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Urea Concentration and Hsp70 Expression in the Kidney of Thirteen-lined Ground Squirrels during Diuresis and Antidiuresis

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Abstract.

During bouts of torpor hibernating animals have greatly reduced metabolic rates leading to profound decreases in body temperature and blood pressure. As a result of these conditions, kidney filtration and the ability to concentrate urine cease. Once a week, however, hibernators rewarm to euthermic body temperatures and regain kidney function. This is associated with rapid changes in extracellular osmotic gradients within the kidney, a remarkable feat but one that is potentially damaging to kidney cells. While hibernators deal with this stress by up-regulating expression of heat shock proteins (HSP's) and protective organic osmolytes, little research has been done to see if hibernating animals can achieve and cope with similar situations during the summer. To address this question we placed a typical hibernator (*I. tridecemlineatus*) on various water intake regimes over the summer to experimentally manipulate vertical osmotic gradients in the kidney, represented by changes in urea concentration. We then measured renal expression of HSP 70 in response to changes in the vertical gradient. Animals rapidly altered the vertical gradients in their kidneys in response to different water intake regimes. This was accompanied by large changes in urine volume and concentration, and maintenance of serum hydromineral homeostasis. Unlike hibernation however, HSP70 expression was up-regulated in response to loss, rather than gain, of vertical osmotic gradients. This difference may be due to an interplay between HSP70 and protective organic osmolytes. Future studies will examine this relationship in closer detail and also evaluate the response of non-hibernating species to similar conditions.

Introduction.

The process of hibernation involves many challenges placed on the animal's body during bouts of torpor and arousal from torpor. Bouts of torpor are characterized by reduced body temperature, depressed heart rate, lower cardiac output and blood pressure, and almost complete immobility (Geiser 2004; Geiser and Ruf 1995). Despite these alterations in physiology, hibernating animals are adept at maintaining skeletal muscle, (Cotton and Harlow 2010), minimizing bone loss (Woida et al 2012), and carefully orchestrating renal perfusion to maintain blood homeostasis (Cotton 2012). There are, however, many gaps in the knowledge of how hibernators achieve these remarkable outcomes. Although we know hibernating animals tolerate large swings in renal perfusion, coupled with major changes in GFR and urine concentrating ability, very little is known about the potential mechanisms involved especially in relation to epithelial cell protection during the massive swings in medullary and papillary osmolality during torpor cycles. Furthermore, nothing is known about the abilities of hibernating animals to tolerate massive changes in kidney function during the summer. Therefore, this study examined the renal responses of a typical hibernator (*I. tridecemlineatus*) to acute changes in water intake during the summer. By understanding how these animals cope with rapid changes in medullary and papillary osmolality during the summer, it may be more fully understand how these animals achieve protection during the hibernation season.

The kidney in the mammalian body is critical for a number of processes: 1) maintaining hydromineral balance 2) achieving acid-base balance, and 3) excreting metabolic waste products. In order to achieve these functions, kidneys filter a huge volume of blood each day, reabsorbing a large portion of the filtrate and excreting a smaller portion as necessary to achieve the previous goals. The major functional unit of the kidney performing these steps is the nephron. The

nephron consists of: Bowman's capsule, proximal convoluted tubule (PCT), loop of Henle, distal convoluted tubule (DCT) and collecting duct. While Bowman's capsule, PCT, and DCT all reside in the outer cortex of the kidney, the Loop of Henle and collecting ducts reside in the innermost portions of the kidney, the medulla and papilla. Taken together, these components of the nephron are collectively responsible for filtering large portions of the plasma volume every day in an effort to maintain consistent plasma volume, osmolality, electrolyte concentrations, and minimize accumulation of metabolic waste products.

Filtration of blood plasma occurs in the glomerulus, with blood supply via the afferent arteriole and drainage via the efferent arteriole. The glomerulus is a highly convoluted bed of fenestrated capillaries that winds around itself within Bowman's capsule. Hydrostatic pressure within this capillary bed is quite high which favors formation of ultrafiltrate. This is mitigated by colloid osmotic pressure within the glomerular capillaries and hydrostatic pressure within Bowman's capsule from previously filtered fluid. The filtered fluid per unit of time that passes into Bowman's capsule is called the glomerular filtration rate (GFR). The GFR is equal to the net filtration pressure (NFP) multiplied by the filtration coefficient (K_f). The GFR is fairly well homeostatically regulated within blood pressures ranging from 80mmHg to 180mmHg, the K_f of the afferent and efferent arterioles can have some profound effects on the GFR. During filtration the blood first passes through fenestra in the endothelial cells before crossing the basement membrane of the capillary, and then passes through the visceral layer of Bowman's capsule. Foot processes of the podocytes in this region form filtration slits that ultrafiltrate must pass through prior to entering the lumen of Bowman's capsule. Contraction of podocytes can reduce K_f and reduce filtration.

After entering Bowman's capsule the filtrate comes into contact with the proximal tubule which reabsorbs 60-70% of filtered sodium and H_2O, processes that are largely unregulated. The Loop of Henle reabsorbs 25% of sodium and through the single effect and countercurrent multiplier generate the corticopapillary gradient, the distal tubule and collecting duct have regulated reabsorption of the last 8% of the filtrate (Sherwood 2013). The most important components for this research will be the loop of Henle and the collecting duct. After passing through the proximal tubule the filtrate reaches the loop of Henle. The loop of Henle is made up of the ascending limb and descending limb. The descending limb begins like the other two components in the cortex then dips down into the medulla and papilla where it forms a sharp "U" and returns via the ascending limb back to the cortex. The loop of Henle is critical for generating a corticopapillary osmotic gradient, a steadily increasing concentration of sodium ions and urea as you move from cortex to papilla, with the most concentrated portions occurring at the very tip of the papilla. The increasing magnitude of this gradient determines maximal urine concentration, so function of the loop of Henle is critical to maintaining blood homeostasis. The descending limb contains aquaporins, making it highly permeable to water, but lacks necessary proteins for sodium transport. In contrast, the ascending limb actively transports sodium out of the tubular lumen into the interstitial space but has zero capacity for water transport, i.e. no aquaporins. Active transport of sodium from the ascending limb lumen to the interstitial space creates the single-effect, an approximately 200 mOsm difference in concentration between the ascending limb lumen and the surrounding interstitial space. Due to the permeability of the descending limb to water, the descending limb lumen comes into equilibrium with the interstitial space. The combination of the single-effect coupled with counter-current flow through the loop

traps solute in the inner medulla and papilla of the kidney, creating an osmotic concentration gradient from the cortex to the papilla of each kidney.

After passing through the loop of Henle, filtrate passes on to the distal tubule followed and collecting duct. Absorption of water and sodium in these two components are under hormonal regulation via the Renin-Angiotensin-Aldosterone System (RAAS) and anti-diuretic hormone (ADH). RAAS is a homeostatic control system to regulate the extracellular fluid volume arterial blood pressure. In response to low levels of any of these factors granular cells on the efferent arteriole (just outside of Bowman's capsule) release the hormone renin into the blood. The release of renin is in response to three inputs to these granular cells. 1) the cells themselves function as intrarenal baroreceptors, when they detect a fall in blood pressure they secrete renin. 2) the macula densa cells on the distal tubule within the juxtaglomerular complex are sensitive to sodium moving past them through the lumen of the distal tubule and a drop in sodium passing by the cells results in a release of renin. 3) the granular cells are innervated by the sympathetic nervous system. When blood pressure falls below normal the baroreceptor reflex increases sympathetic activity which in turn stimulates the secretion of more renin. Renin in the blood is an enzyme to active angiotensinogen into angiotensin I. Angiotensinogen is synthesized by the liver and always circulates the blood plasma in high concentrations. On passing through the lungs via pulmonary circulation, angiotensin I is converted to angiotensin II via angiotensin-converting enzyme (ACE), which is abundant in pulmonary capillaries. Angiotensin II circulates in the blood and causes a few effects before the next step in this pathway. It causes a small amount of vasopressin to be released, thirst (both of which cause an increase in blood plasma volume) and arteriolar vasoconstriction to increase arterial blood pressure. Angiotensin II is the primary stimulus for the release of the functional hormone of this system from the adrenal

cortex; aldosterone. Aldosterone promotes the insertion of additional Na-K-2Cl cotransporters into the luminal membranes of the distal tubule and collecting duct as well as additional sodium potassium pumps into the basolateral membranes as well. The end result is greater passive movement of sodium across the apical surface of epithelial DCT cells and increased pumping of sodium across the basolateral surface of epithelial DCT cells from the cell into the interstitium and blood vessels. Because these segments of the nephron contain aquaporins, net reabsorption of sodium also results in net movement of H_2O. RAAS, then, promotes sodium retention and subsequent water retention in an effort to maintain plasma volume (Sherwood 2013).

The primary hormone that is involved in regulating plasma osmolality and urine concentration is vasopressin or antidiuretic hormone (ADH). ADH is produced by paraventricular and supraoptic neurons in the hypothalamus. Axons from these neurons terminate in the posterior pituitary where they release ADH into the blood stream in response increases in blood osmolality or reductions in blood volume. Vasopressin circulates in the blood and binds to the basolateral membrane of the distal tubule and collecting duct via a V2 receptor. V2 is a g-protein coupled receptor which causes ATP to be converted into cAMP. The increase in cAMP ultimately actives Aquaporin 2 (AQP-2). Once activated AQP-2 will insert into the luminal membrane, increasing the permeability of water to the cell and ultimately resulting in a net reabsorption of water (Sherwood 2013). ADH also allows NA-K-2Cl cotransporters to bind to the apical membrane. This cotransporter being active allows for greater concentrations of sodium to exit the filtrate into the interstitium thus amplifying the single effect and creating a larger corticopapillary gradient. Urea transporters UT-A1 and UT-A2 also bind to the apical and basolateral membranes respectively in the presence of ADH. This allows urea to be reabsorbed into the interstitial space of the papillary where it contributes to the corticopapillary gradient and

countercurrent multiplier (Cai et al. 2010). After passing through the collecting duct the concentrated urine passes to the ureter and finally to the bladder. The actions of ADH therefore increase the corticopapillary gradient and enhance H_2O reabsorption from the collecting duct separate from sodium reabsorption. This results in decreased plasma Na^+ and reduced plasma osmolality.

During bouts of torpor when GFR is at a minimal rate and Q_{10} effects reduce the efficacy of the single effect, corticopapillary gradient also is greatly reduced. The concentration is reduced because when the GFR drops there will be less filtrate being produced via the hydrostatic pressure in the glomerulus and will lower levels of filtrate passing through the loop of Henle the lower amounts of sodium ions and urea can be reabsorbed to create a concentrated corticopapillary gradient needed for the concentration of urine (Clausen and Storesun 1971; Cotton and Harlow 2012). Small hibernators, like the thirteen-lined ground squirrel, go through periods of arousal every 5-15 days (Geiser 2004) to a euthermic state lasting 12-24 hours in which urination occurs (Clausen and Storesun1971; Pfeiffer and Moy 1968). This arousal period causes an interesting problem for these hibernators. If the corticopapillary gradient is not restored during periods of arousal, the animal is at serious risk for dehydration as they will be producing extremely dilute urine. Studies have shown that GFR does not resume until halfway through the arousal period, well after the blood pressure has resumed (Baddouri and Elhilali 1986; Lyman and O'Brien 1960; Moy 1971), which may enable the return of a functional corticopapillary gradient prior to resumption of GFR.

To protect themselves from rapid dehydration, the animal must restore this gradient as soon as possible. During a rapid shift of the corticopapillary osmotic gradient the interstitial space around these cells goes from being isosmotic (relative to plasma) to hyperosmotic (relative

to plasma) in a short time span. The epithelial cells therefore must quickly match intracellular concentrations to those of the interstitium to avoid severe volume change. In addition to volume changes, the high concentration of urea present in the interstitium can enter the cells and produce protein denaturation and possibly even cell apoptosis. Hibernators, and indeed any animal with a corticopapillary gradient, therefore make use of two distinct classes of compounds to prevent these detrimental effects: 1) organic osmolytes 2) heat shock protein 70 (HSP 70)

When epithelial cells in the kidney experience increased intracellular tonicity in response to a hypertonic environment they increase intracellular concentrations of certain organic osmolytes by upregulating uptake and synthesis of these compounds to help counterbalance the high extracellular osmolality (Beck et al. 1998). Tonicity-Responsive Enhancer Binding Protein (TonEBP) is stimulated by alterations in extracellular tonicity (Burg and Garcia-Perez 1992) as well as an increase in intracellular urea concentrations (Kwon et al. 2009). When TonEBP is stimulated it interacts with TonEs in the promoter region of corresponding genes and upregulates their transcription (Miyakawa et al. 1999b; Woo et al. 2000). The genes that are upregulated via this interaction are sodium/myoinositol cotransporter (SMIT) (Rim et al. 2998), the sodium-chloride betaine cotransporter (BGT1) (Miyakawa et al. 1999a), aldose reductase (AR) as well as a phospholipase (Ko et al. 1997; Kwon et al. 2009). SMIT and BGT1 are transporters that allow for the influx of the organic osmolytes myosinositol and betaine respectively, while AR is an enzyme that synthesizes sorbitol and the phospholipase synthesizes glycerophosphorylcholine (GPC) (Kwon et al. 2009).

In addition to organic osmolyte regulation TonEBP is also capable of regulating HSP70 in response to hypertonicity and urea concentration (Woo et al. 2002c). HSP70 protects from the potentially lethal effects of urea that were mentioned above by acting as a chaperone of sorts to

unfolding or unfolded proteins. However, there is another type of upregulation of HSP70 that is specific to high concentrations of urea. The denaturation of proteins is primarily caused by the influx of urea, as well as a number of other factors including heat and stress (Morimoto 1997). These denatured proteins activate heat shock factor (HSF), which bind to heat shock element (HSE) and thus stimulate the transcription of HSP70 (Woo et al. 2002b).

While hibernators precisely match certain organic osmolyte concentrations and HSP70 expression to the magnitude of the corticopapillary gradient during the torpor/arousal cycle, it is not currently known if these animals can achieve similar responses during the summer. The goal of this research was to evaluate summer responses of kidneys from a typical hibernator during acute manipulations in water intake intended to mimic the corticopapillary changes experienced by hibernating animals. To do so, I placed animals into three treatment groups: dehydration group with water deprivation for 48 hours, sucrose group with ad libitum 600 mM sucrose water to maximize water intake, and a combination groups that experienced 48 hours of ad libitum 600 mM sucrose water followed by 48 hours of water deprivation. Specifically, I sought to answer three questions: 1) can hibernators rapidly alter their corticopapillary gradient during the summer? 2) if hibernators rapidly alter corticopapillary gradients, is this accompanied by upregulation of HSP70? and 3) are hibernators able to maintain blood homeostasis during acute changes in water intake during the summer? I predicted that the animals would indeed be able to perform a rapid shift in their corticopapillary gradients and that the response in HSP70 would be comparable to what is documented during hibernation of small rodents, an upregulation of HSP70 in response to increased tonicity and urea concentrations. I also predicted that as a result of the rapid changes in the kidney, animals would be able to maintain blood homeostasis despite experiencing acute changes in water intake. To test these predictions I measured urine output,

urine concentration, kidney osmotic concentration, renal urea concentration and renal HSP70 expression of thirteen-lined ground squirrels, *Ictidomys tridecemlineatus* during acute changes in water intake. I also evaluated markers of blood homeostasis in response to these manipulations.

Methods.

Animal Capture/ Maintenance

Fifteen (2 male, 13 female) ground squirrels, ***Ictidomys tridecemlineatus,*** were trapped using 5 x 5 x 25 cm live traps (Havart, Lititz, Pennsylvania) and peanut butter as bait. All animals were obtained from mixed use agricultural land near Saint John's University (45°35'34.5"N 94°24'27.3"W). To minimize parasite loads, squirrels were sprayed with a flea and tick compound prior to introduction to the lab. They were then placed in a plastic shoebox cage (25cm X 25cm X 50cm), with standard rodent/small animal bedding and an 8 inch section of vinyl downspout inside the cage to simulate their burrows. Once the animals were taken back to the lab they were placed on a metal rack with dimensions 6 feet by 5 feet. Four cages were placed per shelf with three cages on the bottom shelf. Animals were kept on a natural photoperiod and were provided ad libitum food and water. High protein dog food (Iam's Proactive Health Chunks; 25% crude protein, 14% crude fat, 3645 kcal/kg) was used along with sunflower seeds to simulate their high protein diet (Merriman). Once all animals were trapped and placed in the lab, they were each given an injection of 1% solution Ivermectin (Agri-mectin, St. Joseph, MO) at a dosage of 0.4mg/kg body mass to reduce parasite load

Experimental Procedure.

Animals all were placed in the metabolic cages (Tecniplast, West Chester, PA) overnight to acclimate to their new environment (roughly 15 hours) before modulation of water

consumption was altered. During this time period the animals were given ad libitum access to food and water. After this 15 hour period the sucrose group was given ad libitum 600 mM sucrose to mimic a minimal corticopapillary gradient like a hibernator would experience during a bout of torpor. The dehydration group was given no access to water to mimic a maximal corticopapillary gradient as a hibernator would experience during a period of arousal. The combination group underwent a slightly different protocol before entering the cage. These animals were given ad libitum access to 600 mM sucrose water for 48 hours prior to having water removed. 33 of these hours were spent in their standard cages and the last 15 hours were spent in the metabolic cages for acclimation before the dehydration segment of the protocol began. The combination experimental group was to mimic the rapid shift in corticopapillary gradient a hibernator would experience when going from a bout of torpor to a period of arousal (Figure 1).

Before the experimental procedure began a series of trials were performed to observe how the animals respond to the metabolic cages they would be placed in for the duration of the experiment. A preliminary trial of an animal in the metabolic cage indicated that an overnight period of approximately 15 hours was sufficient for animals to acclimate to the metabolic cages in terms of water and food consumption. I did two additional trial tests to make sure the sucrose would indeed coax the animals into consuming additional amounts of water and tested the effects of the combination group to make sure the animals responded relatively well in terms of body mass and activity.

Each animal was given a minimum of two weeks after being trapped before starting the actual experimental procedure. Each trial consisted of four animals in four separate metabolic cages with animals subjected to varying experimental procedures for each trail to help stimulate

random selection. Animals were placed in a Tecniplast metabolic cage to measure water and food consumption and urine output. During the experimental period, urine samples were collected at 0 (immediately following a 15 hour acclimation period) 24 and 48 hours. Urine samples were then stored in a -80°C freezer until analysis of the samples could be completed. Following completion of the experimental protocol, animals were euthanized with nitrogen gas in an airtight container. Serum samples were obtained by cardiac puncture and were allowed to stand at room temperature for 30 minutes then were centrifuged at 4,000rpm. The serum samples were analyzed by an Abaxis II blood analyzer (Abaxis, Union City, CA) to produce a complete blood chemistry profile. Remaining serum samples and kidneys were frozen in dry ice and stored in a -80°C freezer until subsequent analysis. Serum and urine osmolality were measured with an Advanced Instruments model 3320 freeze-point depression osmometer.

Partially thawed kidneys were sectioned into cortex, medulla and papilla and homogenized in distilled water (average dilution factor of 18.8:1) with disposable Biomasher II Tissue Grinders (Kimble Chase, Rockwood, TN). Following osmotic concentration measurements, samples were then analyzed for urea concentration using a colorimetric urea assay (Quantichrom, BioAssay Systems, Hayward, CA). For this assay 50µL and 200µL of working reagent were added to each well of a 96 well plate and incubated for 20 minutes. This plate was then read using Versamax microplate reader (Molecular Devices, Sunnyvale, CA) at 520nm. Following that procedure the other kidney harvested was sectioned in a similar manner, however only the papilla of each kidney was tested for HSP70 concentration using a HSP70 assay (HSP70 ELISA, Enzo Life Sciences, Farmingdale, NY). For this assay the samples were first centrifuged at 1000rmp for 15 minutes at 4 degrees Celsius. 100µL of varying sample and 100µL of buffer was then placed into each well of a 96 well plate and allowed to incubate for

two hours. The wells were then emptied and washed 4 times. This washing was repeated after adding 100µL of yellow antibody and incubating for one hour, and adding 100µL blue conjugate and incubating for another hour. Then 50µL substrate solution was added (and incubated for 15 minutes) followed by 50µL of amplifier solution which incubated for another 15 minutes. Finally the plate was read under a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) at 495 nm.

Statistics.

Blood chemistry, HSP70 concentrations, kidney osmolality, urea concentration, urine volume, and urine concentration comparisons between groups were made using a single factor ANOVA test (Excel Data Analysis Pack) and with Tukey's post-hoc tests used to determine significant difference between groups (Sigmaplot 10.0).

Results.

During the acclimation stage, the dehydration and sucrose groups were supplied with ad libitum water while the combination group was supplied with 600 mM sucrose. This is reflected in daily water consumption values which ranged from 10 – 15ml/day in the dehydration and sucrose groups, up to 28.1 ml/day in the combination group (Table 2). Importantly, there was no significant difference between the dehydration and sucrose groups during the acclimation period ($P = 0.548$) (Figure 2). There was a large degree of variation between individual animals during the acclimation periods, this is to be expected as they were put into a new environment without their synthetic burrows, potentially causing them stress and potentially altering water intake. Following the acclimation period, the sucrose group was supplied with 600 mM sucrose for 48 hours while the dehydration and combination groups had water withdrawn for 48 hours. At the

end of experimental conditions, the sucrose group's average daily water intake (30.9 ml / day) was quite similar to that seen in the combination group during acclimation ($P = 0.802$).

Relative rates of water intake had profound implications on urine output. Although there were no differences in urine output during acclimation between any of the groups ($P = 0.418$), by hour 24 urine output in both the dehydration ($P = 0.034$) and combination groups ($P = 0.035$) was less than the sucrose group (Figure 2). This trend intensified by hour 48 with daily urine output in the dehydration group only 7.9% of the sucrose group ($P < 0.001$) and daily urine output in the combination group only 4.6% of the sucrose group ($P < 0.001$). Overall the combination group experienced the most drastic change in urine output, ranging from 13.15 mL / day during the acclimation period and decreasing to 0.78 mL / day by hour 48. The sucrose animals at hour 48 should have been quite similar to the combination group during acclimation, as they both would have been exposed to 48 hours of sucrose water, and indeed their urine outputs at these two time points were quite similar (Sucrose hour 48 = 16.8 ml / day; Combination acclimation = 13.15 ml / day).

Not surprisingly, water intake also had a profound effect on urine concentration. At the end of acclimation there was no difference in urine concentration between the groups ($P = 0.180$), although the combination group had low urine osmolality (303.8 mOsm) compared to the dehydration (1350.4 mOsm) and sucrose group (1691.2 mOsm). Thereafter, the dehydration and combination groups steadily increased urine osmolality and decreased urine volume, while the sucrose group steadily decreased urine osmolality and increased urine volume (Figure 2). For instance, by hour 48 mean urine concentration for the dehydration group (3937.9 mOsm) and combination group (2868.0 mOsm) were roughly 800% and 600% larger than the final average value of the sucrose group (482.9 mOsm, $P < 0.001$). One striking feature of the changes in urine

concentration was the initial slow change in urine osmolality for the combination group. By hour 24, urine osmolality had only increased to 849.8 mOsm, and was significantly less than dehydration group's urine osmolality at the same time (2478.9 mOsm, $P = 0.044$). Between hour 24 and hour 48, the combination group's urine osmolality increased substantially, although it was still marginally less than the dehydration group ($P = 0.053$).

Maximal urine concentration is theoretically limited by papillary osmotic concentrations with urea being the most common osmolyte. Therefore I expected to see large differences between the groups in terms of overall osmotic and urea concentrations in the kidney after 48 hours of treatment. Indeed the papilla from the dehydration (589.2 mOsm) and combination (581.5 mOsm) groups were very similar in osmotic concentration ($P = 0.979$), while the sucrose group had a much lower osmotic concentration in papilla (375.1 mOsm) that was significantly less than the dehydration ($P < 0.001$) and combination group ($P = 0.001$) (Figure 3A). Similar to overall osmotic concentrations, the dehydration (195.8 mM) and combination (191.7 mM) groups also had much higher ($P < 0.001$ in both cases) papillary urea concentrations than the sucrose group (58.2 mM) (Figure 3B). In summary, both the dehydration and combination groups had sizeable osmotic and urea corticopapillary gradients by hour 48 that were larger than those found in the sucrose group, and correspond to their increased ability to form small quantities of concentrated urine.

Despite extreme changes in water intake over short periods of time, the animals' ability to alter corticopapillary gradients, urine concentration, and urine volume was sufficient to maintain blood chemistry for all markers other than blood urea nitrogen (BUN). We saw a significant increase in BUN for both the dehydration (32.0 mg / dL, $P < 0.001$) and combination group (25.8 mg / dL, $P = 0.001$) relative to the sucrose group (13.2 mg / dL, Table 3). The

statistically similar values across the other components of the blood chemistry profile means that these animals were able to perform homeostasis like they would in hibernation. Throughout the experiment I observed a loss in body weight across all groups. The combination and dehydration groups lost the most weight, however the dehydration group was the only one with a significant loss in body weight from the start of the experiment to the end ($P = 0.014$) (Table 1).

Despite having significantly lower papillary osmotic concentration, the sucrose group had almost double the HSP70 expression of either dehydration ($P = 0.038$) or combination ($P = 0.031$) groups. In fact, if HSP70 concentration is expressed as a function of papillary urea concentration for all study animals, there is a significant negative relationship ($P = 0.015$, $R^2 = 0.404$, Figure 5). In contrast to hibernating animals, summer animals with the most dilute vertical gradient and least urea concentration expressed the highest concentration of HSP70.

Discussion

The maximal urine concentration is limited by the corticopapillary gradient that exists in the kidney. A large gradient can be utilized to induce water reabsorption in the inner collecting duct of the kidney with high concentrations of osmolytes in the urine (Gottschalk 1964). The magnitude of the vertical gradient is regulated by a number of systems the two most important are the Renin-angiotensin-aldosterone system (RAAS) and the release of the hormone vasopressin (antidiuretic hormone or ADH). The RAAS system is initiated when blood volume, blood pressure, or relative sodium concentration in the distal convoluted tubule is low. When any of these variables are low baroreceptors near the juxtaglomerular cells (JG) release renin into the blood. Renin is then converted into angiotensin I by angiotensinogen. Angiotensin I is then converted to the functional component of this system Angiotensin II (Atlas 2007). Angiotensin II signals a variety of different responses from different tissues in the body 1) an increase in

sympathetic activity which induces arteriolar vasoconstriction and increased blood pressure. 2) The start of tubular reabsorption of sodium and chloride ions. 3) The secretion of aldosterone from the adrenal gland which amplifies the reabsorption of sodium and chloride ions. 4) The release of ADH from the pituitary gland which stimulates water reabsorption and increases urea transporters in the collecting duct as well as enhances sodium reabsorption in the ascending limb of the loop of Henley. The four effects listed contribute in the net reabsorption of sodium and chloride ions and of water, however ADH is the primary driver for establishing a concentrated corticopapillary gradient in response to low blood volume or increased plasma osmolality by increasing the urea transporters in the collecting duct and sodium transporters in the ascending limb of the loop of Henle.

As the osmotic gradient and urea levels rise the proteins of the kidney become stressed and begin to unfold as they are not optimized to function under the stress. As a result there are a number of protective compounds that are released in response to the stress these cells are put under 1) organic osmolytes 2) heat shock proteins. Urea destabilizes many macromolecular structures and inhibits functions such as ligand binding. The secretion of certain (GPC and Betaine) organic osmolytes can enhance protein folding and ligand binding which counteracts the concentrations of urea and assist the kidney cells to deal with the stresses of these conditions (Yancey 2005). In addition to these compounds, HSPs are expressed in response to a variety of stresses such as hyperthermia, hypertonicity, and elevated urea concentration. HSP's provide cytoprotection by preventing, or even correcting, the misfolding of proteins to allow the cell to survive in the unfavorable condition it is exposed to (Borkan et al. 1993, Lavoie et al. 1993, Lee et al. 1992, Neuhofer et al. 1998, Welch 1990). They are able to protect cells from the initial

abnormality, repair the protein unfolding as a result to the stress and have a limited ability to produce a state of resistance to subsequent stress the cell may undergo (Beere et al. 2000).

Hibernation induces the two extremes of cortico-papillary osmotic gradient. During periods of torpor blood pressure decreases to levels that are insufficient to sustain GFR (Shipley and Study 1951). Because of this, the cortico-papillary gradients within the kidney disappear (Clausen and Storesun 1971; Cotton and Harlow 2012). Every one to two weeks arousal occurs and the gradient rapidly returns. If the gradient remained dilute, the animal would produce diluted urine and would be at risk for heavy dehydration. During this arousal period and subsequent high osmotic gradients in the kidney, the expression of organic osmolytes and HSP70 drastically increases in response to the stress of the environment on the cells (Cotton 2012). This study was performed to test whether a common Minnesota hibernator, the thirteen-lined ground squirrel, was able to handle acute, summertime water challenges by: 1) rapidly altering their cortico-papillary gradient and 2) matching HSP70 expression to magnitude of corticopapillary gradients. Ultimately, the successful outcome of these events was judged by examining alterations serum markers associated with blood homeostasis.

Urine Volume and Osmolality

Maximal urine concentration in combination with minimal urine output was achieved in the combination and dehydration groups by 48 hours, while the sucrose group had minimal urine concentration with a maximal urine output at hour 48. Urine output is regulated largely by RAAS and ADH. In response to the vast increase in water intake by the sucrose group, activation of the RAAS and ADH secretion would largely decrease in response, due to decreased blood osmolality and an increase in blood volume and blood pressure. Decrease in these two systems would cause a vast increase in urine output, which was significantly higher at hours 24 and 48

($P = 0.035$, $P < 0.001$ respectively). In opposite conditions RAAS and ADH activity increase in response to increased blood osmolality and decreased blood volume and pressure. Since these two systems are increased, urine volume is drastically lowered to oppose these negative effects of dehydration. The sucrose group's average urine concentration was inversely proportional to urine output observed. The combination and dehydration groups average urine osmolality increased inversely proportional to the levels of urine the produced. These two groups achieved a maximal osmotic gradient within 48 hours of dehydration.

Interestingly the combination groups' urine volume decreased at hour 24 before the urine concentration achieved maximal concentration at hour 48 (Figure 2). During dehydration the RAAS system will be upregulated along with ADH secretion. This will cause maximal sodium and water reabsorption in the DCT (RAAS), which aids in reducing urine volume. Additionally, ADH ensures maximal H_2O reabsorption in the inner collecting duct due to increased AQ-2 insertion in the apical membrane of epithelial cells, which aids in reducing urine volume as well as increasing urine concentration. Concentration of urine is, however, limited by the magnitude of the vertical osmotic gradient. The corticopapillary gradient takes time to be established and requires maximal reabsorption of both sodium in the ascending limb (due to ADH regulation of NKCC cotransporters) and urea in the inner collecting duct (due to ADH regulation of UTA-1 and UTA-2 channels). As dehydration time increases, maximal ADH secretion due to low blood volume and increased plasma osmolality results in peak reabsorption of sodium and urea likely establishing a large vertical osmotic gradient by hour 48, coinciding with peak urine concentration. These results identify that the animals appropriately responded to hibernation like transition (diuresis followed by antidiuresis) by a relative rapid shift in urine output and concentration.

Corticopapillary Osmolality and Urea Concentration

As mentioned above, the vertical osmotic gradient is the key component that determines the maximal osmotic concentration for urine. As the gradient is increased the concentration of the urine can therefore also increase, which was observed in my experiment. For instance, the dehydration and combination groups had the largest vertical osmotic gradient and also the highest urine concentrations, while the sucrose group had the smallest vertical osmotic gradient and the lowest urine concentrations. The increase in corticopapillary concentration is highly dependent on the presence of ADH for both maximal sodium reabsorption in the ascending limb and urea reabsorption in the collecting duct, conditions likely experienced in the dehydration and combination groups but not the sucrose group. ADH also regulates water reabsorption in the collecting duct, explaining why the sucrose group's renal osmolality is higher than that of the urine osmolality. My data coincided with the fact that during antidiuresis with elevated ADH, urea accumulates in the renal medulla to very high concentrations (Lee et al. 2011). Lowest urea levels were observed in the sucrose group, while the maximal levels were in the dehydration and combination group. Similarity the concentration of urea increased moving from cortex to papilla in all experimental groups.

Blood Chemistry

As renal urea levels increased due to increased reabsorption of urea, clearance of plasma urea drops resulting in a significant increase in serum BUN levels for the dehydration and combination groups ($P < 0.001$ and $P = 0.001$ respectively). An important note is that the creatinine, sodium and potassium blood serum levels were not significantly different regardless of the experimental conditions. This indicates that the animals were able to alter urine output and concentration sufficiently to maintain blood homeostasis, similar to animals arousing from

hibernation. Although not significant, the sucrose groups' sodium levels trended lower than that of the other two groups ($p = 0.054$) while potassium levels trended slightly higher ($p = 0.16$). While the data suggest that the dehydration and combination treatments pushed the animals to the edge of their homeostatic control range, we can't know for sure without control values from freshly captured animals in the field. The borderline p-values could also be due to the relatively low sample size and lack of power.

Papillary HSP70 Concentration

During periods of arousal after bouts of torpor and increased expression of HSP70 could be due to an increased concentration of urea in the interstitium, however increased expression of HSP70 can be upregulated in response to a number of other factors as well (stress, hypertonicity). Most heat shock proteins are upregulated in response to stress. Heat shock factors (HSFs) are activated in response to nonnative proteins that accumulate in response to a variety of insults (Morimoto 1997). Activation of the HSF bind to heat shock elements (HSE) which then stimulates the transcription of HSP70. A different pathway for the upregulating of HSPs is through the tonicity-responsive enhancer binding protein (TonEBP). This protein is activated in response to hypertonicity and then upregulates the transcription of a specific HSP. These pathways are separate in their nature of response, which allows the HSPs to become active in response to a variety of different responses (Woo et al. 2002b).

In the kidney the major stimulus for the family of heat shock proteins is high intracellular concentration of urea. Urea is a potent protein-unfolding agent that, in the concentrations reached in the renal papilla of many mammals, may lead to destabilization of protein structure and deterioration of protein function (Somero and Yancey 1997, Yancey et al. 1982). HSP 70, by reversibly binding to unfolded or partially unfolded proteins, promotes the reformation of the

correct conformation, thus preventing irreversible loss of function (Fink 1999, Fourie et al. 1994, Hightower and Leung 1998, Neuhofer 2002b, Skowyra et al. 1990, Welch 1992)**.**

However I was surprised to see that my research shows almost perfect contradiction to the current literature on HSPs. It shows that in the case of the renal papilla of thirteen lined ground squirrels there is an inverse relationship between urea concentration and expression of HSP70 (Figure 4). There are a couple of different possibilities on why there was a significantly higher expression in the animals that experienced diuresis rather than antidiuresis. This specific hibernator lives in an environment where it doesn't have access to a lot of water. Under natural conditions, the corticopapillary gradient is most likely at a fairly high level. With this in mind, when the animals were given access to 600 mM sucrose water they drastically increased water intake and diminished the vertical osmotic gradient. Although typically associated with high tonicity, HSP70 expression can also be induced by lower than normal tonicity in the kidney (Neuhofer 2002c). It is also a stress that these cells are not used to and HSP70 is classified as a stress response protein. This may explain why the animals exposed to dehydration showed relatively low levels of HSP70. Another possibility is that there could have been a mismatching of organic osmolytes in response to the loss of the corticopapillary gradient. Under this assumption there would be a high expression of organic osmolytes being produced via the TonEBP prior to the sucrose treatment as the urine osmolalities were on average about 1700 mOsm prior to sucrose treatment which would stimulate the TonEBP pathway. Then when the animals consumed high quantities of the 600 mM sucrose water, the vertical osmotic gradient was greatly diminished. If these organic osmolytes did not leave the cell's fast enough they could have "over stabilized" proteins within the cell in the absence of high urea concentrations. This

"over stabilization" of proteins within the cell could in fact cause increased activity of HSF resulting in upregulation of HSP70 to refold the proteins to their resting state.

In summary my research went against the common knowledge of expression of HSP70 with elevated osmolalities of urea, a negative correlation rather than a positive one. This is not out of the realm of possibilities as described above, there has been at least one other project that has discovered an increase in the expression of HSP70 in a hypotonic environment rather than a hypertonic one (Neuhofer 2002c). It is difficult to get the full picture with my results because I was unable to sample the organic osmolyte concentration in the animals at the end of the experiment. This sample is key because looking at just HSP70 concentration is only one half of the protection response these cells utilize. HSP70 protects the misfolded proteins while the organic osmolytes balance out the tonicity of the cell. Having this one piece of information could have filled in the gaps and created a full picture as to what sort of changes occurred in these animals throughout the experiment. The other issue is that I did not sample a control group straight from the wild to see what resting blood chemistry, HSP70 and urea concentration looked like. This would have allowed me to compare my experimental values to the animal's normal resting values not only the relative relationship we normally observe between HSP70 and urea. Future research will be conducted with the same experimental procedures applied and with non-hibernating rodents to compare the differences between the two classes of rodents.

Human applications of this research would be a stasis-like state for extended space travel. With advances in technology and the recent interest in the science community for space travel there is no doubt going to be a project whose goal is successfully putting a human being in stasis for an extended period of time. In fact some people have already begun to design projects that attempt to take into account the logistics (Ayre et al. 2004). During a hypometabolic stasis-

like state, risk of muscle atrophy and bone loss would be reduced as in hibernators (Cotton and Harlow 2010, Donahue et al. 2005, Tinker et al. 1998). However, the human body would undergo the same types of transitional processes as an animal would undergo in hibernation. For example, the individual would go through a period of time where metabolic functions are lowered (like a bout of torpor) and then switch to a state of high metabolic function after coming out of stasis (like an arousal period). Space stasis is only one application, there is also research being done on perfusing trauma patients with cold saline that would also induce a hypometabolic state to give doctors extra time to operate current research involves testing this on dogs and pigs (Weihs et al 2011, Wu et al. 2006). In either of these examples, a human would experience low body temperature, metabolic rate, and GFR during stasis or cold-saline infusion, much like a bout of torpor. The person would then transition to elevated metabolic rate, increased body temperature, and a return of GFR as stasis was ended or blood temperature was increased, much like an arousal period. As this occurs, the patient would likely regain a vertical osmotic gradient and require adequate upregulation of HSP70 and organic osmolytes to facilitate protection of epithelial cells in the kidney papilla.

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Figure 1.
Overview of procedures for each experimental group. Acclimation periods consisted of 15 hours in the metabolic cages prior to the initiation of experimental water intake: either 48 hours of water deprivation or 48 hours of ad libitum 600 mM sucrose. The combination group had a total of 48 hours ad libitum 600 mM sucrose prior to 48 hours of water deprivation. Blood samples and kidneys were collected at the end of 48 hours.

Figure 2.
Urine parameters during experimental conditions. Urine osmolality did not differ between the three experimental groups following acclimation period, but dehydration and combination groups were statistically different at 24 hours. This was followed by an increase in the concentration of the combination group leading to the dehydration and combination groups having significantly more concentrated urine by hour 48 than the sucrose group. Similarly urine volume did not differ between the three experimental groups following acclimation period, but by hour 48 the dehydration and combination groups were producing substantially less urine than sucrose group. N = 5 for each group, error bars represent standard error of the mean.

Figure 3.
Comparison of kidney osmolality and urea concentration by kidney region. A) average kidney osmolality for all experimental groups . B) average urea concentration for all experimental groups. Cortical osmotic concentration of the combination group was statistically higher than that of the sucrose group, while sucrose medullary osmotic gradients were statistically lower than both the combination and dehydration groups at the medulla and papilla. Medullary urea concentrations of the dehydration group were statistically higher than both the combination and sucrose groups with papillary urea concentrations significantly higher in the dehydration and combination groups than the sucrose group. N = 5 for each group, error bars represent standard error of the mean.

Figure 4.
Papillary HSP70 expression as a function of papillary urea concentration. There was a significant negative relationship between urea concentration and papillary HSP70 expression ($p = 0.015$, $R^2 = 0.404$).

Figure 5.
Average urea concentrations for the three experimental groups across the three kidney sections as well as average HSP70 expression in the papilla region of the kidney. Although sucrose group had significantly lower papillary urea concentrations than either dehydration ($P < 0.001$) or combination groups ($P < 0.001$), the sucrose group had almost double the HSP70 expression of dehydration ($P = 0.038$) or combination groups ($P = 0.031$). N = 5 for each group, error bars represent standard error of the mean.

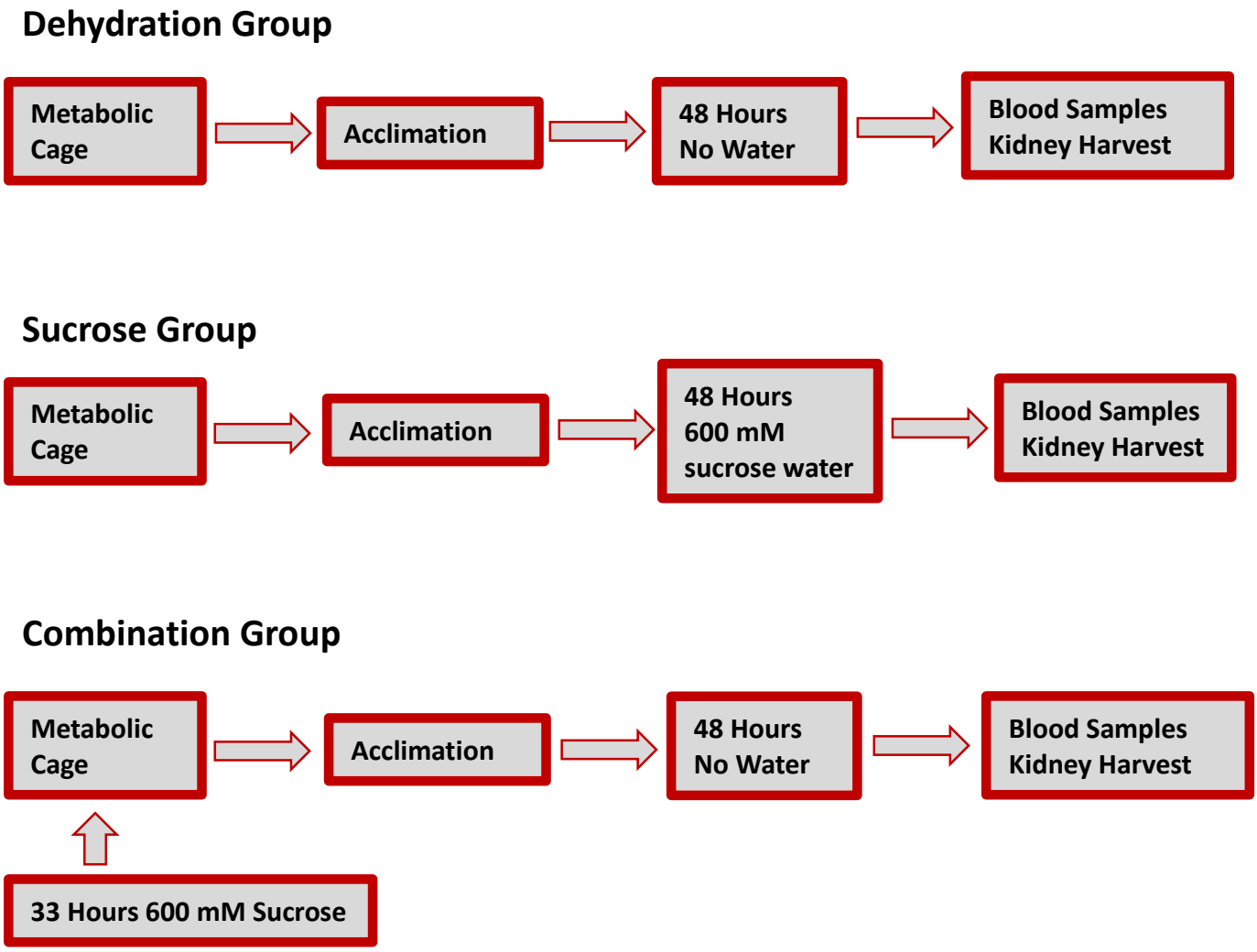

Dehydration Group
Metabolic Cage
Acclimation
48 Hours No Water
Blood Samples Kidney Harvest
Sucrose Group
Metabolic Cage
Acclimation
48 Hours 600 mM sucrose water
Blood Samples Kidney Harvest
Combination Group
Metabolic Cage
Acclimation
48 Hours No Water
Blood Samples Kidney Harvest
33 Hours 600 mM Sucrose

Figure 1.

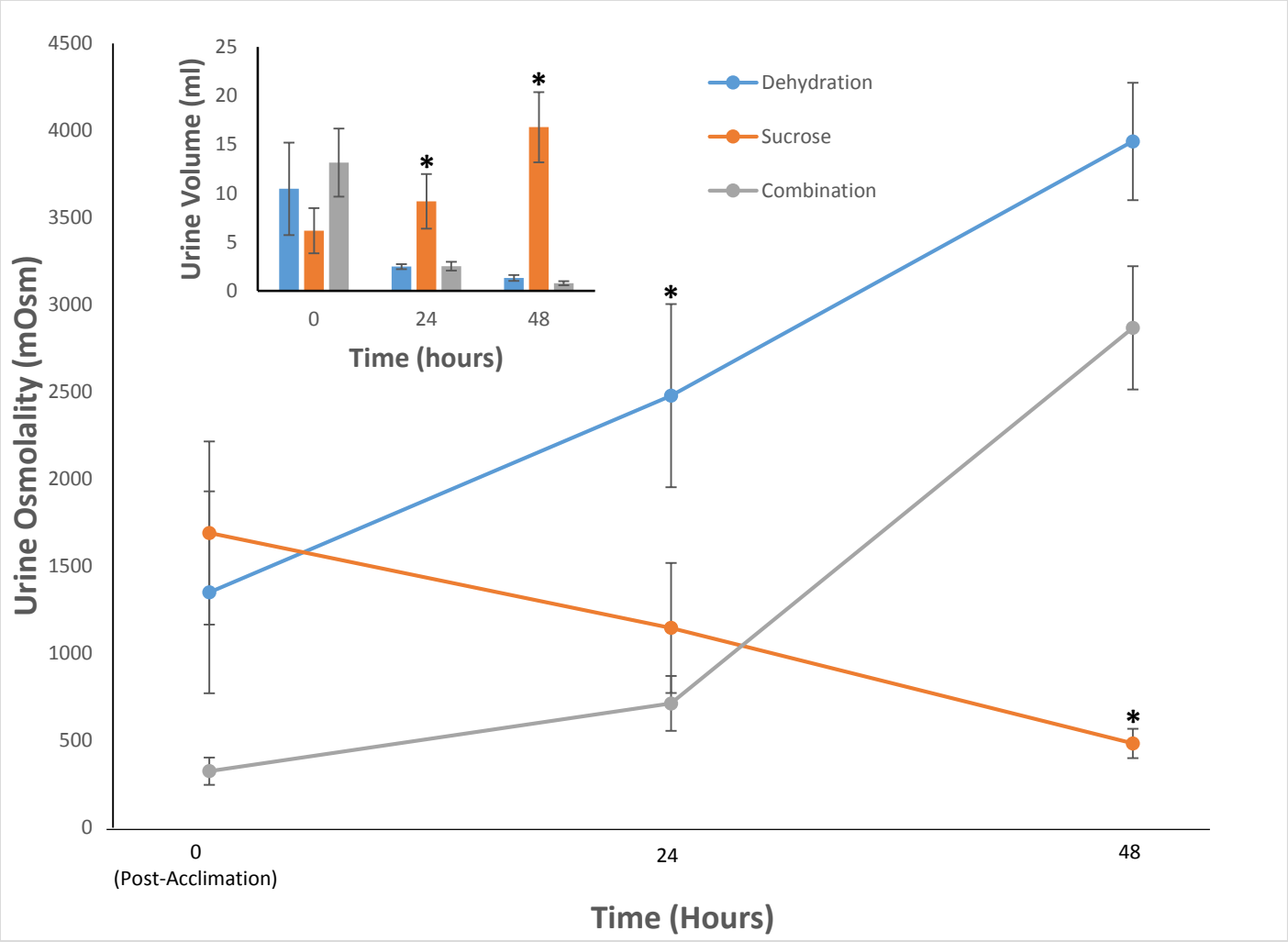
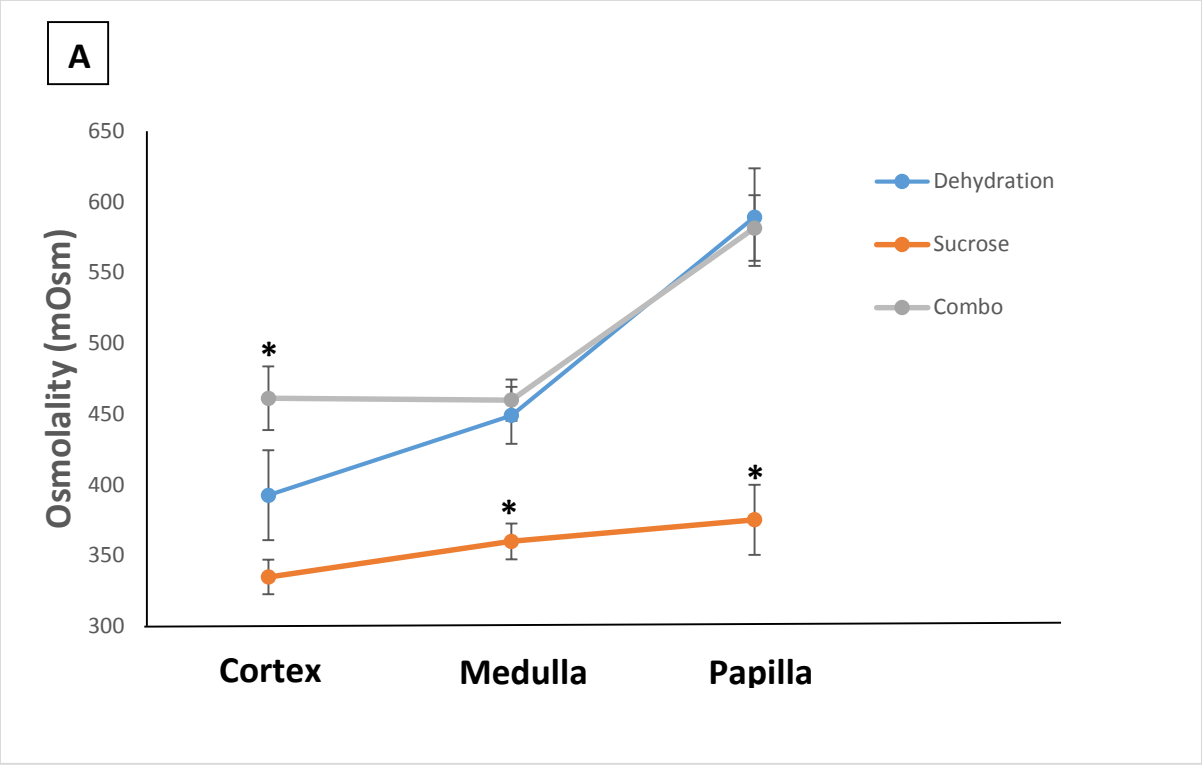

Urine Osmolality (mOsm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
(Post-Acclimation)
24
48
Time (Hours)
Dehydration
Sucrose
Combination
Urine Volume (ml)
25
20
15
10
5
0
0
24
48
Time (hours)
*
*
*
*

Figure 2.


A
650
600
550
500
450
400
350
300
Osmolality (mOsm)
Dehydration
Sucrose
Combo
*
*
*
Cortex
Medulla
Papilla

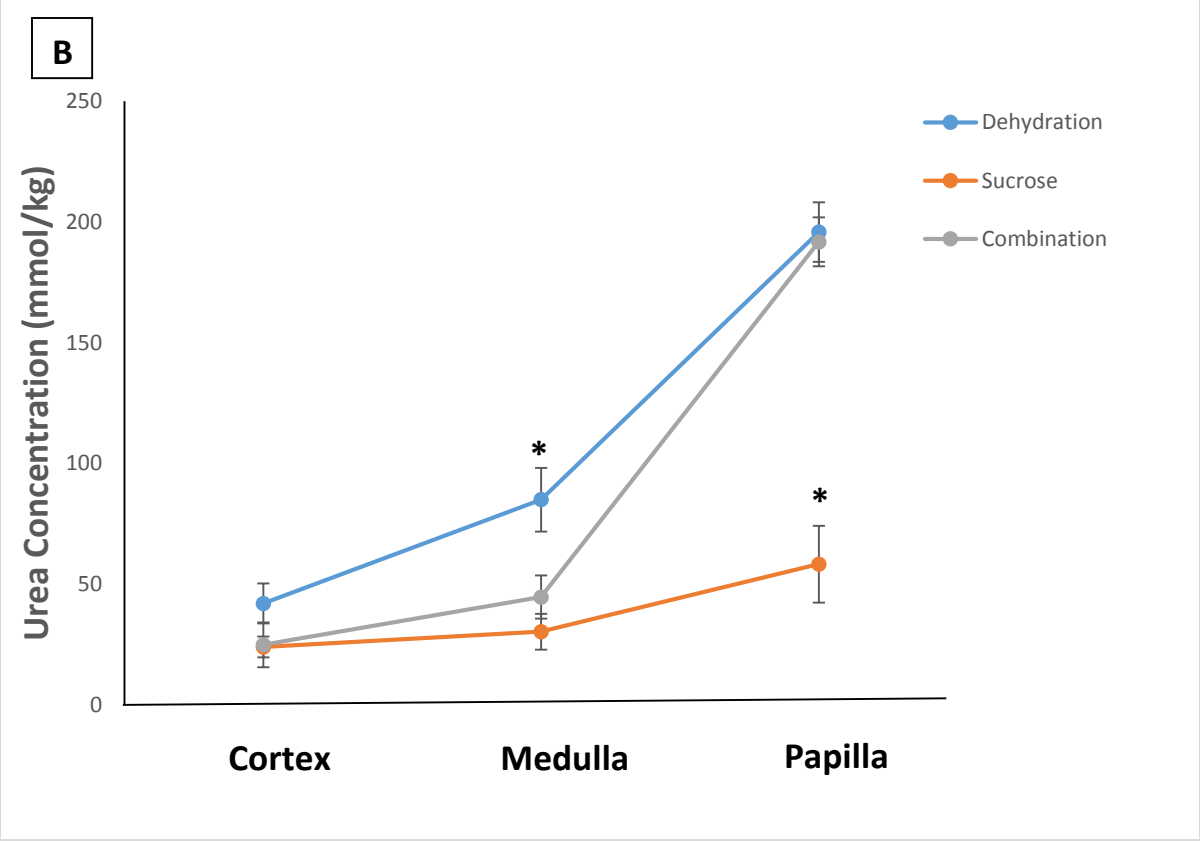

B
250
200
150
100
50
0
Urea Concentration (mmol/kg)
Dehydration
Sucrose
Combination
*
*
Cortex
Medulla
Papilla

Figure 3.

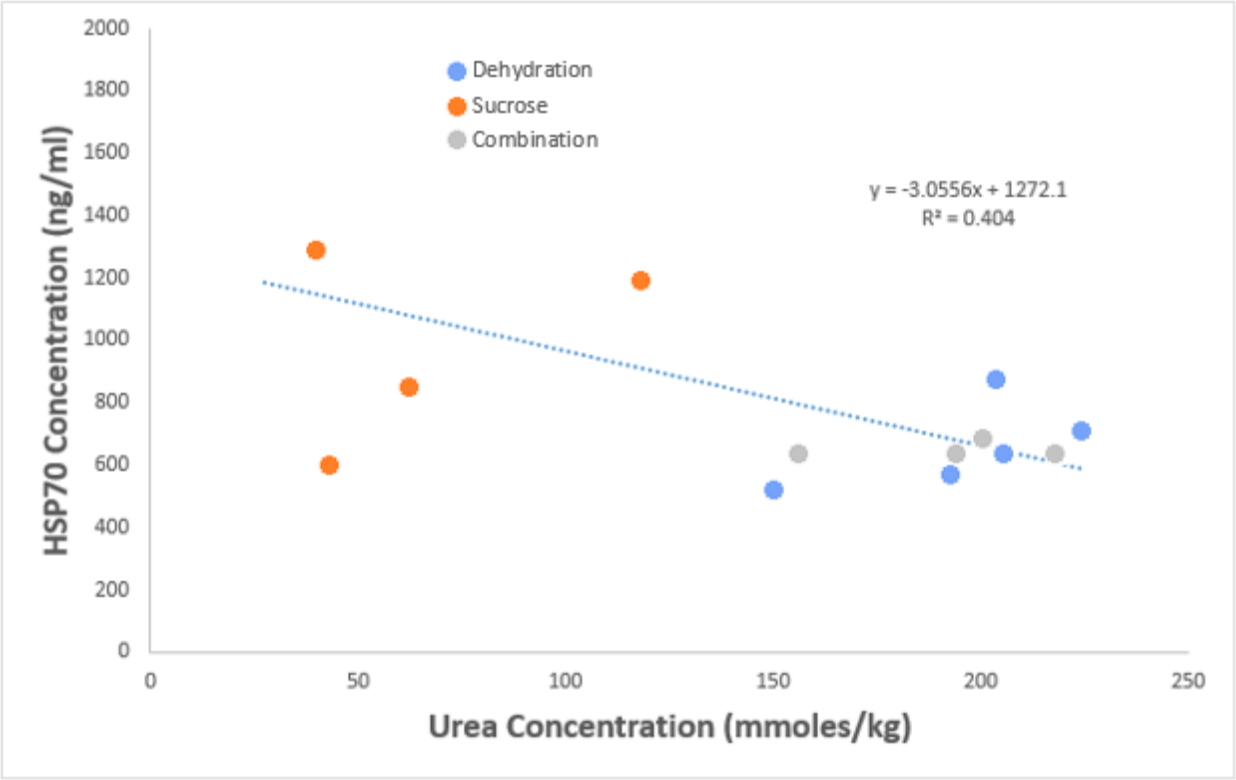

HSP70 Concentration (ng/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
250
Urea Concentration (mmoles/kg)
Dehydration
Sucrose
Combination
y = -3.0556x + 1272.1
R² = 0.404

Figure 4.

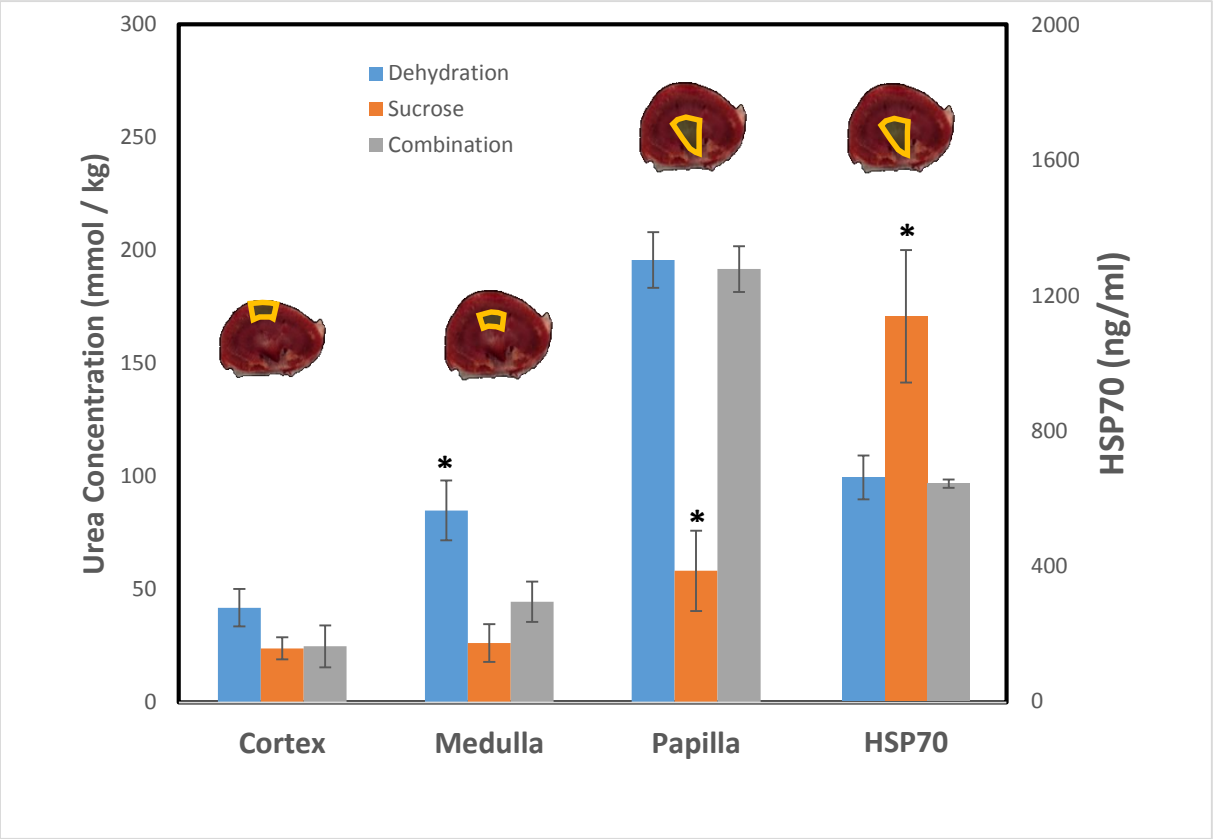

Dehydration
Sucrose
Combination
Urea Concentration (mmol / kg)
HSP70 (ng/ml)
300
250
200
150
100
50
0
2000
1600
1200
800
400
0
*
*
*
Cortex
Medulla
Papilla
HSP70

Figure 5.

Table 1. Mean body mass for experimental groups

Group	Trapping[a] (g)	Start[b] (g)	End[c] (g)	Difference (g)
Dehydration	156.54 ± 7.85	242.88 ± 7.12	214.62± 5..56	-28.26 ± 2.62
Sucrose	162.72 ± 4.21	243.54 ± 17..56	236.54 ± 16.34	-7 ± 2.29
Combination	168.1 ± 12.39	225.86 ± 19.81	197.6 ± 18..13	-28.26 ± 4.82

[a]Denotes body mass within 1 hour of trapping
[b]Denotes body mass at start of experiment
[c]Denotes body mass at end of experiment

Table 2. Average water consumption of dehydration, sucrose and dehydration groups in standard after acclimation and at the end of experimental testing.

Group	End of Acclimation Period (ml)	48 Hours (ml)
Dehydration	14.78 ± 5.86	0
Sucrose	9.64 ± 4.31	30.9 ± 9.8
Combination	28.1 ± 4.36	0

Table 3. Critical differences in blood chemistry data for *Ictidomys tridecemlineatus* following the experimental protocol. The numbers were derived from the average values for the five animals in each group with the standard error reported.

Group	BUN (mg/dl)	CRE (mg/dl)	NA+ (mmol/L)	K+ (mmol/L)	CA (mg/dl)
Dehydration	32 ± 1	0.471 ± 0.06	152.2 ± 2.01	6.62 ± 0.42	11.325 ± 0.17
Sucrose	13.2 ± 1.32	0.4 ± 0.08	148.4 ± 1.03	7.96 ±0.45	11.12 ± 0.14
Combination	25.8 ± 2.37	0.46 ± 0.11	154 ± 1.06	7.3 ± 0.44	11.24 ± 0.17