Role of glucose-induced transcription factor signalling and mitochondrial epigenetics in stress tolerant wood frog, Rana sylvatica

Gurjit Singh
B.Tech. Hon. Kurukshetra University, India, 2010
M.S. University of Houston, USA, 2013

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Doctor of Philosophy
Department of Biology

Carleton University
Ottawa, Ontario, Canada

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Gurjit Singh
ABSTRACT

The freeze-tolerant wood frogs, *Rana sylvatica* are one of only a few vertebrate species in the animal kingdom which are extensively studied to understand vertebrate freeze tolerance. They undergo whole-body freezing during winter and become ice solid with no heartbeat, brain activity and blood flow but amazingly come back to life during spring, unharmed without any major changes in their body. Freeze survival is challenging, with wood frogs facing ischemia due to freezing of blood, dehydration via cell volume reductions due to loss of 60-70% of total body water into extracellular space as well as hyperglycemia, producing a large amount of glucose as a cryoprotectant. Interestingly, wood frogs can also tolerate these stresses independent of freezing. Also, winter survival by wood frogs is associated with a metabolic reorganization to reduce their energy demands to a bare minimum by globally suppressing energy-expensive pathways and selectively regulating genes to prioritize available energy use for pro-survival pathways. This thesis examined the effects of freezing and dehydration-induced hyperglycemic response in selectively inducing transcription factor MondoA in regulating glucose-induced transcription and activating an adaptive transcriptional response to induce stress response via inflammasome activation, mitochondrial dysfunction and mitochondrial epigenetics. The current findings establish MondoA in guiding an adaptive transcriptional response to activate genes regulating glucose homeostasis and circadian rhythm in a tissue-specific manner in the liver during the freeze/thaw cycle. Also, the role of TXNIP (downstream to MondoA) and its PTMs, in activating inflammasome via NLRP-3 in a stress-specific way
during freezing was shown. Moreover, the higher mitochondrial presence of TXNIP did not correlate to protein expression of its downstream targets in inducing mitochondrial dysfunction in any of the stresses, which were attributed to its low/weak binding to TRX-2. Investigating the role of mitochondrial methylation suggests its tissue-specific regulation in the liver and potential role in maintaining a tight regulation of mitochondrial transcriptional and gene expression response. Altogether, findings from this thesis demonstrate that a highly synchronized and intricate control via multiple levels of regulation is present in activating mechanisms that are involved in maintaining cellular milieu during stress in wood frogs.
PREFACE

I designed and performed all experiments outlined in this thesis, analyzed the data, generated the figures, and wrote all chapters. Dr. Kenneth B. Storey provided funding, materials and reagents, guided this research program, performed editorial review, and final approval of all chapters. Janet M. Storey assisted in animal sampling and provided an editorial review of this thesis.

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This study is published in *Molecular Cellular Biochemistry* journal and reproduce in whole as Chapter 2 of the Ph.D. thesis.


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Chapter 4:

This study is published in *Biochemistry and Cell Biology* journal and reproduced in whole to be used as part of Chapter 4 of the Ph.D. thesis. Additionally, 5mC methylation results have been added to the thesis chapter.

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ACKNOWLEDGEMENT

By far, it was the best decision of my life to choose Storey lab for my graduate work after seeing Dr. Kenneth B. Storey’s talk on “Living dead: Metabolic Arrest and Control of Biological Time” on YouTube. The talk, further with some reading on his work in the field of cryopreservation and freeze-tolerant wood frogs, inspired me to apply as a graduate student in his lab. I am forever thankful to Ken for accepting me as a graduate student in his lab on the condition that “you will not burn the frogs,” as I did with burn mice research in my previous job. It has been a tremendous learning journey working under supervision of Ken, not only in science but also in learning to be a better teacher, an effective leader and deal with different people. One piece of advice which will always stay with me was, “research in science is a blue-collar job; dig deep and keep on working hard, and you will be successful.” I am also thankful for all the moments when you were hard on me; unbeknownst, they were the best moments that ultimately made me more confident and resilient. Also, a big thank you for supporting new ideas and experiments and giving freedom to do something that has not been done before but also at the same time mentioning possible risks and other outcomes.

Another huge thank you to my committee members, Dr. Bruce Mckay and Dr. Steve Brooks, who always gave me invaluable insights, suggested improvements and asked challenging questions on experimental design or research ideas during this time.

Also, a very important part of the journey is Jan Storey. I consider her mother of Storey lab, and besides her scientific contributions to numerous manuscripts in Storey lab, she always listened to problems or issues I had and gave the best piece of advice scientifically and non-scientifically when needed. The selfless hard work and all the time she has spent on all graduate students in honing their writing skills, lab management and organization skills and being an anchor in the lab in making the Storey lab this successful is second to none.

Next, I would like to thank Rasha for being a great teacher, friend, and mentor in this journey and for always being there no matter what for “X” number of scientific
questions asked during her time in Storey lab and beyond as well. Also, thanks to Tighe for being a great support and friend, challenging and questioning the science when creating new methods, and always willing to volunteer for most of the new ideas or methods I generated in this time. Many thanks to Storey lab members (past and present) who have been part of this journey and supported from time to time in answering many questions.

Most importantly, I would like to thank my parents for being super supportive, role models of working hard, never giving up and fighting in the face of hard situations, and being selfless in doing things for others. Without their efforts and sacrifices, I might not have been here today and be the first one to get a Ph.D. in our entire family! Also, to my sister, brother-in-law and kids for always supporting me and being extremely excited about my accomplishments.

Finally, I would like to dedicate this thesis to my wife, Madhu and my daughter, Ajooni. Without the constant effort, sacrifice, hard work and selfless care of Madhu, I don’t think this journey would have been possible at all. Thank you for everything you do, which is beyond my capabilities to put in words. Of course, to my daughter Ajooni who was born during my Ph.D. journey and reminds me to smile every day and move on to do better things in life.
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<tbody>
<tr>
<td>5mC</td>
<td>S-methylcytosine</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Adomet</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>AFP</td>
<td>Antifreeze protein</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ AMP-activated protein kinase</td>
</tr>
<tr>
<td>ARRDC4</td>
<td>Arrestin domain containing 4 protein</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein</td>
</tr>
<tr>
<td>ASK</td>
<td>Apoptotic signalling cascade</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>ATP6/8</td>
<td>ATP Synthase Membrane Subunit 6</td>
</tr>
<tr>
<td>bHLHZip</td>
<td>Basic helix loop helix leucine zipper</td>
</tr>
<tr>
<td>BMAL-1/ARNTL</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono-phosphate</td>
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<tr>
<td>CCGs</td>
<td>Clock controlled genes</td>
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<td>CHoRE</td>
<td>Carbohydrate response element</td>
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<td>Chromosome</td>
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<td>CLOCK</td>
<td>Circadian locomotor output cycles</td>
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<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
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<td>Circadian cryptochrome regulator 1</td>
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<td>Cytc c</td>
<td>Cytochrome c</td>
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<td>DCL</td>
<td>MLX cytoplasmic retention domain</td>
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<td>DMP</td>
<td>Dimethyl pimelimidate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNMT</td>
<td>DNA methyltransferase</td>
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<td>Description</td>
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<tr>
<td>DPI</td>
<td>DNA-protein</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FOXO1</td>
<td>Forkhead Box O1</td>
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<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<td>GP</td>
<td>Glycogen phosphorylase</td>
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<tr>
<td>GPa</td>
<td>Glycogen phosphorylase active from a</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>Hexokinase-2</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>INP</td>
<td>Ice nucleating protein</td>
</tr>
<tr>
<td>ITCH</td>
<td>Itchy E-3 ubiquitin-protein ligase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KLF-10</td>
<td>Kruppel like factor 10</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCR</td>
<td>MondoA conserved region</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte enhancer factor-2</td>
</tr>
<tr>
<td>miR</td>
<td>micro RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<td>MLX</td>
<td>Max like protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>MLXIP</td>
<td>MLX interacting protein</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mol wt.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MRD</td>
<td>Metabolic rate depression</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>ND4</td>
<td>NADH dehydrogenase subunit 4</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLRP</td>
<td>NLR Family Pyrin Domain Containing protein 3</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OGA</td>
<td>O-GlycNAcase</td>
</tr>
<tr>
<td>OGT</td>
<td>O-GlcNAc transferase</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffer saline in 0.05% Tween</td>
</tr>
<tr>
<td>PER-1</td>
<td>Period circadian regulator 1</td>
</tr>
<tr>
<td>PER-2</td>
<td>Period circadian regulator 2</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PFKFB-3</td>
<td>6-phosphofructo-2-kinase</td>
</tr>
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<td>PGM</td>
<td>phosphoglucomutase</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKAc</td>
<td>Protein kinase A subunit c</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroanilide</td>
</tr>
<tr>
<td>P-serine</td>
<td>Phosphoserine</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidiene difluoride</td>
</tr>
<tr>
<td>QSQE</td>
<td>Quaternary structure quality estimation</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for AGE</td>
</tr>
<tr>
<td>RIP2</td>
<td>The Receptor-interacting-serine/threonine- protein kinase 2</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error means</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>SSCT</td>
<td>Saline sodium citrate in 0.1 % Tween</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type-2-diabetes mellitus</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline in 0.0% Tween</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TIEG</td>
<td>Transforming growth factor β-inducible early gene 1</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TRX</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin interacting protein</td>
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</tbody>
</table>
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**Figure 2.1:** Schematic diagram representing the involvement of MondoA and MLX under high glucose (G6P) conditions and the feedback loop of the circadian clock. The MondoA:MLX dimer is present in the outer mitochondrial membrane and senses changes in cellular fuel (G6P) and shuttles to the nucleus to activate downstream targets (TXNIP, ARRDC4, HK-2, PFKFB-3 and KLF10) by binding to CHoRE. TXNIP and ARRDC4 negatively regulate glucose uptake via GLUTs whereas the glycolytic enzyme HK-2 facilitates nuclear entry of the MondoA:MLX complex that also binds to the HK-2 promoter. PFKFB-3 is also expressed under control by MondoA and its product (fructose-2,6-bisphosphate) is a potent regulator of glycolytic versus gluconeogenic flux. Circadian genes control downstream targets common to the MondoA-MLX transcription complex (KLF-10, PFKFB-3). Abbreviations are: GLUT, glucose transporter; G6P, glucose-6-phosphate; CHoRE, carbohydrate response element; TXNIP, thioredoxin interacting protein; ARRDC4, Arrestin family protein domain 4; HK-2, Hexokinase 2; PFKFB-3, 6-phosphofructo-2-kinase isozyme 3; KLF-10, Kruppel-like factor-10. *(Image Created with www.BioRender.com)*

**Figure 2.2:** Relative protein expression of MondoA-MLX and the downstream targets (TXNIP, ARRDC4, HK-2, PFKFB3) in liver samples from control, 24 h frozen and 8 h thawed *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. **Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’**. Representative immunoblots are shown below the histogram.

**Figure 2.3:** Relative protein expression of MondoA-MLX and its downstream targets (TXNIP, ARRDC4, HK-2, PFKFB3) in brain samples from control, 24 h frozen and 8 h thawed *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05
accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. **Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’**. Representative immunoblots are shown below the histogram.

**Figure 2.4**: Effects of 24 h freezing and 8 h thaw on the protein expression of BMAL, CLOCK and other proteins involved in circadian rhythm (CRY1, PER1, PER2, KLF-10) in liver of *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. **Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’**. Representative immunoblots are shown below the histogram.

**Figure 2.5**: Effects of 24 h freezing and 8 h thaw on BMAL-CLOCK and relative amounts of (CRY1, PER1, PER2, KLF-10) in brain of *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. **Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’**. Representative immunoblots are shown below the histogram.

**Figure 2.6**: Relative nuclear distribution of MondoA, MLX, BMAL-1 and CLOCK proteins in liver samples from control, 24 h frozen and 8 h thawed *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. **Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’**. Representative immunoblots are shown below the histogram.

**Figure 2.7**: Relative nuclear distribution of MondoA, MLX, BMAL-1 and CLOCK proteins in brain samples from control, 24 h frozen and 8 h thawed *Rana sylvatica*. Data are
mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.

**Figure 2.8:** DPI-ELISA analysis of the DNA-binding ability of MondoA, MLX, BMAL-1, and CLOCK to the E-box consensus sequence using total protein extracts from liver of control, 24 h frozen and 8 h thawed wood frogs. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.

**Figure 2.9:** DPI-ELISA analysis of the DNA-binding ability of MondoA, MLX, BMAL-1 and CLOCK to the E-box consensus sequence using total protein extracts from brain of control, 24-h frozen and 8-h thawed wood frogs. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.

**Figure 3.1:** A) Schematic representation of TXNIP induced inflammasome activation and mitochondrial dysfunction pathway. Under control conditions, TXNIP is in the nucleus while cell’s NLRP-3 inflammasome is not assembled and antioxidant system via TRX-2-ASK-1 is active in the mitochondria. During freezing/dehydration induced hyperglycemic stress, subcellular localization of the TXNIP into cytoplasm and mitochondria activate inflammasome and mitochondrial dysfunction. Both of these pathways activate a stress response either via caspase-1 or via releasing mitochondrial
cytochrome c in the cytoplasm activating cytokine mediated damage signalling response (Image Created with www.BioRender.com).

B) Schematic representation of NLRP-3 assembly. Sensor protein is NLRP-3 upon recognising damage signal response attaches to ASC and arrange inflammatory caspase (caspase-1) which if cleaved is ready to induce inflammatory damage signaling via activating cytokines.

**Figure 3.2:** Relative protein expression of cytoplasmic TXNIP-NLRP3 induced inflammasome and downstream targets (ASC, caspase-1, IL-Beta) in A) liver samples, B) muscle samples, and C) Kidney samples from control and 24H frozen wood frogs. Representative bands are shown below each Figure. Data are mean ± SEM, n= 4 independent biological replicates. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.

**Figure 3.3:** Relative protein expression of cytoplasmic TXNIP-NLRP3 induced inflammasome and downstream targets (ASC, caspase-1, IL-Beta) in A) liver samples, B) muscle samples, C) Kidney samples from control and 40% dehydrated wood frogs. Representative bands are shown below each figure. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.

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**Figure 3.5:** Relative expression of proteins involved in TXNIP-induced mitochondrial dysfunction and activating targets (ASK-1, TRX-2, Cytoplasmic Cyt c) in A) liver samples, B) muscle samples, and C) kidney samples from control and 24 H frozen wood frogs. Representative bands are shown below each Figure. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.

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Chapter 1: General Introduction
1.1 Stress adaptations in the cold

Much of our planet is cold by our standards, and most animal species are unable to endure exposure to the subzero temperatures of winter at high latitudes or altitudes. In advance of subzero temperature exposure, most organisms seek protection from the frigid conditions, that could cause death [1]. Organisms adopt many different strategies for survival. For example, migration is common for many birds; for example, bar-tailed godwits fly ~7000 miles over the Pacific Ocean from Alaska to New Zealand to exploit favorable food options and avoid frozen lands [2]. Other long-distance migrators include insects such as monarch butterflies, sea mammals like humpback whales, or catadromous fish like American eels [3]. Short distance migrations also occur; for example, garter snakes of Manitoba, Canada, gather in large numbers in underground limestone caves and American toads, *Bufo americanus*, dig ~1 m deep in the earth to avoid the frost line and achieve insulation. Other short-distance migrations include some animals that hibernate underwater (e.g. many frogs and turtles) or spend the winter in an aquatic life stages (e.g. dragonfly nymphs) whereas perennial plants cluster their reserves in underground roots and tubers and sacrifice their leafage [4–6].

Interestingly, selected species have evolved to survive cold and the associated stresses that can occur (food scarcity, whole-body freezing, dehydration, low oxygen) [4]. Numerous animals have developed behavioral, biochemical and physiological adaptations to endure subzero temperatures [7]. These strategies include: anhydrobiosis (cryptobiosis), vitrification, freeze avoidance by supercooling, and freeze tolerance. Anhydrobiosis means “without water,” and the process involves lowering
metabolic rate by almost 100% and losing most water, thereby entering a virtual ametabolic state. This survival adaptation occurs in various small invertebrates such as plant-parasitic nematodes and brine shrimp cysts [8]. Some plant species, such as birch and poplar trees, also undergo vitrification that solidifies water, not into a crystal, but into an amorphous glass that holds all the dissolved solutes [9]. These vitrified cells survive hypotonicity stresses, ionic strength and osmotic changes compared to frozen cells [9].

Finally, ectothermic organisms that survive at subzero temperatures and cannot evade frost come under two categories: freeze avoidance, those that endure low temperatures and can maintain bodily functions (although most also enter dormancy), and freeze tolerance, those that allow ice to form in all extracellular fluid spaces, bringing a halt of organ/tissue functions [1, 5, 7]. The freeze avoidance strategy of cold hardiness includes reducing the inoculative freezing and supercooling points of body fluids below the normal environmental minima for that species. Major components to drive these two processes are Anti-freeze proteins (AFPs). For example, Alaskan willow cone gall fly (Thadbophaga strobiloides) larvae, produces AFPs against ice nucleators and along with concentrations of colligative antifreeze (glycerol, trehalose) can undergo supercooling to -56°C[10]. For example, polar teleost fish produce anti-freeze proteins (AFP) that keep them from freezing in ocean waters that can fall to -1.9°C, as compared with freezing point of their body fluids (about -0.5°C) [11].

Furthermore, during this time, there is a rise in dissolved solutes such as glycerol, which stabilizes the protein structure, and trehalose, which maintains the
integrity of the lipid bilayer, helping these organisms survive in these harsh conditions [11–13]. Lastly, freeze tolerance is an overwintering strategy that has developed mainly in poikilotherms such as insects (commonly members of the Coleoptera, Diptera, Hymenoptera, and Lepidoptera), reptiles (e.g., hatchlings of Chrysemys picta, Emydoidea blandingii and Terrapene carolina), amphibians (examples, Rana sylvatica, Hyla versicolor and Pseudacris crucifer) that can tolerate ice forming in extracellular fluid spaces while maintaining intracellular liquids unfrozen [7, 14].

1.2 Overview of Freeze tolerance in wood frogs, Rana sylvatica

The wood frog, Rana sylvatica (R. sylvatica), is amongst the most freeze-tolerant of vertebrates and is the primary model organism for studying freeze tolerance [7, 13, 15]. They are widely distributed across the boreal and eastern deciduous forests of North America, from the southern Appalachian regions of Georgia, USA, spanning all across Canada and all the way into Alaska [16]. They shelter under an insulating cover of leaf litter during the winter, which eventually becomes covered with snow. As the winter temperature penetrates their insulated leaf hibernacula, these animals enter the survival mode, allowing their bodies to freeze when ambient temperature falls below the equilibrium freezing point of their body fluids (~0.5°C)[7]. As much as 65-70% of their total body water can freeze as extracellular ice. The wood frog’s ability to endure internal ice formation, cryopreserving itself and undergoing a state of metabolic rate depression are well documented [7, 16]. Fully frozen wood frogs experience major physiological changes that include cessation of heartbeat, circulation, breathing, muscle movement and brain activity [7]. These animals survive
winter cold for weeks/months with up to two-thirds of their total body water frozen as extracellular ice. The concentration of solutes rises in this space compared to the intracellular space, which pulls the water out of cells [7]. These physio-chemical changes trigger the liver to produce large amounts of glucose (blood and tissue glucose levels can rise as much as 50-fold) and distribute it throughout the body via the bloodstream to be used as a cryoprotective agent [15]. Hence, wood frogs must deal with freezing and its associated consequences, which include a) potential ice mediated physical damage to tissues, organs, or blood vessels, b) cellular dehydration due to water drawn out of the cells into the extra organ or extracellular ice masses, c) hyperglycemia due to huge amounts of glucose entering cells to act as a cryoprotectant, d) ischemia due to freezing of blood plasma which stops blood circulation and anoxia caused by the cessation of breathing and heartbeat [4]. Under these conditions, all cells are challenged in using their endogenous carbohydrate fuel reserves by modulating their glycolytic capacity to produce ATP by anaerobic pathways. So ultimately, freezing induced damage falls under two categories: physical damage to cells/tissue caused by ice formation and as well as changes in energy metabolism that can be caused by freezing and its consequence (example, ischemia, hyperglycemia, cellular dehydration, osmotic/ionic, oxidative stress) [17]. Interestingly, wood frogs can endure these multifactorial consequences of freezing individually as well, such as enduring dehydration or anoxia stress without freezing, which ultimately demarks the differences between freezing and its associated consequences and can depict a complete picture of vertebrate freeze tolerance.
Thawing, which is considered a recovery mechanism from freezing stress in wood frogs during spring, also brings many challenges such as ensuring resumption of breathing and heartbeat. One of the major challenges is the rapid production of reactive oxygen species (ROS) that can damage proteins and trigger pathways such as apoptosis [18]. The primary site for ROS production is the mitochondria (superoxide produced from complex I and complex III), which face significant effects of oxygen limitation during freezing, with a complete shutdown of oxygen-dependent ATP production [19]. However, during thawing with the reintroduction of oxygen, a general ROS stress response can be induced in mitochondria. Usually, a heightened antioxidant response in wood frogs both during freezing and especially during thawing (in anticipation of ROS insults) appears to form a system to prevent most cell damage due to ROS [20, 21].

### 1.3 Major consequences and adaptations to Freeze-thaw tolerance

*Ice Management and Ice nucleation*

Wood frogs are forest-dwelling animals that breed and live near ephemeral pools such as freshwater swamps, cool moist ravines or ditches, freshwater wetlands and hollows on the forest floor where water accumulates from melting snow. During winter, they can neither dig deep underground (as toads can) nor move into deep waters (like leopard frogs or bullfrogs) and ultimately are unable to access frost-free hibernacula. Therefore, during winter, they hide below the insulating cover of leaf litter and snow on the forest floor, where the temperature of their hibernacula generally
does not surpass -7°C even in extreme conditions [22]. They freeze below the equilibrium freezing point (-0.5°C) of their body fluids and allow ice crystals to propagate from the skin’s surface to inside the body to initiate ice nucleation. This is the first important step that initiates freezing in wood frogs and not the low temperature of their hibernaculum. Ice nucleation is triggered by the action of ice-nucleating bacteria on the skin or gut as well as ice-nucleating proteins (INPs) found in wood frog plasma [4, 23]. Within the next 5 minutes, as the ice starts to build up in extracellular compartments, the concentration of solutes rises in this space compared to intracellular space, which pulls water out of the cells [7]. This step helps circumvent an immediate ice surge that occurs when freezing begins from a deeply supercooled state. For example, it takes nearly 12h or more for wood frogs to reach maximum ice content during freezing, providing substantial time for wood frogs to convert liver glycogen reserves into glucose and distribute the sugar throughout the whole body via the blood to be used as a cryoprotective agent [5, 17]. A study done on wood frogs using proton magnetic resonance imaging (MRI) in real-time also showed that during freezing, the movement of ice is from the exterior to inward and requires many hours to complete. Core organs such as the liver, that produces the cryoprotectant, and heart that distributes the glucose to other organs via the blood were the last to freeze [24].

Unlike freezing, in which ice formation initiates from the extremities and moves inward through the frog’s body, thawing occurs more uniformly across the frog’s body [24]. Although, the heart and liver were the last organs to freeze, they are the first to thaw because of their very high glucose concentration [24]. The time of recovery
during experimental thawing was directly proportional to the temperature and duration length of freezing: the greater the length of freezing, the more time it takes to thaw [21,22]. Excess glucose is converted back into glycogen within several days after thawing and can be used again for subsequent freezing cycles in the future [27].

Cryoprotectant synthesis, glycogenolysis and glucose transport

As mentioned above, the liver is one of the major storage organs for glycogen, and its mass accounts for nearly 10% of the total wood frog’s body. During freezing, wood frogs lose roughly half of their glycogen reserves to produce cryoprotectant glucose, which is evident from the increase in the activities of glycogen phosphorylase and glucose-6-phosphatase, two key enzymes of glucose synthesis[28]. Upon ice nucleation at the skin, β-adrenergic receptors stimulate production of enormous amount of glucose from the liver, superseding the normally tight hormonal and homeostatic control, and showing that wood frogs have developed molecular mechanisms to reduce the damage to subcellular components by avoiding non-enzymatic glycation which is the leading cause of diabetes[7, 29–33]. This large amount of glucose produced from the liver acts as a cryoprotectant and provides colligative resistance to cellular dehydration, thereby retaining a minimum cell volume. Due to this biochemical intervention and higher concentration of glucose, freezing is delayed, and the freezing point is further lowered in the core organs of the frog, thereby allowing more time for the glucose to be distributed [4]. For example, during the initiation of freezing, the heart functions by doubling the heart beats to 8.0 beats/min within one minute of the onset of freezing but slows down after about an hour, still
long enough to distribute glucose throughout the body [34]. The average values of glucose levels after freezing exposure have shown to be 388, 198, 121 and 27 µmol/g wet weight in liver, heart, kidney, and leg muscle, respectively, suggesting tissue-specific cryoprotectant content [15].

Interestingly, as mentioned above, thawing occurs at a much faster rate compared to freezing. Still, short thawing periods do not result in a drastic decline in glucose levels, as shown in a study [34]. Usually, the levels of cryoprotectant remain high even after a week of the initial thawing episode, possibly in anticipation of another freeze-thaw cycle for which enhanced cryoprotectant levels might aid in greater survival of wood frogs [15].

The conversion of glycogen to glucose happens in straight pathway with consumption involving three enzymes: glycogen phosphorylase (GP), phosphoglucomutase (PGM) and glucose-6-phosphatase (G6Pase). The β-adrenergic signals sustained over several hours during freezing activate cAMP synthesis, which triggers the release of inactive protein kinase A (PKA) tetramer. PKA further releases a catalytic unit, PKAc, which phosphorylates and activates glycogen phosphorylase kinase to activate GP and its active form GPa to stimulate glucose-1-phosphate production. Under the action of PGM, glucose-1-phosphate is converted to glucose-6-phosphate (G6P) and is transferred to the endoplasmic reticulum. Further, levels of fructose-1,6-bisphosphate did not increase despite an increase in levels of fructose-6-phosphate (F-6-P)[35]. This indicates that the glycolysis is blocked at the level of phosphofructokinase (PFK) during freezing to facilitate glycogenolysis. Thus,
glycogenolysis is a supereminent pathway under freezing conditions in wood frogs [7, 36]. Ultimately, the glucose generated from this pathway is released at the plasma membrane and transported by glucose transporters (GLUT2) that can both export glucose into other organs via blood during freezing and take up glucose again during thawing.

Overall, the use of carbohydrates such as glucose as a source of cryoprotectant and fuel has many advantages under freezing conditions, such as i) glucose can be easily synthesized from glycogen without ATP consumption, ii) it is easy to transport glucose in and out of cells via GLUT transporters via liver iii) glucose can also be converted back to glycogen and stored in the liver when it’s not needed (during late thawing) and iv) it can stabilize biomolecules such as proteins and does not affect their enzymatic activities or function [1]. However, the presence of a large amount of glucose during both freezing and dehydration can amplify the effects of oxidative stress and mitochondrial dysfunction by forming advanced glycation end products [37, 38]. As such, wood frogs must have a line of defense to mitigate the formation of these products to prevent hyperglycemia-induced damage during freezing and dehydration.

Energy production

Since freezing halts breathing, heartbeat and blood flow, oxygen delivery to tissues is halted in frozen frogs and hypoxic and then anoxic conditions soon occur. Surviving anoxia requires several adaptations, such as accessibility to large quantities of fermentable fuels, adjusting ATP utilization downwards to match the reduced
capacity for anaerobic ATP production, and neutralizing anaerobic end products [36, 39]. Under these consequences of freezing conditions, the mitochondrial electron transport chain is reduced, and cells are forced to switch from aerobic to anaerobic metabolism to generate energy. This is evident from a study that found that lactate and alanine rise during freezing, confirming that wood frogs use anaerobic glycolysis to generate ATP under freezing conditions [40]. Also, under freezing stress, accumulation of ice outside of organs leads to cell/organ dehydration that could give rise to insufficient ATP synthesis, increased intracellular osmolality and generation of ROS (although more active during thawing). Under these anaerobic conditions, the wood frog can produce only 2 moles of ATP from the catabolism of glucose compared with aerobic metabolism, where 36-38 moles of ATP are produced per glucose utilizes [41]. This imbalance between energy production and consumption is huge, so wood frogs must reprioritize their energy demands since they won't be able to fulfill all normal cellular functions. Wood frogs do this by coordinated physiological and biochemical responses that allow them to use this finite amount of energy for pro-survival mechanisms while suppressing the energy-expensive processes that not required for survival, a process called metabolic rate depression [7].

*Metabolic rate depression is a complex network of events at transcriptional, post-transcriptional, translational and post-translational levels*

Metabolic rate depression (MRD) involves lowering the metabolic rate to a fraction of the resting metabolic rate and entering a state of hypometabolism or dormancy [42]. As mentioned above, under freezing conditions, wood frogs have an extraordinary
ability and escape mechanism to undergo MRD and promote facultative anaerobic metabolism[43]. MRD is facilitated by global suppression of energy-expensive pathways such as transcription, translation and by prioritizing available energy to selective pro-survival pathways (e.g. anti-apoptosis, antioxidant response, chaperones), as well as shutting down cell-damaging pathways (e.g. apoptosis) and energy-expensive pathways (e.g. cell cycle regulation, cell division and growth)[21, 44–47]. Ultimately, entry into and exit from MRD is a coordinated and complex response which works in synchrony at multiple levels of regulation to enable wood frogs to survive, switch “ON” stress adaptive processes, and abate the effects of detrimental and non-essential processes.

At the transcriptional level, epigenetic markers can alter the availability of DNA to the transcriptional machinery such as by inhibitory controls on transcription factors and/or epigenetic modifications to DNA in order to prevent transcription [33, 48]. For example, the role of DNA methylation and its associated proteins. Indeed, DNA methyltransferases (DNMTs) were found to be upregulated in both in nuclear and mitochondrial liver samples during freezing [33, 48]. These studies suggested a role of methylation levels in tightly regulating selective transcription, aligning with the liver’s metabolic role during freezing [33, 48]. Other epigenetic mechanisms include post-translational modifications of the histone proteins that surround DNA, including acetylation or methylation of histone lysine residues. For example, frozen wood frogs showed reduction in mono-methylation of lysine 4 and lysine 27 on histone H3 protein (H3K4me1, H3K27me1) in skeletal muscle during freezing [49]. Interestingly, both
H3K4me1 and H3K27me1 marks are involved with active gene transcription and euchromatin and downregulation of these can be crucial to freezing survival under a state of MRD. The same study showed downregulation of H3K4me1 but higher levels of H3K27me1 histone methylation mark in freezing liver. This suggests a role of differentially regulated histone methylation that might aid in selectively downregulating certain non-essential pathways while simultaneously maintaining others that are crucial, as in case of the liver which is metabolically a more active tissue compared to skeletal muscle during freezing [49].

Interestingly, this selective transcription regulation can induce the levels of essential transcription factors (TFs) that are fine tuners or “master regulators” of major cell processes or highly selective controllers of specific genes [50]. Their binding to specific DNA motifs (consensus sequences) in the nucleus can influence the expression of genes. For example, the TF MondoA was activated with greater nuclear localization and DNA binding activity under freezing-induced hyperglycemic conditions in wood frog liver, activating downstream genes that are involved in maintaining glucose homeostasis during freezing stress [51]. Another example of transcription factor regulation is myocyte enhancer factor-2 (MEF2) that was shown to regulate multiple genes involved in glucose transport and phosphagen homeostasis under anoxia stress in the wood frogs [52]. The same study also linked an increase in MEF2 nuclear localization to an increase in transcripts of Glucose transporter 4 (glut 4) relating the role of transcription factor activation to regulating transcription of essential downstream targets during anoxia [52]. Lastly, studies have shown the role of an
oxygen-sensitive TF, NF-κB, during freezing and thawing stress in wood frogs and mitigating the effects of ischemia-reperfusion-induced oxidative stress by enhancing downstream antioxidant defences [20]. Under inactive or control conditions, NF-κB is bound by its inhibitor protein (ΙκB) that inhibits its activation and translocation to the nucleus[53]. Upon sensing oxidative stress, ΙκB dissociates and is degraded, facilitating NF-κB entry into the nucleus to activate downstream antioxidant proteins such as manganese superoxide dismutase (MnSOD), an enzyme found in mitochondria that plays a primary role in detoxification of superoxide radicals [54]. Interestingly in the above study, the protein levels of both p50 and p65 subunits of NF-κB were upregulated in liver and skeletal muscle of freezing wood frogs which was in conjunction with MnSOD levels in both the tissues. These results suggested that MnSOD, under the NF-κB axis, could enhance protection from superoxide radicals generated from shrunken cells during freezing [20].

At the root of many of these regulations and adaptations are changes at gene transcript levels which have been shown in multiple studies to induce responses to freezing and its associated stress [17, 36]. For example, screening of a cDNA library of liver from frozen wood frogs showed an upregulation of multiple genes: α and β subunits of fibrinogen, mitochondrial ADP/ATP translocase, and three novel genes (fr10, fr47 and li16) [55–59]. Both Li16 and FR10 were subsequently linked to their potential roles in stabilizing the plasma membrane when water exits cells into extracellular ice crystals. A separate study also showed the role of FR10 in inhibiting ice crystallization in extracellular spaces suggesting a possible role of this protein in
minimizing ice damage to the organs and capillaries [60]. A study focused on freeze responsive genes in mitochondria showed upregulation of ATP synthase membrane subunit 6/8 (ATP6/8), NADH-ubiquinone oxidoreductase subunit 4 (ND4), and 16S RNA during 24 h freezing in both liver and brain of wood frogs. Interestingly, the same study showed a differential response of these genes in component stresses of freezing (anoxia and dehydration), with overall upregulation during anoxia but not during dehydration, thereby linking the role of oxygen levels and mitochondrial gene responses in regulating cellular energetics [61].

At the post-transcriptional level, microRNA (miRNA) has been shown to regulate many cellular functions and control the availability and levels of gene transcripts in many species under both normal and stressed conditions. MiRNAs are short ~22nt long noncoding RNA molecules that have the ability to regulate the fate of their gene transcript targets within cells [62]. They bind with either partial or full complementarity to specific mRNA targets resulting in either translational inhibition or degradation of the mRNA [63]. For example, one study showed tissue-specific regulation of miR-16 in the liver during freezing stress of wood frogs, relating this with translational suppression of downstream targets [64]. The study also showed elevated levels of miR-21 in the liver and muscle during freezing[64], miR-21 being known to explicitly target mRNAs for inflammatory caspases such as caspase-3 and promote anti-apoptotic factors such as apoptotic protease activating factor-1 (apaf-1) during freezing episodes [64]. A study of brains from wood frogs showed an upregulation of miR-451 that can promote protection from anoxia/reperfusion injury whereas reduced
levels of miR-181a-3p were found in the same study during thawing at 5°C and were linked to brain recovery from ischemic conditions via restricting apoptosis [65].

During freezing, the ribosomal machinery is also strictly regulated in wood frogs. For example, a study of frozen wood frogs analyzed AMP-activated protein kinase (AMPK) responses and showed that the kinase activated the translation factor eukaryotic elongation factor 2 kinase (eEF-2K) that in turn led to phosphorylation of eukaryotic elongation factor 2 (eEF-2) to halt protein synthesis [66]. At post-translational levels, reversible modifications that include multiple forms of covalent modifications such as phosphorylation, glutarylation, ubiquitination, methylation, acetylation, SUMOylation and GlcNAcylation are involved in supporting stable modifications of enzymes and proteins and ultimately regulating MRD [67]. For example, carbamoyl phosphate synthetase-1 (CPS-1), an important regulatory enzyme of the urea cycle, is modified post-translationally via lysine glutarylation to regulate the activity of the urea cycle in response to freezing stress [68]. A phosphoproteome study showed differential phosphorylation of key enzymes involved in glycolysis and glycogenolysis in the liver of frozen wood frogs with upregulation of phosphopeptides belonging to GP and PFK2. These have roles in controlling glycogenolysis and place a metabolic block at PFK that inhibits glycolysis and facilitates glucose synthesis during freezing[69]. Another study found that glutamate dehydrogenase (GDH), that regulates amino acid catabolism by the Krebs cycle, has a lower degree of acetylation and ADP-ribosylation under frozen conditions as compared to control wood frogs. Another unique PTM, O-GlcNAcylation, that transfers β-D-N acetylglucosamine to serine and
threonine residue on a protein, has been shown to be involved in ischemia-reperfusion injury, diabetes and neurodegenerative diseases [70]. Hence, overall, reversible protein modifications play a crucial role in regulating MRD by maintaining the activity, stability and expression of various proteins.

As depicted in various studies discussed above, it is clear that an intricate control of metabolic organization is required for wood frogs to survive in a frozen state over the winter by strategically synchronizing multiple levels of regulation to maintain cell integrity, activate pro-survival pathways and mitigate potential cell damaging pathways.

1.4 Freezing and dehydration: biochemical and physiological similarities and differences

The major physiological and biochemical responses are common to both freezing and extensive water loss (dehydration) by wood frogs. In general, amphibians have a high tolerance for variation in body water content due to their highly water-permeable skin. Hence, it is not surprising that amphibians have a high tolerance for dehydration due to evaporative water loss from their bodies, immersion in saline water, or freezing (that removes water from cells and into extracellular ice crystals). For example, similar to freezing, dehydrated frogs (controlled dehydration at 5°C) can endure body water loss of ~50% and respond by stimulating glycogenolysis in liver to synthesize glucose as an osmolyte and export it via blood to other organs [71]. This was also evident from the increase in activity of glycogen phosphorylase a in the liver, along with total phosphorylase activity during dehydration stress. Also, glycolytic
intermediates (G-6-P and F-6-P) rose sharply in dehydrated liver with no change in fructose-1,6 bisphosphate suggesting an inhibitory block on glycolysis at PFK reaction step to promote glycogenolysis and glucose accumulation/export [72]. Cellular ATP levels are also affected when dehydration levels are greater than 25%, and at higher dehydration (50 to 60%) levels, liver and other tissues become hypoxic with the accumulation of lactate [72]. This is because water loss from the blood impairs blood flow and slows delivery to tissues. There is also an increase in osmolality and ionic strength in the cytoplasm and a decrease in cell volume which can cause structural changes to organelles and plasma membranes [71]. Indeed, the commonality between freezing and dehydration responses suggests that natural freeze tolerance adaptations probably developed from pre-existing mechanisms to combat water stress. Yet, there are certain marked differences between the two stresses, making them even more interesting to study separately. For example, although both freezing and dehydration have consequences of total body water loss, the rate of water loss is greater during dehydration, where most of the water is lost to the environment whereas during freezing, water is mainly maintained as ice in extracellular spaces. Also, compared to freezing, wood frogs can undergo dehydration stress many times but can only last for about 7-8 days in dry conditions[71]. Another important adaptation in dehydrating wood frogs is the accumulation of urea, and this observation has also been reported in freeze-tolerant frogs but generally in much lower amounts. Overall, urea can play a role as an osmoprotectant and can mitigate cell volume reductions during dehydration stress [73].
1.5 Objectives and hypothesis

Various studies have established that an intricate control of metabolic organization is required for wood frogs to survive in a frozen state over the winter. The underlying principles of these molecular mechanisms of natural freeze tolerance have applications in medical research in improving cell preservation techniques to benefit the field of organ transplantation and preservation. Moreover, a deep understanding of the role of heightened hyperglycemic stress, oxidative stress response, and pro-survival adaptations under a state of MRD could well serve the fields of diabetes and cancer research. In order to comprehend the role of freezing and its associated stresses in inducing hyperglycemic and oxidative stress responses, this thesis focused on:

1. Understanding the role of glucose-induced transcriptional (MondoA:MLX) and allied networks in maintaining glucose homeostasis.
2. Role of downstream proteins (TXNIP) in activating signal transduction pathways (inflammation and mitochondrial dysfunction)
3. Analysis of the role of mitochondrial DNA methylation and links to stress response pathways.

General hypothesis: Glucose-induced transcription factors and downstream gene responses regulate circadian rhythm, inflammation, mitochondrial dysfunction and epigenetics under environmental stresses (freezing and dehydration) in freeze tolerant wood frogs.
**Objective 1:** Determine the role of the transcription factor MondoA:MLX complex in regulating glucose-dependent gene expression and assess links to circadian rhythm.

**Hypothesis:** MondoA:MLX regulates glucose-induced transcription under hyperglycemic conditions of freezing and links with circadian rhythm proteins to regulate glucose homeostasis (Chapter 2)

Previous studies of wood frogs have shown that even under global transcriptional suppression, selective gene expression continues and allows wood frogs to express specific genes/proteins in response to freezing or its associated stresses. The fine-tuning of gene expression is partly regulated by transcription factors that facilitate the activation of these pro-survival pathways. Glucose metabolism is partly regulated by transcription factors that can act as a “switch” to selectively turn on and off the relevant genes. The regulation that underlies expression of genes/proteins of glucose metabolism affected by these transcriptional changes are not completely understood. Therefore, in this study, I predicted that MondoA (also known as MLX interacting protein), a master regulator of glucose-responsive transcription, along with its binding partner MLX (Max-like protein X), would be activated and would guide an adaptive transcriptional response under changing glucose levels (control vs freezing conditions). Also, disruption of the molecular clock (circadian rhythm) can affect the expression of genes involved in glucose metabolism. Both Kruppel-like factor 10 (KLF-10) and 6-phosphofructo-2-kinase (PFKFB3) are downstream targets of the MondoA:MLX transcriptional network and are also regulated by circadian rhythm. Therefore, I further hypothesized a role of circadian rhythm proteins in regulating levels of these
downstream targets. Ultimately, assessing the link between MondoA:MLX and circadian rhythm can give insights into how wood frogs regulate MondoA:MLX induced glucose metabolism in a circadian-dependent manner under freezing stress.

**Objective 2:** Analyze the role of TXNIP subcellular localization in regulating inflammation and mitochondrial dysfunction during freezing and dehydration stress.

**Hypothesis:** Under cellular stress, TXNIP shuttling to the cytoplasm and mitochondria activates inflammation and mitochondrial dysfunction. The binding of TXNIP to different proteins and post-translational modification enhances its role in activating these pathways. (Chapter 3)

Thioredoxin interacting protein (TXNIP) is a downstream target of the MondoA:MLX complex and a negative regulator of glucose uptake. TXNIP also inhibits antioxidant function by binding to thioredoxin-2 (TRX-2), which is a major regulator of cellular redox signaling and protects cells from oxidative stress in the mitochondria apart from its role as a regulator of glucose metabolism [74]. Overexpression of TXNIP has also been linked to its binding and activating of glucose-induced inflammation in various mammalian disease models [75]. To date, there have been no studies analyzing the role of mitochondrial dysfunction and reporting physiological evidence of tissue inflammation in wood frogs under environmental stress (freezing, dehydration, hyperglycemia). Hence, I hypothesized that TXNIP would play a crucial role in regulating inflammation and mitochondrial dysfunction by interacting to their core proteins, NLR family pyrin domain containing 3 (NLRP-3) and TRX-2, respectively, to
guide the expression of the downstream genes. Also, subcellular localization and post-translational modification drives TXNIP action during stress, so I proposed a role for these to be active in promoting TXNIP action during freezing and dehydration stress. This study will ultimately help us elucidate the role of TXNIP, its location, and whether it is involved in mediating diverse stress responses.

**Objective 3:** Evaluate the role of mitochondrial methylation and link with stress pathways activated in response to freezing and dehydration.

**Hypothesis:** Under low mitochondrial energetics and selective gene transcription, mitochondrial methylation is upregulated to diminish the stress response under freezing and dehydration conditions (Chapter 4).

As described above, freezing and dehydration induced hyperglycemic stress can induce TXNIP and its downstream signalling pathways, including activating proteins involved in mitochondrial dysfunction, which induce stress on mitochondrial machinery. Although low mitochondrial energetics and selective gene transcription have been observed in wood frogs under freezing and its consequences. But, also under freezing/dehydration induced hyperglycemic stress, a heightened ROS production can lead to damage to the cellular components, including mitochondrial DNA (mtDNA), which does not have a robust DNA repair mechanism compared to that of nuclear DNA. DNA methylation machinery not only methylates the fifth carbon of cytosine (5mC) on DNA to arrest transcription, but its regulatory proteins, DNA methyltransferases (DNMTs), can also aid in selective gene expression and combating
ROS induced oxidative stress. Therefore, for this study, I predicted a role of mitochondrial DNMTs, and higher 5mC levels in maintaining a higher methylation state to protect mitochondrial machinery during stress.
1.6 References


Chapter 2: MondoA:MLX complex regulation and links to circadian rhythm
MondoA:MLX complex regulates glucose-dependent gene expression and links to circadian rhythm in liver and brain of the freeze-tolerant wood frog, *Rana sylvatica*

Gurjit Singh and Kenneth B. Storey

Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

Correspondence to K.B. Storey: kenstorey@cunet.carleton.ca
Tel: 613-520-2600, ext. 3678

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2.1 Abstract

The wood frog, *Rana sylvatica*, is one of only a few vertebrate species that display natural freeze tolerance. Frogs survive the freezing of about two-thirds of their body water as extracellular ice over the winter months. Multiple adaptations support freeze tolerance including metabolic rate depression and the production of huge amounts of glucose (often 200 mM or more) as a cryoprotectant that protects cells from freeze damage. To understand how high glucose levels affect gene expression, we studied MondoA, a glucose sensing transcription factor, and its partner MLX (Max-like protein) to assess their ability to modulate the expression of genes involved in glucose metabolism and circadian rhythm. Wood frog liver and brain tissues were analyzed, assessing protein levels, nuclear distribution, and DNA binding activity of MondoA:MLX during freezing (24 h at -2.5°C) and subsequent thawing (8 h returned to 5°C), as compared with 5°C controls. Downstream targets of MondoA:MLX were also evaluated: TXNIP (thioredoxin interacting protein), ARRDC4 (arrestin domain containing 4), HK-2 (hexokinase-2), PFKFB-3 (6-phosphofructo-2-kinase isozyme 3) and KLF-10 (Kruppel-like factor-10). Both KLF-10 and PFKFB-3 are also involved in circadian dependant regulation which was also explored in the current study via analysis of BMAL-1 (aryl hydrocarbon receptor nuclear translocator-like protein 1) and CLOCK (circadian locomotor output cycles kaput) proteins. Our data establish the MondoA-MLX complex as active under the hyperglycemic conditions in liver to regulate glucose metabolism and may also link to circadian rhythm in liver via KLF-10 and PFKFB-3 but not in brain.
2.2 Introduction

Earth is cold by our standards where most animal species including humans are not capable to survive under the frigid temperatures of winters. However, selected species have evolved to survive cold and the associated stresses (food scarcity, whole body freezing, dehydration, low oxygen) [1]. Many of these animals have developed behavioral, biochemical and physiological adaptations that allow them to endure whole body freezing [2].

The wood frog, *Rana sylvatica*, is one of a small group of vertebrates that can tolerate whole body freezing and is the main model organism for studying vertebrate freeze tolerance. Wood frogs are geographically spread across the boreal and eastern deciduous forests of North America, from the southern Appalachian mountains of the USA, across the forests of Canada, and all the way into Alaska [3]. During winter, they hide below an insulating cover of leaf litter and snow on the forest floor, where the temperatures in their hibernacula generally do not descend below about -7°C [4]. The wood frog’s ability to cryopreserve itself is very well documented [2, 5]. They begin to freeze below the equilibrium freezing point of their body fluids (about -0.5°C) and survive the conversion of about two-thirds of their total body water into extracellular ice [2]. Ice crystals typically propagate from the surface of the skin and through the extracellular spaces of the body interior [6]. Initiation of freezing triggers the liver to convert glycogen reserves into glucose and then export the sugar into the bloodstream for distribution and uptake by all tissues as a cryoprotectant. Blood glucose levels can rise from ~5 mM in unfrozen frogs to as high as 300 mM in freezing animals [7].
Glucose is taken up into cells via plasma membrane glucose transporters (GLUTs). Multiple isoforms of GLUTs exist, and have similar amino acid sequences but they differ in tissue specificity and regulatory properties [8]. For example, GLUT 2 is the bidirectional transporter found in liver and in wood frogs is upregulated by the initiation of freezing to mediate both the export of glucose cryoprotectant during freezing and its reuptake after thawing [8].

Winter survival by wood frogs also relies on metabolic reorganization to reduce their metabolic rate to a fraction of their normal rate by globally suppressing energy expensive processes such as protein synthesis, transcription and translation and prioritizing energy use for pro-survival processes [9]. For example, wood frogs enhance both anti-apoptotic and antioxidant defenses (pro-survival pathways) that aid survival and help organs to recover from metabolic insults caused by freeze/thaw [2]. Studies from our lab have also shown that during freezing, selective freeze-responsive genes are expressed in a tissue specific manner aiding wood frogs to survive the freezing stress [9, 10]. By contrast, anabolic processes are generally suppressed; for example, Akt (protein kinase B), that is known to activate many energy expensive pathways (e.g. protein synthesis, cell proliferation) is downregulated during freezing in frog heart, muscle and kidney. However, in liver, the most active organ making cryoprotectant, phospho-Akt (Ser-473 and Th-308) levels were upregulated which indicated a tissue-specific response to aid cryoprotectant synthesis and export [11].

The fine-tuning of gene expression is regulated, in part, by transcription factors (TFs) that facilitate activation of pro-survival pathways. TF proteins are encoded by nearly
10% of the eukaryotic genome and their binding to specific DNA motifs (consensus sequence) influence the expression of genes [12]. TFs can act as “master regulators” of major cell processes or as highly selective controllers of specific genes [13]. For example, the NFAT (nuclear factor of activated T cells) family of transcription factors influence the regulation of metabolic activity during periods of freeze-induced anoxia/ischemia and reoxygenation in wood frogs and also has a role in preventing liver damage [14]. Glucose metabolism is also regulated by the action of many transcription factors that respond to cellular demands for glucose storage or glucose use as a fuel or as a building block for many processes. Hence, we were interested to see how cells in wood frogs respond to changes in glucose levels during freezing.

The present study focuses on the role of the MondoA (MLX interacting protein) and MLX proteins that belong to the basic helix-loop-helix leucine zipper (bHLHZip) family of transcription factors that sense and respond to changes in intracellular energy levels. MondoA and MLX form a heterodimer pair that localize to the outer mitochondrial membrane (OMM) and upon sensing changes in glucose levels (detected as glucose-6-phosphate) they shuttle to the nucleus to guide an adaptive transcriptional response [15, 16]. The nuclear movement of MondoA:MLx depends on the activity of hexokinases. These enzymes catalyze conversion of glucose to glucose-6-phosphate (G6P), the first intermediate of glycolysis. Upon nuclear entry, MondoA regulates the expression of genes including thioredoxin interacting protein (TXNIP) and arrestin domain containing 4 (ARRDC4) that both belong to the α-arrestin protein family [15]. Both TXNIP and its paralog ARRDC4 are direct targets of the glucose
responsive MondoA:MLX complex and act as negative regulators of glucose uptake to maintain glucose homeostasis[17]. MondoA also turns on the transcription of hexokinase-2 (HK-2) that facilitates nuclear entry by MondoA:MLX and PFKFB3 (6-phosphofructo-2-kinase, isozyme 3) that facilitates activation of glycolysis by producing fructose-2,6-bisphosphate (F2,6P2)[18]. F2,6P2 is a key allosteric regulator of 6-phosphofructokinase (PFK-1), the main enzymatic control point in glycolysis [18, 19]. Given that wood frogs are hyperglycemic during the winter freeze and glycolysis is blocked at PFK-1 during freezing, the wood frog model is an ideal candidate for studying the impact of the MondoA-MLX transcriptional network and its downstream targets (TXNIP, ARRDC4, HK-2 and PFKFB-3) on glycolysis and their roles during freezing stress.

Furthermore, cross-talk between glucose homeostasis and the body’s physiological processes, such as circadian regulation, has been established [20, 21]. Most organisms have molecular clocks that operate on a 24 h rhythm to regulate and coordinate many functions of physiology, metabolism and gene expression [21]. Studies have established that disruption of the molecular clock is linked to disturbances in glucose homeostasis [21–23]. The action of the molecular clock is based on successive gene activation in the form of a negative feedback loop. In this loop the CLOCK (circadian locomotor output cycles kaput) transcription factor and BMAL1 (brain, muscle Arnt-like 1) drive the expression of their own inhibitors, the PER (Period Circadian Regulator) and CRY (Cryochrome) proteins [24]. The feedback loop begins with CLOCK and BMAL forming a heterodimer in the cytoplasm and entering the nucleus (Fig 2.1) [21].
These TFs bind to a consensus sequence (the E box CACGTG) to activate the negative regulators of the cycle: CRY-1, CRY-2, PER1 and PER2. CRYs and PERs negatively regulate their own expression and the expression of other clock-controlled genes (CCGs) (Fig. 2.1). This molecular machinery is capable of generating “ripples” of gene expression over a 24 h period which leads to changes in energy metabolism over time (e.g. lipid and glucose metabolism) [21, 25]. One such CCG is KLF-10 (Kruppel-like factor 10) that is known to be stimulated by rising glucose levels and acts as a transcriptional repressor of glucose activated targets (e.g. Mondo, MLX) while also promoting gluconeogenesis and lipid metabolism [26, 27]. KLF-10 is recruited by BMAL-1 and activated by BMAL-1-CLOCK [28]. It has an active involvement in regulating gene expression in gluconeogenesis and glycolysis as well as other regulatory cell functions such as cell proliferation, apoptosis and differentiation in a tissue specific manner [29].

Another CCG is PFKFB-3 whose expression also exhibits circadian rhythmicity and has been shown to be regulated by CLOCK genes [30]. Thus, the circadian clock controls the expression of KLF-10 and PFKFB-3, both direct targets of MondoA [26, 30].

Given the highly unique glucose metabolism of wood frogs that produces the hyperglycemia needed for whole body cryoprotection during freezing, we postulated that TFs involved in the normal regulation of body glucose metabolism must be disrupted or dysregulated. The present study examined the effects of freezing on the MondoA:MLX transcriptional network and downstream glucose-regulating proteins under its control in two crucial organs of wood frogs, liver (that controls cryoprotectant glucose metabolism) and brain (that has a major dependence on glucose as its fuel).
We also report that circadian rhythm bridges with glucose-induced gene transcription of MondoA:MLX via downstream targets during freezing stress in liver but not in the brain of wood frog.

2.3 Methods

Animals

Male wood frogs were collected during April from breeding ponds near Oxford Mills, Ontario. Animals were washed in tetracycline bath and placed in plastic boxes with damp sphagnum moss at 5°C for ~2 weeks prior to experimentation. Control frogs were directly sampled from these conditions. For freezing exposure, frogs were placed in closed plastic boxes lined with a damp paper towel and then transferred to an incubator set at -4°C for 45 minutes. This allowed the frog’s body temperature to cool below -0.5°C (the equilibrium freezing point of wood frog body fluids), and trigger ice nucleation on the skin soon after contact with ice crystals formed on the paper towel. The temperature of the incubator was then raised to -2.5°C and frogs were held at that temperature for 24 h. The 24 h frozen group was sampled at this time point. A second frozen group was transferred back to an incubator set at 5°C to thaw for 8 h before sampling [7]. All frogs were euthanized by pithing, rapidly dissected and tissues were flash frozen in liquid nitrogen before being stored at -80°C until use.

Collection of male wood frogs was authorized by a Wildlife Scientific Collector’s permit from the Ontario Ministry of Natural Resources. All experimental lab protocols
had the prior approval of the Carleton University Animal Care Committee (protocol #106935) and followed the guidelines of the Canada Council on Animal Care.

**Preparation of tissue extracts for total protein measurements**

Total protein extracts were prepared as previously described [14]. Briefly, frozen samples of liver and brain (~50 mg each; n= 4 independent replicates from different animals) for each experimental condition were homogenized in homogenization buffer (1:2 w: v) containing 20 mM HEPES buffer, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na$_3$VO$_4$, 10 mM β-glycerophosphate, a few crystals of phenylmethylsulfonyl fluoride (PMSF), and 1 μL of Sigma protease inhibitor cocktail (Burlington, ON, Canada, cat. no. P1C001.1) per mL of homogenization buffer using a Polytron homogenizer for 15-20 seconds. Homogenates were centrifuged at 12 000 × g at 4°C for 15 min, and supernatants containing soluble proteins were collected. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada), and all concentrations were then standardized to 10 μg/μl by adding calculated small volumes of homogenization buffer. Aliquots of total soluble protein extracts were then mixed 1:1 v:v with 2× loading buffer containing 100 mM Tris-HCl, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, and 10% v:v 2-mercaptoethanol to give final concentrations of 5 μg/μL for liver and brain samples. Samples were then boiled for 5 min, cooled on ice for 5 min, and stored at −80°C until needed.

**Preparation of nuclear protein extracts**
Nuclear protein extracts were prepared from liver and brain samples of four animals for each of the three experimental conditions. Frozen tissue samples (~50 mg) were homogenized (1:5 w:v) in 1X Buffer A (10 mM HEPES, 10 mM KCl, 10 mM EDTA, 20 mM β-glycerol phosphate, pH 7.9) with 10 µL/mL of 100 mM dithiothreitol (DTT) and 10 µL/mL of protease inhibitor cocktail added immediately before use. Tissue was homogenized with a Dounce homogenizer with 3-4 gentle strokes. Samples were held on ice for 25 min and then centrifuged at 12,000 x g at 4°C. Following centrifugation, supernatants were removed and saved as the cytoplasmic fraction. The pellet containing the nuclear fraction was re-extracted in 5X lysis buffer B: 100 mM HEPES, 2 M NaCl, 5 mM EDTA, 50% v:v glycerol, 100 mM β-glycerol phosphate, pH 7.9 with 100 mM DTT and protease inhibitor (1:1000 v:v) added to the cocktail. An aliquot of 250 µL of 5X buffer B was added to each pellet and the pellet was sonicated (Kontes micro-ultrasonic cell disruptor) for 5-10 sec. Samples were incubated on ice for 10 min and then centrifuged for 10 min at 14000 x g at 4°C and the supernatant was saved. Protein concentrations were determined with the Bio-Rad protein assay and concentrations were adjusted to 1.5 µg/µL for control and 3 µg/µL for 24-h frozen and 8-h thaw liver samples and to 3 µg/µL for brain of all three conditions. Samples were stored at −80°C until use. To evaluate the separation of cytoplasmic and nuclear fractions, immunoblotting was carried out as described below and tested with antibodies to α-tubulin (SC- 31779; Santa Cruz Biotechnology) and acetyl-histone H3 (CS-9649; Cell Signaling Technology) to assess any cross contamination. The cytoplasmic fractions of liver and brain showed much higher expression of α-tubulin protein as compared to
the nuclear fractions. Conversely, histone protein levels were much higher in the nuclear fractions from liver and brain as compared with cytoplasmic fractions in both tissues [31].

**Immunoblotting**

To evaluate relative protein expression levels of selected targets in liver and brain, proteins were separated on SDS-PAGE gels. Equal amounts of protein were loaded into each well and gels were electrophoresed using a Bio-Rad Mini Protean III apparatus at 180 V in 1X running buffer (3.2 g Tris base, 18.4 g glycine, 1 g SDS per liter) until the desired separation was achieved.

Proteins were then electroblotted onto polyvinylidene difluoride (PVDF cat no. IPVH00010) membranes by wet transfer in 1X transfer buffer (25 mM Tris pH 8.5, 192 mM glycine, 20% v:v methanol) at room temperature (RT) for 70-120 min at 160 mA. After transfer, membranes were blocked with either non-fat milk (2-5%) or high molecular weight polyvinyl alcohol (2-4 mg/ml) (Sigma- P-1763) diluted in 1X TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.05% v/v Tween 20) for 30 min. Membranes were then washed before probing with specific primary antibodies (1:1000 v:v dilution in 0.5% TBST) at 4 °C for 16 h on a rocking platform.

Membranes were washed for 3 x 5 min using 0.5% TBST, followed by incubation with secondary antibody; depending on the primary antibody, these were horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody, anti-goat IgG secondary antibody, or anti-mouse IgG secondary antibody (1:5000 v:v dilution) for ~30 min.
Following incubation, membranes were washed as described above before visualization using enhanced chemiluminescence (H₂O₂ and luminol). Blots were then stained using Coomassie blue (0.25% w:v Coomassie brilliant blue, 7.5% v:v acetic acid, 50% v:v methanol). The following antibodies were used: MondoA (Bethyl Lab-A303-195A), MLX (R&D systems-AF4186), TXNIP (Novus Biological -54578SS), ARRDC4 (Biobyrt- orb235023), HK-2 (GTX- 111525), PFKFB3 (GTX-122577), BMAL-1 (Bethyl Lab-A-302-616A), CLOCK (Bethyl Lab-A302-618-A), CRY-1 (GTX-132177), CRY-2 (Bethyl Lab-A-302-615A), PER-1(Novus Biological-2-25589SS), PER-2 (Bethyl Lab-A303-109A), and KLF-10 (TIEG-1-Bethyl Lab-A-302-015-1A).

**DNA-protein interaction enzyme-linked immunosorbent assay (DPI-ELISA)**

Biotinylated-DNA oligonucleotides containing the binding element for Mondo A, MLX, BMAL-1, or CLOCK that have canonical E-box sequences 5′-CACGTG-3′ were synthesized by Sigma Genosys (Sigma-Aldrich, Oakville, Canada). The biotinylated probe (5′-biotin-AGTATTTAGCCACGTGACAGTGTA-3′ and complementary probe 5′-CTTACACTGTCACGTGGCTAAATACT-3′)were diluted to final concentrations of 500 pmol/µL with ddH₂O. Forward and reverse probes were mixed in a 1:1 ratio, placed in a thermocycler (Eppendorf Master Cycler gradient) for 10 min at 95°C to hybridize and then allowed to cool to RT. Double-stranded probes were then diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, pH 7.4) and 50 µL of diluted double stranded probe was added (40 pmol DNA/well) to streptavidin-coated wells (Thermo Multiscan Spectrum, Cat. no. 1500-074). Following 1 h incubation at RT, unbound probes were discarded, and the wells were washed twice with 1X PBST wash
buffer (PBS + 1% Tween), and a third time with 1X PBS. Transcription factor-binding buffer (10 mM HEPES, 50 mM KCl, 0.5 mM EDTA, 3 mM MgCl₂, 10 % v:v glycerol, 0.5 mg/ml bovine serum albumin, 0.05 % NP-40, 20 mM DTT, pH 7.9) and 1 µg/µL salmon sperm DNA (Bioshop DNA002) was added to each well containing DNA probe. Then 15-30 µg of protein from liver and brain extracts was added, with the exception of negative control wells which contained all assay components but no protein. Following 1 h incubation with gentle shaking at RT, unbound protein mixtures were discarded, and wells were washed three times with wash buffer.

Mondo A, MLX, BMAL-1 (ES-DK9891) or CLOCK (Abm-Y058186) primary antibodies were diluted 1:1000 v:v in 1X PBST and aliquots of 60 µL/well were added to appropriate wells and incubated for 1 h at RT without agitation. Subsequently, excess antibody was discarded, and the wells were washed three times with wash buffer before incubation with secondary antibody diluted in PBST (1:2000, 60 µL/well) for 1 h. The plate was incubated for 1 h at RT without agitation before the antibody was discarded and wells were washed 4 times with wash buffer. Following incubation, a 60 µL aliquot of tetramethylbenzidine (TMB) (Bioshop TMB 333.100) was added to each well, color was developed for 10–15 min, and then the reaction was terminated by addition of 60 µL of 1 M HCl. Absorbance was measured at 450 nm (reference wavelength of 655 nm) using a Multiskan spectrophotometer. To determine the specificity of the probes, a test run using pooled samples was performed for all antibodies to compare the DNA binding activity of wells containing all assay components with wells that contained no protein, no probe or no primary antibody.
Quantification and Statistics

Bands on Western blots were visualized using a ChemiGenius Bio-Imager (Syngene, Frederick, MD) and band intensities were analyzed using the associated Gene Tools software. Band densities in each lane were standardized against the collective intensity of group of Coomassie-stained protein bands in the same lane. The Coomassie-stained group was prominent and consistent across all the lanes and was well separated from the band of interest. This method has been shown to be more accurate as compared to standardizing band intensities against a housekeeping protein such as GAPDH [32]. Immunoblot band densities were standardized for each condition relative to the corresponding control band densities. Immunoblot data are presented as mean ± SEM, n=4 independent biological replicates. Statistical testing used the RBioplot program [33] to conduct a one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as significant. DPI-ELISA data were also analyzed using RBioplot. The relative DNA binding activity of DPI-ELISAs for 24 h frozen and 8 h thawed groups were determined relative to the control group which was set to 1. A One-Way ANOVA was performed to determine if our experimental groups were significantly different from each other, as only one independent variable (type of stress only) was present.

2.4 Results

Analysis of total protein levels of MondoA and downstream targets

Protein levels of MondoA and its downstream targets were measured using Western blotting to assess their relative expression levels in liver and brain samples from 5°C
acclimated controls, 24 h frozen (at -2.5°C) and 8 h thawed (at 5°C) wood frogs. For analysis, we used mammalian polyclonal antibodies and preliminary tests showed that each reacted with a single band on the gel at the expected molecular weight for that protein.

MondoA protein levels showed a significant increase (1.96 ± 0.3 fold) in liver of 24 h frozen frogs compared to controls but then decreased again during thawed recovery to a value that was just 1.35 ± 0.1 fold over controls and not significantly different from the control value (Fig 2.2). MLX protein levels also increased significantly (2.46 ± 0.5 fold over controls) in liver of 24 h frozen wood frogs and also returned to control values after 8 h of thawed recovery. Levels of the downstream target TXNIP showed a strong upward trend after 24 h freezing and remained significantly higher after the 8 h recovery period (2.21 ± 0.2 fold) as compared to the controls. Levels of the arrestin family protein, ARRDC4, did not change during freezing but had risen significantly (1.8 ± 0.09 fold) after 8 h thawed as compared to control and 24 h freezing values.

Hexokinase-2 (HK-2) protein levels were unchanged over the course of the freeze-thaw cycle whereas PFKFB3 protein levels were suppressed to ~60% of the control value in liver of 24 h frozen frogs but returned to control levels after thawing.

In wood frog brain samples, MondoA and MLX protein levels were higher (1.45 ± 0.13 fold and 1.6 ± 0.2 fold, respectively) in 24 h frozen frogs compared to controls (Fig 2.3). Protein levels of both returned to baseline after 8 h recovery. Levels of TXNIP were significantly higher (1.8 ± 0.2 fold) in 24 h frozen wood frogs compared to controls and remained high after thawing (although not significantly different from either control or
frozen values). ARRDC4 and PFKFB3 levels were unchanged under both frozen or thawed conditions. HK-2 levels were unchanged during freezing but rose 1.3 ± 0.04 fold after 8 h thawed, an increase that was only significant compared to the 24 h frozen group.

**Analysis of total protein levels of BMAL-1-CLOCK and downstream targets in liver and brain samples**

Total protein levels of BMAL-1 and CLOCK and their downstream targets were also measured in liver and brain samples from control, 24-h frozen and 8-h thaw wood frogs. BMAL-1 protein levels were 1.7 ± 0.3-fold higher in liver of 24 h frozen frogs, as compared with controls, but then decreased back to control levels after 8 h thawed (Fig 2.4). CLOCK protein levels remained at control values during freezing but significantly decreased by 40% in the 8 h thawed group. CRY-1 total protein levels showed a rising trend during freezing but increased very strongly (3-fold over controls) in 8 h thawed frogs. PER-1 levels showed an apparent decrease during freezing and increased again after thawing (but neither was significantly different from controls) but values for PER-1 in thawed frogs were significantly higher (~3-fold) compared with the level in liver from frozen frogs. PER-2 protein levels were unchanged in response to freezing but decreased significantly to just 40% of control values during the 8 h thawed recovery. KLF-10 protein levels were unchanged during freezing but showed a significant 50 % decrease after the 8 h thaw, as compared with controls.
In brain, BMAL-1 protein levels remained unchanged during freezing and decreased by 30%, compared with controls, after 8 h thawed (Fig 2.5). CLOCK protein levels were unchanged by freeze-thaw as were levels of CRY-1. PER-1 levels decreased by 30% in brain of 24 h frozen frogs and returned to control values after 8 h thawed, whereas brain PER-2 levels decreased by 60% in frozen animals and remained low after 8 h recovery. KLF-10 protein levels did not change over freeze-thaw in brain.

**Nuclear levels of primary targets of glucose homeostasis and circadian rhythm in liver and brain of wood frogs**

Nuclear extracts were prepared, and Western blotting was used to assess the relative distribution of protein targets in wood frog liver and brain nuclei under control, 24 h frozen and 8 h thawed conditions. Nuclear contents of MondoA and MLX in liver were not affected by freezing but rose significantly after 8 h thawed (1.5 ± 0.04-fold and 2.3 ± 0.4-fold) as compared with control frogs (Fig 2.6). BMAL-1 levels in liver were reduced to 80% of control values during 24 h freezing stress but rose again after 8 h thawed (1.23 ± 0.12-fold) whereas CLOCK protein levels decreased by 40% during 24 h frozen but rose slightly again after thawing. Interestingly, nuclear extracts prepared from wood frog brain showed no significant changes between control, frozen and thawed conditions for any of the four targets tested (MondoA, MLX, BMAL-1 and CLOCK) (Fig 2.7).

**DNA binding activity of MondoA-MLX and BMAL-1-CLOCK in liver and brain samples**
The DNA-binding capacities of MondoA-MLX and BMAL1-CLOCK were also measured in liver and brain samples from control, 24 h frozen and 8 h thawed groups. DNA binding levels of MondoA in liver extracts did not change significantly over the freeze-thaw cycle (Fig 2.8). However, MLX binding decreased during freezing by 40% as compared to controls, and then rebounded after 8 h thawed. DNA binding of CLOCK in liver extracts also decreased by 35% compared with controls after 24 h frozen but rose again after thawing to a value that was ~1.3 fold higher than controls and ~2-fold higher than the frozen value. However, DNA binding activity of BMAL-1 showed no significant change during freezing and was reduced significantly after thawing by about 40% as compared with controls. The DNA-binding capacity of MondoA-MLX and BMAL1-CLOCK in brain samples did not change significantly for any of the 4 proteins assessed over the freeze-thaw cycle (Fig 2.9).

2.5 Discussion

MondoA, is an essential glucose-responsive transcription factor, that along with its binding partner MLX, is responsible for most of the intracellular glucose-induced transcriptional response [17]. An understanding of the MondoA:MLX transcriptional network can shed light on the ways that wood frogs regulate glucose homeostasis and maintain high levels of glucose as a cryoprotectant over the freeze/thaw cycle. Our results showed that in response to 24 h freezing, the total protein levels of MondoA and its heterodimeric partner MLX were upregulated in both liver and brain suggesting that these two binding partners may play a significant role in achieving and/or maintaining hyperglycemic conditions in wood frog tissues (Fig 2.2, 2.3).
Since the transcription factors, MondoA and MLX, are active inside the nucleus, we were interested to see if their nuclear distribution and DNA binding activity was substantially altered during freezing. Our data for liver found that both MondoA and MLX were unaffected in the frozen state but showed a higher nuclear distribution during the 8 h recovery period (1.5 ± 0.04-fold and 2.3 ± 0.4 fold, respectively), compared to the controls (Fig 2.6). Furthermore, DNA binding activity remained constant for MondoA across the freeze/thaw course in both tissues, but liver MLX binding activity levels went down during freezing and bounced back to control levels after 8 h thawed recovery (Fig 2.8). Although total protein levels were higher for MondoA (1.96 ± 0.3-fold) and MLX (2.46 ± 0.5 fold) in liver during freezing, as compared to controls (Fig 2.2), the trends in nuclear levels along with DNA binding activity suggest that wood frogs might be actively using the MondoA/MLX machinery during the thawing recovery phase to aid them in re-establishing normal glucose homeostasis. However, unlike the freeze-induced rapid increase in liver glycogenolysis and glucose export as a cryoprotectant when freezing begins, wood frogs take much longer to restore glucose back into glycogen post-thaw recovery, as long as 8-10 days before glycogen and blood/tissue glucose levels return to normal [34]. This could be adaptive in keeping frogs partially prepared for a subsequent freeze that could occur again soon, especially during autumn or spring when environmental temperatures can vary above/below 0°C. However, the much higher activities of glycogen phosphorylase compared with glycogen synthase in liver are also an important factor in determining relative rates of glycogenolysis versus glycogen synthesis [34, 35]. We have also
proposed previously [2] that there must be an “override” on the normal homeostatic controls that regulate liver glycogenolysis in order to achieve the higher output of glucose as the cryoprotectant. This is remains to be identified but might possibly be linked to modified regulation of genes under MondoA/MLX control.

MondoA and MLX expression during freezing displayed tissue specific trends in brain. Total protein levels of both MondoA and MLX were higher (1.45 ± 0.13-fold and 1.6 ± 0.2- fold, respectively) in 24 h frozen frogs as compared to controls (Fig 2.3). Both nuclear expression and DNA binding activity of both the proteins assessed (MondoA and MLX) did not change in brain during either freezing or thawing (Fig 2.7, 2.9). While brain is a metabolically active organ, it does not contribute to the cryoprotection of other organs as liver does. Since the nuclear levels and DNA binding did not change but total protein levels remained higher for both MondoA and MLX during freezing, this could relate to other functions that these two targets could play inside the cell. One study has shown that MondoA negatively interacts with the mTOR complex 1 (mTOR C1) and inhibits its action [36]. Another study done on brain of wood frogs showed suppression of PI3K/AKT pathways during freezing that would also suppress TOR activity and protein translation to suppress this energy-expensive function in the frozen wood frog model [37]. Therefore, in brain MondoA could be involved as a checkpoint to minimize energy expensive pathways such as mTOR pathway during freezing.

Both TXNIP and ARRDC4 activities are controlled in a MondoA/MLX dependent manner that is tightly connected to glucose uptake [38]. These α-arrestin family proteins are regulated in a reciprocal manner to glucose supply and act as negative regulators of
glucose uptake [39]. TXNIP has been shown to play a major role in liver glucose homeostasis where it acts as a regulator to modulate the movement of glucose via glucose transporters (GLUTs) across the plasma membrane [40, 41]. In our study, the total protein levels of TXNIP in liver rose during freezing and stayed high during thawed recovery, as compared with controls (Fig 2.2). The higher expression trend of TXNIP during recovery aligns with increased nuclear translocation of MondoA/MLX that regulates TXNIP transcription in liver (Fig 2.6, 2.8). The bidirectional glucose transporter, GLUT-2, has also been shown to be induced under freezing stress in wood frog liver as a primary adaptation that fulfills the requirement for rapid efflux of the cryoprotectant [42]. Interestingly, TXNIP has also been shown to directly regulate GLUT-2 activity, which also suggests that TXNIP might be acting as a “switch” under control of MondoA/MLX to regulate glucose levels in and out of liver via GLUT-2 [43].

In brain, total protein levels of TXNIP were also upregulated (1.8 ± 0.2 fold) in 24 h frozen frogs as compared to controls (Fig 2.3). This again correlated with enhanced levels of MondoA and MLX in brain during freezing although nuclear levels and DNA binding activity of these upstream regulators of TXNIP transcription did not change (Fig 2.7, 2.9). This suggests that TXNIP might be regulated in an alternate manner in brain. TXNIP plays a central role during glucose/oxidative stress and has been shown to be upregulated in cerebrovascular and neurodegenerative diseases in brain [44]. In brain, TXNIP also acts as a redox regulator by binding to TRX-1 (thioredoxin-1, an antioxidant protein) and inhibiting its antioxidant activity [45]. It is notable that TRX-1 was downregulated during freezing stress in brain of wood frogs, although the antioxidant
response and protein chaperones involved in freezing were upregulated [46]. This suggested that TXNIP might inhibit the activity of TRX-1 to downregulate its level while not affecting the overall antioxidant capacity. Also, TRX-1 had been shown to regulate the cell cycle and lower levels of this protein are associated with cell cycle arrest which is one crucial factor for metabolic rate depression and long term survival in a frozen state. Hence, TXNIP might be key player in inducing MRD during freezing via actions on TRX-1 in brain [47, 48].

In addition, ARRDC4 protein levels were significantly higher in liver of the 8 h thawed group compared to control and 24 h frozen frogs (Fig 2.2). It is interesting to note that expression of this protein also aligns with MondoA/MLX, as described above. It’s major role in liver also aligns with its paralog, TXNIP, since ARRDC4 has also been shown to be involved in regulating glucose transporters (glucose endocytosis, transport and degradation) [49]. However, ARRDC4 levels did not change during freeze/thaw in wood frog brain (Fig 2.3). As mentioned before, the brain is probably not as metabolically active during freezing as is the liver that must synthesize and export not only large amounts of glucose as a cryoprotectant but also produce/export various other products such as freeze-responsive proteins like FR10 [2]. Brain likely takes up glucose that is circulated from the liver but probably is not producing endogenous glucose.

HK-2, a key glycolytic enzyme is known to be involved in facilitating MondoA/MLX in triggering transcriptional responses by downstream genes. HK-2 levels were unchanged across 24 h freezing and 8 h recovery in wood frog liver (Fig 2.2) but this did not rule out the possibility that reversible phosphorylation of HK-2 (known to occur in
wood frogs) [50] might be a factor in driving nuclear entry of MondoA/MLX under freezing stress. In brain, HK-2 showed differential regulation, levels showing a downward trend after 24 h freezing (but not significantly different from control) but rose above control levels during 8 h recovery (Fig 2.3). HK-2 is an enzyme that is regulated in response to disturbances in glucose metabolism and is involved in many signal transduction pathways. For example, HK-2 has been linked with Akt which links to suppression of apoptosis [51, 52]. Since the trend of studies in freezing wood frog model suggest that apoptotic pathways are downregulated in multiple tissues [12], HK-2 regulation and links to apoptosis suppression will be an interesting area of metabolic control to explore in future studies in the frog model since control over apoptosis could be crucial for long term survival in a frozen state.

Previous studies of wood frog liver have shown that freezing stress results in a strong reduction in PFKFB (also known as PFK-2 or 6-phosphofructo-2-kinase) activity in liver [19]. This enzyme that synthesizes fructose-2,6-bisphosphate (F2,6P₂) a potent activator of PFK-1 (6-phosphofructo-1-kinase) that is regarded as the rate-limiting enzyme of glycolysis. Suppression of PFKFB leads to a strong decrease in F2,6P₂ concentrations thereby inhibiting PFK-1. Indeed, in both liver and brain of wood frogs showed reduced F2,6P₂ concentrations during freezing along with reduced activity of PFKFB [18] which would suppress PFK-1 activity and glycolytic flux and, in liver, help to divert hexose-phosphates into glucose production for export. MondoA/MLX is a known regulator of PFKFB-3 expression [15], but protein expression of PFKFB-3 in 24 h frozen samples was lower (Fig 2.2), as compared with control frogs. This was expected since glycolysis in
freezing wood frogs is inhibited at PFK-1. PFKFB-3 responses to freezing are also consistent with its involvement in cell cycle regulation and cell proliferation, both of which are inhibited during freezing in wood frogs [19]. However, in brain, PFKFB3 levels did not change across the three stresses (Fig 2.3) suggesting that this protein might only be required at basal levels over the freeze/thaw cycle.

**Crosstalk between components of circadian rhythm and glucose-induced transcription link via downstream targets PFKFB-3 and KLF-10**

Circadian rhythm is a complex program of gene expression, that ensures proper oscillations of the expression of other genes such as those involved in glucose homeostasis [53]. Interestingly, while most of the studies on circadian rhythm show changes in gene expression with changes in the light/dark cycle of the day, this system is also responsive to cues from internal peripheral clocks that affect gene expression in multiple organisms [23, 54, 55]. Since wood frogs remain in constant darkness during experimentation (in low temperature incubators) and would also have little or no perception of day vs night during the winter in nature (under forest floor leaf litter and a deep blanket of snow), we were interested in analyzing changes in molecular clock proteins under our experimental conditions. We evaluated clock controlled proteins linked to the MondoA:MLX transcription machinery in both brain and liver over the freeze/thaw cycle, with a particular interest in those involved in glucose homeostasis.

The circadian clock controls the expression of KLF-10 (Kruppel like factor-10) and PFKFB-3, both being direct targets of MondoA [26, 30]. KLF-10 is stimulated by rising
glucose levels and acts as a transcriptional repressor of glucose-activated targets (Mondo, MLX); it also promotes gluconeogenesis and lipid metabolism [26, 27]. The present data for liver shows that KLF-10 had a lower protein expression after 8 h thawed recovery wood from freezing, just 47% of control values (Fig. 2.4). This change was supported by the higher nuclear levels and DNA binding activity of MondoA/MLX in liver of thawed frogs (Fig. 2.6, 2.8). Furthermore, a study of a KLF 10-/- animal model showed that it was involved in inducing apoptosis via activating SMAD2 (pSMAD2) signalling [56, 57] and both SMAD2 and pSMAD2 levels were reduced after the 8 h thaw in liver of wood frogs [58]. This hints that the mechanisms that wood frogs might be inducing during recovery after freezing also keep apoptotic proteins at bay.

KLF-10 also shows strong BMAL-1 dependent expression which is trans-activated by BMAL1-CLOCK levels in liver [28]. We analyzed total nuclear protein levels, as well as DNA binding activity, of both BMAL-1 and CLOCK in liver. Total protein levels of BMAL-1 were higher in 24 h frozen wood frogs (by 1.7 ± 0.3 fold) but decreased to control levels after 8 h thawed (Fig. 2.4). Also, analyzing the active nuclear form of the BMAL1/CLOCK complex, we found that nuclear levels of both BMAL-1 and CLOCK remained low during freezing but rebounded during recovery (Fig. 2.6) although this did not restore KLF-10 protein levels after the 8 h thaw [28]. CRY-1 has also been recognized as an inhibitor of BMAL-1/CLOCK regulation and acts to antagonize KLF-10 activation [28, 59]. Interestingly, although our results showed much higher expression of CRY-1 (3-fold) after 8 h thawed in wood frog liver (Fig. 2.4), we did not see a decline in levels of BMAL-1 and CLOCK expression after thawing (Fig. 2.6). This suggested that
CRY-1 might not be able to regulate activity of BMAL-1/CLOCK solely by itself in thawed animals. PER-1 and PER-2, which together form a repressor complex, were also dysregulated (Fig 2.4) over the freeze/thaw cycle. This suggested that BMAL-1/CLOCK dependent KLF-10 activity might be controlled by another mechanism during freeze/thaw. This was also supported when we analyzed DNA binding activities of both BMAL-1 and CLOCK over the freeze-thaw cycle. It was interesting to note that DNA binding activity by BMAL-1 and CLOCK after 8 h thawed did not match with protein expression responses (Fig 2.6, 2.8). This could hint that posttranslational modifications might also be involved in regulating clock genes to bring expression of KLF-10 down during 8 h recovery. Indeed, such a mechanism has been shown in many studies [28, 60].

In brain, KLF-10 has also been shown to regulate apoptosis [61]. Previous studies from our lab have shown that proteins belonging to the apoptotic pathway are downregulated during freezing in R. sylvatica [51]. In the present study, protein levels of brain KLF-10, CLOCK and CRY-1 were unchanged during freeze/thaw cycle. Only, BMAL-1 levels dropped to nearly 70% (Fig 2.5) of controls during thawed recovery but, on its own, this did not change KLF-10 expression. This was also reflected in the DNA binding and nuclear protein levels of BMAL-1 and CLOCK (Fig 2.7, 2.9). Finally, dysregulated levels of PER-1 and PER-2 were again observed in brain, similar to liver, but this did not lead to a change in the expression of KLF-10 during either freezing or thawing. These results suggest that the clock proteins regulating KLF-10 do not affect its expression in brain over freeze-thaw cycles.
PFKFB-3 levels have also been linked to circadian clock genes [30]. PFKFB-3 protein levels decreased in liver after 24 h freezing but rose again to near control levels after 8 h thawed (Fig 2.2). PFKFB-3 expression has been shown to be regulated mainly by CLOCK but is also further enhanced under BMAL-1 expression [30, 64]. Although total protein levels of CLOCK (Fig 2.4) did not change during freezing as compared to controls, its nuclear levels were significantly lower after 24 h freezing (Fig 2.6) with lower DNA binding activity (Fig 2.8) as well. BMAL-1 nuclear levels were also downregulated during freezing (Fig 2.6), consistent with the above observations. This suggests that downregulation of the BMAL-1/CLOCK transcription complex controls PFKFB-3 expression during freezing.

In brain, the total protein, nuclear distribution and DNA binding levels of CLOCK did not change across the two experimental conditions, compared with controls (Fig. 2.5,2.7, 2.9). Only the total protein levels of BMAL-1 decreased (by 30%) during 8 h recovery (Fig 2.5) while nuclear protein levels and DNA binding levels of BMAL-1 remained unchanged. This correlated with the expression profile of PFKFB-3, which stayed the same across the freeze-thaw exposure.

In conclusion, the current findings provide evidence that the MondoA/MLX transcription factors play a tissue-specific role in regulating glucose homeostasis over the freeze-thaw cycle in wood frogs. This was perhaps not unexpected since liver has a major role in producing and exporting glucose cryoprotectant during freezing (and restoring the sugar as glycogen again after thawing) whereas brain is known as an organ that almost exclusively depends on glucose as a metabolic fuel.
MondoA/MLX was upregulated by freezing and showed higher nuclear protein content and increased DNA binding activity that could upregulate downstream targets including negative regulators of glucose uptake (TXNIP, ARRDC4) and also modify enzymes associated with glycolysis (PFKFB-3). In addition, both KLF-10 and PFKFB-3 protein levels correlated with changes in key circadian proteins, thereby hinting at control by the hepatic peripheral clock in regulating glucose homeostasis via these targets over the freeze/thaw cycle. In brain, although MondoA/MLX were induced during freezing, the effect of the freeze/thaw cycle on nuclear levels and DNA binding activity of these targets suggest that they might have alternative roles in regulating other pro-survival processes such as inhibiting apoptosis. The higher protein levels of one of the negative regulators (TXNIP) in brain during freezing might also be involved in maintaining cryoprotectant levels and inducing metabolic rate depression. Overall, our results suggest that the MondoA/MLX complex is activated in liver under high glucose conditions and may link the molecular clock to metabolism in liver but not in brain.

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2.6 References


2.7 Figures
**Figure 2.1:** Schematic diagram representing the involvement of MondoA and MLX under high glucose (G6P) conditions and the feedback loop of the circadian clock. The MondoA:MLX dimer is present in the outer mitochondrial membrane and senses changes in cellular fuel (G6P) and shuttles to the nucleus to activate downstream targets (TXNIP, ARRDC4, HK-2, PFKFB-3 and KLF10) by binding to CHoRE. TXNIP and ARRDC4 negatively regulate glucose uptake via GLUTs whereas the glycolytic enzyme HK-2 facilitates nuclear entry of the MondoA:MLX complex that also binds to the HK-2 promoter. PFKFB-3 is also expressed under control by MondoA and its product (fructose-2,6-bisphosphate) is a potent regulator of glycolytic versus gluconeogenic flux. Circadian genes control downstream targets common to the MondoA-MLX transcription complex (KLF-10, PFKFB-3). Abbreviations are: GLUT, glucose transporter; G6P, glucose-6-phosphate; CHoRE, carbohydrate response element; TXNIP, thioredoxin interacting protein; ARRDC4, Arrestin family protein domain 4; HK-2, Hexokinase 2; PFKFB-3, 6-phosphofructo-2-kinase isozyme 3; KLF-10, Kruppel-like factor-10. (Image Created with www.BioRender.com)
Figure 2.2: Relative protein expression of MondoA-MLX and the downstream targets (TXNIP, ARRDC4, HK-2, PFKFB3) in liver samples from control, 24 h frozen and 8 h thawed R. sylvatica. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Representative immunoblots are shown below the histogram.
**Figure 2.3:** Relative protein expression of MondoA-MLX and its downstream targets (TXNIP, ARRDC4, HK-2, PFKFB3) in brain samples from control, 24 h frozen and 8 h thawed *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey's post-hoc test with $P<0.05$ accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Figure 2.4: Effects of 24 h freezing and 8 h thaw on the protein expression of BMAL, CLOCK and other proteins involved in circadian rhythm (CRY1, PER1, PER2, KLF-10) in liver of R. sylvatica. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Figure 2.5: Effects of 24 h freezing and 8 h thaw on BMAL-CLOCK and relative amounts of (CRY1, PER1, PER2, KLF-10) in brain of *R. sylvatica*. See Figure 2 for more information. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
**Figure 2.6:** Relative nuclear distribution of MondoA, MLX, BMAL-1 and CLOCK proteins in liver samples from control, 24 h frozen and 8 h thawed *R. sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Figure 2.7: Relative nuclear distribution of MondoA, MLX, BMAL-1 and CLOCK proteins in brain samples from control, 24 h frozen and 8 h thawed *Rana sylvatica*. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Figure 2.8: DPI-ELISA analysis of the DNA-binding ability of MondoA, MLX, BMAL-1, and CLOCK to the E-box consensus sequence using total protein extracts from liver of control, 24 h frozen and 8 h thawed wood frogs. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Figure 2.9: DPI-ELISA analysis of the DNA-binding ability of MondoA, MLX, BMAL-1 and CLOCK to the E-box consensus sequence using total protein extracts from brain of control, 24-h frozen and 8-h thawed wood frogs. Data are mean ± SEM, n=4 independent biological replicates. Statistical testing used one-way ANOVA and Tukey’s post-hoc test with P<0.05 accepted as a significant difference. Histogram bars that share the same letter notation ‘a’ or ‘b’ are not significantly different from each other. Histogram with ‘ab’ means not significant from either ‘a’ or ‘b’. Representative immunoblots are shown below the histogram.
Chapter-3: TXNIP shuttling - a key molecular link in regulating inflammation and mitochondrial dysfunction
3.1 Abstract

Amphibians such as the wood frog, *Rana sylvatica*, are a primary example of a freeze-tolerant vertebrate that undergoes whole body freezing. Their ability to cryopreserve themselves by sequestering 65-70% of total body water as extracellular/extra organ ice and producing large amounts of glucose as a cryoprotectant is well documented. Interestingly, the high glucose levels induced in response to freezing can amplify oxidative stress’s effects (reactive oxygen species, ROS) and induce inflammation and mitochondrial dysfunction. Since both freezing and dehydration stress (independent of freezing) can render wood frogs hyperglycemic, this study focussed on these two stresses to elucidate the role of a scaffold protein thioredoxin interacting protein (TXNIP), which localizes in multiple compartments inside the cell under hyperglycemic conditions and mediate diverse stress responses via interacting with multiple proteins. The results from this study suggest a stress-specific response of TXNIP in inducing the cell-damaging pathway of inflammasome activation via its cytoplasmic localization during freezing but not in dehydration stress. Mitochondrial retention of TXNIP usually leads to its binding to another antioxidant protein, thioredoxin 2 (TRX-2), which activates the dysfunction of this organelle by releasing a mitochondrial protein cytochrome c (Cyt c) into the cytoplasm. Interestingly, a direct role of TXNIP in inducing this pathway could not be established in both freezing and dehydration stress, although an overall downregulation of Cyt c in this pathway across both stresses hinted at minimal damage to the organelle. The overall regulation of TXNIP in regulating both inflammasome and mitochondrial dysfunction was seen to be affected in part by its
post-translational modifications. An *Insilco* model of TXNIP and TRX-2 binding in a frog model suggests that sequential differences in TXNIP could be attributed to its weak binding to TRX-2. Altogether, this study establishes a role for TXNIP in activating inflammasome in a stress-specific way during freezing, while the mitochondrial presence of this protein did not correlate to protein expression of downstream targets in inducing mitochondrial dysfunction damage, which was attributed to PTMs and structural differences of TXNIP.
3.2 Introduction

Poikilothermic animals have developed multiple strategies to regulate their internal temperatures, a factor key to their survival under environmental challenges such as cold or freezing temperatures during winters [1]. Amphibians such as the wood frog, *Rana sylvatica*, are a primary example of a poikilothermic freeze tolerant vertebrate that undergoes whole body freezing and has the extraordinary ability to remain frozen for weeks or months while sequestering 65-70% of total body water as extracellular ice [2, 3]. Interestingly, once frozen, these animals do not exhibit any physiological vital signs like heartbeat, breathing or brain activity. Multiple adaptations at biochemical, physiological and molecular levels allow them to not only survive but thrive under these conditions, since they come back to life unharmed once thawed. These adaptations include managing ice formation in extra-organ or extracellular spaces, cell volume reductions due to loss of 60-70% of total body water into extracellular space as well as producing a huge amount of glucose as a cryoprotectant from the breakdown of liver glycogen [1, 4]. Interestingly, large amounts of glucose are produced (300 mM compared to 5 mM in control frogs) during freezing and this not only provides glucose for cryoprotection but also a substrate for anaerobic energy production (under the ischemic/anoxic conditions of the frozen state) and can also stabilize biomolecules such as proteins and enzymes without affecting their function [1, 3, 5]. However, the presence of this freezing-induced hyperglycemia can also amplify the effects of oxidative stress (reactive oxygen species, ROS) and mitochondrial dysfunction [6, 7]. Also, while undergoing freezing, wood frogs undergo a state of metabolic rate
depression (MRD) to suppress energy expensive pathways (e.g. protein synthesis and cell cycle) and prioritize available energy to combat cell damaging pathways (e.g. apoptosis, oxidative stress) by activating pro-survival pathways (e.g. inducing chaperone and antioxidant responses) [8]. MRD is a complex and coordinated response to freezing (and its associated consequences) involving multiple levels of regulation at transcriptional, post transcriptional, translational and post translational levels [3]. For example, transcription factors (TFs), that are regulators of gene expression involving all cellular processes, can be used to selectively control specific genes under stress conditions [9]. For example, a study of the TF MondoA (MLX-interacting protein) that senses changes in intracellular glucose, showed its involvement during freezing as an adaptive transcriptional response via activating downstream targets such as an alpha-arrestin protein, and TXNIP (thioredoxin interacting protein) [9]. TXNIP plays a major role in negatively regulating glucose uptake, maintaining glucose homeostasis as well as shifting metabolism from oxidative to anaerobic under hypoxic conditions [10–12]. Overexpression of TXNIP under hyperglycemic conditions has also been linked to glucose-induced inflammation in many diseases such as type-2 diabetes [13].

Interestingly, the diverse roles and regulation that TXNIP exerts on different cellular cascades is strongly dependent on its subcellular localization. For example, under basal/control conditions, TXNIP stays in the nucleus, while the cell’s antioxidant defense system via small redox proteins such as thioredoxins (TRX1 in the cytoplasm and TRX2 in the mitochondria, Fig. 3.1A) remain intact and active [13, 14]. Both TRX1 and TRX2 are ubiquitously expressed proteins and control the cell’s response to
oxidative stress [13, 14]. Interestingly, under hyperglycemic conditions, the location of TXNIP in both cytoplasm and mitochondria, allows it to inhibit both TRX-1 and TRX-2, respectively (Fig 3.1A). In mitochondria, this is mainly done by displacing the redox-regulated apoptosis kinase (ASK-1) (Fig 3.1A), a stress-induced mitogen activated protein kinase (MAP3K), that is an important link and marker between oxidative stress and inflammation [15]. The disruption of binding of ASK-1 can reduce TRX-2 levels significantly and promote TXNIP-induced stress [13]. Indeed, this disruption in mitochondria promotes more binding of TXNIP to TRX-2 and inhibits the antioxidant reducing power of TRX-2 that could potentially cause unrestrained ROS levels [13]. Free ASK-1 is phosphorylated and allows release of cytochrome c in the cytoplasm. This can further induce a caspase-3 response and induce mitochondrial dysfunction and an apoptotic cascade[16]. Interestingly, hyperglycemic conditions in the cytoplasm can also enhance association of TXNIP with NLR Family Pyrin Domain Containing protein 3 (NLRP-3), a major protein involved in inflammasome assembly and inflammation (Fig 3.1A)[17, 18]. The inflammasome is a multiprotein complex that initiates an inflammatory response to ROS and triggers release of proinflammatory cytokines IL-1β and IL-18 [17]. The TXNIP-NLRP-3 induced inflammasome is known to be involved in Alzheimer’s disease, insulin resistance, type-2 diabetes and other conditions [19]. The TXNIP-NLRP-3 association modulates the conformation of NLRP-3 and allows it to bind to apoptosis-associated speck-like protein (ASC) that contains a caspase activation and recruitment domain (PYCARD) (Fig 3.1B) [13]. This assembly further combines with pro-caspase-1, causing its transformation to cleaved caspase-1 and leading to the
processing of the cytokine, interleukin-1β [13], that is released as a proinflammatory marker of cellular stress and damage [20].

Finally, it is also of interest to determine if different reversible protein modifications could be involved in the role and function of TXNIP in regulating these pathways. Reversible modifications of proteins are known to play a central role in animals undergoing MRD by coordinating the suppression of a diverse array of cell proteins ranging from metabolic enzymes and structural proteins to transcription factors [21]. For example, in a study of wood frogs, differential levels of phosphorylation of Protein kinase B (also known as Akt) on Ser-473 and Th-308 residues showed their role in selectively activating or reducing the activity of Akt, which has a major role in glucose metabolism, cellular energetics and cell survival [22]. The upregulation of Ser and Thr modifications in liver (which remains active until frogs are completely frozen) compared to downregulation in peripheral tissues (e.g. skeletal muscle) suggested a role in tissue-specific regulation of Akt post translational modifications (PTMs) during freezing [22]. Interestingly, TXNIP serine phosphorylation is also related to its association with NLRP-3 and, thereby, enhances overall inflammatory activity [23]. Another post-translational modification is O-N-acetyl glucosaminylation (O-GlcNAcylation) that involves addition of β-D-N acetylglucosamine to serine and threonine residues of proteins [24]. This PTM is nutrient-sensitive and responds to changing glucose flux in cells. It is controlled by two enzymes: the O-GlcNAc transferase (OGT), that transfers glucose monosaccharide to Ser or Thr residues on target proteins, and O-GlcNAcase (OGA), that removes the sugar [25]. Interestingly, a study showed
that TXNIP was under O-GlcNAcylation control under high glucose conditions that induced the inflammasome [25]. Other studies showed a role for ubiquitination (a PTM involved in degrading target proteins) under high glucose conditions in regulating TXNIP levels to restore the function of Trx protein[26, 27] that could also be required during recovery in frozen/dehydrated frogs.

To date there have been no studies done to analyze the role of subcellular localization of TXNIP in regulating the mitochondrial oxidative stress response and reporting physiological evidence of tissue inflammation in wood frogs. Therefore, the present study focuses on elucidating the role of TXNIP, its binding to other proteins, and its localization involved in mediating diverse stress responses during freezing and dehydration in multiple tissues (liver, skeletal muscle and kidney).

3.3 Methods

Animal treatment

Adult male wood frogs were collected during spring from breeding ponds around Ottawa. Animals were briefly washed in a tetracycline bath and then transferred into plastic boxes lined with damp sphagnum moss for two weeks acclimation at 5°C before experimentation. Control animals were taken from these conditions. Freezing experiments set up as described previously [9]. For dehydration treatment, acclimated frogs were held under dry conditions in containers lined with dry paper towels until 40% of total body water was lost, a survivable condition for this species [28].
Following each treatment, the frogs were euthanized by pithing and selected tissues were collected, flash frozen in liquid nitrogen and stored at -80°C. All the animal experiments had the prior approval of the Carleton University Animal Care Committee (Protocol #106935) and adhered to guidelines set by the Canada Council on Animal Care.

**Preparation of total protein extracts**

Samples of frozen tissues of liver, muscle and kidney (~50 mg each; n = 4 independent replicates from different animals) from 24H frozen and 40% dehydrated wood frogs were homogenised in 1:2 w:v in prechilled homogenization buffer. All steps to make total soluble protein extracts were performed as described before [9, 29].

**Preparation of mitochondrial and cytoplasmic protein extracts**

Mitochondrial protein extracts were prepared as described previously [30] using a commercially available kit: Mitochondrial Isolation Kit for Tissue (for Dounce homogenizer), catalog number: ab 110169, Abcam, Toronto, ON, Canada. Samples from liver, hind leg skeletal muscle and kidney were prepared from the frogs kept at control, frozen and dehydration conditions Briefly, 200-300 mg samples of frozen tissue (n=4 independent repeats from different wood frogs) were rapidly weighed and then washed multiple times in the PBS buffer provided with the kit (chilled at 4°C). Tissue was rapidly minced with dissection scissors (kept on ice) and extra cold wash buffer was removed. The washed tissue was homogenised in 1 mL of cold Isolation buffer with a Dounce homogeniser with number of pestle strokes as recommended by
the manufacturer for each tissue. Samples were then centrifuged as described above to discard the nuclear pellet followed by centrifugation 12000 x g at 4°C for 20 min to isolate the upper cytoplasmic fraction and mitochondrial pellet. The cytoplasmic fraction was removed and stored in separate 2 mL centrifuge tubes and the mitochondrial pellet was resuspended in 500 µL Isolation buffer and 5 µL protease inhibitor cocktail (Bioshop, ON, Canada, cat. No. P1C001.1) and saved as the final mitochondrial fraction. Protein concentrations in both cytoplasmic and mitochondrial fractions were determined using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) and all concentrations were standardized to 2 µg/µL. Samples were stored at -80°C until use. To evaluate the separation of cytoplasmic and mitochondrial fractions, western blotting was carried out and fractions were tested with antibodies characteristic of cytoplasmic (tubulin; ABclonal Cat# AC007) and mitochondrial (citrate synthase; ABclonal Cat# A5713) fractions to assess any cross-contamination (Appendix B, Fig. B1).

Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed to obtain a higher purity sample of TXNIP suitable for testing for PTMs and assess the binding partners of TXNIP (TRX-2, ASK-1 and TRX-1). A chemical cross-linking step was performed for the primary antibody before actual Co-IP could be performed to reduce final contamination by the antibody, usually the heavy (50 kDa) and light chain (25 kDa) of immunoglobulin, since the size of TXNIP is also ~50 kDa. This treatment covalently cross-linked the antibody to protein G beads. Protein G-Plus agarose beads (Santa Cruz, sc-2002) were extracted
from the stock and briefly centrifuged in a micro-centrifuge to separate supernatant from beads. The beads were resuspended in phosphate buffered saline (PBS: 10 mM Na$_2$HPO$_4$, 137 mM NaCl, 2.7 mM KCl, 2 mM KH$_2$PO$_4$, pH 7.4). The beads were kept on a tube rotator and were mixed overnight at 4°C. The next day, the beads were centrifuged as before, and supernatant was removed and replaced with dilution buffer (1X PBS plus 1 mg/ml BSA). The beads in dilution buffer were mixed again on a tube rotator for 10 minutes. The supernatant was removed and 2 µL of antibody (TXNIP: NBP1-54578, 1 mg/mL) diluted in 40 µL of dilution buffer was mixed with the beads for 2 h on tube rotator at 4°C. Beads were then centrifuged to remove supernatant and washed with equal volume of dilution buffer followed by PBS buffer. The supernatant was briefly centrifuged and discarded, followed by addition of a freshly prepared solution of dimethyl pimelimidate (DMP, 6.5 mg/mL) in wash buffer (0.2 M triethanolamine in PBS buffer) with pH adjusted to 8-9 and was then added in a 1:1 ratio and mixed with beads for 30 min at RT. The solution was discarded after above step and washed with wash buffer for 5 min, followed by adding fresh aliquot of DMP again as above. The beads were again washed with the wash buffer. The DMP and wash step was repeated two more times. This step ensures an adequate cross linking between bead and the antibody. After these steps, the supernatant was removed and an equal volume (1:1) of quenching buffer (50 mM ethanolamine in PBS) was added and incubated for 5 min. This step was repeated for 3 times, followed by replacing quenching buffer with PBS for 5 min. Beads were washed with equal volume of 1 M glycine (pH 3) for 3 x 10 min followed by final wash with wash buffer (4 x 5 min) to
ultimate clear the beads of any unlinked antibody. Beads were resuspended in wash buffer and stored until use. This same protocol was used to also prepare two negative controls, one containing IgG (Santa Cruz, sc-2025) and the other with only beads.

Tissue samples of liver, muscle and kidney (150-200 mg) from control, frozen or dehydrated frogs were homogenised in a medium salt buffer (23 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.1 M NaVO₄, 10 mM β-glycerophosphate, 10 µL/mL protease inhibitor). The samples were kept on ice for 5 min and then centrifuged at 12,000 x g for 30 min. The pellet was discarded, and the supernatant was transferred to a fresh 2 mL microcentrifuge tube. Protein concentration was measured using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) and samples were standardized 1.5 µg/µL using the medium salt buffer. An aliquot of 400 µL of this lysate was added to tubes containing beads (in a separate tubes for TXNIP and IgG) after discarding the extra wash buffer from the tubes. Samples were incubated with cross-linked beads for TXNIP, IgG for 4 h at 4°C on a tube rotator. The supernatant was removed after centrifugation in a micro-centrifuge and beads were washed 5 times with 500 µL of medium salt buffer to remove any non-specifically bound proteins. After the final wash the beads were resuspended in medium salt buffer (1:1). An equal volume of 2X SDS buffer (100 mM Tris buffer, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol, pH 6.8) was added to the tubes containing beads and samples were boiled for 5 minutes and cooled on ice to be used for Western blotting.
Western blotting

For immunoblotting, samples of extracted protein containing 20-30 µg of protein (from liver, muscle and kidney samples of control, 24 H frozen or 40% dehydrated) frogs were loaded on SDS-PAGE gels and electrophoresis was carried out using a Bio-Rad Mini Protean III apparatus at 180 V as described previously. The transferred protein membranes were incubated overnight with primary antibodies (diluted at 1:1000 in 1X TBST) at 4 °C. The membranes were exposed using a Chemi-Genius Bioimager (Syngene, Frederick, MD) for viewing chemiluminescent and Coomassie blue bands. The following antibodies were used: TXNIP (Novus Biologicals, NBP1-54578), NLRP-3 (Santa Cruz, Sc-134306), ASC (Abclonal Cat# A16672), Caspase-1 (Novus Biologicals, NBP1-45433), IL-1β (Santa Cruz, Sc-12742), TRX-2 (Abclonal Cat# A12591), ASK-1 (Abclonal Cat# A0283), Phosphoserine (Abclonal Cat#9332), O-GlycNacylation (Santa Cruz Cat# 55623), Ubiquitin (PTM Biolabs Cat# 1106), citrate synthase (Abclonal Cat# A5713), and tubulin (Abclonal Cat# AC007). The membranes were visualized with enhanced chemiluminescence reagents (ECL) and subsequently stained with Coomassie blue followed by imaging again. Coomassie blue staining was used for quantification of relative mitochondrial protein content in each lane and to standardize the intensity of the protein of interest to adjust for any differences in protein loading in each lane or gel.

Caspase-1 activity
Caspase-1 is known to be involved in initiating the apoptotic cascade. A commercial caspase-1 activity kit was used to assess the enzyme via recognition of the sequence YVAD that has a p-nitroanilide (pNA) group attached. The activity of caspase-1 directly correlates with cleavage of pNA from YVAD-pNA and the reaction was quantified spectrophotometrically in a 96 well plate. The assay was performed as per manufacturer’s guidelines. Briefly, tissue samples from liver, muscle and kidney of the wood frogs from control, frozen or dehydration conditions were collected from n=4 individuals. The tissue samples were weighed (20-40 mg) were homogenised in 50 μL of chilled cell lysis buffer using glass Dounce homogeniser. The Bio-Rad assay (Bio-Rad, #500-0005) was used to quantify protein concentration of each sample. To measure caspase-1 activity, ~80 μg of protein from each condition was added with 50 μL of chilled Cell Lysis Buffer and a mixed cocktail of 55 μL (10 mM DTT, 2X reaction buffer, and 5 μL of 4 mM YVAD-pNA substrate). The reaction was incubated at 37° C for 1.5 h and was quantified on 96 well plate at 405 nm.

**Multiple sequence alignment and conserved residues for PTMs**

A full sequence of TXNIP (NCBI accession # XP_002938510.2) from *Xenopus tropicalis* was aligned to the human sequence (NCBI accession # NP_006463.3) using the Clustal Omega multiple sequence alignment tool [31] to seek sequence conservations. The PhosphoSitePlus tool [32] was used to assess serine residues on human TXNIP for phosphorylation (P-serine) and lysine residues for ubiquitination. The sites located on human TXNIP protein for both P-serine and ubiquitination were then located in the conserved and aligned sequence of *Xenopus tropicalis*.
SWISS MODEL OF TXNIP-TRX-2 and structural difference analysis using Chimera

The amino acid sequences of *Xenopus tropicalis* TXNIP (NCBI accession # XP_002938510.2) and TRX-2 (CAJ81685.1) and Human TXNIP (NCBI accession # NP_006463.3) and TRX-2 (NCBI accession # AAH50610.1) were used to predict 3D-structure and association using a homology modelling 3D structure prediction tool, SWISS-MODEL [33]. For both hetero-tetramer models (2 chains of TXNIP, 2 chains of TRX-2), a previous X-ray-crystal validated structure of Human TXNIP and TRX-2 were used. The quality of both SWISS models generated in terms of structural validation were assessed by analyzing QSQE score, Molprobity, and Ramachandran plots. To compare Xenopus and human TXNIP-TRX-2 SWISS models and see their structural differences (interactive visualization and analysis of molecular interactions), the UCSF Chimera molecular modeling system was used [34].

Quantification and statistics

Bands on Western blots were visualized using a Chemi Genius bioimager (Syngene, Frederick, Maryland, USA), and the intensities of Western blot bands were standardized against a group of Coomassie-stained bands (either total, cytoplasmic, or mitochondrial protein as applicable) that were present in each lane and well-separated from the band of interest. This method is more robust and consistent than the expression of glycolytic targets such as GAPDH or other housekeeping proteins, that can change under stress [35]. The RBioplot statistical program was used to conduct unpaired Student’s t-tests to compare the data from control vs. 24 H frozen, or control
vs. 40% dehydrated wood frogs for both Western blotting and Caspase-1 activity assays with $P<0.05$ accepted as significant difference.

### 3.4 Results

**Protein responses of inflammasome activation under to freezing and dehydration stresses**

To assess the responses of the NLRP-3 induced inflammasome and its downstream targets, relative total protein levels of multiple targets were measured in liver, muscle and kidney from control, 24 H frozen, and 40% dehydrated wood frogs. Also, to assess the role of TXNIP in association with NLRP-3, cytoplasmic levels of this protein target were also analyzed.

During 24 H freezing, NLRP-3 protein levels showed a rising trend in liver but were not significantly different from control values (Fig 3.2A), whereas the levels of NLRP-3 decreased significantly in both muscle and kidney to 52% (Fig 3.2B) and 62% (Fig 3.2C) of control levels. ASC, which is a NLRP-3 inflammasome activating adapter protein, showed tissue specific regulation with levels increased during freezing in liver to 2.18 ± 0.2-fold over controls (Fig 3.2A) whereas both muscle and kidney showed significant decreases in ASC levels to 36% and 68% as compared to control wood frogs (Fig. 3.2B, C). Levels of caspase-1 rose significantly during freezing only in liver (by 1.42 ± 0.13-fold) (Fig 3.2A) but remained unchanged in both muscle and kidney (Fig. 3.2B, C).

Interestingly, despite the rise in caspase-1 levels in liver from frozen frogs, the levels of
IL-β remained unchanged (Fig 3.2A). IL-β levels were also unchanged in kidney (Fig 3.2C) whereas in muscle, levels of IL-β were reduced by nearly 70% as compared with controls (Fig 3.2B). The levels of cytoplasmic TXNIP (TXNIP-cyto) conformed with the general trend of the NLRP-3 inflammasome in liver, muscle and kidney during 24 H freezing. Cytoplasmic levels of TXNIP increased significantly in liver of frozen frogs to 1.91 ± 0.32-fold over controls (Fig 3.2A,) whereas TXNIP levels decreased in both muscle and kidney to 38% and 46%, respectively, compared with controls (Fig 3.2B, C).

Cellular dehydration is one consequence of freezing, since about two-thirds of total body water is converted to extracellular ice. Interestingly, dehydration stress also occurs independent of freezing. Amphibians are well-known for their ability to endure variable body water contents as a consequence of their generally highly permeable skin. Wood frogs also activate liver to produce glucose (thereby raising the osmotic pressure within cells) in response to dehydration (whole body water loss) which can make the cellular milieu hyperglycemic. To determine the role of dehydration independent of freezing, total protein levels and cytoplasmic TXNIP levels were assessed in liver, muscle and kidney of control frogs and of 40% dehydrated wood frogs (a level of body water loss that is known to be survivable by wood frogs)[28]. The relative protein expression of NLRP-3 changed significantly only in kidney of dehydrated frogs, rising to 2.31 ± 0.31 over control values (Fig 3.3C) but remained unchanged in both liver and muscle (Fig 3.3A, B). Cytoplasmic TXNIP levels increased only in liver of dehydrated frogs by 1.65 ± 0.18-fold (Fig 3.3A) but remained unchanged in muscle and kidney, as compared with controls (Fig. 3B and Fig 3.3C). Similarly, ASC
levels changed only in kidney, rising by 2.67 ± 0.28-fold (Fig 3.3C) but remained at control levels in both liver and muscle (Fig 3.3A, B). Interestingly, the levels of caspase-1, although unchanged in liver (Fig 3.3A), were significantly increased in both muscle and kidney by 1.4 ± 0.07-fold (Fig 3.3B) and 1.73 ± 0.3-fold (Fig. 3C), respectively, as compared to control frogs. Also, a band for a small proteolytic cleaved caspase-1 at 20 kDa was observed in kidney and showed a 40% rise during dehydration to 1.84 ± 0.18-fold over controls (Fig 3.3C). The levels of IL-β remained unchanged in all three tissues as compared to controls.

*Caspase-1 enzyme activity levels in freezing and dehydration*

Caspase-1 activity was also assessed in liver, muscle and kidney under dehydration stress in addition to protein levels of the enzyme (measured via Western blotting). The level of Caspase-1 activity directly translates to the active form of this enzyme that is sometimes not recognised by measuring autoproteolysis of caspase-1 via Western blotting [36]. During 24 H freezing, caspase-1 activity remained unchanged in liver and muscle but increased significantly in kidney to 2.34 ± 0.37-fold above control values (Fig 3.4A). However, during dehydration stress, caspase-1 activity increased in both liver (by 1.72 ± 0.05-fold) and kidney (by 1.52 ± 0.18-fold) (Fig 3.4B) but did not change significantly in skeletal muscle.

*Responses by targets of mitochondrial dysfunction and cytoplasmic cytochrome c expression during freezing and dehydration*
Protein targets that can indicate mitochondrial dysfunction (TXNIP, ASK-1, TRX-2) were analyzed in isolated mitochondrial fractions. Since TXNIP binding to TRX-2 can displace ASK-1, ultimately leading to release of cytochrome c into the cytoplasm, levels of Cyt c in the cytoplasmic protein fraction were also assessed. Levels of TXNIP were elevated after 24 H freezing in liver by 1.70 ± 0.13-fold (Fig 3.5A), whereas in both muscle and kidney TXNIP levels showed an upward trend but were not significantly different from controls (Fig 3.5B, C). Levels of TRX-2 in liver from 24H frozen frogs showed a similar pattern, rising 1.5 ± 0.02-fold (Fig 3.5A), whereas levels of TRX-2 were unchanged in both muscle and kidney (Fig 3.5B, C). Furthermore, ASK-1 levels were reduced to 56% of control values in liver of 24H frozen frogs (Fig 3.5A), but ASK-1 was unchanged in both muscle and kidney during freezing (Fig 3.5B, C). Cytoplasmic cyt c showed unique responses during 24H freezing in all tissues. Cyt c levels were unchanged in both liver and muscle during freezing (Fig 3.5A, B) but decreased significantly in kidney to 46% of control values (Fig 3.5C).

Under 40% dehydration stress, the levels of TXNIP increased significantly to 1.47 ± 0.16-fold over controls in liver of wood frogs (Fig 3.6A) but levels of TXNIP were unchanged in both muscle and kidney from dehydrated frogs as compared to control wood frogs (Fig 3.6B, C). The levels of TRX-2 rose significantly in both liver and kidney by 1.47 ± 0.16 and 3.63 ± 0.8-fold, respectively, under dehydrating conditions (Fig 3.6A, C), whereas they were unchanged in muscle (Fig 3.6B). ASK-1 levels decreased significantly in both liver and kidney of 40% dehydrated frogs, falling to 56% and 66% of control values (Fig 3.6A, C), whereas levels of ASK-1 remained close to control values.
in muscle (Fig 3.6B). Finally, levels of cytoplasmic Cyt c increased in liver of 40% dehydrated frogs to 1.4 ± 0.1-fold over control values (Fig 3.6A) but decreased significantly in both muscle and kidney to 20% and 66% of control values, respectively, under 40% dehydration stress (Fig 3.6B, C).

**TXNIP-TRX-2 binding during freezing and dehydration stress**

Immunoprecipitated TXNIP was also assessed to analyze binding partners via Western blotting. TRX-2 showed binding affinity to TXNIP in test blots (with pooled samples) in all tissues under both freezing and dehydration stress. Levels of TRX-2 showed a significant decrease in the liver of 24H frozen wood frogs to 57% of controls values (Fig 3.7A), whereas binding to TXNIP immunoprecipitated beads did not change in either muscle or kidney during freezing (Fig 3.7A). Interestingly, in tissue of 40% dehydrated frogs the levels of TRX-2 binding to TXNIP immunoprecipitated beads increased in kidney by 2.08 ± 0.23-fold but did not change significantly in either liver or muscle compared to controls (Fig 3.7B).

**Sequence alignment and conserved residues for PTMs of TXNIP**

Fig 3.8A shows that the TXNIP amino acid sequence of the frog, *X. tropicalis*, is substantially conserved with over 75% residues matching the human TXNIP sequence. Fig 3.8B shows conserved residues for both ubiquitination on lysine (K) residues (highlighted in yellow) and phosphorylation on serine (S) residues (highlighted in green) in *X. tropicalis* compared with the human sequence.

**Post translational modifications (PTMs) of TXNIP during freezing and dehydration**
Post translational modifications on immunoprecipitated TXNIP were assessed by Western blotting using PAN-specific antibodies recognizing serine phosphorylation, O-GlcNAcylation, and ubiquitination modifications. During freezing, only wood frog liver ubiquitination levels rose (by 2.8 ± 0.32-fold compared with controls) for immunoprecipitated TXNIP (Fig. 3.9A). In muscle from frozen frogs, levels of O-GlcNAcylation were reduced to 36% of the control values and remained unchanged for the other two modifications (Fig 3.9B). Interestingly, in kidney from frozen frogs the levels of all three modifications remained close to control values (Fig 3.9C).

Dehydration stress showed a different response to PTMs compared to freezing in all three tissues. In liver from frozen frogs, the phosphorylation of serine residues increased 1.8 ± 0.13-fold as compared to control frogs (Fig 3.10A), whereas in muscle from dehydrated frogs the level of ubiquitination rose by 1.57 ± 0.08 fold above controls (Fig 3.10B). Interestingly, unlike kidney from frozen frogs, kidney from dehydrated animals showed a significant increase in serine phosphorylation, O-GlcNAcylation, and ubiquitination levels to 2.62 ± 0.53-fold, 1.81 ± 0.22-fold and 1.85 ± 0.06-fold above controls, respectively (Fig 3.10C).

**Bioinformatics**

To assess the binding efficiency of TXNIP and TRX-2 observed via co-immunoprecipitation and relate it with functional downstream responses in activating mitochondrial dysfunction, an *in silico* analysis of binding by TXNIP and TRX-2 was generated using a SWISS-MODEL for both the Western clawed frog, *Xenopus tropicalis* (Fig 3.11A) and *Homo sapiens* (Fig 3.11B). In comparison to the human model, the
Xenopus model showed conservation of cysteine (Cys) residues (highlighted in yellow) (Fig 3.11A). Cys 63 and Cys 32 were present in Xenopus whereas Cys 247 residue was only present in the human sequence. An overlapping model of Xenopus (distinct colored chains) compared with the human sequence was also generated (regions matching to Xenopus are highlighted in green) to view conformational matches and mismatches in a TXNIP-TRX-2 (Fig 3.12) model generated for Xenopus.

3.5 Discussion

The α-arrestin family protein, TXNIP, is a scaffold protein that plays a crucial role in negatively regulating glucose uptake, glycolytic flux and oxidative stress via mitochondria in many stress models [18, 37–39]. Indeed, overexpression of TXNIP has been observed under hyperglycemic conditions, that induce ROS damage in cells and has also been related to its role in inducing inflammatory and mitochondrial dysfunction pathways [13, 18, 38, 40]. Another crucial aspect of the role of TXNIP in inducing the cellular stress signalling cascade is its subcellular localization and ability to regulate target protein expression via multiple protein-protein interactions with TRX1 in the cytoplasm and TRX-2 in the mitochondria [13]. Also, under hyperglycemic conditions, the regulation and activation of TXNIP is under control by the MondoA-MLX complex [9]. Furthermore, a role for TXNIP has also been seen in other stress-tolerant animals such as hibernating mammals (a Chilean marsupial, Dromiciops gliroides) and cellular models [41, 42]. These studies suggest that the role and balance of TXNIP are crucial under cellular stress conditions in diverse animal, cell or disease models and can
act as a double-edged sword depending on the conditions and requirements of the cellular milieu in activating stress-inducing or pro-survival pathways. So, in the present study, the aim was to determine the role of active TXNIP in various cellular compartments in activating tissue-specific inflammation and mitochondrial dysfunction under different environmental conditions inducing hyperglycemia (freezing and dehydration) in freeze-tolerant wood frogs.

**Inflammasome activation and caspase-1 activity during freezing and dehydration stress**

Both freezing and dehydration stresses lead to hyperglycemia in wood frogs that can cause ROS-induced damage. Therefore, under these stress-specific conditions, it was of interest to analyze the role of cytoplasmic TXNIP in activating the inflammasome via NLRP-3 in liver, muscle and kidney tissues of wood frogs. Under 24 H freezing stress, the levels of cytoplasmic TXNIP (TXNIP-cyto) were upregulated in liver in conjunction with NLRP-3, whose levels rose above controls but were not significantly different (Fig 3.2A). Interestingly, the levels of ASC, a downstream target and adaptor protein that induces priming of inflammasome formation via NLRP-3 also increased during 24H freezing (Fig 3.2A). ASC also contains a caspase-recruitment moiety to ultimately activate caspase-1 protein, whose levels were also significantly upregulated (Fig 3.2A) [20]. Active caspase-1 is involved in proteolytic cleavage of the inflammatory cytokine IL-β whose levels remained at control levels in liver samples from 24 H frozen frogs (Fig 3.4A) that could be due to unchanged levels of active caspase-1 (Fig 3.4A). Overall, higher expression of NLRP-3 inflammasome proteins hints that wood frogs might keep
this pathway active enough to induce damage responses if required. Also, a study has suggested the role of hyperglycemic ROS-induced NLRP-3 response with another TF, nuclear factor erythroid 2-related factor 2 (Nrf-2) that once active can diminish the levels of inflammatory markers such as IL-β [43, 44]. An active role of Nrf2 has been established in wood frogs and other stress-tolerant animal models [45, 46]. Therefore, in the liver of frozen frogs, the association between NLRP-3 and Nrf2 could potentially maintain the internal cellular milieu via Nrf-2 keeping IL-β levels in check (Fig 3.2A) until damage signalling response is needed to wade of damaged components [47]. In freezing muscle, unlike the liver, a lower response of overall levels of TXNIP-cyto, NLRP-3 and ASC were observed (Fig 3.2B). Despite no change in caspase-1 expression or activity (Fig 3.2B, 4A), a lower expression of IL-β was seen (Fig 3.2B). A lower expression of IL-β does hints at a profile of lower inflammation. This is relevant to the functional role of this peripheral tissue, whose metabolic activity drops early in the freezing process as ice penetrates from the skin surface inwards through the skeletal musculature and finally to the internal organs, liver being the last tissue to freeze due to its very high glucose content. Lastly, the NLRP-3 inflammasome response in the kidney of frozen wood frog samples was similar to that in muscle except for the IL-β response (Fig 3.2C). The levels of cyto-TXNIP were lower in conjunction with NLRP-3 levels observed (Fig 3.2C), which aligned well with the adapter protein, ASC (Fig 3.2C). Despite the downregulation of ASC, the levels of caspase-1 protein (Fig 3.2C) did not change and remained high at control levels, but caspase-1 activity was upregulated (Fig 3.4A). Although, an active caspase-1 levels could not further induce proinflammatory
cytokine IL-β (Fig 3.2C). But, other studies have suggested the role of caspase-1 activity in NLRP-3 independent manner in cell survival pathways as well [48]. For example, a study done in a murine model showed the involvement of Receptor Interacting serine/threonine kinase 2 (RIP2) in inducing caspase-1-induced NF-κB activation. NF-κB is an oxygen-sensitive transcription factor involved in activating the stress response in inflammation [49]. Interestingly, the RIP2/caspase-1 axis is disturbed by ASC as it binds to caspase-1 and shifts the momentum towards inflammation [50]. The decrease in the levels of ASC observed (Fig 3.2C) in conjunction with an overall heightened response of caspase-1 (Fig 3.2C, 4A) could possibly induce a tissue-specific NF-κB activation response such as has previously been shown to have an active role in many different stress-tolerant animal models [51–54].

Cellular dehydration is a consequence of freezing but can also occur independently of freezing stress. Indeed, most amphibians are very susceptible to changes in body water content due to their highly permeable skin. Like freezing, wood frogs respond to dehydration stress by production of large amount of glucose, showing a hyperglycemic stress response [55]. Hence, the present work evaluated both freezing and dehydration separately to demark the dehydration-specific responses involved in activating the TXNIP-induced inflammasome pathway in contrast to the freezing-specific stress responses. Under dehydration stress, liver showed no change in NLRP-3 inflammasome proteins despite an increase in TXNIP levels (Fig 3.3A). Only caspase-1 activity was enhanced (Fig 3.4B). Interestingly, an active caspase-1 (Fig 3.4B) could plays a role in caspase-1 mediated secretion of many proteins involved in cytoprotection and tissue
repair [56]. In dehydrated muscle, the overall response of the NLRP-3 inflammasome proteins and TXNIP-cyto was unchanged compared with control wood frogs (Fig 3.3B). Only caspase-1 protein levels (Fig 3.3B) were upregulated, but its activity remained at control levels under 40% dehydration stress (Fig 3.4B). Interestingly, a study done in mouse muscle cells showed that a lack of caspase-1 levels induces fast twitch muscle fibres with even shorter contraction and relaxation times than required in response to ischemia [57]. During dehydration, wood frogs are not frozen and can use their available substrates and energy to move to better/moist areas nearby, where they don’t have to face the challenge of dehydration. Hence, overall regulation of muscle tissue for movement becomes very important, using both fast and slow-twitch movements, which could be crucial for frog survival. Interestingly, unlike the liver and muscle, the kidney showed heightened NLRP-3-induced signalling despite no change in cyto-TXNIP levels (Fig 3.3C). The kidneys deal mainly with burden of osmoregulation and ionic balance under dehydration stress and induce cellular responses such as heat shock proteins (HSPs) as demonstrated in multiple studies on wood frogs and other dehydration-tolerant anurans [58, 59]. HSPs such as HSP 90 and HSP 70 are known to be negative regulators/inhibitors of the NLRP-3 inflammasome and can help to avoid osmotic stress-induced cell disintegration [60, 61]. Interestingly, a study done with another dehydration-tolerant anuran, *Xenopus laevis*, showed downregulation of both HSP 70 and HSP 90, which would lead to an overall upregulation of NLRP-3 inflammasome proteins (Fig 3.3C), including a heightened caspase-1 expression and activity (Fig 3.3C, 3.4B). Interestingly, despite this heightened response of caspase-1, IL-
β levels remained unchanged (Fig 3.3C). One possible explanation in stagnant IL-β response could be the role of other HSPs such as HSP 27 whose upregulation is directly linked to diminishing or maintaining IL-β levels[62, 63]. Interestingly, the levels of HSP 27 were heightened in the *Xenopus laevis* study mentioned above. This hints at a possible role and crosstalk of different HSPs proteins with the NLRP-3 inflammasome in maintaining the cellular milieu in the kidney under dehydration stress response in wood frogs.

**TXNIP induced mitochondrial dysfunction during freezing and dehydration stress**

The role of TXNIP in disrupting the mitochondrial antioxidant response under freezing or dehydration conditions was explored in liver, muscle and kidney of 24H frozen and 40% dehydrated frogs (Fig 3.5, 6, 7). During 24H freezing, levels of TXNIP in mitochondria were elevated (Fig 3.5A). It is interesting to note that hyperglycemia-induced TXNIP can inhibit the function of TRX-2 by binding to the active site and diminishing its reductase activity in mitochondria[64]. Despite this, protein levels of TRX-2 were induced during 24H freezing (Fig 3.5A). Lower levels of ASK-1, which is usually a binding partner of TRX-2 under non-stressed conditions, hinted that TXNIP could displace it [14]. To assess the role of TXNIP in mediating TRX-2 function, binding between the two was analyzed (Fig 3.7A). Levels of TRX-2 binding to TXNIP were reduced during 24H freezing (Fig 3.7A), which was surprising and did not match with the overall protein expression of TRX-2 (Fig 3.5A). This observation hinted that despite the overexpression of TXNIP in the mitochondria, other forms of regulation of this protein (e.g., PTMs, structural conformations) could be limiting its binding to TRX-2 to a
minimum, needed to mitigate damage to liver mitochondria. This could also be seen in the levels of mitochondrial and cellular damage marker, cytoplasmic marker cytochrome c (Cyto-Cyt c) which remained at control levels during 24H freezing (Fig 3.5A). These observations also aligned with the previous studies done in freezing wood frog model, showing a heightened response of mitochondrial-specific genes and protein responses in the liver likely contributing to keeping overall freeze-induced damage to mitochondria at bay [65]. Unlike the liver, both muscle and kidney samples from frozen wood frogs showed no change in TXNIP-induced signalling to disrupt antioxidant functions (Fig 3.5B, 3.5C) or in its binding to TRX-2 in either tissue (Fig 3.7A). Despite the unchanged protein axis in both tissues, the levels of cytoplasmic cytochrome c (Fig 3.5C) were downregulated in kidney during 24H freezing. Lower levels of cytochrome c mostly occur in the cytoplasm when antioxidant or antiapoptotic responses are upregulated [66, 67]. Interestingly, a study done with the wood frog model showed a heightened response by antioxidant glutathione pools in kidney of 24H frozen frogs [68], which is also known to act on cytochrome c in the cytoplasm and keep it in a reduced or inactive state [69].

During dehydration stress in the liver of the wood frogs, the mitochondrial dysfunction pathway proteins showed a similar trend as frozen with an overall upregulation of both TXNIP and TRX-2 and downregulation of ASK-1 (Fig 3.6A). However, despite upregulated levels of TXNIP and TRX-2, the binding efficiency as seen via co-immunoprecipitation between the two proteins did not change (Fig 3.7B) as compared to control samples. This suggests that TXNIP and TRX-2 binding might not
play a major role in inducing mitochondrial dysfunction damage as was implicated via
the upregulation of the cytoplasmic damage marker Cyto Cyt c (Fig 3.6A). This suggest
that a rise in Cyto Cyt c levels observed here might be via a route independent of TXNIP
and TRX-2 binding [70]. In the case of muscle from dehydrated frogs, the levels of
mitochondrial TXNIP, ASK-1 and TRX-2 remained unchanged (Fig 3.6B). Also, there was
no change in binding efficiency of TXNIP with TRX-2 in muscle of dehydrated frogs (Fig
3.7B). However, the protein levels of Cyto Cyt c were reduced significantly (to below
basal levels) in muscle from 40% dehydrated frogs (Fig 3.6B), hinting that Cyto Cyt c
induced damage signalling remains at bay. Again, muscle from dehydrated frogs was a
more active tissue than muscle from frozen frogs (as mentioned above) and keeping
damage below control levels can ensure better tissue survival under stress.
Interestingly, only kidney samples from dehydrated frogs showed an increase in TXNIP
and TRX-2 binding as compared with controls (Fig 3.7B), even when only TRX-2 levels
were elevated (Fig 3.6C). Furthermore, lower expression of ASK-1 was also observed
but did not correlate with levels of Cyto Cyt c that surprisingly remained low (Fig 3.6C).
This hinted that in samples from dehydrated kidney, overall mitochondrial dysfunction
damage was low, and other proteins or alternate mechanisms might be involved in
keeping damage response at a minimum. Another possibility could be that in kidney
TXNIP-TRX-2 binding remained high in anticipation of a further increase in dehydration
stress and only aids in the induction of Cyto Cyt c levels, that might be under greater
control by other mechanisms or proteins [71]. The overall independence of Cyto Cyt c
expression of TXNIP and TRX-2 binding in freezing and dehydration hints at an
alternate regulation of TXNIP or structural compatibility of TXNIP and TRX-2 (discussed below).

**Post-translational regulation of TXNIP in freezing and dehydration**

The role of PTMs lies in functional proteomics that usually ranges from stabilizing the proteins, aiding in correct folding, conformation, subcellular localizations or interactions with other proteins to induce signalling or respond to cellular disturbances[72]. Interestingly, TXNIP, which is expressed ubiquitously, was found to be highly conserved when the human sequence was compared with another stress-tolerant amphibian, *Xenopus tropicalis* (Fig 3.8A). Assuming the PTMs will be conserved and will induce various effects in either stabilizing or degrading the levels of TXNIP in wood frogs, we assessed their expression levels on TXNIP protein. During 24H freezing stress, a rise in ubiquitination levels in the liver (Fig 3.9A) indicated an overall preference of degradation for TXNIP. Interestingly, ubiquitination as a PTM is active in modifying lysine residues on the protein sequence, as compared with the human TXNIP sequence that showed most of the residues were conserved (Fig 3.8B). Despite this, TXNIP levels were upregulated (Fig 3.2A, 3.5A). A plausible explanation could be that ubiquitin modifications are usually very dynamic and conjugation of the ubiquitin to lysine residues on TXNIP might not always lead to degradation, as the presence of deubiquitinating enzymes associated with the proteasome could detach ubiquitin chains [73]. In muscle of frozen frogs, the decrease in O-GlyNAcylation levels (Fig 3.9B) coincided with an overall downregulation of the TXNIP-NLRP-3 axis (Fig 3.2B). Multiple studies have shown that O-GlyNAcylation increase under hyperglycemic conditions and
have been shown to induce TXNIP to activate the NLRP-3 inflammasome [25, 74]. However, in contrast to both liver and muscle, kidneys from frozen frogs did not show a response to any major PTM tested.

The levels of phosphoserine (P-serine) in liver of dehydrated frogs (Fig 3.10A) were upregulated and this is known to be involved in the degradation of TXNIP via multiple signalling cascades [75–78]. Despite this, overall TXNIP levels (Fig 3.3A, 3.6A) remained high in liver, similar to those in the liver of frozen frogs. Phosphorylation on Ser 308 and Ser 361, known to be conserved (Fig 3.8B), relate to degradation of TXNIP via ITCH signaling induced proteasomal degradation [76, 78]. However, another study showed that a mutation at Ser 298, Ser 300 or Ser 304 sites, also shown to be conserved (Fig 3.8B), can blunt the overall expression of TXNIP [77]. Therefore, the upregulated P-serine levels could actually act in inducing TXNIP levels during dehydration stress. Furthermore, upregulated ubiquitination levels in muscle from dehydrated frogs (Fig 3.10B) could contribute to maintaining the expression of TXNIP at control levels (Fig 3.3B, 3.6B) and this could be involved in downregulating both cell-damaging pathways in the muscle. Finally, in kidney samples from 40% dehydrated frogs, all three PTMs were induced (Fig 3.10C). Ideally, an upregulation of O-glycNAcylation (usually on Ser/Thr residues) under hyperglycemic conditions could promote the TXNIP-induced NLRP-3 axis, that was also upregulated (Fig 3.3C), but a simultaneous upregulation of both phosphorylation and ubiquitination levels hinted at a more intricate form regulation. Interestingly, phosphorylation-induced degradation of TXNIP via ubiquitination is also a well-established concept [79]. Another possibility
could be that TXNIP also has a binding domain (PPXY sequence towards the C-terminal) that is recognized by the Itchy E-3 ubiquitin-protein ligase (ITCH) at lys 63 (also conserved in the amphibian gene) (Fig 3.8B). The binding of ITCH could maintain levels of TXNIP to promote TRX-2 expression (Fig 3.6C) [27, 79]. Another important and interesting fact is that O-GlycNAcylation can also regulate protein ubiquitination via phosphorylation. This is because O-GlycNAcylation can undergo cross-talk with phosphorylation via sharing the same or adjacent residues (Ser/Thr), and O-GlycNAcylation induced phosphorylation can further induce ubiquitination [80, 81].

**Predicted 3D frog and human models of TXNIP-TRX2 decipher structural differences and binding efficiency**

An overall dissimilarity of TXNIP-TRX-2 binding compared to their individual protein levels in the mitochondria (via Co-IP), leads to assessing the role of putative TXNIP structural variations. TXNIP contains cysteine residues (cys 63, cys 247) that aid to bind with TRX (cys 32, cys 35) residues via disulphide bonds [82]. The structural variations of TXNIP due to differences in cysteine residues can disrupt its conformation as well as its binding to TRX-2. For example, cys 63 and cys 247 residues on TXNIP have been shown to form an intramolecular disulphide bond that can mediate conformational change to the structure of TXNIP and affects its binding to TRX chains [82]. This conformational change can further aid in forming a stable TXNIPcys247-TRXcys32 disulphide bond required to activate a downstream stress response [83]. Interestingly, three of these cysteine residues were conserved in the *Xenopus tropicalis* sequence (Fig 3.11A), except for cys 247 that was found in the human but not frog TXNIP sequence (Fig
3.11B). The difference in the residue cys 247, could be the reason not seeing, appropriate conformational changes to TXNIP with respect to its binding to TRX-2 (Fig 3.12). Overall, this is an interesting finding using predictive tools and homology models, showing that a difference in a conformational change in TXNIP could be responsible for its slow/lower response observed in recognizing TRX-2.

**Conclusion**

This study summarizes the role of TXNIP in activating mitochondrial dysfunction and NLRP-3 stress signalling under freezing and dehydration stresses in wood frogs. TXNIP plays a role in activating the NLRP-3 inflammasome in a stress-specific manner, where positive/negative regulation of cytoplasmic TXNIP could regulate an overall NLRP-3 protein response to freezing in all three tissues. By contrast, during dehydration, overall NLRP-3 regulation seems not to be affected. In any case, the data correlate with previous studies with wood frogs that showed that a heightened antioxidant/pro-survival pathway response might act in conjunction under both freezing or dehydration stress to mitigate the stress response signalled by NLRP-3. Interestingly, when mitochondrial localization of TXNIP was assessed, only liver showed a stress-specific response during both freezing and dehydration with an increase in overall TXNIP and TRX-2 levels. However, this increase in expression did not translate into increased binding between the two proteins. An overall decrease or unchanged levels of cytoplasmic cytochrome c hinted at downregulation of this pathways and a role for other players and mechanisms. Post-translational modifications of TXNIP did shed some light on unexpected changes in the expression of the NLRP-3 inflammasome and
mitochondrial dysfunction. A mismatch between mitochondrial TXNIP and TRX-2 levels to their binding levels (via Western blotting) could be attributed to missing a major residue (cys 247) in the frog sequence of TXNIP protein when matched with human sequence via structural bioinformatics webs servers such as homology modelling in SWISS-Models and UCSF Chimera. Overall, this study establish a role for TXNIP in activating inflammasome in stress-specific way during freezing while mitochondrial presence of this protein did not correlate to protein expression of downstream targets in inducing mitochondrial dysfunction damage which was attributed to PTMs and structural differences of TXNIP.
3.6 References


3.7 Figures

A

![Diagram showing the effects of freezing and dehydration on cellular processes involving ROS (reactive oxygen species), TXNIP, NLRP3, and mitochondria. The diagram illustrates the flow of glucose, oxidative stress, and the involvement of cytokine activation.](image-url)
**Figure 3.1:** A) Schematic representation of TXNIP induced inflammasome activation and mitochondrial dysfunction pathway. Under control conditions, TXNIP is in the nucleus while cell’s NLRP-3 inflammasome is not assembled and antioxidant system via TRX-2-ASK-1 is active in the mitochondria. During freezing/dehydration induced hyperglycemic stress, subcellular localization of the TXNIP into cytoplasm and mitochondria activate inflammasome and mitochondrial dysfunction. Both of these pathways activate a stress response either via caspase-1 or via releasing mitochondrial cytochrome c in the cytoplasm activating cytokine mediated damage signalling response. (image created with www.BioRender.com) B) Schematic representation of NLRP-3 assembly. Sensor protein is NLRP-3 upon recognising damage signal response attaches to ASC and arrange inflammatory caspase (caspase-1) which if cleaved is ready to induce inflammatory damage signaling via activating cytokines.
Figure 3.2: Relative protein expression of cytoplasmic TXNIP-NLRP3 induced inflammasome and downstream targets (ASC, caspase-1, IL-Beta) in A) liver samples, B) muscle samples, and C) Kidney samples from control and 24H frozen wood frogs. Representative bands are shown below each Figure. Data are mean ± SEM, n= 4 independent biological replicates. Statistical testing used the Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.
Figure 3.3: Relative protein expression of cytoplasmic TXNIP-NLRP3 induced inflammasome and downstream targets (ASC, caspase-1, IL-Beta) in A) liver samples, B) muscle samples, C) Kidney samples from control and 40% dehydrated wood frogs. Representative bands are shown below each figure. Statistical testing used Student’s t-test with P<0.005. * denotes values that are significantly different from controls.
Figure 3.4: Relative Caspase-1 activity in liver, muscle and kidney samples from A) 24 H frozen, and B) 40% dehydration stressed wood frogs. Statistical testing used Student’s t-test with P<0.005. * - denotes values that are significantly different from controls.
Figure 3.5: Relative expression of proteins involved in TXNIP-induced mitochondrial dysfunction and activating targets (ASK-1, TRX-2, Cytoplasmic Cyt c) in A) liver samples, B) muscle samples, and C) kidney samples from control and 24 H frozen wood frogs. Representative bands are shown below each Figure. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.
Figure 3.6: Relative expression of proteins involved in TXNIP induced mitochondrial dysfunction and activating targets (ASK-1, TRX-2, cytoplasmic Cyt c) in A) liver, B) skeletal muscle, and C) kidney samples from control and 40% dehydrated wood frogs. Representative bands are shown below each figure. Statistical testing used Student’s t-test with P<0.005. * denotes values that are significantly different from controls.
Figure 3.7: Relative protein expression of TRX-2 on TXNIP immunoprecipitated beads comparing liver, muscle and kidney samples from A) 24H frozen, and B) 40% dehydrated wood frogs. Representative bands are shown below each figure. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.
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Figure 3.8: A) Multiple sequence alignment of TXNIP comparing *Homo sapiens* (NCBI accession # NP_006463.3) and *Xenopus tropicalis* (NCBI accession # XP_002938510.2). B) Conserved lysine (K) residues involved in ubiquitination are highlighted in yellow and conserved serine (S) residues involved in phosphorylation are highlighted in green in both sequences.
Figure 3.9: Relative expression of posttranslational modifications of TXNIP in tissues of 24 H frozen wood frogs: **A)** liver, **B)** muscle, and **C)** kidney, as compared to controls. Representative bands are shown below each Figure. Statistical testing used Student’s t-test with $P<0.005$. *- denotes values that are significantly different from controls.
**Figure 3.10:** Relative expression of posttranslational modifications of TXNIP in tissues from 40% dehydrated wood frogs: **A)** liver, **B)** muscle, and **C)** kidney compared to controls. Representative bands are shown below each Figure. Statistical testing used Student’s t-test with $P<0.005$. * denotes values that are significantly different from controls.
Figure 3.11: SWISS homology models assessed by the UCSF Chimera molecular modeling program showing TXNIP and TRX-2 interaction in A) *Xenopus tropicalis* B) *Homo sapiens*. Representative cysteine residues (CYS) are shown with arrows. TXNIP chains are in purple and green and two side chains in pink and gold represent TRX-2. Selected amino acid residues are labeled.
Xenopus-Human TXNIP-TRX models overlap

Figure 3.12: Overlapping model of TXNIP-TRX2 from Xenopus (distinct colored chains) and human. Regions matching to Xenopus are highlighted in green showing conformation and structural changes between the two models.
Chapter 4: Mitochondrial epigenetics regulation
Mitochondrial DNA methyltransferases and their regulation under freezing and dehydration stresses in the freeze tolerant wood frog, *Rana sylvatica*

Gurjit Singh and Kenneth B. Storey

Department of Biology, Carleton University, 1125 Colonel By Drive,

Ottawa, Ontario, Canada K1S 5B6

(This study is published in *Biochemistry and Cell Biology* journal and reproduced in whole to be used as part. Additionally, 5mC methylation results have been added to the thesis chapter which were not published in the original manuscript)

Correspondence to K.B. Storey:  kenstorey@cunet.carleton.ca

Tel: 613-520-2600, ext. 3678

ORCID:  K.B. Storey  0000-0002-7363-1853
4.1 Abstract

Wood frogs are one of a few vertebrate species that can survive whole-body freezing. Multiple adaptations support this including cryoprotectant production (glucose), metabolic rate depression and selective changes in gene/protein expression to activate pro-survival pathways. The role of DNA methylation machinery (DNA methyltransferases, DNMTs, 5mC levels) in regulating nuclear gene expression supporting freezing survival has already been established. However, a comparable role for DNMT methylation machinery in mitochondria has not been explored in wood frogs. We examined the mitochondrial protein levels of DNMT-1, DNMT-3A, DNMT-3B and DNMT-3L, and mitochondrial DNMT activity as well as levels of 5mC methylation marks in the liver and heart to assess the involvement of DNMT machinery in the survival of freezing and dehydration stresses (cellular dehydration being one component of freezing). Our results showed stress and tissue-specific responses by mitochondrial DNMT-1 protein in liver and heart, respectively. During 24h freezing and whole body dehydration, we saw an overall downregulation of mitochondrial DNMT-1, a major protein involved in maintaining methylation levels related to its role in selective transcription of mitochondrial genes as well as antioxidant response. Tissue-specific responses by protein levels of DNMT-3A, DNMT-3B and DNMT-3L, DNMT activity and induced 5mC levels suggested a preference for a higher methylation state in the liver of frozen frogs but an unchanged 5mC response in liver of dehydrated animals suggested an anticipatory response of inducing a state of quiescence with a gradual increase in dehydration stress. Heart showed a different response than liver.
under both freezing and dehydration stresses. The mitochondrial profile for DNMT proteins, DNMT activity, and 5mC methylation levels was mostly reduced or unchanged across the two stresses suggesting a role for stress-specific responses by the mitochondrial machinery according to changes in the cellular environment under each stress.

Keywords: DNMT activity, metabolic rate depression, antioxidant response, mitochondria, mitochondrial proteins.
4.2 Introduction

Wood frogs, *Rana sylvatica*, combat multiple stresses associated with winter whole body freezing (e.g., anoxia, ischemia, cell/organ dehydration, hyperglycemia) by switching “ON” their survival mode to suppress energy expensive processes, undergo a state of MRD, and selectively upregulate selected genes involved with activating pro-survival processes [1]. To reduce their energy expenditure and enter a hypometabolic state, wood frogs have been shown to undergo nuclear transcriptional silencing using various epigenetic mechanisms [2, 3]. Mitochondria are usually central to energy metabolism in all animals but are under stress during freezing (and its associated stresses such as dehydration) and become a liability as they are a primary site for ROS production under anaerobic conditions [4, 5]. Interestingly, studies involving mitochondrial induced stress and allied damage responses have implicated a role for epigenetic modifications of mitochondrial genes such as DNA methylation, in supporting various changes in gene expression and protein levels [6–8]. In addition, studies of wood frogs have also shown differential regulation of gene transcripts and proteins involved with mitochondrial regulation during freezing and its associated stresses, and therefore, an interest in understanding the role of mitochondrial epigenetics in wood frogs was eventuated [4, 5, 9].

Studies from our lab have established a role for nuclear epigenetic mechanisms in the interplay between DNA methylation and DNA methyltransferases (DNMTs) in suppressing nuclear gene transcription in various animal models including wood frogs [3, 10-11]. The role of DNMTs is highly conserved across eukaryotes not only the
nucleus but also in mitochondria [6, 8, 12], where these enzymes chemically modify DNA by adding a methyl group derived from S-adenosyl-L-methionine (Adomet) to the fifth carbon of a cytosine residue to create 5-methylcytosine (5-mC) [13, 14]. Four main DNMT isoforms participate in regulating DNA methylation. DNMT-1 is involved in the maintenance of methylation patterns including methylating newly synthesized DNA [6]. DNMT-3A and DNMT-3B act as de novo methyltransferases that have important roles in regulating gene expression responses during development or disease as well as methylating unmethylated regions of DNA [15]. Finally, DNMT-3L does not have its own methyltransferase activity but aids DNMT-3A and DNMT-3B in enhancing their enzyme activity or affinity for Adomet, and thereby promoting methylation [16].

Interestingly, studies have shown that the translocation of DNMTs to mitochondria also regulates metabolic signalling via 5mC methylation on mtDNA in response to cellular stresses including hyperglycemia, oxidative stress or hypoxia to either initiate or receive signals within the mitochondria [6, 8, 12, 17]. For example, in one study the levels of mitochondrial DNMT-1, and thereby 5mC levels, were affected by the major players involved in oxidative stress such as P-53, suggesting a role of mitochondrial epigenetics in the cellular response to oxidative stress [6]. In wood frogs, the complete mitochondrial genome is a circular molecule of ~17000 bp that codes for 13 protein-coding genes [18] and also shows selective upregulation of the mitochondrial antioxidant response and mitochondria encoded gene expression (e.g. ATP6/8, ND4 and 16S RNA), thereby suggesting a possible role for DNMT machinery in maintaining mitochondrial function under freezing or dehydration stress to maintain
overall cell function. Therefore, the current study focussed on exploring the role
mitochondrial DNMT machinery and gives a snapshot of tissue and stress specific
expression of DNMTs, mitochondrial DNMT activity, 5mC levels and the putative roles
and interplay of DNMTs in regulating methylation patterns in wood frog mitochondria
from liver and heart in response to whole body freezing or dehydration stresses in
wood frogs.
4.3 Methods

Animals

Male wood frogs (5-7 g weight) were collected during the early spring from breeding ponds near Oxford Mills, Ontario. The frogs were washed in tetracycline bath and then held for a 2-week acclimation at 5°C in plastic boxes lined with damp sphagnum moss prior to experimentation. All further steps were performed as previously described [19, 20]. All frogs were euthanized by pithing and tissues were collected quickly and flash frozen in liquid nitrogen before storage at -80°C. Experimental protocols were previously approved by Animal Care Committee (Protocol #106935) of Carleton University and adhered to guidelines set by the Canada Council on Animal Care.

Mitochondria Isolation and preparation of tissue extracts

A commercially available kit (Mitochondrial Isolation Kit for Tissue (with Dounce Homogenizer), catalog number: ab 110169, Abcam, Toronto, ON, Canada) was used to isolate a mitochondrial fraction from frozen tissue samples of liver and heart tissues of control, 24 h frozen and 40% dehydrated wood frogs. All the steps for mitochondrial tissue processing were done on ice and were performed as described before [21]. The mitochondrial fraction and upper supernatant fraction tubes were assessed for protein concentrations using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) and all concentrations were standardized to 4 μg/μL by adding calculated amounts of isolation buffer. The final concentration of aliquots of mitochondrial and supernatant protein extracts were then mixed with 1:1 v:v with 2X loading buffer to give final
concentrations of 2 µg/µL for both liver and heart samples. Samples were then boiled for 5 min, cooled on ice for 5 min and then stored at -80 °C until needed. To assess the separation of supernatant and mitochondrial fractions, immunoblotting was carried out (as described below) and fractions were tested with antibodies for the presence/absence of mitochondrial citrate synthase and cytoplasmic tubulin markers (Appendix B, Fig. B1).

Immunoblotting

For immunoblotting, samples of extracted protein containing 20-30µg of protein (from liver and heart samples of control, 24 h frozen or 40% dehydrated) frogs were loaded on SDS-PAGE gels and analyzed as explained before[19]. The membranes were incubated overnight with primary antibodies (diluted at 1:1000 in 1X TBST) at 4 °C. The membranes were exposed using a Chemi-Genius Bioimager (Syngene, Frederick, MD) for viewing chemiluminescent and Coomassie blue bands. The following antibodies were used (Rabbit polyclonal antibodies): DNMT-1 (ABclonal Cat# A16729), DNMT-3A (GeneTex Cat# GTX129125), DNMT-3B (GeneTex Cat# GTX129127), DNMT-3L (GeneTex Cat# GTX115985), Citrate synthase (ABclonal Cat# A5713) and Tubulin (ABclonal Cat# AC007).

DNMT activity

To assess overall DNMT activity in the mitochondrial fraction of liver and heart samples, a commercially available DNMT activity kit was used (Catalog number: P-3009, Epigentek, N.Y, USA). A pooled mitochondrial sample was used to perform a
dilution test to find the concentration that worked best for the kit. For both liver and heart samples, aliquots of mitochondrial protein (10 µg) were diluted in ~45 µL of 1X Adomet (50X Adomet diluted to 1:50 v:v in DNMT assay buffer) in each well of microplate and incubated 100 min at 37°C. For blank wells, DNMT assay buffer was used and DNMT enzyme (provided with the kit at 50 µg/mL) was used as positive control for the experiment. All samples were run in duplicates. Following incubation, the microplate wells were washed with 1X Wash buffer, followed by incubation with 50 µL of Capture Antibody (1:1000 v:v) for 1 hr. After 1 hr of incubation, the microplate wells were washed again with 1X wash buffer for 4 times, followed by another incubation with 50 µL Enhancer solution (1:5000 v:v) for 30 min. The Enhancer solution was removed and microplate wells were again washed 5 times, followed by addition of 100 µL of a developing solution. A blue color in the sample well (including the positive control) indicated methylated DNA. The reaction was further stopped by adding an equal volume of Stop Solution. The amount of methylated DNA which also correspond to DNMT enzyme activity was measured at 450 nm and by using microplate reader (Multiscan Spectrum, Thermo Labsystems). The absorbance measured at 450 nm was directly proportional to DNMT activity (calculated by using following formula) in control vs 24H freezing and control vs 40% dehydration mitochondrial samples.

\[
\text{DNMT activity (OD/h/mg)} = \frac{(\text{Sample OD} - \text{blank OD})}{(\text{Mitochondrial protein (µg)} \times \text{Incubation time at 37°C (h)})} \times 1000
\]

**Mitochondrial DNA isolation**
MtDNA was isolated using an in-house method (described below) from frozen tissue samples of liver and heart tissues of control, 24 h frozen and 40% dehydrated wood frogs. All the steps for mitochondrial tissue processing were done on ice. Briefly, 150-200 mg of frozen tissue (n=4 independent repeats from different wood frogs) was rapidly weighed and washed in small beaker (on ice) containing cold homogenization buffer (250 mM sucrose, 100 mM Tris-HCl, 10 mM EDTA, pH 7.4). The washed tissue was homogenised in 700 µL of cold homogenization buffer with cold Dounce homogenizer (on ice), with 10-25 pestle strokes (depending on tissue type). The homogenate was then transferred to a microtube (kept on ice) and filled with up to 1 mL of homogenization buffer and kept on ice for 15-20 min. The tubes were then centrifuged at 1500 x g at 4°C for 10 min to pellet cell debris and nuclei which were discarded, and supernatant was saved. The supernatant was transferred to new 2 mL tubes and centrifuged again at 11000 x g at 4°C for 15 min, to isolate crude mitochondrial pellet. For improving extraction and reducing impurities, this step was repeated twice. The pellet was resuspended in in 500 µL high salt buffer and then 80 µL 10% SDS and incubated at 55°C on the heating block for denaturation of proteins as contaminants. The proteins from the last step were salted out as pellet by adding 480 µL 5M NaCl and centrifuged at 11, 500 x g for 20 min in a microcentrifuge at RT. The supernatant from this step was added with ice-cold 95% ethanol in each tube (filled with up to 2mL) and centrifuged again at 11,000 x g for 15 min at 4°C for precipitating the mtDNA pellet. The pellet was washed with 70% ethanol and air dried for 5-10 min. The dried pellet was eluted in 80 µL autoclaved sterile water. To assess the purity of
extracted mtDNA a 260/280 ratio using Take3 micro-volume quantification plate (BioTek, Winooski, VT, USA) was calculated. A ratio of 1.7-1.85 confirmed the quality of initially extracted mtDNA and also provides you with concentration values for each sample. Additionally, mtDNA integrity was also assessed by the presence of a sharp band of intact mtDNA ~16000 bp on a 0.8% agarose gel electrophoresis stained with SYBR Green (Cat #S7563; Invitrogen, Carlsbad, CA, USA). The tubes containing mtDNA were stored at -20°C until use.

**Mitochondrial DNA Dot blots for assessing 5mC levels**

DNA dot blots were used to measure the expression of 5mC variants on mtDNA from frozen tissue samples of liver and heart of control, 24 h frozen and 40% dehydrated wood frogs. Briefly, the extracted mtDNA tubes (from above) were thawed on ice and quantified again on Take3 micro-volume quantification plate (BioTek, Winooski, VT, USA). The samples were standardized to 1-3 ng/µL in Solution I (0.4 mol/L NaOH and 10m mol/L EDTA) and heated at 95°C on a heating block for 10 min. A commercially available positive control (methylated gDNA) and negative control (unmethylated gDNA) was also used and treated as mtDNA. A 96 well Bio-Dot microfiltration apparatus (Biorad; Catalog #. 1706545) was used to perform dot blots using above DNA samples treated with Solution I. The Bio-Dot microfiltration apparatus was rinsed and cleaned with ddH2O before use. A presoaked positively charged nylon membrane and filter paper in 1X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) was set up on the Bio-Dot apparatus, with membrane on top followed by the filter paper covering all 96 wells of the apparatus. The wells were washed with 500µL of ddH2O and water was
filtered out using vacuum. Following this, 50-70 µL of DNA samples were loaded into the wells and were left to filter out through the positively charged membrane via gravity setting on BioDot apparatus. The wells were washed with 0.4M NaOH using vacuum filtration and then membrane was taken out and washed with 1X SSC twice and air-dried. The air-dried membrane was incubated in hot oven for 2 hrs at 80⁰ C. Following incubation, membrane was blocked with 10% not fat dry milk in 1X SSCT (1X SSC, 0.1% Tween-20) for 1 hr and then washed three times with 1X SSCT (5-10 min each). A primary antibody corresponding to 5mC (1:5000 in 1X SSCT, AbClonal Cat#A20599) was added and membrane was incubated overnight for 16 hours. The next day, the primary antibodies were removed, and the membrane were washed as mentioned above, before incubating it with an anti-rabbit HRP-conjugated secondary antibody (1:20,000 in 1X SSCT) for 30 min at RT. The membrane was washed as mentioned above and is visualized using chemiluminescence (H₂O₂ and Luminol). The same membrane was stained in Methylene Blue stain and imaged again to be used for relative quantification of mtDNA and to standardize the intensity of the each dot for loading control to adjust for any differences in loading DNA onto the membrane.

Data analysis and Statistics

Bands on immunoblots were visualized using a ChemiGenius Bioimager (Syngene, Frederick, Maryland, USA) and intensities of immunobands of interest were standardized against a group of Coomassie stained mitochondrial protein bands that occurred in each lane but were well separated from the band of interest. This method is more consistent and accurate compared to standardizing band intensities against a
housekeeping protein such as tubulin [22]. The DNA Dot-blots were visualized using the same imaging system. Data standardization was done for all the dot intensities using mtDNA dots stained with 0.02% w/v methylene blue in 1X TAE buffer (40 mM Tris acetate, 20 mM acetic acid, 1 mM EDTA, pH 8.5) on the same membrane. Statistical testing was performed using the RBioplot program [23] to conduct a Student’s t-test to compare the data from control vs 24 h freezing or control vs 40% dehydrated wood frog samples for both immunoblotting and DNMT activity assays with P<0.05 accepted as a significant difference.

4.4 Results

Effects of freezing on mitochondrial DNA methyltransferase protein levels in liver and heart

The relative protein expression levels of mitochondrial DNMT-1, DNMT-3A, DNMT-3B and DNMT-3L were assessed via immunoblotting in liver and heart from control (5°C acclimated) and 24 h frozen (at -2.5°C) frogs. The relative protein level of mitochondrial DNMT-1 in liver from 24 h frozen frogs was significantly reduced to just 55 ± 11% of the control value (Fig 4.1A) and a comparable response was seen in heart with DNMT-1 protein levels in frozen frogs reduced to just 35 ± 6% of the control value (Fig 4.1B). By contrast, mitochondrial protein levels of DNMT-3A increased significantly in liver of frozen frogs by 3.75 ± 0.82-fold compared with controls (Fig 4.1A) but no significant change occurred in heart (Fig 4.1B). After 24 h freezing exposure, levels of DNMT-3B protein increased significantly in both liver and heart, by 4.27 ± 1.3-fold and 1.92 ±
0.19 fold respectively, as compared with control values (Fig 4.1A, B). The relative protein levels of DNMT-3L changed significantly in liver by $3.39 \pm 0.76$ fold (Fig 4.1A) but remained unchanged in heart (Fig 4.1B).

**Effects of dehydration on mitochondrial DNA methyltransferase protein levels in liver and heart**

Cellular dehydration is one component of freezing since much water exits cells to accumulate in extracellular ice masses. To determine whether cell dehydration influenced mitochondrial DNMT protein expression, the relative expression levels of DNMT-1, DNMT-3A, DNMT-3B and DNMT-3L were assessed via immunoblotting in the mitochondrial fraction of both liver and heart comparing control frogs and 40% dehydrated wood frogs. The relative level of mitochondrial DNMT-1 in the liver rose significantly by $2.29 \pm 0.52$-fold in 40% dehydrated wood frogs (Fig 4.2A) whereas in heart the levels of DNMT-1 decreased to $74 \pm 5\%$ of control values in response to dehydration (Fig 4.2B). Protein levels of DNMT-3A showed a similar trend rising significantly by $2.11 \pm 0.40$-fold in liver (Fig 4.2A) whereas in heart levels decreased to $57 \pm 10\%$, of control values (Fig 4.2B). Interestingly, levels of other mitochondrial proteins DNMT-3B protein did not change significantly in either tissue under 40% dehydration stress whereas the levels of DNMT-3L increased significantly in both liver and heart by $1.87 \pm 0.22$ fold and $1.80 \pm 0.26$ fold respectively (Fig 4.2A, 2B).

**Effect of Freezing on mitochondrial DNMT activity in liver and heart**
The mitochondrial DNMT enzyme activity in the liver was also assessed and correlated well with protein expression levels. DNMT activity in liver increased significantly after 24 h freezing by 2.55 ± 0.36-fold (Fig 4.3A). However, in the heart total activity did not change over the 24 h of freezing exposure (Fig 4.3B).

**Effects of dehydration on mitochondrial DNMT activity in liver and heart**

The mitochondrial DNMT enzyme activity in liver rose under 40% dehydration stress to a level 2.52 ± 0.47-fold higher than control values (Fig 4.4A), whereas in heart the DNMT activity levels was not significantly changed under dehydrating conditions (Fig 4.4B).

**Effects of freezing on 5mC levels in liver and heart**

Relative mtDNA levels of 5mC were assessed in liver and heart from 24 h frozen wood frogs by DNA dot blotting. The 5mC levels in liver rose under 24 h freezing to 1.58 ± 0.06 compared to controls (Fig 4.5A), whereas in frozen heart the 5mC levels remained at control levels (Fig 4.5B).

**Effects of dehydration on 5mC levels in liver and heart**

The 5mC levels remained unchanged and stayed close to control levels in both dehydrated liver (Fig 4.6A) and heart (Fig 4.6B).
4.5 Discussion

Proper mitochondrial function is required to maintain and/or stabilize cellular machinery in response to shifting environmental conditions that can disrupt or damage normal cell processes and homeostasis. In freeze-tolerant wood frogs, mitochondria are one of the most riveting organelles for study. During freezing, mitochondria not only face major stress conditions inside cells (e.g. reactive oxygen species, changing oxygen availability, suppressed or complete shut down of oxygen-dependent ATP production) but, unlike most animals, freeze-tolerant animals must endure not only extracellular freezing of body water but also the accompanying dehydration and anoxia stresses caused by freezing [24]. In most animals, such stresses would trigger apoptosis. The adaptations supporting freezing survival have been shown in multiple studies from our lab including various mitochondria-specific regulatory/adaptive mechanisms that help wood frogs to endure stresses including freezing and dehydration [4, 9, 25]. Even though the normal functions of mitochondria are largely shut down during freezing or dehydration with ATP production shifted to anaerobic glycolysis [26, 27], mitochondria still need protection and to maintain their metabolic systems in order to rapidly restart their functions quickly upon recovery from both the stresses.

So, it became important to determine whether the mitochondrial machinery was preserved and sustained in the frozen as well as dehydrated states. The current study gives a snapshot of the effect of freezing and dehydration on protein levels and activity of the mitochondrial DNMT system as well as methylation of mtDNA (5mC levels) that
has an important role in regulating the expression of mitochondria-encoded genes and the methylation state of mitochondrial DNA. To help assess the role of DNA methylation in coping with freezing or dehydration stress, we first analyzed mitochondrial DNMT protein expression levels in liver and heart tissues of wood frogs. In the liver during 24 h freezing, the levels of mitochondrial DNMT-1 were significantly reduced (Fig 4.1A). Mitochondrial DNMT-1 is recognized as the main maintenance enzyme that methylates DNA [6]. Indeed, the enzyme affects mitochondrial biology under conditions of oxidative stress and elevated ROS (which are also consequences of freezing) [28]. Increased levels of this enzyme in the mitochondria have also been shown to decrease the protein expression of some mitochondrial encoded genes [29]. Oppositely decreased DNMT-1 levels should increase mitogene expression. Interestingly, a separate study from our lab has shown upregulation of mitochondria-encoded genes ATP6/8, ND4 and 16S RNA) during freezing in the liver of wood frogs [4] which aligns with the DNMT-1 downregulation reported here (Fig 4.1A). Although DNMT-1 levels in liver were reduced during 24 h freezing, elevated DNMT-3A & DNMT-3B in mitochondria could underlie the upregulation of mitochondrial DNMT activity (Fig 4.3A) during 24 h freezing. Catalytic mitochondrial DNMT3L also rose in conjunction with the rise in DNMT3A and DNMT3B (Fig 4.1A) and, in addition, upregulation of mitochondrial DNMT-3A and DNMT-3B (Fig 4.1A) could directly increase the 5mC methylation levels of mtDNA (Fig 4.5A) to promote hypermethylation, thereby contributing to an overall suppression of mitochondrial gene expression [8, 30].
In the case of hearts from frozen frogs, DNMT-1 also showed reduced levels (Fig 4.1B) as in the liver. The role of mitochondrial DNMT-1 in the heart also relates to the regulation of oxidative stress [31]. In addition, DNMT-1 levels in mitochondria have been reported to regulate the D-loop (displacement loop) found in the non-coding region of mitochondrial DNA that is essential for regulating mitochondrial copy number and mitochondrial DNA damage under stress [32]. Reduced levels of mitochondrial DNMT-1 suggest that regulation of these processes might also be reduced during freezing but interestingly this also aligns with the higher protein levels of the mitochondrial antioxidant enzyme, superoxide dismutase-2 (SOD-2) reported in heart of frozen wood frogs [33]. SOD-2 has been shown to directly associate with mtDNA [34] and can also bind to the mitochondrial D-loop [35] ultimately providing protection to mtDNA from oxidative damage [34]. Interestingly, the rise in levels of mitochondrial DNMT-3B (Fig 4.1B), despite the lower levels of DNMT-1, along with basal levels of mitochondrial DNMT activity (Fig 4.3B) could be enough to maintain the required overall 5mC methylation patterns which remained at control levels (Fig 4.5B) during freezing stress in wood frog hearts [32, 36, 37].

Cell dehydration stress is a major consequence of freezing and we were interested to determine if it independently affected mitochondrial function via DNMT regulation, in a similar manner to that of freezing. Dehydration of wood frogs also triggers the production of high levels of cryoprotectant (glucose) and leads to major changes in cell volume, osmolality and ATP levels [38]. Interestingly, the trend in mitochondrial DNMT protein expression levels under dehydration stress were very similar to those triggered
by freezing with the overall profile for DNMT protein levels (DNMT-3A, DNMT-3B, DNMT-3L) and mitochondrial DNMT activity in the liver being upregulation of all parameters in response to 40% dehydration (Fig 4.2A, 4A). However, unlike the response to freezing, the levels of DNMT-1 in mitochondria were significantly increased during dehydration (Fig 4.2A). Despite this overall upregulation under dehydration stress, there was no change in the 5mC levels (Fig 4.6A) or changes in the levels of mitochondrial genes as seen during freezing stress [4]. This hinted that alternate roles for the DNMT machinery might be in play besides maintaining mtDNA methylation at basal levels during dehydration (Fig 4.6A). For example, an upregulation of DNMT-1 levels can also negatively regulate mitochondrial microRNAs such as miR-34a, which ultimately diminishes the anti-oxidant response via the Nrf2 axis [28, 39, 40].

Interestingly, other studies from our lab with animal models that also undergo states of metabolic rate depression have shown downregulation of miRNA-34a in the liver [41, 42] along with upregulation of Nrf-2 induced antioxidant responses during dehydration stress [43]. Overall, DNMT expression and activity (Fig 4.2A, 4A) hinted at a preference for stricter regulation in maintaining the cellular stress response during dehydration, while keeping mtDNA methylation (Fig 4.6A) at control levels to regulate genes actively required in the stress response. This could also be because, during dehydration stress, metabolism is still very active and mostly aerobic but over time this changes as the percent dehydration rises above ~35%, when a preference for anaerobic ATP production becomes evident and lactate accumulates [27]. Unlike freezing, dehydration is a gradual process that takes 2-3 days to reach 40% water loss and gives
the frogs ample time to adjust their adaptive strategies [27]. However, despite several days of slow water loss to reach 40% dehydration, an upregulated response from all DNMT proteins including DNMT-1 (compared with frozen liver) could be a response to a slowly changing metabolic state. The levels of 5mC staying close to control levels might be an attempt to keep the mitochondrial machinery in check to induce a state of quiescence to avoid any further damage since at higher dehydration rates wood frogs tend to become hypoxic [38].

Unlike in liver, hearts from 40% dehydrated wood frogs showed downregulation of mitochondrial DNMT proteins (DNMT-1 and DNMT-3A) (Fig 4.2B), whereas only levels of DNMT-3L rose significantly (Fig 4.2B). However, DNMT activity did not change in the hearts of dehydrated wood frogs (Fig 4.4B) and neither did 5mC levels (Fig 4.6B). The overall regulation of mitochondrial DNMTs suggests downplay of the DNMT machinery which is known to drive mtDNA methylation levels during stress. One possible explanation for a relaxed profile for mitochondrial DNMTs in hearts from 40% dehydrated frogs could be that at higher % dehydration values (as mentioned above), hypoxia develops and makes it more difficult for the heart to produce ATP to drive contraction and also to deal with the pumping of thickened blood [27]. This is also evident from the lack of change in mitochondrial proteins or antioxidant proteins during dehydration [4, 33]. Hence, keeping gene methylation levels in check via DNMTs might help to maintain the mitochondrial machinery in the heart under 40% dehydration stress conditions.

**Conclusion**
The present study shows mitochondrial DNMT protein, activity levels and 5mC methylation responses to freezing and dehydration stresses in the liver and heart of freeze-tolerant wood frogs, *Rana sylvatica*. Mitochondrial DNMT-1 protein levels responded in a stress specific manner in the liver and tissue-specific manner in the heart. DNMT-1 regulation in the liver from frozen frogs suggested a role in the enhanced profile of mitochondrial genes as seen previously during freezing in wood frogs whereas mitochondrial DNMT-1 levels during dehydration suggested a possible role in the antioxidant response. Interestingly, the overall responses by mitochondrial DNMT-3A, DNMT-3B and DNMT-3L, and DNMT activity in liver of 24 h frozen frogs coincides with higher 5mC methylation levels, whereas in dehydrated liver it coincides in enhancing antioxidant response, while 5mC levels could be an anticipatory response of inducing a state of quiescence if a gradual increase in dehydration stress is encountered. Heart responded differently under both freezing and dehydration stresses. The mitochondrial profile for DNMT proteins, DNMT activity and 5mC methylation levels was mostly reduced or unchanged across the two stresses. Overall, reduced protein levels of mitochondrial DNMT-1 in both frozen and dehydrated hearts could relate to a similar antioxidant response as seen in the liver whereas levels of mitochondrial DNMT-3B during 24 h freezing and DNMT-3A during 40% dehydration suggested stress-specific responses by the mitochondrial machinery according to the changes in the cellular environment during each stress. The current study suggests a possible role of mitochondrial DNMTs in regulating freezing and dehydration-specific
responses and methylation state that may play a prominent part in promoting wood
frog freeze tolerance.
4.6 References


25. De Croos JNA, McNally JD, Palmieri F, Storey KB (2004) Upregulation of the mitochondrial phosphate carrier during freezing in the wood frog Rana sylvatica: potential roles of


Fig. 4.1: A) Relative protein expression of mitochondrial DNA methyltransferases (DNMT-1, DNMT-3A, DNMT-3B, DNMT-3L) in liver and B) heart samples of control and 24h frozen *R. sylvatica*. Representative immunoblots are shown below the histogram. Data presented are mean ± SEM, n = 4 independent biological replicates. Statistical testing used Student’s t-test with $P < 0.05$ (‘*’), accepted as a significant difference.
**Fig. 4.2:** A) Relative protein expression of mitochondrial DNA methyltransferases (DNMT-1, DNMT-3A, DNMT-3B, DNMT-3L) in liver samples and B) heart samples of control and 40% dehydrated *R. sylvatica*. Representative immunoblots are shown below the histogram. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.
Fig. 4.3: A) Relative total DNMT activity in liver and B) in heart samples of control and 24 h frozen *R. sylvatica*. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.

Fig. 4.4: A) Relative total DNMT activity in liver and B) in heart samples of control and 40% dehydrated *R. sylvatica*. Statistical testing used Student’s t-test with P<0.005. *- denotes values that are significantly different from controls.
Fig. 4.5: A) Relative total 5mC levels in liver, and B) heart samples from control and 24 h frozen *R. sylvatica*. Statistical testing used Student’s t-test with P<0.005. * denotes values that are significantly different from controls.
Fig. 4.6: A) Relative total 5mC levels in liver, and B) heart samples from control and 40% dehydrated *R. sylvatica*. Statistical testing used Student’s t-test with \( P < 0.005 \). *-denotes values that are significantly different from controls.
Chapter 5: General Discussion
5.1 Discussion

Earth is a planet of environmental extremes, yet various animal species survive under these conditions, facing harsh temperatures (both hot and cold), food scarcity, low oxygen, dehydration, or whole-body freezing, that would usually mean death. Freeze tolerance is one of the best and most extreme examples of winter survival strategies. Animals that are freeze tolerant have been shown to activate multiple adaptive responses at physiological, biochemical, and molecular levels, mainly characterized in amphibians, reptiles and insects [1–3]. Of these, wood frogs (*Rana sylvatica*) are one the best studied animal models of vertebrate freeze tolerance and have been widely used to decipher underlying molecular mechanisms that contribute to cryopreservation. The findings from these studies have added substantially to both our knowledge of how animals deal with subzero temperatures and paved the way to improve fields of human stress biology to better understand organ transplant and preservation methods, therapeutic hypothermia, diabetes, and ischemic-reperfusion injury of core organs [4–7].

Freezing survival by wood frogs actually involves dealing with multiple component stresses including cellular dehydration, anoxia, and hyperglycemia. If not managed properly these can induce various stress responses with consequences that could be lethal for wood frogs [8]. Such hazardous consequences for wood frogs range from physical damage to tissues from extracellular ice buildup during freezing, that can also disrupt vascular integrity during thawing, to ischemic/anoxic stress during freezing since breathing, heartbeat and blood circulation are all curtailed [9] Also, all
cells/organs must endure cell shrinkage and dehydration due to the loss of water into extra-organ ice masses as well as hyperglycemia (glucose produced as cryoprotectant) that can also have negative metabolic effects (that are well known to diabetic humans)[10]. Freeze-induced hyperglycemia can also amplify the effects of oxidative stress (reactive oxygen species, ROS) and mitochondrial dysfunction[11, 12].

As an adaptive response to these multiple insults, wood frogs undergo metabolic reorganization including utilizing carbohydrate fuel (glucose) to generate energy anaerobically due to low or no oxygen conditions and reducing their metabolic rate (MRD) to a fraction of basal metabolic rate. Regulation of MRD further helps in globally suppressing energy-expensive processes such as protein synthesis, transcription, and translation and prioritizes the use of available energy for activating pro-survival pathways[13]. For example, while metabolically taxing and energy expensive pathways are impeded [14], heightened gene expression of antiapoptotic and antioxidant proteins can aid survival by helping organs to endure freezing and aid recovery after thawing [1]. Such fine-tuning of gene regulation is largely under the control of transcription factors (TFs), that are “master regulators” inside cells and act to “switch” ON/OFF major cellular processes or act as highly selective controllers of specific genes [15]. Interestingly, glucose homeostasis inside cells is also regulated in part by TFs which activate the downstream genes that can act according to cellular demands to either store glucose or use it as fuel. Also, adaptive regulation of glucose metabolism in wood frogs is evident from various studies done in the past, suggesting that intricate and tight controls of glucose metabolism is one of the chief reasons for winter survival
of wood frogs. The effects of high glucose include colligative resistance to cellular dehydration (during freezing and/or dehydration), high levels of anaerobic fuel, and multiple levels of regulation associated with signalling and metabolic pathways in wood frogs[10, 16–21]. Still, the transcriptional regulation which underlies glucose-dependent gene regulation and its roles in regulating other important processes in wood frogs is not entirely understood.

In this thesis, I aimed to better understand the role of glucose-induced transcription factors and downstream gene responses in regulating allied networks under environmental stresses (freezing and dehydration) in freeze-tolerant wood frogs. For this, I first profiled a glucose-induced transcription factor response (MondoA-MLX complex) to determine if responses by this TF are active during freeze-thaw cycles in various tissues. Then, active TF responses were used to search for the downstream genes activated under this TF, since it is also linked to allied networks that overlap with glucose-induced transcription (Chapter 2). The downstream target, TXNIP, featured in Chapter 2, showed freeze-induced upregulation, which was further explored to establish the role of this protein in activating stress signaling in cytoplasm and mitochondria under both freezing and dehydration stresses (Chapter 3). Downregulation of mitochondrial-induced stress signalling (Chapter 3), paved the way to identify the role mitochondrial epigenetic responses (5mC methylation) via DNA methyltransferases in controlling these stress responses during freezing and dehydration stress (Chapter 4).
5.2 MondoA/MLX complex is activated under high glucose conditions and may link with the circadian clock during freezing

In Chapter 2, I showed tissue-specific regulation of the MondoA-MLX complex over the freeze-thaw cycle in the liver and brain of wood frogs. In response to freezing and a short recovery period of 8 h thawing, my study documented an overall upregulation of total and active nuclear levels as well as DNA binding of both MondoA and MLX in the liver but not in the brain. Considering the role of the MondoA-MLX complex in activating downstream responses (e.g., TXNIP and ARRDC4) and in negatively regulating glucose uptake, these results were expected. Also, the data indicated that liver was the more active tissue during freezing in producing and exporting cryoprotectant as compared to the brain, that actually depends on glucose made by the liver as its metabolic fuel. Furthermore, MondoA-MLX machinery has downstream targets (KLF10 and PFKFB3), that are intricately controlled by circadian clock-controlled genes (CCGs). The circadian clock is known to regulate and coordinate many functions of metabolism and gene expression [22]. Specifically, this molecular machinery generates “ripples” of gene expression, that can lead to a major change in energy metabolism in controlling genes involved in glucose homeostasis, as mentioned above [22, 23]. My interest in this topic was further firmed as the circadian clock not only shows changes in gene expression to light and dark cycles but is also responsive to cues from internal peripheral clocks in multiple organisms when held in constant darkness [24, 25]. Since wood frogs are kept under the dark during experimentation and under layers of leaf litter and snow in the field, assessing changes in the molecular signature
of circadian proteins (that ultimately overlap glucose-induced transcription via common targets of MondoA-MLX), becomes evident. For this, I analyzed the role of major proteins and TFs that drive the circadian machinery, BMAL-1 and CLOCK, using the same approach and experiments mentioned above. In conjunction with the MondoA-MLX response, BMAL-1 and CLOCK also showed similar levels of regulation in terms of active DNA binding, suggesting that these TFs are active over the freeze-thaw cycle. The increased DNA binding of TFs linked with observed protein levels (total and nuclear) in the liver but not in the brain. In addition, both KLF-10 and PFKFB3 proteins correlated with changes in key circadian proteins hinting at the regulation of the hepatic peripheral clock in regulating glucose homeostasis over the freeze-thaw cycle in wood frogs.

5.3 Induced TXNIP levels relate to tissue inflammation and mitochondrial dysfunction during freezing and dehydration stress

In Chapter 3, I analyzed the role of thioredoxin interacting protein (TXNIP), a direct downstream target of the MondoA: MLX complex and a negative regulator of glucose uptake, that was found to be upregulated in tissues of frozen wood frogs (Chapter 2). TXNIP is also an interesting target to study because it has alternate roles inside the cell based on its subcellular localization (cytoplasm or mitochondria) and has been shown to be activated under hyperglycemic stress [26–28]. Cytoplasmic retention of TXNIP under hyperglycemic stress (oxidative stress) has been linked to tissue-specific inflammasome activation (via NLRP-3 protein), whereas mitochondrial translocation of TXNIP under similar conditions activates mitochondrial dysfunction via releasing
cytochrome c (cyt c) in the cytoplasm, a marker for mitochondrial induced damage [28]. Both inflammasome and mitochondrial dysfunction pathways, if induced, can ultimately activate a caspase-mediated cytokine response (interleukin, IL), that is a marker of cellular stress and damage [29]. Interestingly, under both freezing and dehydration stress, the accumulation of large amounts of glucose (as a cryoprotectant) further causes a rise in glucose-induced cytotoxicity, oxidative stress and cell-damaging pathways [30]. Therefore, I was interested in deciphering the overall role of TXNIP, and its subcellular localization in inducing both inflammasome and mitochondrial dysfunction pathways under freezing and dehydration stresses. Firstly, mitochondrial and cytoplasmic subcellular fractions were used to analyze protein levels and distribution of TXNIP across three tissues (liver, muscle and kidney) under freezing and dehydration stresses. Cytoplasmic retention of TXNIP showed an active response and association to activation of NLRP-3 inflammasome in a stress-specific manner during freezing but not in response to dehydration stress. By contrast, mitochondrial localization of TXNIP showed only a stress-specific (freezing) response in liver, along with TRX-2, a mitochondrial antioxidant enzyme and member of the thioredoxin family that plays a crucial role in maintaining cellular homeostasis under oxidative stress [31]. A higher expression of TXNIP hinted at the possibility of its binding to TRX-2 and disrupting TRX-2 antioxidant function. Interestingly, despite higher expression of TXNIP, the actual binding between TXNIP and TRX-2 (analyzed via co-immunoprecipitation) was downregulated. This also aligned with a missing key residue in the TXNIP sequence protein (observed via SWISS-MODEL analysis) that is involved in
forming a disulfide bond with TRX-2. This further supported the observed
downregulation or unchanged levels of TRX-2. Furthermore, the terminal reporter of
this pathway, Cyt c, that is activated under conditions of oxidative stress (when the
TRX-2 axis is disrupted via TXNIP), also showed lower or no change in response in the
cytoplasmic fraction, suggesting that overall mitochondrial damage signaling remains
at the state of quiescence under freezing and dehydration stresses.

5.4 Mitochondrial DNA methyltransferases and 5mC methylation
response may play an important role in promoting wood frog freeze
tolerance

The rationale for this first-of-its-kind study in wood frogs was because changes in
the methylation state of mtDNA have been shown to involve mitochondrial-
induced stress and allied damage responses and support various changes in gene
expression and protein levels [32–34]. Studies with wood frogs also showed
differential regulation of gene transcripts and proteins involved in mitochondrial
regulation during freezing and dehydration. Also, Chapter 3 showed an overall
downregulation of the mitochondrial damage pathway (mitochondrial dysfunction)
in both freezing and dehydration stress. Therefore, Chapter 4 focused on
understanding the role of epigenetic modifications of the mitochondrial genome,
specifically methylation, in regulating gene transcription and gene expression under
freezing and dehydration stresses. The major proteins involved in adding these
methylation marks (5 methylcytosine) onto mtDNA are the DNA methyltransferases
(DNMTs). Overall, the various DNMTs (DNMT-1, DNMT-3A, DNMT-3B, DNMT-3L)
showed a stress-specific response in wood frog liver and a tissue-specific response in the heart under freezing or dehydration stresses. Lower levels of DNMT-1 in liver of frozen frogs were linked with an unrestrained profile of mitochondrial peptides, as seen previously during freezing in wood frogs, whereas DNMT-1 levels in liver from dehydrated frogs hinted at a role in the antioxidant response. Interestingly, the overall responses by mitochondrial DNMT-3A, DNMT-3B and DNMT-3L, and their total DNMT enzymatic activity in liver of 24 h frozen frogs showed a state of higher preferred methylation levels via upregulating 5mC marks on mtDNA. By contrast, in liver of dehydrated frogs, it coincided with an enhanced antioxidant response, whereas 5mC levels did not change, hinting at an anticipatory response that could be active in inducing a state of quiescence if a gradual increase in dehydration stress was encountered by frogs. Overall, this is the first study to analyze mitochondrial DNA methylation levels in wood frogs across various stresses, with findings that emphasize the potential role of methylation in maintaining tight regulation over mitochondrial transcriptional and gene expression machinery under environmental stress conditions.

5.5 Future directions

This thesis provides a broad overview of multiple levels of metabolic regulation in several pathways of wood frogs in response to freezing or dehydration stresses. Further experiments could be done to provide an enhanced view and definitive answers to the results reported in this thesis. First, the results in chapter 2 showed a role of MondoA-MLX under the hyperglycemic conditions of freezing in liver as
guiding adaptive transcriptional response(s) and cross-talk with circadian rhythm to regulate glucose homeostasis. Based on the current findings in liver, a further interesting study could be done on wood frog hepatocytes using a knockout model of MondoA via the CRISPR-Cas9 approach, as done before [35]. There would be two goals in doing so. One is that knockout or knockdown assays could validate the findings of this thesis. Secondly, beside its (MondoA) role in regulating glucose homeostasis in wood frogs, these studies could also unravel other metabolic or signalling pathways that are activated in a tissue-specific manner during freezing, as shown in other studies [36]. The benefit of these findings will be in enhancing our understanding of potential improvements that could be made to current therapeutic approaches in which MondoA has been implicated as a potential drug target [37, 38].

In chapter 3, the role of TXNIP was characterized in terms of subcellular localization in activating stress signaling showing its association with the NLRP-3 inflammasome pathway during freezing. Differences in protein levels of TXNIP were attributed to its PTMs and sequence, that might affect its role in regulating inflammasome and mitochondrial dysfunction pathways under freezing and dehydration stresses. Transcripts could be measured to further enhance understanding of the role of TXNIP in these pathways at transcriptional levels. Although challenges are evident due to the non-availability of the full sequenced genome of wood frogs, this could be a possibility in the near future. This will help in measuring multiple transcripts that are downstream of a target (for example,
TXNIP) in regulating cellular stress response signaling in wood frogs. Another level of regulation to consider is at the post-transcriptional level, specifically analysis of miRNAs, that are highly conserved across species and known to regulate two-thirds of transcription under stress conditions in various stress models [39]. The role of miRNAs on TXNIP regulation can be further explored by measuring the levels of miRNAs that are specifically known to regulate or are regulated by TXNIP under various stress conditions [40–43].

Lastly, in chapter 4, the levels of DNA methyltransferases (DNMTs) and 5mC levels (a modification on mitochondrial DNA) showed stress and tissue-specific responses, with overall upregulation linked to a preference for a higher methylation state of mtDNA. The levels of this epigenetic modification on mtDNA can be validated by using a sequence-based approach such as bisulfite sequencing or single-molecule detecting technologies such as nanopore sequencing [44]. Recently, mitochondrial methylation has started to be recognized as a major epigenetic modification on mtDNA. Recent studies have also reported other epigenetic modifications, such as N6 methyladenine, which is formed by methylation of exocyclic NH₂ group of adenine at position 6, and that can also lead to silencing/activation of gene transcription via methylation[45]. Overall, both my current data and these proposed future directions can shed light on the extent of mitochondrial methylation and its role in regulating transcription in response to stress.

5.6 Final conclusion
The findings in this thesis provide information on novel regulatory pathways and players which are involved in controlling glucose homeostasis, circadian rhythm, inflammasome activation and mitochondrial dysfunction in wood frogs under various environmental stresses. First, this thesis summarizes the role of MondoA in guiding the adaptive transcriptional responses to activate genes regulating glucose homeostasis and circadian rhythm in a tissue-specific manner in the liver but not in the brain of the wood frogs. Moreover, the role of TXNIP was established, from being a downstream target of MondoA to its role in stress-specific regulation of inflammasome activation in multiple tissues under freezing conditions. Furthermore, an overall reduction in mitochondrial dysfunction profile could not be related to active TXNIP levels in the mitochondria, which ideally induces it. However, an overall lower binding affinity of TXNIP to TRX-2 due to the unique sequence (missing a critical residue required for binding to TRX-2) and PTMs were implicated as playing a role in regulating the overall stress response in mitochondria. Finally, a higher mitochondrial methylation profile was seen again in a tissue-specific manner, which correlated with the regulation of mitochondrial transcripts and gene expression involved in oxidative stress repair. Overall, a robust control via mitochondrial epigenetics might ultimately protect the mitochondrial milieu from stress. Altogether, findings from this thesis demonstrate that a highly synchronized and intricate control via multiple levels of regulation is present in activating mechanisms that are involved in maintaining cellular milieu during stress in wood frogs.
5.7 References


2. Storey KB, Storey JM (2012) Insect cold hardiness: metabolic, gene, and protein adaptation 1. This review is part of a virtual symposium on recent advances in understanding a variety of complex regulatory processes in insect physiology and endocrinology, including development, metabo. Canadian Journal of Zoology 90:456–475. https://doi.org/10.1139/z2012-011


Appendix A: List of publications and scientific meetings
PUBLICATIONS RECORD

Published in peer-reviewed journals


Under review (2022)


Ready to submit (2022)


Future manuscripts (2022-2023) (completed projects)

1. Kupakuwana K, Singh G, Storey, K.B. Regulation of methylation and demethylation via DNA methyltransferases during anoxia and dehydration stress in wood frogs.

Scientific meeting (selected)

2. Singh G, Storey K.B. MondoA is a key regulator of sugar-induced gene expression and link to circadian rhythms in frozen wood frog. Poster session presented at: Mechanism and Evolutionary processes at 10th international Congress of Comparative Physiology and Biochemistry; Aug 5-9 2019, Ottawa, ON.
   a. https://doi.org/10.1096/fasebj.31.1_supplement.799.15

Appendix B: Subcellular fractions test blots, representative blots, Co-immunoprecipitation and multiple sequence alignment
Mitochondrial fraction isolation

To assess the separation of cytoplasmic (supernatant) and mitochondrial fractions, immunoblotting was carried out and fractions were tested with antibodies for the presence/absence of mitochondrial citrate synthase and cytoplasmic tubulin markers. Below are fractions isolated from wood frog heart samples under control conditions (at 5°C), cytoplasmic (cyto) and mitochondrial (mito).

A) Mitochondrial marker (Citrate Synthase)

B) Cytoplasmic marker (Tubulin)

C) Coomassie blue stained membrane for citrate synthase

Fig B1. Immunoblots corresponding to of A) citrate synthase and B) Tubulin in cytoplasmic (cyto) vs mitochondrial (mito) fraction isolated from hearts of the wood frogs under control conditions (5°C). C) Coomassie blue stained membrane for immunoblot representing Citrate synthase cyto vs mito protein profiles.
Representative Coomassie (TXNIP, frozen brain)

A)

B)

Fig B2. A) Representative ECL blot showing TXNIP bands (Frozen brain Fig. 2.3) in control, 24H frozen and 8H thaw stress conditions and B) Coomassie blue stained membrane for immunoblot representing total protein levels used to “normalize” the levels of TXNIP expression in each lane.

Given our unique experimental condition (Metabolic Rate Depression), we are presented with an opportunity to utilize constant total protein staining (RED box above) as both a loading control AND an experimental control. Therefore, immuno-band densities in each lane quantified (Fig. B2, A) and then gels were stained with Coomassie blue (Fig. B2, B) and reimaged. Immuno-band densities were then normalized against the collective intensity of a group of Coomassie-stained protein bands in the same lane. The Coomassie-stained group was prominent and consistent across all the lanes.
Co-Immunoprecipitation

Co-Immunoprecipitation was done to isolate the protein of interest from cell lysate (antigen for TXNIP) using an antibody (TXNIP, Novus biologicals: NBP-1 54578) complexed on Protein G beads. The final wash step and elution step separated our protein of interest. Next, the pelleted protein was ran on a western blot apparatus. Following this, antibodies for various PTMs and binding partners were used to see the interaction of our protein of interest.

Fig. B3: Principle of Co-immunoprecipitation with control vs stress samples from freeze tolerant wood frogs.

Fig B4: Test representative blot showing (A) ECL (a single band for TXNIP in red box) and (B) Coomassie stained membrane with a single band for TXNIP represented in red box. A single band at correct molecular weight ~50kDa bound represent successful immunoprecipitation of TXNIP. For negative control and no sample control, a test IgG antibody (anti-mouse) and only
Fig. B5: Representative full blot showing TRX-2 antibody binding to TXNIP (IP-beads) in dehydrated liver of the wood frogs.

Mitochondrial DNA isolation for dot blots

Fig. B6: Representative full electrophoresis gel image showing mtDNA isolation band at ~16000bp. Lane-1 - 4 (control heart mtDNA), Lane 5-8 (Frozen heart mtDNA)
Protein Alignments to use mammalian antibodies

A sequence alignment is performed for amino acid sequence for mammalian antibodies to be used in non-mammalian animal model such as wood frogs. Amino acid sequence alignment represents sequence conservation for immunogen (epitope) sequence (from vendor’s website) between Homo sapiens and Xenopus tropicalis (amphibian sequence).

A) CLUSTAL O(1.2.4) multiple sequence alignment (Citrate synthase)
**Fig B7. A)** Amino acid sequence alignment represents sequence conservation for immunogen (epitope) sequence (1-124 amino acids), **highlighted in yellow** between *Homo sapiens* (accession number NP_004068.2) and *Xenopus tropicalis* (CAJ83375.1) (amphibian sequence). **B)** Amino acid sequence alignment represents sequence conservation for immunogen (epitope) sequence (167-477 amino acids, **highlighted in yellow**) between *Homo sapiens* (accession number NP_004068.2) and *Xenopus tropicalis* (CAJ83375.1) (amphibian sequence). ‘*’ represents amino acid residues which are conserved between the two sequences.
Appendix C: Extra data