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Mitochondrial DNA methyltransferases and their regulation under freezing and dehydration stresses in the freeze-tolerant wood frog, *Rana sylvatica*

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Abstract: Wood frogs are 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 and protein expression to activate pro-survival pathways. The role of DNA methylation machinery (DNA methyltransferases, DNMTs) in regulating nuclear gene expression to support freezing survival has already been established. However, a comparable role for DNMTs in the mitochondria has not been explored in wood frogs. We examined the mitochondrial protein levels of DNMT-1, DNMT-3A, DNMT-3B, and DNMT-3L as well as mitochondrial DNMT activity in the liver and heart to assess the involvement of DNMT in the survival of freezing and dehydration stresses (cellular dehydration being a component of freezing). Our results showed stress- and tissue-specific responses to mitochondrial DNMT-1 in the liver and heart, respectively. During 24 h of freezing and whole-body dehydration, we observed an overall downregulation of mitochondrial DNMT-1, a major protein involved in maintaining methylation levels related to its role in the selective transcription of mitochondrial genes as well as antioxidant response. Tissue-specific responses of protein levels of DNMT-3A, DNMT-3B, DNMT-3L, and DNMT activity in the liver suggested a preference for a higher methylation state in the liver under both freezing and dehydration stress, but not in the heart.

Key words: DNMT activity, metabolic rate depression, antioxidant response, mitochondria, mitochondrial proteins.

Résumé : Les grenouilles des bois sont l'une des rares espèces de vertébrés qui peuvent survivre à la congélation du corps entier. De multiples adaptations permettent cette survie, notamment la production de cryoprotecteurs (glucose), la réduction de la vitesse du métabolisme et des changements sélectifs dans l'expression de gènes et de protéines pour activer les voies favorisant la survie. Le rôle de la machinerie de méthylation de l'ADN (ADN méthyltransférases, DNMT) dans la régulation de l'expression des gènes nucléaires favorisant la survie lors de la congélation a déjà été établi. Cependant, un rôle comparable des DNMT dans les mitochondries n'a pas été exploré chez les grenouilles des bois. Les auteurs ont examiné les niveaux des protéines DNMT-1, DNMT-3A, DNMT-3B et DNMT-3L dans les mitochondries ainsi que l'activité DNMT mitochondriale dans le foie et le cœur afin d'évaluer l'implication des DNMT dans la survie aux deux types de stress provoqués par le gel et la déshydratation (la déshydratation cellulaire étant une composante du gel). Leurs résultats ont montré une réponse par la protéine DNMT-1 mitochondriale spécifique au stress et au tissu dans le foie et le cœur respectivement. Au cours d'une congélation de 24 heures et d'une déshydratation du corps entier, ils ont observé une régulation globale à la baisse de la DNMT-1 mitochondriale, une protéine majeure impliquée dans le maintien des niveaux de méthylation, liée à son rôle dans la transcription sélective des gènes mitochondriaux ainsi que dans la réponse antioxydante. La réponse spécifique au tissu des niveaux des protéines DNMT-3A, DNMT-3B et DNMT-3L et l'activité DNMT dans le foie ont suggéré une préférence pour un état de méthylation plus élevé dans le foie sous les deux types de stress de congélation et de déshydratation, mais pas dans le cœur. [Traduit par la Rédaction]

Mots-clés : Activité DNMT, réduction de la vitesse du métabolisme, réponse antioxydante, mitochondries, protéines mitochondriales.

Introduction

Wood frogs, *Rana sylvatica*, are one of the best-studied models of natural freeze-tolerance; they can survive whole-body freezing during winter, with up to 65%–70% of their total body water frozen as extracellular ice (Storey and Storey 2017). As a consequence of freezing, cells shrink to minimal volumes, and physiological functions (e.g., breathing, heartbeat, nerve activity, muscle movement) halt but return to normal function during spring upon thawing. Extensive studies have gradually illuminated the underlying principles of freeze-tolerance at physiological, biochemical, and molecular levels (Storey and Storey 2013, 2017). To combat the multiple stresses imposed by freezing (e.g., anoxia, ischemia, cell and organ dehydration, hyperglycemia), wood frogs switch "ON" survival mode. Multiple adaptations are upregulated, including the synthesis of cryoprotectants (primarily glucose, but also urea), which help cells maintain their integrity and defend a minimum cell volume when most cell water exits to join growing extracellular ice crystals (Storey and Storey 2017). When ice nucleation on the skin is triggered, the liver immediately starts catabolizing glycogen reserves to produce glucose and exports sugar via the blood for uptake by other tissues. Plasma glucose concentrations have

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been reported to increase from 5 mmol/L in control wood frogs to 300 mmol/L in frozen wood frogs (Storey and Storey 1984, 1986). Frogs also undergo metabolic rate depression to conserve cellular fuels and energetics (Storey and Storey 2017) by strongly suppressing energy-expensive processes, such as protein synthesis and the cell cycle, whereas essential pro-survival pathways remain active (Zhang et al. 2013; Zhang and Storey 2013). The transcription and translation of most genes are repressed in this hypometabolic state.

Studies from our lab have shown a role for epigenetic mechanisms in contributing to this hypometabolic state (Hawkins and Storey 2018; Krivoruchko and Storey 2010; Storey 2015; Zhang et al. 2020). For example, recent studies from our lab have established an interplay between DNA methylation and DNA methyltransferases (DNMTs) for suppressing nuclear gene transcription in freeze-tolerant wood frogs, hibernating mammals such as 13-lined ground squirrels (*Ictidomys tridecemlineatus*), and invertebrates such as the goldenrod gall moth, which are known to avoid extreme freezing temperatures (Tessier et al. 2021; Williamson et al. 2021; Zhang et al. 2020). Interestingly, the conserved role of DNMTs in the chemical modification of DNA by adding a methyl group from S-adenosyl-L-methionine (Adomet) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5-mC) has been well documented in eukaryotes (Denis et al. 2011; Lyko 2018). Four main DNMT isoforms regulate DNA methylation. DNMT-1 is involved in the maintenance of methylation patterns, including the methylation of newly synthesized DNA (Shock et al. 2011). DNMT-3A and DNMT-3B act as de novo methyltransferases that play important roles in regulating gene expression responses during development and disease as well as in methylating unmethylated regions of DNA (Okano et al. 1999). 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 substrate (Adomet), thereby promoting methylation (Jia et al. 2007).

Although encoded in the nucleus, the translocation of DNMTs to the mitochondria and their role in regulating metabolic signaling via methylation in response to cellular stress, including hyperglycemia, oxidative stress, or hypoxia (which either initiate or receive signals within the mitochondria) has recently emerged (Dou et al. 2019; Iacobazzi et al. 2013; Shock et al. 2011; Wong et al. 2013). For example, in a previous study, the levels of mitochondrial DNMT-1 were affected by the major players involved in oxidative stress, such as P-53, suggesting a role for mitochondrial epigenetics in the cell's response to oxidative stress (Shock et al. 2011). Interestingly, in wood frogs, multiple studies have also emphasized the role of mitochondria during freezing stress, which have similar consequences as mentioned above, and the switch to cell survival mode by enhancing antioxidant defenses (against reactive oxygen species (ROS)), upregulating mitochondria-encoded gene expression (e.g., ATP6/8, ND4, and 16 S RNA), and regulating mitochondrial enzymes in a tissue-specific manner (Gerber et al. 2016; Storey et al. 2021; Wu et al. 2018). Therefore, these studies suggest the ability of mitochondria to endure the stresses caused by freezing, in part by affecting epigenetic signatures to modify gene transcription and chromatin accessibility. Since wood frogs readily recover from freezing stress and its two major consequences (cellular dehydration and anoxia), the maintenance of mitochondrial function becomes even more crucial to maintaining overall cell function. Hence, regulatory and preservation mechanisms that affect mito-encoded proteins and mitochondrial DNA (mtDNA) such as mitochondrial DNMTs, are very important. Thus, we identified a need to analyze the roles played by these DNMTs in response to whole-body freezing or dehydration stress in wood frogs. Therefore, the current study focused on exploring the role of mitochondrial DNMTs and provides a snapshot of tissue- and stress-specific expression of DNMTs, mitochondrial DNMT activity, and the putative roles and interplay of DNMTs in regulating methylation patterns in wood frog mitochondria from the liver and heart under these stress conditions.

Methods

Animals

Male wood frogs (5–7 g) were collected during early spring from breeding ponds near Oxford Mills, Ontario. Prior to experimentation, the frogs were washed in a tetracycline bath and then acclimated for 2 weeks at 5 °C in plastic boxes lined with damp sphagnum moss. The control frogs were sampled under these conditions. For freezing exposure, frogs were moved to closed plastic boxes with a damp paper towel on the bottom and transferred to an incubator set at –4 °C for 45 min to cool frogs below their supercooling point and trigger ice nucleation of body fluids. The ice formed on the paper towel ensures highly consistent nucleation of frogs when each frog chills to the supercooling point of its body fluids (Storey and Storey 1984). Subsequently, the temperature was raised to –2.5 °C and frogs were maintained at this value for a 24 h freezing exposure, during which ~65% of the total body water is frozen in extracellular and extra-organ spaces (Storey and Storey 1984). For the dehydration treatment, other frogs were held in dry buckets at 5 °C and allowed to dehydrate over time until a mean of 40% of the total body water was achieved (Churchill and Storey 1994). The average rate of whole-body dehydration was approximately 0.5% of total body water loss per hour. The mass of total body water lost was calculated using the previously described method (Churchill and Storey, 1994). Both freezing and dehydration treatments were fully survivable by wood frogs. All frogs were euthanized by pithing, and tissues were collected quickly and flash-frozen in liquid nitrogen before storage at –80 °C.

The experimental protocols were approved by the Animal Care Committee (Protocol #106935) of Carleton University and adhered to the guidelines set by the Canadian Council on Animal Care.

Mitochondria isolation and preparation of tissue extracts

A commercially available kit (Mitochondrial Isolation Kit for Tissue (with Dounce Homogenizer), ca. No. ab 110169, Abcam, Toronto, ON, Canada) was used to isolate the mitochondrial fraction from frozen tissue samples of liver and heart tissues of control, 24 h frozen, and 40% dehydrated wood frogs. All steps of mitochondrial tissue processing were performed on ice. Briefly, 200–300 mg of frozen tissue (n = 4 independent replicates from different wood frogs) was rapidly weighed and then washed multiple times in cold wash PBS buffer (4 °C) provided with the kit. The tissue was rapidly minced with dissection scissors (kept on ice) and the extra cold wash buffer was removed. The washed tissue was homogenized in 1 mL of cold isolation buffer with a Dounce homogenizer, with the number of pestle strokes recommended by the manufacturer for each tissue. The homogenate was then transferred to a microtube (kept on ice) and filled with up to 2 mL isolation buffer. The tubes were centrifuged at 1000*g* at 4 °C for 10 min to pellet the cell debris and nuclei, which were discarded, and the supernatant was saved. The supernatant was transferred to new 2 mL tubes and centrifuged again at 12 000*g* at 4 °C for 20 min to isolate the upper supernatant fraction and the mitochondrial pellet. Following this step, the pellet was washed in 1 mL isolation buffer (containing 10 μL protease inhibitor cocktail) and centrifuged again at 12 000*g* at 4 °C for 20 min. The washed 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 were measured in both mitochondrial and supernatant fractions using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada), and all concentrations were standardized to 4 μg/μL by adding the calculated amounts of isolation buffer. Aliquots of mitochondrial and supernatant protein extracts were then mixed 1:1 *v*/*v* with 2× loading buffer (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 2 μg/μL for both liver and heart samples. Samples were then boiled for 5 min, cooled on ice for 5 min, and stored at − 80 °C until needed. To assess the separation of the 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 (Figs. S1 and S2[1]).

Immunoblotting

For immunoblotting, samples of extracted protein containing 20–30 μg of protein (from liver and heart samples of control, 24 h frozen, or 40% dehydrated wood frogs) were loaded on SDS-PAGE gels and electrophoresis was carried out using a Bio-Rad Mini Protean III apparatus at 180V in 1X running buffer (25 mM Tris base (pH 8.5), 250 mM glycine, and 0.1% *w*/*v* SDS) for 75–120 min. Proteins on the gels were transferred to PVDF membranes for 75–140 min at 160 mA. After transfer, the PVDF membranes were blocked using non-fat milk (2%) diluted in 1X TBST (20 mM Tris base pH 7.6, 150 mM NaCl, and 0.05% *v*/*v* Tween 20) for 30 min. Membranes were incubated overnight with primary antibodies (diluted 1:1000 in 1X TBST) at 4 °C. The membranes were exposed using a Chemi Genius Bioimager (Syngene, Frederick, MD, USA) to visualize the chemiluminescent and Coomassie blue bands. The following antibodies were used (Rabbit polyclonal antibodies): DNMT-1 (ABclonal cat. No. A16729, RRID:AB_2769201), DNMT-3A (GeneTex cat. No. GTX129125, RRID:AB_10621537), DNMT-3B (GeneTex cat. No. GTX129127, RRID: AB_2885904), DNMT-3L (GeneTex cat. No. GTX115985, RRID: AB_10621537), Citrate synthase (ABclonal cat. No. A5713, RRID:AB 2766471), and Tubulin (ABclonal cat. No. AC007, RRID:AB_2772755). Following primary incubation, the membranes were washed three times in 1X TBST and incubated with a horseradish peroxidase (HRP)-linked goat anti-rabbit IgG secondary antibody (Bioshop, #AP007P.2) diluted at 1:5000 *v*/*v* in TBST for 30 min at room temperature on a rocker. After secondary antibody incubation, the membranes were washed again three times in 1X TBST and bands were visualized with enhanced chemiluminescence reagents (ECL). The same membranes were stained with Coomassie blue and imaged again to be used for relative quantification of mitochondrial protein in each lane and to standardize the intensity of the protein of interest for loading control to adjust for any differences in protein loading in each gel.

DNMT activity

To assess the overall DNMT activity in the mitochondrial fraction of liver and heart samples, a commercially available DNMT activity kit was used (cat. No. P-3009, Epigentek, NY, USA). A pooled mitochondrial sample was used to perform a dilution test to determine the optimal concentration of 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 1:50 *v*/*v* in DNMT assay buffer) in each well of the microplate and incubated for 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 a positive control. All samples were run in duplicate. 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 h. After 1 h of incubation, the microplate wells were washed again with 1X wash buffer four times, followed by another incubation with 50 μL of Enhancer solution (1:5000 *v*/*v*) for 30 min. The Enhancer solution was removed, and the microplate wells were again washed five times, followed by the addition of 100 μL of developing solution. A blue color in the sample well (including the positive control) indicates methylated DNA. The reaction was further stopped by adding an equal volume of stop solution. The amount of methylated DNA, which also corresponds to DNMT enzyme activity, was measured at 450 nm using a microplate reader (Multiscan Spectrum, Thermo LabSystems). The absorbance measured at 450 nm was directly proportional to DNMT activity (calculated using the following formula, (OD, optical density)) in control vs. 24 h freezing and control vs. 40% dehydrated wood frog mitochondrial samples.

$$\text{DNMT activity}\,(\text{OD/h/mg}) = \frac{(\text{Sample OD} - \text{blank OD})}{(\text{Mitochondrial protein}\,(\mu\text{g}) \times \text{Incubation time at } 37\,^{\circ}\text{C}\,(\text{hrs})} \times 1000$$

Data analysis and statistics

Bands on the immunoblots were visualized using a Chemi Genius bioimager (Syngene, Frederick, Maryland, USA), and the intensities of the 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 than standardizing band intensities against housekeeping proteins, such as tubulin (Eaton et al. 2013). Statistical testing was performed using the RBioplot program (Zhang and Storey 2016) 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.

Results

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

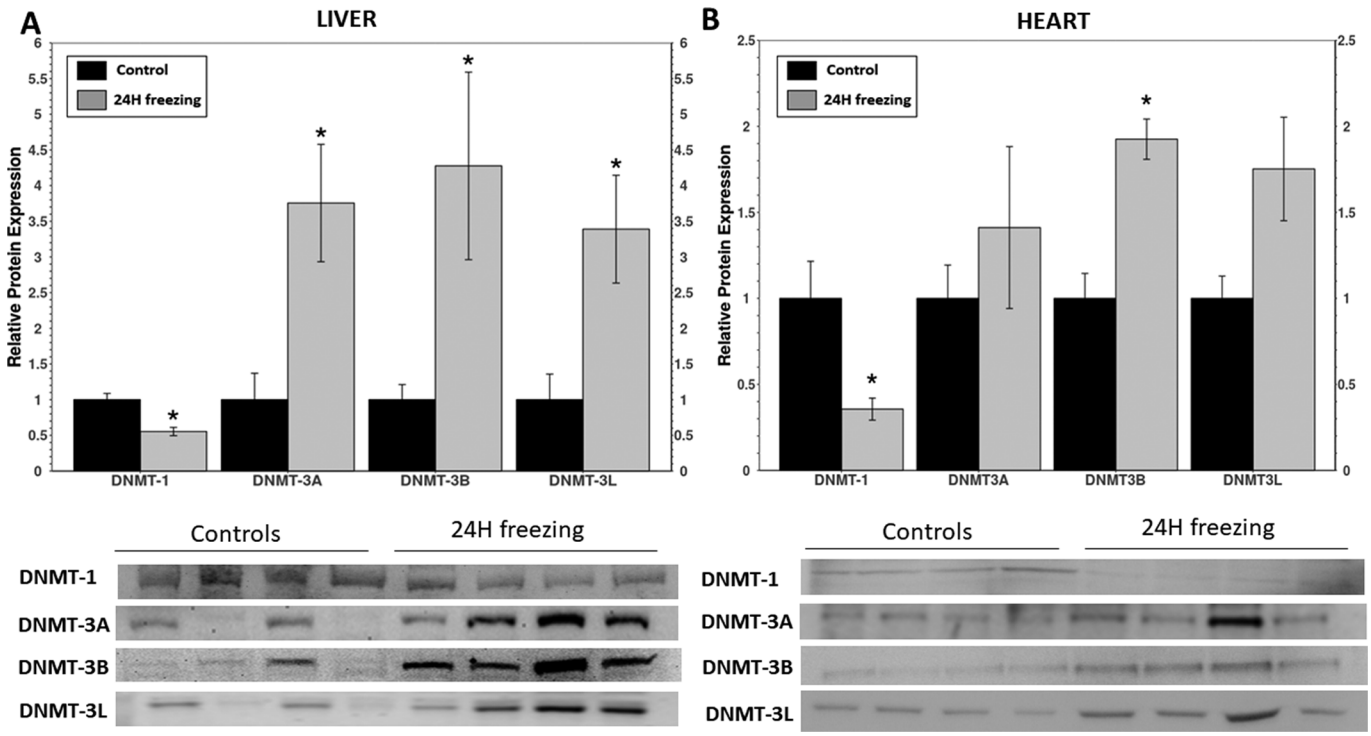
The relative protein expression levels of mitochondrial DNMT-1, DNMT-3A, DNMT-3B, and DNMT-3L were assessed via immunoblotting in the liver and heart from control (5 °C acclimated) and 24 h frozen (at –2.5 °C) frogs. The relative protein level of mitochondrial DNMT-1 in the liver from frogs frozen for 24 h was significantly reduced to 55% ± 11% ($P < 0.05$) of the control value (Fig. 1A), and a comparable response was observed in the heart with DNMT-1 protein levels in frozen frogs reduced to just 35% ± 6% ($P < 0.05$) of the control value (Fig. 1B). In contrast, mitochondrial protein levels of DNMT-3A increased significantly in the liver of frozen frogs by 3.75 ± 0.82-fold ($P < 0.05$), compared with controls (Fig. 1A), but no significant change occurred in the heart (Fig. 1B). After 24 h of freezing exposure, levels of DNMT-3B protein increased significantly in both the liver and heart, by 4.27 ± 1.3-fold and 1.92 ± 0.19-fold, ($P < 0.05$), as compared with the control values (Figs. 1A and 1B). The relative protein levels of DNMT-3L changed significantly in the liver by 3.39 ± 0.76-fold (Fig. 1A), ($P < 0.05$) but remained unchanged in the heart (Fig. 1B).

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

Cellular dehydration is a component of freezing, as 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 the liver and heart compared to control frogs and 40% dehydrated wood frogs. The relative level of

[1]Supplementary data are available with the article at https://doi.org/10.1139/bcb-2021-0519.

Fig. 1. (A) Relative protein expression of mitochondrial DNA methyltransferases (DNMT-1, DNMT-3A, DNMT-3B, and DNMT-3L) in liver and (B) heart samples of control and 24 h frozen *Rana 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.



mitochondrial DNMT-1 in the liver increased significantly by 2.29 ± 0.52-fold ($P < 0.05$) in 40% dehydrated wood frogs (Fig. 2A), whereas in the heart, the levels of DNMT-1 decreased to 74% ± 5% of control values ($P < 0.05$) in response to dehydration (Fig. 2B). Protein levels of DNMT-3A showed a similar trend, rising significantly by 2.11 ± 0.40-fold ($P < 0.05$) in the liver (Fig. 2A), whereas in the heart, levels decreased to 57% ± 10% ($P < 0.05$) of the control values (Fig. 2B). Interestingly, the levels of other mitochondrial proteins, DNMT-3B, did not change significantly in either tissue under 40% dehydration stress, whereas the levels of DNMT-3L increased significantly in both the liver and heart by 1.87 ± 0.22-fold and 1.80 ± 0.26-fold, ($P < 0.05$) (Figs. 2A and 2B).

Effect of freezing on mitochondrial DNMT activity in liver and heart

Mitochondrial DNMT enzyme activity in the liver was also assessed and correlated well with protein expression levels. DNMT activity in the liver increased significantly after 24 h of freezing by 2.55 ± 0.36-fold ($P < 0.05$) (Fig. 3A). However, the total heart activity did not change after 24 h of freezing exposure (Fig. 3B).

Effects of dehydration on mitochondrial DNMT activity in liver and heart

The mitochondrial DNMT enzyme activity in the liver increased under 40% dehydration stress to a level 2.52 ± 0.47-fold ($P < 0.05$) higher than the control values (Fig. 4A), whereas in the heart, DNMT activity levels were not significantly changed under dehydrating conditions (Fig. 4B).

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., ROS, changing oxygen availability, and suppressing or completely shutting down 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 (Gerber et al. 2016). In most animals, this stress triggers apoptosis. Adaptations supporting freezing survival have been shown in multiple studies from our lab, including various mitochondria-specific regulatory/adaptive mechanisms that help wood frogs endure extreme stresses, including freezing and dehydration (De Croos et al. 2004; Storey et al. 2021; Storey and Storey 2020). Even though the normal functions of mitochondria are largely shut down during freezing and dehydration, with ATP production shifting to anaerobic glycolysis (Storey and Storey 1984), mitochondria still need protection and maintenance of their metabolic systems to rapidly restart their functions upon recovery from both stresses. Therefore, it is important to determine whether the mitochondrial machinery is preserved and sustained in frozen and dehydrated states. The current study provides a snapshot of the effects of freezing and dehydration on protein levels and activity of the mitochondrial DNMT system, which plays an important role in regulating the expression of mitochondria-encoded genes and the methylation state of mitochondrial DNA.

To assess the role of DNA methylation in coping with freezing or dehydration stress, we analyzed the mitochondrial DNMT protein expression levels in the liver and heart tissues of wood frogs. The levels of mitochondrial DNMT-1 were significantly reduced in the liver 24 h after freezing (Fig. 1A). Mitochondrial DNMT-1 is recognized as the main maintenance enzyme that methylates DNA and has been shown to act on and serve mitochondrial DNA (Shock et al. 2011). Indeed, the enzyme affects mitochondrial biology under conditions of oxidative stress and elevated ROS levels

Fig. 2. (A) Relative protein expression of mitochondrial DNA methyltransferases (DNMT-1, DNMT-3A, DNMT-3B, and DNMT-3L) in liver samples and (B) heart samples of control and 40% dehydrated *Rana sylvatica*. Representative immunoblots are shown below the histogram. Other information as in Fig. 1.

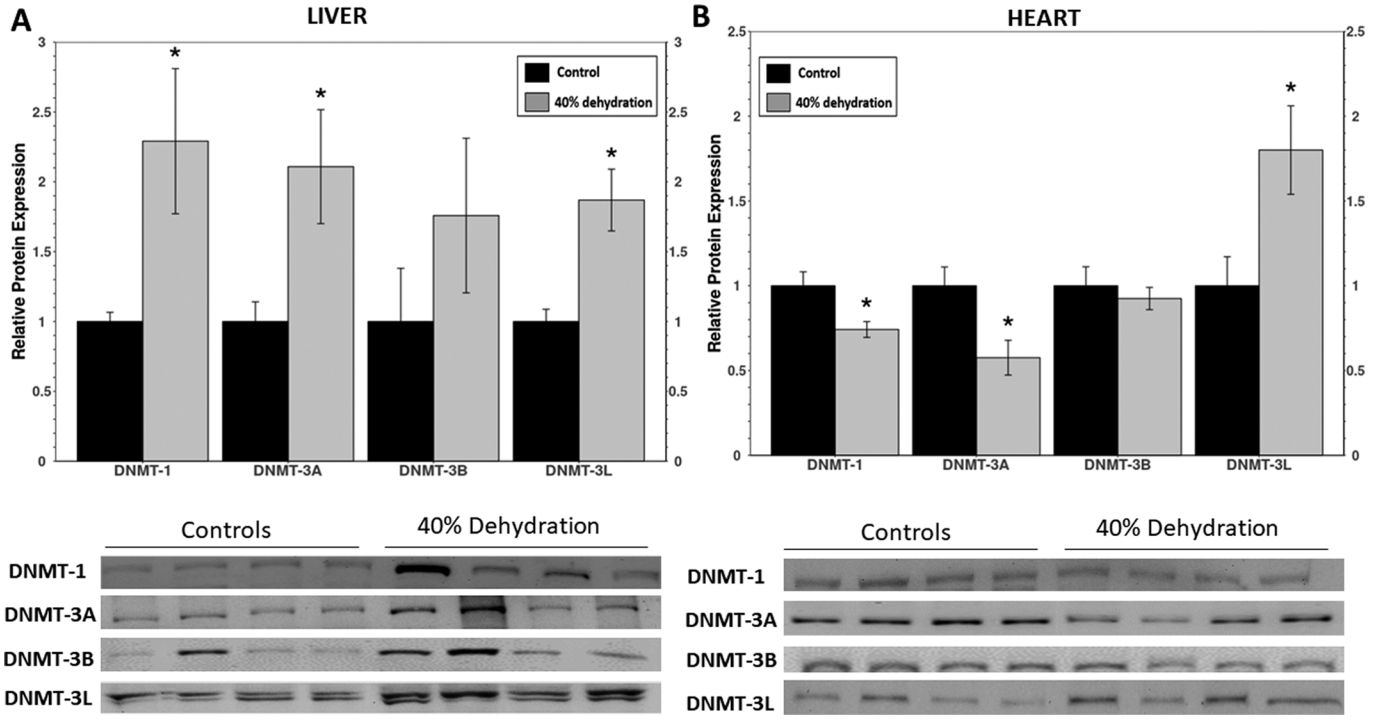
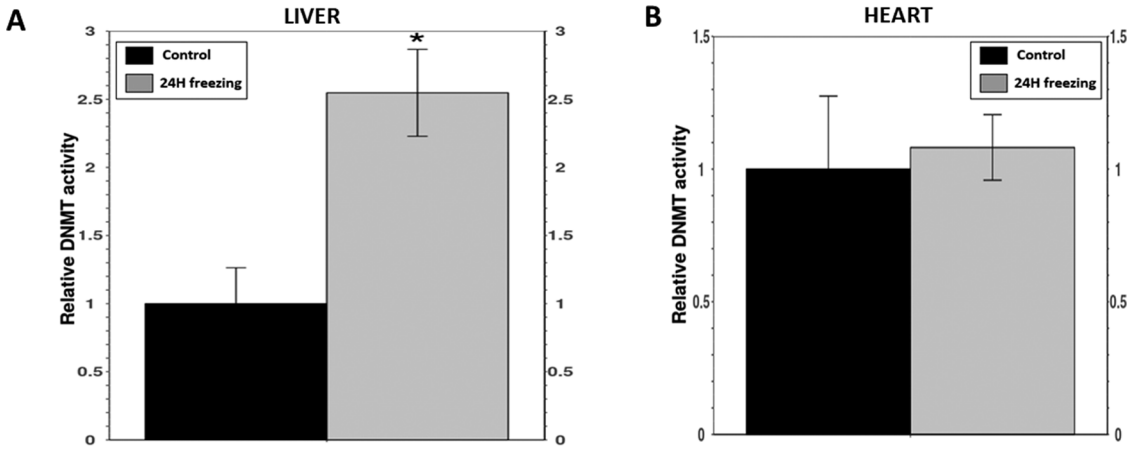


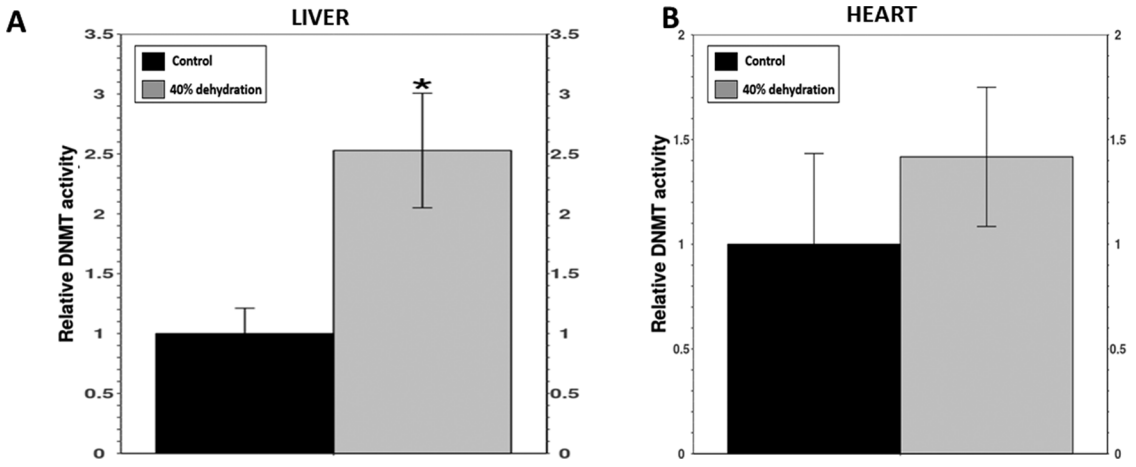
Fig. 3. (A) Relative total DNA methyltransferase (DNMT) activity in liver and (B) in heart samples of control and 24 h frozen *Rana sylvatica*. Other information as in Fig. 1.



(which are also consequences of freezing) (Singh et al. 2017). Increased levels of this enzyme in mitochondria have also been shown to decrease the protein expression of some mitochondrial-encoded peptides (Liu et al. 2020). In contrast, decreased DNMT-1 levels should increase mitogene expression. Interestingly, a separate study from our lab showed upregulation of mitochondria-encoded genes (ATP6/8, ND4, and 16S RNA) during freezing in the liver of wood frogs (Storey et al. 2021), which aligns with the DNMT-1 downregulation reported here (Fig. 1A). Although DNMT-1 levels were reduced during 24 h of freezing, elevated DNMT-3A and DNMT-3B levels in the mitochondria could underlie the upregulation of mitochondrial DNMT activity, which ultimately relates to the amount of methylated mtDNA (5mC levels) (Fig. 3A). Interestingly, catalytic mitochondrial DNMT3L levels also increased in conjunction with an increase in DNMT3A and DNMT3B (Fig. 1A). Overall, selective regulation of DNMT1 could promote crucial genes in maintaining mitochondrial function, while upregulation of DNMT3 (Figs. 3A and 3B) along with DNMT activity indicates a preference for a higher methylation state on mtDNA in the liver (Lopes 2020; Wong et al. 2013), possibly to keep freeze-induced disruption of mitochondrial functions in check so as not to face consequences of cell death and tissue damage (Storey et al. 2021).

In the case of hearts from frozen frogs, DNMT-1 also showed reduced levels (Fig. 1B), similar to those in the liver. The role of mitochondrial DNMT-1 in the heart is related to the regulation of oxidative stress (Kietzmann et al. 2017). In addition, DNMT-1 levels in the mitochondria have been reported to regulate methylation in the D-loop (displacement loop, the non-coding region of

Fig. 4. (A) Relative total DNA methyltransferase (DNMT) activity in liver and (B) in heart samples of control and 40% dehydrated *Rana sylvatica*. Other information as in Fig. 1.



mitochondrial DNA), which is essential for regulating mitochondrial copy number and mitochondrial DNA damage during stress (Bellizzi et al. 2013). Reduced levels of mitochondrial DNMT-1 suggest that the regulation of these processes might also be lower during freezing, but interestingly, this also aligns with the higher protein levels of the mitochondrial antioxidant enzyme, superoxide dismutase-2 (SOD-2), reported in the hearts of frozen wood frogs (Wu et al. 2018). SOD-2 has been shown to be directly associated with mtDNA (Kienhöfer et al. 2009) and can also bind to the mitochondrial D-loop at low methylation levels (Mishra and Kowluru 2019), ultimately protecting mtDNA from oxidative damage (Kienhöfer et al. 2009). Interestingly, the increase in the levels of mitochondrial DNMT-3B (Fig. 1B), despite the lower levels of DNMT-1, along with the basal levels of mitochondrial DNMT activity (Fig. 3B), could be sufficient to maintain the required methylation patterns during freezing stress in wood frog hearts (Barrès et al. 2009; Bellizzi et al. 2013; Grandjean et al. 2007).

Cell dehydration stress is a major consequence of freezing, and we were interested in determining whether it independently affects mitochondrial function via DNMT regulation, in a manner similar to that of freezing. Dehydration of wood frogs also triggers the production of high levels of cryoprotectant (glucose), leading to major changes in cell volume, osmolality, and ATP levels (Churchill and Storey, 1994). Interestingly, the trends in mitochondrial DNMT protein expression levels under dehydration stress were very similar to those triggered by freezing, with the overall profile of DNMT protein levels and mitochondrial DNMT activity in the liver upregulated in response to 40% dehydration (Figs. 2A and 4A). However, unlike the response to freezing, the levels of DNMT-1 in the mitochondria were significantly increased during dehydration (Fig. 2A). Interestingly, this upregulation under dehydration stress was not accompanied by changes in the levels of mitochondrial genes, as observed during freezing stress (Storey et al. 2021). However, upregulation of DNMT-1 levels can also negatively regulate mitochondrial microRNAs, such as miR-34a, which ultimately diminishes the antioxidant response via the Nrf2 axis (Cao et al. 2020; Rippo et al. 2014; Singh et al. 2017). Interestingly, other studies from our lab using animal models that also undergo states of metabolic rate depression have shown downregulation of miRNA-34a in the liver (Biggar and Storey 2015; Hadj-Moussa et al. 2016), along with upregulation of Nrf-2-induced antioxidant responses during dehydration stress (Malik and Storey 2009). Furthermore, the levels of all three mitochondrial proteins (DNMT-3A, DNMT-3B, and DNMT-3L) involved in forming new methylation marks were upregulated (Fig. 2A), suggesting a preference for stricter regulation during dehydration and a state of quiescence for mitochondrial machinery at higher dehydration rates (40% dehydration) (Rosendale et al. 2014). One reason for the overall upregulation of both mitochondrial DNMT protein and DNMT activity in the liver (Figs. 2A and 4A) could be that during dehydration stress, metabolism is still very active and mostly aerobic; however, over time, this changes as the percent dehydration increases above approximately 35%, when a preference for anaerobic ATP production becomes evident and lactate accumulates (Churchill and Storey 1993). Unlike freezing, dehydration is a gradual process that takes 2–3 days to reach 40% water loss and gives frogs ample time to adjust adaptive strategies (Churchill and Storey 1993). Therefore, an upregulated response from all DNMT proteins, including DNMT-1 (compared with frozen liver), could be a response to a slowly changing metabolic state and an attempt to maintain the mitochondrial machinery in a state of quiescence to avoid any further damage because at higher dehydration rates, wood frogs tend to become hypoxic (Churchill and Storey 1994).

Unlike in the liver, hearts from 40% dehydrated wood frogs showed downregulation of mitochondrial DNMT proteins (DNMT-1 and DNMT-3A) (Fig. 2B), whereas only the levels of DNMT-3L increased significantly (Fig. 2B). However, DNMT activity did not change in the hearts of dehydrated wood frogs (Fig. 4B). The overall regulation of mitochondrial DNMTs suggests a downplay of the DNMT machinery, which is known to drive mtDNA methylation levels during stress. One possible explanation for the relaxed profile of mitochondrial DNMTs in hearts from 40% dehydrated frogs could be that, at higher percent dehydration values (as mentioned above), hypoxia develops and makes it more difficult for the heart to produce ATP to drive contraction and to deal with the pumping of thickened blood (Churchill and Storey 1993). This is also evident from the lack of changes in mitochondrial or antioxidant proteins during dehydration (Storey et al. 2021; Wu et al. 2018). Hence, maintaining gene methylation levels 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 and activity levels in response to freezing and dehydration stresses in the liver and heart of the freeze-tolerant wood frog, *Rana sylvatica*. Mitochondrial DNMT-1 protein levels responded in a stress- and tissue-specific manner in the liver and heart, respectively. DNMT-1 regulation in the liver of frozen frogs suggested a role in the enhanced profile of mitochondrial peptides, as seen previously in freezing 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, along with DNMT activity, in the liver were generally upregulated under both 24 h freezing and 40% dehydration stress, which might aid in maintaining the mitochondrial machinery in a state of quiescence by regulating the methylation marks. The heart responded differently to both freezing and dehydration stress conditions. The mitochondrial profiles of DNMT proteins and DNMT activity were mostly reduced or unchanged under the two stresses. Overall, reduced protein levels of mitochondrial DNMT-1 in both frozen and dehydrated hearts could be related 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 that may play a prominent role in promoting freeze-tolerance in wood frogs.

Competing interests

The authors declare there are no competing interests.

Author's contributions

G.S. designed and conducted the experiments, performed data analysis, and wrote the manuscript. K.B.S. contributed to the reagents, materials, and analysis tools. Both G.S. and K.B.S. edited and revised the manuscript. All authors have read and approved the final manuscript.

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