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The Effect of Freeze-thaw Events on DNA integrity in the Gray Treefrog (Hyla versicolor)

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THE EFFECT OF FREEZE-THAW EVENTS ON DNA INTEGRITY IN THE GRAY TREEFROG (*HYLA VERSICOLOR*)

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THE EFFECT OF FREEZE-THAW EVENTS ON DNA INTEGRITY IN THE GRAY TREEFROG (*HYLA VERSICOLOR*)

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THE EFFECT OF FREEZE-THAW EVENTS ON DNA INTEGRITY
IN THE GRAY TREEFROG (*HYLA VERSICOLOR*)

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Freeze-tolerant gray treefrogs survive winter by producing natural cryoprotectants and accommodating ice formation within extracellular spaces. While frozen, gray treefrogs endure hyperglycemia, dehydration, and anoxia due to the halt of all bodily functions. Upon thawing, the frogs' anoxic cells receive a rapid influx of oxygen, which can cause oxidative damage to vital macromolecules including DNA. Previous studies have suggested freeze-tolerant frogs avoid oxidative damage after freeze-thaw events by elevating antioxidant activity, but recent work has shown upregulated DNA repair encoding genes in post-freeze frogs. The objective of this thesis is to assess the cellular costs of freezing by measuring oxidative DNA damage in gray treefrogs (*Hyla versicolor*) as they thaw. Experimental frogs were frozen for 24 hours at -3°C and dissected after 2, 6, or 24 hours of thawing. Both liver and skeletal muscle tissues were excised and examined due to previously reported differences in antioxidant and cryoprotectant capacity. An ELISA was used to detect concentrations of oxidatively damaged guanine in each tissue sample. It was found that oxidative DNA damage within liver tissue did not increase from baseline during the freeze-thaw event. In muscle tissue, damaged DNA concentrations were elevated after 2 hours of thawing but slowly decreased with time. By 24 hours post-freeze, damage levels in muscle tissue returned to baseline, suggesting a full recovery. The absence of freezing-induced damage in the liver and short-lasting damage in the muscle shows that gray treefrogs are well adapted to

mitigate freeze-thaw injury. These results reaffirm that freeze-tolerance is an effective overwintering strategy that is comprised of a complex series of evolutionary adaptations, which deserve further investigation.

I. Introduction

A. Vertebrate Freeze Tolerance

For ectothermic animals, winter survival is complicated by the dependence of their body temperature on environmental temperature. Those that inhabit northern latitudes, where temperatures regularly drop below 0°C, must find strategies to prevent the lethal freezing of body fluids. Most northerly-distributed ectotherms are behaviorally adapted to evade extreme cold altogether. For example, some flying insects migrate south and several snake species occupy underground hibernacula for the winter months (Carpenter, 1953; Froy et al., 2003). Amphibians generally overwinter in aquatic habitats or wet burrows where they can be protected against both dehydration and fluctuating air temperatures (Tattersall and Ultsch, 2008). However, a small number of species are recurrently exposed to subzero temperatures due to their use of poorly insulated refugia. For example, pool frogs (*Rana lessonae*) hibernate beneath moss or detritus on the forest floor and have been observed moving between hibernation sites while temperatures were still near freezing (Holenweg and Reyer, 2000). Winter survival of this species, and several others, is only possible through the evolution of physiological mechanisms that allow them to withstand the freezing cold.

Animals with natural tolerance to subzero temperatures utilize at least one of two adaptive approaches; 1) preventing ice formation by depressing the freezing point of bodily fluids and supercooling (freeze avoidance), and 2) controlling internal ice formation to keep it within extracellular spaces (freeze tolerance) (Voituron et al., 2002; Storey and Storey, 2017). Compared to freeze avoidance, freeze tolerance can allow for

greater cold hardiness and is less energetically expensive, though the risks of this strategy are still being uncovered (Voituron et al., 2002).

Invertebrates have evolved freeze-tolerant mechanisms numerous times, leading to a diversity of employed cryoprotective compounds and extreme cold hardiness with some insect larvae able to survive cryopreservation at -196°C (Stetina et al., 2018; Toxopeus and Sinclair, 2018). In vertebrates, freeze tolerance is rare, and the ranges of temperatures tolerated tend to be less extreme. Among the most cold-hardy vertebrates are the wood frog (*Rana sylvatica*) and Siberian salamander (*Salamandrella keyserlingii*) which can survive freezing at temperatures as low as -18°C and -35°C respectively (Larson et al., 2014; Storey and Storey, 2017).

Amphibians have evolved freeze tolerance at least several times, implying that innate physiological mechanisms in this group contribute to cryoprotective adaptations (Churchill and Storey, 1993; Costanzo et al., 1993; Storey and Storey, 2017). For example, preexisting tolerances for dehydration and anoxia confer the ability to withstand water loss and arrested blood flow due to ice crystallization (Storey and Storey, 2017). Additionally, most freeze-tolerant vertebrates accumulate glucose as a cryoprotective compound, and even amphibians that are not freeze-tolerant have been observed increasing glucose production when exposed to subzero temperatures (Costanzo et al., 1993). Ostensibly, this is part of the vertebrate "fight or flight" response which involves a stress-induced increase in glycogenolysis. Cardioacceleration, also due to stress, helps spread glucose throughout the body (Costanzo et al., 1993). As such, it is probable that the production and mobilization of cryoprotectant that occurs moments before freezing is an augmented stress response.

Freeze-tolerant species prepare for winter by consuming glycogen-rich prey and depressing their metabolic rate to conserve nutrients for eventual cryoprotectant production (Irwin and Lee, 2003; Storey and Storey, 2017). The glycogen which will be catabolized into glucose cryoprotectant is stored in the liver (do Amaral et al., 2018). As the temperature drops and liver output increases, amphibians must simultaneously alter normal homeostatic controls to allow for an extreme hyperglycemic state to ensue (Storey and Storey, 2017). Circulating a large concentration of glucose increases plasma osmolality, which in turn reduces the amount of freezable water (Layne, 1999). As some bodily water begins to solidify, urea also accumulates in response to dehydration (do Amaral et al., 2018). Together, glucose and urea prevent ice formation in intracellular spaces and protect cells against volume reduction (Storey and Storey, 2017).

B. Gray Treefrogs

Hyla versicolor and *H. chrysoscelis* are unique among freeze-tolerant amphibians in that they accumulate glycerol in addition to glucose (Storey and Storey, 2017). *H. versicolor*, the tetraploid gray treefrog, has arisen multiple times from genome duplication events within *H. chrysoscelis*, the diploid gray treefrog (Booker et al., 2020). Apart from different chromosome count, these species are highly similar genetically and phenotypically (Ralin et al., 1983; Booker et al., 2020). Therefore, it is expected that the freeze-tolerant mechanisms of *H. versicolor* and *H. chrysoscelis* are roughly equivalent though no direct comparisons have been done (Layne, 1999; Layne and Stapleton, 2009; Storey and Storey, 2017; do Amaral et al., 2018). In separate experiments, *H.*

chrysoscelis has survived several days at -4.5°C and *H. versicolor* has survived temperatures as low as -6°C (Storey and Storey, 2017).

Although gray treefrogs survive less extreme temperatures than the wood frog, which is the most extensively studied freeze-tolerant amphibian, glycerol has been shown to be the superior cryoprotectant to glucose (Costanzo and Lee, 1991; Storey and Storey, 2017). For example, Costanzo and Lee (1991) froze wood frog blood that was infused with either glucose or glycerol and found that less cell death occurred in the glycerol solution. Additionally, do Amaral et al. (2018) postulate that glycerol, while serving the same function as glucose, can mobilize quicker due to aquaporin compatibility, plus it could contribute to maintaining redox balance and preserving membrane fluidity.

Cold-acclimated treefrogs circulate glucose once exposed to subzero temperatures, but glycerol circulation begins only after the onset of freezing (Layne, 1999; do Amaral et al., 2018). Despite its later start, frozen frogs were found to have a more equal distribution of glycerol throughout their body than glucose, which is found in higher concentrations near the liver (do Amaral et al., 2018). Most notably, glycerol levels were seven times higher than glucose levels in frozen *H. chrysoscelis* leg muscles (do Amaral et al., 2018). Glucose-dependent species exhibit very low cryoprotectant concentrations in skeletal muscle relative to core organs, which evidently allows more cryoinjury to occur in the hind legs (Costanzo et al., 2013; Santos-Santos et al., 2017; do Amaral et al., 2018).

In one study, *H. versicolor* survived after 53% of their bodily water converted into ice (Layne and Jones, 2001). It is estimated that an ice content >70% would be lethal for all freeze-tolerant frogs despite any cryoprotective measures taken (Storey and Storey,

2017). Death can be caused by several types of cryoinjury; for instance, ice accumulation may result in cell volume reduction and changes in electrolyte concentrations (Costanzo and Lee, 2013). Ice crystals may also cause mechanical damage by compressing cells or rupturing membranes.

C. Stress Associated with Freezing

Studies on the post-freeze physical performance indicate that freeze-tolerant mechanisms for averting cryoinjury are limited (Costanzo et al., 1997; Irwin et al., 2003; Santos-Santos et al., 2017). For example, freezing reduced locomotor endurance by 38% in wood frogs, and motor impairment was evident after four days of recovery (Irwin et al., 2003). Another wood frog study found that males would not attempt to breed for several days after thawing although they did gather at a breeding pond (Costanzo et al., 1997). These easily observable impairments in recently thawed frogs likely reflect the physiological stress and buildup of damage incurred by freezing.

As previously mentioned, freeze-tolerant frogs can typically endure 60-70% of their body water being converted into ice (Storey and Storey, 2017). To ensure that ice formation is limited to extracellular spaces, intracellular water is drawn out from cells causing dehydration, ion imbalances, and organ shrinkage (Storey and Storey, 2017). Severe dehydration also causes an increase in blood viscosity and decrease in blood volume, which inhibits oxygen delivery to organs (Hermes-Lima and Zenteno-Savin, 2002). The subsequent anoxia is accompanied by metabolic rate depression and a switch to anaerobic metabolism (Storey and Storey, 2017). Amphibian species are capable of surviving under oxygen stress for several days or weeks; in contrast, most mammals

experience internal injury after only a few minutes in anoxic conditions (Hermes-Lima and Storey, 1996; Hermes-Lima and Zenteno-Savin, 2002).

While frozen, frogs halt all physiological processes, including nerve signaling, circulation, and respiration (Storey and Storey, 2017). Early in the thawing process, the heart regains function and it quickly starts circulating oxygenated blood (Costanzo and Lee, 2013). This rapid re-oxygenation and reperfusion of previously ischemic organs can trigger oxidative damage in macromolecules and lead to severe tissue damage (Hermes-Lima and Zenteno-Savin, 2002). Similar ischemia-reperfusion events are known to lead to heart attack and stroke in humans (Cooke et al., 2003). Hence, it is postulated that freeze-tolerant animals must possess rigorous cellular protective mechanisms in order to thaw without substantial injury (Joanisse and Storey, 1996; Wu et al., 2018).

D. Oxidative Damage

Normal cellular respiration produces reactive oxygen species (ROS) as a byproduct of the electron transport chain (Chatterjee and Walker, 2017). At low levels, ROS may perform various defensive tasks such as instigating apoptosis and protecting cells against microbial pathogens. However, at high levels, ROS can cause oxidative damage to proteins, RNA, DNA, and membrane polyunsaturated lipids (Hermes-Lima and Zenteno-Savin, 2002; Chatterjee and Walker, 2017). The consequences of high ROS levels can be mitigated by restricting mitochondrial activity or by releasing antioxidant enzymes, which will terminate the chemical reactions that lead to oxidation (Chatterjee and Walker, 2017). Despite protective measures, overabundance of ROS and high oxidative damage to biomolecules are omnipresent in disease (Cooke et al., 2003).

The three main ROS of biological significance are hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), and hydroxyl radicals (OH^-), all of which are known to cause oxidative damage if not neutralized (Chatterjee and Walker, 2017). High levels of these reactive species trigger a cellular stress response that includes antioxidant defenses, protein stabilization by chaperones, DNA damage repair, and alterations to energy metabolism (Cooke et al., 2003). Anoxia-tolerant species often have highly adapted antioxidant defenses, which are needed to protect against the upsurge in ROS formed when oxygen is reintroduced into anoxic cells (Hermes-Lima and Storey, 1996; Storey, 2004). Joanisse and Storey (1996) showed wood frogs increase antioxidant enzyme activity during freezing, and their rate of increase is higher than that of a freeze-intolerant relative (*Rana pipiens*), suggesting that high antioxidant defenses are also integral to freezing survival.

Stress-induced chaperones, such as heat shock proteins (HSPs), play a crucial role in stabilizing new proteins and refolding proteins that had been damaged by cellular stress (Storey and Storey, 2017). A heat shock response has been observed in the freeze-tolerant Antarctic midge (*Beligica antarctica*) during thawing, indicating damage to proteins and possibly DNA (Teets et al., 2019). It was also discovered that midges sustain significant tissue damage during freezing, with about 55% of fat cells and 35% of midgut cells lost after 24 hours at -9°C (Teets et al., 2019). Meanwhile, the nature and severity of injury endured by freeze-tolerant vertebrates is poorly studied. Glucose, their main cryoprotectant, has pro-oxidant properties that stimulate the production of ROS and cause the accumulation of advanced glycation end products (AGEs), which can also be damaging to macromolecules (Storey, 2004). Hyperglycemia, along with rapid re-

oxygenation during thawing, perpetrates a buildup of ROS, which could potentially lead to genomic instability, cell death, and mortality (Cooke et al., 2003; Storey, 2004).

Oxidative damage to DNA is especially deleterious, as it can lead to base pair modifications, single stranded breaks (SSB), and double stranded breaks (DSB) (Cooke et al., 2003). Repairing these oxidative DNA lesions is vital for limiting of mutagenesis, cytostasis, and cytotoxicity (Cooke et al., 2003). As such, there are three overlapping pathways to repair oxidatively damaged DNA: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). However, persistent mutations caused by oxidation are associated with a multitude of human diseases, including Alzheimer's, cystic fibrosis, psoriasis, diabetes, rheumatoid arthritis, and various cancers (Cooke et al., 2003).

Previous studies examining oxidative stress in wood frogs were unable to find evidence of post-freeze damage (Joanisse and Storey, 1996; Wu et al., 2018). These studies measured the concentration of antioxidants associated with oxidative damage, but did not measure oxidatively damaged DNA directly. It remains unclear whether freeze-tolerant frogs possess the ability to fully mitigate DNA damage, or if the damage endured cannot be detected by previously used techniques. Additionally, freeze-tolerant frog species may face different levels of damage depending on their cold-hardiness and unique adaptive mechanisms.

E. Objectives

Recent data by do Amaral et al. (2020) show that cold-acclimated and frozen *H. chrysoscelis* upregulate several genes that encode HSP and DNA repair. This is the first

evidence that has emerged pointing toward DNA imperilment during or after freezing in gray treefrogs. The goal of the current study is to quantify and compare the amount of DNA damage present in frozen and post-freeze gray treefrogs.

Specifically, an ELISA was used to measure oxidative DNA damage in unfrozen and frozen frogs that were allowed to thaw for 2 hours, 6 hours, or 24 hours. By observing frogs at these separate time points, estimates can be made for the length of time the DNA damage lasts, as well as the efficiency of adaptive DNA repair mechanisms. Additionally, both liver and skeletal muscle tissue were examined to determine the effects of cryoprotectant concentration and other organ-specific qualities on damage susceptibility. It is hypothesized that the liver, which receives the most cryoprotectant, is better protected from freezing-induced damage than skeletal muscle, which receives the least amount of cryoprotectant (do Amaral et al., 2018). Furthermore, oxidatively damaged DNA levels are expected to decline in both tissue types as the amount of time thawed increases.

II. Methods

A. Experimental Animals

Adult gray treefrogs (n=25) were purchased from Backwater Reptiles Inc., a Texas-based retailer, in September 2020 and shipped to Western Kentucky University. Frogs were housed in plastic shoeboxes with wet foam and fed crickets biweekly until the end of November when frogs were fasted for three weeks to prepare for hibernation. Frog rearing and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Western Kentucky University (submission ID: 18-05).

Species identity was verified using DNA sequence data from the mtDNA NAD1gene with custom PCR primer sequences that contain lineage-specific diagnostic variation capable of distinguishing between Texan *Hyla versicolor* and *H. chrysoscelis* (W.W. Booker, personal communication). The species was determined as *H. versicolor*.

B. Freezing Experiment

The frogs spent two weeks at room temperature before being moved into an incubator (Percival, model: I-66NL), which was initially set at 20°C with a 12:12 h light-dark cycle. The photoperiod was shortened and the temperature was lowered by four degrees every other week (Table 1). Within three months, the frogs were cold-acclimated to 4°C and held in complete darkness. During the acclimatization process, two frogs died of unknown causes.

Frogs remained in darkness and 4°C for three weeks prior to freezing. During this time, each frog was weighed and assigned to a study group. The mean body mass (~6.8 g) was counterbalanced among all four groups (n=6, 5). While the cold-acclimated

control frogs remained in the 4°C incubator, the remaining frogs were placed into ice-filled 16 oz. plastic deli cups to initiate somatic freezing. The temperature inside the deli cups was monitored with a thermocouple, which was attached to the inside of deli lids throughout the freezing process. After three hours on ice, the frogs were transferred to a refrigerated incubator (Thermo Fisher Scientific, model: PR205745R) set to -3°C and left to freeze for 24 hours. Frozen frogs were placed back into the 4°C incubator to thaw.

Each frog's liver and thigh muscles were excised and freeze dried in liquid nitrogen. Tissues were stored at -80°C.The first experimental group was dissected after two hours of thawing, then another group was dissected after six hours. The last experimental group was given 24 hours to thaw and consequently regained consciousness and thus were euthanized via pithing. The control group was also pithed and dissected on the same day as the 24-hour group.

C. Tissue Preparation

DNA was isolated from 30 mg of each collected tissue using Omega Biotek's E.Z.N.A. Tissue DNA kit. The amount of DNA in each sample was quantified with a Qubit fluorometer (Thermo Fisher Scientific) and normalized to 80 μg/mL of DNA per sample. The DNA was precipitated with sodium acetate and ethanol to produce an 8 μg pellet.

The DNA samples needed to be digested with nuclease P1 and alkaline phosphatase, as well as have a pH between 7.5-8.5 to be compatible with the ELISA used in this experiment. Accordingly, the DNA pellets were resuspended in 20 mM sodium acetate and boiled to denature the DNA. A reaction mixture was created with zinc chloride, nuclease P1, and ultrapure water then added to each DNA sample. The samples

were incubated for 30 minutes at 37°C, allowing the nuclease P1 break down the denatured DNA to mononucleotides (Fujimoto et al., 1974). The pH of the samples was adjusted to 7.5-8 by the addition of 1 M Tris-HCl, pH 8. Finally, samples were incubated again at 37°C for 30 minutes with added alkaline phosphatase to dephosphorylate the DNA. Samples were boiled for ten minutes to deactivate all enzyme activity, and stored in -80°C (see Appendix A for full DNA treatment protocol).

D. Assay

The amount of oxidative damage to nucleic acids was measured by a high sensitivity DNA/RNA oxidative damage ELISA kit (Cayman Chemical, Item No. 589320). This assay detects three species of oxidatively damaged guanine: 8-OHdG, 8-OHG, and 8-hydroxyguanine, which are measured cumulatively as the mass of damaged guanine per sample volume. This assay's detection range is 10.3 to 3000 pg/mL.

The 8-OH-dG-AChE tracer provided in this assay competes with oxidatively damaged guanine to bind to a monoclonal antibody. Since the volume of tracer is held constant while the concentration of damaged guanine varies per well, the amount of tracer that can bind to the antibody is inversely proportional to the concentration of damaged guanine. The enzymatic reaction that occurs between the developing reagent and the antibody-bound tracer produces a yellow color, the intensity of which was measured with a spectrophotometer (BioTek Synergy H1).

All DNA samples for this study were diluted by factors of 15 and 7.5 to be within the detectable range for this assay. The plates were allowed to develop for three hours and were read at a wavelength of 410 nm. The resulting logistic four-parameter standard curve was plotted on the website myAssays.com to determine the concentrations of

oxidatively damaged guanine in pg/mL within each sample. Each concentration value was also given a bound/maximum bound ratio, which specifies the amount of bound tracer in that well compared to the maximum binding (tracer control) well. A ratio that strays too far from 0.5 indicates potential issues with that well (e.g., contamination or inexact pipetting). To ensure high quality data, only concentration values with a bound/maximum bound ratio between 0.4 and 0.6 were used in the final data set.

E. Statistical Analyses

Data analyses were performed using R version 4.0.2 (R Core Team 2020) and R-Studio version 1.3.1073 (R Studio Team 2020), and differences with $p \leq 0.05$ were considered significant. Variation in DNA damage was assessed with one-way ANOVA of a linear model with oxidative DNA damage as a quantitative dependent variable and treatment as a categorical independent variable. Separate analyses were performed for each tissue type. A Tukey post-hoc test was used to find differences within test groups for muscle tissue.

Dates (2020)	Temperature (C)	Photoperiod
Sept 28 – Oct 11	20	12L/12D
Oct 12 – Oct 25	16	11L/13D
Oct 26 – Nov 7	12	10L/14D
Nov 8 – Nov 23	8	24D
Nov 24 – Dec 13	4	24D
Dec 14	Froze frogs	

Table 1: The acclimatization timeline used to prepare frogs for freezing.

III. Results

Frozen frogs displayed a blueish coloration and rigid crouched posture symptomatic of extensive tissue freezing (Figure 1). Frogs dissected after thawing for two hours retained sizable ice crystals within their coelomic cavity. No heartbeat was observed and all organs appeared frozen solid except the liver, which contained fluid. Frogs dissected after thawing for six hours were similarly stiff and unresponsive, though ice crystals were absent and livers appeared to have completely thawed. Frogs thawed for 24 hours remained in a crouched posture but had wet skin, clear eyes, and were responsive to tactile stimuli before dissection. Based on the observable heartbeat in 6-hour and 24-hour thawed frogs, it is assumed that all animals survived the freezing event.

Several ELISA samples were removed from the final data set due to extreme bound/maximum bound ratios, which resulted in variable sample sizes per treatment (see Table 2). Compared to muscle tissue, liver tissue had a notably larger range of damaged guanine concentrations (679.6 – 3745 pg/mL) across all treatment groups. The ANOVA of oxidatively damaged guanine concentration across treatments for liver tissue showed no significant variation ($F_{3,12}$=0.023, p=0.995; Table 3; Figure 2). This observed mean damage concentration (2005.9 pg/mL) may reflect a normal baseline for liver tissue as it is not responsive to freezing or thaw time.

The ANOVA of oxidatively damaged guanine concentrations revealed significant differences across treatment groups in muscle tissue ($F_{3, 14}$=7.038, p=0.004; Table 3; Figures 3 & 4). After thawing for only two hours, frozen frogs showed increased oxidative damage compared to the cold-acclimated controls (q=6.39, df=15, p=0.004). The 24-hour post-thaw group had low mean damaged guanine concentration equal to the

controls (q=2.42, df=15, p=0.555) and different from the 2-hour thawed frogs (q=4.71, df=15, p=0.035). At six hours post-thaw, oxidative damage levels were not significantly different from either the control (q=4.03, df=15, p=0.098) or the 2-hour thawed (q=3.23, df=15, p=0.256) groups. Based on the control groups' mean, the baseline oxidatively damaged guanine concentration in muscle tissue is 1483 pg/mL. Freezing increased oxidative damage and damaged guanine concentrations were inversely proportional to time thawed.

Treatment group		Sample size
Liver	Control	4
	2-hour	5
	6-hour	3
	24-hour	4
Muscle	Control	3
	2-hour	6
	6-hour	5
	24-hour	5

Table 2: The number of independent replicates per treatment group included in the data set that was used in statistical analyses. Variation is the result of poor quality ELISA readings being removed from the data.

Tissue	Model	df	SS	MS	F	p
Liver	Treatment	3	71280	23760	0.0234	**0.995**
	Residuals	12	12174283	1014524		
Muscle	Treatment	3	2226537	742179	7.038	**0.004**
	Residuals	14	1476357	105454		

Table 3: ANOVA results comparing oxidatively damaged guanine concentrations between study groups for liver and muscle tissue.

	diff	lwr	upr	p adj
2-C	982.417	315.000	1649.833	**0.004**
6-C	600.680	-88.625	1289.985	**0.098**
24-C	331.950	-388.942	1052.842	**0.555**
6-2	-381.737	-953.278	189.805	**0.256**
24-2	-650.467	-1259.732	-41.201	**0.035**
24-6	-268.730	-901.897	365.437	**0.617**

Table 4: A summary of the Tukey HSD test comparing treatment groups of muscle tissue.

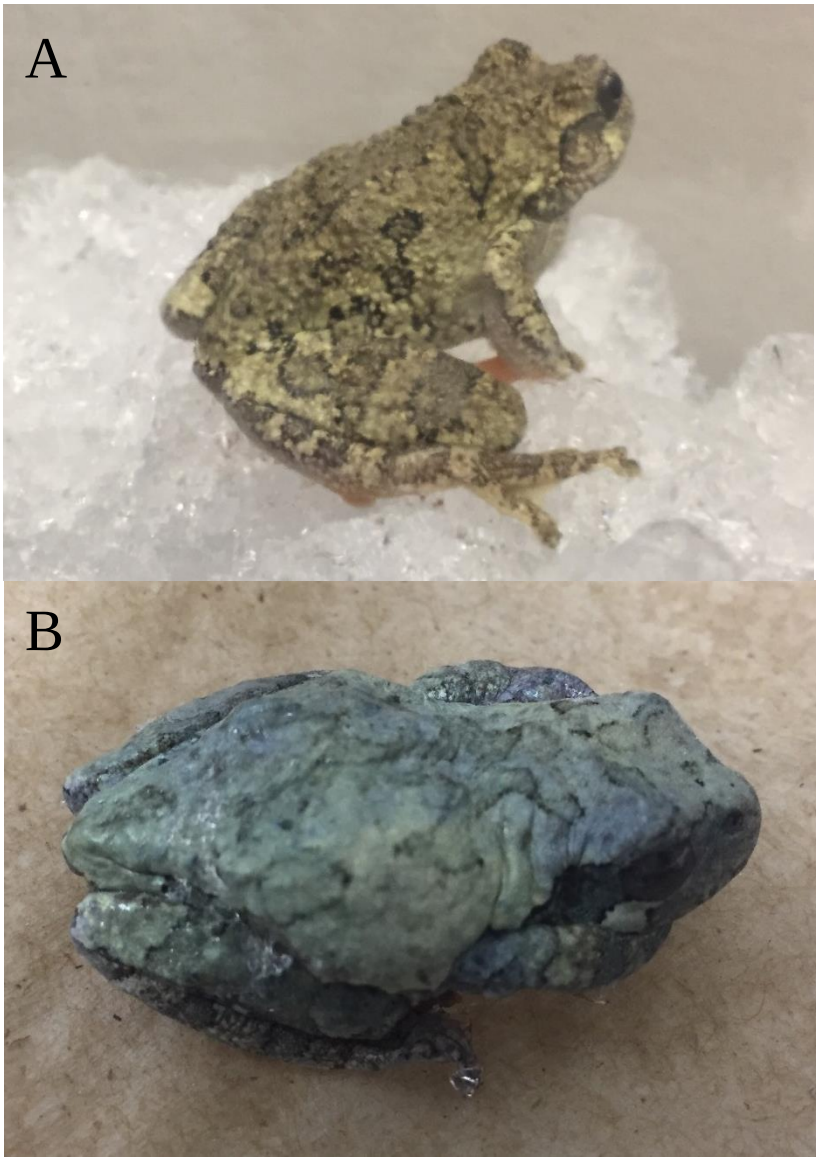

Figure 1: (A) *H. versicolor* immediately after being placed on ice. The frog's vibrant skin, clear eyes, and standing posture indicate somatic freezing has not begun. (B) *H. versicolor* after having frozen for 24 hours at -3°C and thawed for 2 hours at 4°C. The skin has become dull and blue-toned and the eyes are cloudy. The tucked posture of the frozen frog is taken to preserve body moisture (Churchill and Storey, 1993).

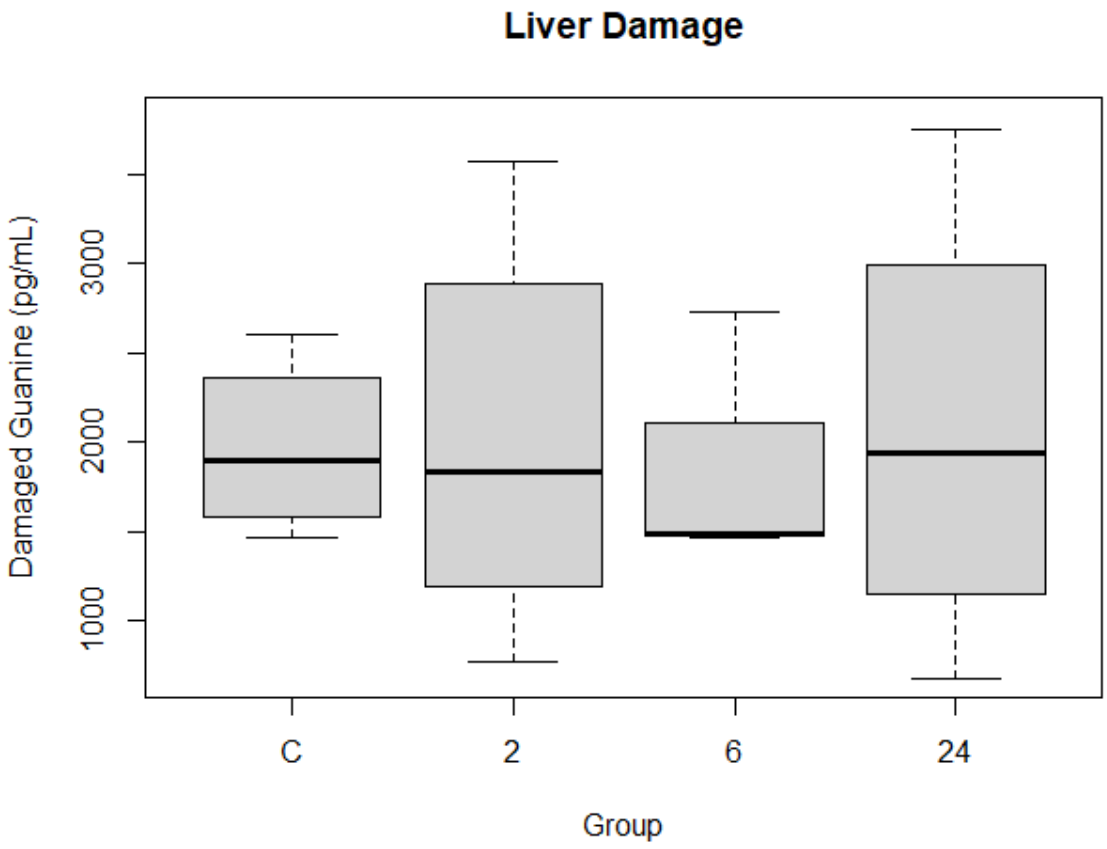

Figure 2: The concentration (pg/mL) of oxidatively damaged guanine found in the liver tissue of the Control (n=4, mean=1967), 2-hour (n=5, mean=2050), 6-hour (n=3, mean=1895), and 24-hour (n=4, mean=2073) thawed groups. A one-way ANOVA showed no significant variation across all groups ($F_{3,12}$=0.023, p=0.995).

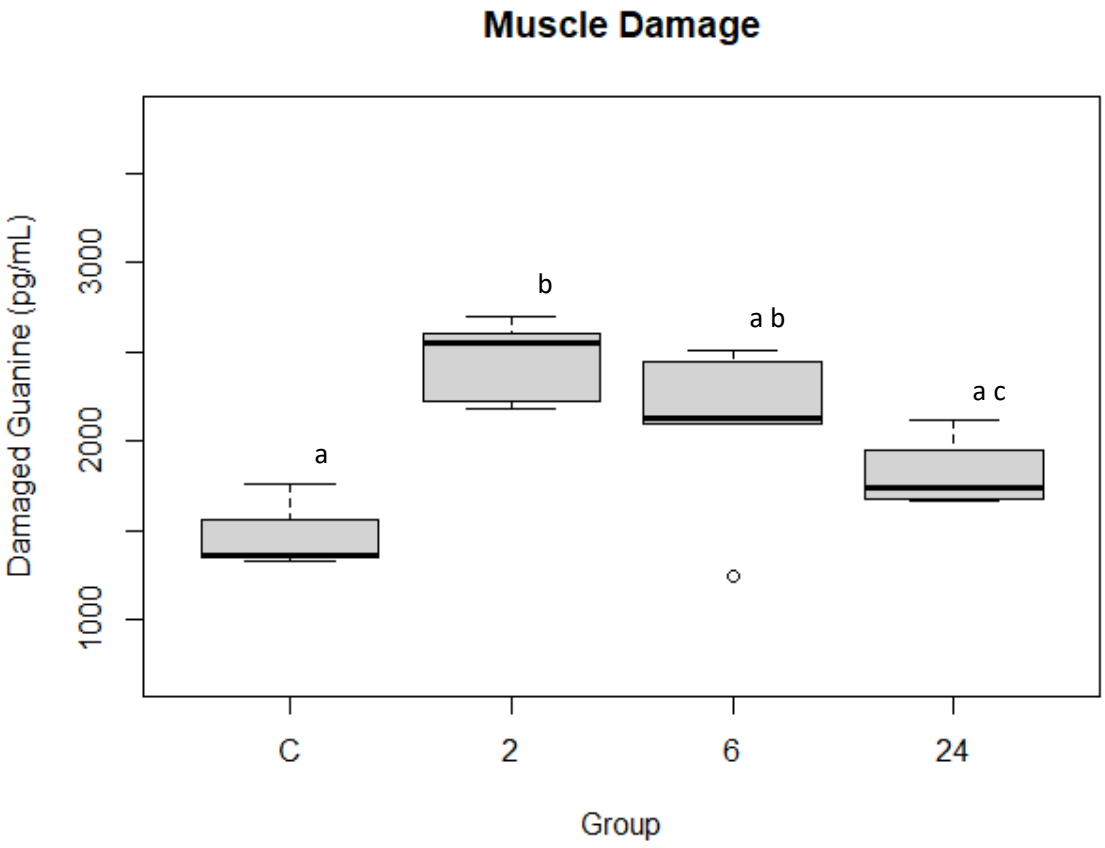

Figure 3: The concentration (pg/mL) of oxidatively damaged guanine found in the muscle tissue of the Control (n=3, mean=1486), 2-hour (n=6, mean=2465), 6-hour (n=5, mean=2084), and 24-hour (n=5, mean=1815) thawed groups. A one-way ANOVA showed variation among some of the groups ($F_{3, 14}$=7.038, p=0.004). Both the control (q=6.39, df=15, p=0.004) and 24-hour thawed group (q=4.71, df=15, p=0.035) are significantly different than the 2-hour thawed group.

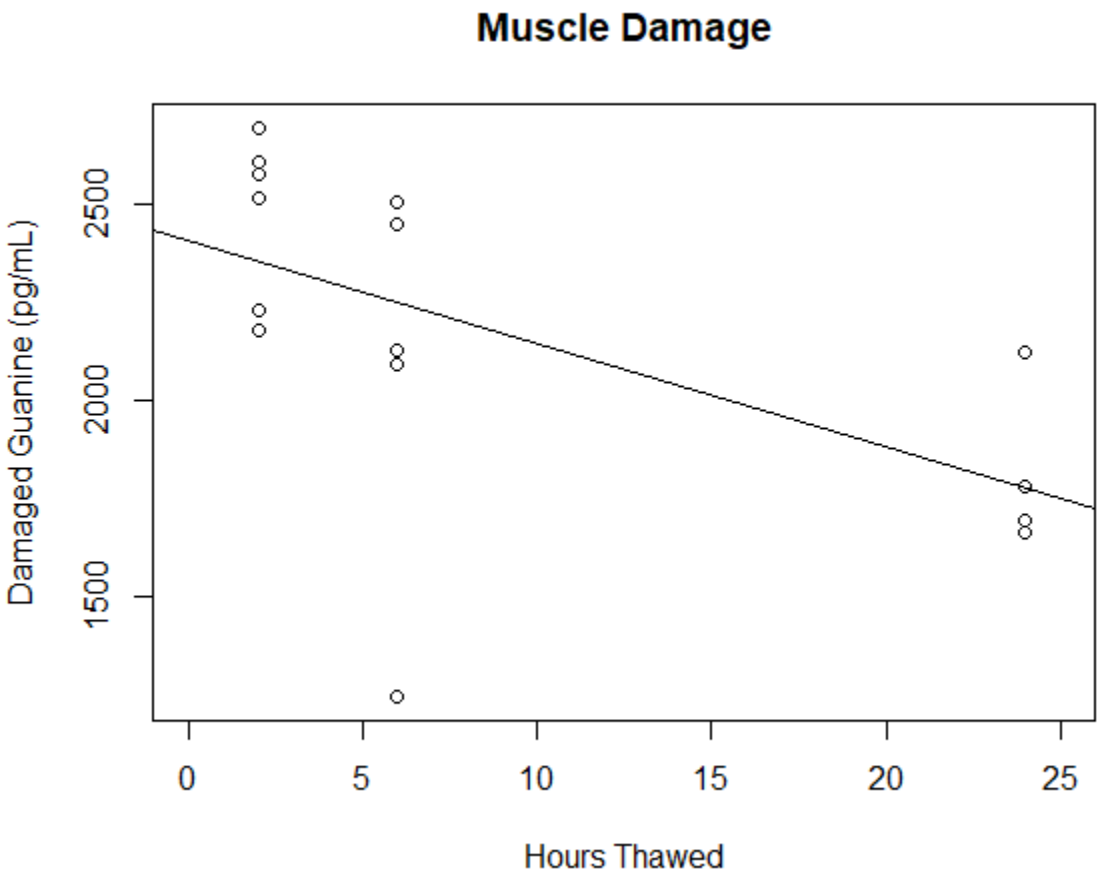

Figure 4: Linear regression showing the rate of decrease in oxidatively damaged guanine over time in muscle tissue (y=-26x+2410). The ANOVA of the regression line showed a significant relationship between the x and y variables ($F_{(1, 13)}$=6.97, p=0.020).

IV. Discussion

The multiple independent evolutions of freeze-tolerance in amphibian lineages reflect its importance to the survival and dispersal of these organisms (Storey and Storey, 2017). Indeed, freeze-tolerant frogs were shown to have a nearly 100% winter survival rate in studies within their natural habitat (Costanzo et al., 2013; Larson et al., 2014). However, the costs of survival and capability to repair ice-induced damage are largely unknown. Aside from mechanical damage caused by ice crystals, cellular components may be harmed by the physiological stress triggered by ice nucleation. For instance, as the animal freezes, its cells become anoxic and unable to oxidize hydrogen ions to fuel the electron transport chain (Joanisse and Storey, 1996). After an extended period of stress and hypometabolism, the reintroduction of oxygen upon thawing can lead to a rapid increase of toxic ROS. Oxidative damage caused by the ROS and exacerbated by the other ice-induced physiological changes could result in cell death and irreversible tissue damage (Cooke et al., 2003; Storey, 2004).

In order to survive freeze-thaw events, freeze-tolerant frogs must wield robust mechanisms for tolerating oxidative stress, hyperglycemia and dehydration at once. The question of whether freeze-tolerant frogs are able to avoid cellular damage altogether or endure some damage as a trade-off to survive winter has been addressed by previous researchers (Joanisse and Storey, 1996; Wu et al., 2018). However, the current study is the first to directly quantify the amount of oxidative DNA damage over a freeze-thaw cycle. While oxidative damage may impact a variety of macromolecules, this study focused on DNA due to alterations in DNA integrity being particularly consequential for cells (Chatterjee and Walker, 2017).

Oxidatively damaged guanine was measured using an ELISA kit which could detect three mutated species: 8-hydroxyguanosine (8-OHG), 8-hydroxy-2'-deoxyguanosine, and 8-hydroxyguanine. To examine a potential change in DNA damage concentration over time, frogs were split into three experimental groups, which were allowed to thaw for either 2, 6, or 24 hours. These time points are physiologically significant based on previous observations in post-freeze wood frogs (Costanzo and Lee, 2013). After two hours of thawing, wood frogs regain their heartbeat—the first sign of their survival. By six hours post-freeze, the frogs' bodies warm up to their thawed-at temperature (4°C), and by 24 hours post-freeze all bodily functions return to pre-freeze conditions (Costanzo and Lee, 2013). It is possible that gray treefrogs thaw at a different rate than wood frogs considering no heartbeat was observed in the 2-hour thawed group in this study.

A. Liver

The impact of freezing events elicited tissue-specific responses as oxidatively damaged guanine concentrations were elevated in muscle tissue but not liver tissue. Damage levels within the liver tissue showed no change in response to freezing or amount of time thawed ($p=0.995$), and had a mean oxidatively damaged guanine concentration of 2005.9 pg/mL across all groups (Figure 2). As ROS are an ordinary product of cell respiration, it is expected that there would be a baseline concentration of oxidative damage, but the liver's baseline was notably higher than that of the muscle (1483 pg/mL).

The liver's resistance to freezing-induced oxidative DNA damage could be the result of several factors that influence susceptibility. Firstly, the liver accumulates more

cryoprotectant than other organs as it is also the source of glucose and glycerol cryoprotectants (Costanzo et al., 2013; do Amaral et al., 2018). While skeletal muscle does contain glycogen stores, there is no evidence that these stores are converted into cryoprotectants during freezing (do Amaral et al., 2018). Hind limbs are the first body region to freeze and thereby the first to experience arrested blood flow (Costanzo et al., 2013). As a result, leg muscles only receive a small distribution of cryoprotectant and spend a long period in an ischemic state. Contrastingly, the liver is the last to freeze and the first to thaw, so it likely spends the least amount of time deprived of oxygen (Costanzo et al., 2013).

Glucose and glycerol cryoprotectants help maintain cell volume and membrane fluidity, which are critical for the cell to avoid permanent injury (Storey and Storey, 2017). However, hyperglycemia facilitates oxidative stress through several pathways; for example, glucose increases the mitochondrial proton gradient, aiding the citric acid cycle to increase mitochondrial ROS formation (Dey and Jagannathan, 2013). High glucose levels also prompt aldose reductase to convert glucose into sorbitol, a process which uses the cofactor NADPH that is needed to generate cellular antioxidants (Dey and Jagannathan, 2013).If hyperglycemia had a large impact on the amount of oxidative damage inflicted, then it would be expected that the body regions with the highest concentration of glucose and/or glycerol should be the most damaged. As the opposite was observed in this study, the effects of hyperglycemia may be negligible in freeze-tolerant frogs, which are known to tolerate nearly 50-fold increases in plasma glucose levels during freezing (Layne and Stapleton, 2009). Additionally, gray treefrogs accumulate more glycerol than glucose, and the extent to which glycerol incites oxidative

damage is unknown (Layne, 1999). Glycerol solutions are commonly used in cryopreservation of livestock sperm and have shown to be effective in protecting DNA integrity post-thaw (Tasdemir et al., 2013). Therefore, it is possible that freeze-tolerant species that only rely on glucose cryoprotectant are more vulnerable to glucose-mediated oxidative damage than gray treefrogs.

Another explanation for this study's results suggests that the liver experiences an outburst of ROS, but avoids oxidative damage via strong defense mechanisms. Due to its role in detoxifying hazardous compounds, the liver contains high levels of low molecular weight antioxidants and enzymes that degrade ROS (Inoue, 1994). As examples, the liver maintains large quantities of glutathione (GSH), superoxide dismutase (SOD), catalase, vitamin C and vitamin E. A normal liver is adapted to efficiently manage ROS accumulation as the hepatic Kupffer cells produce excessive ROS as part of their immunological function (Cesaratto et al., 2004). In freeze-tolerant frogs, the liver is more highly adapted to handle ischemia-reperfusion events than freeze-intolerant frogs (Joanisse and Storey, 1996). In a study comparing *Rana sylvatica* to *R. pipiens*, it was found that *R. sylvatica* livers exhibited greater enzyme activities and higher GSH concentrations during reperfusion (Joanisse and Storey, 1996). It is likely that freeze-tolerant frogs needed to evolve strong anti-oxidative damage defenses to protect the liver due to its indispensable role in winter survival. Not only does the liver provide cryoprotectant at the onset of freezing, but it also plays in important role in clearing access glucose and reestablishing normal metabolic activity post-thaw (Layne et al., 1996).

B. Muscle

For skeletal muscle, the 2-hour thawed group showed a higher concentration of oxidatively damaged guanine than the unfrozen controls ($p=0.004$; Figure 3). Oxidative damage levels among experimental groups decreased as time thawed increased, from a mean of 2465 pg/mL at two hours, to 2084 pg/mL at six hours, to 1815 pg/mL at 24 hours post-freeze (Figure 4). Neither the control nor 2-hour thawed group were significantly different from the 6-hour thawed group ($p=0.098$ and 0.256, respectively), which could indicate healing is initiated near the 6-hour point in thawing. The 24-hour thawed group was also not different from the control ($p=0.555$), but was different from the 2-hour thawed group ($p=0.035$). These results suggest that gray treefrogs experience oxidative damage in their hind leg muscles upon reperfusion, but are able to recover from their injury within 24 hours post-freeze.

Skeletal muscle consists of post mitotic cells, which are prone to accumulate oxidative damage during both intense exercise and extended dormancy (Radak et al., 2001). Relative to most core organs, skeletal muscle is tolerant of ischemia, and human muscles can endure three hours of oxygen deprivation before sustaining permanent injury (Zhou et al., 2018). However, myocytes contain relatively few defenses against oxidative damage and are disposed to necrosis and apoptosis during reperfusion. Though skeletal muscles are less essential for survival than the liver, increased ROS in muscles is associated with symptoms of fatigue and muscle dysfunction (Zhou et al., 2018). The observed elevated oxidative damage in the hind limbs aligns with previous studies that found post-freeze frogs show decreased hopping and swimming performance (Irwin et al., 2003; Santos-Santos et al., 2017).

C. Damage Defenses and Repair

Antioxidants play the most important role in averting oxidative damage, and in humans, abnormalities in antioxidant defense genes were found to be significant indicators of disease development (Cooke et al., 2003). The complex, overlapping antioxidant systems in vertebrate cells execute detoxifying reactions with ROS, ultimately converting ROS into nontoxic oxygen compounds such as H2O and preventing oxidative damage from occurring (Chatterjee and Walker, 2017). Wood frogs have highly adapted antioxidant defenses that are comparable to those of anoxia-tolerant turtle species, which can survive many months submerged in cold water (Hermes-Lima and Zenteno-Savin, 2002; Storey, 2004). Wood frogs also increase antioxidant enzymes prior to freezing, showing preparedness to handle increased ROS during thawing (Joanisse and Storey, 1996). While there have been no investigations of antioxidant activity in frozen gray treefrogs, it is likely that treefrogs have developed similar defenses as the wood frog.

When ROS generation overwhelms antioxidants, oxidative damage will occur and can potentially impact nucleic acids, proteins, and lipids (Chatterjee and Walker, 2017). Nucleotides with a guanine base are particularly vulnerable to oxidative damage due to guanine's low redox potential (Chatterjee and Walker, 2017). Oxidatively damaged DNA may undergo conformational changes, which cause mismatched base pairing (e.g., guanine to adenine), and strand breakages (Cooke et al., 2003). A high volume of oxidatively damaged DNA is tightly associated with disease development, thus, it is necessary for vertebrates to have several pathways to repair such DNA lesions (Cooke et al., 2003).

Damaged DNA bases can be removed via base excision repair (BER) or nucleotide excision repair (NER) (Chatterjee and Walker, 2017). BER corrects minor oxidative distortions of DNA bases by removing the oxidized base and replacing it. Significant lesions and intrastrand crosslinks are repaired by NER, a more complex process involving excision and resynthesis of several nucleotides at once. Additionally, oxidative damage can potentially cause single stranded and double stranded breaks in the DNA backbone, which are particularly difficult to heal. Left unresolved, strand breaks can result in cessation of DNA replication and transcription, deletions and fusions within the DNA, and eventually cell death (Chatterjee and Walker, 2017). Although the highly complex strand repair mechanisms of mammals are popularly studied, mostly in regards to the etiology of human cancers, little is known about how amphibians handle this type of damage.

D. Conclusion

Freeze-tolerant frogs are a unique example of adaptation to extreme environmental conditions in a vertebrate system. Studying the cellular mechanisms that make freeze tolerance a viable overwintering strategy can help with understanding the evolutionary process with which freeze tolerance arose. Additionally, the gray treefrogs in this study were able to avoid substantial oxidative DNA damage in their liver tissue, showing they are highly adapted to survive the ischemia-reperfusion event during thawing. Further investigations of these protective adaptations could potentially benefit organ transplantation and sperm/egg cryopreservation as ischemia-reperfusion injury is a major obstacle in those fields (Tasdemir et al., 2013; Bruinsma and Uygun, 2017).

Meanwhile, the presence of oxidative DNA damage in the legs of thawing gray treefrogs presents another challenge of winter survival for these organisms. Though skeletal muscle is not a vital organ, several researchers have pointed out that locomotor impairment could make post-thaw frogs vulnerable to predation (Irwin et al., 2003; Santos-Santos et al., 2017). Severe oxidative damage is also linked to nearly all mammalian cancers and several diseases, meaning frogs may suffer consequences of the damage long after thawing (Chatterjee and Walker, 2017). However, in this study, gray treefrogs seemed able to repair excessive oxidative DNA damage within 24 hours, which may allude towards advanced repair mechanisms in this species.

Few studies have examined the amount of physiological stress endured by amphibians during the freeze-thaw process. How freeze tolerance compares to other overwintering strategies in terms of survival and nonlethal costs is still ambiguous. For instance, multiple researchers have suggested that harsh freezing events could reduce an individual's fitness, but no studies have demonstrated this in a natural setting (Costanzo et al., 1997; Irwin et al., 2003). As shown by Larson et al. (2014), cryoprotection tends to be more effective when acclimatized in the wild rather than inside of a laboratory. Reduced stress and better preparedness due to natural environmental cues could be correlated with less impairment in post-freeze frogs.

The other well-known adaptive approach for surviving sub-zero temperatures is freeze avoidance, characterized by the release of antifreeze proteins which cause a depression in bodily freezing point (Storey and Storey, 2017). Compared to freeze tolerance, freeze avoidance requires high energy reserves at the time of exposure to freezing temperatures (Voituron et al., 2002). Freeze tolerance seems to be the more

energy efficient strategy, particularly because cryoprotectants may be recycled as metabolic fuel (Voituron et al., 2002; do Amaral et al., 2018). However, freeze tolerance necessitates a highly complex set of adaptations to not only avoid cryoinjury, but also survive after ischemia-reperfusion injury.

The objective of this study was to measure oxidative DNA damage in gray treefrogs during a freeze-thaw event in order to evaluate the cellular costs of freezing. Due to the lack of freezing-induced damage in the liver, and quick repair response in the muscle, it appears that gray treefrogs possess adaptations to minimize freeze-thaw injury. These results demonstrate the complexity of freeze tolerance as an overwintering strategy while also highlighting the need for investigations into the protective mechanisms that freeze-tolerant frogs possess.

E. Future directions

This study used gray treefrogs as a study species, which are moderately cold hardy relative to other freeze-tolerant species (Storey and Storey, 2017). Additionally, *Hyla versicolor* from Texas, which is the southern end of their range, had not previously been tested for freeze tolerance and may be less cold hardy than their northern counterparts. To avoid deaths of individuals, the treefrogs in this experiment were frozen only at -3°C for 24 hours. Pushing the limits of gray treefrog thermal tolerance could yield vastly different results. For instance, longer periods of oxygen deprivation can result in greater ROS accumulation (Zhou et al., 2018). Therefore, frogs that are frozen for many days, or even months, could exhibit much higher oxidative damage levels.

Furthermore, the defenses which allow gray treefrogs to avoid substantial oxidative damage in the liver warrant investigation. Observations of antioxidant activity in thawing liver tissue could elucidate the results of the current study. Information on DNA repair mechanisms in freeze-tolerant frogs could also help explain the recovery from oxidative damage seen in the skeletal muscle. Future studies should aim to compare the cellular defense mechanisms of freeze-tolerant and freeze-intolerant frogs in order to identify which characteristics evolved specifically to avoid ischemia-reperfusion injury.

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VI. APPENDIX A

DNA Digestion Protocol

DNA extractions need to be precipitated to begin digestion and phosphatase treatment as recommended by the kit's instructions. Precipitation was done using sodium acetate and chilled 100% ethanol, which were added in $1/10^{th}$ and 3x proportions to the starting volume of each DNA sample, respectively. Samples were briefly mixed and centrifuged on high for 10 minutes. While viewing the pellet, the supernatant was carefully poured out. The pellet was washed by adding 1 mL 70% ethanol and re-centrifuged for 2 minutes. The supernatant was discarded and remaining droplets were drawn out with a small pipette. Samples were left uncapped to dry for approximately 1 hour.

Dried 8 µg pellets were re-suspended in 100 µL 20 mM sodium acetate (pH 5). Samples were boiled at 90°C for ten minutes to denature the DNA then immediately placed on ice. As samples were cooling, a reaction mixture was made which contained 2 µL 10 mM zinc chloride, 50 µL 5 U/mL nuclease P1 (diluted in 20 mM sodium acetate, pH 5) and 48µL ultrapure water per DNA sample. 100 µL of the reaction mixture was added to each sample, resulting in a solution with a DNA concentration of 40 µg/mL. Samples were incubated for 30 minutes at 37°C. The pH was then adjusted by the addition of 20 µL 1 M Tris-HCL (pH 8). Based on the starting concentration of DNA, 80 µL 10 U/mL alkaline phosphatase (diluted in 50 mM Tris-HCL, pH 8), was added to each sample and incubated again for 30 minutes at 37°C. Samples were again boiled for 10 minutes at 90°C to deactivate the phosphatase. Undiluted samples were stored at -80°C.