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Abstract

Over 80% of the Earth's surface is exposed to seasonal cold temperatures less than 5.0ºC. Ectotherms implement a variety of strategies to survive seasonal, or permanent, cold exposure. Some of the most common overwintering strategies are migration, hibernation, and freeze avoiding behavior. However, freeze tolerance is a minority choice among ectotherms. This strategy permits organisms to survive between 50.0 to 70.0% of their total body water volumes frozen primarily in extracellular spaces for up to several months at a time. Freeze tolerant organisms undergo minimal supercooling of their body fluids to ensure ice formation is slow and produce a wide variety of specific proteins to control the size of ice crystals forming in the body. Freezing gives rise to severe physiological stressors which must be mitigated in order to survive freezing and thawing. While freeze tolerance is not fully understood, a growing body of evidence highlights several core tenants of this complex physiological process. When a non-freeze tolerant organism freezes, osmotic stress caused by the removal of pure water to form ice crystals in the extracellular fluid causes cells to shrink. As ice crystals thaw, water is rapidly reintroduced into the extracellular fluid causing local hypotonicity. Consequently, cells experience a rapid influx of water molecules, inducing acute cell swelling which progresses and ultimately causes cell lysis which leads to irreparable damage to an organism's tissues and organs. Many freeze-tolerant animals combat dehydration stress by the seasonal accumulation or rapid mobilization at ice-nucleation of colligative cryoprotectants that diffuse across cell membranes through specific integral transmembrane proteins in order to limit cellular volume changes. Cope's gray treefrog, *Dryophytes chrysocelis*, is a treefrog capable of freezing 65% of its total body water content for extended periods of time during harsh winter months. This treefrog is unique because it is the only known freeze-tolerant anuran which mobilizes glycerol as a cryoprotectant.

Glycerol diffuses moves across cell membranes through integral transmembrane protein channels called aquaglyceroporins. This thesis presents a comprehensive literature review which focuses primarily on proposed cellular mechanisms that mitigate dehydration stress caused by the formation of pure ice crystals, as well as anoxic and oxidative stresses caused by freezing-induced ischemia and subsequent blood reperfusion during thawing. This thesis also proposes further research to elucidate vital information about the mechanisms permitting *D. chrysoscelis'* freeze tolerance. Finally, the biomedical application of human organ and tissue cryopreservation is discussed, and an argument is presented that glycerol may be a superior cryoprotectant to use in future cryopreservation studies.

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I. Introduction

Cold weather exposes organisms to severe physiological stressors that must be mitigated with naturally developed mechanisms. Surviving in the cold must be a priority for many organisms as more than 80% of the Earth's surface is exposed to seasonal and/or permanent temperatures of less than 5^{o}C (Maayer, 2014). Ectothermic animals survive cold temperatures by utilizing behavioral avoidance, freeze avoidance, or freeze tolerance – the general tenants of these behaviors are shown in **Figure 1** (Sømme, 1999; Storey, 2006; Voituron et al., 2009; Lee, 2010; Storey & Storey, 2017; Toxopeus, 2018). Behavioral avoidance requires an organism taking action to avoid prolonged cold exposure by migrating, hibernating, or burrowing (Storey & Storey, 2017; Toxopeus, 2018), while freeze avoidance involves an organisms' mechanistic reaction to sensing cold temperatures which may include: supercooling, cryoprotective dehydration, and vitrification (depressing freezing point of body fluids, removing water available for freezing, and modifying body fluid composition to prevent ice crystallization respectively; Storey, 2006; Elinsky et al., 2008; Sformo et al., 2010; Storey & Storey, 2017; Toxopeus, 2018). While behavioral and freeze avoidance tend to be common to ectotherms, freeze tolerance is a minority choice for ectotherm overwintering (Storey & Storey, 2017).

Freeze tolerance has been observed across many species of microbes, insects, intertidal invertebrates, small soil invertebrates, as well as vertebrate amphibians and reptiles and involves an organism freezing around 50-70% of its total body water volume primarily in extracellular spaces for up to several months at a time (Storey, 2006; Maayer, 2014; Storey & Storey, 2017; Toxopeus, 2018). Freeze tolerance is a complex,

and integrated, physiological survival strategy that varies among species and, to date, is not fully understood. When frozen, an organism has no respiration or cardiopulmonary function, voluntary muscle movement, transmembrane transport, immeasurable nerve conductance, and experiences hypometabolism (Lee, 2010; Storey & Storey, 2017; Toxopeus, 2018). Freezing also exposes organisms to severe physiological stress including dehydration, hypoxia/anoxia, ischemia, osmotic stress, reduced enzyme activity, increased gas solubility, increased oxidative stress, and impaired cell-cell signaling (Storey & Storey, 2017). Non-freeze tolerant animals die if they freeze, as severe osmotic stress associated with ice formation and thawing cause irreparable tissue damage – shown in **Figure 2a** (Storey, 2006; Voituron et al., 2009; Maayer, 2014; Storey & Storey, 2017; Toxopeus, 2018). Water freezes in the extracellular fluid (ECF) as pure ice crystals. Ice crystal growth removes available solvent in solution, causing the osmolarity of the ECF to increase rapidly, quickly becoming hyperosmotic to nearby cells (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017). In accordance with the principle of osmosis, osmotic pressure pulls water out of cells' intracellular fluid (ICF) into the surrounding hyperosmotic extracellular fluid to maintain equilibrium (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017). The dehydration of cells causes them to shrink, severe shrinkage may damage a cell's cytoskeleton or plasma membrane rendering the cell unviable (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017). When ice begins to thaw, the ECF is flooded with pure solvent, decreasing osmolarity and rapidly becoming hypoosmotic to nearby cells (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017).

Consequently, cells rapidly experience a large influx of water, causing cells to swell (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017). When a cell swells past a critical threshold, the plasma membrane ruptures, killing the cell (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; do Amaral et al. 2017; Storey & Storey 2017). Mechanisms to prevent widespread damage from the compression and lysis of cells by mitigating osmotic stress during freezing and thawing is clearly a priority in freeze tolerant organisms and has been observed in nearly every known freeze tolerant organism – shown in **Figure 2b** (Storey, 1997; Storey, 2006; Krane, 2007; Voituron et al., 2009; Costanzo & Lee 2013; Maayer et al., 2014; do Amaral et al. 2017; Storey & Storey 2017).

II. Principles of Freeze Tolerance

A. Ice Formation & Management:

While the percentage of total body water sequestered as ice in freeze tolerant organisms varies (~45-55% in reptiles, ~50-70% in amphibians, up to ~82% in insects), controlling the rate and location of ice nucleation is integral to surviving freezing and thawing (Storey, 1997; 2006; Voituron et al., 2009; do Amaral et al., 2017; Storey & Storey, 2017; Toxopeus, 2018). Freeze-tolerant organisms usually initiate ice formation at temperatures slightly lower than their body fluid's normal equilibrium freezing point (FP) by supercooling body fluids to a supercooling point (SCP) approximately 2-3^{o}C below the equilibrium FP (Claussen et al., 1990; Storey, 1997; Raymond et al., 2016; Storey & Storey, 2017). Freezing near the equilibrium FP serves to slow the rate of ice formation and prevents large instantaneous ice surges, water instantly converted to ice, giving freeze tolerant organisms a longer time period to implement responses to freezing (Claussen et al., 1990; Storey, 1997; Raymond et al., 2016; Storey & Storey, 2017). When wood frogs are frozen at -2.5^{o}C, it takes approximately 1-hour post-nucleation to confirm freezing, several hours before voluntary muscle movement is lost, and nearly 24 hours before maximal ice content is observed (Storey, 1997; Storey & Storey, 2017). Freeze tolerant organisms' SCPs are varied and dependent upon the individual's physiology and environmental climate, as i nsect SPCs can range from -1^{o}C (*Chymomyza costata* larvae) to as low as -54^{o}C (*Pytho deplanatus;* Alpine Beetles) and reptiles have been observed to supercool down to -15^{o}C (Ring, 1982; Costanzo et al., 1998; Baker et al., 2003; Storey, 2006; Koštál et al., 2011; Toxopeus, 2018). Extensive supercooling can also be a hinderance to surviving freezing, as it exposes organisms to

the risk of lethal instantaneous ice formation (Claussen et al., 1990; Storey, 1997; Raymond et al., 2016; Storey & Storey, 2017). This process is exemplified in the chorus frog *P. triseriata*; when ice nucleation is initiated between -1 & -2ºC, the frogs show 100% survival rate, but when ice nucleation begins around -6ºC, a 0% survival rate is observed (Swanson et al., 1996). It is clear that freeze tolerant organisms need to control the rate of ice formation, partially accomplished through equilibrating to an SCP only a few degrees (Celsius), below the normal equilibrium FP, to allow ample time to implement physiological responses to freezing. Controlling the rate of formation also allows a freeze tolerant organism to control the location of ice within the body.

Ice nucleation is triggered in three ways: an organism contacts environmental ice (inoculating body fluids across an epithelial layer - usually skin), epithelial contact with nonspecific ice-nucleating agents (often produced by microbes in gut or on skin), or through specific action of ice-nucleating proteins synthesized by the organism (Storey & Storey, 2017). Ice-nucleating agents (INAs) are any agent which promotes formation of ice crystals; including dust, minerals, proteins, and macromolecules, INAs are not specifically produced by an organism to initiate freezing (Duman, 2001; Costanzo et al., 2004). Freeze avoiding organisms commonly sequester INAs in intracellular vesicles and organelles during cold hardening or excrete them in feces or urine to prevent freezing (Duman, 2001; Storey & Storey, 2017). However, it is commonplace for freeze tolerant organisms to utilize INAs to trigger freezing, a few examples found in freeze tolerant animals include gut and skin bacteria, some blood proteins, and stored mineral crystals (Duman, 2001; Storey & Storey, 2017). Ice nucleating proteins (INPs) are specifically synthesized by an organism to promote regulated ice formation in freeze tolerant animals

(Davies, 2014; Duman, 2015; Storey & Storey, 2017). Accumulating INAs and INPs allow freeze tolerant organisms to form ice crystals within their bodies at high subzero temperatures (Storey & Storey, 2017). The presence of these two types of ice nucleators give freeze tolerant organisms the capacity to maintain their SCP slightly below the equilibrium FP of their body fluid and freeze in a slow and organized fashion. In contrast, freeze avoidant animals that survive subzero temperatures at a low SCP accumulate antifreeze proteins (AFPs) that prevent ice crystals from growing larger than microscopic in size and limiting exposure to INAs (by sequestering in cells or excreting in waste) to minimize the risk of lethal instantaneous ice formation. AFPs have been identified in many cold-water marine fish as well as a diverse set of freeze-avoiding terrestrial insects but have never been identified in cold-hardy amphibians or reptiles to date (DeVries, 1971; Duman, 2001; Fletcher, 2001; Davies, 2014; Duman, 2014; Storey & Storey, 2017). Controlling ice nucleation in a slow ordered pattern via INPs and INAs at high subzero temperatures gives freeze tolerant organisms more time to implement a coordinated response to freezing to minimize cell damage. Cell damage is most directly minimized by ice formation exclusively in the ECF (Wharton & Ferns, 1995; Sinclair & Renault, 2010; Ali & Wharton, 2014; Raymond & Wharton, 2016; Storey & Storey, 2017). Ice almost always forms in the extracellular spaces of freeze tolerant organisms, the only know exceptions are a few Insects including *Cephus cinctus* wasp larvae; *Celatoblatta quiquemaculata* nymphs and some nematodes (*Panagrolaimus sp.*) under specific conditions (Salt, 1961; Wharton & Ferns, 1995; Sinclair & Wharton, 1997; Sinclair & Renault, 2010; Ali & Wharton, 2014; Storey & Storey, 2017). Intracellular ice formation may damage the cytoskeleton, plasma membrane, and any organelles within a

cell if crystals grow too large (Wharton & Ferns, 1995; Sinclair & Renault, 2010; Ali & Wharton, 2014; Raymond & Wharton, 2016; Storey & Storey, 2017). As previously mentioned, freeze-avoidant organisms minimize INA exposure to prevent nucleation and accumulate AFPs to keep ice crystals microscopic. However, freeze tolerant organisms use many complex mechanisms to prevent cytosolic ice formation including membrane adaptations and osmoregulation which promote ice nucleation in the ECF, and minimize the risk of ice propagation into cytosol.

B. Dynamic Osmoregulation:

The formation of pure ice crystals exclusively in the ECF poses unique challenges to freeze tolerant organisms that must be mitigated to ensure survival of freezing and thawing (Storey, 1995; 2006; Krane, 2007; Storey & Storey, 2017; do Amaral, 2018). Ice crystal formation consequently increases the osmolarity of the ECF as the amount of available solvent decreases against a fixed quantity of solutes in solution (Storey, 1997). Dictated by the principles of osmosis, water effluxes out of cells into the hyperosmotic ECF in an attempt to equilibrate the osmolarity of both fluid volumes (Storey, 1997; Krane, 2007; Costanzo & Lee 2013; Storey & Storey 2017; do Amaral et al. 2018). Congruent with the colligative property of freezing point depression, increasing the osmolarity of a fluid will invariably decrease its FP. As a freeze proceeds, the osmolarity of both the ECF and ICF increase in tandem until the FP of the ICF is equal to the current temperature of the organism's body ([$FP_{ICF} = T_{body}$]Storey, 1997; Krane, 2007; Costanzo & Lee 2013; Storey & Storey 2017; do Amaral et al. 2018). Therefore, the percentage of total body water that freezes in an organism depends upon the temperature of the environment, and quantity of solute held in both the ECF and ICF. The osmotic forces

associated with freezing and thawing directly change cell volume (Storey, 1997; Krane, 2007; Storey & Storey 2017; do Amaral et al. 2018). The loss of water from the ICF occurring in response to a freeze causes cells and organs throughout an animal's body to shrink (Storey, 1997; Krane, 2007; Storey & Storey 2017; do Amaral et al. 2018). If a cell drops below a critical minimum volume, compression may damage the plasma membrane (preventing the phospholipid bilayer from being a functional barrier post-thaw) (Lee, 2010; Toxopeus, 2018). On the other hand, the influx of water into cells associated with ice crystals melting in the ECF causes cells and organs to swell (Storey, 1997; Krane, 2007; Mutyam et al., 2011, do Amaral et al., 2017). If a cell swells past a critical maximum volume, the plasma membrane may rupture (cell lysis) (Storey, 1997; Toxopeus 2018). The cytoskeleton structure within a cell may also be damaged by significant cell compression or expansion, as well as increased ionic strength within the ICF resulting from dehydration (Harrison, 2001; Orrenius et al., 2003; Zachariassen et al., 2004; Teets et al., 2013; Storey & Storey, 2013; Toxopeus, 2018).

Increased ionic strength caused by loss of available solvent during freezing poses additional challenges to freeze-tolerant organisms (Lee, 2010; Toxopeus, 2018). Increasing the concentration of hydrogen cations *in vivo* will lower the pH of an organism's body fluids which may denature proteins, decrease enzyme activity, and even disrupt mitochondrial function (Harrison, 2001; Toxopeus, 2018). Increasing the concentration of calcium ions can disrupt concentration-dependent cell signaling, and even activate unfavorable cellular processes like apoptosis (Orrenius et al., 2003; Teets et al., 2013; Toxopeus, 2018). Increasing ferrous ion concentration can facilitate the formation of reactive oxidative species (ROS) causing oxidative stress (Storey & Storey,

2013; Toxopeus, 2018). High concentrations of trace metals (i.e., copper & magnesium) may lead to cytotoxicity (Zachariassen et al., 2004; Toxopeus, 2018). High concentrations of extracellular potassium ions (Hyperkalemia) can cause impaired muscle function as well as compromised nerve conductance in many vertebrates (MacMillan et al., 2014; Toxopeus, 2018). Therefore, to prevent irreparable tissue damage and death during freezing and thawing, freeze-tolerant organisms must control the percentage of total body water that is sequestered during freezing and limit changes in cell volume during both processes (Storey, 1997; Storey, 2006; Krane, 2007; Voituron et al., 2009; Costanzo & Lee 2013; Maayer et al., 2014; do Amaral et al. 2017; Storey & Storey 2017). The most direct mechanistic way of limiting cell volume change is by mitigating the severe osmotic stress associated with freezing and thawing through dynamic osmoregulation.

Freeze tolerant organisms have adapted vast and varied mechanisms of dynamic osmoregulation to prevent drastic changes in cell volume during a freeze-thaw cycle. One of the most important, and well characterized, adaptations of freeze tolerance is the utilization of low molecular weight metabolites called cryoprotectants (Storey, 1997). There are two general types of cryoprotectants which aid freeze tolerance in unique ways (Storey, 1997). The first category is the colligative cryoprotectants (and will be the main focus of this section) that are accumulated at high concentrations, ranging from 0.2 to 2 M, reduce the percentage of water that freezes in the ECF, preventing a critical reduction in cellular volume during freezing by increasing the osmolarity of body fluids (Storey, 1997). The second category includes membrane protectants that are accumulated at low concentrations, typically less than 0.2 M, and interact directly with the plasma membrane

(Storey, 1997). Membrane protectants stabilize the phospholipid bilayer and maintain membrane fluidity (prevent an irreversible transition to the solid, or gel, state) when the cell is compressed due to volume reduction during freezing (Storey, 1997). Trehalose and proline are the two most common membrane protectants. Both have been extensively studied and confirmed to interact directly with the polar heads of membrane lipids to stabilize the bilayer (Rudolph & Crowe, 1985; Crowe et al., 1987; Storey, 1997). Elevated levels of trehalose and proline have been consistently observed in freeze tolerant insects during the winter. Proline is also a common major intracellular free amino acid in marine invertebrates and its concentration can be changed rapidly in response to osmotic stress. In fact, several common intracellular osmolytes (including free amino acids) accumulated by marine invertebrates have been observed to function as membrane protectants (Loomis et al., 1989; Storey, 1997).

Cryoprotectants may be accumulated seasonally as part of a cold acclimation phase or mobilized rapidly at the onset of freezing (not mutually exclusive) and are utilized by most freeze-tolerant insects and amphibians (Storey, 2017). Notably, the use of cryoprotectants is poorly developed in reptiles, as current research indicates that reptiles do not utilize osmolytes as cryoprotectants during cold acclimation or freezing. Seasonal lactate accumulation ranging from 2 to 10mM in blood plasma was observed in the freeze-tolerant reptile species *C. picta, E. blandingii,* and *M. terrapin* and was previously hypothesized to function as a cryoprotectant (Storey et al., 1988; Churchill & Storey, 1991; Churchill & Storey, 1992; Storey et al., 1993; Hartley et al., 2000; Costanzo et al., 2001; Baker et al., 2003; Costanzo et al., 2004; Dinkelacker et al., 2005; Storey, 2006). However, further studies disproved this hypothesis and found that lactate

conferred no protective effect in these animals. Furthermore, there is no evidence for enhanced metabolite production for cryoprotection in any freeze-tolerant reptile species; nor does plasma osmolality change seasonally, with cold acclimation, or in response to freezing (Storey et al., 1993; Costanzo et al., 2004; Storey et al., 2004; Voituron et al., 2004; Storey, 2006). This observation, though important, does not invalidate the integral role cryoprotectants serve in freeze tolerance as reptiles appear to be the outlier when considering all freeze tolerant organisms, especially insects and amphibians.

The most commonly occurring natural colligative cryoprotectants are carbohydrates in terrestrial animals (Storey, 1997; Storey & Storey, 2017; Toxopeus, 2018). Carbohydrate cryoprotectants are derived from large glycogen reserves which are accumulated during feeding in late summer and early autumn (Costanzo & Lee, 1993; Jackson & Ultsch, 2010; Storey & Storey, 2017; Toxopeus, 2018). Glycogen is stored in the liver of frogs and fat bodies of insects and may be catabolized gradually during a cold acclimation period and retained in cytosol and blood plasma or rapidly at the onset of ice nucleation (Costanzo & Lee, 1993; Storey, 1997; Krane, 2007; Jackson & Ultsch, 2010; Mutyam et al., 2011; Storey & Storey, 2017; Toxopeus, 2018). Glycerol, a polyhydric alcohol derivative of glucose and triacylglycerols (TAGs), is the most common cryoprotectant among insects and is produced in both freeze-tolerant and freeze-avoidant species (Storey, 1997; Toxopeus, 2018). Glycerol is accumulated at high concentrations in insects, *Pyrrharctica Isabella,* for example, accumulates over 800mM of glycerol in the haemolymph (invertebrate analog of blood; Marshall & Sinclair, 2011). Some insect species utilize other polyhydric alcohols (including sorbitol, ribitol, erythritol, threitol, and ethylene glycol), amino acids (proline, arginine) and the disaccharides trehalose and

sucrose in a few instances (Storey, 1997; Toxopeus, 2018). For example, *Hemideina maori* may accumulate up to 300 mM trehalose in haemolymph, and exposure to high concentrations of proline and arginine *in vivo* increased the freeze tolerance of *Chymomyza costata* and even conferred freeze tolerance to *Drosophila melanogaster* (Neufeld & Leader, 1998; Koštal et al., 2011; 2012; 2016). Many species accumulate two or more cryoprotectants, the most common pairing observed in insects is glycerol and sorbitol. Studies of *Eurosta solidaginis* (gall fly) larvae and *Bombyx mori* (silkmoth) eggs imply differential cryoprotective roles of glycerol and sorbitol when used as a pair based upon the observation of independent periods, and rates, of synthesis and accumulation in these freeze tolerant insects (Storey, 1997). Freeze-tolerant bacteria (extremophilic psychrophiles) and freeze-avoidant mesophilic and extremophilic bacteria also utilize carbohydrates, polyhydric alcohols, and amino acids as cryoprotectants. Although different from insects, the most commonly observed cryoprotectants in bacteria include sucrose, mannitol, glycine, and betaine (Chattopadhyay, 2006; Maayer, 2014). Trehalose is also observed to be the primary cryoprotectant in *E. coli* (Chattopadhyay, 2006; Maayer, 2014). Although there are seemingly endless metabolites used as cryoprotectants, glycerol is one of the most popular natural cryoprotectant molecules because it is highly soluble, nontoxic, extremely compatible with central metabolic pathways, yields two osmotically active molecules from one glycogen hexose subunit, and shows optimal conversion efficiency (carbon dioxide is not lost unlike in the biosynthesis of C2, C4, or C5 polyolys; Storey & Storey, 1992; Storey, 1997; Layne, 1999; Irwin & Lee, 2003; Layne & Stapleton, 2009; do Amaral et al., 2018; Toxopeus,

2018). Although, in-spite of its favorability, glycerol is sparsely utilized as a cryoprotectant by only a few amphibian species.

The salamander *S. keyserlingii* as well as the hylid family of frogs (studied in the recently named *Dryophytes* genus) are the only known amphibians that produce glycerol as a cryoprotectant (Berman et al., 1984; Storey & Storey, 1985; 1986; Storey, 1997; Krane, 2007; Mutyam et al., 2011; Storey & Storey, 2017; do Amaral et al., 2018). Glucose and urea tend to be much more commonly used as cryoprotectants in amphibians. Glucose is produced rapidly at the initiation of freezing from liver glycogen stores and distributed from the liver into blood plasma and other organs (Storey, 1987; 1997; Rosendale et al., 2014; Storey & Storey; 2017). The process of freeze dependent glucose mobilization has been well characterized in the *Rana* and *Pseudacris* frog species. Urea, on the other hand, is not synthesized as a direct response to freezing, instead it accumulates as part of a widely developed dehydration response common to many amphibian species (Costanzo et al., 2008; 2008; 2013; Storey & Storey, 2017). Urea has been repeatedly observed to accumulate throughout the year as hibernation sites begin to dry (Hillman et al., 2009; Navas, 2010; Storey & Storey, 2017). To combat water loss and dehydration stresses, amphibians synthesize and circulate urea to increase body fluid osmolarity to slow the loss of water from the body and promote the uptake of water from the soil through the skin (Storey, 1997; Costanzo et al., 2008; 2008; Hillman et al., 2009; Navas, 2010; Costanzo et al., 2013; Storey & Storey, 2017; Mutyam et al., 2018). This mechanism has been identified in toads and frogs that estivate underground (or spend long, dormant, periods underground), as well as freeze-tolerant frogs (Storey, 1997; Costanzo et al., 2008; 2008; Hillman et al., 2009; Navas, 2010; Costanzo et al.,

2013; Storey & Storey, 2017; Mutyam et al., 2018). Freeze-tolerant Alaskan wood frogs showed significant accumulation of urea during cold acclimation periods in late autumn that increased from ~10 to ~86 µmol/mL in blood plasma, and further enhanced acclimation levels under drying conditions (up to ~ 187 µmol/mL; Costanzo et al., 2013). But when frozen for 48 hours, only frog liver showed significant increases in urea concentration (~ 114 to 157 µmol/mL; Costanzo et al., 2013). The production and seasonal accumulation of cryoprotectants alone is not enough to confer freeze-tolerance to an organism. Cryoprotectants produced in liver cells, hepatocytes, need to be circulated throughout the entire body of an organism to increase the osmolarity in all organs, tissues, and blood plasma. Since these colligative cryoprotectants are by definition polar and hydrophilic, they cannot readily diffuse across the semipermeable plasma membranes surrounding every cell in the body. Therefore, a freeze tolerant organism needs to utilize a quick but energetically favorable method to distribute cryoprotectants before freezing causes ischemia.

This problem is mitigated by the presence of integral transmembrane transport and carrier proteins which allow the transmembrane flux of cryoprotectants. The most important classes of transmembrane proteins related to cryoprotectant diffusion *in vivo* characterized in freeze-tolerant vertebrates and focused on in this review include aquaporins, aquaglyceroporins, glucose transporters, and facultative urea transporters.

Aquaporins (AQPs) are highly specific integral transmembrane channel proteins that allow for the facilitated diffusion of water across cell membranes (Krane, 2007). Maintaining fluid homeostasis is imperative for all life processes, not just surviving a freeze/thaw cycle (Krane, 2007). While the osmotically driven transmembrane simple

diffusion of water molecules through the phospholipid bilayer can occur, the process is slow (Krane, 2007). Mammals and amphibians require precise regulation of water, and ion, movement that allows rapid diffusion during processes such as secretion and reabsorption (Krane, 2007). The presence of AQPs in cell membranes increase the permeability of the phospholipid bilayer to water, allowing for a more rapid diffusion rate while decreasing the time necessary for two separate fluid volumes to equilibrate their net concentrations *in vivo* (Preston & Agre, 1991; Preston et al., 1992; Krane, 2007). Aquaglyceroporins (GLPs), are an additional subset of the selective MIP superfamily of integral transmembrane protein channels that allow the facilitated diffusion of glycerol, urea, and other small molecules in addition to water (Hara-Chikuma & Verkman, 2006; Krane, 2007). GLPs are presumed to be crucial in conferring freeze-tolerance to vertebrates that utilize glycerol as a cryoprotectant, as they may allow for the facilitated diffusion of glycerol, and potentially urea, throughout various tissues of an animal (Schmid, 1982; Storey & Storey, 1985; Costanzo et al., 1992; Layne & Jones, 2001; Irwin & Lee, 2003; Krane, 2007; Zimmerman et al., 2007; Mutyam et al., 2011; do Amaral et al., 2017; do Amaral et al., 2020). Specifically, AQPs and GLPs are hypothesized to play a vital role in conferring freeze-tolerance to Cope's gray treefrog, *Dryophytes chrysoscelis*. The history, structure, function, and classifications of AQPs and GLPs, as well as their hypothesized role in anuran freeze-tolerance, will be discussed in greater detail in further sections of this review. While the AQP family of channel proteins are extremely important for allowing rapid osmoregulation during freezing or thawing, animals that utilize glucose as a primary cryoprotectant must depend on an entirely different protein.

In vertebrates, glucose moves across cell membranes through carrier-mediated facilitated diffusion. These glucose-specific carrier proteins are classified as the glucose transporter (GLUT) family. These proteins were first identified in humans (Mueckler et al., 1985), and there are now 14 unique GLUT protein isoforms characterized in mammals (Thorens & Mueckler, 2010). GLUT proteins have been further identified in fish, birds, and amphibians (Wang et al., 1994; Castillo et al., 2009; Rosendale, 2014). This protein family plays an important role in maintaining glucose homeostasis in all vertebrate species, especially during exposure to hypoxia and dehydration stress. During hypoxia, the abundance of GLUT protein is regulated to promote glycolysis, providing an organism metabolic energy and preventing hypoxic injury (Bunn & Poyton, 1996; Lin et al., 2000). Dehydration, glucose deprivation, hyperosmolarity, and high pH are all known to elicit a change in GLUT expression (Ismail-beigi, 1993; Vannucci, et al., 1994; Ramasamy et al., 2001). Although GLUT proteins have been expansively studied in mammalian stress tolerance, little information is available regarding their role in amphibians. Recently, there are four well-known proteins in this family identified in amphibians, and are named GLUT-1, 2, 3, or 4 (Rosendale et al., 2014). The two most extensively studied family members are GLUT-1 and GLUT-3, these proteins mediate unidirectional uptake of glucose into cells independent of insulin. GLUT-1 is widely distributed and expressed in nearly every tissue in amphibians and mammals, while GLUT-3 is mostly localized to neurons. Conversely, GLUT-4 provides insulin-sensitive glucose uptake into cells and is often observed to be present in adipose tissues, skeletal muscle, and the heart. The last family member, GLUT-2, is unique as it allows for the bidirectional transport of glucose molecules into, or out of, cells. GLUT-2 is almost

exclusively found in the liver and intestines. It is understood that GLUT-2 is the primary protein responsible for exporting cryoprotective glucose molecules from the liver into blood plasma during freezing in amphibians (Rosendale et al., 2014). This protein shows low-affinity and high-capacity for glucose transport in mammalian and amphibian liver and has also been observed to increase in abundance in freeze-tolerant wood frogs collected from Alaska, Canada, and Ohio (Storey & Storey, 2017). Furthermore, changes in GLUT-2 expression varies in species, population, seasonal and stress-specific regulatory patterns (Rosendale et al., 2014). The bidirectionality of GLUT-2, also allows for hepatocytic reuptake of glucose during thawing to reduce blood glucose levels, mitigating hyperglycemia (Rosendale et al., 2014). Glucose cannot be easily cleared by the kidneys and is instead converted back into liver glycogen rapidly during and after thawing. Dynamic regulation of GLUT-2 expression in the plasma membranes of hepatocytes is crucial to surviving freezing and thawing in organisms which utilize glucose as a primary cryoprotectant. This protein mediates rapid export of glucose into blood plasma as it is produced in the liver during freezing. Blood plasma glucose then circulates throughout the body and enters tissue cells via GLUT-1 or GLUT-3 to increase the osmolarity of the ICF before freezing causes the cessation of cardiopulmonary function. Additionally, upon thawing, the exuberant levels of glucose still present in the blood plasma mostly enter back into the liver through GLUT-2 to be converted to glycogen (some glucose is excreted in urine), preventing hyperglycemia and associated cell damage (Storey & Storey, 2017). The dynamic regulation of GLUT-2 allows glucose to be used as a cryoprotectant without seasonal accumulation (which would prove dangerous to animals as prolonged hyperglycemia can lead to severe cell damage and

death), instead liver glycogen stores accumulated during late-summer and early-autumn feeding are catabolized as an immediate response to freezing (Rosendale et al., 2014). Liver glycogen-derived glucose cryoprotectants are mostly converted back to glycogen in the liver during thawing, which allows organisms to maintain sufficient glycogen stores necessary for surviving multiple freeze/thaw cycles over the winter season. The GLUT and AQP protein families (including GLP subfamilies) are emphasized heavily in contemporary vertebrate freeze-tolerance research. However, one family of proteins specific to urea, a third common amphibian cryoprotectant, has received much less attention.

Urea accumulates independently from freezing and is associated with a common amphibian response to dehydration stress. However, freezing exposes animals to dehydration stressors and urea is ultimately accumulated and confers a cryoprotective effect in amphibians. Some GLP subtypes have been observed to exhibit permeability to urea, but if urea permeable GLPs are not expressed in an animal, or abundantly expressed in plasma membranes, a new problem arises. Urea is able to cross plasma membranes through facultative urea transporters (UTs), an integral transmembrane glycoprotein which allows for the diffusion of urea (Klein et al., 2012). This family of proteins was first identified in mammals and designated the SLC14A protein family (Klein et al., 2012). Consisting of two subgroups, UT-B (or *Slc14A1*) and UT-A (or *Slc14A2*), UTs are crucial to the kidney's ability to concentrate urine. The UT-B protein was first isolated from mammalian erythrocytes (red blood cells), and to date only two unique isoforms have been identified (Klein et al., 2012). The UT-B isoforms are present primarily in the descending *vasa recta* of the kidney (Klein et al., 2012). Currently 6 distinct UT-A

isoforms have been characterized, 3 of which are localized to the kidney medulla, 2 in the inner medullary collecting duct, and 1 is expressed in the thin descending limb of the kidney (Klein et al., 2012). In amphibians, UTs have been identified in just one toad species (*Bufo marinus*) and four Ranid species (*R. esculenta, R. sylvatica, R. pipens, & R. septentrionalis*; Couriaud et al., 1999; Konno et al., 2006; Sun et al., 2015; Storey & Storey, 2017). Exposing the toad UT to the peptide hormone responsible for regulating various aspects of amphibian water homeostasis (arginine vasotocin) *in vitro* resulted in increased urea uptake and protein expression in cells. The ranid UTs are highly conserved among each other, showing ~95% amino acid similarity, and are substantially expressed in kidney and bladder, supporting urea reuptake if needed (Rosendale et al., 2014). Additionally, UT expression was observed in the skin of *R. sylvatica* and protein levels have been observed to increase seasonally, as well as in response to experimental dehydration (Rosendale et al., 2014). More research must be completed to identify whether additional amphibian species express UT proteins, and their role in dehydration responses as a potential link to freeze-tolerance. In freeze-tolerant amphibians, polar colligative cryoprotectants (commonly glucose, urea, and glycerol) may be accumulated seasonally or produced at the initiation of freezing to increase the osmolarity of body fluids which, in-turn, causes body fluids to supercool and limits the percentage of total body water that may freeze in the ECF. These cryoprotectants then diffuse across plasma membranes facilitated by specific channel or carrier proteins, allowing colligative molecules to quickly disperse throughout the entire volume of body fluids efficiently before freezing results in the cessation of cardiopulmonary function. However, this mechanism is similar to dehydration responses observed across many amphibian species.

In fact, it is presumed that preexisting mechanisms of dehydration tolerance may have been adapted through natural selection to support freeze tolerance in amphibians.

It has long been known that amphibians exemplify the best known capacity for dehydration tolerance among vertebrates and are able to survive losing on average 50-60% of total body water volumes. Interestingly, the mechanisms that allow for severe dehydration tolerance include dynamic osmoregulation to slow water loss and promote water uptake through the skin from surrounding soil. Dehydration responsive osmoregulation is accomplished by production of colligative molecules such as urea or other osmolytes paired with the dynamic regulation of AQPs and other transport proteins in tissue specific manners. Additionally, a seasonal acclimation period has been observed in toad and frog species as underground estivation sites begin drying during the late-summer and early-autumn periods. During this acclimation period, amphibians over-feed to develop extensive liver glycogen stores, kidney function and urine excretion rate is decreased, urea is gradually accumulated in body fluids, and transport proteins are increasingly expressed in cell membranes. These characteristics are nearly identical to physiological changes during cold acclimation in freeze tolerant amphibians. In fact, several studies observing the physiological responses of some freeze-tolerant amphibians to severe dehydration provide evidence that the same mechanisms used for a cryoprotection in response to freezing are utilized in severe cases of dehydration (Storey & Storey, 1986; 1988; 1992; Costanzo et al., 1993; Voituron et al., 2005; 2009 Higgins et al., 2013; Costanzo et al., 2015; Storey & Storey, 2017). When exposed to controlled whole body dehydration stress, the wood frog *R. sylvatica* and spring peeper *P.* crucifer tolerated the loss of ~50-60% of their total body water volumes, nearly identical to the

percentage of body water converted to extracellular ice during freezing (Churchill & Storey, 1993; 1994; 1995). It was also found that these two species responded to dehydration by rapidly mobilizing glucose from liver glycogen, which was subsequently exported to other organs, and significantly more glucose was mobilized in frogs collected in autumn than those collected in the spring (Storey & Storey, 1988). A parallel study investigating the dehydration response of the freeze-intolerant leopard frog *R. pipens*, found that exposure to controlled whole body dehydration, resulted in the loss of ~50% of its total body water volume. Additionally, *R. pipens* exhibited significant liver glycogenolysis, and glucose export to organs throughout the body as a dehydration response (albeit to a lower extent than *R. sylvatica* and *P. crucifer*) (Churchill & Storey, 1993; 1994; 1995). Nonetheless, these studies provide evidence that the metabolic response of mass glucose mobilization from liver glycogen stores is present in both freeze-tolerant and intolerant amphibians. Many questions still remain regarding the origin of vertebrate freeze-tolerance and specific adaptations to repurpose pre-existing mechanisms.

It is generally presumed that pre-existing mechanisms have been adapted to manage ice formation within the body, as well as the ability to survive the prolonged cessation, and subsequent reactivation, of vital processes during both freezing and thawing. The well-developed capacity for ice-formation management and dynamic osmoregulation observed in, and necessary to, freeze-tolerance may have been adapted from pre-existing mechanisms of dehydration tolerance. The ability to implement these mechanisms serve to sequester ice crystals in extracellular spaces, control ice formation in an ordered and slow manner, limit the total volume of water that freezes in an

organism, and tolerate multiple potential freeze/thaw cycles over winter months. However, these adaptations which prevent mechanical and osmotic damage to cells, do not ensure protection form the dangers associated with ceasing all vital body functions for prolonged periods of time, or provide a mechanism for restarting body functions in a coordinated and efficient manner. One potential developmental link may be derived from the impressive hypoxia and anoxia tolerance observed in freeze-tolerant organisms.

C. Hypoxia/Anoxia Tolerance:

When frozen, organisms are exposed to significant hypoxic (inadequate oxygen supply) or anoxic (no oxygen supply) conditions resulting from the cessation of vital body functions and associated ischemia (inadequate blood supply to an organ or body part) throughout the entire body. Prolonged hypoxia and anoxia interrupt aerobic metabolism, and the ensuing lack of ATP will cause metabolic damage as all available blood-bound and cytosolic oxygen molecules are rapidly depleted (Hermis-Lima et al., 2001; Churchill, 2004; Storey, 2004; 2006). However, the reintroduction of oxygen can be equally as deadly (Hermis-Lima et al., 2001; Churchill, 2004; Storey, 2004; 2006). Damage occurring when oxygen becomes rapidly available after a period of ischemia are called reperfusion injuries (Storey, 2006). These injuries are caused when high levels of reactive oxidative species (ROS) are formed when an anoxic tissue experiences a large influx of oxygen (Storey, 2006). A quick burst of ROS in a cell can overwhelm normal antioxidant defenses and degrade macromolecules or trigger cellular apoptosis mechanisms (Storey, 2006). A severe lack of oxygen may also cause acidosis resulting from carbon dioxide levels continually increasing in the blood, without being expired from the lungs. As predicted by the bicarbonate buffer system, this buildup of carbon

dioxide will inadvertently increase the concentration of H^+ ions and decrease blood pH. Therefore, to survive long-term freezing, an organism must survive on endogenous fuel reserves through a well-developed anaerobic metabolic capacity, stabilize macromolecules, prevent the initiation of cellular apoptosis mechanisms, and prevent acidosis. Accordingly, these responses are not unique to freeze-tolerant organisms, as anoxia tolerance has been frequently observed in a wide variety of species (Storey & Storey, 2017).

The most impressive anoxia-tolerant vertebrate is *C. picta,* painted turtle, a facultative anaerobe capable of surviving 3-4 months continually submerged in cold, deoxygenated, water (Jackson & Ultsch, 2010; Storey & Storey, 2017). These turtles have adapted to store enormous amounts of fermentable fuels (primarily liver glycogen), utilize their shell to release calcium and carbonate ions that buffer lactate anions and H^+ (anaerobic metabolism end products) as well as storing large quantities of lactate, and even the capacity for strong metabolic rate depression to as low as ~10% of their normal aerobic metabolic rate (Storey, 2007; Jackson & Ultsch, 2010; Storey & Storey, 2017). The freshwater turtle species, *T. s. elegans* is another excellent facultative anaerobe capable of surviving up to three consecutive months submerged in cold, deoxygenated, water (Jackson, 2002; Storey, 2006). As adults, these turtles are also known to possess the highest levels of antioxidant enzyme activity among all known ectothermic vertebrates (Wilmore & Storey, 1997; 1997; Storey, 2006). The characteristics of these two species would also lend themselves beneficial in long-term freezing survival. In fact, studies of the freeze tolerant frog *R. sylvatica* found that there were significantly higher levels of antioxidant enzymes present in all organs when compared to levels in the freeze-

intolerant leopard frog *R. pipiens* (Joanisse & Storey, 1996; Storey, 2006). Furthermore, *y*-glutamyl transpeptidase (an antioxidant enzyme) increased by ~2.5-fold during freezing in the liver of both *R. sylvatica* and *C. picta* (Hemmings & Storey, 1996; 2000; Storey, 2006). Catalase activity has been observed to increase significantly in the liver of several turtle hatchling species in response to both anoxia and freezing exposure (Dinkelacker et al., 2005; Storey, 2006). Additionally, this study found that species with low capacities for freezing, had a significantly higher increase in liver catalase activity (Dinkelacker et al., 2005; Storey, 2006). This finding provides evidence that constitutive liver activity in freeze-tolerant organisms may be mostly sufficient to mitigate the oxidative stress associated with both freezing and anoxia exposure (Storey, 2006). Further studies have found that inducible reactive oxygen species (ROS) defenses are observed more often in species rarely exposed to anoxic/ischemic conditions (Hermes-Lima et al., 2001; Storey 2006). One example is the garter snake *T. sirtalis*; when exposed to freezing, the activity of both catalase (CAT)and glutathione peroxidase increased significantly in skeletal muscle, however anoxia exposure only increased superoxide dismutase (SOD) significantly in liver (Hermes-Lima & Storey, 1993). More recent studies have confirmed the hypothesis that many freeze-tolerant reptiles and amphibians, as well as species exposed to frequent anoxic periods alone possess high constitutive, or seasonally induced, antioxidant enzyme activities. However, additional anoxia-responsive enhancements of ROS defenses have been observed.

One example of employing anoxia-responsive enhancements of constitutive antioxidant defenses is observed in the freshwater turtle *T. s. elegans*. When exposed to anoxic water submergence, mRNA transcript levels of ferritin (an iron-binding protein)

plus two forms of SOD (both the cytoplasmic Cu/Zn-binding isoform and the mitochondrial Mn-binding isoform) were upregulated in adult turtles (Storey & Storey, 2017). The increased transcription levels were regulated by just one transcription factor, nuclear factor kappaB (NF-$_K$B; Storey & Storey, 2017). Adaptations to defending against environmental oxidation stress are not limited to high constitutive activity levels and upregulating gene transcription (Storey & Storey, 2017). Post-translational modifications of enzymes which may alter the stability and enzymatic properties of a specific enzyme are another route to quickly respond to environmental changes. Recent studies of SOD and CAT enzymes purified from wood frog skeletal muscle demonstrate a role of posttranslational modification in enhancing pre-existing antioxidant defenses during freezing (Dawson & Storey, 2016; Storey & Storey, 2017). When comparing a control group held unfrozen at 5^{o}C to an experimental group frozen for 24 hours at -3^{o}C, no change in mRNA transcript or protein levels were observed between either Cu/Zn-SOD or Mn-SOD. However, the two groups of purified enzymes had significantly different properties which serve to enhance enzymatic function in frozen skeletal muscle (Cu/Zn-SOD: V_{max} increased 1.5-fold; Mn-SOD: lower K_m, and reduced sensitivity to urea-mediated denaturation). Further investigation also revealed that improved functionality of SOD enzymes from frozen skeletal muscle correlated positively with increased phosphorylation on SOD at both serine and tyrosine residues versus SOD from the control group (2.63 & 1.27-fold greater, respectively). Similar to purified SOD, CAT purified from skeletal muscle extracts of frozen wood frogs increased in efficiency as V_{max} increased by 1.5-fold and K_m for hydrogen peroxide decreased by ~36% when compared to the control (Storey & Storey, 2017). Improved efficiency of CAT also

positively correlated to increased enzymatic phosphorylation of serine and tyrosine residues (1.60 & 1.27-fold, respectively) (Storey & Storey, 2017). Post-translational phosphorylation of both SOD and CAT in wood frogs during freezing confers increased enzymatic activity to further enhance the pre-existing high constitutive level of antioxidant defense is mediated by two separate protein kinases (enzyme that hydrolyzes ATP and subsequently transfers a phosphoryl to another protein) which are already known to be associated with other adaptive responses supporting freeze tolerance (Storey & Storey, 2017). The two kinases involved in adapted responses to freezing and thawing are protein kinase A (PKA) and AMP-activated protein kinase (AMPK) which will be discussed in more detail further in this review. In freeze-tolerant organisms, defending against ROS by maintaining high constitutive, or seasonally increasing, antioxidant enzyme activity levels, which may be further enhanced by upregulated transcription of antioxidant enzyme genes, or by posttranslational modifications on pre-existing enzymes as an anoxia exposure response is crucial in preventing reperfusion injuries caused by freeze-derived ischemia and associated anoxic conditions are resolved when an organism thaws (Storey & Storey, 2017). Antioxidant enzymes are a great defense against ROS, and indirectly stabilize macromolecules to ensure cell survival. However, these enzymes alone are not sufficient to protect an organism from ROS molecules denaturing macromolecules, or the numerous stresses associated with extended periods of anoxia. Often, chaperone proteins are also produced to aid in stabilizing macromolecules.

ROS formed by blood reperfusion during thawing is not the only denaturing force an organism is exposed to during a freezing cycle. As previously mentioned in this review, the dehydration experienced during freezing causes cells to shrink and sharp

increases in the osmolarity of both the ECF and ICF. By removing water volume, molecules in the cytosol are packed closely together. This molecular crowding may promote unfavorable interactions between macromolecules, potentially leading to the aggregation of denatured proteins (Toxopeus, 2018). Therefore, freeze-tolerant organisms need to stabilize molecules through mechanisms other than antioxidant enzyme upregulations. As mentioned earlier, freeze-tolerant insects often produce and accumulate membrane stabilizing cryoprotectants during a seasonal cold-acclimation phase (Toxopeus, 2018). Of which, the major intracellular free amino acids proline and arginine are presumed to reduce protein aggregation by clustering together and forming cytosolic chains that physically buffer unfavorable protein-protein interactions (Rudolph & Crowe, 1986; Arakawa & Tsumoto, 2003; Das et al., 2007; Koštal et al., 2016; Toxopeus, 2018). However, a more sophisticated mechanism has been widely observed in freeze-tolerant as well as anoxia-tolerant organisms.

Chaperone proteins facilitate the folding, trafficking, and assembly of new proteins, prevent unfolded protein aggregation, and promote refolding of existing proteins that have folded incorrectly. Many chaperones are expressed constitutively by organisms, while expression of some are induced by physiological stressors. The most extensively studied inducible chaperone proteins are the family of heat shock proteins (HSP), that prevent protein denaturation and aggregation as well as promoting the refolding of denatured or misfolded proteins (King & MacRae, 2015). HSP expression is triggered when an organism is exposed to dramatic temperature changes, osmotic stress, anoxia exposure, and many other stressors (Storey & Storey, 2011; 2012). Several studies primarily focused upon insects and arthropods have concluded that a necessary process in

developing winter cold hardiness is the seasonal, cold-induced, or freeze-responsive upregulation of chaperone proteins (Rinehart et al., 2006; Zhang et al., 2011; Lu et al., 2014). HSPs have been observed to be upregulated under the coordination of heat shock transcription factor (HSF1) in both of the anoxia-tolerant turtle species *C. picta* and *T. s. elegans* in response to low oxygen levels (Storey & Storey, 1997; 2011). In addition to HSPs, anoxia triggers strong increases in mRNA transcription and protein levels of two resident endoplasmic reticulum (ER) chaperone proteins, glucose regulated protein 78 (GRP78) and GRP94 closely associated with the unfolded protein response (UPR) in both turtles (Ramaglia & Buck, 2004; Krivoruchko & Storey, 2010; 2013; 2017). The UPR is a mechanism which manages stresses that affect protein folding/assembly in the ER by suppressing ribosomal protein synthesis in the ER, increasing GRP levels in the ER to improve folding capacity, and in some cases increasing unfolded protein degradation via the proteasome (a protein complex containing protease enzymes that degrades ubiquitin-tagged proteins through peptide bond breaking proteolysis reactions) (Schröder, 2008). There is extremely limited data regarding the UPR in amphibian and hatchling turtle freeze tolerance, but the stress induced HSP and GRP upregulation in adult turtles predict a plausible role of chaperone proteins in freeze tolerance. Data supporting this hypothesis was identified in wood frogs, as HSP70 levels were significantly higher in cold-acclimated winter frog liver, than in warm-acclimated summer frog liver (Kiss et al., 2011; Naicker et al., 2012; Storey & Storey, 2017). Additional evidence was collected from a study of freeze-tolerance in yeast, 19 different chaperone deletion mutants reduced cell viability after a freeze-thaw cycle in the presence and absence of glycerol as a cryoprotectant (Storey & Storey, 1997). Studies on insect freeze-tolerance found that

chaperone proteins play a crucial role in preventing cold-induced depolymerization of actin microfilaments in the cytoskeleton, as chaperonin containing T-complex polypeptide-1 (CCT) was strongly upregulated in onion maggot pupae (*Delia antiqua*) during cold acclimation (Kayukawa & Ishikawa, 2009). Furthermore, the upregulation of chaperone T-complex protein 1 (TCP-1) during cold acclimation was identified in *E. solidaginis*, suggesting the potential chaperone mediated reassembly of dissociated cytoskeleton monomers post-freeze (Voituron et al., 2018). However, more extensive research needs to be completed in order to elucidate the role of chaperone proteins in freeze-tolerance. Stabilizing macromolecules is crucial to maintaining cell viability after enduring a freeze/thaw cycle as well as returning to normal physiological activity after thawing. The capacity of freeze-tolerant organisms to employ high constitutive levels of antioxidant enzyme activity that may be further enhanced by upregulation of transcription for new protein synthesis or posttranslational modifications to enzymes in response to anoxic or freezing conditions to protect against ROS and stabilize macromolecules may have been adapted from pre-existing mechanisms of anoxia-tolerance. Additionally, dynamic regulation of chaperone proteins to stabilize cellular proteins has been observed in anoxia-tolerant vertebrates and insects. Chaperone proteins are presumed to be regulated in many freeze-tolerant organisms, adapted from pre-existing anoxia tolerance mechanisms, but more research must be completed to conclude if a beneficial, consistent, and widespread use of chaperone proteins is observed in freeze-tolerant organisms. Aside from surviving denaturing stress during anoxic and freezing exposure, freeze-tolerant organisms must survive on endogenous fuel reserves for the duration of a freeze.

In order to survive long-term freezing up to several months at a time, an organism must store vast carbohydrate reserves, possess a well-developed glycolytic capacity, optimize facultative anaerobic metabolism, employ hypometabolism, and regulate metabolic enzymes. These requirements are necessary to sustain ATP production under anoxic/frozen conditions and ensure enzymatic function at subzero temperatures, while decreasing the overall need for ATP during a frozen state to prevent entirely depleting carbohydrate stores (Storey & Storey, 2017). When cardiopulmonary function ceases during extracellular ice formation, cells will continue metabolic processes until oxygen levels are depleted, at which point anaerobic glycolysis becomes the main source of ATP production. Many species of both reptiles and amphibians have been observed to accumulate lactate, glycolytic fermentation end product, while frozen, indicating the use of lactic acid fermentation as a main supplier of ATP (Voituron et al., 2002; 2009; Sinclair et al., 2013; Storey & Storey, 2017). Reptiles and amphibians rely on their extensive anoxia-tolerance to aid in freezing survival by minimizing their energetic needs while frozen through metabolic rate depression (MRD), in which an organism enters a hypometabolic state that slows the rate of metabolic reactions through suppressing non-vital, and energetically expensive, bio-synthetic processes such as *de novo* protein synthesis (Voituron et al., 2002; 2009; Sinclair et al., 2013; Storey & Storey, 2017). The combination of strong MRD and the consequences of subzero temperatures on enzyme kinetics greatly decrease the cellular demand for ATP production *in vivo*, which allows freeze-tolerant organisms to survive through anaerobic catabolism of large endogenous glycogen stores accumulated during feeding periods in late-summer and early-autumn (Voituron et al., 2002; 2009; Sinclair et al., 2013; Storey & Storey, 2017). An additional

source of ATP in muscle tissues may be creatinine phosphate reserves and has been observed in wood frogs (Storey & Storey, 1986). Creatinine kinase (CK; an enzyme that catabolizes the reversible phosphorylation of creatinine; creatinine + ATP → creatinine-P + ADP) in wood frog muscle undergoes specific regulation while the animal is frozen (Dieni & Storey, 2009; Storey & Storey, 2017). Temperature effects and posttranslational modifications (PTMs), mainly phosphorylation, increased CK activity by 35% and decreased K_m for creatinine by 39% (with no change in protein quantity) when comparing frogs frozen at -3ºC for 24 hours to unfrozen frogs acclimated to 5ºC (Storey & Storey, 1986). It was determined that two protein kinases, AMP-activated protein kinase (AMPK) and calcium/calmodulin-dependent protein kinase (CaMK), were capable of phosphorylating CK enzymes in response to freezing (Storey & Storey, 1986). AMPK is commonly known as a cellular "energy sensor" and responds to increases in intracellular concentrations of AMP when ATP is scarce - shown in **Figure 3** (Storey & Storey, 1986). The activity of this kinase increased 4.5-fold in wood frog muscle when frozen, suggesting AMPK may be crucial to regulating many enzymes involved in energy metabolism during freezing (Rider et al., 2006). Another enzyme phosphorylated by AMPK in response to freezing in wood frog skeletal muscle is AMP deaminase (AMPD). During physiological stress when ATP is consumed faster than it is produced, AMPD works in tandem with adenylate kinase to stabilize the relative levels of AMP, ADP, and ATP (Storey & Storey, 2017). Mechanistically, AMPD catalyzes the conversion of AMP into IMP and NH_4^+ and adenylate kinase converts 2 ADP molecules into 1 ATP and AMP molecule (reaction of both enzymes in tandem: 2ADP → ATP + AMP → IMP + NH_4^+) (Storey & Storey, 2017). AMPD is regulated in several ways when wood frogs

freeze to increase AMP affinity such as an increase in the myosin-bound form, decrease in temperature (25 to 5ºC), the presence of 250mM glucose, and phosphorylation by four different enzymes (Storey & Storey, 2017). Metabolic control resulting in hypometabolism is implemented to decrease the cellular demand for ATP, allowing an organism to preserve their extensive glycogen stores for several months at a time. MRD is presumed to have been adapted from pre-existing mechanisms in anoxia-tolerant vertebrates and is an extremely complicated process that varies by species involving dynamic regulation of genes, ribosomes, and proteins to suppress non-vital metabolic processes in addition to ensuring existing enzymes involved in vital mechanisms and anaerobic ATP production are able to function under a variety of stressors. Literature regarding MRD's role in freeze tolerance is scarce, but PTMs including phosphorylation are presumed to be integral in maintaining metabolic enzyme function and exerting coordinated metabolic responses. In order to survive long-term freezing periods, an organism needs to possess prolific capacities to respond rapidly to the numerous physiological challenges associated with severe dehydration and anoxia exposure at subzero temperatures. The anuran order of amphibians are exemplary models of mitigating the physiological stressors of freeze tolerance capacities and will be the primary focus of the remainder of this review.

III. Physiology of Anuran Freeze Tolerance

A. Physiological Consequences of Freezing:

Among vertebrates, freeze tolerance is rarely employed as an overwintering survival strategy and has only been observed consistently in one chelonian, squamate, and caudata species as well as several unique anuran species (Berman et al., 1984; Voiiturin et al., 2003; 2009). Freeze tolerant anurans experience varying durations of time frozen and frequency of freeze/thaw cycles per year, which mainly depends on their over-wintering environment. Anurans have been documented to survive in a frozen state for up to several months at a time, and have adapted mechanisms over time to cope with the physiological consequences of freezing; including the cessation of cardiopulmonary function, no voluntary muscle movement, no inter-tissue transport, dehydration and hypoxia/anoxia exposure in all cells and tissues, nerve conductance in both the central and peripheral nervous systems become immeasurable, and ice crystals form in every cavity, lumen, and extracellular space in the body. The majority of research investigating freeze tolerant vertebrates are mechanistic studies focused on anurans native to North America. However, the evolutionary significance of developing freeze-tolerance has received little attention (Voituron et al., 2018). It is known that the capacity to survive up to 65% total body water volume frozen as extracellular ice has evolved several times and at different time periods among anurans (Voituron et al., 2018). It is presumed that freeze-tolerance was adapted from pre-existing mechanisms involved in the well-developed dehydration and anoxia tolerances present in anurans. In fact, anuran freeze tolerance shares common characteristics between all species in spite of being genetically distant.

All freeze tolerant anurans accumulate and maintain large stores of liver glycogen by seasonal overfeeding during late-summer and early-autumn (do Amaral et al., 2018). Anurans also exhibit a specific freezing pattern in which ice crystals grow exclusively in extracellular spaces (Storey & Storey, 2017). These animals minimize supercooling to an SCP approximately 2-3^{o}C below the normal equilibrium FP of their body fluids, in order to minimize the risk of deadly instantaneous ice surges and slow the rate of ice formation to allow an organism time to implement freeze-responsive physiological changes (Storey & Storey, 2017). Ice nucleation begins by contact with external ice or some other INA on the skin and slowly propagates inward over several hours. Ice formation on the skin instantaneously results in a surge of adrenergic signaling that is maintained during several hours as freezing progresses which causes a sharp rise in both respiration and heart rate, as well as initiating cryoprotectant mobilization in the liver (Storey & Storey, 2017). One consequence of increased cardiopulmonary function derived from a surge of adrenergic signaling is a small rise in body temperature that further depresses the rate of freezing (Storey & Storey, 2017). The process of freezing water is also an exothermic process, and may contribute, in part, to the observed increase in body temperature. Nonetheless, this increase in body temperature provides additional time for an animal to mobilize and distribute cryoprotectants throughout the body and implement other necessary physiological changes to survive freezing and thawing. Ice propagation follows a predictable pattern in which ice progressively moves inward from the skin and the liver is always the last organ to freeze, presumably due to possessing the highest concentration of colligative cryoprotectants (Storey & Storey, 2017). The thawing process also follows this pattern in reverse, wherein the liver thaws first, and the skin is the final tissue.

Similar to freezing, thawing is a dynamic process. Initiated when the environmental temperature increases above the body fluid FP (on average between -1.0 and -0.5ºC), thawing then progresses in equilibrium with the temperature of the external environment (Storey & Storey, 2017). Just as sequestering body water as extracellular ice depresses the equilibrium FP of body fluids, reintroducing water into solution as ice melts increases the equilibrium FP of body fluids. Therefore, thawing only progresses to completion if environmental temperature gradually increases above the organism's SCP. Thawing follows additional patterns across all freeze-tolerant anuran species.

Vital signs deactivated by freezing are always restored in a specific order. Heartbeat is recovered first, and blood perfusion to the skin is restored shortly thereafter (Storey & Storey, 2017). Respiration then resumes, characterized by low levels of oxygen consumption at the beginning of the thaw, and higher than normal levels directly after the completion of thawing which eventually normalize (Storey & Storey, 2017). Finally, skeletal muscle reflexes are restored up to 48 hours post-thaw (Storey & Storey, 2017). Motor function is additionally observed to recover in a consistent pattern, in which animals first exhibit a reflex response to pinch, coordinated limb responses to stimulation second, assuming voluntary normal body posture third, and voluntary locomotion is restored last (Storey & Storey, 2017). In order to successfully reactivate organ systems and resume physiological homeostasis, anurans must ensure cells are not destroyed following freezing and thawing by mitigating mechanical damage to cells caused by ice formation and cellular volume changes derived from the associated severe dehydration stress.

B. Ice Management:

Anurans make use of various mechanisms in order to limit ice formation exclusively to extracellular spaces as well as drastically slow the rate of ice formation, providing enough time to implement freeze-responsive physiological changes. One method by which anurans manage ice formation and propagation *in vivo* is minimal supercooling of body fluids, usually to an SCP only 2 to 3°C below the equilibrium FP of their body fluids. Extensive supercooling, often observed in freeze-avoidant animals, substantially increases the risk of experiencing a deadly instantaneous ice surge, in which large volumes of body water freeze instantaneously. Instead, by maintaining a relatively high SCP, anurans mitigate the risk of instantaneous ice surges and exploit the dynamic freezing process by allowing gradual decreases in environmental temperature to dictate what percentage of total body water is sequestered as extracellular ice in a slow and ordered manner. Additionally, anurans are known to utilize many proteins and other agents that interact with ice to prevent or manage crystallization.

Freeze avoidant organisms tend to implement mechanisms designed to prevent ice inoculation. For example, terrestrial arthropods rely on their waterproof exoskeletons (integument) to resist ice inoculation as well as seasonally accumulate colligative cryoprotectants (primarily glycerol) and produce antifreeze proteins (AFPs) which bind ice crystals and prevent them from growing larger than a microscopic size. While the use of AFPs by freeze-tolerant anurans is an area for future research, it is plausible that AFPs may be produced seasonally and localized to the cytosol to protect against intracellular ice formation. More recent work has identified the existence of a novel antifreeze glycolipid (AFG) common to several insects in both freeze tolerant and avoiding species,

as well as two anuran species, Alaskan wood frogs and a European frog *R. lessonae*. AFGs are associated with the apical or extracellular side of the plasma membrane and interact with ice crystals in order to prevent extracellular ice from inoculating ice crystals in the ICF, as well as limit ice recrystallization in the ECF. Ice nucleating proteins (INPs) often play integral roles in anuran freeze tolerance. These proteins are seasonally synthesized and accumulated by the organism specifically to promote the regulated, and ordered, formation of ice in the ECF. Ice nucleating agents (INAs; nonspecific proteins or other molecules which initiate ice formation) are also vital in anuran ice management as natural ice nucleation in frogs primarily results from skin contact with extracellular ice or INA action on epithelial cells in the skin or gut. It has been recently discovered that bacteria such as *Pseudomonas* and *Enterobacter* found in the skin or gut of the wood frog *R. sylvatica* produce INAs that initiate freezing. Both INPs and INAs are crucial to freeze-tolerant anurans as they provide a mechanism to initiate ice nucleation and propagation at high subzero temperatures, allowing anurans to minimize supercooling so that freezing occurs slowly in an ordered manner. Modern genomic work has allowed for the classification of a third-class ice-active proteins. Ice-binding proteins (IBPs), are produced to actively prevent the restructuring of small ice crystals into larger crystals. An IBP recently characterized in wood frogs is the novel freeze-responsive protein (FR10), which is excreted into the ECF in the winter and associates with the plasma membrane to bind ice crystals in the ECF. The freeze-responsive 10 (*fr10*) *gene* was first identified nearly twenty years ago, and studies of *fr10* transcripts have found that gene transcript levels are strongly upregulated in wood frog liver after 24-hour freezing at 2.5^{o}C and increased by 1.8 to 3.8-fold in other tissues including brain, heart lung, testes, and

skeletal muscle. Furthermore, transcription is upregulated in the heart and brain when exposed to dehydration stress, as well as the heart, kidney, and lung under anoxia. INPs and IBPs are not the only proteins that undergo differential expression seasonally, or in response to freezing. A wide range of metabolic enzymes are regulated in order to promote a hypometabolic state during freezing in anurans.

C. Differential Regulation of Energy Metabolism:

Anurans are exposed to anoxic conditions due to the cessation of cardiopulmonary function when frozen for extended periods of time. Freezing-induced ischemia forces cells to depend on anaerobic metabolism of endogenous fuel stores to produce ATP, as available oxygen is rapidly depleted. It is well known that anurans seasonally build extensive liver glycogen stores by late-summer and early-autumn feeding. However, large glycogen stores alone are not sufficient to sustain a frog's high basal demand for ATP, potentially for several months at a time. While the subzero temperatures maintained during a frozen state do impact enzyme kinetics negatively, anurans utilize a process known as metabolic rate depression (MRD) to enter a hypometabolic state as low as 1-30% of the basal metabolic rate. Presumed to have been adapted from pre-existing mechanisms of anoxia tolerance, MRD is an extremely intricate, integrated physiological response induced by freezing in anurans and requires meticulous research before it is fully understood. It is understood that MRD suppresses energy-expensive non-vital cellular processes including ATP driven primary active transport, many forms of biosynthesis, and mitosis/meiosis by reversible phosphorylation and other PTMs on proteins, as well as utilizing microRNAs and sequestering mRNA transcripts in intracellular storage granules or P-bodies to prevent translation of undesired

mRNA transcripts. However, the remainder of this section will focus on the enzymatic regulation of common metabolic enzymes among anurans.

As discussed earlier, AMPK is presumed to play a vital role in the enzymatic regulation of metabolism during freezing. As it reversibly phosphorylates both CK (changes conformation to favor creatinine-P hydrolysis) and AMPD to ensure their function at subzero temperatures under anoxic conditions. These two enzymes (as well as adenylate kinase; AK) function to maintain relatively equal levels of intracellular AMP, ADP, and ATP which is crucial to maintain vital cellular processes and prevent metabolic damage. Additional freeze responsive PTMs to common metabolic enzymes occur to improve the efficiency of anaerobic glycolytic fermentation and prevent metabolites from falling into alternative metabolic routes. Studies of dehydrogenase enzymes have found that PTMs are not limited to rate-limiting enzymes. Multiple dehydrogenase enzymes have been observed to undergo reversible modifications including lactate dehydrogenase (LDH), glutamate dehydrogenase (GLDH), and glucose-6-phosphate dehydrogenase (G6PDH). LDH catalyzes the reversible conversion of pyruvate to lactate (the end product of fermentation), and anoxia-induced phosphorylation and acetylation PTMs have been observed in the liver of anoxia-tolerant red-eared slider turtles. These two PTMs resulted in reduced LDH pyruvate affinity and increased the sensitivity of LDH to high pyruvate concentration as an inhibitor. LDH purified from wood frog skeletal muscle was exposed to five separate PTMs. In dehydrated frogs, acetylation and ubiquitination actually increased LDH's affinity for pyruvate, lactate, and NAD^+ as well as increasing the enzymes sensitivity to urea inhibition. High intracellular concentrations of lactate can lead to acidosis, and the PTMs on LDH may actually serve to decrease the

amount of lactate accumulated in cells during a frozen state. Evidence supporting this presumption lies in the varied concentrations of *L*-alanine (alternative end product of fermentation) accumulated across different tissues of wood frogs. This reversible reaction (pyruvate + *L*-glutamate □ *L*-alanine + *a*-Ketoglutarate) catalyzed by the enzyme alanine transaminase may indirectly become more favorable when PTMs on LDH decrease its substrate affinity, removing pyruvate from the cytosol without increasing intracellular lactate pools. *L*-alanine has been observed to accumulate in tissue specific patterns in frozen wood frogs as the ratio of lactate:alanine buildup was approximately 1:1 in liver, 1:2 in skeletal muscle, with almost no alanine accumulated in the heart and liver. Phosphorylation of G6PDH, may serve to decrease the function of this enzyme, preventing G6P destined for fermentation to be converted into 6P-gluconate and pulled into the pentose-phosphate pathway. Additional enzymatic regulation does exist, as nearly every animal enzyme involved in the major metabolic pathways of glycogen, glucose, urea, and fatty acid metabolism can be subject to PTMs. Current research has barely scratched the surface of the vast intertwined regulatory controls of enzymes resulting from PTMs (such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and O-glcNAcylation), and many studies must be completed before the profoundly precise metabolic control exhibited during freezing can be fully understood. MRD effectively suspends anurans into a hypometabolic state as low as 1-30% of the normal metabolic rate so that extensive liver glycogen stores can provide sufficient ATP while frozen for up to several months. However, anuran glycogen stores do not serve only as an ATP supply while frozen, the colligative cryoprotectants glucose and glycerol are synthesized by glycogenolysis during freezing.

D. Cryoprotectants: *fight, flight, or freeze*

In anurans, the adrenal medulla secretes the catecholamine neurohormone epinephrine into blood at the initiation of ice nucleation on the skin. Epinephrine plays a vital role in cryoprotectant mobilization, especially in species reliant upon glucose production at the initiation of freezing, by quickly activating glycogenolysis in the liver. This process has been extensively studied in the ranid wood frog complex and is shown in **Figure 4**. Activating glycogenolysis to synthesize glucose is a short, ATP-independent, pathway involving only three enzymes: glycogen phosphorylase (GP), phosphoglucomutase (PGM), and glucose-6-phosphatase (G6Pase). *ß*-adrenergic signaling is an element of the commonly known "fight or flight" response in all vertebrates but has been adapted by wood frogs to induce high levels of sustained glucose output from the liver (that can be measured in as little as 2-5 minutes post-nucleation) over the course of several hours during freezing. The binding of epinephrine to integral membrane $ß_2$-adrenergic G-protein channel receptors (GPcR) stimulates the synthesis of cAMP, which causes dissociation of the inactive protein kinase A (PKA) tetramer R2C2, releasing both the regulatory and catalytic (PKAc) subunits. PKAc in turn, phosphorylates glycogen phosphorylase kinase (GPK), which subsequently phosphorylates inactive GP (GP*b*), activating the enzyme (GP*a*) which cleaves hexose units from glycogen polysaccharides. Glucose-1-phosphate resulting from the actions of GP*a* is converted to glucose-6-phosphate (G6P) by PGM. G6P is subsequently transferred to the ER, where G6Pase, anchored to the ER membrane (amino terminal and active site on the lumen side), produces glucose that is released near the plasma membrane. The bidirectional integral membrane carrier protein GLUT-2 exports glucose

from the liver and into the blood, where it is circulated throughout the body. The location of G6Pase expression in the ER is vital in both exporting glucose and minimizing the reconversion of glucose back to glycogen. Glucose needs to be exported from the ER close to the plasma membrane, in order to be quickly exported via GLUT-2, before a cytosolic enzyme involved in glycogenesis binds the glucose molecule. The capacity for continued large-scale glucose output is determined, in part, by the size of liver glycogen stores. On average, wood frogs from Ontario, Canada possess glycogen stores that account for approximately 18% of their total liver mass (Storey & Storey, 2017). Studies between Alaskan and Ohioan wood frogs have found that Alaskan frogs, which can survive freezing at much colder temperatures than Ohioan frogs, maintain liver glycogen stores 3.5-fold higher than Ohioan frogs (Storey & Storey, 2017). Additionally, Alaskan frogs possess a much higher hepatosomatic index (or more massive liver vs. total dry body weight) plus significantly higher levels of β_2-adrenergic receptors in hepatocyte membranes during the early hours of freezing that, over time, fell to low levels of expression in fully frozen and thawed frogs (Storey & Storey, 2017). β_2-adrenergic signaling is continually stimulated in wood frog hepatocytes during the first few hours of freezing to produce extreme hyperglycemic conditions. Intracellular hepatocyte cAMP levels were 2-fold greater during a 24-hour freezing condition compared to an unfrozen control (Storey & Storey, 2017). Plus, PKAc levels were 8-fold and 5-fold greater in hepatocytes of frozen frogs versus control at 1 and 5 hours, respectively, after freezing began (Storey & Storey, 2017). Furthermore, GP and glycogen synthase (GS) protein levels are observed to be lowest during summer months, and highest in winter months in both Alaskan and Ohioan wood frog hepatocytes (Storey & Storey, 2017). Notably, the

freeze-responsive increases in PKA and activated GP were significantly higher in Alaskan frogs when compared to Ohioan frogs (Storey & Storey, 2017). Compared to the freeze-intolerant leopard frog, *R. pipiens*, wood frogs possess both 12-fold and 13-fold higher GP activity and glucose output, respectively, in hepatocytes (Storey & Storey, 2017). Unlike wood frogs, the treefrog complex of anurans utilize glycerol as a primary cryoprotectant instead of glucose.

Glycerol is presumed to be accumulated seasonally by treefrogs as part of a necessary cold acclimation period to provide a base-level cryoprotective effect, and further mobilization of glycerol occurs directly in response to freezing potentially in the liver via β_2-adrenergic signaling – shown in **Figure 4** (do Amaral et al., 2020). Conflicting data regarding the extent of seasonal glycerol accumulation does exist, however this is believed to be a result of varied feeding and cold acclimation protocols among laboratories conducting studies (do Amaral et al., 2020). Glycerol is one of the most common colligative cryoprotectants observed in nature but is sparsely employed by amphibian species, even though glycerol may serve as a superior cryoprotectant molecule. Unlike glucose, glycerol forms two osmotically active molecules from one G6P molecule but is more energy-expensive to synthesize (net input of 1 ATP and NADH) and reconvert to glycogen. However, glycerol is easily cleared by the kidneys and excreted in the urine bypassing the need for reconversion to glycogen in the liver. Additionally, glycerol does not expose an organism to hyperglycemia. This allows freeze tolerant organisms to circulate base levels of the cryoprotectant for extended periods of time while reducing the emphasis on extensive glycogen catabolism at the initiation of freezing. Finally, mobilizing glycerol instead of glucose prevents repetitive exposure to

the cellular stresses associated with hyperglycemia if exposed to several freeze/thaw cycles over the course of one winter. The third well-known cryoprotectant utilized by anurans is also observed to be seasonally accumulated, however the circulation of this molecule is independent of freezing.

Urea contributes to the freeze tolerance of both wood frogs and treefrogs but is not produced as a direct colligative response to freezing. Urea is presumed to be accumulated in response to dehydration (one of the main physiological stressors associated with freeze tolerance) and its synthesis has been consistently observed in dehydration exposure in both unfrozen wood and treefrogs. Urea accumulation occurs naturally as anuran habitats begin to dry during late summer, with circulating urea levels peaking in the winter. This process is widely observed across dehydration-tolerant vertebrate species and is presumed to have been adapted by freeze-tolerant vertebrates to aid in freezing survival as its accumulation is usually seen in tandem with glucose or glycerol production to enhance colligative action during freezing.

While the mechanism has not been fully elucidated, evidence suggests urea production may be a byproduct of cold-induced protein ubiquitination and subsequent degradation (do Amaral et al., 2020). In fish and frogs, transcription of genes associated with the ubiquitin proteasome pathway have been observed to be upregulated in response to cold exposure (do Amaral et al., 2020). Conjugation between ubiquitin and its target proteins promote proteasome binding (do Amaral et al., 2020). Proteasome binding a ubiquitinated target protein results in the degradation of the target protein (do Amaral et al., 2020). The amino acids resulting from proteolytic activity may be used in synthetic pathways of ATP, carbohydrates, and proteins (do Amaral et al., 2020). Although, it is

unlikely that ATP is synthesized from non-carbohydrate sources during freezing due to hypoxic conditions (do Amaral et al., 2020). Additionally, ubiquitin proteasome derived amino acids may act as cryoprotectant molecules on their own, or a source of urea production (do Amaral et al., 2020). Furthermore, analysis of the treefrog *D. chrysoscelis'* hepatic transcriptome revealed that transcripts of several genes involved in amino acid trafficking and amino acid flux were downregulated in both cold and frozen animals (do Amaral et al., 2020). This finding suggests that amino acids derived from proteolytic responses to cold exposure may be accumulated in the liver of *D. chrysoscelis*, necessitating further research to determine if these amino acids are in fact a source of the colligative cryoprotectant urea (do Amaral et al., 2020).

IV. *Dryophytes chrysoscelis*

A. Cope's Gray Treefrog:

Dryophytes chrysoscelis, Cope's gray treefrog, is a freeze tolerant anuran naturally inhabiting an expansive territory covering central and eastern North America. *D. chrysoscelis* and its closely related sister species, *D. versicolor*, are able to survive up to 65% of their total body water volume frozen as extracellular ice crystals. These treefrogs mitigate the physiological stressors associated with freezing and thawing by accumulating high concentrations of the colligative cryoprotectant glycerol, in tandem with urea, presumed to cross cell membranes through a family of integral transmembrane channel proteins called aquaglyceroporins (Schmid, 1982; Storey & Storey, 1985; Costanzo et al., 1992; Layne & Jones, 2001; Irwin & Lee, 2003; Krane, 2007; Zimmerman et al., 2007; Mutyam et al., 2011; Storey & Storey, 2017; do Amaral et al., 2017; 2020). Though conflicting data regarding the accumulation pattern and source of glycerol exists, *D. chrysoscelis* is believed to accumulate glycerol during a necessary cold acclimation period (taking place in late summer and early autumn) to provide a base level cryoprotective effect, which is further elevated by rapid glycerol synthesis and mobilization in response to freezing (Schmid, 1982; Storey & Storey, 1985; Costanzo et al., 1992; Layne & Jones, 2001; Irwin & Lee, 2003; Krane, 2007; Zimmerman et al., 2007; Mutyam et al., 2011; Storey & Storey, 2017; do Amaral et al., 2017; 2020). Glycerol is the most common naturally occurring colligative cryoprotectant utilized by freeze-tolerant and avoiding invertebrates, often in combination with the disaccharide trehalose or the free amino acid proline (Storey, 1997). However, glycerol accumulation is a unique mechanism among vertebrates as the only known animals are the salamander

S. keyserlingii and treefrogs (Storey & Storey, 2017). On the other hand, extensive studies have concluded that the *Rana* and *Pseudacris* anuran species widely rely on glucose as a cryoprotectant (Storey & Storey, 1986; 1988; 1992; Costanzo et al., 1993; Voituron et al., 2005; 2009; Higgins & Swanson, 2013; Costanzo et al., 2015; Storey & Storey, 2017). This molecule is not accumulated seasonally but is rapidly synthesized from liver glycogen stores in as little as 2-5 minutes after the initiation of freezing via β_2-adrenergic signaling (Storey & Storey, 1996). Specific information regarding the integrated mechanisms involved in mitigating the physiological stressors associated with freezing and thawing remains largely unknown among all freeze-tolerant species and requires extensive attention before this awe-inspiring feat of nature can be fully appreciated. However, *D. chrysoscelis* is a promising model for uncovering mechanisms applicable for biomedical research investigating the cryopreservation of mammalian tissues and organs.

V. Aquaporins: Discovery, Function, & Structure

A. Discovery:

Aquaporins (AQPs) are highly specific integral transmembrane channel proteins that allow the facilitated diffusion of water molecules across cell membranes. Since their initial discovery, AQPs have been characterized as a subfamily of the major intrinsic protein (MIP) family that also includes the other homologous transmembrane protein channel subfamilies of aquaglyceroporins and Super-AQPs. The initial study of water transport was catalyzed by the observation that certain amphibian tissues (skin & bladder) are more permeable to water than others (Ussing, 1965; Carbrey & Agre, 2009). The initial observation of high-water permeability of amphibian skin by Hans Ussing and his colleagues was further expanded with the advent of the electron microscope. This technology allowed researchers to visualize structures believed to be protein channels in amphibian bladder that increased in number as the tissue's permeability to water increased (Kachadorian et al., 2000; Carbrey & Agre, 2009). These observations eventually led to the *shuttle hypothesis* in which a tissue's water permeability is regulated by the subcellular localization of water channels. Evidence supporting this hypothesis was found in amphibian bladder samples, protein aggregates were observed to be localized to the cytosol during a state of diuresis (low water reabsorption) and expressed in the plasma membrane during a state of antidiuresis (high water reabsorption) (Wade et al., 1981). Eventually, other water-permeable tissues became subject to research.

Studies with red blood cells (RBCs) headed by A.K. Solomon, suggested pores in the cell membrane were responsible for the low Arrhenius activation energy water transport observed in RBCs (Solomon, 1968). Additionally, Robert Macey et al.

discovered that RBC water movement could be reversibly inhibited in the presence of $HgCl_2$ (Macey et al., 1984). These studies suggested that a protein with free sulfhydryl groups that mercury can access, are responsible for water transport across the cell membrane of erythrocytes. It was not until the late 1980s that the first water channel (AQP1) was incidentally characterized by Dr. Peter Agre while attempting to identify the Rh blood group antigens (Agre et al., 1987; Saboori et al., 1988). The protein was temporarily named channel-like integral protein of 28 kDa (CHIP28) (Smith & Agre, 1991). As more and more CHIP28 homologs were identified in other organisms, the name aquaporin was finally suggested for this water channel protein family in 1993 (Agre et al., 1993; Carbrey & Agre, 2009). Further research aimed at identifying the structure of newly discovered AQPs continued on into the early/mid-2000s, eventually confirming the crystal structures of isolated AQPs (Murata et al., 2000; Fu et al., 2000; Sui et al., 2001; Savage et al., 2003; Gonen et al., 2005). Currently, 17 different isoforms of mammalian AQPs have been identified and characterized. This protein family shares many common characteristics, yet protein functionality and permeability are determined by amino acid substitutions at only a handful of vital positions in the peptide chain.

B. Structure:

Weighing approximately 28-30 kDa, the general structure of the aquaporin (shown in **Figure 5 and Figure 6**) protein is a tetramer consisting of four independent pores (Wspalz et al., 2009). Each individual pore is a monomer and assumes an hourglass shape consisting of 6 transmembrane right-handed helical domains (H1-H6) and 2 short *a*-helical loops – **Figure 5** (Gorin et al., 1984; Wspalz et al., 2009). Loop B connects H2 to H3 and is named HB while Loop E connects H5 and H6 and is named HE. Both the

amino (N) and carboxy (C) terminus jut into the cytosol from the bottom of H1 and H6, respectively (Smith & Agre, 1991; Wspalz et al., 2009). Additionally, each monomer is highly symmetrical resulting from an ancient gene duplication event and can be split into halves, the first consisting of H1-H3 plus the HB loop while the second half contains the H4-H6 plus the HE loop (Verbavatz et al., 1993; Wspalz et al., 2009). Characteristic of AQPs, both HB and HE possess the highly conserved asparagine-proline-alanine (NPA) motif in the loops that are responsible for assuming an hourglass shape (Jung et al., 1994; Wspalz et al., 2009). Each repeat assumes an opposite orientation in the plasma membrane, allowing loop B and E to meet in the center of the bilayer (Jung et al., 1994; Wspalz et al., 2009). Driven by van der Waals interactions, the prolines in the NPA motifs of each loop stack, forming a platform on which HB and HE extend toward the cytosolic and extracellular surfaces of the plasma membrane, respectively (Wspalz et al., 2009). Each monomer is stabilized by the extensive stacking of helices and tetramerization – **Figure 6** (Wspalz et al., 2009). While the protein fold and overall structure is highly conserved among all AQPs, variation in the structure of the pore is the major determinant of the channel protein's selectivity.

An AQP's, permeability and selectivity is controlled by the tightest constriction throughout the protein pore (Wspalz et al., 2009). When a molecule passes through the amphipathic pore of an AQP, it is conducted through the poor by a series of interactions with both amino acid side chains and carbonyl groups on the protein backbone (Wspalz et al., 2009). On average, water molecules propelled through an AQP pore travel a distance of approximately 25Å and encounter two sites which interact strongly with water (Wspalz et al., 2009). The first site is the NPA motif, located equidistant from the

cytosolic and extracellular membrane surfaces, that serves to reorient water molecules, interrupting hydrogen bonding between water molecules that could affect the flow (Wspalz et al., 2009). Additionally, the asparagine (N) residues act as hydrogen donors to passing water molecules to further conduct them through the pore (de Groot & Grubmuller, 2005; Wspalz et al., 2009). The second site of interaction is responsible for determining the channel's selectivity, as it is the narrowest constriction throughout the pore (Wspalz et al., 2009). This constriction, averaging a diameter of approximately 2.8Å in AQPs, is located near the extracellular pore mouth and is formed by four amino acid residues and is named the aromatic residue/arginine (ar/R) constriction (Wspalz et al., 2009). In human AQP1, the ar/R constriction is shaped by phenylalanine at the 56^{th} residue, histidine at 180, cysteine at 189, and arginine at 195 (or Phe56, His180, Cys189, Arg195 respectively; Wspalz et al., 2009). The interactions between these residues confer an extremely hydrophilic site, while also limiting the pore diameter to 2.8Å, identical to the diameter of a water molecule (Wspalz et al., 2009). The residues composing the ar/R constriction are widely conserved among AQPs (i.e., bovine AQP1; Phe58, His182, Cys191, Arg197). However, Cys189 seems to be the exception. Notably, this residue is the site of the previously mentioned free sulfhydryl group where $HgCl_2$ binds the AQP, inhibiting water diffusion (Preston et al., 1993; Wspalz et al., 2009). Aside from the NPA motif and the ar/R constriction, amino acids contained within the AQP's transmembrane pore are mostly hydrophobic residues with polar carbonyl groups oriented inward along one side of the pore forming a backbone that propels water molecules through the protein via hydrogen bonding (Wspalz et al., 2009). Carbonyl oxygens effectively function as a ladder within the AQP pore, as these hydrogen bond acceptor sites are responsible for

directionally conducting water molecules through the pore (Wspalz et al., 2009). Additionally, this continual formation of hydrogen bonds between AQPs and water molecules alleviates the energy required to remove a water molecule from solution and into the pore (solvation energy; Wspalz et al., 2009). AQPs are vital to preserving water homeostasis. This MIP subfamily is presumed to have evolved from an ancient gene duplication event, as AQPs are highly conserved across many genetically distant organisms. However, a relatively small number of amino acid substitutions in the peptide chain dictate the unique functionality and localization observed between AQP homologs and isoforms, all-the-while maintaining a common functionality and structure.

C. Function:

Over time, extremely complex cellular mechanisms for maintaining fluid homeostasis have been selected for and developed in vertebrates. Natural selection has resulted in immense capabilities for both amphibians and mammals to sense and regulate fluid volumes/composition through coordinated mechanisms that allow the precise regulation of both water and ion transporters, a process vital to life (Krane, 2007). While osmotic pressure can drive the simple diffusion of water molecules across cell membranes, the advent of AQPs dramatically increased membrane permeability to, and speed at which, water can diffuse across cell membranes (Krane, 2007). These proteins allow the rapid and selective transmembrane diffusion of water molecules across the phospholipid bilayer crucial to regulated physiological processes such as absorption and secretion Krane, 2007). Every AQP is linked by the general characteristic of allowing the free facilitated diffusion of water molecules across cell membranes through an integral membrane protein channel. However, over 450 members of the MIP superfamily have

been discovered and characterized since Dr. Peter Agre's discovery of AQP1 (originally CHIP28) in the late 1980s across a wide variety of organisms (Krane & Kishore, 2003; Chaumont et al., 2005; Peterson et al., 2005; Tanghe et al., 2006; Suzuki et al., 2007). For the sake of brevity, this section will outline the general function, localization, and regulation of major mammalian AQPs, as they are the point of reference for most comparative characterization studies of AQP homologs in other organisms.

There are 13 characterized mammalian MIP genes, 7 of these genes are AQPs (denoted AQP0, 1, 2, 4, 5, 6, 8), 4 of which are GLPs (denoted AQP3, 7, 9, 10; these proteins will be discussed in depth later in this review), and the last two genes are functionally classified as SuperAQPs (denoted AQP11, 12) (Zardoya, 2005; Gonene & Walz, 2006; Gorelick et al., 2006; Krane, 2007) . The functional permeability of SuperAQPs as well as their physiological role in mammalian fluid homeostasis are unknown and require further research (Morishita et al., 2004; 2005; Itoh et al., 2005; Gorelick et al., 2006). However, AQP11 is known to be expressed in the proximal tubules of the kidney (held in cytosolic vesicles), liver, testis and brain of mammals while AQP12 has been identified in acinar cells of the pancreas (Krane, 2007). AQP0 is expressed in the lens fiber cells of the eye and is permeable exclusively to water, however the protein's observed level of permeability is much lower than other AQPs (Gorin et al., 1984; Dunia et al., 1987; Berry et al., 2000; Francis et al., 2000; Krane, 2007). It may also serve a secondary role in cell-cell adhesion, as extracellular portions of AQP0 are known to bind to extracellular portions of the same protein on other cells (Gorrin et al., 1984; Hasler et al., 1998; Fotiadis et al., 2000; Wspalz et al., 2009). Unlike AQP0, AQP1 is widely distributed constitutively throughout many mammalian tissues including both

the proximal tubule and thin descending limb of the loop of Henle in the kidney, erythrocytes, epithelial cells in capillaries and the cornea, the choroid plexus, ear, lungs, GI tract, skeletal and heart muscle (Denker et al., 1988; Nielson et al., 1993; 1995; Krane, 2007). This protein seems to be the default mammalian AQP based on its high permeability to water and wide range of tissue distribution. There is evidence that AQP1 may not be permeable to just water, this AQP may be permeable to both CO_2 and NO (Wspalz et al., 2009). If confirmed, AQP1 may play an important role in managing the blood's bicarbonate buffer system, since this protein is well known to be expressed on erythrocytes (Wspalz et al., 2009). Additionally, NO is an important signaling molecule known to induce vasodilation by relaxing smooth muscles, and AQP1 may allow erythrocytes to transport NO to smooth muscle in blood vessels and the GI tract (Wspalz et al., 2009). On the other hand, AQP2 is not nearly as widely dispersed throughout mammalian tissues (Wspalz et al., 2009). Localized to the principal cells of the collecting duct and connecting tubules, this AQP is known for its tight regulation by the peptide hormone arginine vasopressin (AVP; or antidiuretic hormone, ADH) (Wspalz et al., 2009). AVP is released into the blood during dehydration conditions, and the resulting signal cascade created in target cells at the collecting duct and connecting tubules (kidney) initiate the trafficking and insertion of cytosolic AQP2, sequestered in intracellular vesicles, into the apical cellular membrane causing increased water reabsorption from the kidneys (Wspalz et al., 2009). AQP4 is also expressed in the kidney (basolateral membrane of collecting duct principal cells and connecting tubules), as well as the retina, ear, airways, lung, GI tract, fast-twitch skeletal muscle, glial cells at the blood brain barrier, and astrocytes (Wspalz et al., 2009). AQP5 is expressed in

salivary and lacrimal glands, trachea, nasopharynx and airway epithelium, alveolar type I cells, ear, eye, placenta, and the pancreas (Wspalz et al., 2009). AQP6 is a unique protein in this subfamily and is localized to intracellular vesicles in type A intercalated cells of the collecting duct in the kidney (Wspalz et al., 2009). This AQP is presumed to play a role in urinary acid secretion due to its low water and selective anion (primarily NO_3^- and Cl^-) permeability when pH drops below 4.0 and is gated by Hg (Wspalz et al., 2009). Finally, AQP8 (localized in the Testis, sperm, GI tract, placenta, proximal tubule and collecting duct of the kidney, airways, liver, salivary glands, glial and neuronal cells, and pancreas) also exhibits unique permeabilities to Urea and NH_3 in addition to water (Wspalz et al., 2009). Of the 7 AQP isoforms identified in mammals, each shows the same basic characteristics with varying permeabilities, mechanisms of regulation, and localization patterns. This motif is also evident when comparing AQPs to GLPs, another important MIP subfamily.

VI. Aquaglyceroporins: Comparative Structure & Function

A. Structure:

Aquaglyceroporins (GLPs) are another MIP subfamily of highly specific integral transmembrane proteins. Unlike AQPs, GLPs permit the facilitated diffusion of glycerol, urea, and other small uncharged molecules across cell membranes in addition to water. The molecular structures of GLPs are surprisingly similar to AQPs in that they are tetramers with 4 individual hourglass shape pores (Wspalz et al., 2009). Additionally, each monomer possesses the 6 right-handed transmembrane helices (H1-H6), the 2 *a*-helical loops (B & E), both the HB and HE domains, the NPA box (except AQP7), and even the ar/R constriction which determines pore selectivity (Wspalz et al., 2009). However, the main structural difference between the two families lies within the composition of the ar/R constriction near extracellular opening of the channel protein pore (Wspalz et al., 2009). As discussed earlier, AQPs share a highly conserved grouping of Phe, Arg, and His residues that interact to produce a hydrophilic constriction approximately 2.8Å in diameter, the same size as a water molecule (Wspalz et al., 2009). However, when comparing the well characterized *E. coli* GLP (GlpF) to mammalian AQP1, the difference between the two ar/R constriction sites are blatant (Wspalz et al., 2009). The ar/R constriction in GlpF consists of tryptophan (Trp48), glycine (Gly191), phenylalanine (Phe200), and arginine (Arg205) (Wspalz et al., 2009). These residues interact to form a substantially more hydrophilic region measuring approximately 3.4Å in diameter, or relatively the same size as a carbon-hydroxyl group (C-OH) present on polyols (multiple hydroxyl groups) like glycerol (Wspalz et al., 2009). In AQP1, the histidine residue (common to water specific AQPs) directly opposite of the arginine

residue confers a hydrophilic edge next to an aromatic residue in the constriction, the strength of which is enhanced by the cysteine residue's sulfhydryl functional group extending down into the pore (Wspalz et al., 2009). In contrast, GlpF (and virtually all GLPs) the lack of histidine and an additional aromatic residue instead of cysteine, while conserving the arginine residue, within the constriction forms a "greasy slide" (Wspalz et al., 2009). This hydrophobic ladder provides GlpF the ability to efficiently conduct glycerol, small linear polyols, and urea through the channel but does compromise water permeability through the pore (Wspalz et al., 2009). In addition to differing ar/R constriction amino acid composition, GlpF is asymmetric and possesses extracellular loops of varying lengths when compared to AQP1 (Wspalz et al., 2009). Five specific amino acid positions (P1-P5) are consistently different between both mammalian and non-mammalian AQPs and GLPs (Wspalz et al., 2009). P1 is contained within the H3 section of the protein and is observed to be nonaromatic in AQPs but aromatic in GLPs (Wspalz et al., 2009). Loop E houses P2 and P3, both of which are small and uncharged residues in AQPs. However, asparagine is observed at P2 in GLPs while either lysine or arginine occupies P3. Both P4 and P5, found in the H6 domain of the protein, are always aromatic in AQPs. Conversely, GLPs conserve proline residues at P4 and a nonaromatic residue at P5. An additional structural difference has been observed in the mammalian GLP AQP7, as the NPA box is not conserved. Instead loop B substitutes proline for an additional alanine (NAA), and loop E contains a serine residue instead of alanine (NPS) (Wspalz et al., 2009). While AQPs and GLPs share similar molecular structures, substituting just a few amino acid residues has an immense impact on channel selectivity. While the physiological roles of several mammalian AQP isoforms have been revealed,

while the functionality of GLPs have proven more challenging to unveil, partially due to the fact that the importance of glycerol to mammals is not fully understood.

B. Function:

GLPs, like AQPs, are highly selective integral transmembrane protein channels that allow the facilitated diffusion of glycerol, urea, and other small uncharged molecules as well as water across the cell membrane. As a subgroup of the MIP superfamily, these proteins share many common characteristics with AQPs, and appear to assume approximately identical molecular structures at a glance. However, upon further examination of sequence homologies, the structural differences within the ar/R constriction site and 5 other amino acid residue positions become apparent. These crucial amino acid positions are conserved across mammalian and non-mammalian species and have been found to consistently determine the functionality of the protein. Among mammals, 4 of 13 characterized MIP genes have been phylogenetically classified as GLPs denoted as AQP3, 7, 9, 10. All 4 of these mammalian GLPs exhibit permeability to urea, glycerol, and water. Additionally, AQP7 and 9 exhibit permeability to arsenite. AQP3 is expressed across many tissues including the kidney's principal cells of the collecting duct and connecting tubules on the basolateral membrane, the airways, lung, GI tract, brain, ear, urinary bladder, cornea, and epidermis. Studies of AQP3 gene knockouts in mice have resulted in diminished capacities to concentrate urine, insufficient skin hydration/elasticity, and even slower wound healing than wild type mice strains. The expression patterns of AQP7 are less cumbersome than AQP3, as this protein has been identified in the testis, sperm, proximal tubule of the kidney, adipose tissues, and skeletal muscle. AQP7 is presumed to play a role in glycerol diffuse from adipose

cells when hydrolysis of triacylglycerols (TAGs) occurs during periods of fasting or extended exercise. Gene knockout studies of AQP7 in mice has resulted in adipocyte enlargement (hypertrophy), increased body fat and weight, as well as insulin resistance and diabetes associated with aging. AQP9 is expressed in the liver, testis, sperm, spleen, leukocytes, kidney, lung, as well as astrocytes and ependymal cells in the central nervous system. This GLP is presumed to play a crucial role in glycerol uptake into the liver from the blood to undergo gluconeogenesis during periods of fasting (Ishibashi et al., 1998; Carbrey et al., 2003; Carbrey & Agre, 2009). AQP9 null mice exhibit slightly elevated blood plasma levels of glycerol and TAGs. However mating leptin-resistant diabetic mice with the AQP9 null mice to produce double mutants, and subsequently exposing them to fasted conditions, results in immensely high accumulation of blood glycerol plus abnormally low blood glucose concentrations (Rojek et al., 2007; Carbrey & Agre, 2009). Finally, AQP10 is localized only in the duodenum and jejunum of the GI tract, but the GLP's functionality remains unknown as information is scarce. Attempts to uncover the specific mechanisms involved in regulating mammalian GLP expression, as well as their physiological role in maintaining homeostasis have proved difficult, partially because we do not fully understand how mammals utilize glycerol. It may then be more productive to divert attention and resources to investigating the roles of GLP orthologs identified in other organisms, specifically vertebrates that possess extensive capacities for managing osmotic stress and have an understood, and heavily emphasized, utilization of glycerol, urea, and/or other small uncharged polyols.

C. Mammalian MIP Superfamily Orthologs in Anurans:

Amphibians are well known for their impressive dehydration tolerance and osmoregulatory capacities. These vertebrates were also the first organisms observed to possess varied levels of water permeability across different tissues that increased as the level of protein pore structures expressed in the plasma membrane increased. This discovery eventually led to Hans Ussing and colleagues developing the "shuttle hypothesis" in the 1960s. Further investigations in mammalian erythrocytes conducted by A. K. Solomon and Robert Macey suggested that a protein pore selective to water containing a free sulfhydryl group to which $HgCl_2$ can bind and effectively inhibit transmembrane diffusion does exist and confers increased water permeability to mammalian erythrocytes. This elusive protein pore was not successfully isolated until the late 1980s when Dr. Peter Agre unintentionally purified this protein, denoted as CHIP28, while searching for the blood Rh antigen protein. However, the name aquaporin was suggested in 1993 as a rapidly increasing number of orthologs were identified among a wide variety of organisms. Today, over 450 different MIP genes have been characterized, several of these genes are present in anurans.

To date, 6 classifications of MIP proteins have been identified in anurans including orthologs of AQP1, 2, 3, & 9 plus two classifications unique to anurans (AQPa1 & AQPa2). AQP1 orthologs have been identified in *D. chrysocelis* (HC-1), *D. japonica* (AQP-h1), *B. marinus* (AQP-t1), & *R. esculenta* (FA-CHIP) (Abrami et al., 1994; Ma et al., 1997; Hasegawa et al. 2003; Krane, 2007; Suzuki et al., 2007; Zimmerman et al., 2007). Similar to the mammalian gene, these orthologs function as a water selective AQP, widely expressed throughout various tissues in the body (Abrami et

al., 1994; Ma et al., 1997; Hasegawa et al. 2003; Krane, 2007; Suzuki et al., 2007; Zimmerman et al., 2007). Orthologs of the mammalian AQP2 gene have only been identified in one anuran, *D. chrysocelis* (Krane, 2007; Zimmerman et al., 2007). This protein (HC-2) functions as a water selective AQP localized to the primary organs of osmoregulation (skin, bladder, and kidney; Krane, 2007; Zimmerman et al., 2007). AQP3 orthologs have been identified in 4 anuran species including *D. chrysocelis* (HC-3), *D. Japonica* (AQP-h3BL), *X. laevis* (AQP3), and *X. tropicalis* (AQP; Schreiber et al., 2003; Akabane et al., 2007; Krane, 2007; Zimmerman et al., 2007). These proteins are commonly expressed in erythrocytes, hepatocytes, skeletal muscle, and the bladder and function as GLPs (Krane, 2007; Zimmerman, 2007). The last mammalian ortholog identified in anurans is HC-9 in *D. chrysoscelis*, which functions like the mammalian AQP9 GLP and is primarily expressed in hepatocytes. AQPa1 is a novel AQP protein identified only in *X. laevis* (Virkki et al., 2002; Krane, 2007). This protein's amino acid sequence shares less than 50% similarity with the closest related mammal AQP sequences (Virkki et al., 2002; Krane, 2007). This AQP also exhibits a unique sensitivity to mercury inhibition, likely due to its unique amino acid structure. Another anuran-exclusive class of AQPs are the AQPa2 group (Virkki et al., 2002; Krane, 2007). This group consists of two AQPs found in *D. japonica* (AQP-h2, AQP-h3) which share high homology between themselves, as well as to AQP-t2 and AQP-t3 found in *B. marinus* (Tanii et al., 2002; Hasegawa et al., 2003; Krane, 2007; Suzuki et al., 2007). AQP-h2 is expressed in both the ventral skin and the urinary bladder, while AQP-h3 is only expressed in the ventral skin in *D. japonica* (Krane, 2007; Suzuki et al., 2007). Expression of both of these proteins is upregulated by arginine vasotocin (AVT;

Hasegawa et al., 2005; Krane, 2007). AVT-induced upregulation is presumed to play a role in *D. japonica's* metamorphosis (transition from aquatic to terrestrial habitat), as co-expression of the AVT receptor, AQP-h2, and AQP-h3 are observed during this period (Hasegawa et al., 2004; Krane, 2007). While data is fairly limited, research on anuran MIPs have shed some insight on their physiological function and regulation, especially in the cold acclimation and freezing/thawing of *D. chrysoscelis.*

VII. Evidence of Seasonal Cold Acclimation & Aquaglyceroporin Facilitated Cryoprotectant Diffusion in *D. chrysoscelis*

A. Cold Acclimation:

D. chrysoscelis (Cope's gray treefrog) is a freeze-tolerant anuran capable of surviving freezing and thawing after a necessary cold acclimation period. Cold acclimation allows freeze-tolerant organisms to survive low temperatures and prepare for the physiological stressors associated with freezing by inducing changes in gene expression, enzyme function, and cell membrane composition (Costanzo & Lee, 2013; Storey & Storey, 2017; do Amaral et al., 2018). *D. chrysoscelis* has been presumed to withstand freezing by accumulating high levels of glycerol and urea during the cold acclimation period, which are able to freely diffuse across cell membranes during freezing and thawing through GLPs. However, data regarding glycerol accumulation has been conflicting. Some studies have suggested that glycerol is accumulated to high levels in response to cold exposure as an anticipating freezing, with no further mobilization upon ice-nucleation (Layne, 1999; Zimmerman et al., 2007). Others have observed no significant increase in glycerol levels in cold-acclimated frogs, even when exposed to cold conditions for extended time periods and is instead mobilized at the initiation of freezing (do Amaral et al., 2018). Likely, the truth probably lies somewhere in the middle as cold acclimation may result in glycerol accumulation but is not failsafe (Layne & Stapleton, 2008; do Amaral et al., 2018; 2020). Glycerol accumulation may be influenced by the frog's individual physiology (energetic and osmotic status), the effects of

decreased periods of light exposure (photophase; naturally occurs during winter as days grow shorter during), low temperatures, fasting, and even experimental methods (i.e. feeding schedule, rate of cooling, temperature of acclimation) (Costanzo & Lee 2005; Layne & Stapleton, 2008; do Amaral et al., 2018). Additionally, the liver is thought to be the main site of glycerol synthesis, however, do Amaral et al., 2018, observed cold treefrogs to possess higher glycerol levels in muscle than the liver and blood plasma in both cold and warm frogs (Irwin & Lee, 2003; do Amaral et al., 2018). This implies low levels of glycerol synthesis in skeletal muscle, likely derived from carbohydrate or lipid catabolism (Marsh & Taigen, 1987). Regardless, more research must be completed in order to determine the precise mechanism of *D. chrysoscelis'* glycerol accumulation, however it is currently presumed that both anticipatory and freeze-responsive glycerol accumulation occur together. In contrast, substantial evidence suggests that urea is accumulated during cold acclimation.

R. sylvatica, a freeze-tolerant wood frog, rapidly produces glucose in response to the initiation of freezing, but additionally accumulates urea in response to dehydration during cold acclimation which further enhances colligative cryoprotection (Clausen & Costanzo, 1990; Storey, 1997; Costanzo & Lee, 2005; Costanzo et al., 2015; do Amaral et al., 2018). Urea is accumulated from protein catabolism during cold acclimation in wood frogs, which is presumed to be the source of urea in cold treefrogs (Costanzo & Lee, 2005; Costanzo et al., 2015; do Amaral et al., 2018). Urea accumulation may be further enhanced by the significant depression of kidney function observed in *D. chrysoscelis* during cold acclimation (Zimmerman et al., 2007; do Amaral et al., 2018). Notably, dehydration of warm-acclimated (WA) does not induce glycerol synthesis,

however mild dehydration of cold-acclimated (CA) frogs may induce protein catabolism, circulating urea and supplying amino acids for glycerol synthesis in the liver (Raymond & Driedzic, 1997; Zimmerman et al., 2007; Costanzo et al., 2015; do Amaral et al., 2018). Consistent with other freeze-tolerant anurans, *D. chrysoscelis* builds large liver glycogen stores during cold acclimation (Storey & Storey, 1985; Costanzo & Lee, 2003; do Amaral et al., 2018). However, the amount of stored glycogen differs between studies likely due to differing housing/feeding conditions (Storey & Storey, 1985; Costanzo & Lee, 2003; do Amaral et al., 2018). After exposure to consistent cold conditions for several months, liver glycogen stores showed no change in size (do Amaral et al., 2018). This suggests that, like other freeze-tolerant anurans, *D. chrysoscelis'* basal metabolism is supported by non-carbohydrate metabolites, such as proteins and lipids during cold conditions (Dinsmore & Swanson, 2008; Costanzo et al., 2013; do Amaral et al., 2016; 2018). However, the maintenance of glycogen reserves does raise concerns as it is believed to be the source of glycerol. As anticipatory accumulation of glycerol via glycogenolysis may compromise the ability to mobilize glucose, and subsequently glycerol at the initiation of freezing. Liver glycogen store maintenance in the liver of CA treefrogs requires additional attention in order to understand the interplay between anticipatory and freeze responsive glycerol mobilization in *D. chrysoscelis*. Aside from the accumulation of colligative molecules, cold acclimation in *D. chrysoscelis* affects kidney function.

The renal system is integral to conserving colligative cryoprotectants in freeze-tolerant anurans (Krane, 2007). Enduring multiple freeze/thaw cycles over the course of one winter deplete anuran liver glycogen stores potentially due to glucose excretion in

urine (Lee & Costanzo, 1993; Layne et al., 1996; Krane, 2007). This poses a serious threat of glycogen depletion, especially in wood frogs as renal tubules in the kidney have dismal capacities for glucose reabsorption that may be overwhelmed under extreme hyperglycemic conditions (Layne et al., 1996; Krane, 2007). To mitigate glucose loss, wood frogs rapidly initiate glycogenesis during thawing to reconvert as much glucose to glycogen as possible while minimizing potential urine excretion and hyperglycemia. These frogs may also reabsorb glucose excreted in the urine via GLUT transporters in the skin. However, gray treefrogs maintain high glycerol blood plasma levels for weeks at a time in anticipation of and during freezing. In order to preserve glycerol levels, *D. chrysoscelis* is presumed to depress the overall rate of kidney filtration as a part of the cold acclimation period. The glomerular filtration rate (GFR) and the urine filtration rate (UFR) of warm, hydrated, treefrogs as well as the decrease in GFR and UFR are consistent among studies (Shoemaker & Nagy, 1977; Shoemaker & Bickler, 1979; Vondersaar & Stiffler, 1989; Zimmerman et al., 2007). Dehydrating warm treefrogs (23^{o}C) by 20% of standard body mass over 48 hours results in GFR to decrease by approximately 84% (226±107 µL/hr to 37± 6 µL/hr), and UFR to drop greater than 97% (85±37 µL/hr to 2±0.06 µL/hr) of the level observed in warm hydrated frogs, without inducing significant changes in plasma glucose or glycerol concentrations (Zimmerman et al., 2007). The observed greater proportional drop in UFR compared to GFR suggests that water reabsorption is heightened, in either the bladder or renal tubules. Both of these tissues are responsive to vasotocin, or the amphibian ADH (Shoemaker & Nagy, 1977; Uchiyama, 1994; Zimmerman et al., 2007). Acclimating treefrogs to cold temperatures (2^{o}C) resulted in the accumulation of ~51mmol/L of glycerol (plus ~2.9mmol/L of

glucose) in blood plasma, a GFR of 6±0.4 μL/hr (<3% GFR of warm hydrated frogs, and ~1/6th GFR of warm dehydrated frogs), and a UFR of 4±0.7 μL/hr (~2-fold higher than warm dehydrated frogs) (Zimmerman et al., 2007). Additionally, urine glycerol excretion rate nearly tripled with cold acclimation (18±10 nmol/hr to 52±6 nmol/hr) versus warm acclimated frogs (Zimmerman et al., 2007). It is possible that cutaneous (through the skin) absorption of glycerol excreted in urine occurs, however the capacity of skin glycerol transport in *D. chrysoscelis* remain unknown (Zimmerman et al., 2007). Water and glycerol reabsorption in the kidney are mediated by AQP and GLP orthologs in *D. chrysoscelis*, which have also been found to be regulated as part of the cold acclimation process.

The AQPs (HC-1 & HC-2) and GLPs (HC-3 & HC-9) in Cope's gray treefrog have been consistently observed to undergo thermal regulation during the cold acclimation period. In fact, HC-1 is presumed to be a homolog of AQP1 because it shares nearly perfect amino acid sequence and the wide-ranging tissue expression patterns as the known AQP1 homolog found in *D. japonica* (a sister species of *D. chrysoscelis*), AQP-h1 (Hasegawa et al., 2003; Zimmerman et al., 2007). HC-1 exhibits high water permeability at both 10 and 23ºC, is inhibited by ~55% in the presence of $HgCl_2$, and also exhibits low permeability to glycerol at 23ºC, but no permeability at 10ºC (Zimmerman et al., 2007). The mRNA expression levels of this protein is temperature sensitive however, as CA treefrogs express increased HC-1 mRNA levels in hepatocytes plus decreased levels in the brain and kidney when compared to WA frogs (Zimmerman et al., 2007). While its nucleotide sequence is divergent from others, HC-2 is believed to be a member of the AQP2 family as it still shares about 78-83% amino acid similarity to other AQP2 proteins

(Zimmerman et al., 2007). As expected, this protein is expressed primarily in the organs of osmoregulation, is presumed to be regulated by amphibian ADH, and retains high water permeability at both warm and cold temperatures (Zimmerman et al., 2007). This AQP's mRNA expression also varies with temperature changes. HC-2 mRNA is found to be highly expressed in the skin of CA treefrogs, compared to a complete absence of mRNA transcripts in the skin of WA treefrogs (Zimmerman et al., 2007). HC-3 is a functional GLP and is most similar to anuran and mammalian AQP3. This protein is a weak water transporter compared to HC-1 and HC-2, and its permeability to water decreases by more than 50% as temperature is decreased from 23 to 10ºC (Zimmerman et al., 2007). However, over this same temperature change, HC-3 maintains high permeability to glycerol (Zimmerman et al., 2007). HC-3 has also been observed to be upregulated in the liver, lung, bladder, gut, brain, muscle, and bladder while being downregulated in the skin of CA treefrogs (Zimmerman et al., 2007). The increased expression in the bladder paired with decreased skin expression under cold temperatures, supports the idea of increased glycerol conservation in the renal system (Zimmerman et al., 2007). Additionally, upregulation of HC-3 in skeletal muscle, erythrocytes, liver, and bladder corresponds to the increased glycerol levels found in the liver and skeletal muscle of CA treefrogs compared to WA frogs (Krane, 2007; Zimmerman et al., 2007). An additional GLP (HC-9) is thermally regulated primarily by increased expression in hepatocytes during cold acclimation periods (Stogsdill et al., 2017; do Amaral et al., 2020). All of these changes in AQP/GLP expression, renal filtration rates, and cryoprotectant management and accumulation during the necessary cold acclimation period all serve to confer a baseline cryoprotective effect to *D. chrysoscelis* without

causing extensive supercooling or metabolic damage. However, while cold acclimation does provide some protection to the physiological stressors of extracellular ice formation the process is not completely understood and requires extensive research. What is known, is that cold acclamation alone is not sufficient to protect Cope's gray treefrog from extensive freezing for extended periods of time. Many additional freeze-responsive mechanisms exist and must be implemented for *D. chrysoscelis* to survive up to 65% of its total body water volume frozen as extracellular ice for up to several months at a time. The liver is an organ thought to be the primary source of freeze-responsive glycerol synthesis, and therefore vital to *D. chrysoscelis'* freeze tolerance.

B. Liver Transcriptome:

In freeze-tolerant anurans, the liver is vital to cryoprotectant synthesis and metabolic regulation. Yet many mechanisms induced by cold acclimation and freezing remain elusive in all freeze-tolerant organisms, including differential gene expression in hepatocytes. However, since the beginning of the "Human Genome Project" in 1990, DNA technology has improved at an exponential rate, all the while becoming less expensive and more accessible to researchers. Successful efforts generated a hepatic transcriptome of 34,936 genes for *D. chrysoscelis* via high-throughput RNA sequencing (RNA-Seq), to examine the differential gene expression of hepatocytes in response to both cold acclimation and freezing (do Amaral et al., 2020). By analyzing differential gene expression at transcript-level, cold acclimation was found to cause the downregulation of 629 genes and upregulation of 1917 genes compared to WA treefrogs while freezing resulted in the downregulation of 1093 genes and upregulation of 2223 versus WA frogs (do Amaral et al., 2020). However, frozen frogs only upregulated 18

and down regulated 7 genes significantly compared to CA frogs (do Amaral et al., 2020). On the other hand, analyzing differential expression at the gene-level highlighted a much lower number of regulated genes in all three comparisons (CA:WA, CA:frozen, frozen:WA; do Amaral et al., 2020). Just 20-44% of genes found in transcript-level analysis were found in the gene-level analysis, while 86-91% of genes in the gene-level analysis were also regulated at the transcript-level (do Amaral et al., 2020). The shared results of the two analyses result in 277 genes downregulated and 382 upregulated in CA versus WA frogs, 530 downregulated and 519 upregulated in frozen versus WA frogs, and no genes downregulated and just 3 genes upregulated in frozen versus CA *D. chrysoscelis* (do Amaral et al., 2020). Of all the genes found to undergo significant differential expression, ~3.6% were non-coding RNAs (do Amaral et al., 2020). This finding suggests that both coding and non-coding RNAs undergo thermal state regulation in the hepatic transcriptome of *D. chrysoscelis* as 7 C/D box small nucleolar RNAs (SNORDs) were upregulated in CA, and 8 were upregulated in frozen frogs, versus WA, 3 H/ACA box small nucleolar RNAs (SNORAs) were upregulated in CA, and 5 were upregulated in frozen frogs versus WA, as well as two micro RNAs (miR-30 & miR-142) which were upregulated in both CA and frozen frogs versus WA frogs (do Amaral et al., 2020). The importance of snoRNAs in cold acclimation and freeze tolerance remains unknown, but their thermoregulation in response to both phenomena suggests they may play a crucial role in managing transcription and translation (do Amaral et al., 2020). It is known that snoRNAs modify rRNAs and other types of RNA molecules, during freezing conditions specific snoRNAs may serve to improve or inhibit ribosomal function as well as guide specific mRNA transcripts to ribosomes for specific proteins, or tag mRNA

sequences for degradation (do Amaral et al., 2020). SnoRNA5 was found to be upregulated in frozen treefrogs, this molecule is associated with reperfusion injury and may play some role in managing the ischemic conditions experienced during freezing (Costanzo & Lee, 1961; Merchen et al., 2014; do Amaral et al., 2020). The upregulation of miR-30 and miR-142 in response to cold exposure provides more evidence that noncoding RNAs play a role in freeze tolerance (do Amaral et al., 2020). The levels of miR-30 have been observed to increase during freezing in the skeletal muscle of the freeze-tolerant wood frog *R. sylvatica* (Bansal et al., 2015; do Amaral et al., 2020). This miRNA has been observed to prevent apoptosis and liver fibrosis (formation of large amounts of scar tissue when the liver attempts to repair damaged cells), but the specific role in freeze-tolerance is unknown (Deng et al., 2010; Zheng et al., 2015; do Amaral et al., 2020). *R. sylvatica* differentially regulates several other miRNAs including miR-21 (liver & skeletal muscle) and miR-16 (liver), however most miRNAs in the brain of this wood frog were found to be downregulated when frozen, suggesting a potential neuroprotective role (Storey & Storey, 2017; Hadj-Moussa & Storey, 2018; do Amaral et al., 2020). Additional research suggests that some miRNAs may actually improve their function under cold temperatures. 11 miRNAs were identified in the anoxia-tolerant *T. s. elegans*, this group was found to have ~640 different target genes at 37ºC, but at 5ºC these 11 miRNAs targeted 1262 different genes (Biggar et al., 2009; 2016; Storey & Storey, 2017). Often, miRNAs function *in vivo* to suppress the translation of their target mRNA preventing biosynthesis of proteins and undesirable cell processes, an ability crucial to an organism suspended in a hypometabolic state (Storey & Storey, 2017). The

hepatic transcriptome found heavy differential expression of genes associated with metabolic processes and glycerol synthesis (do Amaral et al., 2020).

Enzymes involved in the glycerol synthesis pathway, showed significant but surprising differential expression (do Amaral et al., 2020). Genes that promote glycerol synthesis were not upregulated (do Amaral et al., 2020). In fact, neither glycerol-3-phosphate dehydrogenase (G3PDH: DHAP + NADH → G3P + NAD^+) or glycerol-3-phosphatase (G3Pase: G3P → Glycerol + P_i) did not experience any differential regulation (do Amaral et al., 2020). Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate dehydrogenase (GLUD1), and alanine aminotransferase (GPT2) were all downregulated (do Amaral et al., 2020). This implies a reduced role for glycerol synthesis in the accumulation of this molecule in cold and frozen frogs (Driedzic, 2015; do Amaral et al., 2020). However, an enzyme necessary for the metabolism of glycerol in the liver (glycerol kinase: GK) was also observed to be downregulated (do Amaral et al., 2020). It is probable that downregulating GK, reduces the metabolism of glycerol, thereby promoting glycerol accumulation indirectly (do Amaral et al., 2020). One enzyme, glycerol kinase 5, was upregulated but its function in treefrogs is currently unknown (do Amaral et al., 2020). Looking at the transcriptional data of the glycerol synthesis pathway, the data collected infers transcriptional upregulation of genes promoting hepatic glycerol synthesis is not the cause of glycerol accumulation during cold acclimation or freezing (do Amaral et al., 2020). A potential combination of various regulatory mechanism may contribute to glycerol accumulation instead, including diminished glycerol metabolism, production of glycerol from other sources (i.e. fat stores), or tightly regulating the transport and distribution of glycerol (do

Amaral et al., 2020). It also feels necessary to raise the point that not upregulating transcription of enzymes involved in hepatic glycerol synthesis does not invalidate the hypothesis that liver glycogen is the source of glycerol. Many other factors play roles in regulating enzymatic function, PTMs may occur on G3PDH and G3Pase that increase enzymatic function promoting glycerol synthesis, or preexisting mRNA transcripts of enzymes involved in this pathway may be guided to ribosomes ensuring their translation. Where glycerol is synthesized, and how it is accumulated in *D. chrysoscelis* requires extensive research rendering definitive evidence before a conclusion can be drawn. For example, AQP and GLP membrane expression has been shown to increase in response to cold and freezing in *D. chrysoscelis*, however transcripts of both HC-3 and HC-9 showed no significant change in response to cold or freezing in the hepatic transcriptome (do Amaral et al., 2020). This change in expression is presumed to be caused by PTMs which cause GLPs stored in intracellular vesicles to be trafficked to cell membranes (Stogsdill et al., 2017; do Amaral et al., 2020). However, glycerol synthesis is not the only metabolic pathway that undergoes differential expression.

Likely triggered by a signaling cascade at the initiation of freezing, treefrogs mobilize glucose by activating glycogenolysis (Costanzo et al., 1992; do Amaral et al., 2018; 2020). In fact, the anabolic breakdown of glycogen is transcriptionally promoted in hepatocytes (do Amaral et al., 2020). Phosphorylase kinase b (PHKA2 & PHKB2) and the ß-catalytic subunit of protein kinase A (PRKACB) transcripts are upregulated in frozen frogs, potentially promoting glucose and subsequent glycerol synthesis (do Amaral et al., 2020). Glucose export may also be promoted by transcription as G6Pase and pyruvate dehydrogenase kinase 2 (PDK2) by increasing glucose available for hepatic

export and the conversion of pyruvate to acyl-CoA, respectively (do Amaral et al., 2020). Enzymes associated with glycolysis (including PFKM & GAPDH) are downregulated, potentially suppressing glycolysis, consistent with other freeze-tolerant anurans (Storey & Storey, 2017; do Amaral et al., 2020). Notably, an upregulation of hexokinase (HK) and downregulation of glucokinase regulatory protein (GCKR) was seen in hepatocytes, which would actually favor glycolysis (do Amaral et al., 2020). Additionally, low glucose export from the liver has been observed in previous studies of *D. chrysoscelis*, now this observation may be supported by the finding that glucose transporters in hepatocytes were either downregulated or had no change in expression during cold or freezing exposure (Zimmerman et al., 2007; do Amaral et al., 2018; 2020). Overall proteins/enzymes involved in carbohydrate and lipid metabolism were found to be downregulated in response to cold acclimation and freezing (do Amaral et al., 2020). However dynamic gene expression in the hepatic transcriptome suggests that extremely robust intertwined mechanisms of gene and protein regulation impact cryoprotectant accumulation and mobilization in response to cold and freezing exposure require extensive research before they are completely understood (do Amaral et al., 2020). In addition to regulation of metabolic mRNA transcripts, stress related proteins undergo dynamic regulation.

The chaperone proteins HSP70, HSP90AA1, HSPH1, and HSPA5 were all upregulated in cold and frozen hepatocytes (do Amaral et al., 2020). HSP upregulation, as discussed earlier in this review, is associated with cold hardiness and stress responses among many insect, reptiles, and amphibians. Surprisingly, HIF1A (HSP transcription factor crucial to the hypoxia response) which is known to be upregulated in *T.s. elegans*,

C. picta, and *R. sylvatica* did not undergo any transcriptional dynamic regulation in *D. chrysoscelis* hepatocytes (do Amaral et al., 2020). Additionally, genes associated with increased antioxidant defenses were downregulated (CAT, GSTO1, MGST1, NFE2L2) (do Amaral et al., 2020). However, it is unknown if *D. chrysoscelis* possesses high constitutive antioxidant defenses, like other freeze-tolerant anurans, rendering transcription of new proteins unnecessary (do Amaral et al., 2020). Furthermore, genes associated with apoptosis inhibition and other stress responses were found to be generally upregulated (do Amaral et al., 2020). It is clear that Cope's gray treefrog implements dynamic regulation of mRNA transcription in response to cold acclimation and freezing, however further research must be completed in order to develop a broad understanding of how transcription integrates with translation, PTMs, and cell-signaling as well as what tissue specific changes are employed during these responses. In addition to RNA-Seq techniques, studies of erythrocytes harvested from *D. chrysoscelis* have provided insight as to how cryoprotectants and cold acclimation impact survival of freezing and thawing *in vitro*.

C. Erythrocyte Post-Freeze Viability Assays:

A study conducted by Geiss et al. in 2019 aimed to determine how cold acclimation and presence of cryoprotectants affect the percentage of cells in a live erythrocyte culture that survive one freeze/thaw cycle, or the post-freeze viability (pfv), *in vitro* – Results summarized in **Figure 7 and Figure 8**. To accomplish this, blood samples from both WA and CA treefrogs were collected and incubated at 0.0ºC or frozen to -8.0ºC for 30 minutes (cooling rate of -0.35ºC/min) in the presence and absence of cryoprotectants. The cryoprotectants tested in both groups were 150mM glycerol,

glucose, urea, or 83mM of NaCl which were preincubated into PBS solutions of 280mM (Geiss et al., 2019). Cell viability was assessed via hemoglobin leakage assays which determine the percentage of remaining live cells to cells that experienced cell lysis due to osmotic stress by creating a proportion of the amount of hemoglobin that has leaked into solution versus the amount of hemoglobin remaining in erythrocytes (Costanzo & Lee, 1991; Geiss et al., 2019).

It was determined that freezing erythrocytes from WA frogs in 280mM PBS without any cryoprotectants reduced cell viability from 85.1±2.6% all the way down to 18.9±1.3% (Geiss et al., 2019). In contrast, freezing erythrocytes from CA treefrogs with no preincubations resulted in a pfv of 45.8±2.9%, while unfrozen erythrocytes maintained a viability of 88.9±4.9% 18.9±1.3% (Geiss et al., 2019). Therefore, without any cryoprotectants added to solution, CA erythrocytes were naturally 2.4-fold more freeze tolerant than WA erythrocytes (Geiss et al., 2019). Preincubating WA erythrocytes suspensions with 150mM of urea, increased pfv by 2.5-fold (or 47.4±5.2%), a level similar to CA erythrocytes in the absence of cryoprotectants (Geiss et al., 2019). Conversely, the addition of any other cryoprotectant (glycerol, glucose, sorbitol, NaCl) did not significantly improve WA erythrocyte pfv (Geiss et al., 2019). Freezing CA cells with pre-incubations of 150mM urea resulted in a pfv of 71.9±1.6%, and 150mM glycerol resulted in a pfv of 71.6±8.9%, neither of these values were significantly different than CA solutions preincubated with either cryoprotectant (Geiss et al., 2019). Additionally, glucose and the non-permeating cryoprotectants (NaCl, sorbitol) conferred no cryoprotective effect to frozen CA erythrocytes (Geiss et al., 2019). This study shows evidence that freeze tolerance of erythrocytes in *D. chrysoscelis* is conferred by a

necessary cold acclimation phase, that is further enhanced by the accumulation of the cryoprotectants glycerol and urea (Kane, 2007; Zimmerman et al., 2007; Pandey et al., 2010; Mutyam et al., 2011; Stogsdill et al., 2017; do Amaral et al., 2018; Geiss et al., 2019; do Amaral et al., 2020). It is presumed that part of the erythrocyte cold acclimation period is increasing the membrane expression of the HC-3 GLP (permeable to both glycerol and urea), based on the observation that glycerol had no cryoprotective effect on WA treefrogs, and the cryoprotective effect of urea in CA erythrocytes was significantly greater (Kane, 2007; Zimmerman et al., 2007; Pandey et al., 2010; Mutyam et al., 2011; Stogsdill et al., 2017; do Amaral et al., 2018; Geiss et al., 2019; do Amaral et al., 2020). Additionally, HC-3 membrane expression has been found to increase by ~2.5-fold in CA erythrocytes compared to WA (Kane, 2007; Zimmerman et al., 2007; Pandey et al., 2010; Mutyam et al., 2011; Stogsdill et al., 2017; do Amaral et al., 2018; Geiss et al., 2019). HC-9 membrane expression has also been found to significantly increase in *D. chrysoscelis* hepatocytes in response to CA, potentially increasing the capacity for hepatic export and distribution of glycerol throughout the body (Kane, 2007; Zimmerman et al., 2007; Pandey et al., 2010; Mutyam et al., 2011; Stogsdill et al., 2017; do Amaral et al., 2018; Geiss et al., 2019; do Amaral et al., 2020). Over the past 15 years, substantial breakthroughs in understanding the mechanisms conferring freeze-tolerance to Cope's gray treefrog have been made, this treefrog remains largely enigmatic. Future research aimed at specific questions must be completed before we can illustrate specific, integrated, mechanisms that *D. chrysoscelis* depends upon to mitigate the severe physiological stressors associated with freezing and thawing.

VIII. Future Research

A. Research Questions:

Current research of *D. chrysoscelis* has served to uncover more questions we need to answer. The first of which is the site of glycerol synthesis and the method of accumulation, we need to be able to identify the specific source of glycerol synthesis. Is it actually synthesized from liver glycogen stores, or another tissue like skeletal muscle or fat bodies? Or is glycerol synthesized from a variety of tissues? We also need to definitively outline the accumulation patterns of glycerol *in vivo*. Is glycerol accumulated in anticipation of freezing, at the initiation of freezing, or a combination of both? If glycerol is accumulated seasonally and produced at ice-nucleation, is one method more emphasized? In time, we have to describe how metabolism is regulated to produce glycerol. We know the enzymes of the glycerol synthesis pathway are not transcriptionally upregulated, but other enzymes that could metabolize glycerol are downregulated in hepatocytes. What role do non-coding RNAs, translation, PTMs, and chaperone proteins play? Aside from information regarding glycerol, the importance of AQPs and GLPs must be defined.

We must determine if glycerol diffusion through GLPs extends to other cell types. We also need to determine if there are additional GLPs/AQPs in Cope's gray treefrog that have not yet been characterized. We also do not know how cryoprotectant diffusion affects restricted cell types, as erythrocytes exist in a suspension and are free to move about in solution in change volume. On the other hand, restricted cell types such as hepatocytes are more limited in volume changes as they are surrounded by neighboring

cells that cannot move freely. Do cryoprotectants have a weaker protective effect on these cell types? We also need to determine if the effects of cryoprotectants of glycerol and urea are additive, or if a maximum osmolarity limit exists, in which additional colligative molecules offer no more protection. We must also determine how treefrogs anticipate freezing and initiate cold acclimation. Additionally, the epigenetic responses to freezing and thawing have received little to no attention. Do epigenetic changes increase freeze-tolerance over the frog's lifetime? Are they inheritable? The questions we must answer are vast and open ended, only by approaching them piece-by-piece will we find answers. Most of which will be additional questions, but these will be more refined and focused on increasingly narrow topics additionally informed by discoveries in other species and technological innovations that make research more accessible and efficient.

B. Hypothesis & Specific Aims:

Hypothesis 1: Post-freeze cell viability will be enhanced in erythrocytes and hepatocytes from cold acclimated treefrogs vs. warm acclimated frogs when frozen in the presence of cryoprotectants.

Specific Aim 1: Test the post freeze cell viability of erythrocytes and hepatocytes for warm and cold acclimated frogs

in varying concentrations and combinations of glucose, urea, and glycerol.

Hypothesis 2: Expression and membrane localization of glyceroporins HC-3 in erythrocytes and HC-9 in hepatocytes will be increased in cells from cold acclimated vs. warm acclimated animals.

Specific Aim 2: Quantify the subcellular localization of GLPs in cold and warm acclimated erythrocytes and hepatocytes using immunocytochemistry.

C. Proposed Methodology:

Erythrocyte Live Harvest

Erythrocyte samples will be collected at Wright State University, then transported back to the University of Dayton and used in post-freeze viability studies as previously described (Geiss et al., 2019). Blood samples collected from live warm and cold acclimated frogs will be immediately placed in Complete Cell Culture Media (CCCM), at the same temperature to which the frog is acclimated, as previously described (Mutyam et al., 2011; Geiss et al., 2019). An aliquot of blood samples will be used to determine the number of live and dead erythrocytes using Trypan Blue and a hemocytometer to calculate cell viability as a percentage (Krane, 2007; Geiss et al., 2019). RBCs will be separated from the whole blood sample by centrifugation and PBS wash at respective temperature of frog acclimation (Geiss et al., 2019).

Hepatocyte Isolation

Liver tissue will be obtained from warm acclimated treefrogs as previously described (do Amaral, 2015). To isolate hepatocytes, the liver will first be flushed of blood using “frog saline”. Hepatocytes will be harvested by in situ perfusion of the liver, and isolated as described (do Amaral et al. 2015). After successfully isolated, hepatocytes will be stored at the temperature of acclimation until experiments were conducted (do Amaral et al., 2015). An aliquot of isolated hepatocyte suspensions will be tested for erythrocyte contamination, and cell viability calculated as a percentage (do Amaral et al., 2015).

In Vitro Freezing

Prior to freezing, isolated erythrocytes and hepatocytes from warm or cold acclimated experimental groups will be preincubated with varying concentrations and combinations of cryoprotectants. Each group will be suspended in an isosmotic PBS solution as described (Geiss et al., 2019). A cold and warm acclimated suspension containing no cryoprotectants, for both erythrocytes and hepatocytes will serve as the control groups. The cryoprotectants used in the experiment will be glycerol, urea, and glucose. One cryoprotectant will be added alone in suspension, to test individual efficacy of each. Cryoprotectants will also be added in pairs and groups of three in varying ratios to determine if the effect of multiple cryoprotectants is additive, exponential, antagonistic, or logarithmic. To provide consistency in the colligative effect, the total osmolarity of cryoprotectants used to preincubate cell suspensions will always equal 150 mOsM. Each suspension will be tested in the presence or absence of GLP inhibitor, mercury chloride, to examine the functionality of respective GLPs in freeze tolerance. Each group will be tested in triplicates, to increase sample size and calculate a mean value of post-freeze viability. Preincubated suspensions will be frozen by chilling to -8.0ºC on an ethanol bath resting at 0.0ºC, reduced at a rate of - 0.35ºC/min (Geiss et al., 2019). Frozen cells will remain at -8.0C for 30 minutes, then thawed at 4C for 15 minutes as described (Geiss et al. 2019).

Acute Cryoinjury Assessment (Lactate Dehydrogenase Assay)

Post-freeze viability of erythrocyte and hepatocyte suspensions will be assessed via leakage of lactate dehydrogenase (LDH) (do Amaral et al. 2015). LDH is a cytoplasmic enzyme present in all animal cells responsible for the reduction of pyruvate to lactate under anaerobic conditions (BioLegend Inc., San Diego, California). LDH

rapidly leaks out of cells when impaired by stress, injuries, chemicals, or intracellular signaling (BioLegend Inc., San Diego, California). If cells lyse during a freeze/thaw cycle causing the plasma membrane to tear, or rupture, LDH will be present in the extracellular fluid (ECF) in quantifiable amounts (do Amaral et al., 2015). Intracellular and extracellular LDH activity will be determined using a reagent kit (do Amaral et al. 2015). The ratio of ECF LDH activity to total LDH activity will be calculated to estimate LDH leakage of each sample. The data will be replicated in triplets and averaged (do Amaral et al. 2015).

Immunocytochemistry

Immunocytochemistry is a technique used to visualize the subcellular localization of a specific protein by binding its specific antibody to the protein of interest. Then a secondary antibody (labelled with fluorescent dyes) is bound to the protein- antibody complex. The binding pattern of the newly formed protein-antibody-secondary-antibody complex exhibits specific visible light wave absorbances that can be examined with confocal light microscopy to extrapolate subcellular localization of the protein in question. In this experiment, the expression of GLPs HC-3 and HC-9 will be examined in each experimental and control group used in the experiment. Hepatocytes and erythrocytes isolated from warm and cold acclimated treefrogs will be fixed on gelatin coated slides and dried (Mutyam et al., 2011). Slides will be washed and labelled with peptide-derived, rabbit polyclonal antibody against HC-3 or HC-9; then with a goat anti-rabbit fluorescein-conjugated secondary antibody (Vector Laboratories) in 1% blocking serum as described (Mutyam et al., 2011). Immunofluorescence will then be analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope. Fluorescence

intensity representing protein subcellular localization will be analyzed and compared using image J analysis software.

Statistical Analysis

Each experimental group (n=3-4) will be done in triplicates to calculate a viable mean, standard error of the mean (SEM) will also be calculated. A two-way repeated-measures analysis of variance (ANOVA) is necessary to determine if the response of experimental variables is a function of population or treatment, or their interaction (Geiss et al. 2019). Mean values within each population will need to be compared using a one-way, repeated-measures ANOVA followed by a Bonferroni post hoc test (do Amaral et al. 2015).

IX. Significance & Justification

A. Predicted Outcomes & Impact:

The results of this work will also allow us to compare post-freeze viability between two different cell types, erythrocytes and hepatocytes that reside in different *in vivo* environments. In frogs, erythrocytes circulate as single cells in suspension whereas hepatocytes reside within the liver, a structurally restricted multicellular organ composed of different cellular and non-cellular elements. Therefore, these experiments will examine the freeze competence of both suspension and non-suspended cells. The inhibitor and immunocytochemical studies will also allow us to determine the functional requirement of GLPs in cellular freeze competence. The data collected in this experiment will provide new insight to the mechanism of freeze tolerance of D. chrysoscelis. We will produce the first tangible data set comparing the difference in post-freeze viability of hepatocytes (a restricted cell type, limited in its ability to change volume without affecting neighboring cells) to erythrocytes (a suspension cell, unrestricted in changes to volume and shape). We may also present strong evidence that HC-3 and HC-9 are necessary in D. chrysoscelis' freeze tolerance as we test how inhibiting GLPs can affect post-freeze viability rates. In a broad scope, progress in understanding the physiological mechanisms of freeze tolerance is applicable to the biomedical research of human organ cryopreservation. The ability to successfully freeze organs in a safe and accessible manner will serve to extend the potential range of organ recipients and allow for the creation of organ and/or tissue banks alleviating the need for extensive waiting lists for transplants. Glycerol may also be superior to glucose as a cryoprotectant molecule as it does not cause hyperglycemia, does not need to be cleared by the liver through

glycogenesis, and is easily excreted in urine by the renal system. In fact, this molecule is already widely used as a cryoprotectant in the long-term storage of frozen bacteria and animal sperm.

B. Intellectual Merit: Krogh Principle

Dr. August Krogh was a comparative physiologist who won the Nobel Prize in 1920. He claimed that "For many problems there is an animal which it can be most conveniently studied". This statement rings true regarding collaborative attempts to elucidate the cellular mechanisms of freeze tolerance. Anurans are an intuitive model for research regarding osmoregulation and thermoregulation. One reason is that anurans are fairly evolved vertebrates with many similarities to mammals which also exhibit impressive capacities for dehydration and anoxia tolerance. Additionally, the AQPs & GLPs found in Copes' gray treefrog are comparable to mammalian MIPs, and the initial research of AQPs began with observations made in differential anuran tissue water permeability.

C. Broader Impacts: Biomedicine, Cryobiology, & Organ Preservation

The research plan described above will be crucial in gaining new insights into the cellular mechanisms of *D. chrysoscelis'* freeze tolerance. It will provide the first direct comparison of post-freeze viability in suspended and non-suspended cell types as well as the tissue-specific functional requirements of GLPs in *D. chrysoscelis*. Research exploring the mechanisms which permit freeze tolerance in anurans is widely applicable to biomedicine, specifically to the cryopreservation of human organs and tissues. Developing techniques to freeze and thaw human organs and tissues in a safe, efficient, manner will extend the potential range of organ donations and make possible the creation

of organ and tissue banks which would operate in a similar capacity as modern blood banks.

Today, organ and tissue donations are limited primarily by range because human organs and tissue remain viable for only a few hours after being removed from a living donor. As a consequence, when a trauma patient is declared brain-dead, their organs can only be given to a recipient residing in close vicinity to the hospital completing the organ and tissue harvesting procedure. Theoretically, a brain-dead patient could be kept alive in a persistent vegetative state to give the transplant candidate with the highest priority enough time to travel to the donor's hospital. However, doing so would give rise to ethical dilemmas regarding the patient's autonomy and other patients who may be eligible for different transplants from the same donor. An innovative solution to this dilemma is developing safe and efficient procedures which allow healthcare professionals to cryogenically preserve human organs and tissues. This development would effectively give donor organs and tissues infinite shelf-lives. As a result, organs could be harvested, frozen, transported to a distant recipient, thawed, and transplanted into the recipient without any geographical or time-based constraints. Furthermore, organs and tissues can be harvested from eligible organ donors even if no transplant candidates are identified. These organs could be frozen and stored in "organ banks" across the globe and, once an eligible recipient is identified, frozen organs could be transported to hospitals in close proximity to the recipient. Not only does the cryopreservation of human organs and tissue minimize time and distant constraints, but the newfound capacity to harvest and store organs for extended periods of time may directly combat the world-wide shortage of organs available to those in need of life-saving transplants. The unique combination of

colligative cryoprotectants utilized by *D. chrysoscelis* may prove vital to developing safe and effective techniques for humans.

As examined in great lengths previously in the review, Cope's gray treefrog is the only freeze-tolerant vertebrate (aside from one species of salamander) known to seasonally accumulate glycerol as a colligative cryoprotectant. Other anurans, specifically of the *Ranid* family, are known to mobilize glucose as a colligative cryoprotectant at the initiation of freezing by way of β_2-adrenergic signaling. In humans, glycerol may be a superior cryoprotectant molecule for several reasons. First, glycerol produces two osmotically active metabolites from the cleavage of one glycogen hexose subunit while glucose only yields one. Notably, glycerol production is more energy expensive than glucose production. However, energy requirements may prove negligible if glycerol solutions are injected into human organs and tissues prior to freezing. Another advantage is that glycerol does not cause extremely dangerous, and potentially fatal, hyperglycemic conditions. Furthermore, glycerol is less physiologically stressful to remove from circulation in the blood because it is readily converted to glycogen in hepatocytes and is excreted in urine by the renal system. Glucose does not easily pass through the renal system and is removed from circulation by insulin-induced uptake into fat and muscle cells. Glycerol uptake does not need to be induced by pancreatic hormone secretion and can diffuse into cells through GLPs, a family of integral transmembrane channel proteins distributed among a variety of tissues in humans.

X. Reflection

Completing an honors thesis in the midst of a global pandemic has proven to be a more difficult task than expected. I've faced a number of setbacks and disappointments over the last year. Aside from adjusting to the new demands of remote education, I was unable to do any of my proposed lab-based research. I dedicated a large portion of my free time in the fall 2019 and early spring 2020 semesters in Dr. Krane's lab learning various new techniques so that I could complete my research during the summer and fall of 2020. However, unforeseen circumstances would not only prevent me from finishing my training in the spring but also keep me out of the lab for the entire summer. I was not approved to return to the lab until late in the fall of 2020, and at that point Dr. Krane and I decided that there would not be enough time to complete my training, let alone any meaningful research, and still complete my thesis in time. Luckily, we did have a contingency plan so I could do some meaningful work and meet the requirements of the Dean's Summer Fellowship.

After learning I would not be allowed in Dr. Krane's lab in the summer of 2020, we decided that I would author a comprehensive literature review, with the intent to publish, that explores the various cellular mechanisms permitting freeze tolerance among a variety of organisms, the specific mechanisms of Cope's gray treefrog, the goals for future research on this animal, and the biomedical applications of understanding freeze tolerance. Beginning this review felt like I tried to learn to swim by diving into an Olympic pool while wearing a weighted vest. Not only did I have to learn everything I possibly could about freeze tolerance over the course of a few months, I had to learn how to read academic journal articles, process that information, organize it into coherent

arguments, and then synthesize a narrative from this knowledge. The only truly relevant course I had taken prior to this summer was a year of organic chemistry and one-semester courses of biochemistry and physiology. As a result, the majority of my research process was dedicated to learning the biological and statistical topics presented as common knowledge within journal articles. On top of that, I was studying for the MCAT most of the summer because my testing date got delayed from March 5th all the way to June 28th. So, my summer consisted of me sitting at a desk between twelve and fifteen hours a day, six days a week. I guess the best time to do that is during a pandemic, but by July the social isolation began to wear on me, and I was starting to experience severe burn-out. I significantly cut-back on my working hours in the second half of the summer and right around that time the Black Lives Matter movement had increasingly become the center of media focus. As I began digesting more and more information about the protests across the country, I found the way information was delivered through the news strange. Everything seemed to disingenuously focus on property damage occurring during protests, and not the actions which caused the nation to protest or how the police's escalations during protests incite riots – even though 93% of BLM protests have incurred no damage to any public, private, or personal property. I was curious as to why no one was talking about the countless innocent lives lost as a result of police violence, and hyper focusing on people stealing from multi-billion-dollar corporations who have insurance specifically for these situations. I began educating myself and opened my eyes to the brutal history of racism in America, and how historical precedence has created lasting, and pervasive, impacts on a wide variety of oppressed minorities in the United States. I also began to educate myself politically, and I learned about the serious flaws of

American society and partisan legislation which actively works to maintain the status quo without improving the material conditions of the countless number of American citizens living at or below the poverty line. It seems to me that neither party truly wants to change anything, one party chooses to actively ignore systemic issues in this nation while the other just offers empty slogans without taking any action.

My own efforts to educate myself this summer inspired me to take action. I couldn't go out into the streets to protests because I was in close proximity with a few immunocompromised people on a daily basis, but I recognized that I have a unique position of power. I am the president of Alpha Epsilon Delta (AED), an honors society for over 100 brilliant pre-health professional students, and I decided to help do my part to make sure that my peers learned what I had. The American healthcare industry itself is systemically racist. While it is true that the CDC has declared racism to be a public health epidemic and mainstream attention has been given to the fact that BIPOC face a COVID mortality rate at least twice as high as white Americans, a very small number of US citizens, especially those who are white, cis gendered, heterosexuals, truly understand how deeply rooted racism is in our healthcare system. As a straight white man, I understand that no matter how hard I try I will never be able to truly understand the realities of racial discrimination and sexism. Yet at the same time, my demographic status, which does limit my ability to understand these issues, also puts me in a position where my advocacy cannot be immediately dismissed through the same bigoted and misogynistic tropes used to silence minorities and women when they demand equality. Working closely with Dr. Scheltens, we decided to make racism in healthcare a central theme in AED's meetings this year. We hosted a variety of speakers who focused

specifically on these issues, organized a book club that read the phenomenal novel “Black Man in a White Coat” by Damon Tweedy, MD, and provided anti-racism training to our members as well as exercises to recognize implicit biases while practicing medicine. I did what I could to make ensure that the flaws of American healthcare were made indisputable to our members.

As a future physician, I intend to dedicate my life not only to always treating my patients to the best of my abilities but to be an unflinching advocate for the necessary reforms to ameliorate the long and deadly practices which disproportionately harm women, POC, and members of the LGBTQ+ community to this day. I do not think it is possible for one to be a good physician without demanding every single one of their patients is offered the same level of human dignity, access to care, and quality of care regardless of socioeconomic status. I personally believe the most direct way to begin actualizing this goal is by implementing a universal, single payer, healthcare system in the United States. I believe this thesis project has sufficiently prepared me for the next chapter of my professional career so that I may become and extremely successful student in medical school.

I now have the tools to approach a new complex topic, evaluate primary sources, and use this knowledge to synthesize a detailed narrative. In addition to this ability, I can identify research questions which remain unanswered within a particular field and propose methodologies that may uncover answers to said questions. I believe I have the capacity to complete proposed methodologies based upon my prior lab protocol training, even though I was unable to complete any hands-on work myself in this thesis. Aside from practical skills, I have a newfound confidence in myself. I am deeply satisfied with

the work I have completed. Starting this thesis, I felt in over my head and was honestly unsure if I would actually be able to finish; but here I am, approaching 25,000 words. The sense of responsibility, determination, discipline, and self-motivation I developed while writing this thesis will be crucial to my success for the rest of my life. For the first time in my life, I feel like I am truly prepared for medical school and that I was undoubtedly made to become a physician. The only thing that could ever hold me back from achieving greatness is myself.

XI. References

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XII. Figure Legends

Figure 1: The Core Tenants of Organism Responses to Seasonal Cold Temperature. Figure 1 shows four major responses to seasonal exposure to cold temperatures in an organism's environment. The first response is migration and occurs when an organism moves to a warmer habitat for the winter to avoid cold temperatures and returns to the original habitat when temperatures rise. The next three responses involve an organism remaining in its habitat during seasonal cold temperature. Hibernation occurs when an organism enters a state of seasonal hypometabolic activity after building large endogenous fuel stores and implementing metabolic rate depression. Organisms which exhibit freeze avoidance behavior prevent ice nucleation by supercooling their body fluids, mainly through the production of antifreeze proteins (AFPs) and cryoprotective dehydration in order to increase the osmolarity of body fluids. Together, these mechanisms work to implement freezing point depression. At the same time, freeze avoiding organisms modify their cell membrane composition to maintain structural and functional integrity of cells. Freeze tolerance is a minority choice among ectotherms and is accomplished by allowing 50-70% of an organism's total body water volume to freeze primarily in extracellular spaces in a slow and controlled manner by way of minimal supercooling (~ 1.0^{o}C below the normal freezing point of an organism's body water) as well as the production of a wide variety of proteins which control ice crystal formation and maintain vital cellular processes. Freeze tolerant organisms also combat a wide variety of physiological stressor as a result of freezing. Freezing causes dehydration stress which is mitigated by dynamic osmoregulation through the production of colligative cryoprotectants and their transmembrane diffusion through specific proteins to

combat osmotic stress. Freezing also causes ischemia which induces anoxic conditions and oxidative stress in frozen animals, which is mitigated by implementing hypometabolism, strict enzymatic regulation to prevent wasting energy on non-vital cellular processes and ensuring the function of crucial metabolic enzymes during cold conditions, building large stores of endogenous liver glycogen as a source energy, and regulating protein synthesis by way of genetic control to preserve energy. Oxidative stress is directly minimized by implementing strict regulation of apoptosis mechanisms, increasing antioxidant defenses, and the production of chaperone proteins to ensure translation of target proteins.

Figure 2A: Freezing and Thawing in Non-Freeze Tolerant Cells Resulting in Osmotically Induced Cellular Lysis. In figure 2a, box 1 (far left) is a suspended cell in an isotonic solution with non-penetrating solutes (red triangle) and water permeable integral transmembrane protein channels (pink cylinders). Box 2 (middle left) shows the early stages of freezing as pure ice crystals form in the extracellular fluid (ECF). This removes available solvent from the ECF, increasing its osmolarity and rendering the solution hypertonic to the cell. Based on the principle of osmolarity, water is pulled out of the cell into the hypertonic ECF causing the cell to shrink. Box 3 (middle), freezing has completed, cell volume has significantly reduced in an attempt to equilibrate. Box 4 (middle right) shows ice crystals rapidly thawing in the ECF. As water thaws, the ECF is rapidly diluted and results in local hypotonicity of the ECF. Resulting from hypotonic conditions, water rapidly influxes into cells, causing acute swelling. Box 5 (far right) shows acute cell swelling progressing and ultimately causing cell lysis.

Figure 2B: The Colligative Cryoprotectant Glycerol and its Facilitated Diffusion through Glyceroporins May Prevent Severe Changes in Cellular Volume During Freezing and Thawing. In figure 2b, box 1 shows a suspended cell in an isotonic solution containing both penetrating (green circles = glycerol) and non-penetrating solutes (red triangles) as well as water specific and glycerol specific (green cylinders; aquaglyceroporins) integral transmembrane protein channels. Box 2 shows the initiation of freezing. In response to the increasing hypertonicity of the ECF, glycerol moves into the cell to increase intracellular osmolarity which mitigates water movement into the ECF. Box 3 shows more glycerol entering the cell to further increase ICF osmolarity as freezing progresses while significantly preventing large water volume loss within the cell. Box 4 shows glycerol moving out of the cell in response to local hypotonicity of the ECF preventing a large influx of water into the cell. Box 5 shows the cell and ECF returning to isotonic conditions after thawing has completed.

Figure 3: The Function of AMP-Activated Protein Kinase in the Synthesis of ATP. Figure 3 shows active AMPK phosphorylating creatinine kinase (CK) and AMP deaminase (AMPD). The top pathway shows phosphorylated CK (CK-P) converting creatinine-P and ADP into creatinine and one ATP. The middle pathway shows adenylate kinase converting two molecules of ADP into one molecule of ATP and AMP. Phosphorylated AMPD (AMPD-P) then converts AMP into one molecule of inosine monophosphate IMP and an ammonium cation (NH_4^+). The bottom pathway shows the net conversion resulting from AMPK-P activation of CK and AMPD which produces two ATP molecules from three ADP molecules.

Figure 4: The Metabolic Pathways of Glucose and Glycerol Synthesis in Hepatocytes. In figure 4, the first process in both pathways is epinephrine binding to a $ß_2$-adrenergic GPcR which activates the G protein. The activated G protein then activates Adenyl cyclase which converts ATP into cyclic AMP (cAMP), beginning the G-protein signaling cascade. The newly formed cAMP activates protein kinase A (PKA becomes PKA_c). PKA_c activates glycogen phosphorylase kinase (GPK) by phosphorylation (becomes GPK-P). GPK-P phosphorylates inactive glycogen phosphorylase (GP_b) converting it into the active form (GP_a). GP_a then cleaves 1 hexose subunit from a hepatocytic glycogen chain. Glucose 1-phosphate (G1P) is then converted to glucose 6-phosphate by phosphoglucomutase (PGM). G6P can enter either pathways. On the right-hand side is the gluconeogenesis pathway. G6P is transported into the endoplasmic reticulum where it interacts with glucose 6-phosphatase (G6Pase). G6P is converted into glucose, exported out of the endoplasmic reticulum near the cell membrane where it is subsequently exported out of the cell via the GLUT-2 glucose transporter protein. Conversely, G6P can enter the Glycerol synthesis pathway where G6P is converted into Fructose 6-phosphate (F6P) where it undergoes standard glycolysis reactions until Dihydroxyacetone is produced (DHAP). DHAP is converted to glyceraldehyde 3-phosphate (G3P) by glyceraldehyde 3-phosphate dehydrogenase (G3PDH). G3P is then converted into glycerol where it exits the cell through integral transmembrane protein channels (GLPs). In the case of Cope's gray treefrog, glycerol would exit hepatocytes via HC-9. Furthermore, G3P may enter the lactate fermentation pathway shown in the bottom left corner.

Figure 5: The General Structure of Human AQP1 Monomer in an Open and Closed Conformation. In figure 5, the top image shows the open conformation of an AQP1 monomer. Repeat 1 is composed by H1-H3, the intracellular amino terminus of the protein, Loop A (connecting H1 and H2), Loop B which contains the NPA motif (connecting H2 and H3). The NPA motif contribits to the pore's hourglass structure and is represented by the box in the top right corner. Repeat 1 is connected to Repeat 2 by Loop C. Repeat 2 contains H4-H6, Loop D (connecting H4 and H5), Loop E which contains the NPA motif and a cysteine residue acting as the binding site for mercury chloride which inhibits diffusion (connecting H5 and H6), and the intracellular carboxy terminus. The bottom image shows the closed monomer. In the center of the monomer is the creation of the hourglass structure as a result of the reactions between the NPA motifs of Loop B and Loop E. Additionally, both terminal ends of the protein point into a cell's cytoplasm adjacent to each other as H1 and H6 come together to create the closed conformation.

Figure 6: The General Structure of Human AQP1 Tetramer in Closed Conformation. The top image in figure 6 shows human AQP1 in tetrameric form. The tetramer is composed of 4 monomers. 2 monomers are oriented in the same direction and are connected to the other 2 monomers via interactions between H4 amino acid chains. The protein possesses 4 independent pores that allow water diffusion. The bottom image shows the same tetrameric AQP1 from a top-down view of the protein's extracellular surface.

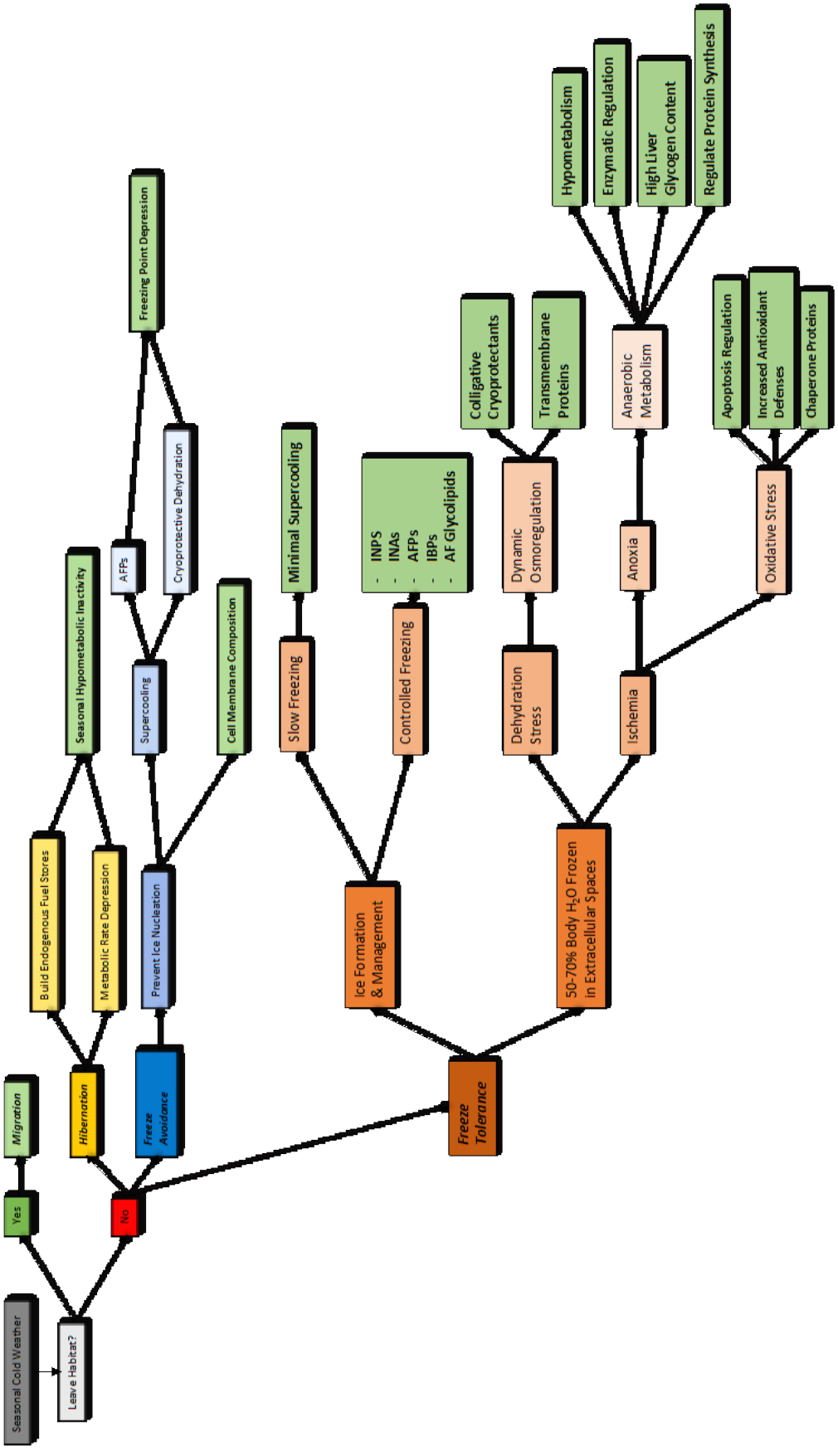
Figure 7A: Post-Freeze Viability of Erythrocytes from Warm Acclimated Animals is Improved by Urea Alone While Erythrocytes from Cold Acclimated Animals is Improved by Both Urea & Glycerol. Adapted from: Geiss L et al. Postfreeze viability of

erythrocytes from Dryophytes chrysoscelis. *J Exp Zool Part A Ecol Integr Physiol* 331: 308–313, 2019. The figure on the top shows that freezing erythrocytes from warm acclimated animals in a PBS control decreases post-freeze viability by approximately 80.0% and was only significantly improved by the addition of urea into the PBS suspension. The bottom image shows that freezing erythrocytes from cold acclimated animals in PBS decreased post-freeze viability by approximately 50.0% and was significantly improved by both urea and glycerol.

Figure 7B: Erythrocytes from Cold Acclimated Animals Exhibit Increased Post-Freeze Viability vs. Erythrocytes from Warm Acclimated Animals; Enhanced by Either Glycerol or Urea. Adapted from: Geiss L et al. Postfreeze viability of erythrocytes from Dryophytes chrysoscelis. *J Exp Zool Part A Ecol Integr Physiol* 331: 308–313, 2019. This figure shows that erythrocytes of cold acclimated frogs exhibit higher post-freeze viability rates versus cells from warm acclimated animals among every measure, and that glycerol and urea were the only osmolytes to significantly enhance post-freeze viability of cold acclimated animal erythrocytes while urea was the only osmolyte to improve warm acclimated post-freeze viabilities.

Chapter XIII: Figures

Figure 1:



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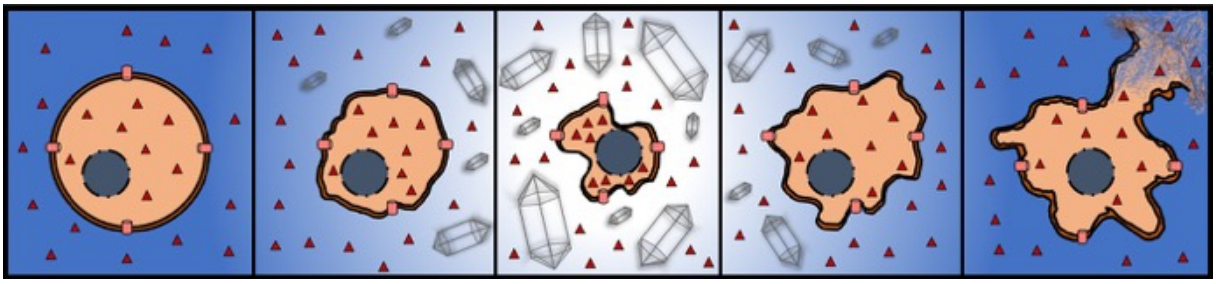

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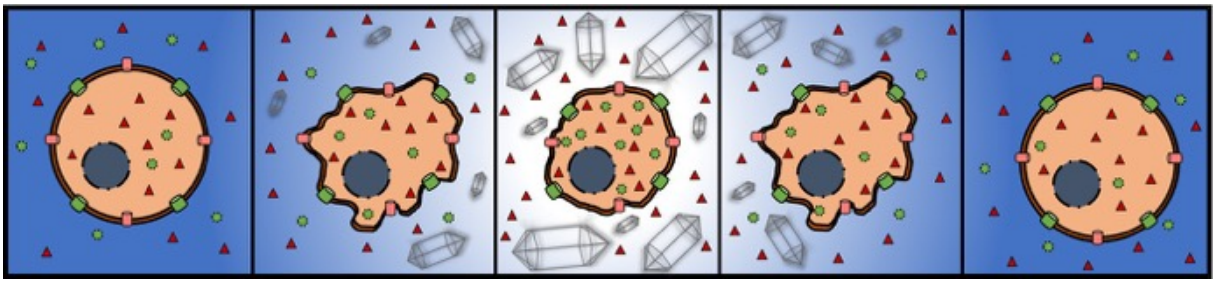

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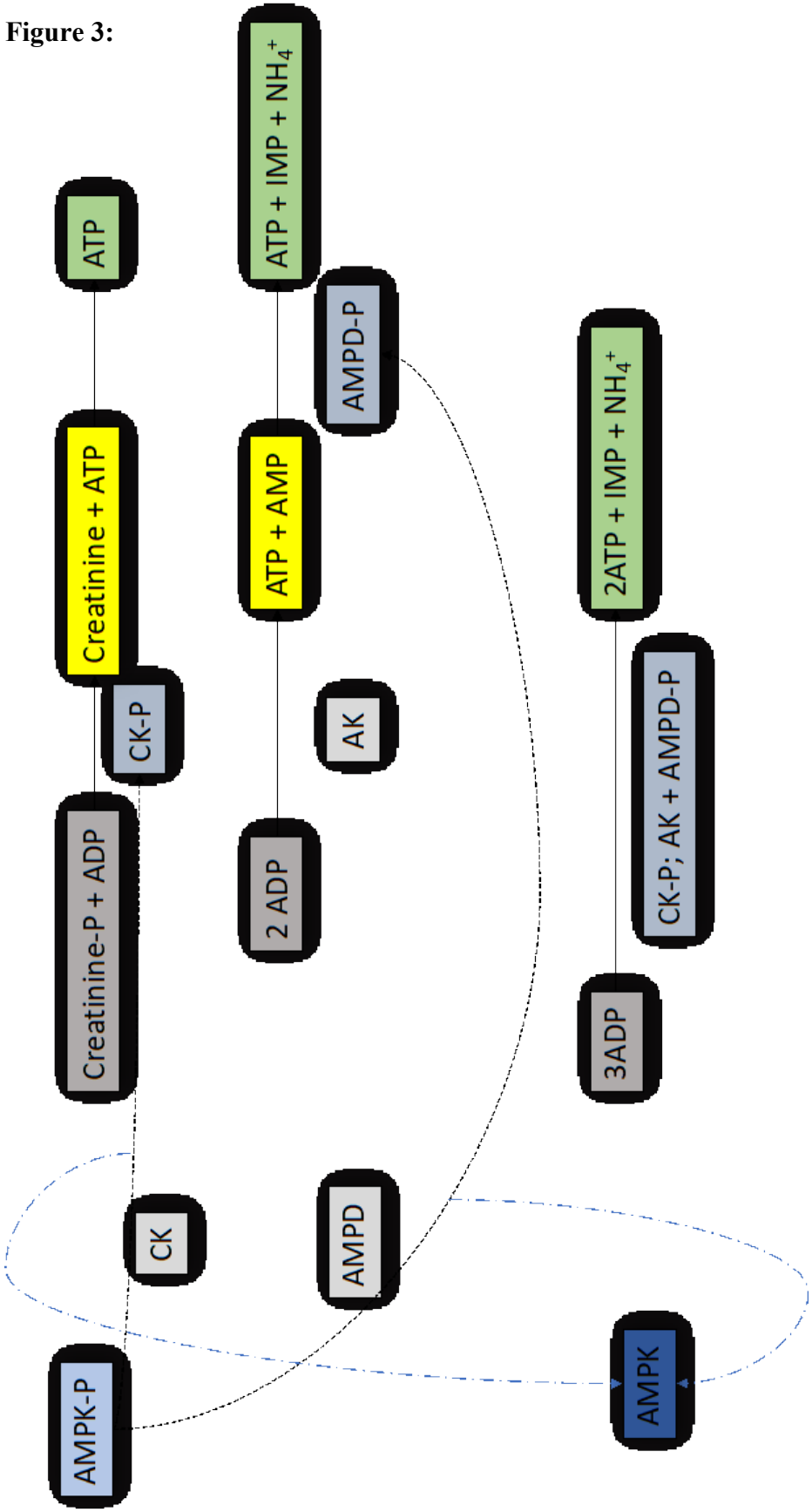

ATP
ATP + IMP + NH_4^+
AMPD-P
Creatinine + ATP
ATP + AMP
2ATP + IMP + NH_4^+
CK-P
AK
CK-P; AK + AMPD-P
Creatinine-P + ADP
2 ADP
3ADP
CK
AMPD
AMPK-P
AMPK

Figure 4:

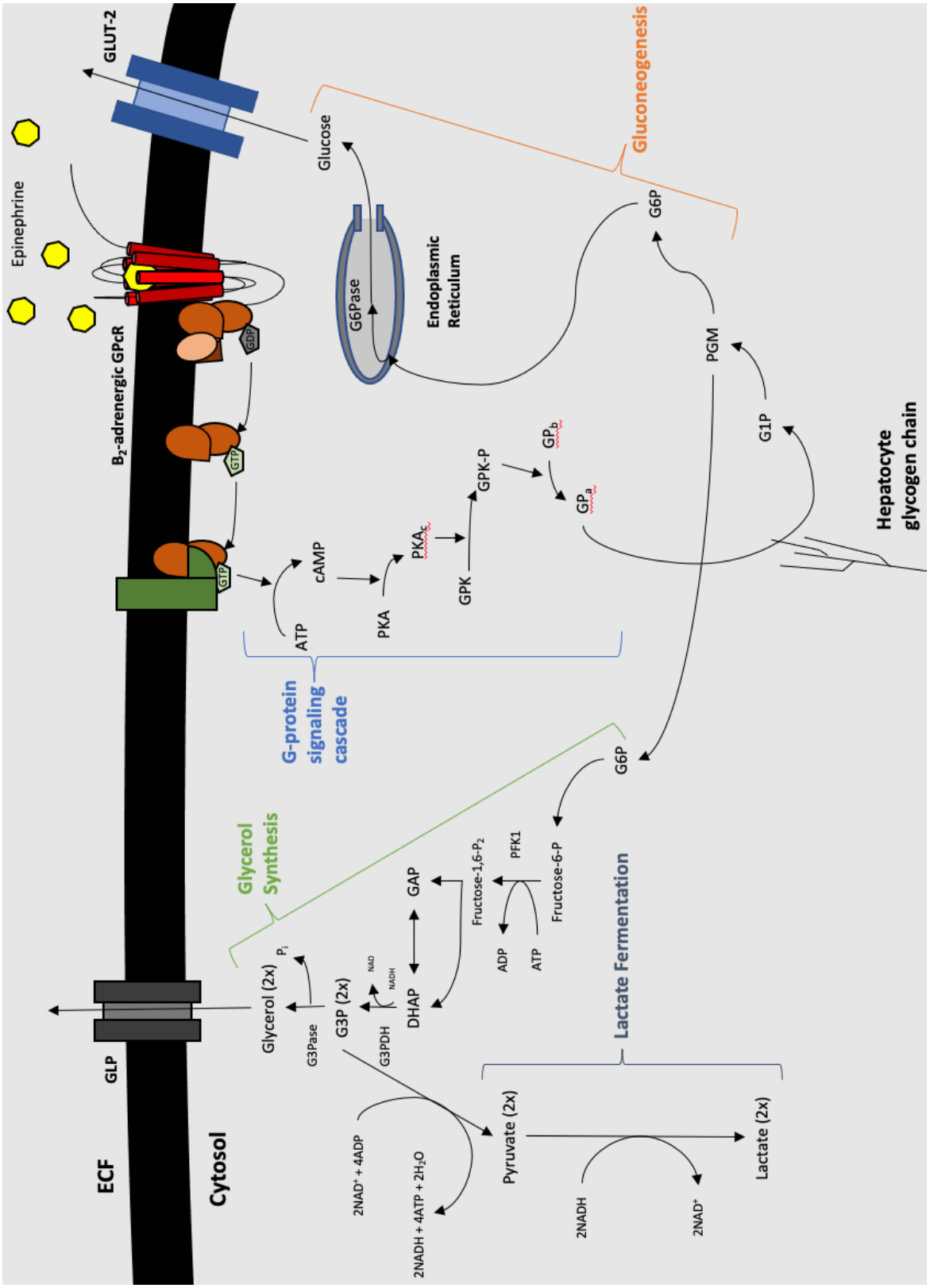
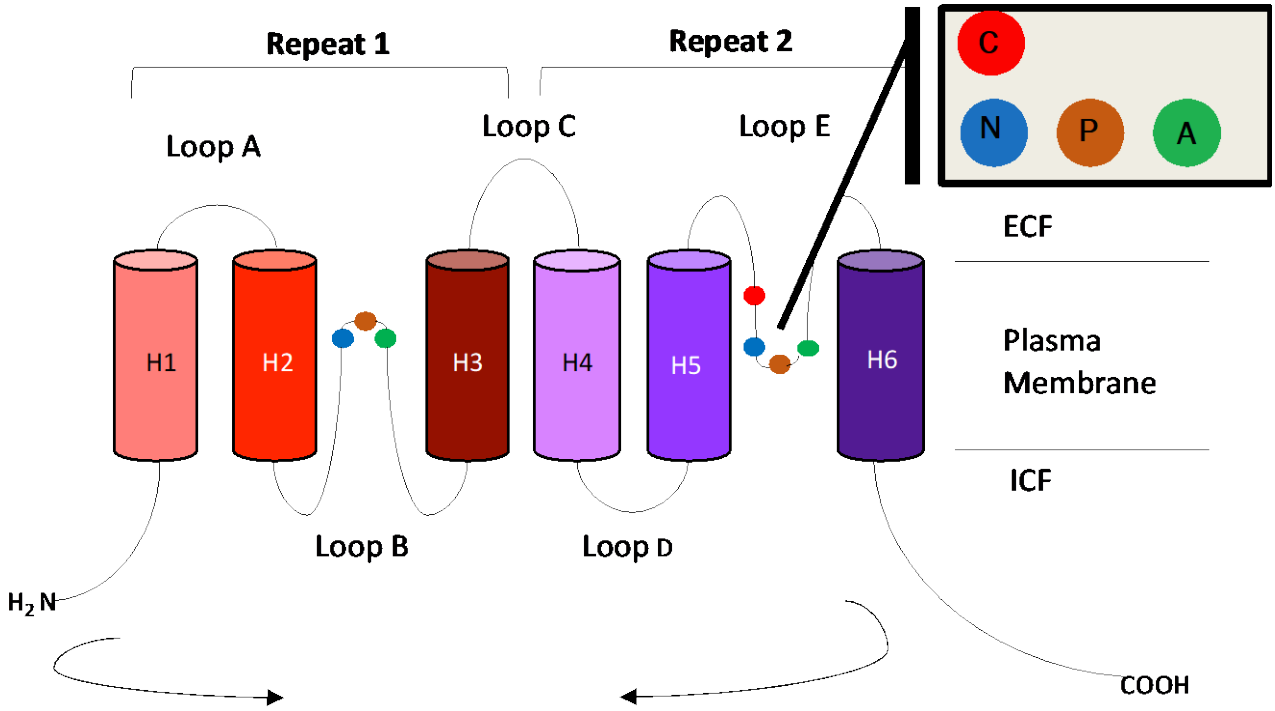

GLUT-2
Epinephrine
B_2-adrenergic GPcR
GDP
GTP
GTP
Glucose
Gluconeogenesis
G6Pase
Endoplasmic Reticulum
G6P
PGM
G1P
Hepatocyte glycogen chain
ATP
cAMP
PKA
PKA
GPK
GPK-P
GP_b
GP_a
G-protein signaling cascade
Glycerol Synthesis
G6P
Fructose-6-P
PFK1
ADP
ATP
Fructose-1,6-P_2
GAP
DHAP
NAD
NADH
G3PDH
G3P (2x)
G3Pase
P_i
Glycerol (2x)
Lactate Fermentation
GLP
ECF
Cytosol
$2NAD^+ + 4ADP$
$2NADH + 4ATP + 2H_2O$
Pyruvate (2x)
2NADH
$2NAD^+$
Lactate (2x)

Figure 5:


Repeat 1
Repeat 2
Loop A
Loop C
Loop E
C
N
P
A
ECF
Plasma Membrane
ICF
H1
H2
H3
H4
H5
H6
Loop B
Loop D
H_2N
COOH

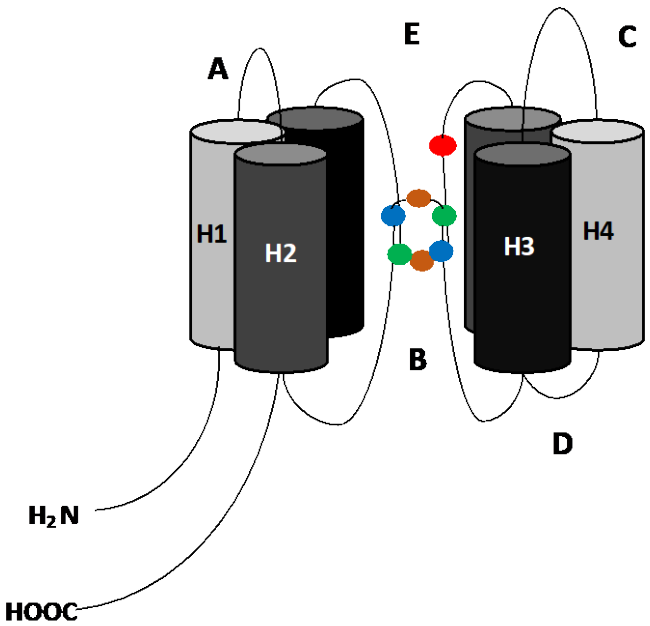
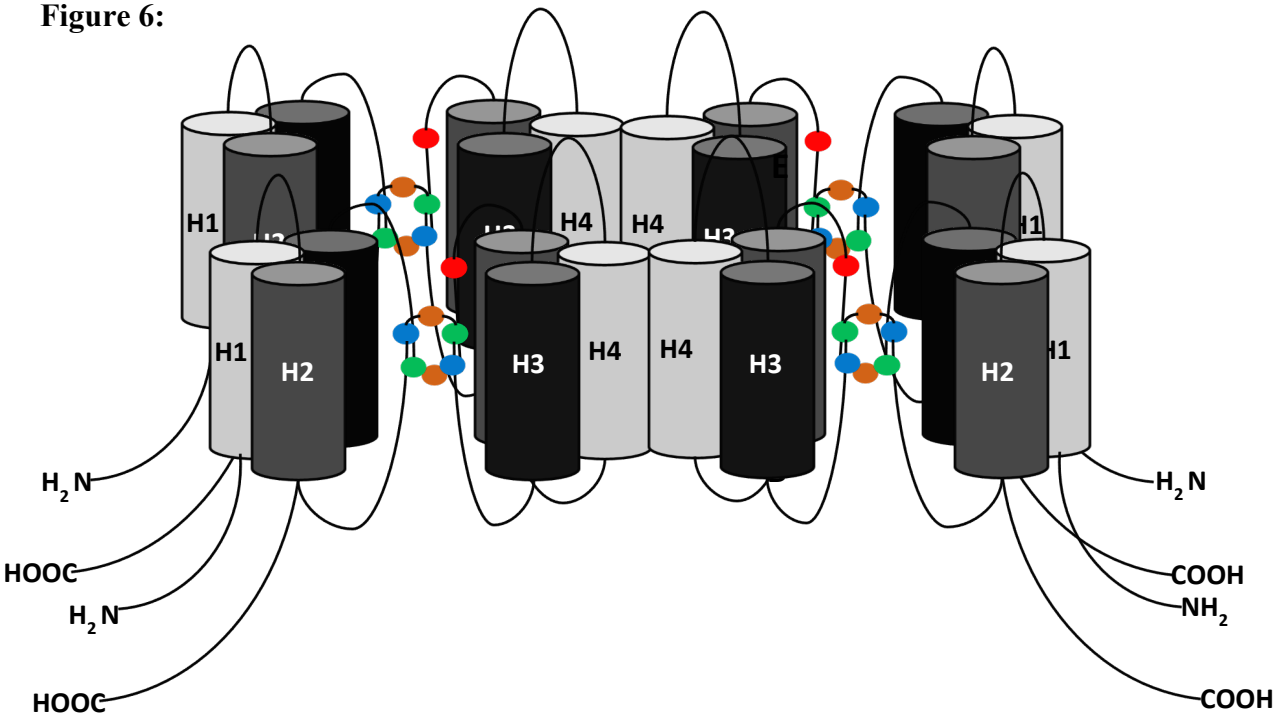

A
E
C
H1
H2
H3
H4
B
D
H_2N
HOOC

Figure 6:


H1
H2
H3
H4
H4
H3
H4
H4
H3
H1
H2
H1
H2
H1
H_2N
HOOC
H_2N
HOOC
H_2N
COOH
NH_2
COOH

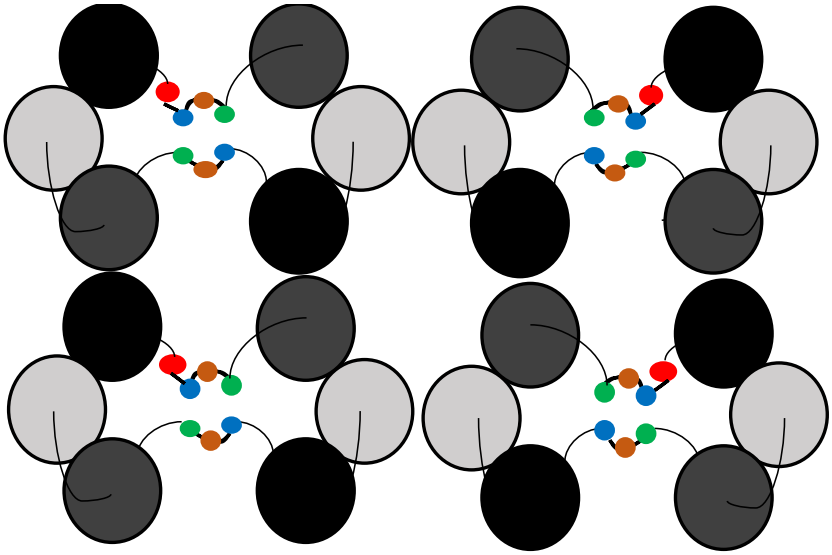
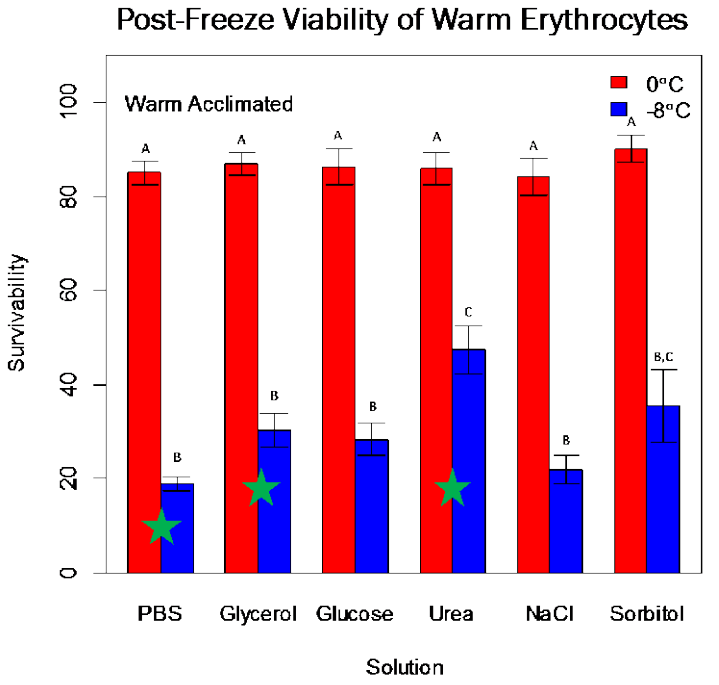


Figure 7A:


Post-Freeze Viability of Warm Erythrocytes
Warm Acclimated
0°C
-8°C
Survivability
0
20
40
60
80
100
A
B
A
B
A
B
A
C
A
B
A
B,C
PBS
Glycerol
Glucose
Urea
NaCl
Sorbitol
Solution

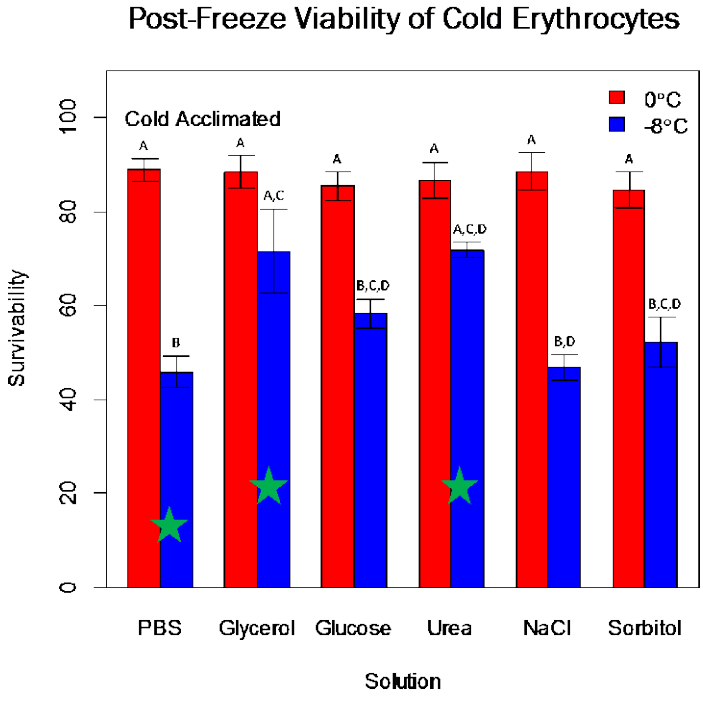
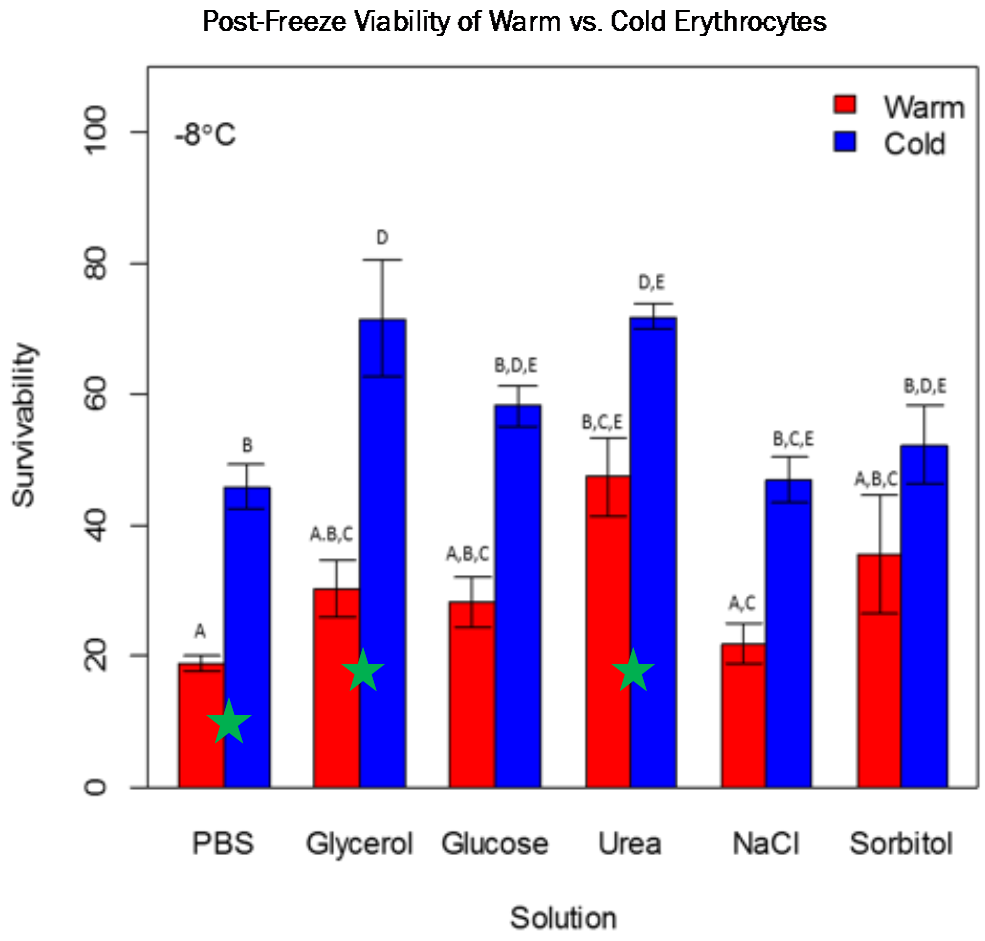

Post-Freeze Viability of Cold Erythrocytes
Cold Acclimated
0°C
-8°C
Survivability
0
20
40
60
80
100
A
B
A
A,C
A
B,C,D
A
A,C,D
A
B,D
A
B,C,D
PBS
Glycerol
Glucose
Urea
NaCl
Sorbitol
Solution

Figure 7B:


Post-Freeze Viability of Warm vs. Cold Erythrocytes
-8°C
Warm
Cold
Survivability
0
20
40
60
80
100
A
B
A,B,C
D
A,B,C
B,D,E
B,C,E
D,E
A,C
B,C,E
A,B,C
B,D,E
PBS
Glycerol
Glucose
Urea
NaCl
Sorbitol
Solution