THE EFFECT OF ARGinine VASOTOCIN ON CRYOPROTECTANT PRODUCTION DURING FREEZING IN THE PACIFIC CHORUS FROG, Pseudacris regilla

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THE EFFECT OF ARGinine VASOTOCIN ON CRYOPROTECTANT PRODUCTION DURING FREEZING IN THE PACIFIC CHORUS FROG, 

Pseudacris regilla

A Thesis
Presented to
The Graduate Faculty
Central Washington University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science,
Biology

by
Ben Ingels
February 2017
CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

We hereby approve the thesis of

Ben Ingels

Candidate for the degree of Master of Science

APPROVED FOR THE GRADUATE FACULTY

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Dr. David Darda

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Dr. Kevin Archer, Dean of Graduate Studies
ABSTRACT

THE EFFECT OF ARGinine VASOTOCIN ON CRYOPROTECTANT PRODUCTION DURING FREEZING IN THE PACIFIC CHORUS FROG,

Pseudacris regilla

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February 2017

Freeze Tolerance is an adaptation seen amongst several species of amphibians as a way of surviving during wintering months. Use of cryoprotectants is seen in all freeze tolerant species (glucose/glycerol). Synthesis and transportation mechanisms that regulate this process are not yet fully understood. Arginine vasotocin is a neurohypophyseal peptide known to have glycogenolytic effects as well as regulate several key processes involved in fluid and solute transportation. Although one prior study suggested that AVT is not involved in responses to freezing, I measured AVT levels during freezing and injected an AVT antagonist to see if it would affect the production of cryoprotectants within liver and thigh muscle during freezing. There was a dramatic increase in systemic AVT levels (p=0.05) during freezing by Pseudacris regilla. The AVT antagonist caused a dramatic reduction (~50%) in hepatic glucose and glycerol concentrations. These results demonstrate that AVT is involved with the process of cryoprotectant sourcing during freezing events in Pseudacris regilla.
This journey is a result of a 4 years, 2 projects, 5 advisors and my countless friends and family being there in support. To Dr. Daniel Selski, who first put the spark in my mind the idea of grad school was even possible and offered me the opportunity to work in his lab - thank you. To my P.I., Dr. Jason Irwin, you gave me the confidence to keep on trudging through, and allowed me to join your lab in a time of need. Your continued support over the years, day and night made this all possible. To Drs. Tom Cottrell and Jim Johnson, thank you for allowing me to keep pursuing my endeavors and providing a position for me over the years. To Dr. Wayne Quirk: you were my four leaf clover during times of turmoil. Thank you to going for bat for me, I am forever grateful. To Dr. David Darda: thank you for your continued support over the years, and being my master Shi-Fu for A&P. The rest of the CWU faculty- so many of you have in some way helped to make this project complete, I am grateful for everything. To three cohorts worth of colleagues: so many friendships, so many necessary breaks, so many great adventures. To my closest friends and family- thank you for your continued support and encouragement as I disappeared away into my project, I am glad to have you all in my life. To my best friend, my better half Emily Maulden- your continued love and support was the primary fuel (outside the coffee) that kept me driven to complete this project. If it wasn’t for you I am not sure this would have ever been written, I am forever grateful.
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CHAPTER I
INTRODUCTION

Winter in the inland Pacific Northwest exposes animals to harsh, cold conditions, including subzero temperatures for extended periods of time. Life has developed many strategies to adapt to such conditions. For warm-blooded or endothermic animals, the choices are numerous. Some indigenous species, such as shorebirds and caribou, choose to simply migrate to more favorable environments. Others, such as silver-haired bats and hummingbirds (Carpenter and Hixon, 1988; Falxa, 2007), have developed physiological adaptations that allow them to temporarily lower their metabolic rate and core temperature to avoid excessive heat loss and conserve energy, a process known as torpor. Hibernation is a strategy able to extend this decrease in metabolic activity/body temperature for extended periods of time (Marchand, 2013).

For ectotherms, which are dependent on environmental heat, the choices are more limited. Exposure to cold, and the near unavoidable contact with ice, brings a series of challenges for survival. Ice formation is detrimental to life in many ways: it can cause physical damage to the cell by intruding through cellular structures, can cause cellular dehydration by loss of fluids to the growing ice crystals and denature proteins (Muldrew and McGann, 1988). Other osmoregulatory processes can be adversely affected by ice formation. For example, dissolved solutes are excluded from growing ice crystals which may result in harmful accumulations within the unfrozen fluids (Muldrew and McGann, 1988).
Certain amphibians living within these northern regions have developed a novel adaptation of tolerating the freezing of body fluids within the organism to survive (Schmid, 1982), an adaptation referred to as freeze tolerance. Currently, freeze tolerance has been observed in 12 amphibian species. These include the Gray Treefrog (*Hyla versicolor* and *H. chrysoscelis*), the Wood Frog (*Rana sylvatica*) and the Pacific Chorus Frog (*Pseudacris regilla*) (Schmid, 1982; Croes and Thomas, 2000; Costanzo *et al.*, 2013). The Siberian Salamander *Salamandrella keyserlingii*, and the Schrenck newt *Salamandrella schrenckii*, have also been documented as having this unique adaptation (Berman *et al.*, 2009). During freezing, cardiac, respiratory and brain activity cease or severely decrease (Storey and Storey, 1988; Costanzo *et al.*, 1995). These functions reanimate upon thawing, with full function returning in one to four days (Costanzo *et al.*, 1995; Irwin *et al.*, 2002).

Understanding the mechanisms behind freeze tolerance reveals the use of specific low-density molecules (glucose, glycerol, urea) as cryoprotectants to preserve cell/organ integrity, limit absolute dehydration and control ice formation within the organism (Costanzo *et al.*, 1992; Storey and Storey, 1992, 1996). The ability to endure massive organ/tissue dehydration is also observed in these freeze-tolerant amphibians. During freezing, organs may lose up to 60% of their fluid content, largely due to the formation of ice crystals (Lee *et al.*, 1992). As the cells dehydrate, intracellular solute concentrations increase dramatically, resulting in an effective decrease in freezing temperature, reducing and controlling ice formation (Lee *et al.*, 1992).
Cryoprotectants

The use of cryoprotectants to aid in the survival of freezing has been observed in all freeze-tolerant amphibians. Amphibians demonstrating freeze tolerance use glucose and glycerol as the main substrates for cryoprotection (Storey and Storey, 1996). Ice formation initiates a sudden and rapid increase in the synthesis of these cryoprotectants, which are assumed to be primarily sourced from liver glycogen (Storey and Storey, 1996). Muscle glycogen is generally preserved for localized metabolic needs and lacks the necessary enzymes to convert their stores into free glucose for systemic use (Van Shaftingen, 2002). These liver glycogen stores are built up prior to the wintering period (Storey and Storey, 1996). Once inoculated with ice through skin contact, the liver begins a rapid breakdown of glycogen to build up cryoprotectant levels and disperse them to the tissues through the bloodstream (Costanzo et al., 1992).

Cryoprotectants aid in protecting and stabilizing cell membranes from ice damage, and aid in the osmotic regulation required during freezing (Amaral et al., 2015). As cells begin to lose their fluids to ice in extracellular spaces, the concentration of cryoprotectants in and around the cell increases. This change creates an osmotic gradient that prevents complete dehydration and allows ice formation to occur at a pace slow enough for cellular stability and regulation to occur (Costanzo and Lee, 1994; Croes and Thomas, 2000; Healas, 2014). Ice sheets begin to form around tissues and within the abdominal/lymphatic cavities where up to 70% of the total body water can be locked up in ice crystals (Layne and Lee, 1987). The sequestration of ice in the lymphatic spaces helps to limit the amount of ice forming in other areas where physical damage to organs
and tissue could occur (Lee and Costanzo, 1998). Urea may also play a protective role in amphibians by guarding the osmotic concentration, stabilizing cell membranes, cell structures and macromolecules (Storey and Storey, 2004; Costanzo and Lee, 2008). Amphibians accumulate urea under times of inadequate environmental water as a means of combating dehydration (Jørgenson, 1997). Dehydration due to ice accumulation should therefore trigger a very similar osmotic stress response. In experiments with the wood frog *Rana sylvatica*, frogs exposed to high levels of urea saw a dramatic increase in both cell/tissue survivability *in vitro* as well as increased recovery time of neuromuscular functions (Costanzo and Lee, 2008). More research is needed to understand the extent of urea accumulation during freezing, variations between freeze-tolerant species and to what degree of protection urea offers during freezing.

**Pacific Chorus Frog; *Pseudacris regilla***

The Pacific Chorus Frog, *Pseudacris regilla* (formerly *Hyla regilla*), is a species that has a range that extends from central British Columbia to Baja California. These frogs are found as far east as Montana and Nevada and all along the West Coast (Duellman and Sweet, 1999; Stebbins, 2003). It was first reported as freeze tolerant in 2000 (Croes and Thomas, 2000). *P. regilla* has been observed overwintering beneath leaf litter, where it is exposed to weather conditions (Irwin, unpub. data). Limited studies have examined the freezing ability of *P. regilla* across its range, but it has been observed that Washington State sea-level populations share the same level of tolerance as higher elevation populations (Healas, 2014). Glucose and glycerol concentrations seem to vary
amongst populations of *P. regilla*, which could be linked to local adaptations, but more research is needed in this area (Healas, 2014).

**Glycogen, Glucose, Glycerol: Sources of Cryoprotectants**

There are several biochemical pathways by which cryoprotectants may be produced (Fig 1.1). The first step of glucose production is glycolysis: the break-down of glycogen to free D-glucose (10% net) and a large amount of glucose-1-phosphate (90% net) (Gerin and Van Shaftingen, 2002). The enzyme involved in this process is glycogen phosphorylase, which acts to break the α1-4 glycosidic bond of the glycogen chain via phosphorylation, giving an end-product of glucose-1-phosphate. Glycogen debranching enzyme then acts as a transferase on the α1-4 glycosidic bond, as well as influencing glucosidase activity on the α1-6 glycosidic bonds via hydrolysis, leaving an end-product of unbound D-glucose. The third enzyme involved in glycogenolysis is phosphoglucomutase, which converts glucose-1-phosphate to the more widely used glucose-6-phosphate. Within the liver, glucose-6-phosphate interacts with glucose-6-phosphatase (G6Pase) to produce free D-glucose. G6Pase is unique, being a membrane-bound enzyme found within liver and kidney endoplasmic reticulum (Gerin and Van Shaftingen, 2002).
The initiation of freezing (via inoculation by ice) has been shown to increase the concentration of glucose in both the blood and liver of amphibians (Storey and Storey, 1996). Within four minutes of the detection of ice formation, liver and blood glucose levels rose to 3-6fold within the species *Rana sylvatica*. This study concluded that the stimulation of glucose release is a result of extracellular signaling mediated by β-
adrenergic receptors within the plasma membrane of hepatocytes (Storey and Storey, 1996). Glycogen phosphorylase activity is stimulated by hormones such as epinephrine and glucagon, both of which increase cAMP levels and trigger cAMP-dependent protein kinase, PKA (Dieni et al., 2011). The β-adrenergic-blocker propranolol prevents the release of glucose in frozen frogs, whereas the α-adrenergic blocker phentolamine had no effect on glycogenolytic activity or glucose output (Storey and Storey, 1996). This finding is more evidence the mechanism of release of glucose is mediated via β-adrenergic reception (Storey and Storey, 1996; Dieni et al., 2011), linked to a cAMP intracellular messenger system.

Aquaporins

Aquaporins (AQPs) are a family of proteins that form selective water channels within the plasma membranes of various cells within the organism (Ogushi et al., 2010). A subgroup of AQPs act to help facilitate the transportation of small solutes such as glycerol and urea through selective pores. This family is known as glyceroporins or aquaglyceroporins (GLPs) (Zimmerman et al., 2006; Ogushi et al., 2010). The discovery of AQPs was in the 1990s (Agre et al., 1993); although they were previously speculated to exist (Brown et al., 1980; De Sousa and Grosso, 1982). AQPs have been observed across a myriad of taxa; ranging from bacteria to vertebrates (Suzuki et al., 2007).

Within amphibians, specifically, 20 AQPs cDNAs have been identified and exist in six clusters with two being unique to the amphibians (Suzuki et al., 2007). Amphibian AQPs are expressed in the epithelium of the vasculature along the pelvic patch skin,
urinary bladder, and within the kidney (Tani et al., 2002; Hasegawa et al., 2003; Ogushi et al., 2010). Epinephrine and AVT have been shown to regulate several families of AQPs/GLPs within amphibians (Ogushi et al., 2007; Ratycz, 2014).

Arginine Vasotocin (AVT)

Arginine vasotocin (AVT), one of two peptides released by the neurohypophysis in amphibians (Boyd, 2006), is homologous to arginine vasopressin in mammals. AVT has a variety of effects in amphibians, including osmoregulation, behavior/reproduction, and cardiovascular regulations. The osmoregulatory actions of AVT are on several key organs including the kidney, bladder and skin. AVT acts on the skin by increasing the water permeability by regulating expression of aquaporins within the epithelium, specifically in the ventral pelvic area (Hasegawa, 2003). Within the kidney, AVT causes vasoconstriction of preglomerular arteries which reduces glomerular filtration rates by as much as 85% (Boyd, 2006). In the bladder, AVT acts to stimulate movement of water from the bladder back into the plasma and is accompanied by the increased expression of aquaporins (Jo and Harris, 1995). AVT has also been observed to increase levels of cAMP through impacted tissues (Janssens et al., 1985; Jo and Harris, 1995).

There are three main receptor subtypes identified for AVT in the amphibian (Boyd, 2012). The VT1aR is specific for AVT and closely mirrors the V1a Vasopressin receptor found in mammals. The VT1bR differs from its VT1aR counterpart in its exclusive expression in the brain and pituitary gland. This subtype closely resembles the V1b Vasopressin receptor in mammals. Both of these subtypes have been shown to
couple with the protein kinase-C mediated signaling pathway (Boyd, 2012). The other vasotocin receptor type, VT2R, has a high affinity for AVT (Boyd, 2006) and is most similar to the V2 Vasopressin receptor in mammals. Unlike the VT1 receptors, VT2R uses a cAMP signaling pathway (Boyd, 2012). Hydrins are believed to be variants of pro-AVT processing and play key roles in fluid absorption/reabsorption in the skin and bladder (Michel et al., 1993).

AVT has also been shown to stimulate glycogenolysis within the liver and increases the activity of glycogen phosphorylase α activity and cAMP (Ade, et al., 1995; Boyd, 2006). AVT stimulates glycogenolysis via VT2R receptor activation (Janssens et al., 1986). Research has also shown insulin can inhibit this AVT-induced glycogenolysis through reduction of cAMP levels within axolotl livers (Janssens and Grigg, 1992). Preliminary research has also shown the AVT V1a/V2 receptor antagonist, β-Mercapto-β, βcyclopenta-methylenepropionyl1-, O-ET-Tyr2, (Val, Arg8)) Vasopressin, reduced liver glucose levels in frozen Pacific Chorus Frogs (Pense, 2011).

To date, there has been a lack of research in understanding AVT’s role in cryoprotectant release and regulation during freezing in freeze-tolerant amphibians. In this study, I first wanted to verify Croes and Thomas’s findings that P. regilla was a freeze-tolerant species utilizing glucose and glycerol as cryoprotectants. I also wanted to confirm my suspicions that AVT plays a role in the freeze-tolerance by seeing if there was an elevation in systemic AVT during freezing. I then wanted to see how cryoprotectant accumulation would be affected if the effect of AVT was inhibited during freezing. Measuring systemic levels of AVT during freezing would be a first, and provide an
evidence base for the theory AVT is involved during freezing. By measuring glucose and glycerol concentrations from hepatic and muscular tissues during freezing in both a control group and an AVT inhibited group, I would gain powerful insight into how AVT impacts cryoprotectant accumulation during freezing. I suspected to see elevations of AVT during freezing. I also expected suppressing AVT within freezing frogs would suppress accumulations of cryoprotectants as well.
CHAPTER II
MATERIALS/METHODS

Specimen Collection/Storage

I collected frogs from two sites: Stampede Pass, Washington (GPS: 47°18’36.4”N 121°18’13.6”W) and Table Mountain, Washington (GPS: 47° 14’ 36.4” N 120° 33’33.4”W). Frogs were collected during August-September by hand and net. I held frogs in a secured vinyl-outdoor enclosure (8x12x24ft), with small mesh sub-enclosures (3x3x3ft). The enclosures were located at Engelhorn pond on the campus of Central Washington University. Frogs were fed *ad libitum* on mineral powder-dusted crickets twice weekly. Dead or ill frogs were immediately removed from the enclosures. On December 5th (before weather was cold enough to induce freezing), I relocated frogs into plastic box enclosures containing substrate and leaf litter sourced from the outdoor enclosure area. Enclosures were stored in a climate-controlled 4°C storage room. Once indoors, frogs began a fasting period and remained in these conditions until experiments began in February or March. All procedures were performed and done with approval by the CWU Institutional Animal Care and Use Committee (protocol number A031505).

Experimental Design

Male frogs were chosen at random and split evenly into control or experimental groups. I injected all frogs in the dorsal lymph sac with either 10µL of physiological saline (control group) or 10µL of 5mg/mL AVT-Antagonist (β-Mercapto-β, βcyclopenta-
methylenepropionyl<sup>1</sup>, O-ET-Tyr<sup>2</sup>, (Val, Arg<sup>8</sup>) vasopressin (Sigma V4253) in physiological saline (experimental group). This compound is a potent antagonist for the V1a and V2 AVT receptors. Once injected, I placed the frogs in 50mL centrifuge tubes along with a small amount of ice to induce inoculation and a soft-foam plug was inserted to immobilize specimen. Tubes were then placed into an ice bath and held for 30mins prior to freezing.

Freezing Protocol

I placed the 50ml centrifuge tubes with the ice-inoculated frogs into a computer-controlled ethanol bath (Thermo RTE-740) pre-cooled to 0.5°C. I used a real-time temperature data logger (Omega DAQPRO5300) probe placed into one control frog tube to monitor temperature around the frog. Temperature was then cooled to -0.8°C, after which a controlled cooling rate of -0.04°C·h<sup>-1</sup> was maintained until a temperature of -2.5°C was reached. The frogs were held at this temperature for four hours, then removed from the bath and immediately dissected to remove liver and femoral muscles. Liver and thigh musculature were weighed and subsamples were removed and weighed to determine water content. The tissues and the carcass were then stored at -80°C until needed for physiological analyses.

AVT Immunoassay Extraction Preparation

Frozen and unfrozen frogs were removed from storage at -80°C were used for the AVT Immunoassay. I removed and weighed any large visible ice crystals from abdomen
of each frog. Decapitation of each frog at the base of the occipital condyles was performed to ensure only systemic AVT levels were measured and no pituitary stores would be calculated into my results. The remaining carcasses were frozen using liquid nitrogen and ground to a fine uniform powder with a mortar and pestle. Tissues were prepared using an enzymatic extraction kit (Peninsula Labs product #S-5000) on ~750mg of carcass powder, initially homogenized in 3mL Buffer A solution (1% trifluoroacetic acid). I then spun down the homogenate at 13.5k, 2°C for 30 minutes and collected the supernatant and froze overnight. Once the supernatant was thawed, it was centrifuged at 13.5k for 10 minutes. I extracted 800ml of the new supernatant and ran it through SEP-column filters and washed per instructions. The eluent from each filter was freeze dried for 48 hours, then reconstituted with 500μL EIA buffer. This solution was used to determine circulating AVT levels in the Immunoassay.

AVT Immunoassay Protocol

Immunoassay was performed using instructions provided by Peninsula Lab’s AVT-EIA Kit (Product # S-1239.0001). I added 50μL of either EIA buffer solution, standard, or sample to each well of a pretreated 96-well plate, followed by 25μL of antiserum and a one hour incubation at room temperature. 25μL of rehydrated Bt-tracer in EIA buffer was added to each well and given an additional two-hour incubation. The plate was carefully washed and dried with 300μL of EIA buffer five times before receiving 100μL of streptavidin-HRP in each well, followed by a 60-minute incubation. The wash washed five times, 100μL of TMB-solution added and allowed incubate for an
additional 45 minutes. Samples were read at 540µm wavelength (Turner SP-870 spectrophotometer), then again at one hour at 450µm wavelength after the addition of 100µL 2N hydrochloric acid.

Metabolite Assay Preparation

I homogenized liver and thigh musculature in ice-cold 0.6 mol·L⁻¹ perchloric acid in 2mL micro-centrifuge tubes, then spun at 13.5k for 5 minutes before being neutralized with 1mol·L⁻¹ potassium bicarbonate. I extracted the supernatant from each tube and placed it in a new, labelled -80°C-safe micro-centrifuge tube. As sample of the raw homogenate was placed in a labeled, -80°C-safe micro-centrifuge tube for measurement of glycogen content. All other samples were placed in a -80°C cold storage until needed for assays.

Free glucose Assay

I measured glucose using an assay kit (Sigma-Aldrich, product no. GAGO-20). Assays involved adding 50uL of the tissue extract to 1mL PGO-solution in a 2.0mL cuvette and allowing it to react for 45mins in darkness. I read absorbance at 450µm (Turner SP-870 spectrophotometer) and calculated glucose concentration was calculated based on comparison to a standard of known concentration (400mg/L).
Glycogen Assay

Glycogen assays were performed on 100µL aliquotes of raw homogenate neutralized with 50µL of 1mol·L⁻¹ potassium bicarbonate. I then added 500µL of a 1:39 aspergillus-enzymatic digest solution (Sigma Aldrich, CAS 9032-08-0) to the tubes and incubated for 2 hours at 40ºC. After which I added 250µL of 0.6N perchloric acid followed by 375µL 1mol·L⁻¹ potassium bicarbonate. Tubes were centrifuged at 13.5k rpm for 10 minutes and analyzed using the Sigma-Aldrich free-glucose assay kit GAGO-20 as described above. Glucose levels obtained from this assay, minus the results of the free-glucose test gave us the total new glycogen concentration. I tested the efficiency of the glycogen digest by using 55mg of bovine glycogen to 2.5mL of 0.6N perchloric acid and assaying the glycogen concentration using the same procedures as used for glycogen assay for tissues described above, I saw a ~99% efficiency rate.

Glycerol Assay

Glycerol was measured using the free-glycerol reagent kit from Sigma-Aldrich (catalog no. F6428). I added 50µL of ultra-purified water, glycerol standard, or extracted supernatant to 800µl of free-glycerol reagent in appropriately labeled 2mL centrifuge tubes. Samples were then incubated for 15mins at ambient temperature. I read the samples at 540nm and compared it to standards of known concentration (200mg/L).
Statistical Analysis

All data were log-transformed for statistical analysis to pass normality and equal-variance testing. Welch’s 2 sample t-tests were performed to look at differences between control and experimental groups, whereas analysis of covariance (ANCOVA) with bodyweight as the covariate was run to account for differences in body size amongst samples. All tests were performed in MiniTab 17.
CHAPTER III

RESULTS

Mean bodyweights (Fig 2.1) were not significantly different between control and experimental frogs (t = 0.07, df=14, p= 0.949). Control frogs showed a mean bodyweight of 3.69 ± 0.37g (n=8) while my AVT-antagonist treatment group averaged 3.65 ± 0.42g (mean ± SEM, n=9). This being the case, differences seen in cryoprotectant were not influenced by bodysize.

![Figure 2.1 Mean bodyweight from control and AVT-antagonist treatment P. regilla.](image)

Cryoprotectant Results

While investigating glucose production, the AVT-antagonist group produced ~50% of the glucose in liver samples compared to control frogs (Fig 2.2). Control frogs showed a mean glucose level of 703±66µMol/g dry mass (n=8) while my AVT-
antagonist frogs showed a concentration of 364±68µMol/g dry mass (n=9). This difference of mean hepatic glucose levels between control and the antagonist treated group was statistically significant (t= 3.58, df=14, p= 0.003).

This trend did not hold true, however, within thigh samples (Fig 2.3). Free glucose levels within the AVT-antagonist treated group were not significantly different from the control in the thigh samples (t= -0.36, df=10, p= 0.728). Control frogs averaged 26±6µMol/g dry mass (n=8). Amongst my AVT-antagonist treated group, mean thigh glucose levels measured 28±3µMol/g dry mass (n=9).
Hepatic glycerol levels of the AVT-antagonist frogs showed a 40% reduction compared to control frogs (Fig 2.4). Control frogs showed a mean hepatic glycerol level of 5.1±0.8μMol/g dry mass (n=8), AVT-antagonist group 3.2±0.7μMol/g dry mass (n=9). Liver glycerol production was significantly influenced by body size as well (f= 12.06, df=1, p=0.004). Thigh glycerol levels (Fig 2.5) showed no significant difference between control and AVT-antagonist groups (t=1.21, df=1, p=0.247). Control value for thigh glycerol were 3.2±0.6μMol/g dry mass (n=8). AVT-antagonist values 2.2±0.5μMol/g dry mass (n=9). Bodyweight contributed significantly to glycerol levels as well, with larger
frogs producing larger amounts of glycerol (f= 7.15, df=1, p = 0.019).

I found mean hepatic glycogen levels within control frogs of 644.6±234.2µMol/g dry mass (n=8) (Fig. 2.6). In my AVT-antagonist group, values produced a mean of 676.6±171.1µMol/g dry mass (n=9). These results produced no significant difference. Knowing that muscular glycogen is utilized primarily for localized energy demands as it lacks the G6Pase enzyme (Gerin and Van Shaftingen, 2002), I felt it was unnecessary to measure glycogen content within thigh samples.

![Figure 2.4 Mean hepatic glycerol concentrations from control and AVT-antagonist treatment P.regilla.](image-url)
AVT Immunoassay results

I observed that frozen frogs had over double the measurable AVT levels over unfrozen control frogs (0.42±0.1 ng/g vs. 1.04 ±0.65ng/g; t=0.05, p=0.05) (Fig. 2.7).

Figure 2.5 Mean thigh glycerol concentrations from control and AVT-antagonist treatment *P.regilla*.
Figure 2.6 Mean hepatic glycogen from control and AVT-antagonist treatment *P. regilla*.

Figure 2.7 Mean AVT concentration from unfrozen control and frozen *P. regilla*.
CHAPTER IV
DISCUSSION

Freezing and Cryoprotectant Accumulation in *Pseudacris regilla*

Freeze-tolerant amphibians use glucose and glycerol as cryoprotectants to combat the stressors associated with freezing (i.e., ice formation, dehydration: Storey and Storey 1996; Irwin and Lee, 2003; Costanzo et al., 2013). Freezing drives up hepatic glucose levels nearly 20-fold in *Hyla versicolor* and *Hyla chrysoscelis* (Irwin and Lee, 2003). In *Rana sylvatica* hepatic glucose levels went from ~3µMol/g wet wt. to 236µMol/g wet wt. with freezing (Storey and Storey, 1996). Similarly, a Northern California population of *P. regilla* exhibited a 5 to11-fold increase in liver glucose levels during freezing, with levels reaching ~100µMol/g wet wt. (Croes and Thomas, 2000). I saw even higher hepatic glucose levels, reaching ~266µMol/g wet wt. (Fig. 2.2). My results are comparable to cryoprotectant accumulation with several other known species of freeze-tolerant amphibians, suggested that the glucose produced in cryoprotective as it is in other amphibian species.

Because glycogen is the dominate source of glucose, I measured the glycogen content within *P. regilla* and compared it to previous works. I saw similar patterns as in previous investigations, with my population showing a mean glycogen level of around 245µMol/g wet wt. (Fig. 2.6) and Californian *P. regilla* producing 362µMol/g wet wt. (Croes and Thomas, 2000). Seasonal and geographical variations in glycogen stores have been well documented within amphibians, including those known to be freeze-tolerant and other members of the genus *Pseudacris* (Pinder et al., 1992; Dismore and Swanson,
Knowing that variation is likely to be seen with different populations of *P. regilla*, seeing a marginal variation like I did between my finding vs. Croes and Thomas would be likely and would not suggest any less capacity for freeze tolerance.

Croes and Thomas’s study also noted a 2 to 3-fold increase in glycerol, another cryoprotectant used by freeze-tolerant amphibians (Zimmerman *et al.*, 2007). My results showed glycerol accumulations around one third of that seen in the work of Croes and Thomas with *P. regilla* reaching only 1.92μMol/g wet wt (5.10μMol/g dry wt) (Fig. 2.4). Again, seasonal and geographical variation may very well explain the difference measured by Croes and Thomas vs. my findings within *P. regilla*.

Arginine Vasotocin, Freezing and Cryoprotectants

The concentration of circulating AVT levels has never been studied in freeze-tolerant amphibians. In this current study, I measured a significant increase in systemic AVT levels within frozen *Pseudacris regilla*. Frozen frogs had more than double the systemic AVT levels of control frogs (0.42ng/g vs. 1.04ng/g) (Fig. 2.7). This supports my hypothesis that AVT is involved during freezing, and is the first experiment to show an elevation of AVT during freezing in any freeze-tolerant amphibian.

Although never directly measured before, there was much reason to predict that freezing would increase AVT concentrations. Studies have shown internal organs lose up to 60% of their water content during freezing (Lee *et al.*, 2002) which would activate physiological responses to dehydration like the release of AVT. For example, frogs and
axolotls elevate in systemic AVT when faced with osmotic stress events like dehydration (Maejima et al., 2008; Uchiyama et al., 2014). AVT affects water movement by acting on the permeability of tissues throughout the body, including the integumentary and urinary systems (Tanii et al., 2002; Hasegawa et al., 2003; Suzuki et al., 2006; Ogushi et al., 2010). Several families of aquaporins are directly mediated by AVT as well (Suzuki et al., 2006). Aquaporins affect the movement of not only water, but small solutes such as glycerol and urea. It has already been seen in several freeze-tolerant species that glycerol elevations occur during freezing (Croes and Thomas, 2000; Zimmerman et al., 2007). It has also been observed that elevations of AVT-mediated aquaporins do occur during freezing (Zimmerman et al., 2007). Freezing would induce both osmoregulatory challenges the need for cryoprotectants, both of which could be provided by AVT. Indeed, my results confirm this hypothesis: AVT increases during freezing and is likely being used to stimulate cryoprotectant production and movement, along with its involvement in osmoregulation.

My study is the first to observe a direct relationship between AVT and cryoprotectant accumulation during freezing. Under influence of the AVT antagonist, I saw a dramatic decrease in hepatic glucose concentrations (702.6 vs. 363.8 µMol/g dry wt). This is consistent with other studies demonstrating the glycogenolytic effects of AVT in amphibians (Janssens et al., 1985; Ade et al., 1995). This effect is linked to AVT increasing the activity of glycogen phosphorylase (GP), the initiating enzyme of glycogenolysis (Ade et al., 1995; Boyd, 2006).
AVT also plays a role in glycerol production. The AVT antagonist reduced glycerol accumulation within the liver by ~40% from 5.1 to 3.1 µMol/g dry wt. Glycerol can be obtained through two mechanisms, glycolysis or lipolysis. There is no research to date that has measured the extent to which each pathway is involved with regards to total glycerol output during freezing in amphibians. This study, as well as others, have already shown glucose production is affected by AVT during freezing within *P. regilla*. Glycerol production may also be bound by same relationship, as glucose and glucose 6-phosphate are precursors in the glycolytic production of glycerol.

Glycerol movement may also be influenced by AVT. Glycerol must be allowed to travel through cells to necessary areas to play its cryoprotective role, and the movement of glycerol is in large part facilitated by AQPs/GLPs under regulatory control by AVT (Boyd, 2006; Suzuki *et al*., 2006; Krane and Goldstein, 2007; Ogushi *et al*., 2010). Without upregulation of these necessary transportation mechanisms, it is likely the movement of glycerol could also be directly influenced by AVT. It is also possible this could be a factor in the reduction of total hepatic glycerol levels seen in my AVT antagonist-treatment frogs. With AVT inhibited, both production and movement of glycerol can be effected and again I measured a significant reduction in hepatic glycerol under these conditions.

I hypothesized that liver glycogen levels would be higher after exposure to the AVT antagonist, as these frogs would use less glycogen to produce the lower levels of cryoprotectants observed. Mean glycogen levels for control frozen frogs came in at 644.6±234.2µMol/g dry mass, while AVT-antagonist treated frogs produced
676.6±171.1µMol/g dry mass. The lack of statistical difference in glycogen levels seen in my results is likely due to the natural variation seen amongst individuals. It is clear AVT does play a role in cryoprotectant accumulation during freezing in *P. regilla* and further investigation into understating the biochemical/physiological processes involved certainly are merited.

**AVT, Epinephrine & cAMP signaling**

Epinephrine (β-adrenergic) activity has also been documented in influencing the levels of cryoprotectant accumulation in various freeze-tolerant species (Ade *et al.*, 1995; Storey and Storey, 1996; Amaral *et al.*, 2015). Similar studies have also shown that with the use of propranolol, a β-adrenergic antagonist, frogs saw a significantly reduced accumulation of hepatic glucose during freezing (236µMol/g wet wt. vs 130µMol/g wet wt.) (Storey and Storey, 1996). This reduction is in hepatic glucose is very similar to that observed within my AVT-antagonist group. The rise in glycogenolysis through increased AVT-mediated, GP activity has been shown to be linked directly to an increase in cAMP concentration within the tissues (Ade *et al.*, 1995; Janssens *et al.*, 1985). Indeed, in both AVT and β-adrenergic activation of glycogenolysis, the cAMP intracellular pathway mediates glycogenolysis (Janssens and Grigg, 1992). Similarly, I speculate that AVT is acting along with epinephrine to influence cAMP concentrations within tissues, which increases activity of GP to produce more glucose during freezing events. AVT may also be enhancing beneficial water movements as several families of AQPs and GLPs in freeze-tolerant tree frogs have also been shown to be regulated by both AVT and
epinephrine, including the HC-3 GLP (Zimmerman et al., 2007; Ogushi et al., 2009; Ratycz, 2014).

My results indicate that AVT and epinephrine have overlapping effects on cryoprotectant production. Since both of these glycogenolytic responses are mediated by cAMP, future work to measure cAMP fluctuations -especially after administration of the AVT antagonist and/or propranolol - during freezing may help us understand the extent AVT and epinephrine have on responses to freezing. Can glucose production be suppressed to control levels when exposed to both AVT-antagonist and propranolol? How is glycerol production and movement affected when exposed to various combinations of AVT and epinephrine in tandem? Future work using both AVT and β-adrenergic agonists/antagonists in various combinations might provide powerful insight into the intermingled relationship of AVT and epinephrine affect amphibian freeze tolerance.

Overall, this is the first study to both confirm elevations in systemic AVT during freezing in P. regilla and that cryoprotectant outputs are suppressed when AVT is inhibited during freezing. Both provide useful evidence and offer more insight into understanding the biochemical processes involved in freeze-tolerance.
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