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# Hibernator and non-hibernator responses to acute changes in water intake

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# Hibernator and non-hibernator responses to acute changes in water intake

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**Project title:** Hibernator and non-hibernator responses to acute changes in water intake

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*Abstract*

Hibernating animals undergo dramatic changes in metabolic rates during torpor. One of most notable changes in these animals is the ability to maintain blood pressure and perfuse certain organs. Consequentially, blood perfusion to the kidneys is greatly decreased and the ability to concentrate urine halts. However, about once a week, torpor is interrupted and the animal becomes active to rewarm itself about once a week. This activity induces rapid regeneration of the extracellular osmotic gradient of the kidney, and allows urine to be concentrated. Nonetheless, regaining the extracellular osmotic gradient creates a potentially fatal consequence to the kidney cells. To combat the adverse effects of regaining an osmotic gradient, the animals significantly increase protective mechanisms within their kidneys, such as heat shock proteins (HSP) and organic osmolytes. It is well known that these rapid changes occur during cold-seasons, and little research has been done to compare these protective mechanisms within hibernating animals during the summer. To address this question, we worked in conjunction with a previous researcher to compare the data that has been done on a typical hibernator (*Ichtidomys Tridecimlineatus*) to data of a non-hibernator (*Rattus Norvegicu*s). Rattus Norvegicus was placed under various water intake regimes to facilitate changes in the vertical osmotic gradient of the kidney. We then measured renal expression of HSP70, papillary urea, sorbitol, and the glomerular filtration rate in response to changes in the vertical osmotic gradient. Experimental treatments led to expected changes in urine volume and concentration for the rats, and serum homeostasis was largely maintained. GFR significantly decreased in the dehydration groups compared to the 600mM sucrose groups. The expression of HSP70 was not significantly different in any of the rat groups, but there was increased sorbitol concentrations as papillary urea concentrations increased during combination treatment.

## *Introduction*

Hibernation in heterothermic mammals and birds is characterized by pronounced temporal reductions in body temperature, energy expenditure, water loss, and other physiological functions (Geiser, 2013). Core body temperature in these hibernating animals can drop from 37°C to just above ambient temperatures. Respiratory rates decrease from 100-200 breaths per minute to ~4-5 breaths per minute. Heart rates may also drop from 200-300bpm to ~3-4bpm, thus significantly decreasing cardiac output of the hibernator (Carey, Andrews, & Martin, 2003). This decreased cardiac output affects the animal's ability to sustain an adequate blood pressure during bouts of torpor. As mean arterial blood pressure (MAP) falls below 80mmHg, decreased hydrostatic pressure in the glomerular capillaries begins to negatively impact filtration (Cotton, 2012a; Sherwood, 2010). At a MAP of 40mmHg and below, researchers have shown an abolished ability to filter plasma through the glomerulus which results in a GFR of nearly 0 (Lesser, Moy, Passmore, & Pfeiffer; 1970; Tempel & Musacchia, 1975). Without glomerular filtration, cortico-papillary gradients within the kidney are lost. Thus, the ability to concentrate urine is also abolished (Clausen & Storesun, 1971; Tempel & Musacchia, 1975).

Consequentially, without the ability to filter through the glomerulus, waste products may accumulate in the plasma and animals may lose the ability to regulate pH. With the loss of a cortico-papillary gradient, animals may also experience disruptions in hydromineral balance and osmotic regulation due to the inability to excrete concentrated urine. However, despite these dramatic physiological changes, the hibernating animal is still able to maintain constant blood chemistry homeostasis. (Carey, et. al., 2003; Jani, Martin, Jain, Keys, & Edelstein, 2013).

During arousal, the animal's cortico-papillary osmotic gradient within the kidneys returns, and the ability to concentrate urine is consequentially reestablished when the animal reaches euthermic levels (Baddouri & Elhilali, 1986). However, there is evidence suggesting that GFR does not resume until midway through an arousal bout (Baddouri & Elhilali, 1986), preventing severe dehydration. Before GFR is regained, gradients are rapidly regenerated during arousal, often within a few short hours, creating a hyperosmotic environment for the kidney cells (Baddouri & Elhilali, 1986). High concentrations of intracellular salts and crowding of large intracellular molecules greatly affect the structure and activity of proteins, DNA, and other cellular macromolecules in the kidney medulla and papilla. (Burg, Kwon, & Kultz, 1997). The epithelial cells therefore must quickly become isosmotic with the extracellular environment to avoid severe shrinkage in volume and intracellular crowding. In addition to osmolality changes, the high concentration of urea present in the extracellular space can freely enter the cells and cause protein denaturation. This creates the necessity of having protective mechanisms quickly upregulated to avoid fatal consequences.

*Organic Osmolytes and Heat Shock Proteins*

To combat the harmful effects of a hyperosmotic environment, the kidney cells sequester compatible and counteractive osmolytes. Compatible osmolytes balance extracellular concentrations of sodium that are increasingly abundant from cortex to papilla (Burg, 1997; Yancey, 2005). Common compatible osmolytes in the kidney include sorbitol and *myo*-inositol. Sorbitol is synthesized within kidney epithelial cells by an enzyme known as aldose reductase (AR) (Burg & Ferraris, 2008; Garcia-Perez and Burg 1991; Kwon, Lim, & Kwon, 2009) while *myo*-inositol is dependent on membrane transporters (SMIT transporter) for cellular uptake from

the extracellular space (Burg & Ferraris, 2008; Garcia-Perez and Burg 1991; Kwon, et. al., 2009). Counteracting osmolytes also balance extracellular osmotic gradients, but they also protect intracellular proteins from urea-induced denaturing (Beck, Guder, & Schmolke, 1998; Burg, Ferraris, & Dmitrieva, 2007; Garcia-Perez & Burg, 1991; Jani, et. al., 2013; Neuhofer & Beck, 2006). Glycerophosphorylcholine (GPC) and betaine are the most notable counteracting osmolytes in the mammalian kidney (Neuhofer & Beck, 2005). Renal cells synthesize GPC within the cell, much like sorbitol (Burg & Ferraris, 2008; Garcia-Perez and Burg 1991; Kwon, et. al., 2009). GPC is a product of degradation from phosphatidylcholine (Zablocki, Miller, Garcia-Perez, & Burg, 1991). The enzyme responsible to catalyze the degradation reaction is known as neuropathy target esterase (NTE). Betaine depends on cellular uptake via transporter proteins in the cell membrane, similar to *myo*-inositol (BGT1 transporter) (Buck & Ferraris, 2008; Garcia-Perez and Burg 1991; Kwon, et. al., 2009).

In addition to organic osmolyte regulation, kidney cells also rely on heat shock proteins (HSPs) to counteract the detrimental effects of urea on the cells (Burg, et. al., 1997; Neuhofer, Fraek, & Beck, 2002). HSPs act as chaperones to proteins allowing proteins to remain folded in the correct conformation by binding reversibly to the hydrophobic side chains, removing mis-folded proteins, and assisting in refolding the proteins (Fink, 1999).

*Osmolyte and HSP Regulation*

Cells of almost all organisms accumulate organic osmolytes when exposed to hyperosmolar environments, most often in the form of high salt or urea (Burg & Ferraris, 2008). There is sufficient evidence suggesting that the main contributor to the regulation of the above osmolytes

and HSP is a tonicity- responsive enhancing binding protein (TonEBP) (Burg, et. al., 2007; Burg & Ferraris, 2008; Kwon, et. al., 2009; Neuhofer, et. al., 2002). The upregulation of TonEBP can be induced by hyperthermia, hypertonicity, and many other environmental stresses (Alfieri, Bonelli, Petronini, & Borghetti, 2002). The most dramatic upregulation occurs in hypertonic environments in addition to high urea concentrations. (Burg and Ferraris, 2008).

TonEBP promotes accumulation by transcriptional stimulation of plasma membrane transporters, SMIT and BGT1, and increase synthesis of GPC and sorbitol within the cell. (Burg & Ferraris, 2008; Kwon, et. al., 2009). The upregulation of these transporters allows their respective molecule to enter the cell and accumulate to create an isosmotic environment – balancing out the concentrations of sodium within the renal medulla. In non-hibernators, these mechanisms take a large amount of time relative to the time hibernators take to accumulate HSPs and organic osmolytes (Burg, et. al., 2007). During hibernation, these changes of protective osmolytes are during a short amount of time, within 1-2 hours.

It is well known that these rapid changes in cortico-papillary gradients occur during hibernation seasons, but to date only one study has looked at changes in organic osmolytes from torpor through arousal (Cotton, 2012b), and little research has been done to compare these protective mechanisms within hibernating animals during the summer. To address these deficiencies in the literature, we decided to investigate how the kidneys of a typical hibernating species in summertime (*Ictidomys tridecimlineatus*) would handle acute changes in water intake compared to a typical non-hibernating species (*Rattus norvegicus*). To do so, we experimentally altered water intake over a 48 hour period. At each 24h mark starting at time 0, I measured changes in urine volume, urine concentration, and vertical urea gradients within the kidney. I also measured concentrations of protective osmolytes to examine whether or not hibernators and

non-hibernators vary in their ability to rapidly sequester protective osmolytes and protect renal tissue.

I predicted that the hibernating animals would be able to increase vertical osmotic gradients more rapidly during transition from acute diuresis to acute anti-diuresis than the non-hibernating animals under acute water intake manipulation. We believe that the response in a specific HSP most prevalent in the renal papilla - HSP70 - would be comparable to what is documented during hibernation of small rodents when cortico-papillary osmotic gradients return during arousal. Sorbitol within the papilla is predicted to also increase as urea gradients become steeper. Our predictions are based on hibernating states and the animal's subsequent ability to rapidly regenerate these protective solutes during times of anti-diuresis (Cotton, 2012b). Blood chemistry is also predicted to be maintained during acute changes in water intake. Without the ability to concentrate urine, blood composition will suffer as there will be a buildup of waste products in the plasma.

## *Methods*

### *Animal Housing*

Fifteen lab rats, *Rattus norvegicus*, were housed in 20m X 30cm X 30cm individual cages with metal wire bottoms that were suspended from the racks to allow excrement to fall through. Excrement was collected on rimmed metal sheets under the cages that were filled with aspen bedding. These individual cages were placed on a metal rack with dimensions 6ft X 5ft. Each

shelf contained four individual cages, and each rack contained four shelves, holding a total of sixteen individual cages. Animals were kept on a 12h daylight/ 12h darkness photoperiod and were provided *ad libitum* food (Purina Lab Diet 5001, Largo, FL, USA) and water.

Fifteen 13-lined ground squirrels, *Ictidomys tridecemlineatus,* were captured on prairie land just north of St. John's University, Minnesota during the summer months. The squirrels were trapped using 5 x 5 x 25 cm live traps (Havahart, Lititz, Pennsylvania) using peanut butter as bait. Following the trapping of each ground squirrel, they were immediately given an injection of 1% ivermectin solution (Agri-mectin, St. Joseph, MO) and sprayed with Adams Flea and Tick Spray in the lab. The ground squirrels were then placed in 25cm X 25cm X 50cm plastic shoebox cages, with standard rodent/small animal bedding and an 8in section of vinyl downspout inside the cage to simulate their tunnels. The ground squirrels were kept on a natural photoperiod of 12h daylight/12h darkness and were provided *ad libitum* food (Iam's Proactive Health Chunks; 25% crude protein, 14% crude fat, 3645 kcal/kg) and water. Ground squirrel trapping and housing was accomplished by O'Gara (2015). .

*Experimental Procedure*

Animals were housed under lab conditions for at least two weeks prior to the experiments. After two weeks, the animals were then placed in metabolic cages (Tecniplast, West Chester, PA.) overnight (15h) before the experiment began. Animals were placed in metabolic cages to measure water and food consumption, and urine output. To allow animals to acclimate to the metabolic cage, they were maintained with *ad libitum* food and water during the

15h overnight period. Each animal was then tested under one of the following conditions, five rats and five ground squirrels per group:

1. **Dehydration**

   After the 15h acclimation period, animals were water deprived (no water present in the metabolic chambers) for a period of 48 hours to stimulate antidiuresis and maximize the vertical osmotic gradient in kidneys.

2. **Sucrose**

   After the 15h acclimation period, animals were given *ad libitum* 600 mM sucrose water for a period of 48 hours to stimulate diuresis and minimize the vertical osmotic gradient in kidneys.

3. **Combination**

Animals in the combination group were given ad libitum 600mM sucrose water within their normal cages for a total of 33 hours prior to moving into the metabolic cages for the 15 hour acclimation period. During the 15h acclimation period, *ad libitum* 600mM sucrose water was continued, giving a total of 48 hours on the 600mM sucrose water just like the sucrose group. After the 15h of acclimation, 48h of water deprivation followed to stimulate a rapid shift from diuresis to antidiuresis. We utilized the combination group to examine how the animals would respond to the hibernation-like condition of rapidly transitioning from no urea gradient and associated inability to form concentrated urine to having a large urea gradient and concomitant ability to form concentrated urine.

Rats and ground squirrels were assigned the above groups randomly.

In a separate experiment, the remaining five additional ground squirrels and five additional rats were provided *ad libitum* water throughout the entirety of the experiment with no manipulations to their regular eating or drinking patterns. Animals received congruent treatments to their experimental counterparts including a two-week acclimation period, and a 15h acclimation period in the metabolic cages. Urine volumes and concentrations were averaged over a 48h period. Urea, Sorbitol, HSP70, urine creatinine, blood chemistry values were all collected identically to the experimental groups.

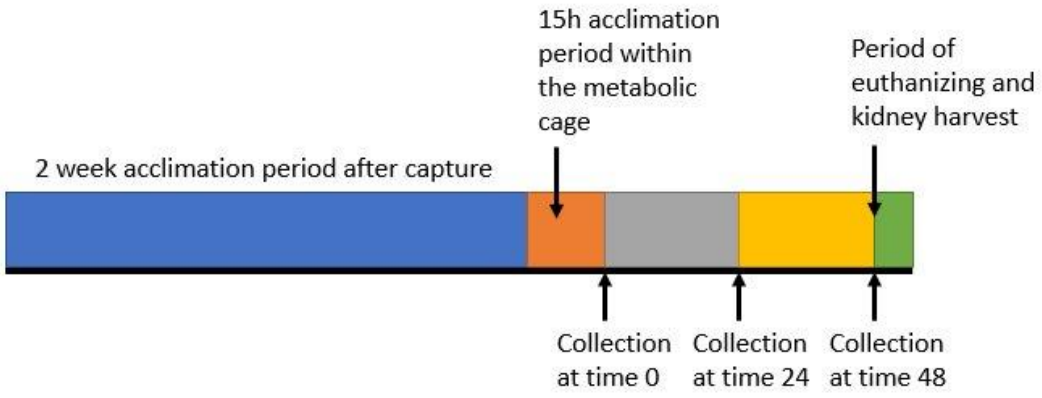


**Figure 1:** Timeline of research protocol.

During the experimental period, urine samples were collected at 0 (after 15 hour acclimation period), 24, and 48 hours. O'Gara (2015) collected all ground squirrel urine samples for the present study. Urine samples were then stored in a -80ºC freezer until the samples could be analyzed. After the last urine samples were collected at 48 hours, animals were euthanized

with nitrogen gas in an airtight container. 2-4mL serum samples and both kidneys in the animals were harvested. Serum samples were obtained by a cardiac puncture with BD 23 gauge needles. O'Gara (2015) collected all ground squirrel serum samples for the present study. Serum samples then stood at room temperature for 30 minutes to form clots, then centrifuged at 4,000 rpm for 10min.

*Blood Chemistry and GFR Calculations*

Each serum sample was then analyzed by an VetScan VS² blood analyzer (Abaxis, Union City, CA). Parameters included average blood urea nitrogen (BUN), creatinine, sodium, and potassium.

We estimated GFR based on creatinine clearance (Sherwood, 2010). Serum creatinine concentrations were obtained via the VetScan VS2 blood analyzer, while urine creatinine from the 48 hour sample was measured using a commercial creatinine assay (Cayman Chemical Company, Ann Arbor, MI). The plate was read at an initial absorbance of 495, and again at 495 after the addition of acid solution.

$C_{CR}$ : creatinine clearance (GFR)

$U_{CR}$: creatinine concentration at 48h

V: 24h urine volume (24-48h experimental period)

$P_{CR}$: plasma creatinine concentration at 48h

$$C_{CR} = \frac{U_{CR} \times V}{P_{CR}}$$

*Kidney Osmolyte Concentrations*

After the collection of both kidneys in each animal, one kidney was partially thawed from the -80ºC degree freezer and sectioned into 3 sections - cortex, medulla and papilla (Figure 1a-c) then homogenized in distilled water with disposable Biomasher II Tissue Grinders (Kimble Chase, Rockwood, TN).

Each homogenized sample was then analyzed in duplicates for urea concentration using a commercial colorimetric urea assay (Quantichrom, BioAssay Systems, Hayward, CA.). The plate was then read using Versamax microplate reader (Molecular Devices, Sunnyvale, CA) at 520nm. O'Gara (2015) collected all urea data on the ground squirrels for the present study. Sorbitol concentrations within the papilla were analyzed in duplicates using a commercial colorimetric sorbitol assay (Quantichrom, Bioassay Systems, Hayward, CA.) and read at an absorbance of 565nm.

The second partially thawed kidney was only sectioned for the papilla to analyze HSP70 concentration using a commercial ELISA (enzyme-linked immunosorbent assay) HSP70 kit (HSP70 ELISA, Enzo Life Sciences, Farmingdale, NY). The plate was read under a Versamax microplate reader at 495 nm. O'Gara (2015) collected all HSP70 data on the ground squirrels for the present study.

*Statistics*

Each data collected was analyzed based on treatments and species of animals. Means of each group of sucrose, dehydration, combination, and control animal were calculated and separated by species. Standard error of the mean was also calculated based on treatment and species.

Analysis of variance (ANOVA) was used to examine differences between treatment groups within the species. Tukey multi-comparison tests were used to determine specific groups that were different from each other. Unpaired T-tests were used to examine differences across species within the sucrose and combination groups of each parameter. All statistical analysis was carried out in Sigmaplot (Systat Software Inc, San Jose CA) with significance accepted at $p < 0.05$.

## *Results*

### *Urine Output and Concentration*

Experimental manipulation of water intake in the animals proved very effective at altering urine output and concentration. By hour 48, the sucrose and dehydration groups in the rats had large differences in both urine volume (sucrose $\bar{x}$ = 21.56mL, dehydration $\bar{x}$ = 3.48mL, $p < 0.001$) and urine concentration (sucrose $\bar{x}$ = 957.5mOsm, dehydration $\bar{x}$ = 3126.5mOsm, p=0.020, Figure 3). Lab rat urine output was highest in the sucrose group and lowest in the dehydration group at 48 hours (Figure 3). Significance at 48h was also detected between rat sucrose groups compared to combination groups (sucrose $\bar{x}$ = 21.56mL; dehydration

$\bar{x}$ = 5.98mL p=0.003). At 24h urine volume the dehydration group produced significantly less urine than the sucrose group (dehydration $\bar{x}$ = 11.62mL; sucrose $\bar{x}$ = 32.46mL p=0.036) in the rat. The combination group also produced less urine than the sucrose group within the rat species at 24h (combination $\bar{x}$ = 10.94mL; sucrose $\bar{x}$ = 32.46mL p=0.013).

Similarly, there were substantial differences between sucrose and dehydration ground squirrel groups for both urine volume (sucrose $\bar{x}$ = 16.78mL; dehydration $\bar{x}$ = 1.32mL, p=0.001, Figure 4) and concentration (sucrose $\bar{x}$ = 482.9; dehydration $\bar{x}$ = 3937.9, p=0.001, Figure 4) when compared to each other at 48h. Unlike the rat group, combination ground squirrels were unable to reach dehydration urine concentration levels by 24h (combination $\bar{x}$ = 712.6mOsm; dehydration $\bar{x}$ = 2478mOsm, p=0.03, Figure 4) At 48h combination ground squirrels were still unable to reach dehydration urine concentration levels (combination $\bar{x}$ = 2868mOsm; $\bar{x}$ = 3937.9mOsm, p=0.052, Figure 4).

No differences were seen between squirrel sucrose group at 48h and combination group at 0h (p=0.323). However, there were differences between the same time points and groups for rats (p=0.036), signifying that the combination group was more diuretic than the 48h sucrose groups.

*Urea*

In concert with changes in urine volume and concentration, the manipulation of water intake also dramatically changed urea gradients within the kidneys of both rats and ground squirrels. In general, urea concentrations increased from cortex, to medulla, to papilla. Sucrose and dehydration groups had significantly different urea concentrations in the papilla for both rats (sucrose $\bar{x}$ = 260.9mOsm; dehydration $\bar{x}$ = 732.8mOsm, p=0.030, Figure 5) and ground squirrels (sucrose $\bar{x}$ = 94.6mOsm; dehydration $\bar{x}$ = 195.8mOsm , p=0.021, Figure 6). However, there were

no significant differences between the combination and dehydration groups for neither rat (combination $\bar{x}$ = 672.4mOsm; dehydration $\bar{x}$ =732.8mOsm, p=0.925, Figure 5) nor ground squirrels (combination $\bar{x}$ = 191.7mOsm; dehydration $\bar{x}$ =195.8mOsm, p=0.991, Figure 6). One other difference I consistently noticed was a higher papillary urea concentration in rat kidneys as compared to ground squirrel kidneys. For example, urea concentrations in dehydration rat papillae averaged 732.8 mOsm/kg number while urea concentrations in dehydration ground squirrel kidneys averaged 195.8 mOsm/kg.

*Papillary Sorbitol*

Despite seeing substantial changes in urine output and associated urea gradients in the kidneys, I did not detect corresponding changes in sorbitol concentrations within kidney papillae. No significant differences were detected among papillary sorbitol values for rats (sucrose ($\bar{x}$ = 965.2nmol/μL) to dehydration ($\bar{x}$ = 732.3nmol/μL: p=0.972 ; sucrose ($\bar{x}$ = 965.2nmol/μL to combination ($\bar{x}$ = 2770.7nmol/μL: p=0.225 ; dehydration ($\bar{x}$ =732.3nmol/μL to combination ($\bar{x}$ =2770.7nmol/μL: p=0.158; Figure 5) nor ground squirrels (sucrose ($\bar{x}$ =1897.0nmol/μL to dehydration ($\bar{x}$ = 2775.8nmol/μL: p=0.680 ; sucrose ($\bar{x}$ =1897.0nmol/μL to combination ($\bar{x}$ = 2563.7nmol/μL: p=0.799 ; dehydration ($\bar{x}$ =2775.8nmol/μL to combination ($\bar{x}$ =2563.7nmol/μL: p=0.977; Figure 6). While there seemed to be a positive correlation between squirrel papillary urea concentration and papillary sorbitol concentrations, the rats showed no such trend.

*HSP70*

No significant differences were detected among papillary HSP70 values for rats (sucrose $\bar{x}$ = 142.9ng/mL; dehydration $\bar{x}$ = 128.3ng/mL , p=0.769 ; sucrose $\bar{x}$ = 142.9ng/mL; combination $\bar{x}$ = 160.1ng/mL, p=0.693 ; dehydration $\bar{x}$ = 128.3ng/mL; combination $\bar{x}$ = 160.1ng/mL: p=0.341; Figure 5). Ground squirrels only showed significance between the sucrose and combination group (sucrose $\bar{x}$ = 1147.4ng/mL ; combination $\bar{x}$ = 758.3ng/mL ,p=0.021, Figure 6), but not sucrose to compared to dehydration (sucrose $\bar{x}$ = 1147.4ng/mL ;dehydration $\bar{x}$ = 660.8ng/mL, p=0.101, Figure 6), or dehydration compared to combination (dehydration $\bar{x}$ = 660.8ng/mL; combination $\bar{x}$ = 758.3ng/mL, p=0.551, Figure 6). Overall, comparing rats to ground squirrels, squirrels had a much higher concentration of HSP70 than rats (p= 1.03E-05) in the sucrose groups. In general, HSP70 was the least expressed in animals receiving the dehydration treatment for both species (Figure 5, 6).

*Glomerular Filtration Rate (GFR)*

No significant differences were detected among GFR for rats (sucrose $\bar{x}$ = 2.47mL/min; dehydration $\bar{x}$ = 1.01mL/min , p=0.259 ; sucrose $\bar{x}$ = 2.47mL/min; combination $\bar{x}$ = 1.85mL/min, p=0.754 ; dehydration $\bar{x}$ = 1.01mL/min; combination $\bar{x}$ = 1.85mL/min: p=0.627; Figure 7). No significant differences were detected among ground squirrels (sucrose $\bar{x}$ = 1.12mL/min; dehydration $\bar{x}$ = 0.40mL/min , p=0.077 ; sucrose $\bar{x}$ = 1.12mL/min; combination $\bar{x}$ = 0.40mL/min, p=0.100 ; dehydration $\bar{x}$ = 0.40mL/min; combination $\bar{x}$ = 0.40mL/min: p=0.100; Figure 7). However, GFR trended toward higher values in the sucrose groups and lower values in the dehydration groups for all species.

*Blood Chemistry*

No significant differences were seen across any blood chemistry value for rats (BUN: p=0.182, Creatinine: p=0.259, K+: p= 0.382, Na+: p=0.142, Table 2). However, sucrose ground squirrels had significantly lower values for BUN compared to dehydration (p=<0.001) and combination (p=0.001). No other values were significantly different (Creatinine: p=0.889, K+: p=0.155, Na+: p=0.054, Table 2). Overall trends in BUN tended to be higher mean values in the dehydration groups, and lower mean values in the sucrose group. Creatinine values showed no significant trend in the rats, but ground squirrels had the largest mean values of plasma creatinine in the combination group. Na+ trends in the rats and ground squirrels were higher mean values in the dehydration groups and lower mean values in the sucrose group. No overall trends were seen in mean K+ plasma values for both species.

## *Discussion*

During bouts of torpor, cardiac output and blood pressure decrease to levels that are insufficient to sustain GFR. As a result, the cortico-papillary gradients within the medulla of the kidney disappear (Baddouri & Elhilali, 1986, Cotton, 2012a). However, during periods of arousal, urea gradients rapidly return and enable animals to concentrate urine to prevent dehydration. Yet, this rapid change in gradients submits the epithelial cells of the kidney to a large degree of stress, and requires an equally rapid accumulation of organic osmolytes and heat shock proteins. Interestingly, in a typical non-hibernating animal, the process of accumulating protective compounds takes many hours, sometimes up to 12h (Burg, et. al., 2007). Hibernators

who arouse from torpor, however, can accumulate protective compounds much quicker, on the order of a few hours (Cotton 2012b).

The purpose of the present study was to examine hibernating and non-hibernating species and their responses to acute changes in water intake during the summer. We were particularly interested in hibernator's and non-hibernator's ability to rapidly change their vertical osmotic gradient in their kidneys to concentrate urine during a time of lost gradients. We were also curious to see if there were appropriate changes made with the upregulation of HSPs, and protective osmolytes.

Our hypothesis predicted that hibernators would be able to upregulate HSP70 concentrations more rapidly in the papilla and express HSP70 more abundantly. We also expected hibernators to shift their cortico-papillary gradient during water deprivation treatments more rapidly compared to non-hibernators. In non-hibernators, these changes take many hours, unlike the hibernator's ability to rapidly regenerate during early arousal (Burg et. al., 2007; Cotton 2012b). Based on the results of the present study, our results did not support our hypothesis.

*Urine output and concentration*

Urine outputs of both lab rats and 13-lined ground squirrels were as expected, producing minimal urine output after 48h of water deprivation and maximal urine outputs after 48h of water loading. The differences between sucrose groups and combination and/or dehydration groups were found to be significant across both species. During periods of water deprivation, urinary output decreases to conserve water and prevent dehydration (Sands & Leyton, 2009).

In the present study, it was thought that animals in the sucrose group would produce similar urine concentrations when compared to combination animals at hour 0. We saw no statistical significance between the squirrels within these groups, however, there was a statistical significance within the rats in the mentioned groups. Although the sucrose group at hour 48 had higher urine concentrations then combination animals at hour 0, significant changes were still seen across water regimes. True concentrations of urea in the papilla are probably more accurately modeled in the hour 0 combination group rather than the 48 hour sucrose group.

The main contributors to water reabsorption is a hormone known as vasopressin (or antidiuretic hormone, ADH), and the renin-angiotensin aldosterone system (RAAS). In times of water deprivation, RAAS increases both sodium and water reabsorption in the distal convoluted tubule. This aids in reducing urine volume and maintaining normal blood pressure to allow plasma to continuously be filtrated. In addition, ADH increases the number of AQ-2 channels that are inserted on the apical membrane of the epithelial cells in the distal convoluted tubule (DCT) and collecting duct (Sherwood, 2010). This allows water to be reabsorbed into the plasma and reduces urine volume. In addition, ADH increases the phosphorylation and the plasma membrane accumulation of urea transporter (UT) -A1 (Fröhlich, Klein, Smith, Sands, & Gunn, 2004). and UT-A3 (Shayakul, Steel, & Hediger. 1996) This allows a urea gradient within the papilla to be established and concentrated urine to be produced. Together, the RAAS and ADH mechanisms allow for a very small amount of concentrated urine to be produced.

Animals that consumed excess water, such as the sucrose group, had a higher urinary output to compensate for the water loading (Murillo-Carretero, Ilundain, & Echevarria, 1999). Low osmolarity within the plasma is the primary factor for decreasing ADH, while higher

plasma volume and high blood pressure decrease the RAAS system. This allows water to pass through the DCT and the collecting ducts and be excreted.

In the present study, it was expected that the hibernators (ground squirrels) would be much quicker at increasing urine concentration and decreasing urine volume than non-hibernators (rats). While both species significantly decreased urine volume during the first 24h in the combination group (rats p=0.039, Figure 3; squirrels p=0.049, Figure 4) and dehydration group (rats p= 0.037, Figure 3; squirrels p= 0.243, Figure 4), only the combination rats compared to the combination squirrels significantly increased urine concentration (p=0.003, Figure 3) during time 0-24. Perhaps this is indicative of varying structures of vasa recta and the loop of Henle. Differing structures can produce urine that is highly concentrated within a few days. Bankir (1985) mentions that the capability of producing concentrated urine does not necessarily mean that the animal is able to concentrate urine fast. Animals with poor medullary insulation may be slower at producing highly concentrated urine. Increased medullary insulation is associated fusion of the vascular bundles into larger bundles and incorporation of the short-loop thin limbs among their vessels (Bankir, 1985). This in turn decreases dissipation by preventing the escape of inner medullary solutes and favoring their reentry into descending structures as explained above. This improves the "insulation" of the inner medulla. In addition, number of long looped nephrons and relative development of the three areas within the kidney may contribute to the animal's ability to conserve water. Therefore, it may be possible that there are differing renal morphologies between the squirrels and the rats.

In addition, hibernators are experiencing intense alterations of blood pressure during arousal (Cotton 2012a). The factor of blood pressure may be an important influence of the rate at which hibernators are able to decrease urine output and increase urine concentration.

*Urea*

During torpor bouts, ground squirrels experience a complete loss of their cortico-papillary urea gradients (Baddouri & Elhilali, 1986; Lesser et al., 1970; Cotton 2012b) which results in a complete inability to produce concentrated urine. However, during mid arousal, urea gradients are reestablished and the ability to concentrate urine completely returns. In the present study, combination ground squirrels mimic hibernation-like conditions in which they presumably rapidly transition from no urea gradient and associated inability to form concentrated urine to having a large urea gradient and concomitant ability to form concentrated urine.

Urea data within each species was largely expected. Animals who received the water deprivation treatments had higher papillary urea concentrations than the sucrose animals. Data from the present study agreed with previous research by Blessing and colleagues (2008), and Zhang and colleagues (2002) suggesting elevated vasopressin during antidiuresis elevated urea transporter (UT) – A1 and UT-A3 transporters, which allows urea accumulation within the papilla.

Higher urea concentrations were seen in the lab rats compared to the ground squirrels. This trend extends from cortical urea concentrations to papillary urea concentrations. However, despite the lower urea gradients in the ground squirrels, they were able to produce a much more concentrated urine than the rats in the dehydration groups. One explanation for this phenomenon is that it is likely that parts of the papillary urea concentrations were diluted with urea concentrations of the medulla due to errors in sectioning the kidney. This would undoubtedly underestimate the true urea concentration within the ground squirrel papilla. It may also be the

case that ground squirrels rely less on a urea gradient for concentrating urine, and are more dependent on a sodium gradient, much like sand rats (*Psammomys obesus*) (Imbert, deRouffignac, Philippe, & Deiss, 1976).

*HSP*

Contrary to previous research (Alfieri, et. al., 2002; Burg, et. al., 1997; Neuhofer, et. al., 2002) many authors have shown HSP to have the highest expression during times of hypertonic stress in the kidney. For example, during times of dehydration. In addition, Neuhofer et.al. (2002) states that HSP72 is most abundant in the renal papilla. The squirrels who received the sucrose treatment produced larger amounts of HSP than the animals who received the dehydration treatment. This is an unexpected occurrence, as several studies have shown HSP70 to be upregulated during antidiuresis and times of hypertonic stress. However, only one study has suggested that HSP70 can also be upregulated during times of water stress causing hypotonic environments in small mammals (Neuhofer, et. al, 2002). During times of water loading, the extracellular environment may become hypotonic relative to the intracellular space. Organic osmolytes in this situation create the potential to over-stabilize proteins, perhaps prohibiting free movement and function. HSP in this case may have the potential to refold proteins in confirmations that allow free function and movement.

*Sorbitol*

Sorbitol values were not significantly different between treatment groups. Although sorbitol seemed to positively correlate with increasing papillary urea trends in the ground squirrels,

no specific trend was followed in the rats. This may be indicative of the evidence presented by Burg & Ferraris (2008) suggesting that osmolytes are regulated independently and sometimes in compensation for another. Different species may also rely more on one organic osmolyte than another. Future research in this area may benefit from analyzing the full suite of organic osmolytes that accumulate in the renal papilla since there is little research done on the accumulation of organic osmolytes.

*Blood Chemistry*

Blood chemistry within the rats and ground squirrels were largely maintained despite the dramatic changes in urea gradients within the renal papilla. This coincides with the findings of Cotton (2012b) and Mogharabi & Haines (1973). No significant differences in blood chemistry were detected among rats. There were trends in Na+ concentrations and K+ concentrations, however. Rats who received water deprivation treatments (dehydration and combination) had higher Na+ concentrations than the sucrose groups. In addition, water deprivation animals also had slightly lower plasma K+ concentrations than the sucrose group (Table 1). These trends are as expected based on Mogharabi and Haines' (1973) research on water deprivation and rats. Animals receiving water deprivation treatments are driven into negative water balance. The magnitude of negative water balance is dependent on the animal's ability to concentrate and minimize urine. The ability to successfully minimize water loss is shown in their plasma Na+ concentrations.

The blood chemistry values of the ground squirrels produced insignificant differences, however significance was found within the sucrose ground squirrels vs. combination and

dehydration BUN data. Mean values for BUN in the ground squirrel sucrose group were significantly lower than the dehydration and combination animals. Similar trends with sodium and potassium were also seen in ground squirrels.

Ground squirrels overall had a much higher BUN than the rats did. This may be indicative of better urea recycling in the ground squirrels. During times of antidiuresis, high levels of ADH stimulate urea transporters, enhancing urea reabsorption into the blood (Sherwood, 2010). In addition, differences between blood chemistry were seen in control animals. Control rats tended to show a close relationship with blood chemistry values with the sucrose groups, while control squirrels showed similar values to the dehydration group.

When arousing from torpor, hibernators must regain their cortico-papillary gradient within a matter of hours to avoid dehydration and waste accumulation. The present study simulates conditions within the kidney that would mimic the abolishment and rapid regain of the cortico-papillary gradient. However, we induced this gradient over a period of days instead of hours. In the present study, there was no stimulation for the 13-lined ground squirrels to produce a gradient over a matter of hours like they would during arousal in hibernation.

*Implications*

Mammalian hibernators are said to demonstrate nature's version of organ preservation (Jani et. al, 2013). There has been interest in mimicking hibernating states to explore the biological mechanisms that permit hibernating mammals to survive for months at extremely low ambient temperatures, with no food or water, and awaken from their hibernation without apparent organ injury (Ratigan & McKay, 2016). A key area for hibernation research could be in

long-term space travel in which humans would undergo a hypometabolic stasis for months at a time (Ayre, Zancanaro, & Malatesta, 2004). Understanding the mechanisms in which hibernators can rapidly regenerate their cortico-papillary sodium and urea gradient, in conjunction with understanding associated protective mechanisms is pertinent to successful hibernation.

Methodological implications relative to this study surround the sectioning of kidneys. The differences in body size, and thus kidney size, posed a difficult obstacle in sectioning adequate sections of each kidney. The smaller size of the ground squirrel may have caused different sections of the kidney to dilute others. For example, some of the papilla may have been contaminated with the medulla. This contamination may produce results that dilute the true value of the papilla.

*Future direction for research*

Future research in this area should include a full profile of organic osmolytes that accumulate in each animal. Understanding the full suite of organic osmolytes is essential to understanding which osmolytes are more actively used in difference species and understanding the sorbitol data from the present study. In addition to surveying for the full profile of organic osmolytes, understanding other hibernators within the area may lead to a better understanding of how more hibernators are able to manipulate their urea gradients and corresponding osmolytes to different water regimens.

Another potential option for research in this area is utilizing furosemide to clear sodium gradients within the kidney. Furosemide is a loop diuretic that rids mammals of their sodium gradient within the kidney. Following the loss of a sodium gradient, researchers may then

administer ADH injections to quickly stimulate kidneys to reestablish a gradient. This would allow researchers to analyze true loss of a sodium and urea gradient, followed by a rapid regeneration. In the present study, the urea gradient was completely abolished in the sucrose groups and time 0 of the combination groups, however, the sodium gradient was likely intact. Researchers may find results that differ from the present study with the addition of clearing the sodium gradient within the kidney, since it TonEBP is stimulated primarily by a hypertonic (high sodium) environment.

## *Conclusion*

During summer months, rats were shown to alter urine concentration faster than the ground squirrels could by hour 24. This data suggests drastically different abilities of ground squirrels during the summer than during hibernation seasons. Ground squirrels and other small hibernators have been known to alter urine faster than most non-hibernating animals to minimize water loss. Despite the differences in urine concentration, no differences were seen between urine volume, or blood chemistry within either species. Ground squirrels had higher concentrations of HSP's for all treatment groups compared to rats, and the ground squirrels' papillary sorbitol concentrations more closely matched the urea gradients compared to the rats. Based on the ground squirrel's ability to accumulate these protective compounds to match their urea gradient, there is a possibility that the ground squirrels' kidneys are less likely to be damaged by acute changes in water intake.

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*Figures and captions*

**Table 1:** ***Rattus norvegicus*** **and** ***Ictidomys tridecemlineatus*** **control values**

| | **Laboratory Rat** | **Ground Squirrel** |
|---|---|---|
| **Urine Volume (mL/day)** | 26.23 ± 6.34 | 7.66 ± 1.94 |
| **Urine Concentration (mOsm)** | 1228.13 ± 316.50 | 2622.37 ± 355.08 |
| **Papillary Urea (mOsm/kg)** | 343.17 ± 120.09 | 201.12 ± 57.79 |
| **Papillary HSP (ng/mL)** | 161.03 ± 20.35 | 465.00 ± 66.58 |
| **Papillary Sorbitol (nmol/mL)** | 676.21 ± 252.69 | 29367.12 ± 7639.06 |
| **GFR (mL/min)** | 2.12 ± 0.57 | 0.57 ± 0.14 |
| **BUN (mg/dL)** | 16.6 ± 2.66 | 26.50 ± 1.54 |
| **Creatinine (mg/dL)** | 0.52 ± 0.05 | 0.44 ± 0.07 |
| **Na+ (mmol/L)** | 142.6 ± 1.78 | 150.34 ± 6.64 |
| **K+ (mmol/L)** | 7.5 ± 0.41 | 7.72 ± 0.37 |

**Notes:**
-Data are presented as means ± SEM.
-N = 5 for all treatment groups.

**Table 2:** ***Rattus norvegicus*** **and** ***Ictidomys tridecemlineatus*** **blood chemistry.**

| | BUN (mg/dL) | Creatinine (mg/dL) | Sodium (mmol/L) | Potassium (mmol/L) |
|---|---|---|---|---|
| **Laboratory Rat** | | | | |
| Sucrose | $16 \pm 2.9^{A}$ | $0.5 \pm 0.1^{A}$ | $144 \pm 1.2^{A}$ | $7.4 \pm 0.3^{A}$ |
| Dehydration | $22 \pm 1.3^{A}$ | $0.4 \pm 0.1^{A}$ | $148 \pm 2.0^{A}$ | $6.8 \pm 0.4^{A}$ |
| Combination | $20 \pm 1.4^{A}$ | $0.5 \pm 0.1^{A}$ | $147 \pm 1.0^{A}$ | $6.9 \pm 0.2^{A}$ |
| **Ground Squirrel** | | | | |
| Sucrose | $13 \pm 1.3^{B}$ | $0.4 \pm 0.1^{A}$ | $148 \pm 1.0^{A}$ | $8.0 \pm 0.4^{A}$ |
| Dehydration | $32 \pm 1.0^{A}$ | $0.4 \pm 0.1^{A}$ | $152 \pm 2.0^{A}$ | $7.0 \pm 0.4^{A}$ |
| Combination | $26 \pm 2.7^{A}$ | $0.5 \pm 0.1^{A}$ | $154 \pm 1.2^{A}$ | $7.3 \pm 0.5^{A}$ |

**Notes:**
-All comparisons are within columns.
-Data that do not share a common letter are significant at alpha <0.05
-Data are presented as means ± SEM.
-N = 5 for all treatment groups.
-O'Gara (2015) collected all serum sample data for the ground squirrels in the present study.

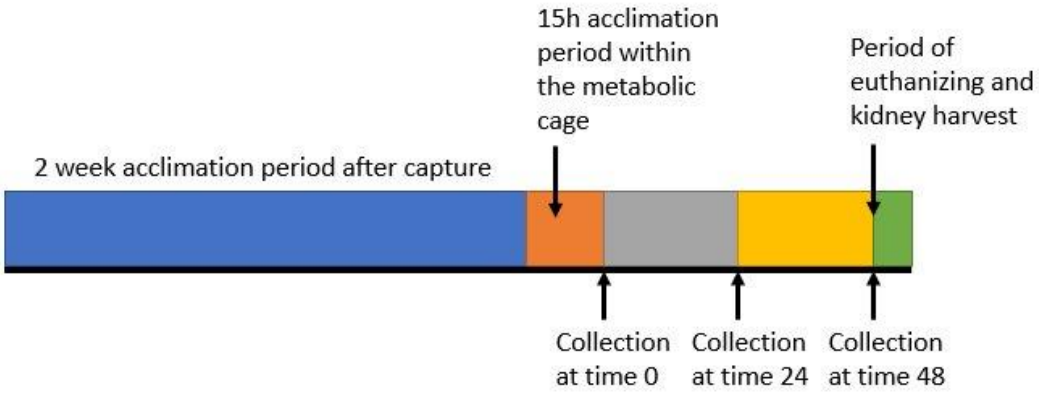



**Figure 1:** Timeline of research protocol.

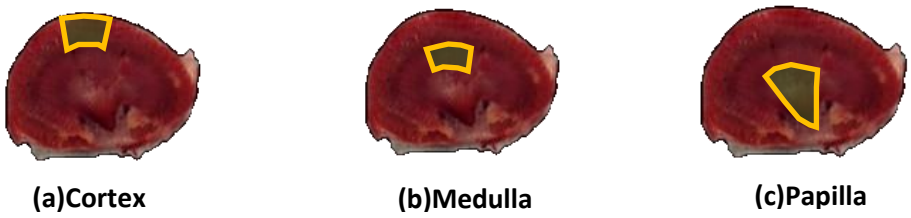



**Figure 2a-c:** Sagittal sections of mammalian kidneys. Highlighted sections correspond to areas analyzed urea concentration, HSP70, and sorbitol.

**Rattus norvegicus urine volume and urine concentration over 48h**

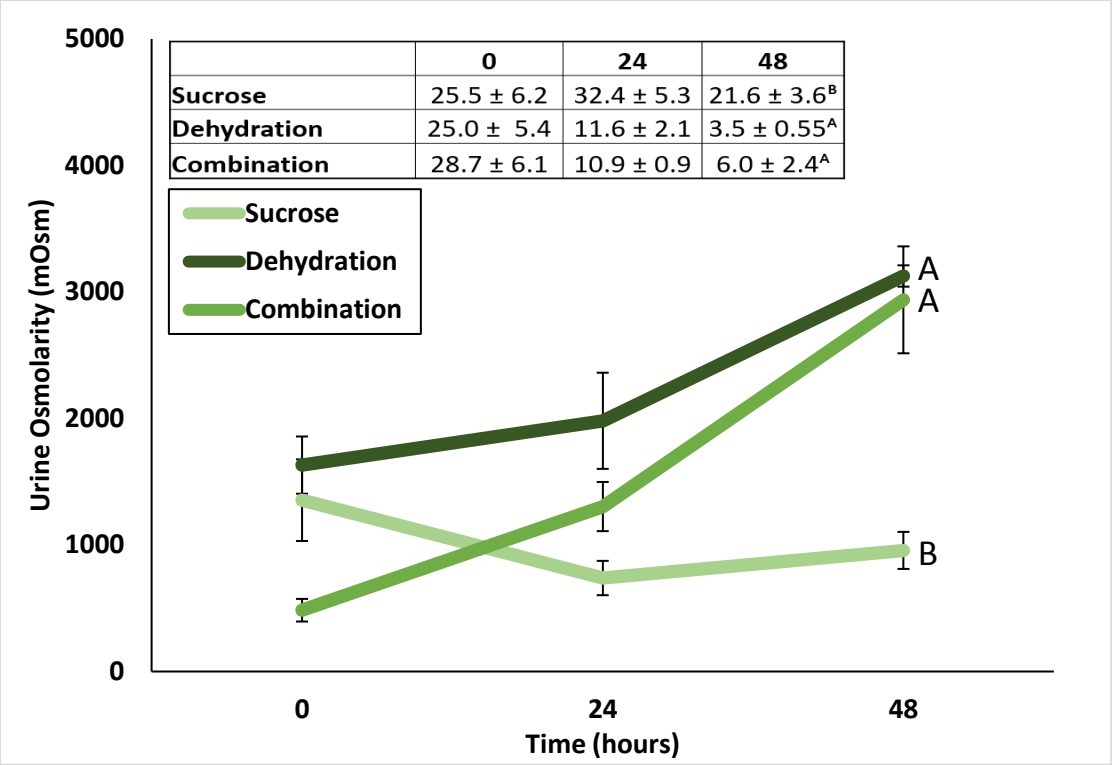
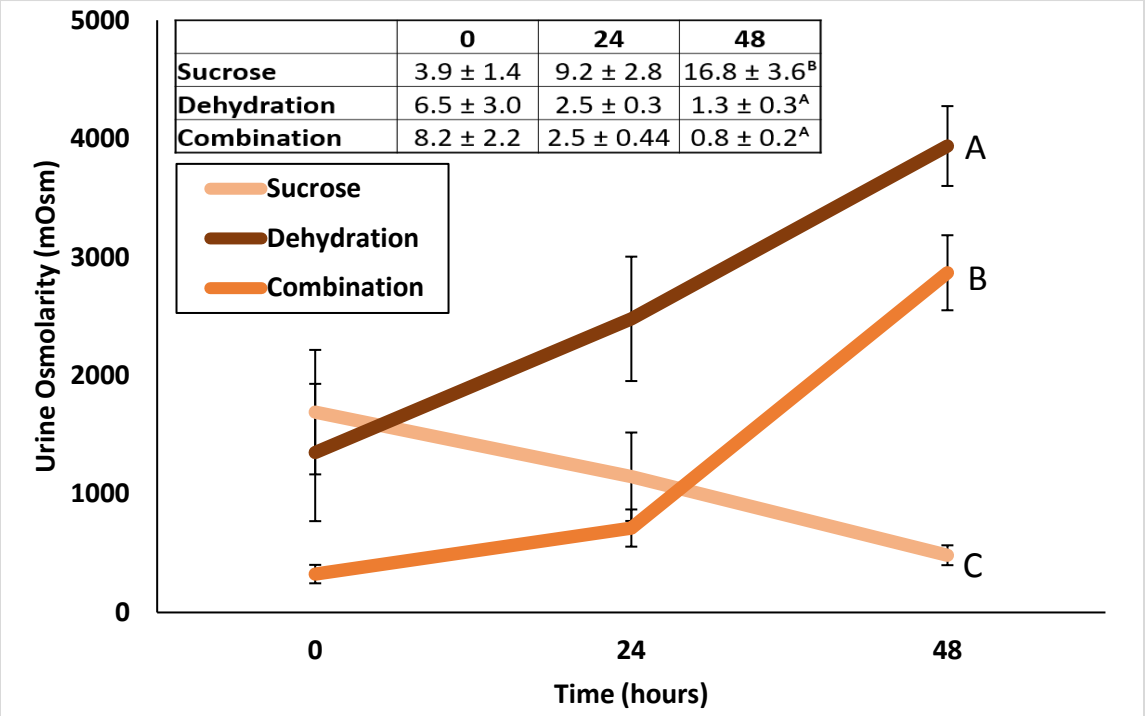



**Figure 3:** *Rattus norvegicus* urine concentration over 48h. Inset table represents urine volumes in mL/day. Urine samples were taken from each treatment group every 24h. N = 5 for each treatment. Error bars represent standard error of the mean. Letters indicate significant differences among groups.

**Ictidomys tridecemlineatus urine volume and urine concentration over 48h**




| | 0 | 24 | 48 |
|---|---|---|---|
| Sucrose | 3.9 ± 1.4 | 9.2 ± 2.8 | 16.8 ± 3.6$^{B}$ |
| Dehydration | 6.5 ± 3.0 | 2.5 ± 0.3 | 1.3 ± 0.3$^{A}$ |
| Combination | 8.2 ± 2.2 | 2.5 ± 0.44 | 0.8 ± 0.2$^{A}$ |

**Figure 4:** *Ictidomys tridecemlineatus* urine concentration over 48h. Inset table represents urine volumes in mL/day. Urine samples were taken from each treatment group every 24h. N = 5 for each treatment. Error bars represent standard error of the mean. Different letters indicate significant differences between groups. Urine volume and concentration was collected by O'Gara (2015).

**Rattus norvegicus urea, HSP, and sorbitol concentrations**

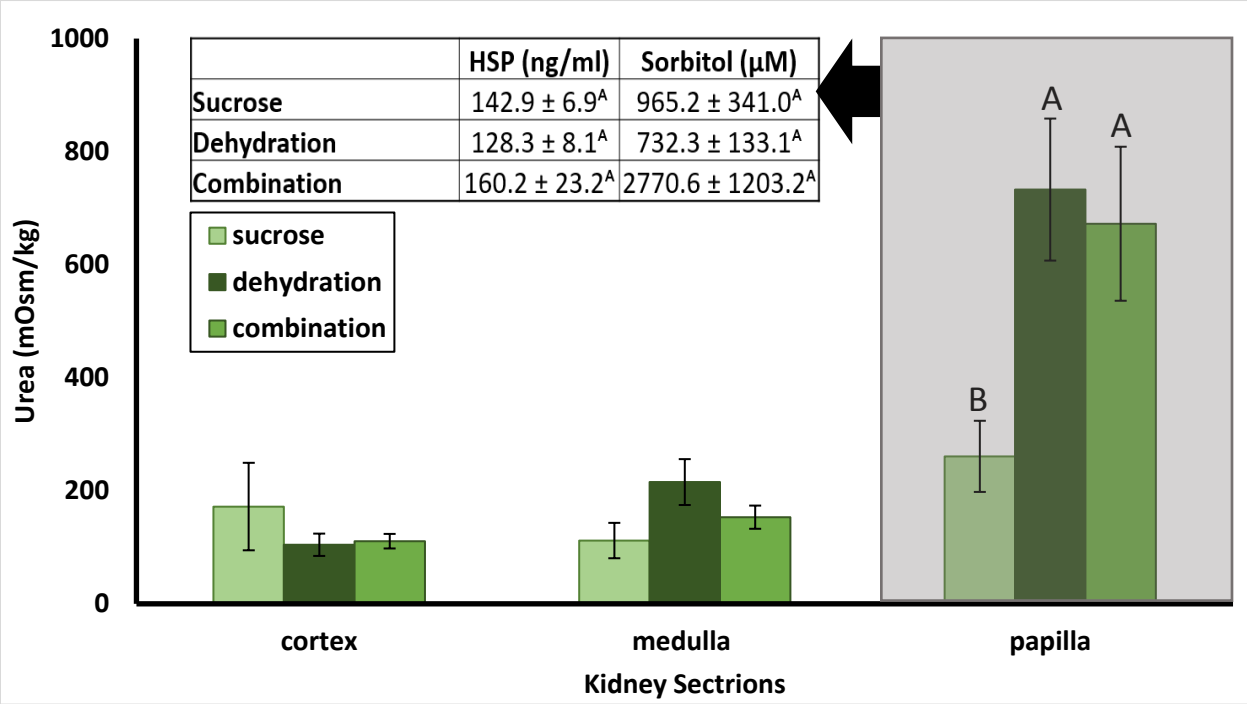



**Figure 5:** Urea concentrations within each section of the *Rattus norvegicus* kidney. Inset table represents mean concentrations of sorbitol and HSP70 within the papilla. N = 5 for each treatment. Error bars represent standard error of the mean. Letters indicate significant differences among groups.

***Ictidomys tridecemlineatus* urea, HSP, and sorbitol concentrations**

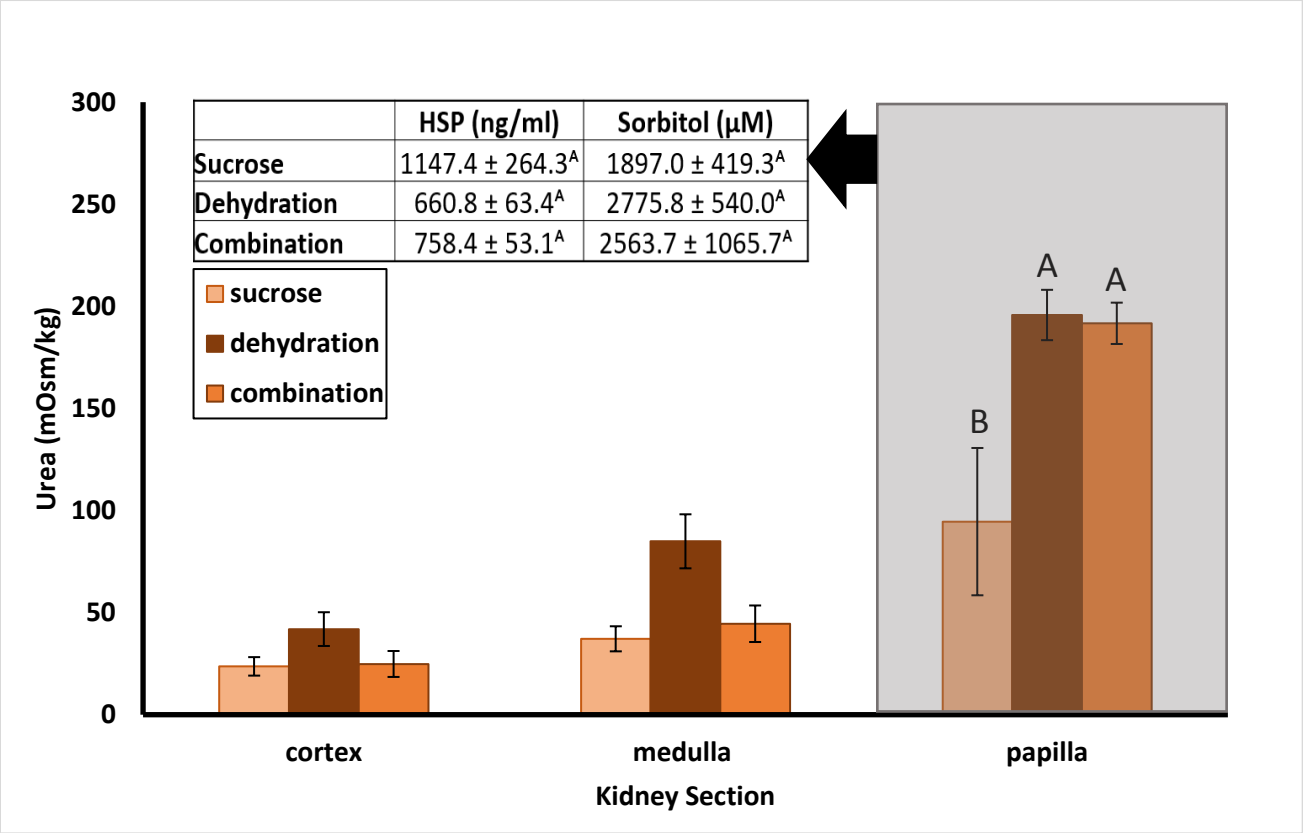



**Figure 6:** Urea concentrations within each section of the *Ictidomys tridecemlineatus* kidney. Inset table represents mean concentrations of sorbitol and HSP70 within solely the papilla. N = 5 for each treatment. Error bars represent standard error of the mean. Different letters indicate significant differences between groups. O'Gara (2015) collected data on urea concentration of the cortex, medulla, and papilla.

**Glomerular filtration rates among Rattus norvegicus and Ictidomys tridecemlineatus**

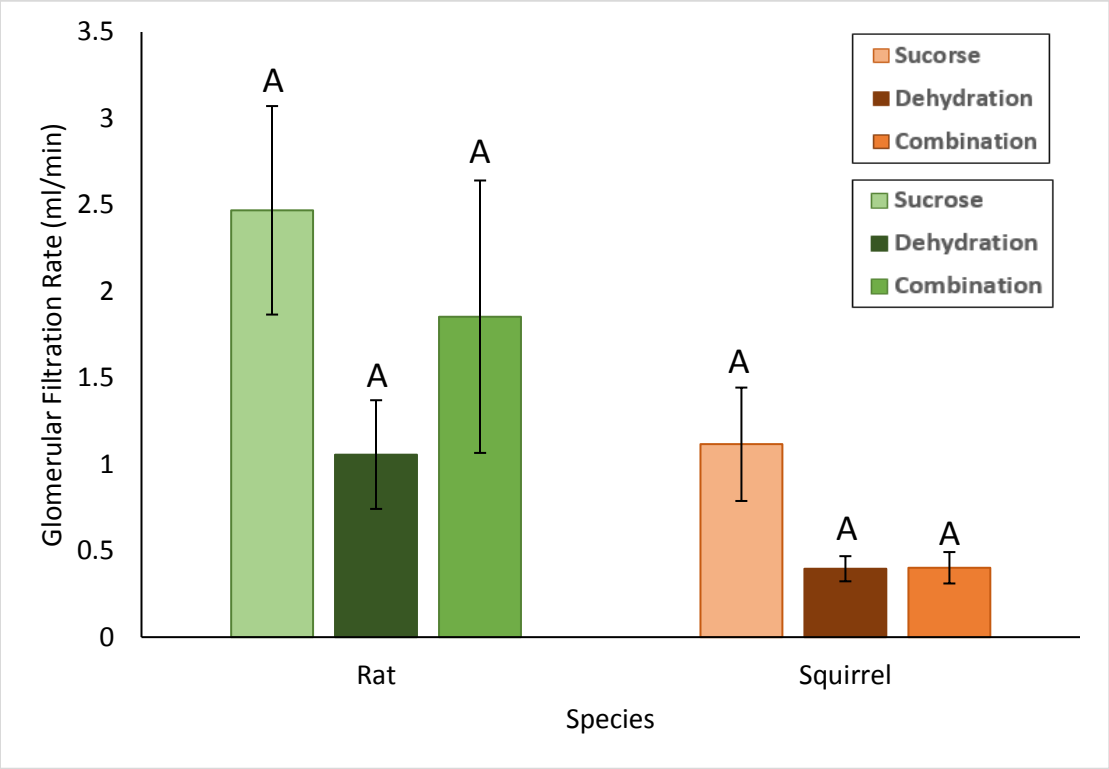



**Figure 7:** Glomerular Filtration Rate of *Rattus norvegicus* and *Ictidomys tridecemlineatus under various treatments.* N = 5, error bars represent standard error of the mean. Different letters indicate significant differences between groups.