Response of DNA and Histone Lysine Methylation Regulators During Anoxia-Reoxygenation and Dehydration-Rehydration in Wood Frog (*Rana sylvatica*) Skeletal Muscle

By

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ABSTRACT

The ability of wood frogs, *Rana sylvatica*, to survive freezing and clinical death during harsh northern winters necessitates well-grounded mechanisms for managing extreme anoxia, dehydration, and reperfusion damage. These stresses are major instigators of pathogenesis yet to be overcome by humans. Contemporary efforts in the field are focused on interrogating stress-specific mediators that play a cytoprotective role in vital tissues such as skeletal muscle, providing valuable information to the biomedical community. Herein, the potential role of DNA and histone lysine methylation enzymes are examined in wood frog skeletal muscle in response to 24 h anoxia and 40% dehydration independently, and recovery from both stresses. This thesis demonstrates a condition-specific response of many epigenetic methylation regulators, highlighting some conserved similarities in comparison to prior freeze-thaw models. These findings support an integral role of epigenetic regulators in survival of hypometabolic stresses, most prominently during recovery stages. These cytoprotective effects are likely attributed to functional roles in transcriptional suppression during hypometabolism and activation during recovery, but also alternative roles based on known interactions with regulators of the cell cycle and repair pathways.
ACKNOWLEDGEMENTS

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day have piled into a mountain of knowledge I can rely on. You have been the glue that holds my sanity together, but also a continual source of positivity and optimism, for which I will be forever grateful. To my other current and former lab- and work-mates, I’ve been blessed for the time we’ve shared, and I will never forget such an amazing crew of researchers and educators. I can only hope our paths will cross again, as it’s been an absolute pleasure to work with and learn from you all. Lastly, I’d like to thank my family, friends, and my better-half Taylor, for standing by me without question throughout this journey. Your support and motivation have been unaltering throughout my life, and it is because of you that I have taken on such a challenge to better myself. I hope that in doing so I will be able to help and inspire others as you have. I wish everyone the best, and all hail the wood frog!
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
<th>Chapter/Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>TITLE PAGE</td>
</tr>
<tr>
<td>ii</td>
<td>ABSTRACT</td>
</tr>
<tr>
<td>iii</td>
<td>ACKNOWLEDGEMENTS</td>
</tr>
<tr>
<td>v</td>
<td>TABLE OF CONTENTS</td>
</tr>
<tr>
<td>vi</td>
<td>ABBREVIATIONS</td>
</tr>
<tr>
<td>ix</td>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>x</td>
<td>LIST OF APPENDICES</td>
</tr>
<tr>
<td>1</td>
<td>CHAPTER 1: General Introduction</td>
</tr>
<tr>
<td>2</td>
<td>1.1 Introduction to the Wood Frog</td>
</tr>
<tr>
<td>3</td>
<td>1.2 Freeze-Associated Stresses: Anoxia, Dehydration, Reperfusion</td>
</tr>
<tr>
<td>5</td>
<td>1.3 Metabolic Rate Depression and Metabolism</td>
</tr>
<tr>
<td>8</td>
<td>1.4 General Objective and Hypothesis</td>
</tr>
<tr>
<td>9</td>
<td>CHAPTER 2: Materials and Methods</td>
</tr>
<tr>
<td>10</td>
<td>2.1 Frog Preparation</td>
</tr>
<tr>
<td>10</td>
<td>2.2 Anoxia Experimentation</td>
</tr>
<tr>
<td>11</td>
<td>2.3 Dehydration Experimentation</td>
</tr>
<tr>
<td>11</td>
<td>2.4 Total Protein Isolation</td>
</tr>
<tr>
<td>12</td>
<td>2.5 Western Blotting</td>
</tr>
<tr>
<td>14</td>
<td>2.6 Statistics</td>
</tr>
<tr>
<td>15</td>
<td>CHAPTER 3: Response of DNA Methylation Regulators to Anoxia and Dehydration</td>
</tr>
<tr>
<td>15</td>
<td>3.1 DNA Methylation</td>
</tr>
<tr>
<td>19</td>
<td>3.2 Objective and Hypothesis</td>
</tr>
<tr>
<td>20</td>
<td>Results</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

5caC 5-carboxylycytosine
5fC 5-formylycytosine
5hmC 5-hydroxymethylcytosine
5mC 5-methylcytosine
ANOVA Analysis of variance
APS Ammonium persulfate
ASH2L ASH2 like histone lysine methyltransferase complex subunit
ATP Adenosine triphosphate
BER Base-excision repair
COMPASS Complex of proteins associated with Set1
DNA Deoxyribonucleic acid
DNMT DNA methyltransferase
EDTA Ethylenediaminetetraacetic acid
G6P Glucose 6-phosphate
GI Gastro-intestinal
H3K36 Histone H3 lysine 36
H3K4 Histone H3 lysine 4
H3K9 Histone H3 lysine 9
H4K20 Histone H4 lysine 20
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF Hypoxia inducible factor
HRP Horse radish peroxidase
kDa Kilodalton
KDM Lysine demethylase
KMT Lysine methyltransferase
MBD Methyl-CpG-binding domain
MeCP2 Methyl-CpG binding protein 2
MLL Mixed-lineage leukemia
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD</td>
<td>Metabolic rate depression</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>NCP</td>
<td>Nuclear core particles</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NSD</td>
<td>Nuclear receptor–binding SET domain</td>
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<tr>
<td>NuRD</td>
<td>Nucleosome remodeling deacetylase</td>
</tr>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PI3K</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly vinyl alcohol</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RBBP5</td>
<td>Retinoblastoma binding protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SET</td>
<td>Su(var)3–9, enhancer of zeste, and trithorax</td>
</tr>
<tr>
<td>SMYD2</td>
<td>SET and MYND domain containing 2</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SUV39H1</td>
<td>Suppressor of variegation 3-9 homolog 1</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TDG</td>
<td>Thymine DNA glycosylase</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′′-Tetramethylethane-1,2-diamine</td>
</tr>
<tr>
<td>TET</td>
<td>Ten-eleven translocation</td>
</tr>
<tr>
<td>UHRF1</td>
<td>Ubiquitin like with PHD and ring finger domains 1</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: Relative Changes in Protein Levels of DNA Methylation Enzymes in Response to 24 h Anoxia and 4 h Recovery as Compared with Controls ........................................ 22

Figure 2: Relative Change in Protein Levels of DNA Methylation Enzymes in Response to 40% Dehydration and 100% (8 h) Recovery as Compared with Controls ...................... 24

Figure 3: Relative Change in Protein Level of Histone Lysine Methylation Enzymes in Response to 24 h Anoxia and 4 h Recovery as Compared with Control ............................ 39

Figure 4: Relative Change in Protein Level of Histone Lysine Methylation Enzymes in Response to 40% Dehydration and 100% (8 h) Recovery as Compared with Controls... 41
LIST OF APPENDICES

Appendix A: Antibody Supplier Information................................................................. 63

Appendix B: Optimized Immunoblotting Conditions.................................................. 64

Appendix C: Loading Control for Immunoblotting..................................................... 65

Appendix D: Representative Immunoblots.................................................................. 67
CHAPTER 1:

General Introduction
1.1 Introduction to the Wood Frog

Adaptation of prolonged whole body freezing has only evolved in a handful of terrestrial-dwelling vertebrate species, including seven species of frog (*Rana sylvatica*, *R. arvalis*, *Pseudacris crucifer*, *P. triseriata*, *P. maculata*, *Hyla versicolor*, *H. chrysoscelis*), two salamanders (*Salamandrella keyserlingi*, *S. schrenckii*), five turtles (*Chrysemys picta*, *Emydoidea blandingii*, *Malaclemys terrapin*, *Terrapene carolina*, *T. ornata*), and one reptile (*Lacerta vivipara*) (Storey & Storey, 2017). Importantly, survival of freezing necessitates well-established cytoprotective mechanisms for managing prolonged cellular exposure to clinically-relevant insults such as dehydration, anoxia, and reactive species. The best-studied of the freeze-tolerant vertebrates is the wood frog (*R. sylvatica*, also known as *Lithobates sylvaticus*), an ideal model organism for understanding molecular response to such freeze-associated stresses. Freeze tolerance has facilitated geographic expansion of the wood frog across a wide range in North America, into regions too cold for many other organisms to survive, including the Arctic Circle. Adults dwell on land in wooded areas but benefit from the ability to mate early in the breeding season, using meltwater pools that accumulate on the forest floor during spring thaw. Over winter, these frogs survive clinical death under modest leaf- and snow-cover, enduring days to months immobilized in extracellular ice, while respiration, circulation, and neural activity are suspended (Layne & Lee, 2011; Storey & Storey, 2017). The frogs can also experience periods of rewarming in response to local weather conditions, which introduces oxidative stresses reminiscent of acute stroke damage, an effect that is most prominent during spring thaw (Elsayed et al., 2020; Joanisse & Storey, 1996; Storey et al., 2021). There are several geographically distinct subtypes of wood frog (Alaska, Ontario, Ohio), with those
living more northerly exhibiting greater magnitudes of resilience than their southern counterparts. Nonetheless, they all share a miraculous ability to emerge unscathed by conditions that tie closely into human pathogenesis, morbidity, and mortality (Storey & Storey, 2017).

1.2 Freeze-Associated Stresses: Anoxia, Dehydration, Reperfusion

The formation of ice in the body cavity is lethal to most species due to physical perforation of organs and cells by ice crystals as well as cell dehydration and shrinkage due to water loss into extracellular ice. Many adaptations that are specific to freeze survival have been thoroughly reported (Storey & Storey, 2017). Briefly, physical perforation is mitigated by production of ice-nucleating and ice-binding proteins that facilitate controlled ice formation in the extracellular spaces, and membrane protectants that are recruited to restabilize and protect the cell surface. Cell shrinkage due to water loss is also counterbalanced by the synthesis and accumulation of high concentrations of glucose inside the cells that provide osmotic resistance to water loss into extracellular ice crystals. However, survival of freezing requires not only preventing direct damage from ice crystals, but also biochemical adaptations to the anoxic and water-deprived conditions within cells that freezing imposes. Freezing induces a substantial loss in intracellular fluid, which is shuttled out through aquaporins to compensate for lost liquid supplies that become frozen in the extracellular space. Severe dehydration, osmotic forces from high intracellular concentrations of solutes, and cell shrinkage must be managed to prevent lysis. In dehydration-exposure experiments, these frogs can endure loss of up to 60% of body water, for around a week outside of the hibernaculum (Churchill & Storey, 1993). In comparison, a loss of as little as 2% of body water by mass in humans can manifest
impairments, with several studies highlighting the association between even moderate dehydration and elevated mortality, and a myriad of cardiovascular, neurological, renal, and gastrointestinal diseases (El-Sharkawy et al., 2015; Riebl & Davy, 2013). Hindering water supply to cells is detrimental not only to integral processes like maintenance of cell volume and integrity, but also shuttling nutrients, signaling molecules, and wastes.

Furthermore, as the blood freezes, and pulmonary and cutaneous breathing slow to a halt, so do oxygen-reliant processes, inducing a state of anoxia. In anoxia-exposure experiments the frogs can endure several days under N₂ gas independently of freezing, and days to months of chronic hypoxia during the natural winter freeze-thaw process (Holden & Storey, 1997). Deprivation of oxygen can have dramatic consequences in non-tolerant mammals like humans, with asphyxiation occurring within minutes (Watanabe & Morita, 1998). Chronic hypoxia is known to contribute to cancer progression, cardiovascular dysregulation, and various other diseases (Chen et al., 2020). Resumption of perfusion and waste removal, and the subsequent reintroduction of high levels of reactive species during recovery also pose several challenges to the cell, as evidenced by ischemia-reperfusion injuries observed in human stroke victims (Orellana-Urzúa et al., 2020). Organs such as the skin, skeletal muscle, stomach, and GI tract, succumb to the effects of stresses like freezing earlier than the kidneys, brain, and heart, and the liver that is the last organ to freeze due to its vital role in protectant production (Storey, 1987; Storey & Storey, 2017). Hence, these organs are studied independently to interrogate potential tissue-specific responses. Of particular interest to the present research is the response of skeletal muscle, which is integral to wood frog mobility, and highly sensitive to stress, commonly implicated in human myodegenerative diseases. Humans and most
mammals lack the adaptive regulatory processes required to prevent uncontrolled protein production and degradation in response to conditions such as inactivity, disease (cachexia), and even natural aging (sarcopenia), which can manifest in death of the muscle cells and loss of the tissue (Evans, 2010). Dehydration in human skeletal muscle can indirectly produce metabolic damage, as aerobic respiration becomes hindered.

As such, there is significant value in understanding how biochemical effectors may be responding to dehydration and anoxia, in valuable tissues such as skeletal muscle, which may provide additional clinical relevance beyond cryobiological considerations, while also potentially identifying some common myoprotective effectors.

1.3 Metabolic Rate Depression and Metabolism

Past research efforts have identified several major multi-stress responsive adaptations required by the wood frog for survival. A major hallmark of exposure to any energetically constrained environment is the use of metabolic rate depression (MRD). During MRD processes unnecessary for survival are selectively downregulated as a means of energy conservation, leaving few integral (housekeeping) processes to be maintained or adaptively upregulated (Storey & Storey, 2004). Sufficient energy provision to any organism is dependent on nutrient intake and subsequent ATP availability, which must meet or exceed ATP use to sustain life of the cell. Cold climates typically mean limited food supply, but this need not worry a frozen organism. Wood frogs feed on arthropods, gastropods, and small invertebrates during the summer months, and store excess fuel in the form of glycogen, adipose tissue, and muscle. Under normoxic conditions, potentially including periods of winter rewarming, it has been proposed that frogs may preferentially (or more efficiently) catabolize lipids and to some
extent proteins, to sustain energetic needs (Kiss et al., 2011; Sinclair et al., 2013); lipid-derived glycerol can be used in gluconeogenesis, and fatty acids in β-oxidation to produce acetyl-CoA, as oxygen is available in the mitochondria to efficiently accept electrons at the terminus of oxidative phosphorylation. However, basal metabolic rate is reduced substantially while frozen, as indicated by a near 90% reduction in CO₂ production using respirometry (Sinclair et al., 2013). Further, in the absence of oxygen and/or extracellular fluids for delivery, aerobic processes become unsustainable, leaving anaerobic catabolism as the primary means of energy production, at a fraction (~5%) of the efficiency. These ischemic conditions limit energy substrate options to cell-derived sugars and amino acids. As a result, wood frogs adaptively employ MRD to suppress aerobic/mitochondrial processes and many other energetically-expensive physiologic processes (breathing, moving, thinking), and cell pathways (cell signaling, immune response, proliferation, ion shuttling) (Storey et al., 2021). The energy that can be produced under hypometabolic conditions is used sparingly for only those processes necessary for survival. One such multi-stress responsive protective process leveraged by the wood frog is that of glucose production, mobilization, and uptake via glycogenolysis and gluconeogenesis. Wood frogs stock up on liver and skeletal muscle glycogen supplies during the warmer months and catabolize hepatic glycogen using glycogenolysis to supply the rest of the body with glucose over the winter (do Amaral et al., 2016; Storey, 1987). Levels of blood glucose in frozen frogs (~257 umol/mL) extend well-beyond those of diabetic humans (~7 umol/mL), but for good reason; a significant proportion of this cryoprotectant is distributed before the blood can freeze, and is taken up by organs/cells during freezing (Storey & Storey, 1986). Freezing leads to anoxia and necessitates the use
of anaerobic glycolysis to produce ATP, as supported by elevated levels of anaerobic metabolites like alanine and lactate (Storey, 1987). Another key benefit of glucose to the wood frog is its role as a cryoprotectant. Glucose serves with other osmolytes like urea to cushion the inward pressures and stabilize the cell interior (Costanzo et al., 1993; Costanzo & Lee, 2005). In skeletal muscle, tight regulation of metabolic enzymes is observed during stress response, with glucose catabolism-associated enzymes like hexokinase (phosphorylates glucose to form G6P) and 6-phosphogluconate dehydrogenase activities being reduced during freezing (Cowan & Storey, 2001). This regulation is even more prominent during thaw, during which many repressed enzymes resume function, sometimes to a greater degree than the pre-freeze period to accommodate necessary repairs (Cowan & Storey, 2001; Sinclair et al., 2013; Storey & Storey, 1986). Some of these changes during thawing can be attributed to stress-responsive transcription factors, such as the hypoxia-inducible factor (HIF), and overall support a reconversion to glycogen of the glucose cryoprotectant and the lactate produced from anaerobic glycolysis while frozen (Storey et al., 2023). While there exists a role for unique and novel proteins, and mutations that may preserve enzyme function under stressed conditions, large fluctuations in metabolic rate and activity necessitate substantial remodeling of the metabolic environment to accommodate, which are largely driven by a myriad of reversible changes (Sinclair et al., 2013; Storey & Storey, 1986). Aspects such as post-translational modification of proteins, activation and repression of stress-responsive transcription factors and miRNA, expression of alternative splice variants, and alterations in other transcriptional and translational regulatory factors govern the proteome during stress-recovery response. While the use of MRD, and
glucose as a protectant are fairly ubiquitous in the body of the wood frog, tissues can also behave differently in response to stress, adding an additional layer of complexity.

1.4 General Objective and Hypothesis

Contemporary efforts have highlighted a potential protective role in processes such as DNA methylation, and histone modifications such as lysine methylation of the histone tails, in response to freeze-recovery and glucose-loading. However, the response of these processes has not yet been examined in wood frog skeletal muscle in response to dehydration-rehydration, or anoxia-reoxygenation, independently of freezing. As such the goal of this present research is to examine potential changes in the levels of several enzyme regulators of both DNA methylation, and histone lysine methylation using immunoblotting. It is expected that enzymes involved in DNA and histone lysine methylation will change in wood frog skeletal muscle, in response to 24 h anoxia/4 h reoxygenation, or 40% dehydration/100% (8 h) rehydration, relative to unexposed controls. Identification of the regulatory elements that govern the integral and adaptive processes that facilitate wood frog freezing survival may hold the key to new doors in human organ/tissue/cell preservation, disease treatment, space travel, and anti-aging technologies.
CHAPTER 2:

Materials and Methods
2.1 Frog Preparation

Male wood frogs (*Rana sylvatica*) weighing 5-7 grams were captured from vernal breeding pools (~5°C) located in wooded areas around southern Ottawa. They were received at Carleton University in snow-insulated boxes, subsequently washed in a tetracycline bath (Dominion Veterinary Laboratories) to remove potential microbial contaminants, and reallocated into ventilated plastic containers lined with damp sphagnum moss. There were approximately 20-25 specimens per container, and in simulation of natural early spring conditions the frogs were held at 5°C without food, for approximately 7-10 days preceding experimentation. Control frogs were sampled directly from this state.

2.2 Anoxia Experimentation

For anoxia experiments, plastic jars (700 mL) were chilled on ice and prepared with a layer of damp paper towel on the bottom that was wetted with distilled water that had been previously bubbled with 100% N₂ gas. Jar lids had two syringe ports; one port was connected to a nitrogen gas line, while the other was left unblocked to vent old air during filling. Nitrogen gas was then flushed through the jars for 20 min. Subsequently, frogs were quickly placed into jars (6-8 specimens per jar), followed by nitrogen gas flushing that was continued for another 30 min. The gas ports were then sealed with parafilm, and the jars with frogs were placed in a 5°C incubator for 24 h. After 24 h, half of the jars were placed on ice, again flushed with nitrogen gas, and the 24 h anoxia exposure frogs were sampled quickly from this condition. Frogs in the remaining N₂ gassed jars were then transferred to new jars (5°C) with ports uncovered, and left to breath normal air for 4 h, followed by sampling as the 4 h recovery frogs.
2.3 Dehydration Experimentation

For dehydration experiments, groups of 5-6 frogs were weighed individually and placed in tall dry buckets lacking lids, which were then set in an incubator (5°C) to initiate gradual desiccation through evaporative water loss. Periodically, frogs were removed and reweighed quickly. Percentage of body water loss was calculated based on the change in mass using the following equation: 

\[
\frac{M_i - M_d}{M_i \times \%H_2O}
\]

where \(M_i\) is the initial mass of an individual, \(M_d\) is the mass at final weighing, and \(\%H_2O\) is the percentage of total body mass that is water in control frogs; this value was previously calculated as 80.8 ± 1.2\% for *R. sylvatica* (Churchill & Storey, 1994). The frogs typically lose ~0.5-1\% of body water per hour under these experimental circumstances. Half of the frogs were sampled when body water loss reached ~40\%. The remaining frogs were placed into buckets containing water, filled to a depth of ~0.5 cm, and left in an incubator (5°C) until fully rehydrated (~8 h). Once the initial mass had been fully recovered, frogs were sampled as the 100\% rehydrated group.

All frogs were euthanized by pithing, and hind leg skeletal muscle was immediately excised (as were other tissues), and frozen in liquid nitrogen, followed by storage at -80°C for subsequent use. Care, experimentation, and euthanasia procedures comply with the Canadian Council on Animal Care guidelines and were approved by the Carleton University Animal Care Committee (protocol #106935). Note that all experimental conditions are naturally survivable by wood frogs.

2.4 Total Protein Isolation
Samples (independent biological replicates of n=5 for control conditions, n=4 for stress and recovery conditions) were quickly weighed and crushed with a mortar and pestle, under liquid nitrogen to ensure tissues remained frozen. Tissue samples were then immediately homogenized 1:2 w/v in ice-cold homogenization buffer: 20 mM HEPES, 200 mM NaCl, 0.1 mM EDTA, with 10 mM NaF, 1 mM Na₃VO₄, and 10 mM β-glycerophosphate, pH 7.4. Additionally, 1 mM phenylmethylsulfonyl fluoride and 1 µL Sigma Protease Inhibitor Cocktail (BioShop, cat. # PIC00.1) were added, prior to homogenization with a Dounce homogenizer, ensuring minimal heat production. Homogenates were then centrifuged at 12,000 rpm for 15 min (4°C) to pellet undesirable cellular debris, and the total soluble proteins contained in the supernatants were collected. Using a Bradford Assay (BioRad, cat. # 5000006), to measure protein concentration in the supernatant, absorbances at 595 nm were read in triplicate by a MR5000 microplate reader. Soluble protein concentrations were then obtained following subtraction of blank values, and were then standardized using a standard curve generated by known concentrations of bovine serum albumin. Using homogenization buffer, sample concentrations were standardized to 5 µg/µL, prior to combination with 2× Laemmli buffer (100 mM Tris base, 4% w/v lauryl sulfate (SDS)), with 20% v/v glycerol, 0.2% w/v bromophenol blue, and 10% v/v 2-mercaptoethanol, at 1:1 v/v. To further encourage denaturation for ideal electrophoretic separation samples were boiled for 10 min, mixed by vortex, and immediately stored at -40°C. Total protein isolates were used for relative quantification of all Western Blotting enzyme targets.

2.5 Western Blotting
Samples were loaded on discontinuous SDS-PAGE gels (8-15%, previously optimized for selected target proteins) for electrophoretic separation and immunoblotting. Stacking gels (5% acrylamide v/v, 1.5 M Tris buffer pH 8.8), and separating gels (8-15% acrylamide v/v, 1 M Tris buffer pH 6.8) were prepared with 0.1 % SDS, 0.1 % APS, and 0.1 % TEMED, and loaded with equal volumes (10-50 μg) previously optimized for the particular target protein. Additionally, 5 μL of PiNK Plus Pre-stained Protein ladder (FroggaBio; Cat. # PM005-0500K) or BLUelf Pre-stained Protein ladder (FroggaBio; Cat. # PM008-0500) were loaded into one well of each gel. After loading, electrophoretic separation was carried out using a BioRad Mini Protean III system (BioRad Laboratories, Hercules, CA, USA), at 180 V for 30-180 min (previously optimized for the target protein), in a Tris-glycine running buffer (25 mM Tris-base, 190 mM glycine, 0.1% w/v SDS, pH 8.5). Proteins were then electroblotted from resolved gels at 160 mA for 50-180 min in a Tris-glycine transfer buffer (25 mM Tris-base, 190 mM glycine, 10% v/v methanol, pH 8.5), onto 0.45μm PVDF membranes. To minimize non-specific binding, membranes were blocked on a rocker with 3-10% skim milk (30 min) or 1 mg/mL PVA (MW 30,000–70,000, 30-60s), in 1× TBST (20 mM Tris-base, 140 mM NaCl, 0.05% Tween-20), then washed in 1× TBST (3× 5 min). Primary antibodies (see Appendix A: Antibody Supplier Information) were diluted (1:1000-1:5000 v/v, previously optimized for each target) with 1× TBST, with 0.02% sodium azide, and added to membranes for probing overnight at 4°C. Membranes were then washed with 1× TBST (3× 5 min), and probed on a rocker (30 min at room temperature) with HRP-linked goat anti-rabbit secondary antibody (cat. # APA002P, BioShop Canada Inc., Burlington, ON, Canada) at 1:5000-8000 v:v in TBST. After final washing in 1× TBST (3× 5 min), membranes were
washed in hydrogen peroxide and luminol 1:1 v/v, and visualized by chemiluminescent detection with a ChemiGenius Bio Imaging System (Syngene, Frederick, MD, USA). Exposed membranes were then stained (0.25% w/v Coomassie brilliant blue, 7.5% v/v acetic acid, 50% methanol), and re-visualized to provide loading standards for normalization. Quantification by densitometry was carried out using ChemiGenius Bio Imaging System with GeneTools Software (Syngene, Frederick, Maryland, USA). Target chemiluminescent protein band densities were standardized using combined intensities from target-independent proteins that were expressed consistently across all conditions, as a loading control.

2.6 Statistics

Data are expressed as mean ± SEM, with n=5 and n=4 independent biological replicates in control and experimental (anoxia-reoxygenation, dehydration-rehydration) conditions, respectively. Significant differences between conditions and data visualization were completed with RBioPlot Statistical Package (Zhang & Storey, 2016), with differences being accepted at p<0.05 using a one-way ANOVA and Tukey’s post-hoc test (α<0.05). A Levene test was used to confirm that the variance in protein levels among control and experimental groups was equal.
CHAPTER 3:
Response of DNA Methylation Regulators to Anoxia and Dehydration
3.1 DNA Methylation

Methylation of cytosine residues in DNA is a well-known mechanism of developmental and homoeostatic regulation within cells, and a pillar of the epigenetics field. This reversible modification is often associated with transcriptional repression, and aberrations in DNA methylation are observed during tumorigenesis. A potential role of DNA methylation in stress-recovery of animals has also been observed, and merits further focus. Methylation is a process by which a methyl group (CH₃) is added to nucleic acids or proteins by methyltransferase enzymes (writers), whereas demethylation involves the removal of a methyl group by demethylase enzymes (erasers). Methylated cytosines in DNA can be recognized by enzymes (readers) that can alter chromatin state and transcription factors, often in an inhibitory manner that reduces the expression of methylated targets. In the context of DNA methylation, methyl groups donated from S-adenosyl methionine (SAM) are covalently attached to the 5’ carbon of cytosine bases, producing 5-methylcytosine (5mC).

There are three primary DNA methyltransferases (DNMTs) responsible for adding methyl groups to cytosines in DNA; these are DNMT1, DNMT3A, and DNMT3B. These all contain a catalytic domain at the C-terminus, and a regulatory domain at the N-terminus. DNMT1 is functionally distinct in that it exhibits greater affinity and catalytic activity at hemi-methylated DNA and, as such, is primarily involved (along with the cofactor UHRF1) in passage of parent methylation marks onto daughter strands during DNA replication, or repairing discrepant methylation sites, earning the designation ‘maintenance’ methyltransferase (Hermann et al., 2004; Mortusewicz et al., 2005; Pradhan et al., 1999). DNMT3A and DNMT3B are often referred to as ‘de novo’
methyltransferases as they lack affinity for hemi-methylated DNA, and are instead responsible for a majority new methylation marks (Okano et al., 1999). Some evidence suggests that the de novo and maintenance methyltransferase activities may not be mutually exclusive to these roles (Feng et al., 2010; Jair et al., 2006). The de novo methyltransferases, possibly to the greatest extent DNMT3A, act in concert with the non-catalytic cofactor, DNMT3L, that has been observed to stimulate their function (Chédin et al., 2002; Hata et al., 2002; Suetake et al., 2004). DNMT3A and DNMT3B also appear to have some functional differences based on flanking region affinity, leading to preferences for major or minor satellite repeats (Gao et al., 2020; Mao et al., 2020; Wienholz et al., 2010). The effect of DNA methylation can manifest as a result of structural changes to DNA that alter transcriptional accessibility, but also via changes in binding affinity of transcription factors. Methyl-CpG domain-binding proteins (MBDs), such as MBD1, MBD2, and MeCP2 can recognize 5mC and associate with histone modifying enzymes to form a condensed chromatin state, suppressing local accessibility (Klose & Bird, 2006). A majority of transcription factors tested that can recognize 5mC are functionally influenced by methylation status, in some cases demonstrating greater affinity for methylated rather than reduced forms (Yin et al., 2017). As such, while DNA methylation is commonly repressive, this is not always the case. The last set of DNA methylation regulators are the demethylases, that facilitate the removal of CH3 from 5mC in either an active or passive manner.

New and unmodified DNA during replication undergoes DNMT1-mediated methylation of the daughter strand, due to recognition of 5mC in the parent strand by the DNMT1-UHRF1 complex. In some cases, demethylation can occur during replication
(passive demethylation), for example by DNMT1 or UHRF1 exclusion from the nucleus, that leaves daughter strands unmethylated, and thus the methylated forms are diluted (Messerschmidt et al., 2014). However, a majority of DNA methylation is due to the effect of thymine DNA glycosylase (TDG), and three methylcytosine dioxygenases called Ten-Eleven Translocation enzymes (TET1-3). These use Fe(II)/2-oxoglutarate in a series of 5mC oxidations, producing primarily 5-hydroxymethylcytosine (5hmC), that can be further reduced to 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Ito et al., 2011). The high specificity of the DNMT1-UHRF1 complex for 5mC, but not for these oxidized intermediates, allows for passive demethylation of daughter strands during replication (Hashimoto et al., 2012). Alternately, active demethylation employs the DNA repair enzyme TDG, which has a wide variety of known roles in cells including excising uracil or thymine from guanine following mispairing (GU, GT), but also glycosylation of many bases and intermediates, interaction with nuclear receptors, and even direct interactions with DNA to alter chromatin structure (Onabote et al., 2022). Active demethylation by TDG involves recognition of 5-fC or 5-caC, that can be excised by TDG, and fixed through base excision repair (Onabote et al., 2022; H. Wu & Zhang, 2014).

DNA methylation most frequently occurs symmetrically at palindromic CpG dinucleotides (CG:GC), however, ‘non-CG’ methylation of cytosines adjacent to other bases in gametic, stem, and brain cells have also been observed (He & Ecker, 2015; Lister et al., 2009; Ramsahoye et al., 2000). CpGs are variably abundant depending on location in the genome, but in developed humans a majority are methylated with the exception of those in loci of high-CpG density (called CpG islands), that are found in ~70% of
promotor regions, mostly at housekeeping or cell-specific genes (Deaton & Bird, 2011; Hodges et al., 2011; Larsen et al., 1992; Saxonov et al., 2006). Whereas this may be the case in humans, the expression and/or activity of many of these genes is adaptively regulated in stress-tolerant organisms, these mechanisms generating many questions for contemporary researchers. Methylation in low-CpG density regions primarily reflects inhibition of transposable elements (intragenomic parasites), that localize away from exons in the genome (Hodges et al., 2011; Larsen et al., 1992).

Overall, research on DNA methylation demonstrates its importance in development, and processes like X-inactivation, and cell determination, but there is also evidence of adaptive regulation of DNA methylation in response to hypometabolic stresses. For example, 5mC levels are notably higher during estivation in sea cucumbers, under anoxia exposure in red-ear slider turtles, during hibernation in thirteen-lined ground squirrels, and even in response to freezing in wood frog skeletal muscle, but not in the more active frog tissues like liver, or in response to glucose-loading independently of freezing (Biggar & Storey, 2014; Wijenayake & Storey, 2016; Zhang et al., 2020; Zhao et al., 2015). Additionally, the means by which DNA methylation is elevated during MRD seems to remain inconsistent across species, stress, and even tissue type, with few studies examining the response of both methyltransferases and DNA demethylases in these contexts.

3.2 Objective and Hypothesis

As such, the goal of the present research was to determine any potential changes in expression of a variety of well-known regulators of DNA methylation, including writers (DNM3A, DNMT3B, DNMT1, and cofactor DNMT3L), readers (MeCP2,
MBD1, and MBD2), and erasers (TDG, TET2, TET3), in response to stresses that lead to hypometabolic responses. For the wood frog model, the experimental conditions investigated were 24 h anoxia and 4 h reoxygenation, and 40% dehydration and 100% (~8 h) rehydration. The target wood frog tissue was skeletal muscle that is highly susceptible to stress and known to show adaptive changes in DNA methylation.

Hypothesis: Proteins involved in the regulation of DNA methylation, which is a known mechanism of controlling the accessibility and expression of genes, will demonstrate differences in expression in response anoxia-reoxygenation, and dehydration-rehydration in *R. sylvatica*.

**Results**

To determine the potential responses of DNA methylation regulators to stress conditions, the relative protein levels of four DNA methyltransferases (DNM3A, DNMT3B, DNMT3L, and DNMT1), three methyl-binding domain containing proteins (MeCP2, MBD1, and MBD2), and three DNA demethylases (TDG, TET2, and TET3) were assessed in wood frog skeletal muscle using immunoblotting. Experimental conditions were (a) 24 h anoxia at 5°C (b) 4 h reoxygenation after anoxia (5°C)(Figure 1), (c) 40% dehydration at 5°C, and (d) 100% (~8 h) rehydration (5°C)(Figure 2), all compared to unexposed controls (5°C).

**3.3 DNA Methylation Enzyme Levels: Anoxia**

Following both 24 h anoxia exposure and 4 h reoxygenation, the protein levels of *de novo* and passive DNA methyltransferases (DNMT3B, DNMT3A, DNMT3L, and DNMT1)(Figure 1) remained unchanged relative to controls, and between conditions.
During recovery, levels of the reader MBD2 were reduced by 32% as compared with anoxia levels, however no other significant changes among the other readers were detected. The eraser TDG was found to be repressed during both anoxia by 26.9%, and recovery by 29%, relative to control levels. Two bands were identified at the expected molecular weight for the DNA demethylase TET3, a smaller (S) and larger (L) form, that were quantified independently. A reduction in only the smaller TET3-S by 52% was observed during recovery, relative to anoxic values.
Figure 1: Relative Changes in Protein Levels of DNA Methylation Enzymes in Response to 24 h Anoxia and 4 h Recovery as Compared with Controls. (A) Histogram of the relative quantities of DNA methylation pathway regulators (writers: DNMT1, DNMT3A, DNMT3B, DNMT3L, readers: MeCP2, MBD1, MBD2, and erasers: TET2, TET3, TDG) in wood frog skeletal muscle (hind leg) extracts, comparing control, 24 h anoxia exposure and 4 h reoxygenation, as detected by immunoblotting. (B) Representative example of immunoblots. Data are mean ± SEM (n=5 independent protein isolations from separate animals for control conditions (lanes 1-5), n=4 for 24 h anoxia (lanes 6-9) and 4 h recovery conditions (lanes 10-13). Data were analyzed using analysis of variance (p<0.05) with a post-hoc Tukey test (α<0.05). Means with a letter in common indicate no statistically significant difference was detected following the Tukey test.
3.4 DNA Methylation Enzyme Levels: Dehydration

In response to 40% dehydration and full rehydration (~8 h at 5°C), compared to unexposed controls (5°C), the relative protein levels of several enzymes were assessed (Figure 2). Of the two DNMT3B bands that were detected around the expected molecular weight, the smaller form exhibited a reduction in expression during recovery by 58%, relative to levels in dehydrated frogs. The demethylase TDG was elevated by 1.46-fold during recovery compared to control levels, whereas TET2 was downregulated by 77% during recovery, as compared to dehydrated levels.
Figure 2: Relative Change in Protein Levels of DNA Methylation Enzymes in Response to 40% Dehydration and 100% (8 h) Recovery as Compared with Controls. (A) Histogram of the relative quantities of DNA methylation pathway regulators (writers: DNMT1, DNMT3A, DNMT3B, DNMT3L, readers: MeCP2, MBD1, MBD2, and erasers: TET2, TET3, TDG) in wood frog skeletal muscle (hind leg) samples, compared to 40% dehydration and 8 h rehydration, as detected by immunoblotting. (B) Representative example of immunoblots. Data are mean ± SEM (n=5 independent protein isolations from separate animals for control conditions (lanes 1-5), n=4 for 100% dehydration (lanes 6-9) and 8 h recovery conditions (lanes 10-13). Data were analyzed using analysis of variance (p<0.05) with a post-hoc Tukey test (α<0.05). Means with a letter in common indicate no statistically significant difference was detected following the Tukey test.
Discussion

Previous research identified an important role of DNA methylation in response to freeze-thaw in tissues such as the brain, liver, and muscle, demonstrating a need for these processes during MRD. Skeletal muscle is highly sensitive to damage and myopathies in non-tolerant organisms under similar conditions. Research also highlights stress- and tissue- distinct behaviour among enzymatic regulators, and the sometimes unclear mechanisms by which the DNA methylation state is altered. These findings are made more complex by differences in other potent mechanisms of epigenetic regulation, such as histone tail modifications and miRNA, which also have distinct roles in stress-recovery of many tissues. Histone lysine methylation is closely linked to DNA methylation to a greater extent than other histone tail modifications such as acetylation and ubiquitylation. Dysregulation of epigenetic methylation events is etiologically involved in pathologies like cancer and, thus, understanding how wood frogs might regulate these effectors during stress may yield important insights. Herein, the relative levels of a variety of well-studied DNA and histone lysine methylation regulators were measured in skeletal muscle using immunoblotting, and compared to unexposed controls (5°C, unfed). The four experimental conditions chosen (40% dehydration-100% rehydration, and 24 h anoxia-4 h reoxygenation) are clinically-relevant, and survivable stresses that wood frogs experience naturally during freeze-thaw.

While anoxia has a direct effect on substrate availability for oxidative processes, metabolic insufficiency during extreme dehydration is a consequence of increased blood viscosity and ischemia. The 40% dehydration condition may be insufficient to fully induce cellular anoxia, as hypoxia biomarkers were not observed until 50-60%
dehydration, but metabolic effects such as MRD of non-core tissues, and glycogenolysis in the liver occur quickly and in a preparatory manner after only 10% water loss (Churchill & Storey, 1993). Tissue-specific glucose distribution alone suggests that survival of 40% dehydration requires activation of processes required to mitigate dehydration-specific stresses such as cell-shrinkage, osmotic pressure, and high concentrations of intracellular solutes, and reestablishing homeostatic gradients during recovery. The adaptive use of glucose as a cryoprotectant is thought to stem from ancestral desiccation survival strategies based on the response of glucose to dehydration, independent of freezing (Holden & Storey, 1997). In contrast, 24 h anoxia- 4 h reoxygenation imposes metabolic constraints and oxidative insults that are distinct from those instigated by 40% dehydration-rehydration (Storey et al., 2021; Wu et al., 2018). In both cases, well-orchestrated regulation of protein expression is required for survival.

The present research supports the notion of a dynamic and complex role for DNA and histone lysine methylation regulators, in response to different stresses in skeletal muscle. DNA and histone lysine methylation enzymes exhibited a differential response to dehydration-rehydration when compared to anoxia-reoxygenation, and previous freeze-thaw models. Although DNA and histone lysine methylation marks were not measured directly, important information can be gathered from the observed changes in expression of associated regulatory enzymes, justifying further research to better-establish their presumptive roles.

3.5 Response of DNA Demethylation Regulators to Stress

TDG
The active DNA demethylase, TDG, was downregulated during 24 h anoxia exposure, an effect that was sustained during 4 h reoxygenation. This response is consistent with the expected increase in methylation under hypometabolic stress, but not with the expected decrease in methylation during recovery. Furthermore, suppression of TDG, was not observed during 40% dehydration exposure, but in contrast was elevated above control levels during recovery from dehydration. It may be that suppression of TDG during anoxia-reoxygenation is important to the preservation of existing cytosine modifications in muscle, even after full (4 h) reoxygenation. Further, the adaptive increase of TDG only during recovery from dehydration supports a potentially important role of this protein in the rehydration response. In the context of freeze recovery in muscle, resumption of complex locomotor activity is one of the last physiologic processes to resume, and thus wood frogs may benefit from maintaining the hypometabolic state or suppression of oxidative processes past four hours in this tissue to ensure perfusion first to core organs (Layne & First, 1991). Wood frogs remain relatively inactive in the hibernaculum for hours-to-days after thawing, before engaging in higher-level movements during spring thaw. Processes like blood perfusion necessitate re-establishment of extracellular fluid levels and osmotic gradients before oxygen can be circulated, which may explain the positive response of TDG levels only to rehydration but not to reoxygenation. In a wood frog freeze-thaw model, 5mC levels were elevated in muscle during freezing, but fell back to control levels during thaw, which coincides a previous report of a reduction in 5hmC during freezing, and restoration to near-control levels during thaw (Zhang et al., 2020). However, these fluctuations could not be explained solely by changes in DNMT levels, as only the maintenance methyltransferase
DNMT1 and the non-catalytic cofactor DNMT3L were elevated during freezing, but not their activity (Zhang et al., 2020). As such, it was previously predicted that a reduction in demethylation regulators may play an important role in freeze-thaw survival. The suppression of TDG under low oxygen conditions supports this hypothesis, however reoxygenation did not appear to stimulate recuperation of TDG levels, which suggests alternate means of activating reoxygenation-specific genes. In contrast, a role for TDG in active demethylation of rehydration-specific genes is plausible, but the regulation of TDG may have a different role beyond active DNA demethylation during rehydration. This is supported by previous observations that implicate formation of a complex with TET1, which has been notably absent in amphibian models, as a necessary cofactor for active demethylase activity (Weber et al., 2016). TDG has many other known functions, and has recently been observed to interact directly with DNA to modify chromatin structure, an effect that is diminished by the presence of 5mC (Deckard & Sczepanski, 2021). This demonstrated ability of TDG could be important for coordinating site-specific activation of rehydration-specific genes that remain to be oxidized by TET enzymes.

Another means by which TDG can affect transcription is through known interactions with various nuclear receptors, often in a stimulatory manner, including those that respond to various hormones such as androgens, estrogen, glucocorticoids, progesterone, peroxisome proliferation factors, and retinoids (Onabote et al., 2022). These interactions could be important to the recovery process. As perfusion resumes, transduction of extracellular hormone signals into the nucleus and subsequent recruitment of transcription factors is needed to reestablish homeostatic balance between the intracellular and extracellular spaces. For example, the production and distribution of
glucose that is observed in response to freezing and dehydration, but not anoxia, is initiated quickly by adrenergic signals from the brain, which engages hepatocyte glycogen catabolism (Storey & Storey, 2017). A similar process may be occurring in muscle in response to rehydration, which may involve TDG-nuclear receptor interactions. Although less-common, TDG may also be involved in preserving genomic integrity during rehydration, due to its role in removing thymine and uracil from mispairs in DNA and RNA, respectively (Wiebauer & Jiricny, 1990). Recovery from stress in wood frogs is characterized by resumption of normal processes and repairing damage, as indicated by changes in proteins involved in the cell cycle, apoptosis, and double-strand break repair (Gerber et al., 2016; Lung & Storey, 2022; Zhang & Storey, 2012). As such, it is plausible that TDG is needed to correct thymine mispairs during this rehydration period when proliferative processes have resumed. Future research should interrogate some known downstream targets of these pathways in wood frogs under anoxia and dehydration stress models to clarify the functional role of TDG during dehydration.

**TETs**

Active DNA demethylation requires first oxidation of 5mC by TET enzymes, in order to generate recognizable 5hmC and subsequent intermediates for removal by TDG and base-excision repair (BER) (Ito et al., 2011). Differential regulation of TET proteins also appeared to occur in a stress- and tissue-specific manner; for example, the smaller isoform of TET3 (TET3-S) was reduced to levels observed for controls during reoxygenation, whereas TET2 was reduced during rehydration. By contrast, prior research on wood frog brain demonstrated an increase 5hmC during recovery from freezing, an effect that was primarily attributed to increases in TET2 and TET3 levels
(Bloskie & Storey, 2022b). This conflicting behaviour of demethylases during stress responses may be a consequence of unique requirements of the brain as compared to muscle, but with similar functional implications. The activity of TET enzymes is dependent on availability of α-ketoglutarate, generated by isocitrate dehydrogenase, which is sustained through stress-recovery in multiple wood frog models, in part due to its role in antioxidation (Mattice et al., 2023). Reducing the expression of specific demethylases may provide a means by which genes unnecessary to survival remain methylated during metabolic rate depression, leaving important effectors susceptible to transcriptional regulation. In another frog model (Xenopus laevis), erasers such as TET1 were also not found, but TET2 and two isoforms of TET3 were observed. The larger isoform of TET3 was noted to contain a DNA binding domain (CXXC) that imparts affinity for unmethylated cytosines, which is thought to contribute an alternate means of transcriptional regulation based on localization at several gene promoters (Xu et al., 2012). This domain is absent in the smaller isoform but still retains demethylase activity. Importantly, TET2-knockout in mice and fish had an inhibitory role on muscle regeneration (Cheng et al., 2023; Wang et al., 2021). Thus, suppression of TET3-S and TET2 appears to highlight a potential means of preserving site-specific methylation patterns during reoxygenation and rehydration, respectively, in a tissue that remains inactive for a longer duration during recovery as compared to core organs. This may repress transcription of distinct genes during reoxygenation and rehydration.

Overall, the profile of demethylation regulators appears to be complex, and is regulated differently by stress in this tissue. Herein, the observed reduction in TDG during anoxia was expected to promote metabolic rate depression, and conserve existing
cytosine marks, or may be adaptively reduced due to its role in other unnecessary and/or energetically demanding processes like cellular repair or hormone signaling. The sustained reduction in TDG during recovery from anoxia highlights a potential means by which resumption of oxidative processes may be delayed in this tissue. The elevation in TDG back to near-control levels during rehydration suggests active demethylation (or some alternative interaction) may be important to the rehydration response. A reduction in TET3-S and TET2 during recovery from anoxia and dehydration, respectively, may represent a means by which existing methylation marks are conditionally preserved at specific loci.

3.6 Response of DNA Methylation Regulators to Stress

MBD2 and DNMT3B-S

Presently, the current results also provide further support for a condition-specific response by other methylation regulators, including the reader MBD2 which was reduced during reoxygenation, and the small form of the writer DNMT3B (DNMT3B-S) which was reduced during rehydration. The repressive function of DNA methylation is in-part due to the methyl-CpG-binding domain containing proteins, such as MBD2, that notably can interact with other chromatin modifying proteins like the nucleosome remodeling and histone deacetylation (NuRD) complex, and directly with methylated and unmethylated DNA, to influence transcription factor function (Wood & Zhou, 2016). However, these interactions appear to be complex and dynamic. It is expected that a reduction in MBD2 may be required for transcriptional activation of reoxygenation-specific genes. Similarly, a reduction in the writer DNMT3B-S during rehydration can be expected to reduce additions of new methylation marks at associated loci, thus promoting the expression of
downstream rehydration-specific genes. Dysregulation of DNMT3B is commonly observed in cancers, an effect that may be due to interactions with important signal transduction molecules that promote dysregulated growth and metastasis (Man et al., 2022; Naghitorabi et al., 2013). Thus, suppression of DNMT3B-S during recovery from rehydration may promote transcription of survival-associated processes, rather than dysregulated proliferation. Some signal transduction pathways that DNMT3B is known to influence have previously been shown to be involved in regulating wood frog stress models, including NF-κB, STAT3, PI3K-Akt, and Notch pathways (Douglas et al., 2022; Gupta & Storey, 2022; So et al., 2020; Zhang & Storey, 2014). However, research also suggests an accessory role of some DNMT3B forms, the suppression of which is expected to achieve a similar purpose (Duymich et al., 2016).

Overall, the protein levels of many regulatory enzymes involved in DNA methylation appeared to behave differently in response to anoxia-reoxygenation, in comparison to dehydration-rehydration, highlighting some important stress-responsive changes that may be contributing to survival of the wood frog under these conditions.
CHAPTER 4:
Response of Histone Lysine Methylation Regulators to Anoxia and Dehydration
**4.1 Histone Lysine Methylation**

DNA undergoes significant structural reorganization to facilitate processes such as replication, repair, and differential expression. During transcriptional repression, euchromatin is condensed into less accessible heterochromatin, wherein nucleosomes organize around core histone protein octamers, forming tight coils. Epigenetic mechanisms like DNA methylation play an important role in this process, but, so does the modification of histone proteins. In particular, methylation of histone lysine residues is closely associated with DNA methylation and has a demonstrated role in animal stress-responses, highlighting a need for further research in the context of other clinically-relevant stresses. There are four types of core histone proteins (H2A, H2B, H3, and H4), and one linker histone protein (H1). These core histones possess a globular carboxyl domain, and a flexible amino domain that is subject to post-translational modification. The post-translational modification of histone tails is largely associated with chromatin rearrangement that alters accessibility of transcriptional effectors to DNA, highlighting an important means of regulating gene expression.

Histone lysine methylation is facilitated by methyltransferases (KMTs) that associate with specific residues of a core histone protein tail, allowing for regulation of accessibility to DNA in a well-controlled manner (Rea et al., 2000). This occurs primarily on H3 and H4 histone tails, the lysine (K) residues of which can be mono-, di-, or tri-methylated (me1, me2, me3) (DeLange et al., 1973). A majority of histone KMTs responsible for these modifications contain a Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain, that can facilitate the removal of a methyl group \((\text{CH}_3)\) from S-adenosyl methionine (SAM), and the covalent addition of up to three methyl groups to a lysine
residue at the ε-nitrogen; forming ε-N-monomethyllysine (Kme1), ε-N-dimethyllysine (Kme2), and ε-N-trimethyllysine (Kme3).

Many KMTs have been identified in a variety of organisms, and associate in a diverse manner with other proteins, forming dynamic complexes that regulate chromatin and nucleosome formation (Li et al., 2023). They can be broadly organized into classes based on residue specificity, and further by the complexes that they form with other proteins (Li et al., 2023; Mohan et al., 2012). Most relevant to the present research are several well-characterized KMTs, that associate with functional lysine residues, including those at which methylation generally induces a transcriptionally-active (H3K4, H3K36) or inactive (H3K9, H4K20) state (Black et al., 2012). For example, the H3K4 methylation is managed by methyltransferases in the mixed lineage leukemia (MLL, or KMT2) family, such as SET1A, that functions in conjunction with binding proteins like Retinoblastoma-Binding Protein 5 (RBBP5) and ASH2-Like Histone Lysine Methyltransferase (ASH2L), to comprise a functional complex of proteins associated with Set1 (COMPASS) (Li et al., 2016; Qu et al., 2018). Another mark associated with transcriptional activation, H3K36 methylation, is regulated by nuclear receptor–binding SET Domain (NSD, or KMT3) family proteins, such as SET2, but also other methyltransferases like SET and MYND Domain Containing 2 (SMYD2). These KDMs associate with other nuclear core particles (NCPs) and exhibit affinity for the nucleosome, at which binding enables catalytic activity of the methyltransferases (Li et al., 2021). A repressive modification, H3K9 methylation, also exhibits diverse interactions with the nucleosome and other binding partners, and is known to be regulated by KMT1-family KMTs, such as suppressor of variegation 3–9 homologue 1 (SUV39H1).
In contrast to other residues discussed, methylation of H4K20 is attributed exclusively to the effect of SET8 (the only known enzyme in the KMT5 family), that functions most effectively at the nucleosome complex to exert a repressive effect (Nishioka et al., 2002). The substrates of several KMTs (such as SMYD2 and SET7) are not always mutually-exclusive and many KMTs have important non-canonical roles in methylation of other non-histone proteins.

The transcriptionally-active or repressive effect of histone lysine methylation manifests in part as a result of detection of these modifications by ‘readers’, that are sensitive to the extent of methylation and residue loci (Black et al., 2012; Zhang & Reinberg, 2001). Similarly, several histone lysine demethylases (KDMs, or ‘erasers’) have also been described, that associate with the removal of methyl groups at specific loci (Black et al., 2012).

Whereas histone tails may be subject to a variety of other modifications, the methylation of histone lysine residues is closely linked with DNA methylation (Rose & Klose, 2014). These important and reversible modifications are commonly implicated in development and cell-type specific processes. There is also support for adaptive regulation in animals as a response to stress. Transcriptionally repressive histone lysine profiles have been observed in hibernating squirrels and anoxic turtles, and most importantly, in wood frog freeze-thaw models (Biggar & Storey, 2014; Morin & Storey, 2006; Wijenayake et al., 2018).

In wood frog brain, repressive H3K9me and H3K27 were reduced during recovery from freezing, effects that were attributed to changes in several KMTs (Bloskie & Storey, 2022a). Freeze-thaw in wood frog liver and muscle elicited a reduction in
transcriptionally-active marks like H3K4me in both tissues. However, other marks responded in a tissue-specific manner such as in liver where an increase in transcriptionally-permissive H3K36me was observed during freeze-thaw, or upregulation in repressive H3K27me during thaw. In addition, skeletal muscle underwent a reduction in H3K27me in response to freeze-thaw (Hawkins & Storey, 2018).

**Objectives and Hypothesis**

As such, the goal of the present research was to examine the relative levels of several representative histone lysine methyltransferases (SET1A, SMYD2, SUV39H1, SET8, and SET7/9) and associated complex proteins (RBBP5, ASH2L), and evaluate their responses to 24 h anoxia and 4 h reoxygenation, or 40% dehydration and 100% (~8 h) rehydration, in wood frog skeletal muscle.

Hypothesis: Proteins involved in the regulation of histone lysine methylation, which is associated with alterations in access to DNA and transcription, will demonstrate differential protein levels following exposure to anoxia-reoxygenation, and dehydration-rehydration in *R. sylvatica*.

**Results**

To determine the potential response of histone lysine methylation regulators, the relative protein levels of five histone lysine methyltransferases (SET1A, SMYD2, SUV39H1, SET8, and SET7/9) and two cofactors (RBBP5 and ASH2L), were assessed in wood frog skeletal muscle using immunoblotting, following exposure to 24 h anoxia and 4 h reoxygenation (5˚C)(Figure 3), 40% dehydration and 100% rehydration (~8 h) rehydration (5˚C)(Figure 4), as compared to unexposed controls (5˚C).
4.2 Histone Lysine Methylation Enzyme Levels: Anoxia

Following exposure to anoxia and reoxygenation (Figure 3), relative to controls, only two histone lysine writers exhibited a change in relative protein expression. The levels of SMYD2 were higher by 1.8-fold during recovery, relative to control levels. Of the two bands detected following probing for ASH2L, the smaller form (ASH2L-S) exhibited a similar increase by 1.6-fold during recovery, relative to control levels.
Figure 3: Relative Change in Protein Level of Histone Lysine Methylation Enzymes in Response to 24 h Anoxia and 4 h Recovery as Compared with Controls. (A) Histogram of the relative quantities of histone lysine methylation pathway regulators (methyltransferases: SET7, SET8, SETD1A, SUV39H1, and SMYD2, associated complex proteins: RBBP5 and ASH2L), in extracts of wood frog skeletal muscle (hind leg) samples, compared to 24 h anoxia and 4 h reoxygenation, as detected by immunoblotting. (B) Representative example of immunoblots. Data are mean ± SEM (n=5 independent protein isolations from separate animals for control conditions (lanes 1-5), n=4 for 24 h anoxia (lanes 6-9) and 4 h recovery conditions (lanes 10-13). Data were analyzed using analysis of variance (p<0.05) with a post-hoc Tukey test (α<0.05). Means with a letter in common indicate no statistically significant difference was detected following the Tukey test.
4.3 Histone Lysine Methylation Enzyme Levels: Dehydration

In response to dehydration and rehydration stresses, the relative levels of four histone lysine regulators appeared to change (Figure 4). Two targets were identified following probing for the methyltransferase SET8, and both small and large forms exhibited elevated levels by 1.6-fold, and 1.5-fold, respectively during recovery, relative to controls. The two forms of the cofactor ASH2L behaved differently under dehydration stress, but similarly during recovery. In response to dehydration, the levels of the small form of ASH2L (ASH2L-S) were indistinguishable from controls, but the large form, ASH2L-L was reduced by 63%. During recovery, both small and large forms of ASH2L were lower by 53% and 64%, respectively, relative to control.
Figure 4: Relative Change in Protein Level of Histone Lysine Methylation Enzymes in Response to 40% Dehydration and 100% (8 h) Recovery as Compared with Controls. (A) Histogram of the relative quantities of histone lysine methylation pathway regulators (methyltransferases: SET7, SET8, SETD1A, SUV39H1, and SMYD2, associated complex proteins: RBBP5 and ASH2L), in extracts of wood frog skeletal muscle (hind leg) samples, compared to 40% dehydration and 8 h recovery, as detected by immunoblotting. (B) Representative example of immunoblots. Data are mean ± SEM (n=5 independent protein isolations from separate animals for control conditions (lanes 1-5), n=4 for 40% dehydration (lanes 6-9) and 8 h recovery conditions (lanes 10-13). Data were analyzed using analysis of variance (p<0.05) with a post-hoc Tukey test (α<0.05). Means with a letter in common indicate no statistically significant difference was detected following the Tukey test.
Discussion: Response of Histone Lysine Methylation Regulators to Stress

Like DNA methylation, histone lysine methylation is an important epigenetic modification that is known to regulate transcription. Prior studies have supported an adaptive role for histone lysine methylation in the animal stress response, providing support for a similar role under these conditions in skeletal muscle of wood frogs. Herein, no changes in histone lysine methylation regulators were observed during anoxia exposure, but during reoxygenation SMYD2 was increased. This KMT is associated with transcriptional activation at marks such as H3K4 and H3K36, but also methylates lysine residues on many non-histone proteins. In the context of histone methylation, the increase in SMYD2 during reoxygenation supports a potential role in activation of reoxygenation-specific genes. In previous research on wood frogs, freezing produced a reduction in SMYD2 in liver, an effect that was sustained during thaw and coincided with reductions in H3K4 methylation (Hawkins & Storey, 2018). These differences may be attributed to the unique requirements of liver, however, there were no observed changes in SMYD2 in muscle during freeze-thaw. Associated marks such as H3K4me were also reduced in response to both conditions, conflicting with the presently suggested behaviour. Freezing induces an anoxic state, and thus it is unknown why reoxygenation independently might stimulate an increase in SMYD2, but not recovery from freezing. One explanation may be that survival processes normally responding to freeze-thaw may alleviate the need to elevate SMYD2, whereas in the absence of thaw-associated transcriptional activation, SMYD2 may be required. In a study of ground squirrel skeletal muscle, SMYD2 was reduced during prolonged torpor, but recovered during arousal, coinciding with an increase in H3K4 and H3K36 methylation (Watts & Storey, 2019). Like anoxia-
reoxygenation in wood frogs, squirrel hibernation does not require effectors to mitigate cold-associated insults (body temperature cools to near ambient while in torpor), highlighting a potential role of SMYD2 in recovery from hypoxia/anoxia in the muscle of wood frogs and ground squirrels. This response may also be absent or delayed in freeze-thaw wood frog models to prioritize recovery from freeze- or dehydration-associated processes and perfusion, which is a prerequisite of oxygen delivery to tissues.

Alternatively, SMYD2-mediated methylation is known to occur on non-histone proteins that are regulated in other wood frog stress models, including p53 and several chaperone proteins (Hawkins & Storey, 2018; Storey & Storey, 2019; Yi et al., 2019). Importantly, methylation increased at p53K370 during freeze-thaw in wood frog muscle, an effect known to be regulated in-part by SMYD2 (Chandramouli et al., 2019). While this potential link justifies further research, the role of SMYD2 in recovery from anoxia in this tissue may be unrelated to its effect on lysine residues of histones, but instead might be important to regulation of non-histone pathways.

The COMPASS complex that works in conjunction with KMTs to methylate marks like H3K4 requires binding partners such as ASH2L. Herein, two forms of ASH2L were detected, the smaller one (ASH2L-S) being elevated during reoxygenation. This supports a potential role of ASH2L-S during recovery from anoxia, possibly via increased methylation at transcriptionally-permissive histone lysine residues. By contrast, the larger form (ASH2L-L) was reduced during dehydration, an effect that was sustained during recovery. These findings for dehydration-rehydration closely align with previous observations in the freeze-thaw wood frog model, wherein ASH2L and associated H3K4 methylation were reduced in response to both conditions (Hawkins & Storey, 2018). This
response, like the adaptive use of glucose as a cryoprotectant, may stem from preexisting ancestral adaptations to desiccation survival present in other amphibians, which are leveraged in the freeze-thaw response of the wood frog.

Similarly, only one change in KDMs was observed during dehydration-rehydration. An increase in both forms of SET8 was observed during rehydration, which suggests an increase in associated methylation at H4K20, an effect that is usually linked to processes such as repair or transcriptional repression and cell cycle regulation (Corvalan & Coller, 2021). However, in the freeze-thaw model, SET8 was reduced in response to freezing, an effect that was sustained during thaw. It is unknown why this response also occurs during rehydration, independent of freezing. Perhaps the downstream effects of SET8 during rehydration may be achieved by an alternative means, independent of H4K20 methylation (as SET8 is the only known enzyme that can methylate H4K20). SET8 also has alternative functions in modifications of other non-histone proteins and pathways, including p53 and Wnt, which could instigate important growth and regenerative processes (Yang et al., 2021). Overall, these changes in histone lysine methylation regulators point towards stress-specific regulation of many effectors.
CHAPTER 5:

General Discussion
5.1 Future Directions

The present study shows a positive role for enzymes that regulate DNA and histone modifications to promote wood frog survival under extreme stress conditions. Some potentially undetected changes may have remained statistically insignificant due to high levels of intraspecies target protein variability, primarily among the control frogs, an effect that appeared to lessen under experimental conditions, warranting further examination. The present data supports further exploration of transcriptional and translational controls that provide stress-specific responses not only to low oxygen and low water conditions, but that can also mediate highly complex stresses such as those that underlie freeze tolerance. Future research should assess direct indicators of methylation, such as 5mC and 5hmC, and methylation levels at specific histone lysine residues. Present attempts to examine such marks using immunoblotting were likely impeded during the genomic DNA and histone extractions stages, which may have failed due to high levels of myofibrillar proteins present in muscle tissues. Beyond detection of global changes, site-specific methylation patterns in DNA may also help determine the functional effect of DNA methylation, that can be loci-dependent. DNA methylated at promoters is commonly repressive, whereas modification of the gene body can have a positive effect on transcription. Additional information could also be gathered through interrogation of post-translational modifications and enzyme activity under these conditions, since factors like oxygen availability and high concentrations of solutes that may influence enzyme function (Schübeler, 2015). Overall, future studies should examine these important biomarkers, and potential alternative targets, to clarify the role of these
methylation regulators. The present research supports the conclusion that these pathways are carefully regulated in expression, and as such justifies further research.

**5.2 Summary of Changes Observed During Anoxia-Reoxygenation**

In summary, several changes in the expression of various regulators of epigenetic methylation events were observed, and were unique to the experimental stresses imposed. These indicate a condition-specific response, most prominently during recovery stages. Under anoxia exposure, a reduction in TDG (the only significant change observed in this condition) is expected to contribute to the hypometabolic state, by reducing associated processes such as active demethylation and DNA repair, or by repressing interactions directly with chromatin, transcription factors, or nuclear receptors. This protein was not previously assessed in muscle of a freeze-thaw model, however, a role for such demethylation regulators is implicated. The sustained reduction in TDG during 4 h reoxygenation may similarly suppress these energetically expensive processes in skeletal muscle to ensure perfusion first to core organs during the recovery process, or to prioritize other myo-protective pathways. The downregulation in TET3-S during reoxygenation supports this notion, as this is expected to preserve existing DNA methylation marks, indicating that alternative means of activating reoxygenation-specific processes is required in this tissue. A reduction in the reader MBD2 could serve this role, given its involvement in recognizing methylated DNA and altering the function of transcription factors at these sites, and as a co-repressor in the chromatin-modifying NuRD complex. Similarly, two regulators of histone lysine methylation (SMYD2 and ASH2L-L) showed adaptive upregulation during reoxygenation; SMYD2 may be important to transcriptional activation during recovery due to its role in permissive
methylation of H3K4, H3K36, or other non-histone survival factors such as P53K370 and HSP-family chaperones. ASH2L-L may have a similar positive effect on transcription, being a member of the COMPASS complex that methylates H3K4 to promote accessibility.

The changes observed during anoxia-reoxygenation in skeletal muscle were different from those noted during dehydration-rehydration exposures, highlighting many condition-specific changes, and a few that appear to respond similarly to freeze-thaw in prior experiments. However, the response of some regulators during dehydration-rehydration seemed to more closely resemble the profiles observed during past freeze-thaw studies of wood frog muscle, as compared to anoxia-reoxygenation.

5.3 Summary of Changes Observed During Dehydration-Rehydration

Only one significant change was detected following exposure to 40% dehydration. A downregulation in ASH2L-L was observed and is expected to lessen transcription during dehydration by reducing COMPASS-mediated H3K4 methylation. During rehydration, ASH2L-L levels remained reduced, and ASH2L-S levels were also downregulated. This effect directly conflicts with the observed upregulation of this protein that was observed during reoxygenation, but aligns closely with previously noted behaviour in a muscle freeze-thaw model, highlighting a dehydration-rehydration specific pathway that is likely leveraged for survival of freeze-thaw. Another response that conflicted directly with observed behaviour during reoxygenation was the upregulation in TDG during 100% (8 h) rehydration. Adaptive upregulation of TDG during rehydration is expected to be important to recovery from freezing and dehydration, but not anoxia, primarily due to its role in active demethylation. While the regulation of these
dehydration-rehydration responsive enzymes appeared to be also involved in the freeze-thaw response, many other changes observed during the rehydration processes appeared to be unique to this condition. For one, TET2 levels were reduced, which is expected to protect existing methylation marks from demethylation at associated loci, since they may be unnecessary to the recovery process (whereas a similar effect is thought to be achieved by TET3-S during reoxygenation). DNMT3B-S levels were reduced during recovery from dehydration, which could reduce new methylation events at associated loci, or act in a more accessory role, to achieve a similar purpose of promoting transcriptional accessibility of necessary genes. Lastly, SET8 was upregulated during rehydration, which could promote repair-associated processes based on the ability of this KMT to methylate H4K20, or by other interactions with cell-cycle and regenerative pathways. However, these findings directly conflict with the observed downregulation in prior freeze-thaw models, which suggests a potentially unique role in rehydration in the absence of thaw-associated recovery effectors.

5.4 Conclusion

Overall, the present research highlights a differential regulation of DNA and histone lysine methylation regulators, based on identified stress-specific differences in responses between anoxia-reoxygenation and dehydration-rehydration models, in wood frog skeletal muscle. These changes are even more apparent when compared to previous research on freeze-thaw models, with some common mechanisms, and several unique alterations in relative expression being noted. Future research could clarify the potential role of some of these regulators in wood frog survival and could potentially provide new
insights that could be applicable to understanding or expanding biomedical efforts that aim to leverage these processes in humans for therapeutic benefit.
REFERENCES


Hodges, E., Molaro, A., Dos Santos, C. O., Thekkat, P., Song, Q., Uren, P. J., Park, J.,


APPENDICES
## Appendix A: Antibody supplier information

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## Appendix B: Optimized Immunoblotting Conditions

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Appendix C: Loading Control for Immunoblotting

The present research employs the use of a previously-published method to standardize the loading of immunoblots by a subset of total proteins in the same lane, that are well-distinguished from the target region of interest, and consistently expressed among conditions (Welinder & Ekblad, 2011). ECL band intensities were standardized by the sum of intensities of multiple distinguished bands, from Coomassie-stained blots, in order to reduce shortcomings noted with traditional methods that use a single housekeeping gene (Bettencourt et al., 2020; Eaton et al., 2013). The present method effectively controls for variations in the sample that may occur during loading or preparation, and the same subset of proteins is used in the normalization of each lane for consistency.
Figure C1: Representative Coomassie-stained blot (above), and corresponding RBBP5-probed ECL (below). Protein loading was standardized using the summed intensity of several Coomassie-stained blot bands from the same lane as the target.
Appendix D: Representative Immunoblots

Figure D1: Representative ECL bands for DNMT3L (dehydration-rehydration), MBD1 (anoxia-reoxygenation), RBBP5 (dehydration-rehydration), and RBBP5 (anoxia-reoxygenation), in wood frog skeletal muscle, as compared to unexposed controls.