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Abstract

By utilizing an extreme physiological adaptation known as freeze-tolerance, Cope's gray tree frog, *Dryophytes chrysoscelis*, freezes and then subsequently thaws up to 65% of its extracellular fluid to survive the winter. During these periods of freezing and thawing, erythrocytes (RBCs) of *D. chrysoscelis* utilize a protein, aquaglyceroporin HC-3, that facilitates transmembrane flux of both water and cryoprotective glycerol to mediate osmotic adjustments. RBCs from cold-acclimated tree frogs up-regulate HC-3 protein expression, which coincides with more abundant membrane localization and higher levels of glycosylation. However, the functional significance of HC-3 glycosylation on membrane localization and cellular freeze tolerance is currently not known. We hypothesize that anticipatory glycerol accumulation observed in cold-acclimated tree frogs contributes to enhanced post-translational modification of HC-3 via N-linked and O-linked glycosylation, and that HC-3 glycosylation is important in subcellular trafficking of HC-3 from the Golgi to the membrane. RBCs from warm-acclimated *D. chrysoscelis* were separated into three categories: freshly isolated RBCs (FI), RBCs cultured in complete cell culture media for 48 hours (CCCM), and RBCs cultured in CCCM containing 0.156M glycerol for 48 hours (CCCM+G). Densitometric analyses of

immunoblots specific for HC-3 showed a 3.5-fold and 1.9-fold average increase in glycosylated HC-3 (60-120 kDa) from RBCs cultured in CCCM+G as compared to FI RBCs and RBCs cultured in CCCM, respectively. Western blots of RBC proteins treated with PNGase F resulted in a 1.3-fold average decrease in glycosylated HC-3 compared to control proteins. However, protein treatment with the O-Glycosidase and Neuraminidase mix did not appear to change the abundance of glycosylated HC-3, indicating that HC-3 is post-translationally modified via N-linked glycosylation but not O-linked. Additional results were collected using scanning laser confocal microscopy and HC-3 localization was measured in mean fluorescent intensity (arbitrary units) using ImageJ software (N=4-6 cells per experiment). For RBCs cultured in CCCM+G, immunofluorescence intensity of HC-3 in the plasma membrane was 21.7 times greater than HC-3 immunofluorescence in the cytosol ($P<0.05$). In contrast, immunofluorescence intensity of HC-3 in the cytosol was 3.2 times greater than HC-3 immunofluorescence in the membrane for FI RBCs ($P<0.01$). There was no difference in HC-3 immunofluorescence intensity between the membrane and cytosol in RBCs cultured in CCCM ($P>0.05$). Using an *in vitro* cell culture system, we have successfully recapitulated cold-acclimated *in vivo* HC-3 expression patterns by focusing solely on the influence of a glycerol-induced hyperosmotic environment on RBCs of *D. chrysoscelis*. Thus, a potential correlation between cryoprotective glycerol, increased HC-3 N-linked glycosylation, and enhanced HC-3 membrane localization has been identified.

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UNIVERSITY of DAYTON

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Chapter I: Introduction and Literature Review

Physiological Homeostasis and Osmoregulation

The coordinated adjustments of body temperature, water content, pH and other physiological variables within an organism that maintain the relatively steady conditions in the body are collectively referred to as "physiological homeostasis" (Anderson, 1977; Cannon, 1929). Homeostasis suggests that the living being is stable, and it must be stable to endure the changing environmental forces that surround it. The environmental factors under homeostatic control that affect cells include osmolality, temperature, and pH, while the materials that cells need that are kept under homeostatic control include nutrients, water, sodium, calcium, other inorganic ions, oxygen, and hormones (Cannon, 1929).

In multicellular organisms, the term osmoregulation refers more specifically to the regulation of the osmolality of an organism's body fluids through the adjustment of water and solute concentrations in the extracellular fluid (ECF) and intracellular fluid (ICF) (Danziger and Zeidel, 2014). If there are alterations in the osmolality of the ECF that disrupt physiological homeostasis within an organism, this can impact the physical structure of cells and tissues and the function of biological molecules (Bourque, 2008). Similarly, in unicellular organisms, alterations between the intracellular fluid and the external environment can affect the proper execution of critical cellular functions. Therefore, osmoregulation is an important physiological process that protects all organisms (Johnson, 2009).

There exist two well-established mechanisms through which osmoregulation is facilitated. Osmoconformers—such as jellyfish, mussels, and some insects—exert minimal effort to resist osmotic changes and instead adopt ICF osmolality values that

reflect those of their surrounding conditions. In contrast, osmoregulators—such as humans and some species of freshwater fish—have developed biological processes that help facilitate the maintenance of the internal environment osmolality near a particular set value in the face of opposing osmotic forces (Bourque, 2008).

One of the main mechanisms present within all organisms that helps to facilitate osmoregulation is osmosis—the passive diffusion of water from a solution of lower concentration to a solution of higher concentration. The diffusion of water occurs through the semi-permeable biological membrane of cells (Lodish et al., 2000). Biological membranes are composed of a double layer of lipid molecules that normally consists of a polar head group and two hydrocarbon tails in which membrane proteins are embedded. This bilayer is fluid and its individual lipid molecules are amphipathic, containing both hydrophilic and hydrophobic ends. Hydrophilic species form more favorable hydrogen bonds with water because they contain charged groups or uncharged polar groups (Alberts et al., 2002). Thus, because of this amphipathic nature, water is able to passively diffuse through biological membranes via osmosis from a solution of a lower non-penetrating solute concentration to one of a higher non-penetrating solute concentration (Lodish et al., 2000).

Another mechanism through which cells regulate internal volume in response to osmotic stress is through facilitated diffusion, which uses membrane channel proteins that are embedded within the lipid bilayer (Preston et al., 1992). It is thought that through osmosis water moves at a slower rate of diffusion across cell membranes as compared to when water travels through membrane channel proteins that are selective for water (Lodish et al., 2000). However, a membrane channel protein specific for osmotically

driven transmembrane water flux—now known as an aquaporin—was not discovered until 1992 by Dr. Peter Agre and his team of scientists.

The Discovery of Aquaporins

In 1985, Gheorghe Benga and his coworkers discovered the first signs of a water channel protein in the human (RBC) membrane (Benga et al., 1986, Kuchel, 2006). The experiment that suggested the presence of a membrane protein specific for water movement used *p*-chloromercuribenzenesulfonate (*p*CMBS), a known inhibitor of water transport across the membranes of human red blood cells. Additionally, the experiment was designed so that *p*CMBS could react only with surface accessible sulfhydryl groups. Based on the results of the experiment, Benga and his team deduced that because *p*CMBS inhibits water transport, and it is bound to proteins in the RBC membrane, the existence of channel proteins specific for water was likely (Benga et al., 1986; Benga, 2012).

However, it was not until 1992 that the first water channel protein was definitively defined, characterized and expressed by Dr. Peter Agre and his team of scientists at Johns Hopkins University (Preston and Agre, 1991; Preston et al., 1993). In 1988, Dr. Agre and his co-workers, while working on the Rhesus blood group antigens, unexpectedly discovered a unique membrane protein they referred to as CHIP28 (channel integral membrane protein of molecular weight 28kDa). At the time, they hypothesized that this protein played a role in the connection of the skeleton of the membrane to the lipid bilayer (Denker et al. 1988). After searching through the literature for possible clues into the function of this "orphan" protein, Dr. Agre and his team decided to clone the complementary DNA of CHIP28 and compare its amino acid sequence to other proteins within the DNA database (Preston and Agre, 1991). Following much debate and

discussion with his team about the possible function of CHIP28, Dr. Agre and his team finally hypothesized that this protein was the long sought after water channel protein.

To test this hypothesis, the team injected the complementary RNA of CHIP28 into *Xenopus laevis* oocytes, which are known to be fairly water impermeable, and compared the injected oocytes to control oocytes. An incredibly exciting difference was observed: when dropped in distilled water, the control oocyte remained the same size, but the oocyte injected with CHIP28 complementary RNA had swollen and lysed (Preston et al., 1992). Therefore, it was concluded that CHIP28 was indeed a water channel protein and was named Aquaporin 1 (AQP1) (Preston et al., 1993). It would later be established as a member of the major intrinsic protein (MIP) family of integral membrane proteins. Dr. Peter Agre was a recipient of the Nobel Prize in Chemistry in 2003 for his landmark discovery of the aquaporins (Agre, 2003).

The Molecular Structure of Aquaporins

Aquaporins are considered relatively simple proteins in terms of their structure and function when compared to other ion channels and solute transporters. Most of the structural features of AQPs have been identified through the use of high-resolution X-ray crystallography (Verkman, 2013; Verkman and Mitra, 2000). Each of the four ~30kDa AQP monomers that make up a tetramer consists of six membrane-spanning helical domains, referred to as H1-H6, and two short helical segment loops (Figures 1 and 2). HB surrounds cytoplasmic vestibules while HE surrounds extracellular vestibules and these vestibules are joined through a central and tight aqueous pore (reviewed in Verkman, 2011). There exists a special motif consisting of Asn-Pro-Ala (NPA) in both HB and HE that overlaps in the center of the pore (reviewed in Agre, 2006).

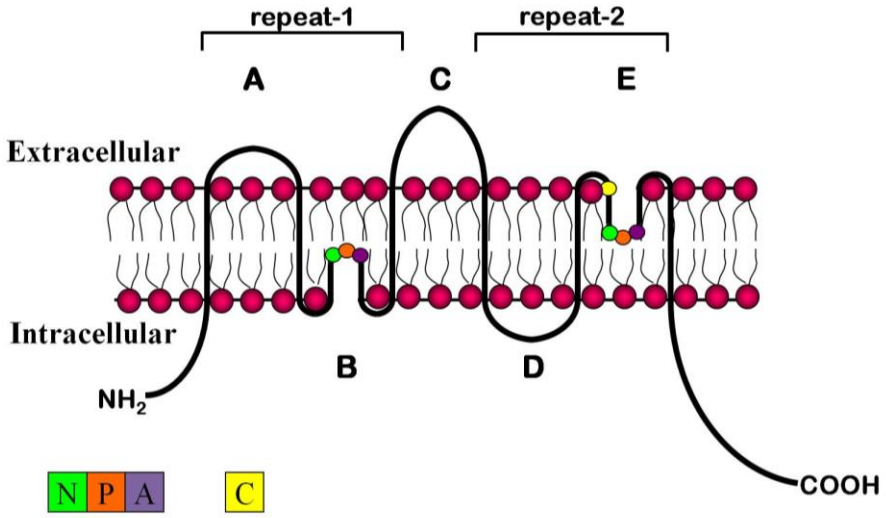

Figure 1. Aquaporin 1 Two-Dimensional Structure. Pictured is the two-dimensional structure of an AQP1 monomer. The two NPA motifs in the first intracellular loop (left) and third extracellular loop (right) fold into the membrane to form the functional water pore (Krane et al., 2007).

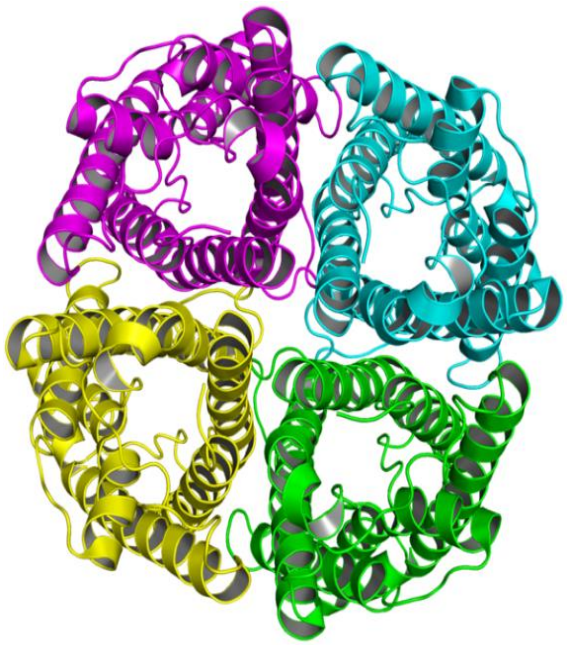

Figure 2. Three-Dimensional Structure of the Aquaporin 1 Tetramer. Pictured is the three-dimensional structure of the AQP1 tetramer. Four visible water pores can be viewed in this complex. This image was generated using SWISS-MODEL.

Evolution of Aquaporins

Because water is vital for life, a vast assortment of membrane integral protein channels (MIPs), or AQPs, of 26-35 kDa have evolved within living organisms to facilitate the movement of water into and out of cells (Abascal et al., 2014). As mentioned previously, Dr. Peter Agre and his team discovered the first MIP: AQP1. However, since this discovery, over 450 members of the ancient MIP family have been characterized and various biological and physiological roles are continuously being identified for them. It seems that since MIPs play an important role in osmoregulation, they are ubiquitously expressed throughout the living kingdoms and indirectly impact a number of critical cellular processes.

MIPs are comprised of six transmembrane helical segments per monomer. Interestingly, these two helical segments present two remarkably similar halves, suggesting that these water channels arose by the internal duplication of a three-membrane segment (Pao et al., 1991). Furthermore, studies of the MIP family indicate that there are two major phylogenetic subfamilies of MIPs: AQPs, which facilitate the passage of water, and aquaglyceroporins (GLPs), which facilitate the passage of water and small solutes such as glycerol and urea. After researchers identified the first prokaryotic aquaporins, it became evident that aquaporins within eukaryotes had also evolved from archaeal and bacterial domains (Finn and Cerdà, 2015).

Aquaporins are in many microbial organisms but show the most numerous and diverse amounts in plants and vertebrates. In contrast, GLPs are absent in plants, but exist as a single copy in microbes and up to four paralogs in vertebrates. In plants, AQPs are divided into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast

intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and uncategorized X intrinsic proteins (XIPs) (Shivaraj et al., 2017). Since plants do not contain GLPs, NIPs are considered a water and glycerol channel co-option to the GLPs found in microbes and vertebrates (Chaumont and Tyerman, 2014). In fact, a phylogenetic analysis indicated that NIPs evolved through a separate path different from GLPs in mammals, indicating a horizontal gene transfer from microbes to plants resulting in the emergence of the NIP subfamily (Zardoya et al., 2002; Zardoya, 2005).

Aquaglyceroporins, Their Molecular Structure, and Their Function

The transmembrane flux of glycerol across cell membrane has been well established and includes simple diffusion as the primary route (Laforenza et al., 2016; Lages and Lucas 1995; Lucas et al. 1990). However, another well-known route through which glycerol movement occurs across the membrane is via a subfamily of proteins within the aquaporin family: the aquaglyceroporins (Hara-Chikuma and Verkman, 2006). Aquaglyceroporins, or GLPs, are members of the MIP family and are known to facilitate the transmembrane flux of both water and organic compounds such as glycerol, urea, and other small molecules such as NH_3 and NH_4^+ (reviewed in Krane and Goldstein, 2007; Engel and Stahlberg, 2002).

The structural differences between AQPs and GLPs can be best described by examining *GlpF*, one of the most widely known GLPs isolated from *E. coli* (Hénin et al., 2008). Like AQPs, *GlpF*, when crystallized, appears organized into tetramers with only subtle structural differences within the selectivity filter (SF) for water (Jensen et al., 2001; Thomas et al., 2002). Five amino acid positions between AQPs and GLPs located in transmembrane helix 3, extracellular loop E, and transmembrane helix 6 are different

(reviewed in Krane and Goldstein, 2007). As mentioned earlier, for AQP1, the SF is located in the center of the pore and is gated by a narrow constriction region of 2.8Å in diameter defined by hydrophilic residues. However, for *GlpF*, the constriction region is 3.5Å wide and defined by more hydrophobic residues (Nollert et al., 2001; Thomas et al., 2002). Therefore, the main barriers opposing glycerol permeation through AQPs but not GLPs are steric in nature, mainly due to the more constricted pore size in AQPs at the SF. Furthermore, a key arginine residue that backs the SF of AQPs, but not GLPs, has been found in a conformational state that acts as a gate and blocks substrate flow through the channel. Combined, these structural differences contribute to AQPs specificity for water and GLPs specificity for water, glycerol, urea, and other small solutes (Wang et al, 2005).

AQPs & GLPs in Mammals

There are thirteen known water channels in mammals (AQP0-AQP12). An evolutionary comparison of these mammalian MIP sequences reveal that AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8 are members of the water specific aquaporin subfamily, while AQP3, AQP7, AQP9, and AQP10 are classified as the water and glycerol specific subfamily—aquaglyceroporins (Gomes et al., 2009; reviewed in Krane and Goldstein, 2007). The expression and localization amongst mammalian AQPs differs vastly. Some AQPs, such as AQP1 and AQP4, are expressed in a variety of tissues and exhibit diverse physiological roles. For example, AQP1 is expressed in the proximal tubule, choroid plexus and corneal epithelium, and AQP4 is expressed in the retina and glial cells at the blood brain barrier. Other AQPs, such as AQP12, are expressed in predominantly the same locations. For example, AQP12 is expressed predominantly in pancreatic acinar cells (Kitchen et al., 2015).

Aquaporins are involved in a diverse set of physiological roles within mammals (Hoffert et al., 2000, Jeyaseelan et al., 2006). For example, AQP2 acts as a water channel for water reabsorption as part of the vasopressin dependent urinary concentration mechanism, demonstrating that AQPs play a major role in the transport of fluid across epithelial cells (Day et al., 2014, Nielson et al., 2000, Tamarappoo and Verkman, 1998). Aquaporin 4 is involved in the facilitation of brain water accumulation in patients with cytotoxic edema, and is therefore targeted for expression knock-down in patients with this condition (reviewed in Verkman, 2011). In contrast, AQP4 is involved in the clearance of excess brain water in patients with vasogenic edema, and is therefore a target for upregulation in patients with this condition (Verkman, 2011). Additionally, it has also been shown that impaired vision, hearing, and olfaction occurred in mice lacking AQP4, indicating that AQP4 is involved in networks of neural signaling (Lu et al., 2008). Aquaporins are also suspected to be involved in cell migration following the observation that an AQP1 deletion in mice reduced the growth and vascularity of implanted tumors (Saadoun et al., 2005).

Post-translational Modifications and Intracellular Trafficking of AQPs/GLPs

Most AQP/GLP gene expression is controlled by temporal and tissue-selective expression via mechanisms within transcription and translation, and most AQP/GLP proteins function, structure, and stability are affected by post-translational modifications (reviewed in Krane and Goldstein, 2007). Post-translational modifications (PTMs) occur following the completion of translation and aid in cellular mechanisms such as the mediation of proper protein folding and the direction of the newly translated protein to a

distinct cellular location such as the nucleus or the plasma membrane (Prabakaran et al., 2012). Aquaporin 2 is most widely studied in terms of PTM influences on AQP/GLP function and localization (Vagin et al., 2009).

Aquaporin 2 has been shown to be modified via two of the most common PTMs: phosphorylation and glycosylation. Phosphorylation is the reversible bonding of a phosphate group to a protein that is catalyzed by a kinase and, in AQP2, it has been shown that phosphorylation at serine 256 may be required for regulatory exocytosis of AQP2 (Fushimi et al., 1997; Prak et al., 2008). In addition to phosphorylation, AQP2 has been shown to be modified via glycosylation. Glycosylation is defined by various types of glyosidic linkages to protein structures. The two main types of glycosylation are N-linked and O-linked glycosylation (Moeller et al., 2011; Öberg et al., 2011). The bonding of a glycan to the amino group of an asparagine on a protein is referred to as N-linked glycosylation, while O-linked glycosylation is the bonding of monosaccharides to the hydroxyl group of serine or threonine residue (Figure 3; Wang et al., 2013).

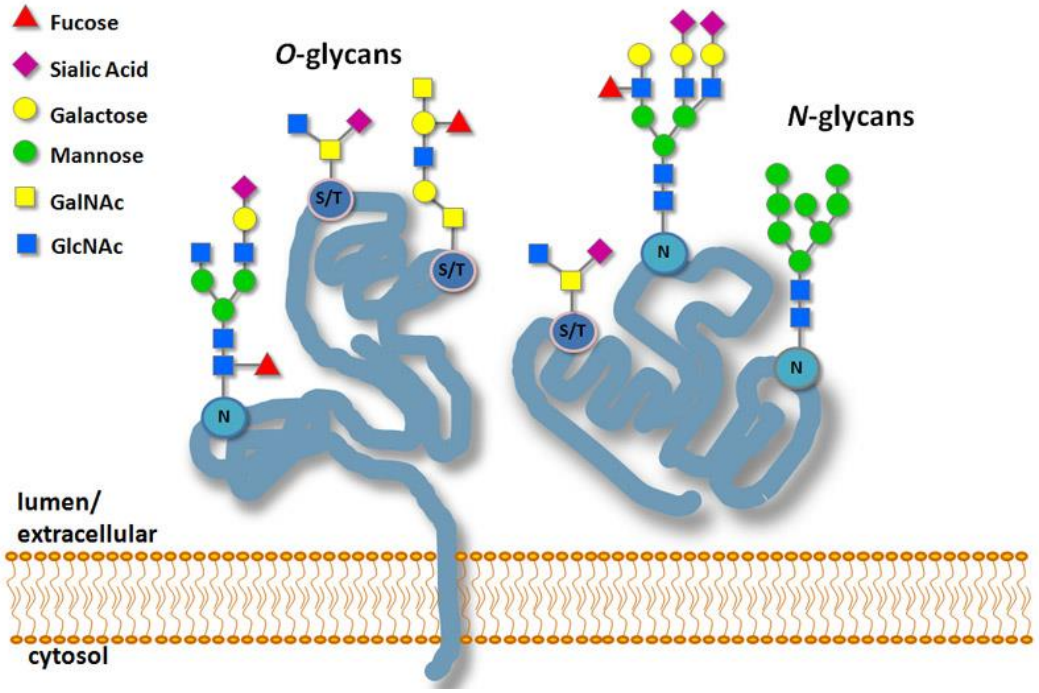

Figure 3. Membrane Proteins with N- and O-Linked Glycans Attached to their Extracellular Surfaces. O-linked glycans are attached at a serine or threonine residue. N-linked glycans are attached at an asparagine residue (Öberg et al., 2011).

The role of N-linked glycosylation has been well-documented for the AQP2 protein. In fact, it has been demonstrated that when the covalent bonding of N-glycans to AQP2 is inhibited, the AQP2 protein travels to the Golgi complex but fails to insert into the plasma membrane. These results indicate that the covalent attachment of an N-glycan to AQP2 protein influences AQP2 localization and as a result, AQP2 function (Hendricks et al., 2003; Potter, 2003; Potter et al., 2005). Additionally, the removal of N-glycans from many apical proteins, similar to the inhibition of N-glycosylation in AQP2, has been shown to decrease their abundance in the apical membrane (Vagin et al., 2004; Vagin et al., 2009). This again suggests that N-linked glycosylation is required for apical

distribution of many other membrane proteins in absorptive and secretory polarized epithelia (Helenius and Aebi, 2004; Molinari, 2007).

One explanation for these findings is that N-glycosylation is required for apical sorting of these glycoproteins. However, the mechanisms for apical sorting remain poorly undefined and understood. Alternatively, another explanation for the significance of N-glycosylation on membrane localization is since N-glycosylation is necessary for proper folding of proteins for them to exit the ER, the lack of N-glycans could result in ER retention. Among the many studies that support the relationship between N-glycan attachment and membrane localization, it is important to note that apical localization of a number of membrane glycoproteins is not affected by changes in N-glycosylation (Vagin et al., 2009).

AQPs & GLPs in Anurans

Both semi-aquatic and terrestrial anuran amphibians experience evaporative water loss across their thin skin while traveling away from the water and have therefore developed osmoregulatory systems for maintaining fluid homeostasis (Suzuki et al., 2007). One such system that the majority of mature anuran amphibians have evolved is the employment AQPs/GLPs to absorb water across the ventral pelvic skin and reabsorb it from urine in the urinary bladder, termed the water balance response or antidiuretic response (Ogushi et al. 2010; Suzuki et al., 2015; Uchiyama et al., 2006). Except for AQP6 and AQP12, anuran orthologs for all of the other mammalian AQPs have been identified. Additionally, there have evolved two anuran-specific AQPs—AQPa1 and AQPa2 (Suzuki and Tanaka, 2009). Orthologs of AQPa1 include the *X. laevis* AQP and

the AQPxlo from *X laevis oocytes*, while AQPa2 orthologs include AQP-h2, AQP-h3, AQP-t2, and AQP-t3 (Hasegawa et al., 2003, Tanii et al., 2002, Virkki et al., 2002).

Aquaporin HC-1 and AQP HC-2 from *Dryophytes chrysoscelis*, a freeze tolerant anuran, are orthologs of mammalian AQP1 and AQP2, while Aquaglyceroporin HC-3 is a GLP and is an ortholog with AQP3 (Zimmerman et al., 2007). Expression and regulation of HC-1 was shown to be temperature-sensitive (cold vs. warm) in the brain, liver, and kidney tissue. Aquaporin HC-2 was isolated from the urinary bladder and HC-2 mRNA was detected primarily in organs of osmoregulation such as the skin and kidney (Zimmerman et al., 2007). Furthermore, frogs that were acclimated to cold conditions had high levels of HC-2 expression in the skin, whereas no HC-2 expression was observed from the ventral skin of warm-acclimated frogs.

Freeze Tolerance

The majority of vertebrate animals seek refuge from the cold by migrating or hibernating during the winter, thereby avoiding the freezing temperatures all together. Other organisms utilize a strategy known as supercooling, which is the ability to remain unfrozen even when temperatures fall below the freezing point in their bodily fluids. However, a more extreme adaptation exists for organisms to survive the winter: freeze tolerance, which refers to the ability to endure the freezing of up to 50-65% of total body water into extracellular ice, the interruption of vital processes such as heart beat and breathing, cell shrinkage, elevated osmolality, anoxia/ischemia, and potential physical damage from ice (Storey and Storey, 2017). Despite the fact that hibernation, migration, and supercooling are far less physiologically demanding and stressful events for living organisms, freeze tolerance has evolved within a variety of diverse groups of life such as

insects, microbes, plants, reptiles, amphibians, and small soil invertebrates, all of which tolerate freezing to survive seasonal sub-freezing temperatures (Storey and Storey, 2017; Voituron et al., 2009; Ali and Wharton, 2014).

There are a few principal requirements for success in natural freeze tolerance: 1.) ice formation must be formed exclusively in the extracellular compartments and the damage from ice crystals must be kept at a minimum, 2.) the progression of freezing must occur gradually, 3.) cell volume decrease and loss of water must be held to a certain threshold, and 4.) cells must survive and maintain homeostasis (Storey and Storey, 1988). Several molecular adaptations exist that support freeze tolerance, including a lower metabolic rate (1-30% of normal resting rate), an optimization of anaerobic ATP production, ice binding proteins and freeze-specific gene/protein/enzyme controls, and the use of carbohydrate cryoprotectants to help maintain fluid homeostasis (Storey and Storey, 2017).

Freeze Tolerance in Anurans

There currently exist seven known species of anurans that are freeze tolerant: *Hyla versicolor, Dryophytes chrysosclies, Rana sylvatica, Hyla crucifer, Pseudcris triseriata, Pseudacris maculate,* and *Rana arvalis* (Shearman and Maglia, 2014; Storey and Storey, 2017). These amphibians choose humid, well-protected sites for the freezing process because if their water permeable skin comes in direct contact with the air, deadly dehydration can occur (Storey and Storey, 1988). The use of cryoprotectants in freeze tolerance is ubiquitous among these seven species of amphibians—*H. versicolor* and *D. chrysoscelis* accumulate and distribute glycerol as a cryoprotectant, whereas the other five species use glucose as a cryoprotectant. The general consensus is that the northern

populations of *H. versicolor* and *D. chrysoscelis* undergo a seasonal accumulation of glycerol to begin preparing for freezing, but they have also been observed to rapidly synthesize glycerol and or glucose on demand as needed (Pittman et al., 2008; Storey and Storey, 2017). However, species such as *R. sylvatica* and *H. crucifer* have been predominantly observed to rapidly synthesize glucose during the onset of freezing.

As mentioned previously, *Dryophytes chrysoscelis,* an amphibian found throughout the eastern United States, uses mainly glycerol as a cryoprotectant. Glycerol helps to avoid damage from extracellular ice crystals my helping to maintain fluid homeostasis. Glycerol and water permeates the cellular membranes of *D. chyrososcelis* through the GLP HC-3, and this ultimately contributes to the success of natural freeze tolerance (Figure 4; Goldstein et al., 2010, Pandey et al., 2010).

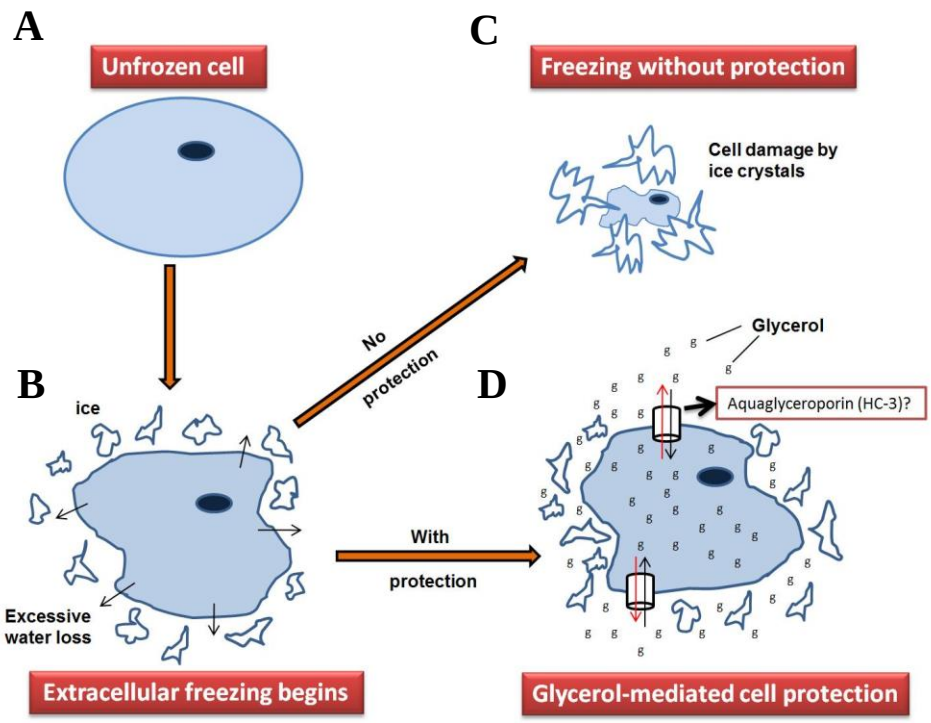

Figure 4. Schematic of Glycerol Mediated Freeze Tolerance in *D. chrysoscelis*. An unfrozen cell from *D. chrysoscelis* is shown (A). As extracellular ice crystals form, excessive water loss occurs (B). Without the distribution of glycerol both intracellularly and extracellulary, cells of *D. chrysoscelis* shrink because of osmotic pressures forcing water to rush out of the cell (C). Additionally, ice crystals grow large and physically damage the cells (C). However, with the protection of cryoprotective glycerol, there is minimal cell volume loss and ice crystal formation (D).

Aquaglyceroporin HC-3 mRNA is expressed in several tissues, some of which show an upregulation of HC-3 expression in various tissues of cold-acclimated frogs as compared to warm-acclimated frogs (Zimmerman et. al, 2007; Goldstein et al., 2010). Furthermore, HC-3 protein has also been shown to be highly expressed in erythrocytes

from *D. chrysoscelis*, where it is more profuse in cold-acclimated erythrocytes as compared with warm-acclimated erythrocytes (Mutyam et al., 2011).

To better understand the mechanisms that influence HC-3 expression and its possible functional roles in glycerol transport in *D. chrysoscelis*, an *in vitro* erythrocyte cell culture system in which HC-3 protein expression could be dynamically regulated was developed and utilized (Mutyam et al., 2011). The initial experiments completed using this *in vitro* cell culture system demonstrated that HC-3 is differentially expressed in the membrane vs. the cytosol in cold-acclimated frogs as compared to warm-acclimated frogs. Additionally, it was shown that there was a greater amount of glycosylated HC-3 in the cold-acclimated tree frogs as compared to the warm acclimated tree frogs. Combined, the above findings concerning HC-3 regulation and expression led to the hypothesis that HC-3 could be involved in some of the mechanisms that contribute to natural freeze tolerance in this organism through facilitating the transmembrane flux of water, glycerol, urea, and other small solutes during periods of freezing and thawing (Mutyam et al., 2011).

Hypothesis

Freeze tolerance, although a remarkable evolutionary adaptation, leads to the rapid formation of extracellular ice crystals and induces immense osmotic disturbance between the ECF and ICF for cells. It is hypothesized that AQPs/GLPs are differentially regulated and expressed within freeze tolerant organisms to facilitate the transmembrane flux of both water and small solutes (cryoprotectants), contributing to the maintenance of fluid homeostasis during periods of freezing/thawing (Goldstein et al., 2010; Mutyam, 2013). More specifically, it is likely that in erythrocytes (RBCs) of *D. chrysoscelis*, GLP HC-3

mediates the transmembrane flux of both water and cryoprotective glycerol (Mutyam et al., 2011). The previous findings of Mutyam et al. 2011 showing enhanced HC-3 expression in the membrane vs. the cytosol in RBCs from cold-acclimated tree frogs, combined with increased amounts of glycosylated HC-3 in cold-acclimated frogs compared to warm-acclimated frogs, have led to the hypothesis that the accumulation of glycerol and the post-translational glycosylation of HC-3 may both be important in the trafficking and insertion of HC-3 into the membrane of RBCs from *D. chrysoscelis.* Thus, the focus of this thesis is to further explore these potential regulating factors—cryoprotective glycerol and HC-3 post-translational glycosylation—on HC-3 expression in RBCs of *D. chrysoscelis* using a previously established *in vitro* cell culture system.

The famous comparative physiologist, August Krogh, once stated, "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929). Thus, the comparative analysis of AQPs/GLPs in the natural freeze tolerant anuran, *Dryophytes chrysoscelis*, provides an excellent model for gaining insights into how multicellular organisms survive freezing and may yield important information for the cryopreservation of larger tissues and organs in humans in the future.

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Chapter II: Characterization of the Glycosylation of Aquaglyceroporin HC-3 in Erythrocytes from the Freeze-Tolerant Anuran, *Dryophytes chrysoscelis*

Abstract

By utilizing an extreme physiological adaptation known as freeze-tolerance, Cope's gray tree frog, *Dryophytes chrysoscelis*, freezes and then subsequently thaws up to 65% of its extracellular fluid to survive the winter. During these periods of freezing and thawing, erythrocytes (RBCs) of *D. chrysoscelis* utilize a protein, aquaglyceroporin HC-3, that facilitates transmembrane flux of both water and cryoprotective glycerol to mediate osmotic adjustments. RBCs from cold-acclimated tree frogs up-regulate HC-3 protein expression, which coincides with more abundant membrane localization and higher levels of glycosylation. However, the functional significance of HC-3 glycosylation on membrane localization and cellular freeze tolerance is currently not known. We hypothesize that anticipatory glycerol accumulation observed in cold-acclimated tree frogs contributes to enhanced post-translational modification of HC-3 via N-linked and O-linked glycosylation, and that HC-3 glycosylation is important in subcellular trafficking of HC-3 from the Golgi to the membrane. RBCs from warm-acclimated *D. chrysoscelis* were separated into three categories: freshly isolated RBCs (FI), RBCs cultured in complete cell culture media for 48 hours (CCCM), and RBCs cultured in CCCM containing 0.156M glycerol for 48 hours (CCCM+G). Densitometric analyses of immunoblots specific for HC-3 showed a 3.5-fold and 1.9-fold average increase in glycosylated HC-3 (60-120 kDa) from RBCs cultured in CCCM+G as compared to FI RBCs and RBCs cultured in CCCM, respectively. Western blots of RBC proteins treated

with PNGase F resulted in a 1.3-fold average decrease in glycosylated HC-3 compared to control proteins. However, protein treatment with the O-Glycosidase and Neuraminidase mix did not appear to change the abundance of glycosylated HC-3, indicating that HC-3 is post-translationally modified via N-linked glycosylation but not O-linked. Additional results were collected using scanning laser confocal microscopy and HC-3 localization was measured in mean fluorescent intensity (arbitrary units) using ImageJ software (N=4-6 cells per experiment). For RBCs cultured in CCCM+G, immunofluorescence intensity of HC-3 in the plasma membrane was 21.7 times greater than HC-3 immunofluorescence in the cytosol ($P<0.05$). In contrast, immunofluorescence intensity of HC-3 in the cytosol was 3.2 times greater than HC-3 immunofluorescence in the membrane for FI RBCs ($P<0.01$). There was no difference in HC-3 immunofluorescence intensity between the membrane and cytosol in RBCs cultured in CCCM ($P>0.05$). Using an *in vitro* cell culture system, we have successfully recapitulated cold-acclimated *in vivo* HC-3 expression patterns by focusing solely on the influence of a glycerol-induced hyperosmotic environment on RBCs of *D. chrysoscelis*. Thus, a potential correlation between cryoprotective glycerol, increased HC-3 N-linked glycosylation, and enhanced HC-3 membrane localization has been identified.

Introduction

In contrast to other physiological adaptations that avoid the numerous stresses generated by exposure to sub-freezing temperatures—such as super- cooling and freeze avoidance—organisms adopting freeze tolerance are able to regulate the formation of ice crystals within the body (Storey et. al, 1988). *Dryophytes chrysoscelis*, commonly known as Cope's gray tree frog, is a freeze tolerant organism that undergoes a period of thermal

acclimation before the winter in preparation for the process of whole body freezing and thawing. In part, the physiological mechanisms that contribute to this remarkable freeze-tolerant adaptation is the accumulation of high concentration of extracellular glycerol, which is mainly mobilized from hepatocytes in the liver (Zimmerman et al., 2007). During cold-acclimation, glycerol that is accumulated traverses cellular plasma membranes and is thus present in both the intracellular and extracellular fluid. Here, it serves as a cryoprotectant to moderate osmotic shifts in water that develop during the rapid formation of extracellular ice crystals. Additionally, glycerol also functions to slow the formation of extracellular ice crystals through interrupting hydrogen bonding between adjacent water molecules and is also thought to stabilize the structures of a variety of biomolecules during the freezing and thawing process (Storey and Storey, 2017).

One pathway that glycerol can cross plasma membranes in bulk supply is through the facilitation via aquaporin/aquaglyceroporin (AQP/GLP) members of the Major Intrinsic Protein Family (MIP) (Thomas et al., 2002). Aquaporins/Aquaglyceroporins are transmembrane channels that facilitate osmotically driven water and/or water and small solutes such as glycerol and urea into and out of cells. Many amphibian orthologs to mammalian AQPs/GLPs have been discovered and, in addition, two anuran-specific aquaporins seem to have evolved to mediate physiological adjustments to variations in the surrounding environment, which often changes from aquatic to terrestrial for amphibians (Tanii et al., 2002; Virkki et al., 2007, Zimmerman et al., 2007; Suzuki and Tanaka, 2009; Ogushi et al., 2010).

Four AQP/GLPs (HC-1, HC-2, HC-3, and HC-9) have been discovered, isolated, and characterized from *D. chrysoscelis*. HC-1 and HC-2 are known to be aquaporins,

while HC-3 and HC-9, orthologs of mammalian AQP3 and AQP9, are known to function as aquaglyceroporins (Stogsdill et al, 2017; Zimmerman et al., 2007). It has previously been demonstrated that HC-3 mRNA and protein is expressed and dynamically regulated depending on thermal acclimation conditions in a variety of tissues within *D. chrysoscelis* (Mutyam et al., 2011; Zimmerman et al., 2007; Goldstein et al., 2010). Furthermore, HC-3 protein is more highly expressed in erythrocytes from cold-acclimated tree frogs as compared to warm-acclimated tree frogs, suggesting that HC-3 may play an integral role in transmembrane water and solute flow during freeze tolerant periods (Mutyam et al, 2011; Goldstein et al., 2010). The previous establishment of an *in vitro* erythrocyte cell culture system presents an opportunity to explore how RBCs from *D. chrysoscelis* function with varying expression patterns of HC-3 and to understand the physiological mechanisms regulating that expression (Mutyam et al., 2011).

Findings from initial experiments performed using this erythrocyte cell culture system indicated that RBCs cultured with the addition of glycerol—the main cryoprotectant accumulated and distributed throughout freezing and thawing—express an increased abundance of N-linked glycosylated HC-3 protein. Additionally, these experiments indicated qualitatively that HC-3 membrane localization was enhanced in RBCs cultured with the addition of glycerol. In the current study, we sought to accomplish the following: further characterize HC-3 glycosylation, quantitatively assess the influence that a glycerol-induced hyperosmotic environment has on HC-3 membrane localization, and begin to examine the effects of glycerol on the total glycoprotein profile of erythrocytes from *Dryophytes chrysoscelis*. The results from this investigation contribute to the understanding of the overall regulatory elements involved in

aquaglyceroporin HC-3 expression and localization, and ultimately aid in human understanding of the mechanisms involved in freeze tolerance.

Materials and Methods

Animals

Male gray tree frogs of the freeze-tolerant anuran species, *Dryophytes chrysoscelis,* were identified and collected from ponds in southwestern Ohio. Species identification was based on trill frequency. The animals were then transferred to constant-temperature rooms at Wright State University in Dayton, Ohio. During the summer months, animals were kept in lighting conditions that correlated with the natural light cycle—16 hours light, 8 hours dark—and were moved to climate controlled rooms with the onset of winter. Warm-acclimated frogs were used in these experiments and were singly housed, maintained at 21°C with a 12:12-hour light cycle, and fed crickets three times per week. They received water *ad libitum*. The methods of collection, housing procedures, and experimental protocols for the care and use of *D. chrysoscelis* were approved by the Institutional Animal Care and Use Committee (IACUC) at Wright State University.

Erythrocyte Cell Culture System

Erythrocytes were selected as an appropriate *in-vitro* cell culture model based on their nucleation, metabolic activity, and because they were able to be repetitively harvested from the same organism in a non-lethal manner. Approximately 100 µl of blood was collected from warm-acclimated *D. chrysoscelis* through an axillary puncture and collected in heparinized capillary tubes. Blood was added to a 15 ml conical tube containing 10 ml of complete cell culture media (CCCM, 250 mOsM: RPMI 1640 medium supplemented with L-glutamine, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B, and 5% fetal bovine serum) and placed in a styrofoam cooler to control

for temperature fluctuations. This styrofoam cooler was then transferred from Wright State University to the University of Dayton for further experimentation. Conical tubes were centrifuged at 1000 *x g* for 15 minutes and the supernatant was removed removed. Pelleted erythrocytes were re-suspended in 10 ml of CCCM and a total cell count was performed using a hemocytometer. The cells were then re-suspended at one million cells per milliliter in CCCM or CCCM containing 0.156 M glycerol and these volumes were added to a 25 cm^2 Corning flask. This glycerol concentration was chosen for two reasons: 1.) the plasma glycerol concentration in cold-acclimated gray tree frogs reaches greater than 100 mM, which further increases to greater than 400mM upon freezing (Storey and Storey, 1985; Layne and Jones, 2001); and 2.) the addition of 0.156 M glycerol raises the osmolarity of the cell culture media to 400 mOsM, roughly the plasma osmolarity in cold-acclimated tree frogs. Suspension cultures were kept at a 45° angle on a plate shaker at 190 rpm at 22-25°C and the media was replenished every 24 hours throughout the 48 hours of the experiment. Viability was quantified by trypan blue exclusion dye staining at 0, 24, and 48 hours.

Deglycosylation

Total cellular proteins were isolated from cultured erythrocytes in a RIPA buffer at 0 hours and 48 hours. Twenty micrograms of protein were used and the protocols for Peptide-N-glycosidase F, O-glycosidase and Neuraminidase, or Protein Deglycosylation Mix II were followed per the manufacturer's instruction (New England Biolabs). These enzymes and enzyme mixes were chosen because they are specific for catalyzing the release of N-glycans, O-glycans, and N- and O-glycans, respectively. Control and enzyme-treated proteins were size fractionated using SDS-PAGE and immunoblotted as described below.

Western Blotting

HC-3 protein expression was analyzed by Western blotting using previously established methods (Mutyam et al., 2011). Roughly $1x10^6$ erythrocytes were collected from cultures at 0 hours and 48 hours and centrifuged at 1000 *x g* for 10 minutes. Pelleted cells were re-suspended in RIPA buffer and lysed by three consecutive freeze-thaw cycles (1 min on dry ice and 1 min at 37°C). Total protein concentration in each of the three samples was quantified using the Pierce BCA Protein Assay Reagent Kit according to the manufacturer's instruction (ThermoScientific). Twenty-five µg of protein was size fractionated by SDS-PAGE on a 12% denaturing polyacrylamide gel and electro-transferred to polyvinylidene difluoride membranes (SequiBlot; Bio-Rad). Western hybridization was carried out overnight at 4°C using a peptide-derived, monospecific rabbit polyclonal antibody raised against HC-3 (0. 44mg/mL; Goldstein et al., 2010), or mouse-anti-β-actin antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotech) or goat anti-mouse secondary antibody (Santa Cruz Biotech), respectively. Immunoreactive signals were detected using chemiluminescence substrate (West Pico SuperSignal) and visualized on X-ray film (Kodak Film) with multiple exposures in a dark-room.

Immunocytochemistry

For freshly isolated erythrocytes, erythrocytes cultured in CCCM for 48 hours, and erythrocytes cultured in CCCM+G for 48 hours, 20 µl of the cell suspension was applied to a gelatin-coated slide and dried at room temperature. Immunocytochemistry was performed as described (Mutyam et al., 2011). Slides were washed and labeled with goat anti-rabbit fluorescein- conjugated secondary antibody specific for HC-3 (Vector Laboratories) diluted 1:1,000 in 1% blocking serum. Cells were treated with RNase

(4mg/mL; Promega) for 5 minutes and stained with propidium iodide as per the manufacturer's instruction (Sigma-Aldrich). Immunofluorescence was analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

Total Glycosylation Stain

The impact of glycerol on the total glycoprotein profile of erythrocytes was assessed via the Molecular Probes® Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit. This kit provides a powerful method for staining glycoproteins on gels through reacting with periodate-oxidized carbohydrate groups. This stain was used on an SDS-PAGE gel following RBC protein size fractionation, and control proteins used were the CandyCane™ molecular weight standards, which contain a mixture of glycosylated and non-glycosylated proteins. The manufacturer's protocol was followed to carry out this method (ThermoFisher Scientific).

Statistics

The abundance of glycosylated HC-3 protein expression (normalized to b-actin) in erythrocytes from warm-acclimated frogs cultured in CCCM+G for 48 hours is represented as a percentage of normalized glycosylated HC-3 expression in freshly isolated erythrocytes from warm-acclimated frogs and in erythrocytes from warm-acclimated frogs cultured in CCCM alone for 48 hours. The analysis of the quantification of glycosylated HC-3 was done using densitometry on UVP software and an average value was calculated for each of the three conditions—Freshly Isolated (FI), erythrocytes cultured in CCCM for 48 hours (M), and erythrocytes cultured in CCCM+G for 48 hours (G)—where n=2 observations.

The abundance of glycosylated HC-3 protein expression (normalized to β-actin) in erythrocytes from warm-acclimated frogs in each condition that were treated with

PNGase F is represented as a percentage of normalized glycosylated HC-3 expression in erythrocytes from warm-acclimated frogs in each conditions that were not treated with PNGase F. The analysis of the quantification of glycosylated HC-3 was done using densitometry, through which we calculated two average values for the amount of glycosylated HC-3; one value for all three conditions with PNGase F, one value for all three conditions without PNGase F—where n=2 observations.

To determine whether HC-3 protein was localized in the membrane of RBCs or the cytosol of RBCs, Image J software was used. A line generated using Image J was drawn across each of the RBCs from each condition (FI, n=4; M, n=5; G, n=6), and the data points were graphed. A standard membrane was determined across all RBCs (10% of the non-zero data points, 5% from each side of the cell). Statistics were performed using a one-way Anova with post-hoc Tukey HSD test through the R program and average values of HC-3 immunofluorescence intensity from the cytoplasm and membrane were inputted into the program.

Results

Increased abundance of Glycosylated HC-3 in Erythrocytes Cultured in Media Containing Glycerol

Prior experiments have demonstrated differential regulation of HC-3 expression in multiple tissues depending upon the thermal acclimation state of *Dryophytes chrysoscelis* (Mutyam et al., 2011; Zimmerman et al., 2007; Goldstein et al., 2010; Pandey et al., 2010). Additionally, through the establishment of an erythrocyte cell culture system, Mutyam and colleagues (2011) showed differential HC-3 express patterns depending on time and media composition of cell cultures. In the present study, Western blotting of RBC proteins from freshly isolated (FI) warm-acclimated frogs, proteins from RBCs

cultured in Complete Cell Culture Media for 48 hours (CCCM), and proteins from RBCs cultured in Complete Cell Culture Media with the addition of 0.156M glycerol for 48 hours (CCCM+G) showed 3.5-fold and 1.9-fold average increase in the abundance of glycosylated HC-3 (60-120 kDa) from RBCs cultured in glycerol containing media compared to FI RBCs and RBCs cultured in CCCM alone (Figures 5, 6).

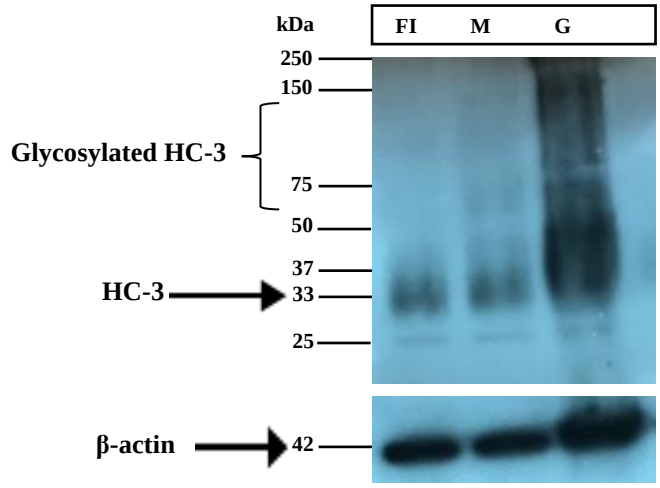

Figure 5. RBCs cultured with the addition of glycerol show an increased abundance of glycosylated HC-3. Proteins were isolated from Freshly Isolated RBCs (FI), RBCs cultured in CCCM for 48 hours (M), and RBCs cultured in CCCM for 48 hours with the addition of 0.156M glycerol (G) and immunoblotting was directed towards HC-3. β-actin (42 kDa) expression served as a gel loading control.

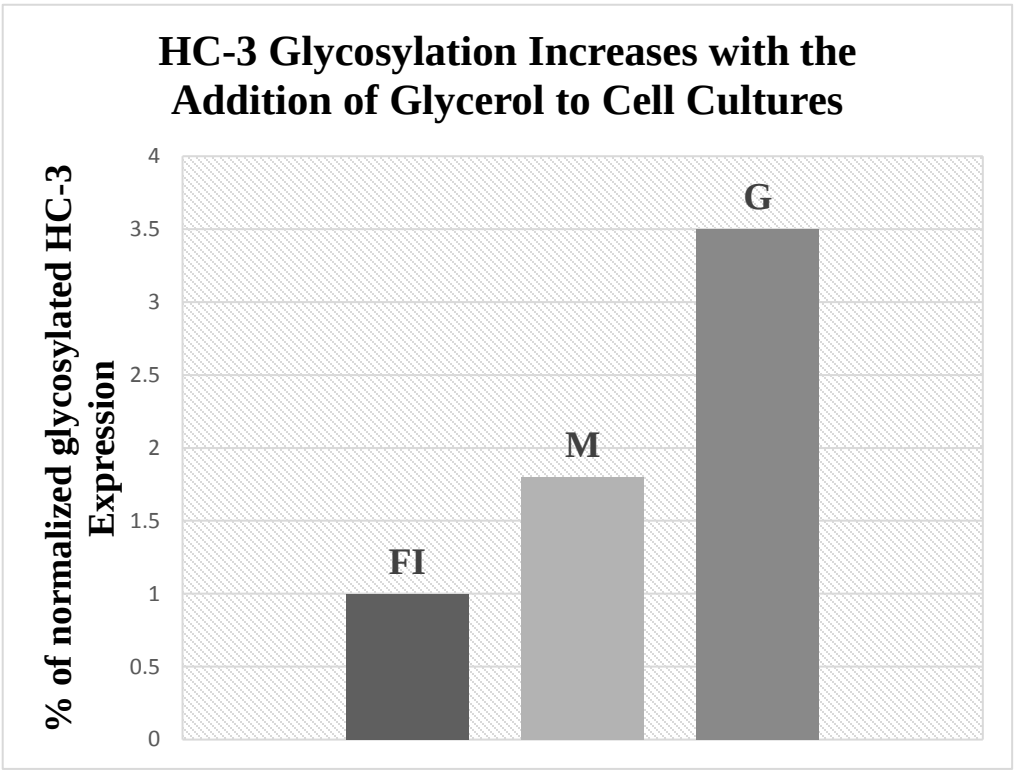

Figure 6. HC-3 glycosylation is enhanced with the addition of glycerol to cell cultures. Densitometric analyses of immunoblots specific for HC-3 showed a 3.5-fold and 1.9-fold average increase in glycosylated HC-3 (60-120 kDa) from RBCs cultured in CCCM with 0.156M glycerol for 48 hours (G) as compared to Freshly Isolated RBCs (FI) or RBCs cultured in CCCM alone for 48 hours (M), respectively.

Evidence for HC-3 N-linked Glycosylation, but Not O-linked Glycosylation

Results from experiments conducted on AQP3, the mammalian orthologue of HC-3, showed deglycosylation of AQP3 with PNGase F, resulting in increased electrophoretic mobility of AQP3 on SDS PAGE (Roudier et al., 2001). These findings contributed to the hypothesis that HC-3 may be N-linked or O-linked glycosylated and it was indeed shown that the treatment of RBC protein lysates with PNGase F, an enzyme that catalyzes the release of N-linked glycan moieties from glycoproteins, subsequently resulted in the

collapse of the high molecular weight "smear" into discrete immunospecific bands at 23 kDa, 31.5 kDa, and 35 kDa (Mutyam et al., 2011). However, to further characterize the glycosylation of HC-3, treatment of protein lysates with O-glycosidase and Neuraminidase, a pair of enzymes that catalyzes the release of O-linked glycan moieties from glycoproteins, was executed.

RBC protein lysates were treated with Protein Deglycosylation Mix II, a set of enzymes specific for catalyzing the release of both N- and O-linked glycans. Results from this experiment showed a decreased amount of glycosylated HC-3 from protein lysates treated with the enzymes as compared to control proteins, indicating that HC-3 could be N- or O-linked glycosylated (Figure 7).

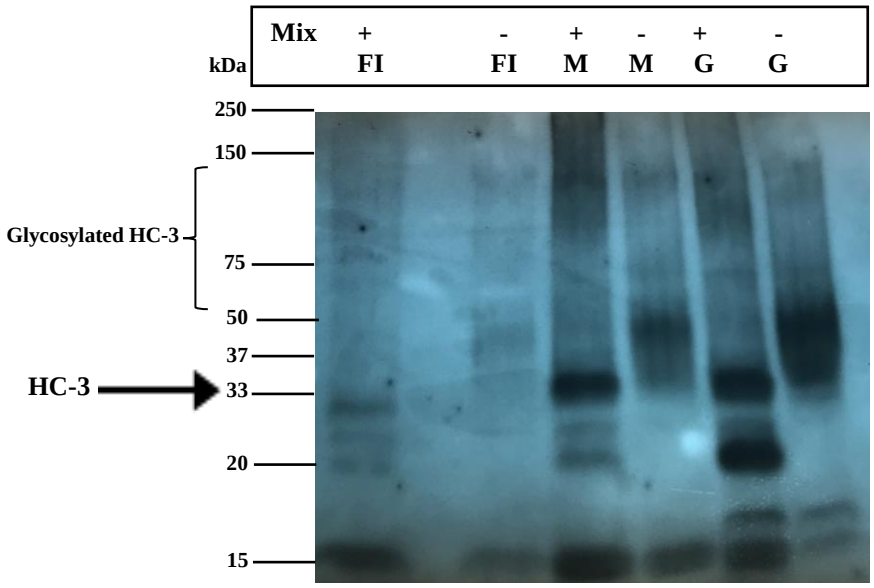

Figure 7. Enzymatic Digestion of HC-3 with the Protein Deglycosylation Mix II resulted in the disappearance of high molecular weight species. RBC proteins from FI RBCs, RBCs cultured in CCCM for 48 hours (M), and RBCs cultured in CCCM with the addition of 0.156M glycerol for 48 hours were treated with and without the Protein Deglycosylation Mix II, a mix of enzymes that cleave both N- and O-linked glycans.

Next, the results attained through the deglycosylation experiments executed by Mutyam and colleagues (2011) were recapitulated, showing that treatment of protein lysates from RBCs with PNGase F resulted in a 1.3-fold average decrease in glycosylated HC-3 in RBC proteins compared to control proteins (Figures 8, 9).

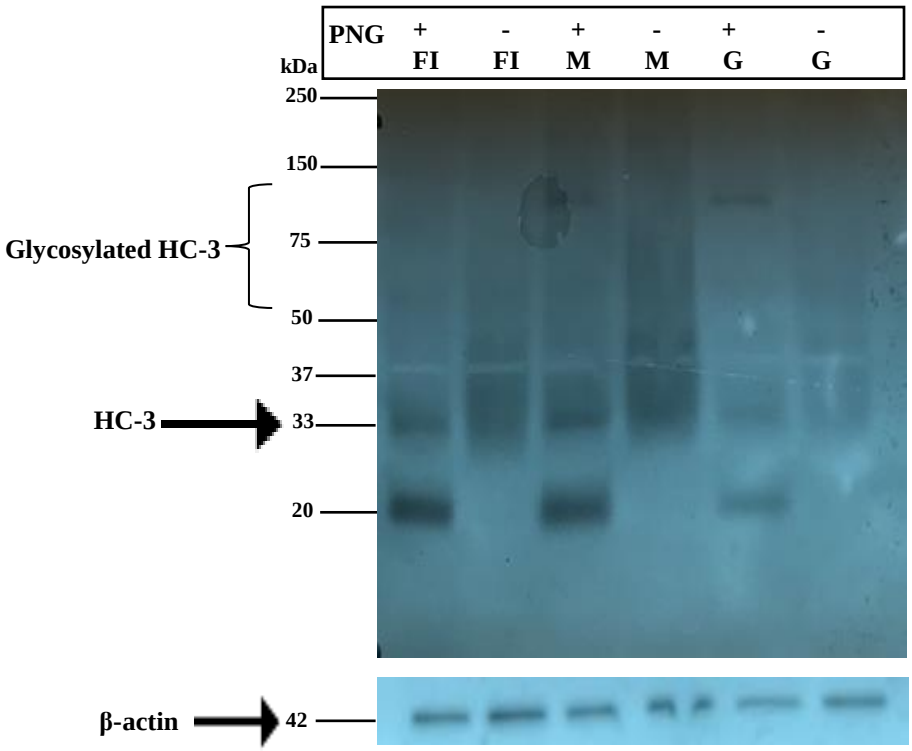

Figure 8. Enzymatic Digestion of HC-3 with PNGase F resulted in the disappearance of high molecular weight species. RBC proteins from FI RBCs, RBCs cultured in CCCM for 48 hours (M), and RBCs cultured in CCCM with the addition of 0.156M glycerol for 48 hours were treated with and without PNGase F, an enzyme that cleaves N-linked glycans.

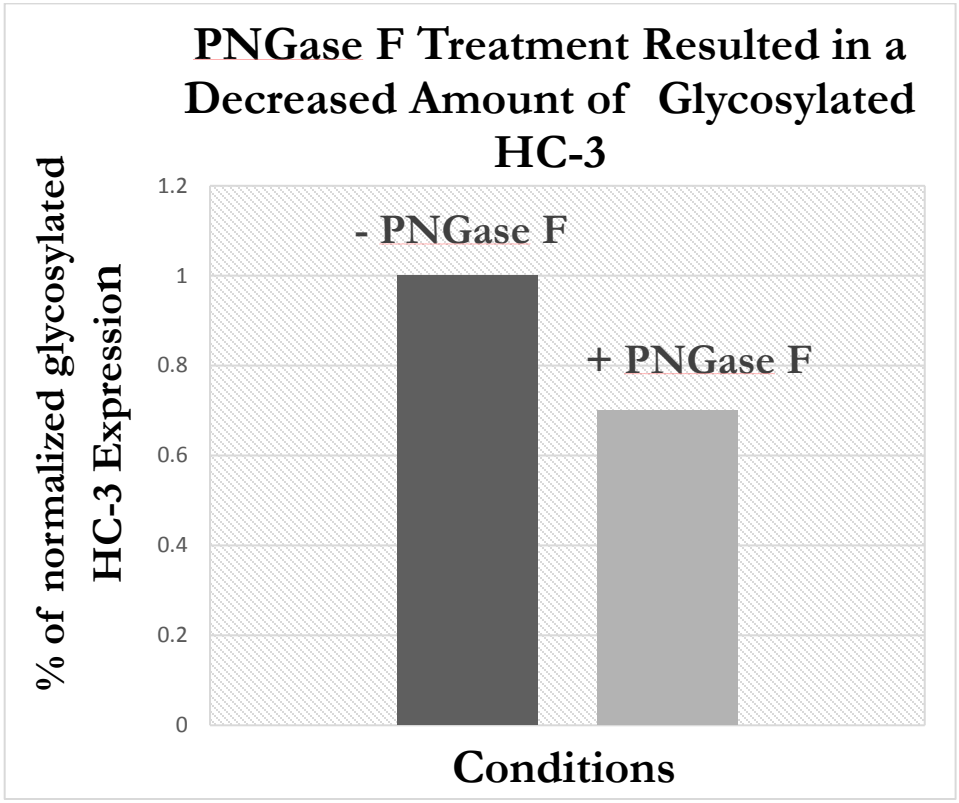

Figure 9. Enzymatic digestion with PNGase F resulted in a decreased amount of glycosylated HC-3. Densitometric analyses of immunoblots specific for HC-3 resulted in a 1.3-fold average decrease in glycosylated HC-3 in RBC proteins treated with PNGase F compared to RBC proteins that were not treated with PNGase F.

Treatment of protein lysates with O-glycosidase and Neuramindase, however, did not appear to affect the abundance of glycosylated HC-3, indicating that HC-3 is N-linked glycosylated but not O-linked (Figure 10).

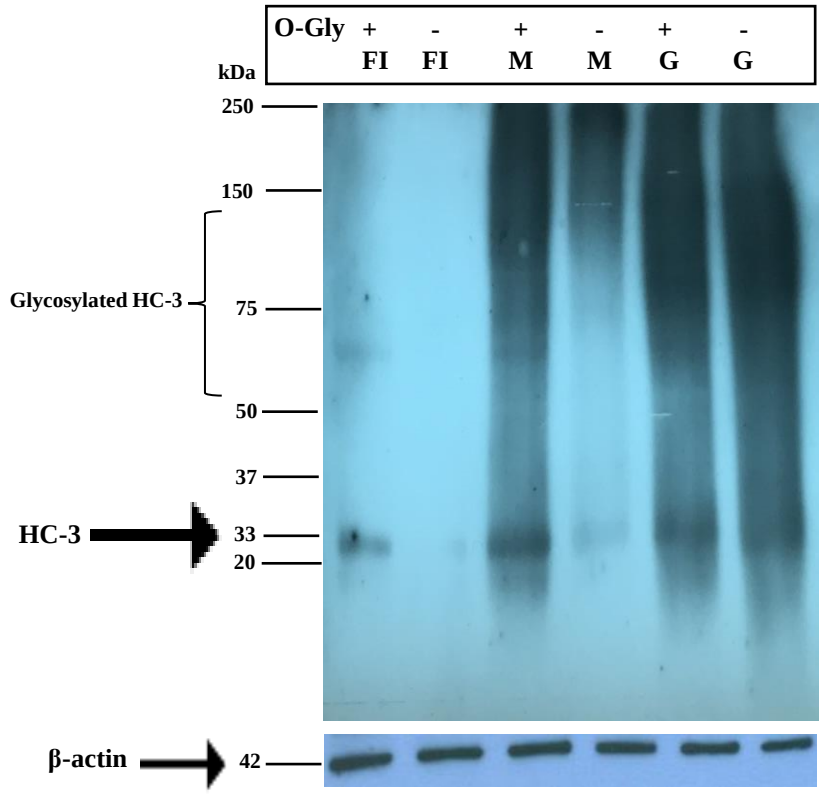

Figure 10. Enzymatic digestion with O-glycosidase and Neuraminidase did not result in the disappearance of high molecular weight species. RBC proteins from FI RBCs, RBCs cultured in CCCM for 48 hours (M), and RBCs cultured in CCCM with the addition of 0.156M glycerol for 48 hours were treated with and without O-glycosidase and Neuraminidase, enzymes that that cleaves O-linked glycans.

Enhanced HC-3 Membrane Localization in Erythrocytes Cultured in Media Containing Glycerol

As previously stated, it is known that HC-3 is differentially regulated depending on the thermal acclimation state of *D. chrysoscelis* (Mutyam et al., 2011; Zimmerman et al., 2007; Goldstein et al., 2010; Pandey et al., 2010). Aquaglyceroporin HC-3 was found to be significantly more robust in the plasma membrane vs. the cytosol in cold-acclimated tree frogs as compared to warm-acclimated tree frogs (Mutyam et al., 2011). Because glycerol naturally accumulates intracellularly and extracellularly in *D. chrysoscelis*

during cold acclimation periods as part of its freeze tolerance strategy, and the distribution of glycerol into and out of cells is likely through aquaglyceroporins, we explored whether the addition of glycerol to the media could regulate changes in HC-3 localization. Immunofluorescence of HC-3 in the cytosol was 3.2 times greater than HC-3 immunofluorescence in the membrane for Freshly Isolated RBCs (Figures 11, 12, 13; $P<0.01$.) There was no difference in HC-3 immunofluorescence intensity between the membrane and cytosol in RBCs cultured in CCCM (M) for 48 hours (Figures 11, 12, 14; $P>0.05$). In contrast, for RBCs cultured in CCCM+G for 48 hours, immunofluorescence intensity of HC-3 in the plasma membrane was 21.7 times greater than HC-3 immunofluorescence in the cytosol (Figures 11, 12, 15; $P<0.05$).

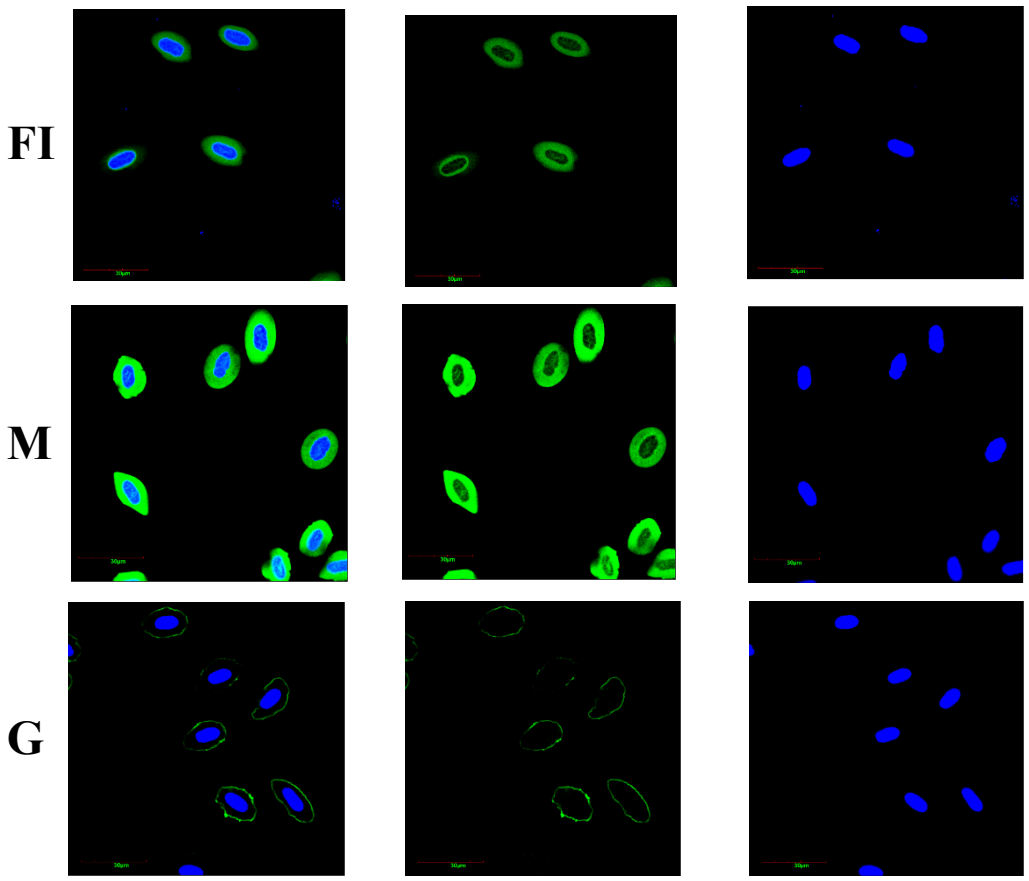

Figure 11. HC-3 membrane localization is enhanced as compared to HC-3 cytosolic localization in RBCs cultured with the addition of glycerol. RBCs were Freshly Isolated (FI), cultured in CCCM for 48 hours (M), or cultured in CCCM with the addition of 0.156 M glycerol for 48 hours (G). Immunocytochemistry, Scanning Confocal Microscopy, and ImageJ Software were used to identify and quantify HC-3 intensity found within the various culture conditions.

To determine whether HC-3 protein was localized in the membrane of RBCs or the cytosol of RBCs, Image J software was used. A line generated using Image J was drawn across each of the RBCs from each condition (FI, n=4; M, n=5; G, n=6), and the data points were graphed. A standard membrane was determined across all RBCs (10%

of the non-zero data points, 5% from each side of the cell), and an average value from the membrane and cytosol from RBCs in each condition were averaged and inputted into the statistical program "R" (Figure 13).

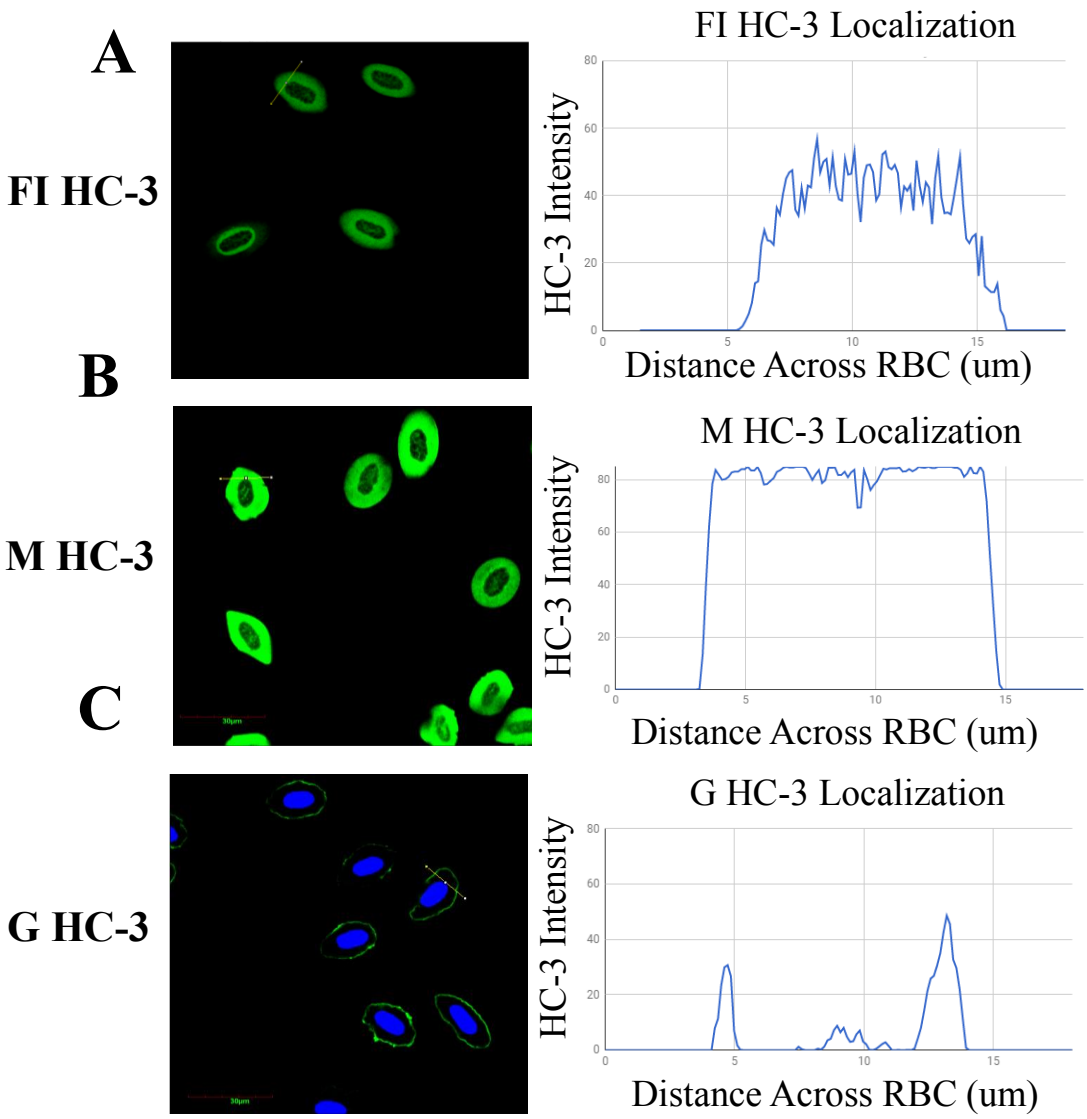

Figure 12. Image J Analyses. The membrane was distinguished from the cytosol by using Image J software and graphing HC-3 immunofluorescence intensity across each of the red blood cells. A standard percentage was determined to be the membrane in all cells. For FI RBCs, n=4 cells (A); for RBCs cultured in CCCM for 48 hours, n=5 (B); for RBCs cultured in CCCM+G for 48 hours; n=6 (C).

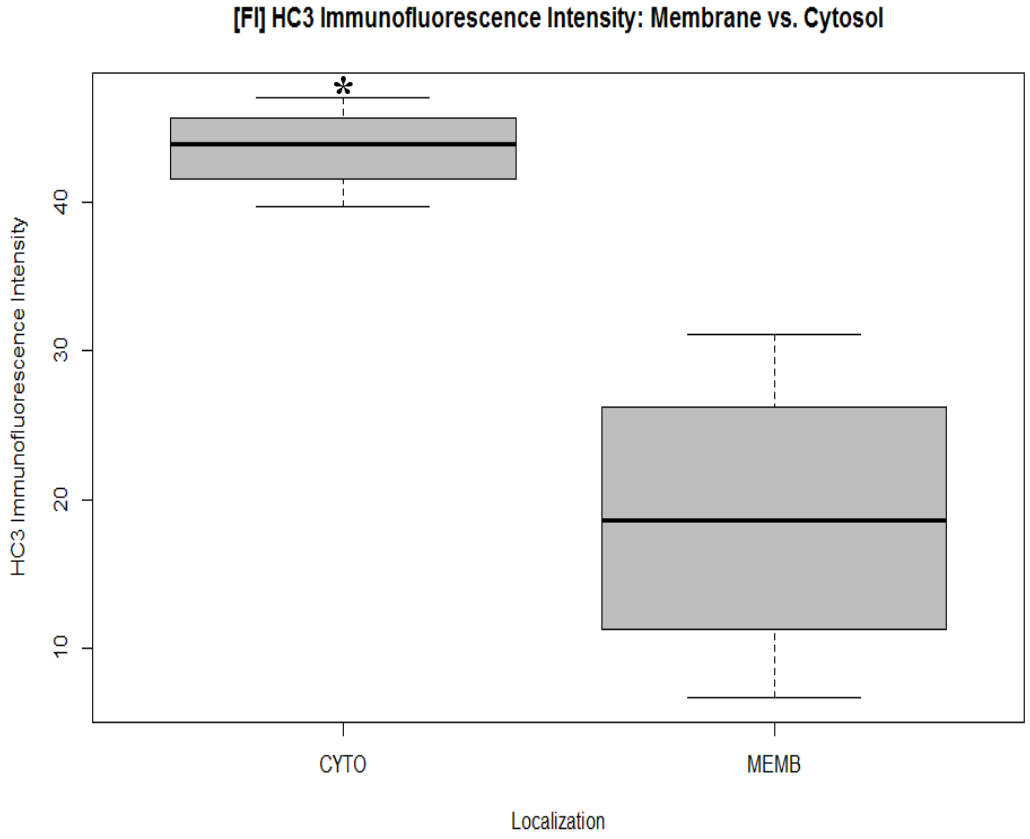

Figure 13. Immunofluorescence of HC-3 in the cytosol was 3.2 times greater than HC-3 immunofluorescence in the membrane for Freshly Isolated RBCs (FI) (*P*<0.01). RBCs from each culture were analyzed using ImageJ software to quantify HC-3 intensity throughout the cell. Statistics were performed using a One-way Anova with post-hoc Tukey HSD test through the R program.

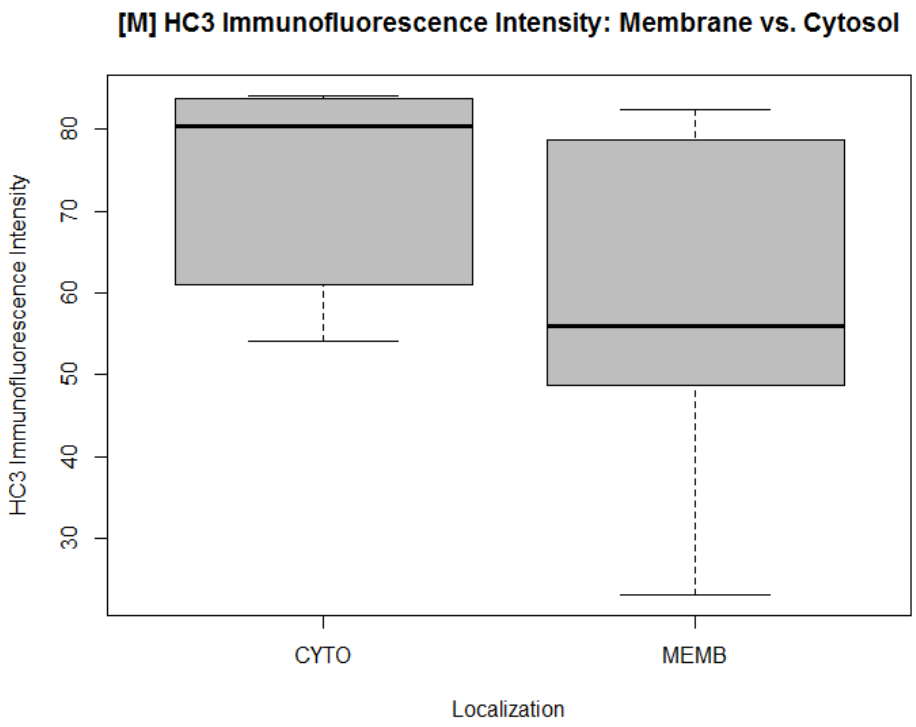

Figure 14. There was no difference in HC-3 immunofluorescence intensity between the membrane and cytosol in RBCs cultured in CCCM (M) for 48 hours (*P*>0.05). RBCs from each culture were analyzed using ImageJ software to quantify HC-3 intensity throughout the cell. Statistics were performed using a One-way Anova with post-hoc Tukey HSD test through the R program.

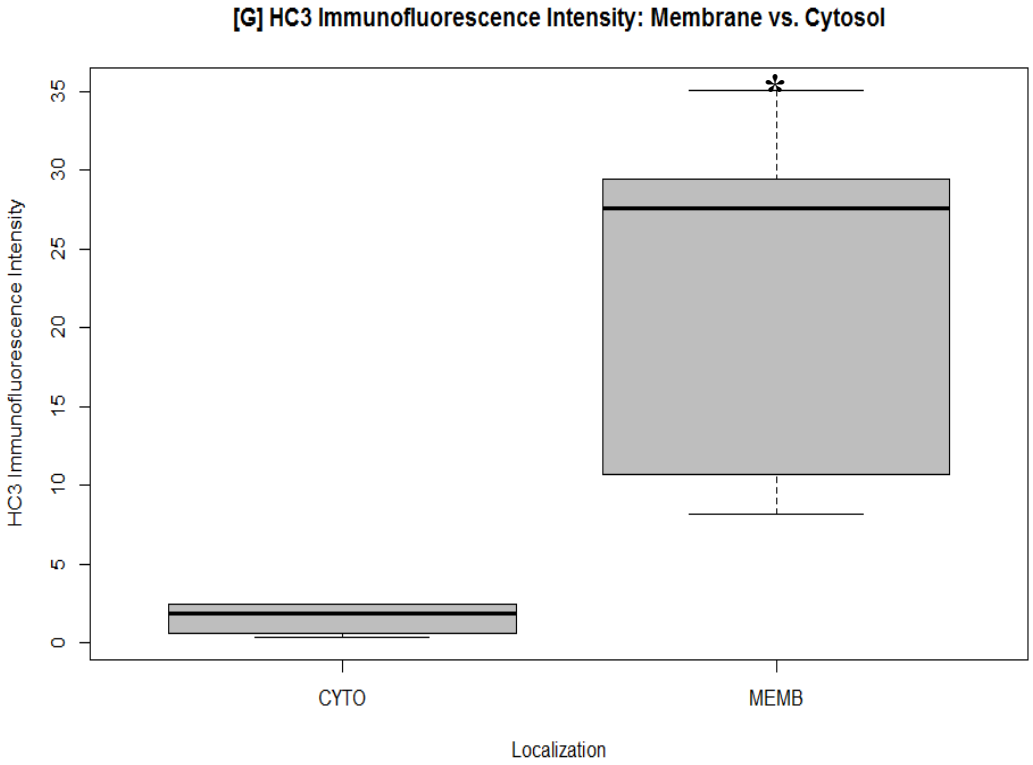

Figure 15. RBCs cultured in CCCM+G for 48 hours (G), immunofluorescence intensity of HC-3 in the plasma membrane was 21.7 times greater than HC-3 immunofluorescence in the cytosol (*P*<0.05). RBCs cultured in CCCM+G for 48 hours were analyzed using ImageJ software to quantify HC-3 intensity throughout the cell. Statistics were performed using a One-way Anova with post-hoc Tukey HSD test through the R program.

Total Glycosylation Stain Results

It was hypothesized that because the addition of glycerol to cell cultures resulted in an increased abundance of glycosylated HC-3 protein, the addition of glycerol to cell cultures would also upregulate the expression of more glycosylated proteins throughout the cells. Further experiments are required to address this hypothesis, however, RBCs cultured in media or media with the addition of glycerol show an enhanced expression of glycosylated proteins as compared to FI RBCs (Figure 16). This indicates that a variation

in the total erythrocyte glycoprotein profile emerges simply by culturing RBCs for 48 hours.

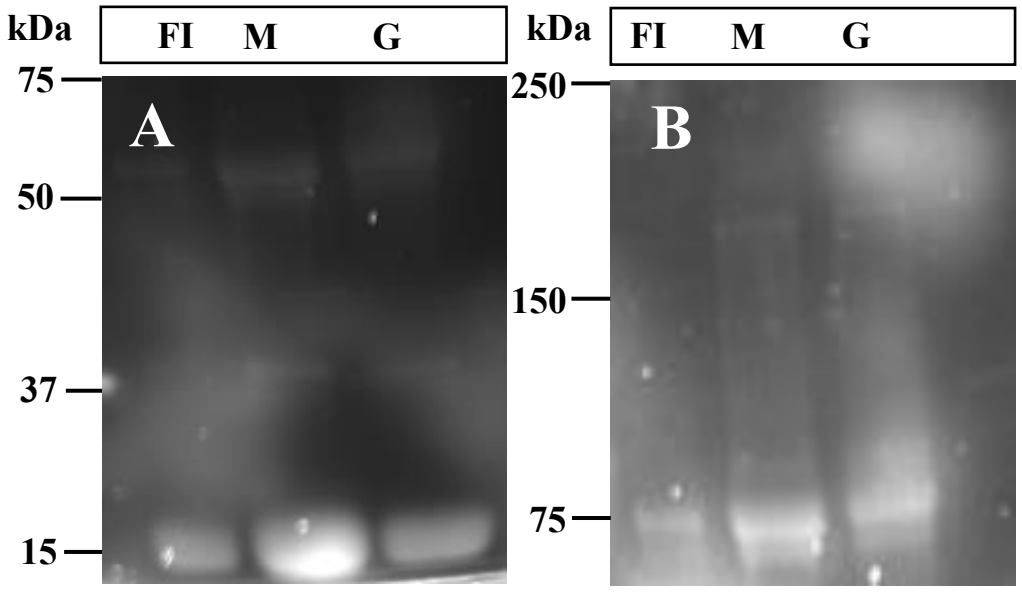

Figure 16. Freshly Isolated erythrocytes (FI) show differential expression of glycoproteins as compared to erythrocytes cultured in CCCM for 48 Hr (M) and erythrocytes cultured in CCCM + G for 48 Hr (G). (A) represents the bottom half of the gel (15-75 kDa), while be represents the top half of the gel (75-250 kDa). Proteins from all RBCs were size fractionated by SDS-PAGE and stained with the Pro Q Emerald 488 Glycoprotein Staining Kit (Thermo-Fisher Scientific).

Discussion

An understanding of the functional role of aquaglyceroporins in animal physiology has only recently begun to emerge, with much of the research to date occurring on mammalian models. For example, it is now known that AQP3 plays an important role in the hydration of the stratum corneum and would healing, AQP7 and AQP9 are involved in obesity, and AQP7 had been shown to regulate triglyceride metabolism (Hara et al., 2002; Maeda et al., 2008; Maeda et al., 2004). Furthermore, AQP3 is expressed mammalian erythrocytes and has been shown to contribute to erythrocyte glycerol

permeability (Liu et al., 2007; Beitz et al., 2009). Thus, it is evident that the study of aquaglyceroporins function and regulation will inevitably further human understanding of basic but important physiological process.

However, GLP function and regulation are significantly less studied in anurans. Investigations over the past fifteen years have led to the understanding the AQPs/GLPs are integrated into many essential physiological mechanisms involving fluid regulation and solute distribution within amphibians. Here, it is hypothesized that AQPs/GLPs may be an important factor in the success of the physiology behind freeze tolerance—a process that calls for immense solute and water redistribution—in Cope's gray tree frog, *Dryophytes chrysoscelis*.

Aquaglyceroporins in other life forms have previously been shown to be involved in freeze tolerance. For example, AQP3 enhances the survivability of cryopreserved mouse oocytes, over-expression of aquaporins in yeast leads to enhanced resistance to cellular damage from freezing, and in several freeze-tolerant organisms AQPs have been shown to be responsible for mediating water and solute flow across membranes during freezing (Tanghe et al., 2002; Edashige, et al., 2003; Izumi et al., 2006; Philip et al., 2008).

Thus, based on the previously stated evidence, it is hypothesized that the AQP3 anuran ortholog, GLP HC-3, expressed more abundantly in cold-acclimated *D. chrysoscelis*, is involved in the transmembrane mediated flux of both water and glycerol during periods of rapid extracellular ice crystal formation and ultimately contributes to this organism's freeze tolerant nature. This hypothesis was confirmed by Mutyam and colleagues (2011) once they discovered that erythrocytes harvested from cold-acclimated

frogs showed an increased abundance of native HC-3 protein expression, enhanced HC-3 membrane localization, and upregulated HC-3 post-translational glycosylation as compared to erythrocytes from warm-acclimated frogs.

Following this discovery, Mutyam et al. successfully established an *in vitro* erythrocyte cell culture system that allowed for further investigation into the function and regulation of GLP HC-3 (2011). Initial experiments resulted in the finding that the addition to glycerol to cell cultures led to an increased abundance of glycosylated HC-3 and enhanced HC-3 membrane localization, similar to the expression patterns found in the cold-acclimated frogs. In this study, the previously established *in vitro* erythrocyte cell culture system was used to further characterize the glycosylation of HC-3, quantify the effect of glycerol on HC-3 membrane localization, and also begin to examine the impact glycerol has on the total glycoprotein profile of erythrocytes from *D. chrysoscelis*.

Erythrocytes cultured with the addition of glycerol to the media showed a 3.5-fold and 1.9-fold average increase in glycosylated HC-3 as compared to freshly isolated erythrocytes and erythrocytes cultured in media alone. These results recapitulate those previously reported and further confirm the evidence for a glycerol-dependent post-translational glycosylation of HC-3 (Mutyam et al., 2011). However, because the osmolarity of the media was different between the three culture conditions, it is not possible to know whether the resulting effects are specific to glycerol or are the result of cellular responses to hyperosmolarity in general. With this held in mind, it is still possible these results indicate glycerol-mediated HC-3 membrane localization in erythrocytes from *D. chrysoscelis*. Further studies are needed to confirm this hypothesis.

Additionally, it was shown that treatment of erythrocyte protein lysates with PNGase F, an enzyme that catalyzes the release of N-glycans, resulted in the disappearance of high molecular weight species in immunoblots directed towards HC-3. However, treatment of protein lysates with O-glycosidase and Neuraminidase, enzymes that catalyze the release of O-glycans, did not appear to result in the disappearance of high molecular weight species. These results indicate that HC-3 is modified via N-linked glycosylation, but not O-linked.

Furthermore, the immunocytochemistry experimental results indicate that HC-3 membrane localization is increased 21.7-fold as compared to HC-3 cytosolic localization in RBCs culture with the addition of glycerol ($P<0.05$), while freshly isolated erythrocytes showed an opposite pattern and RBCs cultured in media alone showed no difference in HC-3 localization. Combined, these results along with the Western blotting results, indicate that a glycerol-induced hyperosmotic environment leads to a higher abundance of glycosylated HC-3 and a higher amount of HC-3 in the membrane. It is unclear, however, whether the HC-3 located in the membrane is glycosylated or not. Further studies are needed to address this question.

Finally, freshly isolated RBCs and RBCs in culture with or without glycerol displayed differential erythrocyte glycoprotein profile, indicating that the addition of these cells to a culture of 48 hours upregulates the number of glycoproteins found within the cells. Thus, taken together, the data from this study successfully recapitulate *in vivo* HC-3 expression patterns found in cold-acclimated frogs and a potential relationship between cryoprotective glycerol, increased HC-3 N-linked glycosylation, and enhanced HC-3 membrane localization has been identified.

Acknowledgements

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Chapter 3: Future Directions and Broader Implications

Future Directions

The establishment of an *in vitro* erythrocyte cell culture system by Mutyam and colleagues (2011) opened up many opportunities to investigate the cellular mechanisms suspected to be involved in the mechanisms of freeze tolerance in Cope's gray tree frog, *Dryophytes chrysoscelis*. From initial experiments, it was found that erythrocytes cultured in media containing glycerol—the main cryoprotectant used in *D. chrysoscelis*—led to an increased abundance of N-linked glycosylated HC-3 and enhanced HC-3 membrane localization as compared to control cells (Mutyam et al., 2011). These results match similarly to the HC-3 expression patterns found in the cold-acclimated frogs as compared to warm-acclimated frogs. Taken together, these two sets of data suggest a potential relationship between cryoprotective glycerol, HC-3 glycosylation, and HC-3 membrane localization that requires further investigation.

The experiments we set out to conduct in this study were aimed at further characterizing the glycosylation of HC-3, quantitatively describing the influence that glycerol had on HC-3 membrane localization, and examining the impact that glycerol had on the total glycoprotein profile of proteins from RBCs of Cope's gray tree frog. While our studies preliminarily completed these objectives, the role of HC-3 glycosylation in membrane trafficking is still unknown. Previous researchers demonstrated that when the covalent bonding of N-glycans to AQP2 was inhibited, the AQP2 protein traveled to the Golgi complex but failed to insert in the plasma membrane (Hendriks et al., 2004). Based on this experiment and other similar experiments, we hypothesize that the addition of sugar moieties to HC-3 via

glycosylation also aids in the trafficking and membrane insertion of the HC-3 protein in erythrocytes of *D. chrysoscelis*, and is thus critical for cellular freeze tolerance.

One avenue through which future researchers could test this hypothesis is by the use of tunicamycin (Figure 17). Tunicamycin belongs to a class of nucleoside antibiotics composed of uridine, an 11-carbon disaccharide called tunicamine, and a fatty acid of variable length, branching, and saturation. Tunicamycin was initially discovered in *Streptomyces lysosuperificus*, and additional similar compounds were found later on in other microorganisms. Its name comes specifically from its antiviral actions, which inhibits viral coat ("tunica") formation (Varki et al., 2017).

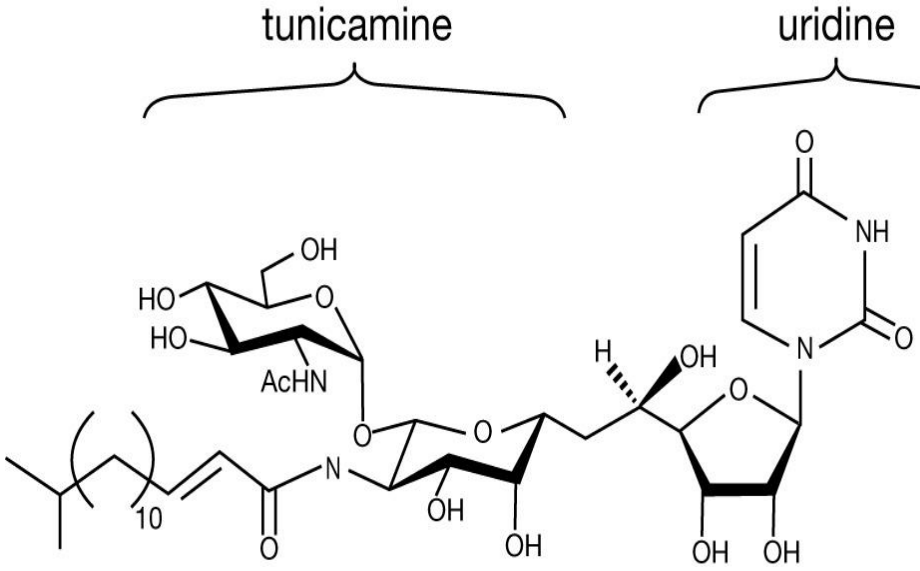

Figure 17. The Molecular Structure of Tunicamycin. Pictured above is the molecular structure of the N-linked glycosylation inhibitor, tunicamycin (Esko et al., 2017)

Tunicamycin inhibits N-glycosylation in eukaryotes by blocking the transfer of *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to dolichol-P (catalyzed by

GlcNAc phosphotransferase; GPT), which limits the formation of dolichol-PP-GlcNAc in the lumen of the ER of cells. Tunicamycin has been a popular tool used to study the role of N-glycans in glycoprotein secretion and function (Varki et al., 2017). Therefore, it is an excellent tool that can be used to investigate the role of the N-linked glycosylation of HC-3 in membrane trafficking.

By culturing erythrocytes of *D. chrysoscelis* using the previously established *in vitro* cell culture system with and without the addition of tunicamycin and conducting downstream biochemical experiments with Western blotting and immunocytochemistry, one could address the efficacy of tunicamycin in the inhibition of N-linked glycosylation and the impact of the inhibition of N-linked glycosylation on HC-3 membrane localization. Following these experiments, a functional freeze assay of cells cultured with and without the addition of tunicamycin could be executed to directly relate the significance of HC-3 N-linked glycosylation with RBC freeze tolerance in Cope's gray tree frog. It is advisable to test the cell type at various concentrations of tunicamycin before choosing a set experimental dose. In addition to cell type, the concentration of tunicamycin used likely depends on type of media used (Elbein, 1987).

However, it is important to note that the addition of tunicamycin to cell cultures may have a wide range of effects, given that it is inhibiting N-linked glycosylation of proteins ubiquitously throughout the cells if administered at the correct concentration. Depending on the concentration dose, tunicamycin may have no effect on cellular function, or it may disrupt the function of important glycoproteins, leading to cell death. Therefore, it is important to test for cellular viability through the cell culturing periods to ensure proper cellular function.

Glycosylation in Disease

Glycans are compounds consisting of a large number of monosaccharides linked glycosidically, and are one of the major constituents that make up the cell. Furthermore, they are the most widespread biopolymers, made of saccharides that are bonded to newly translated proteins within the cell secretory pathway (Ohtsubo and Marth, 2006). Because of the vast amount of diversity in sugar monomer structure and intersaccharide bonding, combined with the variation in glycan attachment sites on proteins, the intricacy of the glycome hugely surpasses that of the proteome. In fact, almost every mammalian cell membrane protein is post-translationally modified via glycosylation (Lauc et al., 2016).

Glycans are involved in a variety of molecular processes, including but not limited to protein folding, molecular trafficking, and modulation of receptor activity (Ohtsubo and Marth, 2006). Furthermore, all human cells are surrounded with a thick layer of glycans bonded to proteins and lipids found within the membrane, known as the glycocalyx, which is at least 10-1000 times the density of the actual cell membrane. The glycocalyx represents a figurative cell's fingerprint, or an identifier for the human body to distinguish the "self" from the "non-self." In fact, unknown glycan distributions found on tissues that were transplanted or diseased cells can be identified by glycan receptors found in the cell membrane, which then initiate specific systemic immune responses to protect the cell (Varki, 2015).

Since glycans are involved in many biological processes, many molecular disruptions in glycan synthesis are continuously being identified as causes of human diseases (Freeze, 2006). For example, endocytosis and trafficking to lysosomes are usually found to be involved in the process of breaking down biomolecules such as proteins and glycans. Disruption of this catabolic process can stem from malfunctions of glycosidase enzymes, which lead to diseases

such as Gaucher's, Niemann-Pick type C, Sandhoff's, and Tay-Sachs diseases (Ohtsubo and Marth, 2006). Additionally, the hereditary disorders of glycosylation (HDGs) include a number of diseases dealing with metabolic defects that are associated with errors in distinct parts of glycan synthesis (Carchon, 2004). Currently, at least 20 unique genes involve mutations that influence glycosyltransferase and glycosidase enzyme activities, leading to HDGs (Aebi, 2001). Based on this knowledge, glycan variants found within a cell can now be used as certain disease markers and present a diagnostic tool in medicine as well as potential therapeutic targets for downstream treatments.

Looking at the glycoprotein profile of a human shows promising contributions to the future of personalized medicine, which aims to individualize diagnosis and treatment down to the molecular level (Almeida and Kolarich, 2016). Considerable efforts in research applying to the human genome and proteome has begun to improve personalized medicine, however protein glycosylation markers have not been fully taken advantage of and much more research needs to be conducted. As mentioned previously, changes in the glycan pattern on a protein are frequently associated with human diseases (Thaysen-Anderson et al., 2015). Thus, in the context of diseases such as cancer or inflammatory conditions, glycoproteins and the glycans associated with them are now being used as potential markers and therapeutic targets. Furthermore, if associations between glycan patterns on proteins, certain diseases, and their respective medical treatments are understood, glycosylation inscriptions specific to proteins can provide insights to an individual's health (Figure 18).

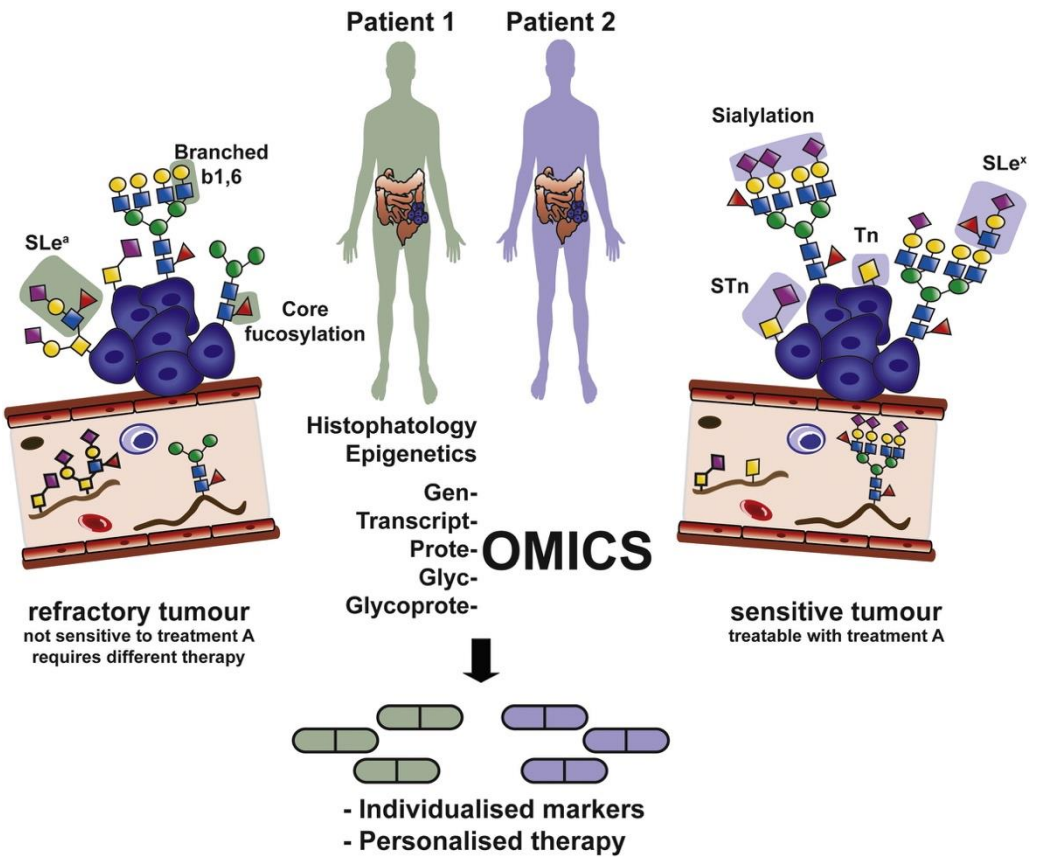

Figure 18. The Glycosylation of Proteins in Personalized Medicine. Glycosylation inscriptions provide a reflection on an individual's health and can aid in personalized treatment. Ultimately, the combination of glycomics & glycoproteomics will come together to personalize medicine at the molecular level. This image demonstrates the necessity of knowing the glycosylation inscription tumor within a patient, since the specific treatment done for that individual may be different than the treatment of the same kind of tumor from a different patient (Almeida and Kolarich, 2016).

Immunoglobulin G (IgG) is a crucial and well-known glycoprotein in the human immune system. IgG is the most numerous immunoglobulin in the serum of humans and is categorized into four subclasses that exhibit variations in their protein sequence, but all four subclasses share a very highly conserved N-glycosylation site at Asn297 (Figure 20) (Xue et al., 2013). IgG N-glycans have an important role in modulating IgG function and activity, and

because of this IgG glycosylation is being studied closely in relation to the development of disease (Figure 19).

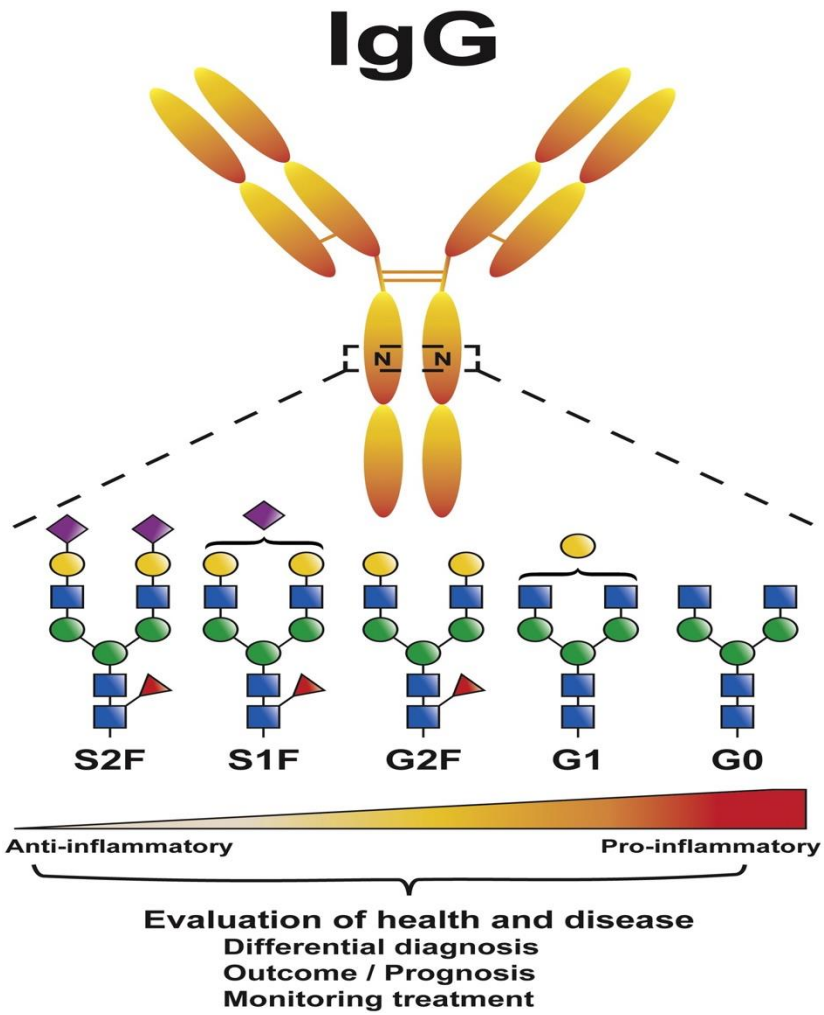

Figure 19. N-glycosylation regulates the inflammatory implications of IgG. The increased abundance of galactosylated and/or sialylated N-glycans promotes anti inflammatory properties, whereas more afucosylated structures increase pro-inflammatory properties. These N-glycan patters provide tools in diagnosis and prognosis for certain diseases. Sialactose (S), Fucose (F) and Galactose (G) are labeled in the figure above, indicating the respective number of each of those saccharides (Almeida and Kolarich, 2016).

As biomedical knowledge surrounding glycobiology grows, strategies for detecting and understanding glycan production and variation will continually advance.

The size of the human genome related to protein glycosylation relative to what is understood about glycosylation suggests that we have much to discover in the field of glycan function (Lauc et al., 2016).

Cryopreservation of Tissues

Cryopreservation uses extremely low temperatures to protect structurally intact living cells and tissues for an extended amount of time. The type of cell or given cells throughout different species, mammalian in particular, influences biological reactions and survival during the freezing and thawing cycle. The process of conventional cryopreservation goes as follows (1): mixing of cryoprotective agents or cryoprotectants (CPAs) with cells or tissues prior to exposure to extremely cold temperatures; (2) cooling of the cells or tissues and subsequent storage; (3) warming of the cells or tissues; and (4) withdrawal of CPAs from cells or tissues following the thawing process. From these major steps, it is evident that the correct use of CPAs is an important aspect of cryopreservation and critical in improving the viability of the cell or tissue being cryopreserved. Cryoprotectants, as mentioned in Ch. II, reduce the damage of freezing. These CPAs are best suited for cryoprotection if they are biologically appropriate, membrane permeable, and have minimal cellular toxicity. Various CPAs are used currently, including glycerol—the main cyroprotectant used by *D. chrysoscelis*—DMSO, ethylene glycol, and trehalose (Jang et al., 2017).

Cryopreservation can be applied to human health and medicine in some of the following ways: (1) cryopreservation of cells or organs; (2) cryosurgery; (3) biochemistry and molecular biology; and (4) *in vitro* fertilization (IVF). Benefits of using cryopreservation include the widespread banking of cells for human leukocyte antigen

typing specific for the process of organ transplantation and also in the increase in time available for successful cell and tissue transplantation to the correct medical centers. Additionally, the long-term banking of stem cells is the first logical step towards the direction of engineering tissues, which can lead to advancements in the regeneration of soft tissue function and in the treatment of known diseases that are without a medical therapy (Jang et al., 2017).

More specifically, the cryopreservation of human oocytes, embryos, sperm, testicular tissue, and hepatocytes could greatly influence human health and medicine in the future. For example, the very first documented case of embryo cryopreservation for fertility preservation occurred in 1996, with the application of an IVF to a woman who was diagnosed with breast cancer before she underwent chemotherapy treatment. Additionally, the cryopreservation of mature oocytes is an evidence-based technique that helps to preserve the reproductive capabilities of a human. A retrospective study of 11,768 cryopreserved human embryos that had gone through at least one thawing period from 1986 to 2007 showed results that suggested that the duration of storage on clinical pregnancy had no significant influence on pregnancy or live birth rate. Furthermore, adult stem cells have the ability to differentiate into several types of certain cells and can be obtained from various locations, and several of these stem cell types have a potential application in regenerative medicine (Jang et al., 2017). Thus, it is clear that the fields of tissue engineering, regenerative medicine, and cell and tissue transplantation are hugely dependent on the capability to preserve and transport these stem cells without changing their genetic makeup or cellular structure (Jang et al., 2017)

Although the applications for cryopreservation in the healthcare field are numerous, there are some limitations that still exist. For example, the metabolism of cells is extremely slow at low temperatures, which leads to changes in lipids and proteins that may disrupt proper cellular function and structure (Karlsson et al., 2008). Also, if used in too great of concentrations, CPAs themselves can be damaging to cells. For example, DMSO could impact the stability of chromosomes, leading to possible tumor formation (Pegg, 2010). Finally, the ice crystals themselves can be physically damaging to cells if formed in high enough amounts and around or in cells, so control of the size and location of ice crystal formation is a challenging problem that needs more research to understand properly (Pegg, 2010).

Taking into account the applications of cryopreservation in human health and disease and our current limitations in the success of this technique, it is clear that research in this area is both valuable and necessary to make advancements in tissue engineering, stem cell preservation, and cell, tissue, and organ transplantation methods. Returning to the "Krogh Principle," coined by August Krogh, a famous Danish Comparative Physiologist who won the Nobel Prize for Physiology or Medicine in 1920: "For many problems, there is animal on which it can be most conveniently studied." These words ring true for the problems within cryopreservation discussed above and the potential to solve these problems through studying an animal that has evolved successful mechanisms to make it naturally freeze tolerant—*Dryophytes chrysoscelis*.

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Chapter IV: Personal Reflections

I began my undergraduate career with one end goal in mind: getting accepted into medical school. However, I had no idea what a doctor actually was, what being a "pre-med" student really meant, and I didn't understand the amount of work and dedication it would take to become a successful medical school applicant. I've always valued the importance of spending time with friends and living a balanced life, so my initial efforts in college were focused on doing well in my coursework and enjoying the social scene at the University of Dayton. My first year of college went by in a hurry, but I had fulfilled what I set out to do that year: excel in my classes as a top student, become involved in a few organizations, and surround myself with friends who shared a similar mindset in terms of living a balanced lifestyle full of working hard and having fun.

Over the summer following freshman year, I chose to shadow few physicians, volunteer at a local hospital, work at a few restaurants as a waiter, and catch up with high school friends I so desperately missed during the school year. But before I knew it, sophomore year had arrived. That year was full of more challenging courses, including the course every pre-medicine student hears horror stories about: Organic Chemistry. To my surprise, though, I genuinely enjoyed taking O-Chem because it was so challenging, requiring me to study much more than I ever had to previously do for any other course. Additionally, before that semester started I made it a goal of mine to become more involved and I did so through becoming a Supplemental Instruction Leader for General Chemistry. Through this job, which required me to hold two sessions per week outside of class aimed at helping the students better understand class material, I came to the realization that I possessed a passion for teaching and thoroughly enjoyed the process of

explaining abstract concepts and helping students reach "aha" moments with difficult material.

As first semester of sophomore year came to an end, I started to more seriously contemplate my future and the medical school application process. I had heard in many seminars my Pre-Medicine major offered that engaging in research was valuable for students wishing to attend medical school. At first, I had no interest in taking on a research project because not only was I disinterested in research because of the dread I had for laboratory courses in Chemistry, Biology, and Organic Chemistry, but also because I didn't understand the relationship between scientific research and clinical medicine. I saw the two fields as mutually exclusive and believed that as a physician, I would be taking care of patients, not doing research. However, because I saw many of my peers doing research and learned that if I were to attend a competitive medical school, I should at least try research out.

As I scrolled through the University of Dayton Department of Biology Faculty Page, I stumbled upon Dr. Carissa Krane and her work with Aquaporin 5 and its relationship to asthma. Growing up with asthma my whole life, I thought participating in a project that was medically relevant and personally related to my life would be both valuable and interesting. So, I emailed Dr. Krane to set up a meeting. My exact words in the email I sent to her on December 10th, 2015 were, "Dr. Krane, Hello, my name is Dante Pezzutti and I am currently a sophomore Pre-Med major here at the University of Dayton. I will be the SI leader for Biology 152 next semester for Dr. Rhoads. After speaking with Dr. Rhoads, she recommended that I contact you in regards to a research position within your lab. The research you are performing on aquaporins and the etiology

of asthma interests me greatly since I personally have been affected by asthma throughout the entirety of my life. If it is possible, I would love to meet with you before the end of the semester to discuss the possibility of helping out in your lab. Get back to me when you can and I look forward to speaking with you!" I can only help but smile at my naïve understanding of research at the time and the over-complicated way in which I wrote a now seemingly simple email. Little did I know the meeting that would follow with Dr. Krane would mark the beginning of a life and career changing experience.

I arrived back on campus following Christmas break sophomore year and immediately set up a meeting with Dr. Krane to discuss the possibility of me doing research in her lab. To this day, I can vividly remember walking into her office extremely nervous and unsure of my decision to set up this meeting in the first place. I walked through the door and saw Dr. Krane sitting at her computer, intently focused on a Microsoft Word document. I quietly knocked on the door and she looked over and smiled at me and said, "Hi, come on in." We shook hands, introduced ourselves to each other, and I timidly sat down. I began asking about the research in her lab and what kind of projects were available for me to work on. To my surprise, the project I was originally interested in dealing with asthma was not being worked on in the lab at the time. However, other projects dealing with a freeze tolerant frog were available, which sounded equally as interesting to me. Up until that point in my life, I never knew that there existed organisms that could literally freeze and thaw themselves. It sounded like something out a science-fiction movie and I certainly wanted to be involved in some way.

Then, after we discussed the research behind the frog and I indicated that I was interested in working on a project in the lab, something peculiar happened. I asked Dr.

Krane how much time she asked of her students to spend in the laboratory so that I could get an idea of the time commitment research would be. She responded by essentially saying, "What do you want to get out of this experience? Are you looking to be an assistant on a project and simply provide technical assistance, or would are you trying to win the David Bruce Award?" I was unsure of how to answer this question and quite frankly can't remember how I responded, but the meeting concluded with her telling me to meet with Raphael Crum, an Honors thesis student that was working in the lab, to discuss his experiences in the lab and the amount of time he dedicates towards research. Later on, I would find out that the David Bruce Award is the most prestigious research award that The American Physiological Society offers to undergraduate students across the country.

Following the meeting, I emailed Raphael and set up a meeting with him at the Kennedy Union Dining Hall. Again, I was extremely nervous to meet with Raphael but I conjured up the courage to sit down with him and eat dinner. I could tell immediately that Raphael was intelligent and deeply involved in his project. The confidence and charisma he carried with him as he spoke about his research was both admirable and inspiring. We had a very nice discussion about the work he puts into the lab and the time commitment it would be if I were to become independent in my own project. Towards the end of our discussion, Raphael emphasized that I should really think deeply about my commitment to the lab because if I wanted it to be a valuable experience, I needed to dedicate a big chunk of my time to the project I assumed for my Honors thesis. Although it was intimidating to hear the actual number of hours that Raphael consistently dedicated to working in the lab (40 hours a week sometimes), his enthusiasm and excitement for

independent research is ultimately what sold me. The idea that I could possess the ability to ask a question and pursue an answer to that question through independent scientific investigation was far too attractive to me to turn down. It would be a whole new type of learning, far different from the classroom style learning I was used to, and I was excited to take on the challenge. I thanked him for taking the time to meet with me and we parted ways for the time being.

Because Raphael was the only one in the lab at the time and was not only working on a completely different project but also busy studying for the MCAT that semester, I was not able to start working on my project until the fall of my junior year. When I returned in the fall of 2016, I began learning techniques necessary for my project from Loren, the graduate student in the lab, and Dr. Clara do Amaral, a post-doctoral fellow that was working in the lab for the next year. I quickly found out how difficult basic science research was. Learning techniques took such a long time, and I found out that even when I mastered one technique, there was another technique waiting to be mastered after that. The learning never stopped.

I developed more persistent patience, overcame adversity, and learned the importance failing. Failing, in my eyes, was always something to avoid. It's stigmatized in the academic world and so I tried to stay as far away from failing as possible my whole life. However, in research, failure is around every corner. I learned to be comfortable with it and learn from my mistakes, not regret them. So many times I would encounter a problem within a protocol that I could have never foreseen before the start of the experiment, and it was only by going through the motions did I figure out the solution. Experiential learning was something that textbooks and classrooms never taught me that

research did, and I progressively began to understand more and more how an experience in research was valuable to an aspiring physician.

Towards the end of that semester, Dr. Krane suggested that if I were interested in staying at UD for the summer to do research, then I should apply for a 2017 American Physiological Society Undergraduate Summer Research Fellowship (APS UGSRF). This award would be given to 24 students across the country and would provide a $4,000.00 stipend for a 10-week long research experience, as well as $1,500.00 in travel money to attend the annual Experimental Biology meeting in San Diego, California in April of 2018. I never thought that I would actually receive this award, but I went ahead and applied for it hoping that I would be fortunate enough to receive it. Luckily, Raphael received this same award in 2015 and agreed to help me write the personal statement and project description that went into the application. I submitted the application in mid-January and found out in April that I had won! I was beyond thrilled and actually couldn't believe it. Up until that point in my life, I had never won any prestigious awards, especially at the national level. I can remember running to Dr. Krane's office and celebrating with her. She told me, "You know what this means for your medical school application right? This puts you in a different category than the majority of your peers. You should be proud of yourself." I thanked her for all of her support and advice towards my application and relished in the fact that I would get to dedicate an entire summer to my project.

That summer is what really solidified my passion and love for research. I encountered many more failures than I did successes, but this only made me want to do research more. I was constantly working towards the perfect experiment and towards

getting the results Dr. Krane and I set out to achieve at the beginning of the summer. I continually found research to be difficult but incredibly rewarding. Outside of my time spent in the laboratory, the APS UGSRF offered opportunities for the fellows to write a blog about their summer research experiences and have that blog published. We also had the opportunity to explain our research to one another via videos and read articles that discussed the scientific process and developing a hypothesis. Overall, my experiences that summer were formative and ultimately contributed to my motivation to pursue a career as a physician-scientist faculty member at a medical school. The idea that I can not only treat patients in the future, but also engage in bench-to-bedside translational research and teach the next generation of physicians, combining a multitude of activities that I thoroughly enjoy, seems like the perfect career for me.

The culmination of my Honors thesis project is the completion of this manuscript, something that I never would have thought I could accomplish when I began my undergraduate studies. Research under Dr. Krane has taught me so much more than just the information about my specific project or how to use certain techniques to answer physiologically related questions. It's taught me how to work independently and collaborate with others. It's given me self-confidence and a deeper appreciation for the scientific process. It's taught me the value in experiential learning and constructive criticism. It's taught me how to balance my time and work under stressful conditions. It's taught me to shoot for the stars and push myself in every aspect of life. And most importantly, it's taught me how to fail.

I cannot express how incredibly grateful I am to have had the honor to work under Dr. Krane for these past two years. She truly has changed my life and instilled in

me a fire that will burn forever. She believed in me when I did not believe in myself, and she provided me the opportunity to pursue something I was truly passionate about. Most importantly, she taught me how to think independently, something I learned that I was not very good at before starting my Honors thesis research project. I could not have asked for a better mentor to work under, and I am blessed to have met and worked with Dr. Krane. I can only hope to be like her in the future.

I am now proud to say that I was recently awarded the Barbara M. Horwitz and John M. Horowitz Outstanding Undergraduate Abstract Award given to 30 students by the American Physiological Society and am thus eligible to compete for the Barbara M. Horwitz and John M. Horowitz Excellence in Undergraduate Research Award (The David Bruce Award) at the Experimental Biology meeting on April 22nd, 2018. I get chills thinking about when Dr. Krane asked me what my intentions were in research in our very first meeting, asking if I wanted to win The David Bruce Award, and now I actually have the opportunity to win that same award.

This fall I will be attending my dream school—The Ohio State University College of Medicine—to pursue my M.D. degree. I'm also considering matriculating into the M.D./Ph.D. following my first year of medical school because of the impact research has had on my life. I look back on my four years at the University of Dayton and could not be more proud of what I've accomplished, the relationships I've formed with friends and faculty, and the person I've become. But I recognize that this is merely the beginning, and I could not be more excited for what lies ahead.