Cortisol Regulation of Aquaglyceroporin HC-3 Protein Expression in the Erythrocytes of the Freeze Tolerant Tree Frog Dryophytes chrysoscelis

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Cortisol Regulation of Aquaglyceroporin HC-3 Protein Expression in Erythrocytes from the Freeze Tolerant Tree Frog *Dryophytes chrysoscelis*

Honors Thesis
Maria P. LaBello
Department: Biology
Advisor: Carissa M. Krane, Ph.D.
April 2019
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Dryophytes chrysoscelis

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Abstract

Dryophytes chrysoscelis, commonly known as Cope’s gray treefrog, is a freeze tolerant anuran that freezes up to 65% of extracellular fluid during winter to survive. Glycerol is presumably used as a cryoprotectant during a period of cold-acclimation to protect cells from permanent damage due to hypoosmotic stress upon freezing and thawing. The passage of glycerol and water during cold-acclimation is mediated through aquaglyceroporin HC-3 in the nucleated erythrocytes (RBCs) of D. chrysoscelis. This thesis analyzes the mechanisms in which D. chrysoscelis prepares for cold-acclimation and glycerol synthesis. Cortisol is a stress hormone known to respond to osmolarity and metabolic challenges and regulate aquaporins; however, the role of cortisol in regulating anuran HC-3 protein expression and subcellular localization, and implications for mediating anticipatory glycerol synthesis and freeze tolerance remain to be determined. We hypothesize that cortisol exposure regulates HC-3 protein expression and subcellular localization. Freshly isolated RBCs were cultured in complete cell culture media (CCCM) and cortisol for 2, 4, and 6 hours at two separate concentrations, 1.0 and 0.1 μg/ml. Another group of RBCs was incubated with CCCM for 24 hours in culture before the 4 and 8-hour incubation with cortisol concentrations of 0.01, 0.1, and 1.0 μg/ml. Densitometric analyses of immunoblots specific for HC-3 for RBCs that had undergone the 24-hour incubation before cortisol exposure showed a 3.7-fold increase in native HC-3 from RBCs cultured in 0.01 μg/ml cortisol for 4 hours, 40-fold increase in native HC-3 from RBCs cultured in 0.1 μg/ml cortisol for 4 hours, and 21-fold increase in native HC-3 from RBCs cultured in 1.0 μg/ml cortisol for 4 hours compared to RBCs cultured in 0 μg/ml for 4 hours. HC-3 protein abundance increased in RBCs exposed to 0.01 μg/ml cortisol for 8 hours by 2.4-fold from the 4-hour time point. The abundance of
glycosylated HC-3 (60-150kDa) increased by 3.1-fold, 5.8-fold, and 5.3-fold for RBCs exposed to 0.01, 0.1, and 1.0 μg/ml cortisol for 4 hours. The abundance of glycosylated HC-3 increased by 0.8-fold for RBCs treated with 0.01 μg/ml cortisol for 8 hours. A variation in HC-3 protein abundance was observed for freshly isolated RBCs. The subcellular localization and fluorescent intensity (arbitrary units) of the HC-3 protein were analyzed via scanning laser confocal microscopy and immunocytochemistry using ImageJ software. Perinuclear localization of the HC-3 protein was observed in RBCs exposed to 0.1 and 1.0 μg/ml cortisol for 4 hours and membrane localization for RBCs exposed to 0.1 μg/ml cortisol for 8 hours. Fluorescent analysis of RBCs exposed to 0.01 and 1.0 μg/ml cortisol for 8 hours exhibit enhanced HC-3 intensity in the cytosol compared to control RBCs. Therefore, there is a potential correlation between cryoprotective glycerol, freeze tolerance, and the role of cortisol in regulating HC-3 protein expression and subcellular localization.

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Chapter 1: Introduction and Literature Review

Physiological Homeostasis and Osmoregulation

The ways in which plants and animals continually defend and sustain essential physiological variables is defined as homeostasis (Cannon 1929). An organism striving to maintain physiological homeostasis has anticipatory mechanisms equipped to minimize disruptions and deviations of bodily standards (reviewed in Kuster et al. 2011). A stable internal environment accounts for steady bodily pH; temperature; osmolality; and concentrations of ions, hormones, and other physiologically active substances (Cannon 1929). The internal, biological environment essentially attempts to ubiquitously preserve a state of equilibrium through coordinated and orchestrated physiochemical signaling mechanisms (Cannon 1929).

Particularly, osmoregulation represents a physiological process that mediates bodily homeostasis. During this process, osmoreceptors detect variations in osmolality between the intracellular fluid (ICF) and extracellular fluid (ECF) of a cell (Bourque 2008). Osmoreceptors synchronize water retention patterns and are utilized to regulate thirst and vasopressin release, for instance (reviewed in Danziger and Zeidel 2015). Water flows across the plasma membrane of cells to account for differing ICF and ECF osmolalities; however, cellular structural integrity and biochemical systems might be affected due to differences in cell volume and ionic strength from changes in water flow (Bourque 2008).

One mechanism of osmoregulation refers to the osmotic flux of water molecules across a semipermeable membrane, also known as osmosis. Osmosis is the movement of a solvent, such as water, through a semipermeable membrane, a living cell for
example, into a solution of higher solute concentration to equalize the concentrations of solute on both sides of the membrane. A semi-permeable membrane partitions two fluids with differing osmolalities to permit the passage of water to the side of the membrane with a higher non-penetrating solute concentration (reviewed in Maher and Maenab 2018). Water molecules passively move through the semipermeable phospholipid bilayer of a cell. The two hydrophobic, hydrocarbon tails and singular hydrophilic head on a phospholipid confer the structural integrity of a biological cell membrane in which water can pass via osmosis (reviewed in Waterhouse and Farmery 2018). An additional method of osmoregulation includes facilitated diffusion which facilitates transmembrane movement via an integral membrane protein (reviewed in Waterhouse and Farmery 2018).

The Discovery of Aquaporins

Before the discovery of the integral membrane protein aquaporins (AQPs), scientists hypothesized the transport of water across hydrophobic membranes happened through non-specific leakage (reviewed in Finn and Cerdà 2011). In 1986, Benga and his group of scientists detected what is now considered AQP1 in the human erythrocyte plasma membrane (Benga et al. 1986). He addressed previous research that identified erythrocyte water permeability is significantly decreased after incubation with sulfhydryl reagents $p$-chloromercuribenzoate (PCMB) and $p$-chloromercuribenzenesulfonate (PCMBS) (Benga 2003; Benga 2012; Benga 1986). Since the sulfhydryl reagent bound to the membrane, Benga and his team of scientists hypothesized that, “a minor membrane protein that binds PCMBS is involved with water transport” (Benga 2003).
Discovered at Johns Hopkins University in 1991, the first water channel CHIP28 (channel-like integral protein of 28 kDa) was identified by Peter Agre and his team of researchers (Preston and Agre 1991; Preston et al. 1993). CHIP28 was isolated from Rhesus blood group antigens and initially deemed as a contaminant among the Rh polypeptide. The 28 kDa polypeptide contaminant was known for water permeability and abundant within renal proximal tubules (Denker et al. 1988). An ex vivo injection of the CHIP28 transcript into Xenopus laevis oocytes trailed by exposure to osmotic pressure provided direct evidence of water channel function. The injected and control oocytes were compared and monitored for water permeability after contact with distilled water. Unlike the control oocytes where osmotic water permeability was unaltered, oocytes with CHIP28 RNA injections “swelled and ruptured” (Preston et al. 1992). The Human Genome Organization officially rendered CHIP28 as AQP1 (Agre 1997). Agre was awarded the Nobel Prize in Chemistry in 2003 for the discovery of AQP1 (Agre 2003). AQP1 is a member of the membrane intrinsic proteins (MIPs) constituting integral membrane channel proteins that expedite the transport of small solutes and water according to amino acid homology to the lens fiber (reviewed in Krane and Goldstein 2007; reviewed in Abascal et al. 2014).

Integral membrane proteins, such as the AQP water channels, are embedded within the amphipathic phospholipid bilayer. AQPs are used for the rapid and regulated movement of water, compared to osmosis, the much slower method of trafficking water. Biological plasma membranes with AQPs are 100X more permeable to water compared to membranes without AQPs (reviewed in Gena et al. 2011). Since Peter Agre and his coworkers unearthed the AQP, PubMed had chronicled over 6000 articles with the tag
“aquaporin,” a publication rate of about one scientific paper per day (reviewed in Alleva et al. 2012).

**Aquaporin Structure and Function**

The structure of AQP1 was uncovered by colleagues of Agre at Kyoto University in Japan and at the University of Basel in Switzerland (Smith and Agre 1991). Purification methods were simplified through the pre-extraction of membrane vesicles with N-lauroylsarcosine and reconstituted into lipid bilayers by dialysis. Uniform lattices were formed while the protein withstood 100% of its water transporting function. The structure was clearly visible using 3D electron microscopy due to the electron density map produced (Walz et al. 1994).

Being a member of the MIP superfamily, the AQP is distinguished by an integral membrane pore through the biological lipid bilayer (reviewed in Krane and Goldstein 2007). The hourglass structural model of the AQP illustrates a subunit in the homotetramer that retains an individual aqueous pore (Kozono et al. 2002). There are six tilted transmembrane domains with intracellular cytosolic amino and carboxy termini oriented 180° to each other (Kozono et al. 2002; reviewed in Krane and Goldstein 2007). Each monomer contains six transmembrane helical domains, H1-H6 and the helical segments HE and HB (reviewed in Verkman 2011). Each of the four monomers convene to yield a tetramer within the cell membrane. Water selectivity varies from channel to channel partially due to electrostatics and steric factors (Verkman and Mitra 2000). Two tandem sequence repeats each encode three transmembrane helices with a hemipore connecting the second and third helices (reviewed in Kozono et al. 2002). The hemipore
loops equip an Asn-Pro-Ala (NPA) motif on opposite sides of the lipid bilayer (reviewed in Krane and Kishore 2003; reviewed in Krane and Goldstein 2007).

Depending upon the physiological stimuli, the transmembrane flux of water may require a flow that enters or exits the cell; therefore, the water-selective channel must account for the bi-directionality of water flow (reviewed in Agre and Kozono 2003). The Grottnus effect occurs, or an exceptionally profuse conduction of protons, when the columns of water molecules are linked by hydrogen bonds. Similarly to the effect of conducting electrons through a copper wire, the hydrogen bonding of water allows for the steadfast exchange of protons between water molecules in bulk solution; thus, the water molecules pass through the pore leaving the protons behind (Roux et al. 1996). The water molecules pass through the pore in single file without hydrogen bonding to each other (reviewed in Agre and Kozono 2003).

**Aquaporin Evolution**

Describing the animal evolution of water usage, physiologist Robert Pitts stated, “Crawling out on dry land…terrestrial forms were faced with…opposite problems…with respect to water. Fluid conservation, rather than fluid elimination, was the major concern. Terrestrial vertebrates modified and amplified their existing systems to salvage precious water…” (Pitts 1968). An enhanced understanding of the diverse array of functions and morphologies associated with the MIP superfamily can be better understood through an evolutionary framework.

The MIP superfamily is divided into two subfamilies, the AQP water channels and aquaglyceroporin (GLP) channels (reviewed in Abascal et al. 2014). Among these two subfamilies, evolutionary history indicates increased diversity and complexity due to
gene duplication and divergence events. Analyses of animal MIPs indicate that maximum diversity has been achieved for the total number of AQP subfamily paralogs (AQP0 to AQP12) due to several rounds of whole genome duplication in fish and land vertebrates (Zardoya 2005; Tingaud-Sequeira et al. 2010). The diversity of GLP paralogs (AQP3, 7, 9, and 10) within vertebrates is also seemingly due to successive rounds of entire genome duplication early in its evolutionary history (Zardoya 2005). Many animal and plant species express a variety of orthodox AQPs and GLPs. Yeasts generally have one or two orthodox AQPs and/or a GLP. *Escherichia coli* employs the glycerol transporter *GlpF* and the orthodox *AqpZ*, whereas archaea have only one AQP (reviewed in Gena et al. 2011). While many scientists hypothesize that AQP/GLP familial complexity has increased over the course of evolutionary history, other postulations however imply the ancient diversity of MIP families experienced many independent losses among unicellular eukaryotes, fungi, bacteria, archaea, and vertebrate, and invertebrate animals (reviewed in Abascal et al. 2014).

**Mammalian Aquaporins and Aquaglyceroporins**

Researchers have identified 13 AQPs (AQP0-12) in mammalian endothelia, epithelia, and other cell types (reviewed in Krane and Goldstein 2007; reviewed in Verkman 2011). The functional diversity of these water channels ranges from regulating brain-fluid homeostasis, triglyceride cycling between the liver and adipocytes, renal water balance, maintaining the structural integrity of the eye lens, and more. These AQPs are sized from 27 kDa (AQP8) to 37 kDa (AQP7) (Kitchen et al. 2015).

Overall, AQPs are classified into three categories, orthodox AQPs, GLPs, and unorthodox AQPs. The orthodox AQPs include AQP0, 1, 2, 4, and 5, which are only
permeable to water (reviewed in Abdel-Sater 2018). AQP0, for instance, is the foremost proteinaceous component of isolated lens junctions and maintains eye transparency (reviewed in Abdel-Sater 2018). Mutations of AQP0 are correlated with cataracts and defects of lens development (Shiels and Bassnett 1996). AQP1 is localized throughout the body but primarily in the kidneys, red blood cells (RBCs), lungs, liver, skin, peripheral nervous system, and central nervous system (CNS) (reviewed Abdel-Sater 2018). AQP1 null mice had defective renal water absorption and fluid secretion processes which decreased urine concentration (Ma et al. 1998). The vasopressin-regulated water transporter, AQP2, determines water permeability in the collecting ducts of the kidneys (Fushimi et al. 1993). During mammalian dehydration, AQP2 expression in the collecting duct improves water retention, and thus increases urine concentration (Rojek et al. 2006). This AQP is also expressed in the inner ear (Mhatre et al. 2002; Taguchi et al. 2007)). Another AQP, AQP4 is expressed in CNS astrocytes at the blood-brain and ependymal-CNS barriers. Water passes through these barriers in response to osmotic pressure gradients (reviewed in Verkman 2011). Furthermore, AQP4 is also responsible for reducing neural swelling and cytotoxic swelling in the spinal cord (Voituron et al. 2009). Isolated and characterized in salivary and lacrimal glands and alveolar pneumocytes, AQP5 potentially adjusts for the discharge of tears and saliva and humidifies the airways (King et al. 1997; Connolly et al. 1998). Mice deficient in AQP5 experienced defects in salivary gland secretion, compromised sweat gland function, and asthma (Krane et al. 2001; Krane et al. 2001; Nejsum et al. 2002).

The GLPs, AQP3, 7, 9, and 10, transport water and other organic compounds like glycerol, urea, glucose, and ammonia (reviewed in Hara-Chikuma and Verkman 2006).
Their pores are less constricted, compared to the AQPs (2.8 Å for GLP pore size vs 2.2 Å for AQP pore size), and more hydrophobic residues line the pore (Sui et al. 2001). The mammalian epidermis is hydrated by AQP3 and expressed in the conjunctiva of the eye, gastrointestinal and respiratory tracts, and spleen (reviewed in Gena et al. 2011; reviewed in Abdel-Sater 2018). Arsenite uptake is mediated through AQP7 and remains expressed in adipocyte cell membranes that are involved in fat and glucose metabolism (Ishibashi et al. 1997; Liu et al. 2002). Characterized by low permeability to water, AQP9 mediates arsenite uptake and has been isolated and characterized from the lungs, testis, liver, spleen, and brain astrocytes and ependymal cells (Liu et al. 2002; reviewed in Krane and Goldstein 2007). The movement of many non-charged particles is facilitated via AQP9, including urea, polyols, purines, and pyrimidines (Tsukaguchi et al. 1998). While its physiological function remains unknown, AQP10 lacks high permeability to water despite expression in the small intestine (reviewed in Krane and Goldstein 2007).
Figure 1. Three-Dimensional Structure of the Aquaporin 3 Homotetramer. Pictured above is an image of the AQP3 homotetramer from *X. laevis* produced using SWISS-MODEL (sequence generated from Schreiber et al. 2000).

The unorthodox AQP6s are classified as AQP6, 8, 11, and 12 with AQP11 and 12 having less well-known transport properties. Poorly permeable to water unless pharmacologically activated by Hg$^{2+}$, AQP6 is presumed to function as an anion channel allowing the passage of chloride and nitrate. This AQP is primarily restricted to intracellular regions, such as in the kidney collecting duct cells, where it may additionally function to contribute to intracellular vesicular swelling (Yasui et al. 1999; Ikeda et al. 2002). The protein AQP6 was cloned as a homolog from the human and rat kidney cDNA library, originally termed WCH3 and hKID (Ma et al. 1993). Named “aqua-ammoniaporin” for its ability to transport ammonia, AQP8 has been detected to channel hydrogen peroxide and urea in addition to water in hepatocytes (Calamita et al. 2001;
Saparov et al. 2007). Overall, AQP8 is expressed in a myriad of tissues of organs, including but not limited to the kidneys, liver, airway, gastrointestinal tract, salivary glands, and testis. Initially cloned in the mouse and rat, AQP8 is permeable to urea but not glycerol (reviewed in Takata et al. 2004). The superaquaporin AQP11 is detectable in retinas across a variety of animal species, considered to be a major retinal AQP (Amann et al. 2016). After evaluating AQP11 function in oocytes from *Xenopus*, water, glycerol, urea, and ion permeability were unable to be identified regardless of pH condition (Gorelick et al. 2006). Predominantly localized intracellularly, AQP12 is expressed in pancreatic acinar cells, though lacking expression in the plasma membrane of *Xenopus* oocytes which makes transport properties relatively difficult to characterize (Itoh et al. 2005). AQP11 and AQP12 only share 20% sequence homology with other MIP members (Gorelick et al. 2006).

**Anuran Aquaporins and Aquaglyceroporins**

Numerous terrestrial and semi-aquatic anurans experience evaporative water loss through their skin and intake water through their skin via puddles and other moist areas (reviewed in Ogushi et al. 2010). They reabsorb water from urine stored in the bladder and tubular fluid in the kidneys (reviewed in Suzuki et al. 2007). Considering the expression of AQPs in these osmoregulatory organs, it is hypothesized that AQPs play a key role in anuran adaptation to terrestrial life (reviewed in Suzuki et al. 2007; Suzuki and Tanaka 2010).

After phylogenetic analyses, anuran AQPs were split into six clusters: types 1, 2, 3, and 5 as well as the anuran-specific types a1 and a2, where the “a” designates anuran (reviewed in Suzuki et al. 2007; Suzuki and Tanaka 2010). Type-a1 AQPs are from
AQPx10 *X. laevis* oocytes and another *X. laevis* AQP. Type-a2 AQPs include orthologs AQP-h2, AQP-h3 from *Hyla japonica*, and AQP-t2 and AQP-t3 from *Bufo marinus* (Suzuki et al. 2007).

Other AQPs were discovered in a freeze tolerant anuran *Dryophytes chrysoscelis*, also known as Cope’s gray treefrog. HC-1, homolog of the mammalian AQP1; HC-2, homolog of the mammalian AQP2; HC-3, homolog of the mammalian AQP3; and HC-9, homolog of the mammalian AQP9 have been identified in *D. chrysoscelis* (Zimmerman et al. 2007; Stogsdill et al. 2017). The protein HC-1 is widely expressed among tissues though varies across amphibian species (reviewed in Pandey et al. 2010). The protein HC-2 was isolated from osmoregulatory organs like the skin, bladder, and kidneys (Zimmerman et al. 2007). The protein HC-9 was detected in the liver, stomach, bladder, fat, heart, brain, intestines, and other organs (Stogsdill et al. 2017). The GLP HC-3 is expressed broadly in tissues as well as erythrocytes from *D. chrysoscelis* (Zimmerman et al. 2007; reviewed in Pandey et al. 2010). Cold-acclimated *D. chrysoscelis* shows a greater abundance of native and glycosylated protein in comparison to warm-acclimated animals (Mutyam et al. 2011).

**Introduction to Freeze Tolerance**

While some species utilize hibernation, migration, or supercooling strategies upon the onset of winter weather, freeze tolerant organisms employ another survival adaptation to maintain homeostasis. Hibernation, migration, and supercooling strategies are preferred mechanisms of freeze avoidance among animals. Supercooling as a freeze avoidance strategy is endured to prevent freezing while sustaining temperatures below the equilibrium freezing point of their body fluids (reviewed in Storey and Storey 2017).
Fifty to sixty-five percent of extracellular water freezes into ice. Despite other simpler methods to seek asylum from the winter months, numerous species of arthropods, insects, soil invertebrates, reptiles, amphibians, bacteria, and other microbes employ freeze tolerant mechanisms (reviewed in Ramløv 2000; reviewed in Holmstrup 2014; reviewed in Storey and Storey 2017).

Specifically, the seven tenets of freeze tolerance represent the physiological conditions that must be fulfilled to successfully tolerate freezing. Primarily, ice formation is contained within extracellular compartments while lacking permeation into intracellular regions. Second, the supercooling point occurs where ice forms at temperatures slightly below the equilibrium freezing point. The triggering of ice formation ensues because of contact with environmental ice, epithelial contact with nucleators, or via the action of ice-nucleating proteins. Next, ice-binding proteins are present to ward cells from the damaging effects of ice crystal formation. Following, extracellular solute concentrations increase since solutes are excluded from ice crystal formation. Body fluid osmolality is thus dependent upon the freezing point of intracellular fluids. Lastly, cryoprotectants are released to combat the freezing of at least 50% of bodily fluids to resist cell lysing (reviewed in Storey and Storey 2017).

**Anuran Freeze Tolerance**

Currently, seven anurans are known to exhibit freeze tolerance: *Dryophytes versicolor* (North American tree frog), *Pseudacris crucifer* (spring peeper), *Pseudacris maculate* (boreal chorus frog), *Rana sylvatica* (wood frog), *Rana arvalis* (moor frog), and *Dryophytes chrysoscelis* (Cope’s gray tree frog). These anurans either live arboreally or on the forest floor surface covered with leaf litter, rocks, logs, and snow during freezing
(reviewed in Storey and Storey 1986; reviewed in Storey and Storey 2017). They endure prolonged freezing where ice content achieves equilibrium.

The fatal dangers of freeze tolerance risk potential ECF ice buildup causing dehydration, cell lysing, and a total loss of interorgan transport through the bloodstream (reviewed in Storey and Storey 2017). The cryoprotectants potentially used by anurans, such as glucose, urea, and glycerol, mediate osmotic pressure gradients from the freezing of extracellular free water. Cryoprotective solutes prevent permanent cell damage from osmotic lysis by regulating cell volume during freezing and thawing. Cryoprotectants eliminate the injurious effects of freezing, potentially synthesized from stored amounts of liver glycogen or adipocyte triglycerides which is converted to glucose via glycogenolysis during freezing for select freeze tolerant anurans, such as *Rana sylvatica* (Layne and Richard 1995; reviewed in Larson et al. 2014). Five freeze tolerant anurans employ glucose as a cryoprotectant, synthesized from hepatic glycogen stores. *R. sylvatica*, for instance, circulates glucose concentrations between 200 and 500 mM during freezing (Layne and Kefauver 1997; Costanzo et al. 2013; reviewed in Storey and Storey 2017). *D. chrysoscelis* mobilizes the cryoprotectants glycerol, glucose, and urea (Layne 1999; Zimmerman et al. 2007; do Amaral et al. 2018).

The model organism of this thesis, *D. chrysoscelis*, is native to central and southwestern Ohio and survives freezing with bodily temperatures as low as -8°C (Layne and Richard 1995). Considered semi-aquatic anurans, they start life as aquatic tadpoles and convert to terrestrial adults (Papadopoulos et al. 2008). Their freeze-thaw cycles correspond to seasonal climate changes (Layne and Richard 1995). *D. chrysoscelis* experiences a suspension of conventional physiological and biochemical functions,
including metabolic rate, heart rate, renal function, breathing, brain activity, and involuntary muscle contraction. Circulating glycerol concentrations have been recorded up to 100mM+ prior to freezing and greater during freezing conditions (Zimmerman et al 2007; Goldstein et al. 2010; do Amaral et al. 2018). The anticipatory mobilization of glycerol regulates cell volume and stabilizes biomolecules only to be reabsorbed and conserved by the kidneys (Zimmerman et al. 2007; Layne and Stapleton 2009; do Amaral et al. 2018). Glycerol is arguably a superior cryoprotectant to glucose due to compatibility with protein function, reduction of free radicals during anoxia, preservation of membrane fluidity, and maintenance of redox balance (do Amaral et al. 2018).

**Rationale for an Erythrocyte Cell Culture System**

The *in vitro* RBC culture system employed in this thesis is well-established in the literature (Mutyam et al. 2011) and more suitable to analyze the mechanism in which *D. chrysoscelis* prepares for cold-acclimation and glycerol synthesis and regulates HC-3 protein expression. HC-3 is highly expressed in the RBCs of *D. chrysoscelis*, enabling the researcher to regulate gene expression and observe the gene regulatory mechanisms that influence dynamic HC-3 expression (Zimmerman et al. 2007; Goldstein et al. 2010; Mutyam et al. 2011; Geiss 2018); therefore, researchers can observe the functional relevance at a molecular level that can be examined.

Additionally, the RBCs respond to environmental cues in culture (Mutyam et al. 2011). RBCs from *D. chrysoscelis* are nucleated and demonstrate active metabolic activities. Equipped with organelles and biomolecules, the researcher can observe functionality. The RBCs also possess a replenishing ability which deters the need to sacrifice any animals for samples.
The Structure and Function of Cortisol

Within the nuclear receptor superfamily, there are over 150 members spanning from worms to humans. Class I receptors are the receptors of interest for this thesis, which group together the steroid hormones, including cortisol (reviewed in Mangelsdorf et al. 1995). Cortisol is synthesized and secreted in a rhythmic and circadian manner though augmented upon stress. This lipid soluble hormone is commonly identified by its four linked carbon rings derived from cholesterol (reviewed in Charmandari et al. 2005). The transportation of cortisol throughout the bloodstream is facilitated by corticosteroid-binding globulin (reviewed in Oakley and Cidlowski 2013). Since cortisol is a lipid-soluble steroid in an aqueous environment, the carrier protein is required to transport the steroid to the lipid bilayer of the target cell of interest.

The structure of the glucocorticoid receptor (GR) contains three domains: the N-terminal transactivation domain, the central DNA-binding domain—which recognizes and binds target DNA sequences called glucocorticoid-responsive elements (GREs)—and the C-terminal ligand binding domain (reviewed Oakley and Cidlowski 2013). The GR transcription factor lies within the cytoplasm when inactive (reviewed in Fuller 1991).

The hypothalamic-pituitary-adrenal (HPA) axis regulates the secretion of the glucocorticoid hormone cortisol. Corticotropin-releasing hormone (CRH) in the hypothalamus stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary. ACTH receptors on the adrenal cortex bind ACTH which acts on the zona fasciculata within the adrenal cortex to synthesize and release cortisol. These tropic levels are insured with a negative feedback system, where an excess of cortisol can
potentially shut down the HPA axis thus preventing the overproduction of cortisol (reviewed in Charmandari et al. 2005).

Regarding the glucocorticoid signaling pathway, lipid-soluble cortisol passes through the membrane lipid bilayer. The stress hormone binds a GR and enters the nucleus furnished with a nuclear localization signal. The GR-cortisol transcription factor is pleiotropic, or in other words multi-functional (reviewed in Oakley and Cidlowski 2013). Once in the nucleus, the GR-cortisol transcription factor binds DNA on a GRE to repress/induce the transcription of target genes. GREs are present in a concentration-dependent manner (reviewed in Oakley and Cidlowski 2013). Clinically, cortisol is the most abundant endogenous anti-inflammatory and immunosuppressive glucocorticoid. This stress hormone is typically prescribed to treat redness, itching, discomfort, skin conditions, and autoimmune disorders (reviewed in Oakley and Cidlowski 2013).

**Stress Responses and Cortisol**

Based on results from previous studies, cortisol is a known neuroendocrine hormone shown to respond to dehydration challenges, mediate between hypometabolism and the need for energy, aid in osmolarity challenges, and regulate AQPs and GLPs. Regarding long-term physiological stress, cortisol was demonstrated to be present in high concentrations during dehydration. In humans, physical exercise places the body under physiological stress. Studies suggest that cortisol concentrations increase when fluid intake is restricted (Costello et al. 2018). An enhanced hydration state, and decrease in physiological stress, correlates to lower bodily cortisol concentrations; whereas, bodily cortisol concentrations increase during dehydration stress. Cortisol fluctuations also mirror the changes in aldosterone and reflect the increased physiological strain resulting
from hypovolemia—which freeze tolerant anurans, such as *D. chrysoscelis*, potentially experience during freezing and thawing (Costanzo et al. 2013; van Rosendal et al. 2015).

The glucocorticoid hormone cortisol is generally released in response to low blood glucose levels, mediating metabolic rate depression and the need for energy (Muthuraman et al. 2014). Cortisol secretion enhances lipolysis in peripheral tissues and promotes glycogenolysis and gluconeogenesis in the liver. In rat adipose tissue, glucocorticoids increased the activity of lipolytic agents to activate triglyceride lipolysis (Ottosson et al. 2000).

During the life cycle of a teleost fish, a metamorphosis transpires where lampreys, eels, and salmons migrate from marine freshwater (FW) to the saltwater (SW) oceans or more upstream (Listiyani et al. 2018). In hypotonic environments, such as FW, the organism intakes water via osmosis and loses Na\(^+\) and Cl\(^-\) ions through diffusion. In hypersaline environments, like SW, the teleost loses water and stores Na\(^+\) and Cl\(^-\) ions (Hourdry 1995). These fish undergo multifaceted morphological, physiological, and behavioral changes as well. This smoltification stage for teleost fish is regulated by cortisol. Commonly referred to as the “SW-adapting hormone” in teleost fish, cortisol is strongly correlated with maintaining internal electrolyte balance when adapting to SW environments (Choi et al. 2013). Transferring the teleostean European eel *Anguilla anguilla* into SW, for instance, induced a transient increase in circulating cortisol concentrations (Martinez et al. 2005). Smolting increase salinity tolerance due to the Na\(^+\)/K\(^+\)-ATPase (Choi et al. 2013). Increased Na\(^+\)/K\(^+\)-ATPase activity in the gill epithelium of *A. anguilla* osmotically regulates solute concentration in the hypersaline
environment (Martinez et al. 2005). The master control hormone to initiate the activity of smolting, and thus Na\(^+\)/K\(^+\)-ATPase activity, is cortisol.

As the “SW-adapting hormone” in teleost fish, cortisol is also strongly correlated with mediating osmotic pressure gradients via AQPs when adapting to SW environments (Hourdry 1995). Increased AQP3 mRNA expression was observed in the intestinal tissue of sockeye salmon _Oncorhynchus nerka_ upon SW adaptation, mimicked through cortisol exposure (Choi et al. 2013). The protein AQP3 in the intestines provides more control over the inflow of salt through water absorption (Aoki 2003). Scientists also hypothesize that AQP3 may act as a conduit in the basolateral membrane for the release of water to the serosal fluid and the prevention of cell swelling (Cutler and Cramb 2002). This expression pattern with AQP3 was similar for _A. anguilla_; the gilthead sea bream, _Sparus aurata_; and the European sea bass, _Dicentrarchus labrax_ (Choi et al. 2013). Particularly for _D. labrax_, a several fold increase in AQP3 expression in the gill filaments was observed (Giffard-Mena et al. 2007). Increased AQP3 mRNA expression in the kidneys was also observed for tilapia (_Oreochromis mossambicus_) and Atlantic salmon (_Salmo salar_) (Watanabe 2005; Tipsmark et al. 2010; Choi et al. 2013). The mRNA expression of AQP8 peaked during smoltification of _S. salar_ in the intestinal tissue which implies ammonia absorption from intestinal chyme (Handlogten et al. 2005; Tipsmark et al. 2010).

The protein AQP1 is the membrane water channel responsible for fast water changes ubiquitously expressed in the kidneys, gills, and intestines of teleost fish (Choi et al. 2013). Cortisol release was correlated with increased AQP1 mRNA expression in the basolateral membrane of gill chloride cells in _A. anguilla_ (Hourdry 1995). Decreased
Aqp1b, isoform of mammalian AQP1, transcripts were observed across the apical brush border of a subset of tubules among renal epithelia in the kidneys after FW to SW transfer among the American eel Anguilla rostrate (Martinez et al. 2005). The decrease in expression may suggest that although teleost Aqp1b seems to specialize in water uptake, other osmoregulatory roles such as water absorption across the rectal epithelium alter expression patterns in somatic tissues (Raldua et al. 2008). Other hypotheses dictate that select cells and tissues become “tighter” in relation to the osmotic flux of water upon SW adaption; hence, there is a reduced need for a high glomerular filtration rate and tubular ion and water transport implemented during diuresis. Cortisol infusion in the FW-adapted silver eel Arisoma mellissii induced an increase in AQP1 expression in esophageal cells. Cortisol mediates the AQP1 concentrations expressed to potentially protect the epithelial cells from the damaging effects of shrinking and swelling due to water filtration across the lumen (Martinez et al. 2005).

The Stress Response in Anurans

Corticosterone—a glucocorticoid stress hormone derivative of cortisol in semi-aquatic amphibians—is structurally similar to cortisol without the extra hydroxyl group (Figures 2A and 2B; Buchholz 2015). Cortisol promotes glycogenolysis and gluconeogenesis in the liver which could explain how freeze tolerant anurans like D. chrysoscelis have the fuel reserves without feeding while frozen. Since cortisol release is correlated with increased lipolysis, this could be potentially occurring in peripheral tissues to support the production of cryoprotectants during freezing and thawing in addition to glycogenolysis or gluconeogenesis.
Figure 2. Structures of Cortisol and Corticosterone. A) Structural formula of cortisol generated using MolView. B) Structural formula of corticosterone generated using MolView.

**Tissue Cryopreservation**

The accidental discovery of cryoprotection by C. Polge, A.U. Smith, and A.S. Parks in 1948 demonstrated that fowl spermatozoa survive subzero temperatures, up to -70°C, upon glycerol exposure (Polge et al. 1949). It is now considered the classical freezing approach, but since the initial discovery of cryoprotective methods, cryoprotective agents (CPAs) and other variables have been manipulated to preserve the viability of biological cells and tissues. Cryoprotective concentration, temperature, rate of cryoprotectant removal, and cell/tissue type represent some of the manipulated conditions. CPAs, such as glycerol, as implemented by Cope’s gray treefrog; DMSO; dimethyl sulfoxide; and propanediol alter freezing behavior. Water transport, nucleation, and ice crystal growth are also affected in the process (reviewed in Pegg 2002; reviewed in Jang et al. 2017). The following procedure outlines the integration of CPAs in cells or tissues: (1) mixed with cells or tissues before cooling, (2) the cells or tissues are cooled,
(3) the cells or tissues are warmed, and (4) CPAs are removed from the cells or tissues after thawing (reviewed in Jang et al. 2017).

Among the grand diversity of cryoprotective biomedical applications, cryosurgery and *in vitro* fertilization (IVF) are common (reviewed in Jang et al. 2017). Cryosurgery involves using intense cold temperatures to destroy diseased tissue. A cryoprobe introduces liquid nitrogen as a freezing agent to treat the diseased tissue (Allington 1950). IVF exemplifies a reproductive medical technology that cryopreserves mature oocytes and improves reproductive capacity (Mazur 1970). Largely, cryopreservation is a stepping stone toward tissue engineering and tissue regeneration (reviewed in Jang et al. 2017).

The limitations of cryoprotection primarily involve CPA toxicity, which otherwise breach or damage cell membrane function and/or impair enzyme function (Best 2015). For example, 10 ml of 50% glycine per kilogram induces renal failure in rats through inflammation, oxidative stress, and apoptosis despite its nontoxic capabilities at lower concentrations (Korrapati et al. 2012). N-acetylcysteine attenuates glycerol-induced kidney injury by regulatory MAPKs and Bcl-2 family proteins at concentrations deemed too high (Kim et al. 2010). Cell-associated deviations in lipids and proteins could potentially result in the impairment of cellular activity and structure (reviewed in Jang et al. 2017).

**Cortisol and Transplantation**

Corticosteroids, such as cortisol, have been administered as necessary agents for immunosuppression regimens practically since the inception of clinical transplantation (Bell et al. 1971). Some of the first heart transplant patients received corticosteroid
treatments due to the anti-inflammatory and immunosuppressive effects (reviewed in Baran et al. 2018). Corticosteroid treatment after transplantation is utilized to prevent the rejection of a transplanted tissue or organ; however, corticosteroid administration suppresses the HPA axis and potentially causes negative biochemical and physiological side effects, such as adrenal insufficiency from chronic steroid therapy (reviewed in Baran et al. 2018).

**Hypothesis**

Previous research by Mutyam et al. 2011 demonstrating increased HC-3 membrane expression in the RBCs of cold-acclimated tree frogs establishes potential connections between freeze tolerance and AQP expression in *D. chrysoscelis*. Previous results indicate that there are correlations between cortisol regulation and AQP expression, specifically among teleost fish (Choi et al. 2013, Cutler et al. 2002, Giffard-Mena et al. 2007, Handlogten et al. 2005, and Martinez et al. 2005). Cortisol aids in osmolarity challenges, dehydration stress, metabolic challenges, and regulates AQPs and GLPs, which are predominantly all tenets of freeze tolerance experienced among freeze tolerant anurans like *D. chrysoscelis*. Based upon these observations, we hypothesize that a potential relationship exists between cortisol regulation, freeze tolerance, and GLP expression within *D. chrysoscelis*. The focus of this thesis is to further explore the regulatory mechanisms of HC-3 protein expression in *D. chrysoscelis* erythrocytes. In this thesis, a well-established *in vitro* cell culture system is used to explore the potential cortisol-induced regulatory mechanisms of HC-3 expression.

In total, renowned physiologist August Krogh, who developed the Krogh Principle, once stated, “the route by which we can strive toward the ideal is by the study
of the vital functions in all their aspects throughout the myriads of organisms” (Krogh 1929). The Krogh Principle is applicable to *D. chrysoscelis* and the study of freeze tolerance as a model for understanding human organ cryoprotection and transplantation.
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Chapter 2: Cortisol Regulation of Aquaglyceroporin HC-3 Protein Expression in Erythrocytes from the Freeze Tolerant Tree Frog *Dryophytes chrysoscelis*

**Abstract**

*Dryophytes chrysoscelis*, commonly known as Cope’s gray treefrog, is a freeze tolerant anuran that freezes up to 65% of extracellular fluid during winter to survive. Glycerol is presumably used as a cryoprotectant during a period of cold-acclimation to protect cells from permanent damage due to hypoosmotic stress upon freezing and thawing. The passage of glycerol and water during cold-acclimation is mediated through aquaglyceroporin (GLP) 3 ortholog HC-3 in the nucleated erythrocytes (RBCs) of *D. chrysoscelis*. This thesis analyzes the mechanisms in which *D. chrysoscelis* prepares for cold-acclimation and glycerol synthesis. Cortisol, a neuroendocrine steroid hormone, is a stress hormone known to respond to osmolarity and metabolic challenges and regulate aquaporins (AQPs); however, the role of cortisol in regulating anuran HC-3 protein expression and subcellular localization, and implications for mediating anticipatory glycerol synthesis and freeze tolerance remain to be determined. We hypothesize that cortisol exposure regulates HC-3 protein expression and subcellular localization. Freshly isolated RBCs were cultured in complete cell culture media (CCCM) or CCCM + cortisol (0.1 or 1.0 μg/ml) for 0, 2, 4, and 6 hours. Another group of RBCs was incubated in CCCM for 24 hours prior to exposure to cortisol (0.01, 0.1, or 1.0 μg/ml for 0, 4, or 8 hours). An inter-individual variation in HC-3 protein abundance was observed for freshly isolated RBCs. The RBCs incubated for 24 hours prior to 0.01, 0.1, or 1.0 μg/ml
cortisol exposure for 4 hours showed an increase in native and glycosylated HC-3 protein abundance with cortisol relative to no cortisol exposure. The enhanced abundance of native and glycosylated HC-3 seen at the 4-hour exposure declined by the 8-hour exposure to cortisol, except for the 0.01 μg/ml cortisol culture. These data suggest that there is a time-dependence of cortisol-enhanced HC-3 protein expression for RBC cultures treated with 0.01, 0.1, and 1.0 μg/ml cortisol. The 0.01 μg/ml cortisol culture showed enhanced native and glycosylated HC-3 protein abundance from 4 to 8 hours, by 2.4-fold and 0.8-fold respectively. Immunocytochemistry analysis revealed perinuclear localization of the HC-3 protein in RBCs exposed to 0.1 and 1.0 μg/ml cortisol for 4 hours and plasma membrane localization for RBCs exposed to 0.1 μg/ml cortisol for 8 hours. The results of this study show that cortisol regulates HC-3 translocation from the cytoplasm to the perinuclear region and plasma membrane of the cell. Thus, these data suggest that cortisol regulates HC-3 protein abundance, glycosylation state, and subcellular localization in *D. chrysoscelis* RBCs.

**Introduction**

Compared to other organisms that implement hibernation, migration, or supercooling strategies upon the onset of winter weather, the freeze tolerant *Dryophytes chrysoscelis*, also known as Cope’s gray treefrog, suspends conventional physiological and biochemical functions and tolerates freezing of up to 65% of its extracellular fluid (reviewed in Storey and Storey 2017). Among the seven known freeze tolerant anurans *D. chrysoscelis* is native to central and southwestern Ohio, surviving freezing with bodily temperatures as low as -8°C (reviewed in Layne and Richard 1995). Anticipatory mobilization of the cryoprotectant glycerol regulates cell volume and stabilizes

Utilized for the rapid and regulated movement of water, aquaporins (AQPs) are present in osmoregulatory organs and tissues. The major intrinsic protein family (MIP) is divided into two subfamilies, the AQP water channels and aquaglyceroporin (GLP) channels (reviewed in Abascal et al. 2014). The GLPs transport water and other organic compounds like cryoprotective glycerol, urea, glucose, and ammonia (reviewed in Storey and Storey 2017).

Four AQPs were identified and characterized from *D. chrysoscelis* HC-1, HC-2, HC-3, and HC-9, orthologs of respective AQP1, AQP2, AQP3, and AQP9. HC-1 and HC-2 are considered AQPs whereas HC-3 and HC-9 function as GLPs (Zimmerman et al. 2007; Stogsdill et al. 2017). The GLP HC-3 is the only known protein to be expressed in erythrocytes (RBCs). RBCs cultured with glycerol regulate membrane trafficking of HC-3 from the cytosol of the plasma membrane similarly to enhanced membrane expression in RBCs from cold-acclimated frogs which naturally accumulate glycerol in vivo during cold-acclimation (Mutyam et al. 2011). The HC-3 protein is highly expressed in cold-acclimated tree frogs versus warm-acclimated tree frogs, implicating the involvement of HC-3 for the transmembrane flux of water and glycerol during freezing and thawing (Goldstein et al. 2010; Mutyam et al. 2011). However, the gene regulatory mechanisms involved in acclimation-state responsive protein expression are not yet known.
The neuroendocrine steroid hormone, cortisol, is synthesized and secreted in a rhythmic and circadian manner though augmented upon stress (reviewed in Charmandari et al. 2005). Based on results from previous studies, animals have been shown to respond to dehydration by releasing cortisol, mediate metabolic and osmolarity challenges, and regulate AQPs and GLPs, which are all predominantly tenets of freeze tolerance experienced among freeze tolerant anurans like *D. chrysoscelis*. There are relationships between freeze tolerance and AQP expression, specifically among anurans. Cortisol has been shown to regulate AQP expression, specifically among teleost fish (Cutler and Cramb 2002; Handlogten et al. 2005; Martinez et al. 2005; Giffard-Mena et al. 2007; Choi et al. 2013). In this study, we examined the effect of cortisol exposure on HC-3 protein expression and subcellular localization in the RBCs from *D. chrysoscelis*. Specifically, we examined HC-3 expression, the abundance of native and glycosylated protein and the subcellular localization in response to cortisol concentration and time. Results from this present study can provide insight for understanding the regulatory elements involved in HC-3 expression and localization.
Methods and Materials

Animals

Collected from Caesar Creek State Park in southwestern Ohio, treefrogs were transferred to Wright State University (WSU) Laboratory Animal Research Center in Dayton, OH. Methods of ethical handling and animal upkeep were approved by the WSU Institutional Animal Care and Use Committee. The warm-acclimated gray tree frogs used in these experiments were fed crickets three times per week or based upon physiological need, exposed to naturally occurring diurnal cycles, individually housed, and kept at ambient temperatures of 21°C.

Erythrocyte Complete Cell Culture System

Blood was drawn from the brachial artery of warm-acclimated gray tree frogs. Blood was collected in a heparinized capillary tube and placed into a 15 ml conical tube supplemented with 10 ml complete cell culture media (CCCM: DMEM 1X; 10-014-CV). The samples were transferred from WSU to the University of Dayton in a styrofoam container to prevent temperature fluctuations. The conical tube was centrifuged at 1000 x g for 15 minutes to remove the supernatant and resuspended in 10 ml CCCM. Cells were counted using a hemocytometer and resuspended at a concentration of 1 million cells/ml in 25 cm² Corning flasks with CCCM. The protocol for keeping the erythrocytes nourished, sterile, and protected outside of the anurans’ bodies in vitro as previously described (Mutyam et al. 2011). Erythrocytes were divided into two separate groups: 24-hour incubation in culture at 21°C before cortisol exposure or exposure to cortisol upon fresh isolation. Cortisol concentrations and time course were chosen based on published research (Breves et al. 2016; Khansari et al. 2017). The incubated erythrocytes were
cultured with 0, 0.01, 0.1, or 1.0 μg/ml cortisol for 0, 4 or 8 hours. Media was replenished and viability was quantified with Trypan blue exclusion dye at time points 0, 24, 28, and 32 hours. The freshly isolated erythrocytes were immediately exposed to 0, 0.1, or 1.0 μg/ml cortisol for 2, 4, or 6 hours. Media was replenished and viability was quantified with Trypan blue exclusion dye at time points 0, 2, 4, and 6 hours.

**Protein Isolation and Quantitative Analysis**

The RBC proteomes were isolated by first collecting 1x10^6 cells/ml from cultures at all time points and cortisol concentrations among incubated and freshly isolated samples. The RBCs were centrifuged for 10 minutes at 1000 x g and lysed with three consecutive freeze-thaw cycles consisting of two minutes on dry ice and two minutes at 37°C. A bicinchoninic acid protein assay (BCA) was performed using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific), quantifying the protein in the RBCs.

**Western Blotting Analysis**

Western blotting analysis was utilized for quantifying HC-3 protein expression through previously established methods (Mutyam et al. 2011). Twenty μg of total RBC protein was size-fractionated by SDS-PAGE (4-20% Mini-PROTEAN TGX Precast Protein Gels, BioRad) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (SequiBlot, BioRad). Equivalency of protein concentration and loading, and protein integrity was determined by visual inspection and densitometric analyses of Ponceau S stained membranes (ThermoFisher Scientific). Overnight Western hybridization was performed using a peptide-derived, rabbit polyclonal antibody against HC-3 (0.44 μg/ml; (Goldstein et al. 2010) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution, Cell Signaling Technology).
Immunoreactive signals were detected using a chemiluminescent substrate (GE Life Sciences) and visualized on X-ray film with multiple exposures.

**Immunocytochemistry**

Immunocytochemistry analysis was employed to visualize the subcellular localization of the HC-3 protein in cultured erythrocytes as previously described (Mutyam et al. 2011). RBC samples were collected at a concentration of 1x10⁶ cells/ml from cultures at all time points and cortisol concentrations among incubated and freshly isolated samples. RBC samples were centrifuged at 1000 rcf at 4°C for 10 minutes, the supernatant was removed, and the samples were resuspended in 1X PBS and centrifuged at 1000 rcf at 4°C for 10 minutes. After removing the supernatant, the RBC samples were resuspended in PLP solution and kept on a shaker at 190rpm at 20°C for 20 minutes. The RBC samples were centrifuged at 1000 rcf at 4°C for 10 minutes, the supernatant was removed, and the samples were resuspended in 1X PBS. The cycle of centrifugation, supernatant removal, and resuspension in 1X PBS was followed twice more. Five μl cell suspensions were fixed on gelatin-coated slides and dried at room temperature. Slides were washed and labeled with a peptide-derived, rabbit polyclonal antibody against HC-3 (0.2 μg/ml in 1% blocking serum) then with a goat anti-rabbit fluorescein-conjugated secondary antibody (Vector Laboratories) with a 1:1000 dilution in 1% blocking serum. Immunofluorescence was analyzed via the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

**Statistics**

The abundance of native and glycosylated HC-3 protein expression (normalized to Ponceau S staining) from the incubated and freshly isolated RBCs cultured in cortisol are
represented as fold-changes of normalized native and glycosylated HC-3 protein expression. Protein abundance was quantified using densitometry analysis on UVP software for each of the following culture conditions after 24-hour incubation at 21°C—0, 0.01, 0.1, and 1.0 μg/ml cortisol for 4 and 8 hours—where n=1 for each condition. Protein abundance from freshly isolated RBCs was quantified using densitometry on UVP software for each of the following culture conditions—0, 0.1, and 1.0 μg/ml cortisol for 2, 4, and 6 hours—where n=3 for each condition. Image J software was utilized to quantify the immunofluorescent intensity of the HC-3 protein along a cross-sectional line in confocal images of immunostained RBCs. Data points were graphed based upon lines that were drawn across each of the erythrocytes from each condition (4-hour and 8-hour exposures to 0, 0.01, 0.1, and 1.0 μg/ml cortisol after 24-hour incubation at 21°C in culture) using Image J software (n=2-11 cells per experiment).

**Results**

**Increased Native and Glycosylated HC-3 Abundance in RBCs Cultured in Cortisol After 24-Hour Incubation and a Variation in Native and Glycosylated HC-3 Abundance for Freshly Isolated RBCs**

The previously established erythrocyte (RBC) complete cell culture system indicated that native and glycosylated HC-3 protein expression differs depending on time spent in culture and media contents (Mutyam et al., 2011). This current study employed Western blotting to analyze native and glycosylated HC-3 protein abundance from RBCs in culture treated with 0, 0.01, 0.1, and 1.0 μg/ml cortisol for 4 and 8 hours after 24-hour incubation at 21°C (Fig. 1). Compared to 4-hr controls, native HC-3 protein expression increased by 3.7-fold for RBCs treated with 0.01 μg/ml cortisol, by 40-fold for RBCs
treated with 0.1 μg/ml cortisol, and by 21-fold for RBCs treated with 1.0 μg/ml cortisol for 4 hours. Native HC-3 protein expression increased by 6.1-fold for RBCs treated with 0.01 μg/ml cortisol for 8 hours, compared to the 4-hour control sample. Native HC-3 protein expression decreased by 0.8-fold for RBCs treated with 0.1 μg/ml cortisol and by 0.5-fold for RBCs treated with 1.0 μg/ml cortisol for 8 hours, compared to the 4-hour control sample (Figs. 1, 2).

Glycosylated HC-3 protein expression (60-150 kDa) from RBCs cultured with 0.01 μg/ml cortisol for 4 hours increased by 3.1-fold, compared controls (Fig. 3). RBCs treated with 0.1 and 1.0 μg/ml cortisol for 4 hours showed 5.8-fold and 5.3-fold increases in glycosylated HC-3 protein expression as compared to the 4-hour control (Fig. 3). Glycosylated HC-3 protein expression from RBCs cultured with 0.01 μg/ml cortisol increased by 3.9-fold, by 2.6-fold for RBCs cultured with 0.1 μg/ml cortisol, and by 2.4-fold for RBCs cultured with 1.0 μg/ml cortisol for 8 hours, compared to the 4-hour control sample (Fig. 3).
Figure 1. Western blotting to determine relative HC-3 native and glycosylated protein abundance. Proteins were isolated from RBCs cultured with 0, 0.01, 0.1, or 1.0 μg/ml cortisol for 4 and 8 hours after 24-hour incubation at 21°C in culture. Ponceau S staining was used as the gel loading control.
Figure 2. HC-3 native protein expression during 4-hour and 8-hour exposures to cortisol after 24-hour incubation at 21°C. Densitometric analysis of immunoblots specific for HC-3 showed a 3.7-fold increase in native HC-3 (60-120 kDa) from RBCs cultured 0.01 μg/ml cortisol, 40-fold increase from RBCs cultured in 0.1 μg/ml cortisol, and 21-fold increase from RBCs cultured in 1.0 μg/ml cortisol upon 4-hour exposure after incubation for 24 hours at 21°C. Native HC-3 protein expression decreased for all cultures upon 8-hour exposure to cortisol in culture, except for the RBCs cultured in 0.01 μg/ml cortisol with a 2.4-fold increase from the 4-hour time point.
Western blotting was used to analyze native and glycosylated HC-3 protein abundance from freshly isolated RBCs in culture treated with 0, 0.1, and 1.0 μg/ml cortisol for 2, 4, and 6 hours at 21°C for n=3 frogs (Fig. 4). Compared to the RBCs
cultured in 0 μg/ml cortisol for 4 hours, an inter-individual variation in native and glycosylated HC-3 protein expression was observed across samples from the three frogs.

Figure 4. Western blots of HC-3 protein abundance from freshly isolated RBCs cultured with the addition of cortisol. RBCs were cultured in 0, 0.1, or 1.0 μg/ml cortisol for 2, 4, and 6 hours in culture compared to control proteins. The abundance of native and glycosylated HC-3 varied from frog 1 (A), frog 2 (B), and frog 3 (C). Ponceau S staining was used as the gel loading control.

(Figs. 4, 5, 6).
Cortisol-Enhanced HC-3 Membrane Localization is Time-and-Concentration-Dependent

Immunocytochemistry was performed on *D. chrysoscelis* RBCs exposed to 0, 0.01, 0.1, and 1.0 μg/ml cortisol after a 24-hour incubation at 21°C in culture to observe the effects on the subcellular localization of HC-3 within the RBCs. There was no difference in the immunofluorescence of HC-3, compared to the control cells, for RBCs cultured in 0.01 μg/ml cortisol for 4 hours (Fig. 6, 9A, 9B). However, fluorescent intensity analysis of cross sections of RBCs exposed to 0.01 μg/ml cortisol for 8 hours exhibit enhanced HC-3 intensity in the cytosol compared to control RBCs (Fig. 6, 9A, 9B).
Fluorescent intensity analysis of RBC cross sections exposed to 0.1 μg/ml cortisol for 4 hours demonstrate two fluorescently intense peaks with perinuclear localization (Fig. 7, 10B). Additionally, fluorescent analysis of RBCs exposed to 0.1 μg/ml cortisol for 8 hours demonstrate two fluorescently intense peaks which are localized to the membrane (Fig. 7, 10C). Cells cultured in CCCM and treated with 1.0 μg/ml cortisol for 4 hours exhibit less intense, perinuclear localization compared to control cells (Fig. 8, Table 1).

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<th>Time</th>
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**Figure 6.** HC-3 membrane localization is enhanced compared to HC-3 cytosolic localization in RBCs cultured with the addition of cortisol. RBCs were cultured in 0.01 μg/ml cortisol for 4 and 8 hours. Immunocytochemistry, scanning confocal microscopy, and ImageJ software were used to quantify HC-3 intensity under the various culture conditions.
11A, 11B). In addition, fluorescent analysis of RBCs exposed to 1.0 μg/ml cortisol for 8 hours exhibit enhanced HC-3 intensity in the cytosol compared to control RBCs (Fig. 8, 11A, 11C).

<table>
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<th>Time</th>
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Figure 7. HC-3 membrane localization is enhanced compared to HC-3 cytosolic localization in RBCs cultured with the addition of cortisol. RBCs were cultured in 0.1 μg/ml cortisol for 4 and 8 hours. Immunocytochemistry, scanning confocal microscopy, and ImageJ software were used to quantify HC-3 intensity under the various culture conditions.
Figure 8. HC-3 membrane localization is enhanced compared to HC-3
cytosolic localization in RBCs cultured with the addition of cortisol. RBCs were
cultured in 1.0 μg/ml cortisol for 4 and 8 hours. Immunocytochemistry, scanning
confocal microscopy, and ImageJ software were used to quantify HC-3 intensity
under the various culture conditions.
Figure 9. Image J analysis. Image J software was used to examine and graph HC-3 immunofluorescence intensity across the *D. chrysoscelis* RBCs cultured in 0.01 μg/ml cortisol for 4 hours (B) and 8 hours (C) compared to 0 μg/ml cortisol for 4 hours (A).
Figure 10. Image J analysis. Image J software was used to examine and graph HC-3 immunofluorescence intensity across the *D. chrysoscelis* RBCs cultured in 0.1 μg/ml cortisol for 4 and 8 hours.
Figure 11. Image J analysis. Image J software was used to examine and graph HC-3 immunofluorescence intensity across the *D. chrysoscelis* RBCs cultured in 1.0 μg/ml cortisol for 4 and 8 hours.
**Discussion**

Among the four AQPs identified and characterized from *D. chrysoscelis*, HC-3 is the only known GLP expressed in nucleated RBCs. It is suggested that *D. chrysoscelis* regulates the transmembrane flux of water and cryoprotective glycerol through the GLP 3 ortholog HC-3 protein; thus, contributing to the freeze tolerant mechanisms of *D. chrysoscelis* (Zimmerman et al. 2007; do Amaral et al. 2018). Further studies observed that RBCs from cold-acclimated treefrogs have an increased abundance of HC-3 compared to RBCs from warm-acclimated treefrogs, which contributes to the treefrogs’ inherent freeze tolerant capability. Culturing the RBCs with glycerol using the in vitro erythrocyte complete cell culture system resulted in HC-3 protein expression and subcellular localization patterns akin to those discovered in cold-acclimated treefrogs (Mutyam et al. 2011). Based upon previous research, cortisol is termed the “saltwater (SW)-adapting hormone” in teleost fish strongly correlated with mediating osmotic pressure gradients via AQPs when adapting to SW environments (Hourdry 1995). Cortisol stabilizes these osmotic pressure gradients by regulating AQPs/GLPs in teleost fish, including AQP3 among others, which suggests cortisol may also be involved in regulating HC-3 protein expression (Cutler and Cramb 2002; Handlogten et al. 2005; Martinez et al. 2005; Giffard-Mena et al. 2007; Choi et al. 2013).

This study employed the previously-established erythrocyte complete cell culture system to further explore the potential cortisol-induced regulatory mechanisms of HC-3 expression. Specifically, this study aimed to characterize the regulatory mechanisms of HC-3 protein expression and subcellular localization and explore the potential correlation between cortisol exposure and HC-3 protein expression.
In this study, *D. chrysoscelis* RBC cultures were exposed to different cortisol concentrations, implicating cellular physiological and biochemical conditions *in vivo*. Cortisol concentrations and timeframe variables were chosen based upon cortisol culture experiments established from previous research (Breves et al. 2016; Khansari et al. 2017). After 24 hours in culture at 21°C, RBCs cultured with 0.01, 0.1, and 1.0 μg/ml cortisol showed increased native and glycosylated HC-3 abundance upon 4-hour exposure to cortisol; particularly, 3.7-fold, 40-fold, and 21-fold for native HC-3 expression and 3.1-fold, 5.8-fold, and 5.3-fold for glycosylated HC-3 expression compared to control RBCs exposed to 0 μg/ml cortisol for 4 hours. The abundance of native HC-3 is enhanced by 6.1-fold for RBCs treated with 0.01 μg/ml for 8 hours, whereas cultures treated with 0.1 and 1.0 μg/ml cortisol for 8 hours had fold-decreases of 0.8-and-0.5-fold, compared to control RBCs exposed to 0 μg/ml cortisol for 4 hours. The abundance of glycosylated HC-3 is enhanced after exposure to 0.01, 0.1, and 1.0 μg/ml cortisol for 8 hours; particularly, 3.9-fold, 2.6-fold, and 2.4-fold compared to control RBCs exposed to 0 μg/ml cortisol for 4 hours.

Except for the culture treated with 0.01 μg/ml cortisol, the abundance of native and glycosylated HC-3 exposed to cortisol for 8 hours is less than the 4-hour cultures. These data suggest a time-dependent effect for RBC cultures exposed to cortisol concentrations of 0.1 and 1.0 μg/ml, where the abundance of native and glycosylated HC-3 decreases over time from 4 to 8 hours. The 0.01 μg/ml cortisol culture suggests a time-dependent effect where the abundance of native and glycosylated HC-3 increase over time from 4 to 8 hours. To our knowledge, this is the first study to correlate cortisol regulation and HC-3 protein expression.
In terms of the HC-3 protein abundance for the freshly isolated RBCs, an interindividual variation in protein abundance was observed. The treefrogs are wild-caught; therefore, treefrog age, genetic composition and previous environmental exposures among other variables are unknown. Additionally, the circulating levels of cortisol through their bloodstreams in vivo can vary from organism to organism upon time of blood draw, which could alter the cortisol concentration exposed in culture. Any variations in HC-3 protein abundance could be correlated to these important differences.

Moreover, data from immunocytochemistry analysis in this study suggests that cortisol regulates the translocation event of HC-3 from the cytoplasm to the perinuclear region of the cell. RBCs cultured in 0.1 and 1.0 μg/ml cortisol for 4 hours exhibited enhanced HC-3 perinuclear localization compared to control samples treated with 0 μg/ml cortisol for 4 hours that exhibited cytoplasmic HC-3 localization. These results, combined with the Western blotting analysis showing fold-increases in native and glycosylated HC-3 abundance, indicate that a cortisol-induced environment, specifically with a concentration of 0.1 and 1.0 μg/ml, leads to an increased abundance of native and glycosylated HC-3 and a greater amount of HC-3 in the perinuclear region. Perinuclear localization of specific proteins, potentially including HC-3, is dynamic and dependent upon cellular stress (Shaiken and Opekun 2014). Bearing this in mind, since cortisol is a stress hormone, and the action of freezing and thawing is a considerably stressful event, perinuclear localization of the HC-3 protein could potentially be part of the stress response in the freeze tolerant D. chrysoscelis.

Furthermore, the immunocytochemistry experimental results indicate that HC-3 plasma membrane localization is observed for RBCs treated with 0.1 μg/ml cortisol for 8
hours as compared to HC-3 cytosolic localization in RBCs cultured with the addition of 0 μg/ml cortisol for 4 hours. Combined with results from Western blotting analysis, where there was a 2.6-fold increase in glycosylated HC-3 when treated with 0.1 μg/ml cortisol compared to control cells with 0 μg/ml cortisol for 4 hours, cortisol exposure leads to a greater amount of HC-3 in the cell membrane. Overall, however, more research has to be conducted to determine whether the HC-3 perinuclear or plasma membrane localization is regulated by glycosylation.

Combined, these data suggest that cortisol regulates HC-3 protein abundance, glycosylation state, and subcellular localization in D. chrysoscelis RBCs, and may contribute to the physiological response that occurs naturally in the frog as a component of freeze tolerance. Understanding the gene regulatory mechanisms prompting dynamic HC-3 expression presents a model for comprehending tissue cryoprotection and tissue banking.

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Chapter 3: Future Directions and Broad Impacts

Future Directions

Indicated by previous research, erythrocytes (RBCs) from the freeze tolerant anuran *Dryophytes chrysoscelis* exhibit glycerol permeability and express the aquaglyceroporin (GLP) HC-3 (Goldstein et al. 2010). An in vitro RBC culture system was established to characterize HC-3 expression in RBCs from warm- and cold-acclimated treefrogs and denotate dynamic HC-3 protein expression (Mutyam et al. 2011). Other experiments demonstrated the connection between cortisol regulation and AQP expression in response to external osmolarity changes, specifically in teleost fish (Cutler and Cramb 2002; Handlogten et al. 2005; Giffard-Mena et al. 2007; Tipsmark et al. 2010; Choi et al. 2013). Results from this thesis integrate bodies of literature to establish a prospective relationship between freeze tolerance, GLP HC-3 protein expression, and cortisol regulation; however, further examination is required to depict these complex correlations.

The current experiments performed in this study focused on furthermore characterizing cortisol as contributor to the regulatory mechanisms prompting HC-3 protein expression and subcellular localization; though, the data gathered from this research prefaces the information that is still yet to be determined. Researchers previously observed increased AQP3 mRNA expression (in the gills) and AQP8 mRNA expression (in the intestines) of sockeye salmon *Oncorhynchus nerka* upon saltwater (SW) adaptation after cortisol exposure (Choi et al. 2013). These results indicated that AQP3 mRNA levels increase during the salinity change from freshwater (FW) to SW environments like previous studies in the European eel, *Anguilla anguilla*, (Martinez et
al. 2005) and gilthead sea bream, *Sparus aurata* (Raldua et al. 2008). AQP8 mRNA levels also increased in the intestines of Atlantic salmon, *Salmo salar*, (Tipsmark et al. 2010) and Japanese eel, *Anguilla japonica*, (Kim et al. 2010) upon SW transfer. Based on these experiments, and results from the current study, we hypothesize that *in vitro* cortisol exposure also induces an increase in HC-3 mRNA levels in the RBCs of *D. chrysoscelis*, essential for cellular freeze tolerance.

Future researchers could address the efficacy of cortisol in the enhancement of HC-3 mRNA levels through the implementation of real-time quantitative polymerase chain reaction (RT-qPCR). This method measures the level of HC-3 gene regulation and HC-3 gene expression and quantifies HC-3 mRNA abundance. Synthesized from the RNA, the first strand of cDNA will be used as a template in RT-qPCR with the relative amount of HC-3 mRNA transcripts being compared across cortisol-treated and control RBCs.

The *in vitro* RBC culture system employed in this thesis is well-established and suitable to analyze the mechanisms in which *D. chrysoscelis* prepares for cold-acclimation and glycerol synthesis and regulates HC-3 protein expression. The RBC culture system mimics conditions *in vivo* thus permitting the implication that *in vitro* conditions simulate events that occur in the live animal (Mutyam et al. 2011). Nevertheless, this research could be strengthened by potentially observing cortisol levels in warm-acclimated and cold-acclimated treefrogs *in vivo* using an enzyme-linked immunosorbent assay (ELISA). An ELISA provides physiologically relevant data, quantifying HC-3 expression in response to cortisol exposure as experienced by live treefrogs.
Furthermore, this research could move beyond experimenting with cortisol and freeze tolerance on the cellular level. A researcher could instead focus on the connection between cortisol and freeze tolerance at the organ level. A scientist could perfuse an organ, kidney for example, from Dryophytes chrysoscelis, Cope’s gray treefrog, with glycerol to be cryoprotected with viable organ and cellular function. The experiment could then involve perfusing the kidney with cortisol to measure a functional component that would render D. chrysoscelis freeze tolerant.

The fundamental characteristics of employing these experiments rest upon the notion that the phenomenon of freeze tolerance is naturally-occurring in D. chrysoscelis; whereas using knockout models to attain data and make observations may lack physiological and/or biochemical relevance in living organisms. This characteristic enhances the value of the comparative model, which contributes to the usefulness and applicability of freeze tolerant mechanisms employed by D. chrysoscelis to human biomedicine and organ cryoprotection.

We once again arrive at the Krogh Principle, a fundamental notion captured by Nobel-winning physiologist August Krogh, stating, “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). Firmly supported by the Krogh Principle, notable advances for cryoprotecting large, whole tissues are conceivably gained via the comparative model of analysis. Studying AQP's and aquaglyceroporins in naturally freeze tolerant anurans provides us with those insights, such as the relatively small treefrog—Dryophytes chrysoscelis.
Cortisol in Disease

In 1956, Hungarian endocrinologist Hans Selye considered “stress” to be the effects of anything jeopardizing homeostasis. He observed that, while the stress response represents an evolved, adaptive process, severe, prolonged stress may cause tissue damage and disease (reviewed in Schneiderman et al. 2005). Cortisol, a stress hormone, affects numerous body systems and is implicated in bone growth; arterial pressure regulation; immune and nervous functions; carbohydrate, fat, and protein metabolism; and the stress response. Although stress can be beneficial and necessary in certain scenarios, chronic stress negatively impacts the immune, dermatologic, nervous, gastrointestinal, neuroendocrine, and cardiovascular systems (reviewed in Garcia-Leon et al. 2018).

While cortisol could potentially be involved in suppressing the bodily organ systems in *D. chrysoscelis* during freezing and thawing, there are implications that cortisol is partially linked to prompting the onset of freeze tolerance according to this study. This data aligns with previous research regarding the use of cortisol as an effective reagent for immune suppression among transplant patients to prevent tissue rejection (reviewed in Baran et al. 2018). The act of freezing and thawing could be considered a stressful event for the treefrogs and stimulate the release of the stress hormone, cortisol. Cortisol could perchance be utilized as a biomarker for the onset of freeze tolerance in *D. chrysoscelis*, similarly to how cortisol levels are tested as a biomarker for stress and other physiological diseases such as adrenal insufficiency, Cushing’s syndrome, and Addison’s disease in humans.
Freeze Tolerance and Climate Change

The core body temperature of amphibians varies with ambient conditions (reviewed in Groff et al. 2016). Ohio’s mid-latitude location—interior and far from the east coast—results in a climate with a range of temperatures which includes warm, humid summers and cold winters. This pattern is starting to shift since environmental temperatures in the 2000s have been warmer than any historical period. The average annual temperature in the state of Ohio has warmed by 1.1°F (0.7°C) since 1990 (NOAA National Centers for Environmental Information 2017). Due to the continual rise in emissions, environmental scientists project historically unprecedented warming by the end of the 21st century, which also affects precipitation patterns. Temperatures increase with every coming year, meaning more rain and less snow. From 1958 to 2012, rainfall increased in the state of Ohio by 37% (NOAA National Centers for Environmental Information 2017).

The environmental changes associated with global warming and climate change have potential implications for the survival of *D. chrysoscelis*. Considering the sizeable population of treefrogs residing in Ohio, the effects of climate change could mean a loss of freeze tolerant competencies or extinction for the species. The naturally-occurring, selective pressure from the cold winter climate influences the freeze tolerant phenotype as a mechanism to minimize damage from ice crystal formation and to tolerate freezing. Genetic selection of freeze tolerance could potentially render a mutation no longer responsive to thermal cues. This was similarly observed among the budding yeast, *Saccharomyces cerevisiae*, where only 12% survived freeze-thaw stress. Most freeze-thaw tolerant strains were found in the Northeastern US suggesting that freeze-thaw
tolerance was selected for in cold climates but lost in other isolates. Adaptive loss of aquaporin paralogs were found in natural populations of *S. cerevisiae* in more ambient climates as well (Kvitek et al. 2008; Will et al. 2010).

In total, the nature of the experiments carried out in this study along with previous literature support the significance of contributing to the body of research pertaining to freeze tolerance. Investigating freeze tolerance and the manners in which *D. chrysoscelis* employs freeze tolerant mechanisms reinforces our enhanced understanding of endocrine diseases, tissue cryoprotection, tissue banking, genetic shifts, and even global warming and climate change.
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Chapter 4: Personal Reflections

According to my family, I am considered the “guinea pig” for being the only person to venture into the healthcare field and medicine. My family consists of business executives, bank tellers, tailors, musicians, cooks, and more, which seemed like I was treading in uncharted territory considering my aspirations for the medical field. I knew since middle school that I wanted to enter the world of medicine as a physician. Moving through high school, I felt reassured of my appetite for medicine through my interest and academic success in the sciences. The biomedical applications laced throughout my coursework kept me enthralled and piqued my curiosity for understanding how organisms and their bodily systems and function and respond to varying environments. Although I felt certain about my vocation as a doctor, I did not know what trying to prepare and matriculate into medical school looked like on-the-ground, that is until I went to the University of Dayton.

Throughout my four years of being a Pre-Medicine major and Sociology minor at the University of Dayton, I took a deep dive into the variety of experiential learning opportunities to strengthen my perception of the daily encounters one experiences as a physician in the medical field. This meant multiple involvements in my local Dayton and UD communities, which eventually made this venture into healthcare not so ambiguous or unfamiliar. I volunteered as a Hospital Elder Life Program volunteer at Miami Valley Hospital to prevent older adult patients 70 years of age and older from developing hospital-acquired delirium. I also volunteered as a Child Life volunteer at Dayton Children’s Hospital in the Long-Term Care Unit. I joined Alpha Epsilon Delta (AED): The National Pre-Professional Health Honor Society as a sophomore, became elected
Pledge Master as a junior, and now currently hold the position of Vice President. I also accelerated my involvement since sophomore year as a member of Beta Beta Beta: The Biology Honor Society, where I held the position of Long-Term Service Volunteer Coordinator as a senior. I also became an orientation leader as a sophomore and junior, promoted to team leader as a junior to help train other orientation leaders. After being a teaching assistant to sociology professor Dr. Jamie Small, I fostered an ardor for the discipline of sociology, interested in the integration of biomedical science and sociology. I was a teaching assistant to Dr. Kathleen Scheltens, Director of Pre-Medical Programs, and shadowed physicians in the field covering 11 subspecialties. While I remain proud of my accomplishments and toiled to reach this point in my academic career, none of these experiences prepared me for my adventure in research.

Throughout my coursework, I enjoyed engaging in lab work that accompanied science lectures and based these sentiments on my initial choice to engage in the research process. Supposedly, research strengthens a medical school application as well as an understanding of the relationship between research and clinical medicine, but this was obscure to me. I started mulling over the decision to potentially pursue research during the summer before my junior year. My journey began with the click of a mouse as I shifted from webpage to webpage on the UD website. Searching and reading through the descriptions of the research interests held by dozens of professors, I encountered Dr. Carisa Krane. Her name was familiar since I recognized it from my fall schedule—I would take her Physiology class in the months to come. I immediately became fascinated with her work based upon the titles of her publications dealing with cryopreservation, aquaporins, and freeze tolerance. The application of organ transplants hit home for me
since members of my family have relied on this medical technology in the past. Additionally, Dr. Krane’s research seemed distinct and unique—I never heard of a frog that survives freezing during the winter and thaws during the spring. I sent her an email, which was one of the longest emails I ever wrote, regarding why I wanted to join her lab and increase my involvement in the world of research. Looking back on when I wrote that email, my understanding of the research process and overall experience was rudimentary.

My first few months in the lab represented a blur of new and complex information. The lab space itself was overwhelming in the best way possible with different parts all moving at once—even in the fridge! I instantly felt like I was starring in the middle of a science fiction movie surrounded by clear flasks shaking pink fluids, huge shelves of industrial-looking chemicals, and a fume hood with an ultraviolet light source. I got the chance to speak with the laboratory technician, graduate student, and undergraduate students in the lab, Barb, Loren, and Dante, who helped shape my understanding of the expectations and commitment. I shadowed them as they performed countless experiments, watching and notetaking.

The second half of my junior year, which passed as quickly as it came, revolved around building my thesis. Choosing the particular topic of inquiry was a new and challenging hurdle, where I learned the basics of freeze tolerance as it relates to the model organism of interest, *Dryophytes chrysoscelis*, also known as Cope’s gray treefrog. Learning proper lab practices and optimizing lab techniques took months but were essential before I could actually begin any research directly related to my thesis. This was one of the most surprising aspects of the research process based upon my experience.
After writing my thesis proposal, I decided to apply for the 2018 Daniel P. Arnold Memorial Scholarship, an award dedicated to a senior Honors student from the College of Arts and Sciences who exemplifies Danny’s character and commitment to research. Danny was a Pre-Medicine major who passed away before graduating and was a significant part of the UD community. I applied for this award because I thought that if I had the opportunity to personally meet and know Danny that we would have been great friends considering how much we have in common. Danny was a member of AED, I am the Vice President. Danny had ambitions of attending medical school to become a doctor, as do I. Lastly, Danny was a member of Dr. Krane’s lab, a feat we once again both share. Danny’s passing is near and dear to my heart, especially since it could have been me or any of my friends at UD. It was senseless. Behold my surprise and utter gratitude upon discovering that I was chosen as the sole recipient of the award. It is an honor to represent the values that Danny cherished every day, and I am proud and motivated to respect his legacy. This means a lot to me, especially considering my involvement in the Danny Arnold 5K Committee. I was touched when I was asked to be one of the keynote speakers at the 2019 Honors Student Symposium Reception, where I shared my sentiments regarding Danny’s story, and how the scholarship impacted my academic achievements.

During the spring semester of my junior year, Dr. Krane encouraged me to apply for the 2018 College of Arts and Sciences Dean’s Summer Fellowship, given by the Office of the Dean for students to perform research over the summer. Being a recipient of this fellowship was a privilege, along with being a co-lead for the 2018 STEM Summer Lunch Club. Along with my lab partner, Elizabeth, every Friday we planned...
and organized lunch with other members of the undergraduate research community at UD. We discussed relevant topics from GRE/MCAT/DAT preparation to inviting guest speakers to elaborate on tissue scaffolding and regeneration research. Elizabeth and I coordinated with the Office of the President, and President Spina invited the STEM Summer Lunch Club to his home. I appreciated how he recognized the contributions that the undergraduate STEM researchers were making to campus over a beautiful lunch. Plus, the event was plenty of fun for members, permitting everyone to relax and step away from their work for a few hours. Both the Dean’s Summer Fellowship and the STEM Summer Lunch Club opened my eyes to the greater community of people involved in research. I was previously unaware of the magnitude of the industry. While I performed experiments individually, I was in good company since there were almost 100 students performing undergraduate STEM research during the summer.

The multi-step, trial-and-error optimizations continued throughout the summer before my senior year, where the fall semester marked the official start to my senior thesis project. During the start of the academic year, it became the responsibility of Elizabeth and I to run the lab with Dr. Krane, ensuring that the lab was maintained with proper upkeep. I learned about the technical aspects of running a lab daily such as keeping the list of chemicals stocked up-to-date, receiving packages, and refilling the liquid nitrogen tank, a task specific to our lab.

Overall, my experience performing research at the undergraduate level inspires me to continually pursue research in medical school. I want to contribute to the literature to perhaps help society move forward and effectively save lives. While I studied the scientific method and different research techniques employed by scientists from years
past, the actual voyage through a research project is rather unique on-the-ground. After spending long, arduous hours in the lab, the best feeling was getting to see the Western blot or immunocytochemistry images showing that the hard work and effort ended in success. I felt overjoyed after getting the result of my first clean Western blot, showing it to each member of my family. They did not know what it was, but it did not matter, for I knew the value of that image regardless. This experience taught me how to be more self-reliant and solve problems individually. I learned how to be an independent thinker and ask questions that I did not even think about asking previously. I learned that I have stamina and endurance, where I truly pushed myself out of my comfort zone. When I urgently needed a chemical for an experiment, but it was not brought to the Department of Biology, for example, I went to Shipping and Receiving and picked up the package on my own. I learned that a pivotal part of the research process is actually the communication aspect, where the entire project would be futile without effectively explaining one’s findings to the public. I learned how to collaborate with other researchers in the field and value constructive criticism.

Throughout this process, my family supported me every step of the way. I want to thank my parents, Rita and Joseph, and my sisters, Teresa and Serena. My unfamiliarity with the medical field was a blessing in disguise. I compensated by participating and diving deep into my local community. I relied on the guidance of UD faculty, staff, and students who facilitated my transition into the college, pre-medical experience. Thank you to my research mentor, Dr. Carissa Krane, for her direction and advice, she provided major support throughout my undergraduate, pre-medical journey. I will always be grateful for the opportunity of being a student researcher in her lab.
Moving forward, with my senior Honors thesis and overall time at UD coming to a close, I am excited to await the next chapter of my life that lies ahead. The memories I forged on UD campus are forever part of me and helped me mature as a person. The Dayton and UD communities provided me with many blessings and I will strive to give back to the communities throughout the course of my life. I am confident in myself as I look ahead, now understanding what it takes to be a leader. I am moving on from UD, ready to confront what life will bring me next.