CCAC). Adult rats (256g–568g) and hamsters (80g–130g) were acquired from commercial breeders (Envigo and/or Charles Rivers) and housed at 20 ± 2 °C all year round. Captive-born adult 13-lined ground squirrels were used in this study. These animals were born in captivity and raised until adulthood (6+ months) under a 12:12 light:dark cycle. Ground squirrels were divided into three hibernation states: summer euthermia, hibernation, and interbout euthermia. All squirrels were kept in a temperature-controlled chamber (20 ± 2 °C) on a photoperiod that matched the daily photoperiod in Vancouver, Canada during the active period and at 5± 2 °C in 24 h dark during the hibernation period (November-April). Summer euthermic animals (230g–316g) were fully active and sampled in July. Winter animals (150–270g) were previously intraperitoneally implanted with RFID temperature chips (IPTT-300; 1 mm × 3 mm, < 0.5 g) and scanned with a DAS-8017 reader (Biomedic Data Systems, DE, USA) as described by Sprenger and Milsom (2022). Animals were confirmed to be in hibernation when body temperature was 1-2 °C above environmental chamber temperature. Hibernating squirrels were sampled when they were confirmed to have been in torpor for at least 48 hours, which was done by daily monitoring and ensuring that wood chips placed on the hibernating animals remained undisturbed, indicating inactivity. Hibernating squirrels entered IBE after gentle agitation and were sampled after at least 12 hours. Only animals that were fully active with a body temperature around 37 °C were sampled during IBE. Only females of each species/state were used.

5.2.2 Hepatocyte isolation

Primary hepatocytes were isolated by using a modified two-step collagenase perfusion method as described by Shen et al., 2012. The inferior vena cava was cannulated in situ near the origin of the renal veins using a 21-gauge needle. To contain the perfusion buffers within the hepatic circulation, the vena cava was clamped with a hemostat slightly cranial to the
The hepatic portal vein was cut for drainage. The liver was first perfused with 150 ml of Ca\(^{2+}\) and Mg\(^{2+}\) free Hank’s Balanced Salt Solution (HBSS) supplemented with 0.9 mM MgCl\(_2\), 0.5 mM EDTA, 25 mM HEPES at 15 ml/min. Subsequently, the liver was perfused with 150 ml of HBSS with Ca\(^{2+}\) and Mg\(^{2+}\) with 200 units/ml of Collagenase I (Worthington Biochemical, NJ, USA) at 25 ml/min. Both solutions were warmed to 37 °C throughout the perfusion procedure, with the exception of the hibernating animal. To best match *in situ* conditions, the perfusion buffers of hibernating animals were kept at 10 °C, which resulted in a lower yield of hepatocytes due to reduced collagenase activity.

The liver was then removed and placed into 50 ml of collagenase HBSS with 2.5 μg/ml of DNase I and gently dissociated using a cell scraper. The dispersed hepatocytes were incubated at 37 °C for 15 minutes with gentle stirring with a magnetic stir bar. The cell suspension was then filtered through a 100 μm cell strainer and suspended in 40 ml of William’s complete medium (William’s E medium supplemented with 2 mM L-glutamine, 5% fetal bovine serum (FBS), 100 nM insulin, 100 nM dexamethasone, 100 IU/ml penicillin, and 100 mg/ml streptomycin, pH 7.4). The cell suspension was centrifuged at 500 x g for 10 minutes at 4 °C. The supernatant containing cellular debris was discarded and the pellet containing hepatocytes was resuspended in 40 ml of William’s complete medium with 2.5 μg/ml of DNase. The cells were centrifuged at 100 x g for 10 minutes at 4 °C three times, with the supernatant discarded each time. Finally, the hepatocyte pellet was resuspended in pH adjusted William’s complete medium to yield a cell suspension of 2.5 x 10^5 cells/ml. Due to the temperature sensitive nature of pH, aliquots of William’s complete medium were first incubated at either 37, 25 or 10 °C, and subsequently adjusted to pH 7.0, 7.4, and 7.8.

According to the manufacturer’s guidelines, William’s E medium maintains a physiological pH range (pH 7.2 – 7.4) when gassed with 5-10% CO\(_2\). However, through pilot studies, I established that the pH of William’s E medium was best maintained consistent throughout the experiment when the medium was adjusted with HCl or KOH. Initial cell viability was quantified by 0.4% trypan blue exclusion, and only preparations with viability above 80% were used in the subsequent experiment to quantify eEF-2 phosphorylation state.
5.2.2.1 Enzyme linked immunosorbent assay of eEF-2 phosphorylation

The phosphorylation state of eEF-2 was measured using a semi-quantitative colorimetric enzyme linked immunosorbent assay (ELISA) kit (EKC1985, BosterBio, CA, USA). Phosphorylated eEF-2 was quantified relative to the expression of non-phosphorylated eEF-2 using primary antibodies specific to phospho-eEF-2 (Thr56). Briefly, three sterile 96-well cell culture plates were used for each temperature incubation (37, 25 and 10 °C), and were coated with poly-L-lysine. Each well was seeded with 40,000 primary hepatocytes in triplicates for each pH treatment or control wells. Each plate was incubated for four hours at its respective temperature. This time frame was chosen as previous studies have demonstrated that primary hepatocytes adhered to the surface of the wells in this time (Charni-Natan & Goldstein, 2020). Subsequently, hepatocytes were permeabilised and fixed in 4% formaldehyde solution and assayed by closely following the manufacturer’s guidelines.

Due to the limited viability of primary cells and potential loss of the hibernating phenotype in culture conditions, prolonged incubation times were undesirable. Previous studies and my own pilot study using an intracellular pH assay kit (BCFL, AM; Abcam Cambridge, UK) have demonstrated that pH_i mirrors pH_e within 4 hours of incubation (Pollock, 1984). I also validated that the hibernating phenotype is maintained in primary hepatocytes by assaying Na^+-K^+ ATPase activity, as it has been shown to be suppressed in hibernating ground squirrels in comparison to active animals (MacDonald and Storey, 1999). I confirm that Na^+-K^+ ATPase activity remained suppressed in hepatocytes of hibernating ground squirrels 4 hours post isolation (Figure 5.1).

5.2.3 ATPase activity

Non-specific total ATPase activity was measured in the liver according to the protocol developed by McCormick (1993) and modified by Moyes et al. (2021). Briefly, liver samples were homogenised in 50 mM HEPES, 1 mM EDTA, and 0.05% sodium deoxycholate. Samples were centrifuged at 2000xg for 1 minute at 4 °C, and the supernatant was used for subsequent assays. All samples were assayed within one hour of homogenisation. Aliquots of
the assay medium (50 mM HEPES, 5 mM MgCl₂, 5 mM ATP, 50 mM NaCl, 25 mM KCl, 4 U/ml LDH, 5U/ml PK, 0.2 mM NADH, and 2 mM PEP) were first incubated at either 37, 25 or 10 °C, and subsequently adjusted to pH 7.0, 7.4, and 7.8. The assay was carried out with 10 μL of liver homogenate and 200 μL of assay medium in duplicates per sample. Absorbance was measured at 340 nm for 10 minutes. Enzymatic activity is expressed as nmol/min/mg and was corrected to protein content of each homogenate. Protein content was determined using a Bradford assay (Bio-Rad, ON, Canada).

Although the protocols were initially established for the quantification of Na⁺-K⁺ ATPase activity specifically through the use of ouabain (a highly specific Na⁺-K⁺ ATPase inhibitor), I observed an unusually high ouabain insensitivity in liver homogenates. Previous studies have recommended the use of 0.5 - 5 mM of ouabain (Moyes et al., 2021), but this concentration range was not sufficient to inhibit Na⁺-K⁺ ATPase activity of liver homogenates in my hands. I empirically determined that 5 mM ouabain was sufficient to inhibit Na⁺-K⁺ ATPase activity in gill homogenates of teleost fish and in isolated primary hepatocytes (for the purpose of confirming the hibernating phenotype as aforementioned), but 5 mM ouabain did not inhibit Na⁺-K⁺ ATPase activity in liver homogenates. Through preliminary dose-dependency experiments, I established that 40 mM was sufficient to achieve maximum Na⁺-K⁺ ATPase inhibition in liver homogenates at 25 °C. This concentration, which is substantially higher than that used in several previous studies in mammalian tissues, surprisingly did not noticeably reduce ATPase activity at 37 °C (Moretti et al., 1991; Moyes et al., 2021). As a result, I was unable to determine the proportion of total ATPase activity that was specific to Na⁺-K⁺ ATPase activity at 37 °C. Furthermore, as such a high concentration of ouabain exceeds the maximum solubility of ouabain at 10 °C, I observed immediate precipitation that interfered with the spectrophotometric assay. Therefore, Na⁺-K⁺ ATPase specific activity was only quantifiable at 25 °C and I report total ATPase activity for 37, 25 and 10 °C. Na⁺-K⁺ ATPase specific activity data at 25 °C should be interpreted with caution nonetheless, as unknown non-specific, off-target biochemical effects may be present due to the excessively high concentration of ouabain used in the assay.
5.2.4 Statistical analysis

All data were analysed in Graphpad Prism v8 (CA, USA). A repeated measures two-way ANOVA or mixed effects model (in cases where some individual animals were missing values) were used to test the effects of pH and temperature on total ATPase activity within a state/species. A Tukey’s multiple comparison test was subsequently carried out to identify means of total ATPase activity at different pH within each temperature that was significantly different from another. A repeated one-way ANOVA was carried out within a state/species to identify the effects of pH on Na⁺-K⁺ ATPase activity at 25 °C. When a significant effect of pH was noted within a state/species, a Tukey’s multiple comparison test was carried out to identify the means of Na⁺-K⁺ ATPase activities at different pH that differed from another. P-values below 0.05 were considered to indicate significant differences and all data are displayed as the mean ± standard error of the mean.

5.3 Results

5.3.1 eEF-2 phosphorylation

Although there was variation in the proportion of phosphorylation of eEF-2 (between 34 - 57%), I did not observe any pH or temperature effects in any species or metabolic state (Figure 5.2). However, in the rat and summer euthermic squirrels, I observed a suggestive (but not significant) effect of temperature (p = 0.0883 and 0.0852, respectively). Similarly, I observed a near significant interaction effect between pH and temperature in the summer euthermic squirrel (p = 0.0763). However, I was unable to observe any conclusive effect of either temperature or pH on phosphorylation state of eEF-2 in any species or metabolic state (n=3-6).

5.3.2 ATPase activity

In the rat, only temperature had a significant effect on total liver ATPase activity in the rat (p < 0.0001), while pH did not (Figure 3). Na⁺-K⁺ ATPase specific activity at 25 °C also was not affected by pH in the rat (Figure 4). In the hamster, temperature had a significant effect
on total ATPase activity in both metabolic states (euthermia $p < 0.0001$; hibernation $p = 0.003$). pH had a significantly lowered total ATPase activity in euthermia ($p < 0.0001$) but only had a suggestive (but insignificant) effect in hibernation ($p = 0.0763$). There was an interactive effect between temperature and pH in the euthermic hamster with pH only decreasing ATPase activity at the warm temperatures (25 and 37 °C). Na$^+$-K$^+$ ATPase specific activity was also decreased at low pH in eutheremic hamsters, but not during hibernation. pH and temperature affected ATPase activity in all metabolic states of the ground squirrel with low pH inhibiting activity at every assay temperature (10, 25, and 37 °C). There was an interaction effect between pH and temperature in all metabolic states of the ground squirrel. pH had a significant effect on Na$^+$-K$^+$ ATPase specific activity at 25 °C and during hibernation and interbout euthermia in the ground squirrel.

5.4 Discussion

The results from Chapter 3 suggest that euthermic animals could exhibit relative acidosis as pH$_i$ increased very little with decreasing body temperature. Although this acidosis appears to be reversed in steady state hibernation, it is possible that as animals transition from euthermia to hibernation (especially during early stages of entrance when T$_b$ is still relatively high), intracellular acidosis of euthermic animals may contribute to metabolic rate suppression. Although there was no effect of acidosis on mitochondrial ATP supply (Chapter 4), it is plausible that ATP demand may be reduced by acidosis during entrance into hibernation, and thereby contribute to metabolic rate suppression. I hypothesised that acidosis may contribute to metabolic rate suppression during hibernation by downregulating the most ATP consuming cellular processes, namely protein synthesis and ion homeostasis. The results suggest that downregulation of protein synthesis by phosphorylated eEF-2 was not mediated by changes in pH. However, cellular ATPase activity was inhibited by low pH, and this pH effect was unique to the hibernating species. The inhibitory effect of low pH on ATPase activity was present at warmer temperatures (25 and 37 °C) only in the facultative hibernator, but was present at all temperatures (10, 25 and 37 °C) in the obligate hibernator, regardless of state.
5.4.1 eEF-2 phosphorylation

Lower pH did not increase phosphorylation of eEF-2 in hepatocytes incubated in media between pH 7.0 – 7.8 at any temperature (Figure 5.2). This is contrary to previous studies in which protein synthesis was shown to decrease during hibernation through the increased activity of eEF-2K, which phosphorylates eEF-2 and thereby inhibits the elongation phase of translation (Frerichs et al., 1998; Chen et al., 2001). Furthermore, protein phosphatase 2A (PP2A) activity is known to decrease during hibernation, leading to decreased rates of eEF-2 dephosphorylation.

Based on previously published methodologies intracellular pH (pHᵢ) was expected to vary to match cell culture media pH (pHₑ) after 4 hours of incubation. Staples (1995) reported pHᵢ of hepatocytes from golden mantled ground squirrels changed from 6.88 – 7.0 to match pHₑ (7.1) within 15 minutes of incubation. Furthermore, Pollock (1984) also reported equilibration of pHᵢ to pHₑ in rat hepatocyte cultures after two hours of incubation in similar culture conditions to this study (in HEPES buffered media in the absence CO₂). Through pilot studies, I also confirmed that the different cell culture media pH were reflected in the pHᵢ of hepatocytes (Supplementary Figure 5.1). It was necessary, however, to incubate the hepatocytes for four hours to allow the cells to adhere to the surface of the 96-well plate for downstream quantification of eEF-2 phosphorylation state (Shen et al., 2012).

Nonetheless, the lack of pH effect on phosphorylation of eEF-2 was unexpected, given that Dorovkov et al. (2002) observed an increase in isolated eEF-2K activity at low pH (6.4) after ten minutes of *in vitro* reaction at 30 °C. In this study, I did not observe any effect of pH on eEF-2 phosphorylation at a similarly warm temperature (37 °C), or any other assay temperature. It is conceivable that intact live cells require longer to manifest changes in phosphorylation state of eEF-2 than that required in *in vitro* reactions with purified/recombinant proteins. Phosphorylation of eEF-2 was significantly increased in mice embryonic fibroblasts (MEFs) after 24 hours of incubation at 30 °C in media between pH 6.0 – 7.4 (Xie et al., 2015). This suggests that for changes in eEF-2 phosphorylation state to manifest, prolonged incubation is required, beyond what is required to observe changes in
pH. However, prolonged culturing of primary hepatocytes isolated from hibernating animals is likely to reverse the hibernating phenotype and thus was not plausible for this study. Future experiments may benefit from empirically determining the optimal incubation time that preserves the hibernating phenotype yet is sufficient enough for changes in eEF-2 phosphorylation state to be observed.

Furthermore, in contrast to previous studies, the average % of eEF-2 phosphorylation was lower in the hibernating animals compared to IBE animals at all temperatures and pH levels (with the exception of pH 7.4 and 7.8 at 25 °C). Chen et al. (2001) reported an increase in phosphorylated eEF-2 in the brain and liver during hibernation compared to IBE. This suggests that the hibernating phenotype in hepatocytes may not have been maintained during the isolation processes, despite the observation that the Na\(^+\)-K\(^+\) ATPase activity remained lower in hepatocytes isolated from hibernating 13-lined ground squirrels compared to IBE animals (see section 5.2.2.1).

5.4.2 ATPase activity

As cellular ion homeostasis is consistently reported as one of the most ATP-demanding processes, I hypothesised that ATPase activity would decrease in hibernation to conserve cellular ATP and that this suppression may be mediated by acidosis.

5.4.2.1 Hibernation state effects on ATPase activity

My hypothesis was only partially supported by the data from the facultative hibernator. Neither total ATPase activity nor Na\(^+\)-K\(^+\) ATPase activity were suppressed in hibernating hamsters compared to euthermic hamsters at any assay temperature (Figure 5.2 and 5.3). Similarly, Goldman & Willis, 1973 also reported that the cerebral cortex Na\(^+\)-K\(^+\) ATPase activity of hibernating Syrian hamsters was not significantly suppressed compared to euthermic hamsters at 38 °C. Furthermore, in this study, total ATPase activity at 10 °C was higher in hibernating hamsters than in euthermic animals. This was also consistent with the findings of Goldman and Willis (1973) in which cerebral cortex Na\(^+\)-K\(^+\) ATPase activity was greater in hibernating hamsters when assayed at 10 °C. In Syrian hamsters, Na\(^+\)-K\(^+\) ATPase
activity at low temperatures appears to be associated with prolonged cold acclimatisation rather than hibernation per se, as non-hibernating hamsters acclimatised for 3 - 4 weeks in the cold exhibited Na\(^{+}\)-K\(^{+}\) ATPase activity at 5 °C that was comparable to that of hibernating animals. Cold acclimatisation has been shown to increase cholesterol content in the phospholipid bilayer of several mammalian species, including the Syrian hamster, and is considered to be an adaptive response to maintain membrane fluidity at low temperatures (Cress & Gerner, 1980; Sicart et al., 1986). As Na\(^{+}\)-K\(^{+}\) ATPase activity is known to increase with higher cholesterol content, I speculate that the increase in Na\(^{+}\)-K\(^{+}\) ATPase activity of hibernating hamsters in this study is the result of increased cholesterol content acquired during the pre-hibernation acclimatisation to cold ambient temperature (Garcia et al., 2019; J. Zhang et al., 2020). In non-cold tolerant species such as humans, decreased Na\(^{+}\)-K\(^{+}\) ATPase activity at low temperatures is known to result in increased Na\(^{+}\) accumulation and loss of ion homeostasis (Mohr et al., 2020). Thus, higher Na\(^{+}\)-K\(^{+}\) ATPase activity in hibernating hamsters at 10 °C compared to euthermic animals (that have not been exposed to the cold) appears to be a beneficial trait acquired during cold-acclimatisation to avoid cell death through ion dysregulation at cold temperatures.

In contrast, data from the obligate hibernator are contrary to my findings in the facultative hibernator. Total ATPase activity in the liver was reduced by 50% in 13-lined ground squirrels during hibernation when compared to IBE animals. This degree of suppression was found in golden mantled ground squirrels where hibernating animals exhibited a 50% reduction in Na\(^{+}\)-K\(^{+}\) ATPase activity compared to IBE (MacDonald and Storey, 1999). This hibernation induced reduction of ATPase activity in obligate hibernators, however, appears to be tissue specific, as Na\(^{+}\)-K\(^{+}\) ATPase activity in the heart did not differ between hibernation and IBE in the golden mantled ground squirrel (MacDonald & Storey, 1999). Therefore, it is possible that facultative hibernators, such as the Syrian hamster, may also exhibit suppressed ATPase activity in tissues other than the liver. To my knowledge, there are no comparative studies that investigate the tissue specific suppression of cellular ATPases in facultative hibernation, and how it may differ to the pattern observed in obligate hibernators.
Hibernation induced suppression of ATPase activity was temperature dependent in the 13-lined ground squirrel, as total ATPase activity at 37 °C (pH 7.4) was only modestly suppressed during hibernation compared to summer animals (14%) and IBE animals (11%) (Figure 5.3). My findings are consistent with the findings of Staples (1995), as cellular respiration rate attributed to Na⁺-K⁺ ATPase in hepatocytes from golden-mantled ground squirrels also did not differ between hibernation states when assayed at 37 °C. This potentially suggests that at 37 °C, the mechanisms that downregulate ATPase activity during hibernation are reversed. As increased phosphorylation has been shown to suppress Na⁺-K⁺ ATPase activity in hibernation, it is conceivable that increased activity of protein phosphatase at 37 °C may result in dephosphorylation of Na⁺-K⁺ ATPase (MacDonald and Storey, 1999). Suppression of total ATPase activity during hibernation was greater when temperature was lowered to 25 °C. Total ATPase activity was reduced by 43% when compared to summer animals and 38% compared to IBE animals at 25 °C (pH 7.4). This reduction in total ATPase activity during hibernation can be attributed, at least partially, to the substantial reduction in Na⁺-K⁺ ATPase activity during hibernation (~50% decrease compared to summer and IBE animals; Figure 5.4). When temperature was reduced further to 10 °C, total ATPase activity was 16% reduced compared to summer animals and 40% reduced compared to IBE animals. I was unable to identify whether this reduced total ATPase activity of hibernating animals at 10 °C was due to the decrease in Na⁺-K⁺ ATPase activity at 10 °C as the high concentration of ouabain required to inhibit Na⁺-K⁺ ATPase precipitated out and interfered with the spectrophotometric assay (see methods in section 5.2.3).

The data suggest that ATPase activity is decreased during hibernation, and this inhibition is readily reversed in IBE. The decrease in ATPase activity in obligate hibernators at low temperatures may be reflective of (but not limited to) the decreased need for active ion regulation, as passive ion movement through the plasma membrane is also known to decrease at low temperatures due to thermal effects on membrane fluidity (Hazel, 1995). Furthermore, lowering temperature has been shown to have a greater effect on reducing passive ion loss in red blood cells of 13-lined ground squirrels (i.e., a greater Q₁₀ value), compared to non-hibernating species such as guinea pigs and humans (Glynn, 1956; Kimzey & Willis, 1971).
Thus, greater passive inhibition of Na\(^+\) influx at reduced body temperatures in 13-lined ground squirrels may allow intracellular ion balance to be maintained while Na\(^+\)-K\(^+\) ATPase activity is suppressed during hibernation to limit ATP consumption.

5.4.2.2 pH effects on ATPase activity

In euthermic hamsters, low pH significantly decreased total ATPase activity at 37 and 25 °C (Figure 5.3). At 25 °C, acidosis induced inhibition of Na\(^+\)-K\(^+\) ATPase activity may be partially responsible for the decrease in total ATPase activity observed at low pH. I did not observe a low pH induced reduction in total ATPase activity at 10 °C, which may be consistent with the idea that passive thermal effects are sufficient to lower metabolic rate in hibernators when body temperature is substantially lowered, while active mechanisms, such as acidosis, are necessary at higher body temperatures. However, there was no pH effect on either total ATPase or Na\(^+\)-K\(^+\) ATPase activity in hibernating hamsters (Figure 5.4). This implicates a hibernation state (or cold-acclimatisation) dependent change in the effect of pH on ATPase activity in this species that requires further investigation.

In the obligate hibernator, low pH decreased total ATPase activity in all hibernation states and at all assay temperatures (Figure 5.3). However, in summer and hibernating animals, this low pH induced suppression of total ATPase activity was not due to a reduction in Na\(^+\)-K\(^+\) ATPase activity at low pH. Na\(^+\)-K\(^+\) ATPase activity at 25 °C was unaffected by pH in summer animals, while the lowest Na\(^+\)-K\(^+\) ATPase activity was observed at the highest assay pH (7.8) during hibernation (Figure 5.4). This implicates both a metabolic state dependent difference in the effect of pH on Na\(^+\)-K\(^+\) ATPase activity between animals in the same season, (hibernating and IBE animals), as well as a seasonal difference between animals in the same active metabolic state, but in different seasons (between summer and IBE animals). The inhibitory effect of low pH on Na\(^+\)-K\(^+\) ATPase activity during IBE is perhaps not surprising given the pH sensitive nature of enzyme-substrate binding kinetics. However, the mechanisms underlying the lack of pH effects on Na\(^+\)-K\(^+\) ATPase activity during summer and hibernation need further investigation. The fact that acidosis does not suppress Na\(^+\)-K\(^+\) ATPase activity suggests that other cellular ATPases are responsible for the reduction in total
ATPase activity at low pH in summer euthermia and hibernation. Notably, Ca\(^{2+}\) ATPase is responsible for \(\sim\)10\% of cellular respiration rate, similar to the proportion of O\(_2\) consumption attributed to Na\(^{+}\)-K\(^{+}\) ATPase (Buttgereit & Brand, 1995, Staples, 1995). To my knowledge, there has been no explicit report on the effect of pH on Ca\(^{2+}\) ATPase activity in hepatocytes. Nevertheless, mammalian vacuolar Ca\(^{2+}\) ATPase isoforms in skeletal muscle, cardiac muscle, cerebellum and platelet cells have been shown to decrease maximal activity at low pH (6.0), due to a reduction in affinity for Ca\(^{2+}\) (Wolosker et al., 1997). Thus decreased Ca\(^{2+}\) ATPase activity at low pH may be responsible for the overall decrease in total ATPase activity at low pH in all states of the 13-lined ground squirrel, although this requires further investigation in future studies.

As T\(_b\) decreases drastically in hibernation, passive thermal effects aid in the inhibition of metabolism. As such, the synergistic effect of both lowered temperature and relative acidosis would further inhibit ATP consumption by cellular ATPases. Lowering temperature from 37 °C to 10 °C, and pH from 7.4 to 7.0 result in a 90\% and 96\% reduction in ATPase activity in the facultative and obligate hibernator, respectively. However, as with any isolated enzymatic activity assay \textit{in vitro}, these findings should be interpreted with caution as the inhibition of an enzyme does not necessarily indicate the downregulation of the pathway \textit{in vivo}. This is exemplified by the findings of Chapter 4, where despite the decrease in ETS complex activity at low pH, intact mitochondrial respiration in fact increased at low pH. In agreement, Hand and Somero (1983) also emphasises the decrease in PFK activity at low pH is only to be viewed as a mechanism of specific inhibition of enzymatic activity, rather than a means of reducing O\(_2\) consumption of the whole animal.

Although acidosis did not increase eEF-2 phosphorylation state, acidosis does appear to inhibit cellular ATPase activity in hibernating species, but not in the rat. However, the inhibitory effect of acidosis on ATPase activity was state dependent in the facultative hibernator and only present during euthermia. On the other hand, acidosis ubiquitously inhibited ATPase activity in all metabolic states of the obligate hibernator. This suggests that the mechanism suppressing ATP consumption may not be universal in all forms of heterothermy. Further research is required to tease out which ATPases are specifically
responsible for the reduction in total ATPase activity in acidosis as the activity of Na\(^+-\)K\(^+\) ATPase was not inhibited by acidosis in summer and hibernating 13-lined ground squirrels.
Figure 5.1. Na⁺-K⁺ ATPase activity at 25 °C in isolated primary hepatocytes at pH 7.4. Statistical significances as determined by Tukey’s post-hoc test are indicated (** *= p < 0.001; ** *= p < 0.01; *= p < 0.05).
Figure 5.2. Relative amount of phosphorylated eEF-2/eEF-2 in primary hepatocytes at pH 7.0 ( ), pH 7.4 ( ), and pH 7.8 ( ). No statistical differences were noted.
Figure 5.3. ATPase activity in liver homogenates at pH 7.0 ( ), pH 7.4 ( ), and pH 7.8 ( ). *P*-values for the statistical models are indicated on each panel. Statistical significances between pH at each temperature as determined by Tukey’s post-hoc test are indicated (**= p<0.0001; ***= p<0.001; **= p<0.01; *= p<0.05).
Figure 5.4. Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity in liver homogenates at 25 °C and pH 7.0 ( ), pH 7.4 ( ), and pH 7.8 ( ). P-values for the effect of pH within a species are indicated on top of each panel. Statistical significances between pH as determined by Tukey’s post-hoc test are indicated (****=p<0.0001; ***=p<0.001; ** = p<0.01; *=p<0.05).
Chapter 6 – General discussion

6.1 Thesis summary

The overarching objective of this thesis was to determine whether acidosis may be an inhibitory mechanism that contributes to active metabolic rate suppression during hibernation. I adopted an integrative approach to understanding the effects of acidosis on ATP supply and demand during hibernation by examining O$_2$ transport, and subcellular O$_2$ consumption and ATP demand. I carried out a three species comparison between the non-hibernating rat (Rattus norvegicus), the facultative hibernator, the Syrian hamster (Mesocricetus auratus) and the obligate hibernator, the 13-lined ground squirrel (Ictidomys tridecemlineatus) to elucidate whether the observed pH effects may be associated with either form of hibernation, or a common trait present in rodents. Data from Chapter 2 indicate that hemoglobin of hibernators exhibit a reduced temperature sensitivity and increased Bohr effect relative to the rat. This may ultimately enhance O$_2$ offloading at the tissues and be beneficial for meeting the increased O$_2$ demand during arousal. The results of Chapter 3 show that while there may be CO$_2$ retention during entrance into hibernation and a relative blood acidosis during steady state hibernation, acidosis is not apparent in steady state hibernation in any tissue in either the facultative or obligate hibernator. They indicate that intracellular buffering in facultative hibernators retains the activity of proteins with changing temperature throughout the year while that in seasonal hibernators changes rapidly between states during the hibernation season. Chapters 4 and 5 show that while mitochondrial respiration was not inhibited by low pH, the activity of enzymes involved in ATP synthesis and ATP consumption (ETS complexes and cellular ATPases, respectively) were inhibited at low pH. This inhibitory effect of low pH was unique to hibernators.

In this chapter, I aim to integrate the results from the four experimental chapters to examine the effects of acidosis during hibernation at multiple levels of biological organisation, spanning from the whole organismal level down to the subcellular level. I also aim to comprehensively discuss the controversial yet long-standing hypothesis put forward by Malan et al. (1985) that acidosis selectively occurs in quiescent tissues of hibernators to
suppress metabolic rate. Lastly, I suggest future research directions that would further our understanding of the acid-base state of hibernators.

6.2 What is the role of acidosis in hibernation?

A reduction in ventilation precedes the decrease in metabolic during entrance into hibernation, indicating a reduction in the RER. This reduction in RER has been well documented in several species of hibernators over several decades, and has also been recently demonstrated in our lab with 13-lined ground squirrels (Bickler, 1984b; Snapp & Heller, 1981; Sprenger & Milsom, 2022). As CO$_2$ is retained, this decrease in RER leads to an acidosis of the blood, which has also been consistently reported in several species of hibernators (Table 1.1). Using anesthetised golden mantled ground squirrels, Bharma and Milsom (1993) demonstrated that artificial acidosis of the blood decreases whole animal metabolic rate. This observation supported the idea that hypoventilation during entrance into hibernation was involved in metabolic rate suppression, although the mechanism was unknown at the time (Bharma & Milsom, 1993). Since then, very few studies have been conducted on hibernators to examine how acidosis affects the main function of blood, which is to transport respiratory gases (Maginniss & Milsom, 1994).

To understand the role of blood acidosis in suppressing metabolic rate in hibernators, Chapter 2 of my thesis investigated the effect of acidosis on HbO$_2$ binding kinetics. Hibernators exhibited a greater pH sensitive hemoglobin (Bohr effect) compared to the non-hibernating rat and literature values of other non-hibernating species. While greater HbO$_2$ binding is often considered a molecular adaptation for increasing O$_2$ transport in animals with high aerobic demands and/or in hypoxia, the converse effect of high HbO$_2$ binding affinity has received less attention, especially in the context of hibernation (Clementi et al., 2003; Coletta et al., 1992; Revsbech et al., 2013). A high HbO$_2$ affinity impedes O$_2$ offloading at the tissues. To compensate for this high Hb O$_2$ binding affinity that may decrease the diffusion gradient at the tissues, some animals are known to exhibit cellular mechanisms for intracellular acidification. This maximises O$_2$ offloading as H$^+$ acts as an allosteric regulator of Hb which decreases HbO$_2$ binding affinity. While it is conceivable that
limiting O₂ offloading with a high HbO₂ binding affinity may contribute to metabolic rate suppression in hibernation, this would require the demonstration of tissue hypoxia during hibernation. Tissue hypoxia has only been demonstrated to occur in the brain of arousing Arctic ground squirrels as indicated by an increase in hypoxia inducible factor-1 (HIF-1α) (Ma et al., 2005). However, HIF-1α is downregulated during steady state hibernation compared to periods of interbout arousal (IBE), indicating tissue hypoxia does not occur during hibernation (Ma et al., 2005). Furthermore, the data from Chapter 2 of my thesis suggest that the combination of blood acidosis, greater Bohr effect, and a smaller temperature sensitivity may serve to enhance O₂ offloading in hibernators to minimise the deleterious effects of the intrinsically high HbO₂ binding affinity in hibernating species.

Malan et al. (1985) suggested that the relative acidosis resulting from CO₂ retention during entrance into hibernation extended beyond the blood, and was also present in quiescent tissues (such as the skeletal muscle and brain) during hibernation. The limited data in the literature suggest that heterotherms (both in hibernation and daily torpor) exhibit acidosis during hibernation, which in turn changes the ionisation ratio of histidine residues, rendering proteins less active (Malan et al., 1985; Nestler 1990). Therefore, acidosis was suggested to be a mechanism of metabolic rate suppression in these quiescent tissues, while maintaining the same degree of relative alkalinity in functional tissues such as the heart and liver maintained cardiac contractility and gluconeogenesis, respectively. While this notion has been widely cited in the literature, empirical data supporting it has been rather sparse (Bharma & Milsom, 1993; Hand & Somero, 1983; Malan et al., 1985; Malan & Mioskowski, 1988; Milsom & Jackson, 2011; Nestler, 1990b). Furthermore, as tissue acid-base state has only been assessed in two heterothermic species (the European hamster and edible door mouse), it was previously unclear whether the proposed role of acidosis in suppressing metabolic rate was a unique trait to heterotherms and dependent on metabolic state, or a common trait exhibited by homeotherms and heterotherms alike (Malan et al., 1985; Nestler 1990). The aforementioned studies were also unable to distinguish between temperature and metabolic state effects on pH; as measurements were made in two steady states (hibernation and euthermia) and reported at the respective body temperatures (10 °C for hibernation, 25 °C for daily torpor, and 37 °C for euthermia). Therefore, in Chapter 3 of my thesis, I
investigated whether tissue specific acidosis occurred in hibernation, and how temperature affected pH in each species/state.

The work presented in this thesis contradict the findings of Malan et al. (1985) and Nestler (1990). In both the facultative and obligate hibernators, I found no evidence to suggest that quiescent tissues undergo acidosis during hibernation compared to euthermia, while the heart and liver do not. I found no evidence of acidosis in any tissue. As such, although previous studies have demonstrated CO\textsubscript{2} retention during entrance into hibernation causes a concomitant relative acidosis of the blood, the data from Chapter 3 suggest that relative acidosis of the blood does not cause acidosis in tissues during hibernation. Note that acidosis would cause a cell-wide response that could lead to the up/downregulation of multiple pathways. For example, proteolysis in skeletal muscle is known to be upregulated under acidotic conditions in other mammalian species (May et al., 1986; Mutsvangwa et al., 2004). This would be detrimental for shivering thermogenesis in arousing hibernators (Hindle et al., 2014). As such, to lower metabolic rate of specific tissues in hibernation, it would be more bioenergetically favourable to downregulate specific pathways, such as oxidative phosphorylation, through mechanisms that allow for more fine-tuned regulation (such as post-translational modifications), rather than acidifying the whole tissue.

However, as I sampled all animals in steady state, any transient acidosis that occurred in the tissues at the early stages of entrance into hibernation and subsequently reversed on arousal will have been overlooked. This is conceivable, given that active metabolic rate suppression is most necessary in the early stages of entrance into hibernation when T\textsubscript{b} is still high. To get around this, future studies will benefit from carrying out a time course analysis of tissue pH\textsubscript{i} continuously using pNMR spectroscopy. In Chapters 4 and 5, I examined whether acidosis, if it did occur transiently, could decrease mitochondrial oxygen consumption for ATP synthesis, and/or ATP consumption by decreasing protein synthesis and/or ATPase activity. The findings from Chapter 4 suggest that even if a transient acidosis did occur and was not detected in steady state (as in Chapter 2), mitochondrial respiration would not decrease. However, data from Chapter 4 and 5 collectively suggest that acidosis may play a role in decreasing enzymatic activity in hibernators, both at the ATP supply (ETS complex activity)
and ATP demand (ATPase activity) level. The inhibitory effect of pH on enzymatic activity was uniquely present in the two hibernating species, and not present in the rat. However, as discussed in Chapter 5, it is uncertain whether these inhibitory effects of low pH on enzymatic activity would contribute to metabolic rate suppression in hibernators in vivo.

Taken together, the data in my thesis indicates that hibernators exhibit a greater physiological response to changes in pH (both at the blood and cellular level) in comparison to the rat. In the rat, O$_2$ offloading from hemoglobin was comparatively less sensitive to changes in pH (smaller Bohr effect; Chapter 2), while mitochondrial respiration, ETS enzyme activity (Chapter 4) and ATPase activity (Chapter 5) were not affected by changes in pH. Currently, there are not enough species comparisons in the literature to explain whether the species difference in physiological responses to acidosis seen in my study are associated with heterothermy. It is conceivable that acidosis plays a protective role, rather than a role in metabolic rate suppression in hibernation as has been traditionally assumed. The limited data available suggest that extracellular acidosis increases cell survivability in mouse neuronal cells, and in ATP-deprived rat hepatocytes (Gores et al., 1988; Kacho et al., 2014). As mice undergo facultative daily torpor with food deprivation while rats are strict homeotherms, it appears that the protective role of acidosis on cell survivability is not unique to heterotherms. Although much more research is required to understand the role of acidosis during hibernation, my thesis concludes that acidosis likely does not contribute to whole animal metabolic rate suppression during hibernation.

6.3 Future directions

As an initial approach to understanding the role of acidosis on suppressing metabolic rate during hibernation, I focused on how O$_2$ transport in the blood and subcellular O$_2$ demand may be affected by acidosis. Although the role of acidosis during hibernation does not appear to limit O$_2$ transport or O$_2$ demand, there is much potential for future research as this means that the role of CO$_2$ retention and the resulting acidosis of the blood during entrance may have a different physiological role than those investigated in my thesis. My thesis only looks
at just a fraction of the physiological processes that may be affected by changes in pH, and there is much potential for future work.

First, better resolution on the time course of acquiring intracellular acidosis during entrance into hibernation is required. To my knowledge, with the exception of the study by McArthur et al. (1990), pH measurements of blood and/or tissues have only been made in steady state hibernation or during periods of IBE. The findings of Chapter 3 do not rule out the occurrence of a transient acidosis in tissues that may occur during early entrance, as all measurements were made in steady state. It is possible that tissue pH\textsubscript{i} initially mirrors the acidosis that is known to occur in the blood of hibernators. This tissue acidosis may occur when the RER is transiently decreased when T\textsubscript{b} is around 34 °C and then reversed by the time the animal reaches steady state hibernation with T\textsubscript{b} around 10 °C (Sprenger, 2021). In the only study of continuous pH measurement in hibernating animals, McArthur et al. (1990) recorded the change in pH\textsubscript{i} in skeletal muscle of Columbian ground squirrels over the course of arousal by using \textsuperscript{31}P NMR spectroscopy. This study demonstrated that the relative degree of alkalinity of blood to pH\textsubscript{nw} increases as T\textsubscript{b} increases, indicating that upon arousal, there is a reversal of the acidosis that was present in steady state hibernation. This is again contrary to the findings of Chapter 3 of this thesis. As such, further investigation to resolve the time course of changes in intracellular acid-base state between tissues, and between different species of hibernators is warranted.

Secondly, given that the blood undergoes acidosis during hibernation, but tissues do not, this implicates reduced sensitivity of the central and peripheral CO\textsubscript{2}/pH chemosensors that regulate pH\textsubscript{e}, while pH\textsubscript{i} is regulated independent of pH\textsubscript{e}. Blood pH is homeostatically regulated by CO\textsubscript{2}/pH sensing central chemoreceptors in the brain, as well as peripheral chemoreceptors in the carotid body and in major vessels such as the aorta (Nattie, 2006). Typically, cells in both central and peripheral pH chemosensitive areas (such as the carotid body and medulla) sense changes in pH\textsubscript{e} as pH\textsubscript{i} of these cells closely mirrors that of the extracellular environment (Buckler et al., 1991; Erlichman & Leiter, 1997). Earlier studies suggested that low pH\textsubscript{e} inhibits the regulatory mechanisms that regulate pH\textsubscript{i} in CO\textsubscript{2}/pH chemosensing cells, thereby allowing pH\textsubscript{i} to reflect changes in pH\textsubscript{e} (Erlichman and Leiter,
As sensitivity to blood CO$_2$/pH appears to have decreased in hibernation, it is plausible that the low pH$_e$ induced inhibition of pH$_i$ regulatory mechanisms in chemosensing cells is downregulated during hibernation.

On the other hand, the mechanisms behind pH$_i$ sensing and regulation in non-chemosensory cells, such as those examined in Chapter 2, appear to remain functional during hibernation. This would allow pH$_i$ to be sensed and regulated independently of pH$_e$. While it is conceivable that pH$_i$ regulating proteins such as the Na$^+$/H$^+$ exchangers (NHE) could act as the pH$_i$ sensor, this does not appear to be the case. Previous studies have demonstrated that these acid-base regulating ion transporters alone do not respond sufficiently to acid-base perturbations for them to be effective pH$_i$ sensors (Aronson et al., 1982; Gluck, 2004; Tresguerres et al., 2010). This indicates that the ion exchangers are themselves activated by an upstream pathway that senses changes in acid-base state. The protonation state of imidazole side chains of G-protein coupled receptors (GPCR) and protein tyrosine kinase 2 (Pyk2) have been implicated in pH$_i$ sensing (Damaghi et al., 2013; Li et al., 2004; Preisig, 2007). Whether these suggested pathways are active during hibernation and responsible for maintaining the same degree of relative alkalinity to pH$_{nw}$ in hibernation as in euthermia requires further investigation. Future experiments using live primary cells isolated from hibernators could reveal the mechanisms of pH$_i$ sensing and regulation that remain functional even in states of reduced temperature and metabolic rate suppression.

Lastly, the three species comparison in my thesis revealed that facultative hibernators during euthermia, before the pre-hibernation acclimatisation period exhibited the same physiological responses to acidosis as the hibernating 13-lined ground squirrels. This was despite the fact that hamsters are phylogenetically more closely related to the rat than squirrels (Blanga-Kanfi et al., 2009). My findings imply that the facultative hibernator, which is capable of entering hibernation at any time of the year may possess physiological traits associated with hibernation all year round, whereas the obligate hibernator may require seasonal remodelling to manifest these traits. In this case, the “two-switch” model for hibernation that has been typically applied to describe the seasonal (first switch between seasons, or before and after cold exposure) and metabolic state dependent changes (second switch between hibernation and IBE) that occurs in many species of hibernators (including the 13-lined ground squirrel)
may not apply to the Syrian hamster, and possibly to other facultative hibernators. Nonetheless, I do recognise that the three-species comparison in this thesis does not conclusively indicate whether this is a hibernator unique trait that is universally observed in all hibernating species.

It was a challenge throughout my research to induce the hamsters into hibernation, as very few (4) animals entered hibernation. Similar difficulties in inducing hamsters to hibernate have been reported by Trefna et al. (2017). It appears that some commercial colonies of hamsters may be losing the hibernation phenotype through generations of inbreeding. Due to the scarcity of these animals, all their tissues were frozen and archived and therefore, experiments using live cells from hibernating hamsters were not possible. In Chapters 2 and 5, I present data with small sample sizes for hibernating hamsters using the limited number of archived tissue samples. To more conclusively remark on the effect of hibernation on pH, (Chapter 2) and enzyme activity (Chapter 5), a larger sample size of hibernating hamsters, and hamsters during IBE are required. Furthermore, future research on isolated intact mitochondria or live hepatocytes from hibernating hamsters would further our understanding of the mechanisms behind metabolic rate suppression in facultative hibernation. This would be a great addition to the literature, as the vast majority of hibernation research has been centred around a few species of obligate hibernators, usually ground squirrel species, that readily enter hibernation in the laboratory (MacCannell & Staples, 2021). Comparative studies that investigate the physiological differences between facultative and obligate hibernators are rare, with the only notable example being comparisons between the facultatively hibernating black-tailed prairie dog, *Cynomys ludovicianus*, and the closely related obligate hibernator, the white-tailed prairie dog, *Cynomys leucurus* (Harlow & Braun, 1995; Harlow & Frank, 2001). There is a great need for more comparative studies with species of both facultative and obligate hibernators, as well as non-hibernating homeothermic species, to understand whether the effects of acidosis seen in the hamster and ground squirrel in my thesis are traits associated with heterothermy.

Despite previous speculation, my thesis collectively did not support the idea that acidosis is involved in reducing metabolic rate during entrance or steady state hibernation.
Understanding the regulatory mechanisms behind metabolic rate suppression during hibernation is complex, as it requires a carefully orchestrated global reduction in metabolism while still matching O₂ supply and demand. In addition, the rapidly changing metabolic demand between hibernation and arousal (i.e., not steady state) further complicates our understanding of the regulatory mechanisms that govern metabolic rate suppression in hibernators. The well-documented blood acidosis in hibernators may contribute to increased O₂ offloading to meet the increased O₂ demand during arousal. Furthermore, acidosis may reverse the inhibitory mechanisms that suppress mitochondrial respiration during steady state hibernation, as acidosis had a stimulatory effect on liver mitochondrial O₂ consumption in hibernating species, but not in the rat. To further understand the role of acidosis in hibernation, prospective studies focusing on animals as they transition between states will be necessary as my thesis was limited to examining animals in steady states (euthermia and hibernation).
References


Appendix: Supplementary materials

**Supplementary Table 4.1.** \( Q_{10} \) values of state 3 respiration with glutamate + malate as substrate. Values were calculated from the mean respiration rate at each temperature and pH as different individuals were used at each temperature.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.0</th>
<th>pH 7.4</th>
<th>pH 7.8</th>
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</thead>
<tbody>
<tr>
<td><strong>Rat</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>37-25°C</td>
<td>0.8</td>
<td>0.6</td>
<td>0.9</td>
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<tr>
<td>25-10°C</td>
<td>2.3</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td>37-10°C</td>
<td>1.4</td>
<td>1.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

| **Euthermic hamster**|        |        |        |
| 37-25°C             | 0.9    | 0.9    | 1.2    |
| 25-10°C             | 2.1    | 2.6    | 1.7    |
| 37-10°C             | 1.4    | 1.7    | 1.4    |

| **Summer euthermic**  |        |        |        |
| **ground squirrel**   |        |        |        |
| 37-25°C             | 1.1    | 1.5    | 1.8    |
| 25-10°C             | 2.2    | 2.0    | 1.8    |
| 37-10°C             | 1.6    | 1.7    | 1.8    |

| **Hibernating**  |        |        |        |
| **ground squirrel** |        |        |        |
| 37-25°C             | 1.4    | 1.5    | 1.7    |
| 25-10°C             | 2.0    | 1.7    | 1.6    |
| 37-10°C             | 1.7    | 1.6    | 1.6    |

| **Interbout euthermic** |        |        |        |
| **ground squirrel**   |        |        |        |
| 37-25°C             | 0.9    | 0.8    | 0.9    |
| 25-10°C             | 1.7    | 1.5    | 2.1    |
| 37-10°C             | 1.3    | 1.1    | 1.4    |
**Supplementary Table 4.2.** $Q_{10}$ values of state 3 respiration with succinate as substrate. Values were calculated from the mean respiration rate at each temperature and pH as different individuals were used at each temperature.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.0</th>
<th>pH 7.4</th>
<th>pH 7.8</th>
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<tbody>
<tr>
<td><strong>Rat</strong></td>
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</tr>
<tr>
<td>37-25°C</td>
<td>1.5</td>
<td>1.4</td>
<td>1.4</td>
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<tr>
<td>25-10°C</td>
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<td>2.6</td>
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<td>37-10°C</td>
<td>1.9</td>
<td>2.0</td>
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<td><strong>Eutheremic hamster</strong></td>
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<tr>
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<td>1.7</td>
<td>1.5</td>
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<tr>
<td><strong>Summer euthermic ground squirrel</strong></td>
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<tr>
<td>37-10°C</td>
<td>2.3</td>
<td>2.4</td>
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<tr>
<td><strong>Interbout euthermic ground squirrel</strong></td>
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<tr>
<td>37-25°C</td>
<td>1.8</td>
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<td>2.4</td>
<td>1.8</td>
<td>1.9</td>
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</tbody>
</table>
Supplementary Figure 4.1. $Q_{10}$ values of complex I, II, and V activity calculated between 37 – 10°C, 37 – 25°C, and 25 – 10°C, and pH 7.0 ( ), 7.4 ( ), and 7.8 ( ). $P$-values for two-way ANOVA are indicated on each panel.
Supplementary Figure 5.1. Calibration curves to confirm intracellular pH (pH\textsubscript{i}) of hepatocytes from hibernating (A) and IBE (B) 13-lined ground squirrels were reflective of pH of the culture media. Solid red circles denote calibration standard curves generated by clamping hepatocytes with calibration buffers of verified pH for 15 minutes. pH\textsubscript{i} of hepatocytes incubated with culture media of different pH (pH\textsubscript{e}) for 15 minutes were interpolated from the calibration standard curves using a 4-parameter logistics model. RFU = relative fluorescence units (Ex/Em=505/535 and 430/535). n=1 for each metabolic state.