12-2019

The Acute Effect of Water Intake on Glucose Regulation in Low Drinkers

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The Acute Effect of Water Intake on Glucose Regulation in Low Drinkers

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Health, Sport, and Exercise Science

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December 2019
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ABSTRACT

Previous evidence suggests the hormone arginine vasopressin (AVP) may be a modifiable factor contributing to hyperglycemia. Significant differences in urine concentration and copeptin, a marker for AVP, have been observed between low and high water drinkers. **Purpose:** The purpose was to investigate the acute effect of adequate water intake on glucose regulation in low drinkers. **Methods:** 7 healthy (5 males, 2 female) low drinkers were recruited using a water frequency questionnaire (WFQ), spot and 24-h urine sample (age 43±6 y, BMI 30.9±3, WFQ volume 823±403 $mL \cdot d^{-1}$, 24 h U_{osm} 961±105 $mmol \cdot kg^{-1}$, copeptin 8.17±3.05 $pmol \cdot L^{-1}$). During two experimental protocols, participants remained in the laboratory for 11 h and were provided either the Institute of Medicine's (IOM) recommended amount of water excluding food (males: 3 L, females: 2 L) or an amount representing the bottom quartile of water consumption observed in the National Health and Nutrition Examination Survey (NHANES) (males: 0.5 L, females: 0.4 L). Food was provided to participants and standardized to body weight (100 $kJ \cdot Kg^{-1}$) using a consistent ratio of macronutrients. **Results:** 11 h urine volume was significantly higher in the high water trial ($P < 0.001$). 11 h UOsm was significantly higher in the low water trial ($P < 0.001$). Plasma osmolality was acutely lower as a result of increased water intake ($P = 0.007$). Copeptin was suppressed as a result of high water intake ($P = 0.019$). Glucagon was similar between trials ($P = 0.372$), however, there was a main effect of water intake on cortisol ($P = 0.009$). No differences in plasma glucose were found due to water intake (P = 0.07). **Conclusion:** Acute increases in water intake do not reduce post-prandial plasma glucose responses in low drinkers, however, cortisol may be acutely reduced.

DEDICATION

There is no other place I would begin sharing my gratitude than with my advisor, mentor, but more importantly, friend, Dr. Stavros Kavouras. Your compassion and drive not only inspired me throughout this degree, but will continue to do so throughout my career. For all of the conversations filled with knowledge, respect, and laughter I cannot thank you enough.

Thank you to my committee for your mentorship and guidance throughout this process. Your expertise and passion for your profession will have a lasting impact on my career as a scientist.

To all my lab mates in Fayetteville and Phoenix. All of the laughs, hard work, and friendships put the heart behind this experience. I will always cherish the relationships we have built.

To all my friends, I can honestly say your support and understanding throughout this degree has been nothing short of amazing. All of you contributed in some way to helping me realize this goal. Knowing I had such a close group of people there for me when needed was a comfort I did not and will not take for granted.

To my family. Anything I can write with these few words will fall utterly short in expressing my thanks to you. Dad, your work ethic, perseverance, and sense of respect was instilled in me early on and will continue to be a driving force throughout my life. Mom, the compassion, love, and hard work that you express every day is incredible to witness and I will always strive to do the same. To my sister, Samantha. All the qualities of Mom and Dad are present in you, but your courage has inspired me since I knew what the word meant. I would not have pursued this degree if not for you.

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INTRODUCTION

Global prevalence of chronic metabolic dysfunction is increasing. This pathology, often characterized by impaired glucose regulation, presents a public health and economic burden partly attributable to diabetes and associated complications. Recent worldwide data from the International Diabetes Federation indicate 425 million people aged 20-79 years live with diabetes (International Diabetes Federation, 2017). Although non-modifiable risk factors such as genetics, age, and sex play a role, several lifestyle factors significantly contribute to increasing prevalence rates including obesity, poor nutritional practices, and physical inactivity (Defronzo, 2009).

The principal pathophysiology of diabetes is characterized by insulin resistance in both muscle and liver cells and beta cell failure. As shown in previous literature, up to 80% of beta cell function in individuals with impaired glucose tolerance preceding diabetes is already lost (Abdul-Ghani, Tripathy, & DeFronzo, 2006). Furthermore, the risk of cardiovascular complications is increased prior to basal glycemic levels indicative of the disease (Cho et al., 2018; Coutinho, 1999; Laakso, 1999). These data emphasize the importance of recognizing at risk individuals before diabetes is diagnosed and implementing lifestyle modifications for primary prevention. Due to global health and economic burdens, it is important to pinpoint preventative strategies that remove contributing factors and supplement primary prevention of diabetes.

Vasopressin, also known as anti-diuretic hormone, is part of a complex homeostatic system regulating the tonicity of body fluids. Along with oxytocin, vasopressin is stored abundantly by magnocellular neurons originating in the hypothalamus and is released from the posterior pituitary. Target receptors of AVP are found in many tissues including the kidney, liver, pancreatic islets, and vascular smooth muscle. In normal, healthy adults the plasma

concentration of AVP lies between 0.5 and 2.5 $pg \cdot mL^{-1}$, however these levels fluctuate due to several factors (Robertson, 2013). The main regulator of AVP concentration is the tonicity of body fluids, which is mediated by cells known as osmoreceptors. These receptors sense plasma osmolality and respond at a certain threshold leading to a directly proportional increase in vasopressin (Moses, 1978; G. L. Robertson, Shelton, & Athar, 1976; Weitzman & Fisher, 1977). As plasma osmolality increases, the anti-diuretic properties of AVP are activated ultimately increasing water reabsorption in the kidney and limiting further rise in plasma osmolality.

Several non-osmotic factors also influence the concentration of AVP. To begin with, decreases in blood pressure or blood volume lead to increases in AVP, however, this stimulus is much less sensitive than the osmotic influence mentioned above (G. L. Robertson, 1983). Nausea also has a strong influence. Emetic sensation, regardless of actual vomiting, elicits profound increases in circulating AVP as compared to basal levels (Rowe, Shelton, Helderman, Vestal, & Robertson, 1979). Furthermore, AVP concentrations have been shown to vary between different phases of the menstrual cycle. In a key study, researchers found that during the luteal phase the threshold for AVP release was decreased resulting in a lower basal plasma osmolality as compared to the follicular phase (Spruce, Baylis, Burd, & Watson, 1985). Despite these influences, body fluid tonicity is tightly regulated due in large part to the actions of osmoreceptors and AVP.

Previous evidence suggests arginine vasopressin (AVP) may be a modifiable factor contributing to hyperglycemia. Kavouras recently defined a state of low water intake in which urine osmolality is elevated and there is no change in total body water, thirst, or plasma osmolality as “underhydration” (Kavouras, 2019). In this state, AVP is working to protect plasma osmolality at the level of the kidney as evident by lowered urine volume and increased

urine concentration (Armstrong & Johnson, 2018). In a classic paper written by Robertson et al., the sensitivity of this system is emphasized. The data demonstrate a 1% increase in plasma osmolality above the osmoreceptors threshold results in a 1 $pg \cdot mL^{-1}$ increase in plasma vasopressin. The rise in plasma AVP has a significant and positive correlation with urine osmolality (G. L. Robertson et al., 1976). In previous studies, significant differences in urine concentration and copeptin, a marker for AVP, were found between low and high water drinkers (Lemetais et al., 2018; Perrier et al., 2013).

As outlined by DeFronzo's "ominous octet," hyperglycemia leading to type 2 diabetes is characterized by 8 key factors including increased glucagon secretion leading to inappropriate hepatic glucose production (Defronzo, 2009). This idea was supported in previous data showing basal plasma glucagon is elevated in type 2 diabetics (Matsuda et al., 2002). Furthermore, numerous studies indicate therapies designed to blunt the effect of glucagon are likely candidates for treatment of type 2 diabetes (Petersen & Sullivan, 2001; Triplitt & DeFronzo, 2006).

Hyperglucagonemia has been observed with low water intake and increased vasopressin. In a 2017 study, 37 healthy participants were provided 3 $L \cdot d^{-1}$ of water or underwent ad libitum water intake for one week in random order. In participants defined as water-responders as indicated by the top tertile of copeptin, there was a significant negative association between water intake and glucagon (Enhorning et al., 2017). In a key early study, Spruce et al. infused vasopressin at 25 $pmol \cdot min^{-1}$ for 30 min and 75 $pmol \cdot min^{-1}$ for 60 min. After 30 minutes of infusion, plasma glucose increased from the basal measurement of 4.9±0.1 to 5.2±0.2 $mmol \cdot L^{-1}$. This increase was continued during the high dose of vasopressin as plasma glucose reached 5.7±0.2 $mmol \cdot L^{-1}$. Interestingly, there was a temporally congruent rise in glucagon (basal: 38±8, 30 min: 79±20 $pg \cdot L^{-1}$) with no significant change in insulin suggesting alpha cell stimulation.

Analysis of glucose tracing data in this study suggests the rise in plasma glucose was due to increased rate of appearance. These data allow for the possibility of increased hepatic glucose production due to higher glucagon concentration (Spruce, McCulloch, et al., 1985).

Type 2 diabetes is characterized by a progressive development of insulin resistance as a result of impaired suppression of endogenous glucose production and impaired glucose uptake. A concerted effort has been made to identify variables contributing to hyperglycemia, some of which are costly to remedy. However, lifestyle modifications continue to be a main focus of current research, especially before diabetes has been diagnosed. Considering elevated AVP may be an easily modified variable contributing to hyperglycemia, it is important to identify if adequate water intake can significantly reduce postprandial glucose responses as compared to low water intake.

SPECIFIC AIMS

Specific Aim 1: To examine the acute effect of adequate water intake on plasma glucose in low drinkers.

Primary Outcome: Plasma glucose area under the curve during the entire protocol and the meals.

Research Hypothesis 1: Plasma glucose will be lower in the high water intake trial as compared to the low water intake trial.

Specific Aim 2: Identify the effect of adequate water-intake on glucoregulatory factors glucagon, insulin, and cortisol.

Primary Outcome: Glucagon, Insulin, Cortisol during the meals.

Research Hypothesis 2: Glucagon concentration will be lower in the adequate water-intake trial as compared to the low-water intake trial indicative of lower endogenous glucose production while there will be no change in insulin or cortisol levels.

LITERATURE REVIEW

Relationship Between AVP and Copeptin

AVP, also known as antidiuretic hormone, releases from the posterior pituitary in response to rising plasma osmolality. Through stimulation of renal vasopressin 2 (V2) receptors, AVP contributes to water reabsorption via aquaporin 2 in the renal collecting ducts. As water reabsorption in the kidney increases, more fluid returns to the bloodstream ultimately limiting further elevation of plasma osmolality.

Despite AVP being one of the first hormones discovered, current assays present many challenges. Namely, low circulating concentration of AVP necessitates extreme sensitivity in sample collection and processing. Additionally, the assay requires extraction of AVP from plasma, which depending on the assay used presents difficult challenges. The hormone derives from a larger precursor molecule known as pre-provasopressin comprised of neurophysin II, AVP, and the C-terminal glycopeptide copeptin. Copeptin has been shown to release in a 1:1 ratio with AVP, thus 1 $pmol \cdot L^{-1}$ of copeptin reflects 1 $pmol \cdot L^{-1}$ of AVP. It is stable in vitro and is, therefore, much easier to analyze as compared to AVP (Fenske et al., 2018; Heida et al., 2017). In a 2006 study, copeptin and AVP were measured in 39 patients with sepsis and 71 healthy controls. The researchers observed a significant positive correlation between AVP and copeptin ($r = 0.78$, 95% CI: 0.69-0.84: $p < 0.001$). Furthermore, the stability of copeptin was measured at both room temperature and 4˚ C across 14 days in serum samples. Percentage of copeptin recovery after 7 days at room temperature was 93.3%-102.7% while at 4˚ C the percentage recovery after 14 days was 103.7% of the original value (Morgenthaler, Struck, Alonso, & Bergmann, 2006). Roussel and his colleagues investigated the relationship between plasma vasopressin and copeptin in 500 participants ultimately concluding copeptin and AVP

were positively and significantly correlated (Roussel et al., 2014). The relationship between AVP and copeptin has also been defined in varying states of plasma osmolality. Balanescu and colleagues measured plasma osmolalities, copeptin, and AVP levels at baseline, after oral water intake, and during and after IV infusion of 3% saline in 20 healthy participants. The researchers concluded there was a strong correlation between AVP and copeptin regardless of increased or decreased concentrations of plasma (Balanescu et al., 2011). Considering this evidence, copeptin is considered a valid surrogate marker for the highly unstable and difficult to measure vasopressin molecule.

Water Intake and Copeptin

Several studies have investigated the effect of water intake on copeptin. In a 2018 prospective study, Lemetais et al. stratified eighty-two healthy adults according to their estimated consumption percentage of the European Food Safety Authority's (EFSA) recommended intake volumes. For 6 weeks, participants consuming the lowest percentage of EFSA reference values had their water-intake matched to those consuming the highest percentage. After the intervention, copeptin decreased significantly from 5.18 to 3.90 $pmol \cdot L^{-1}$ (Lemetais et al., 2018). In a 2017 study, Enhorning et al. observed a 39% reduction in copeptin following rapid ingestion of 1 L of water. In the same study, participants increased water-intake by 3 $L \cdot day^{-1}$ in addition to habitual intake for one week resulting in 15% lower copeptin values as compared to a control week (Enhorning et al., 2017). Unlike the two aforementioned studies that suggest decreased copeptin in response to higher water-intake, copeptin has also been shown to increase in response to lower water-intake. In 2007, Szinnai et al. restricted 24 healthy adults of water for 28 hours resulting in an increase of copeptin from 4.6±1.7 to 9.2±5.2 $pmol \cdot L^{-1}$ (Szinnai et al., 2007). Another experiment conducted by Johnson et al. measured directly the response of AVP to water

intake in low and high water drinkers. Baseline plasma AVP in high (n=14) and low (n=14) drinkers was measured over three days followed by four days of modified water intake and one recovery day. During four days of controlled water intake, 24 h TWI in high drinkers was reduced from 3.2±0.6 to 2.0±0.2 $L \cdot d^{-1}$ resulting in a significant increase in plasma AVP. 24 h TWI was increased in low drinkers from 1.6±0.4 to 3.5±0.1 $L \cdot d^{-1}$ resulting in a significant decrease in plasma AVP (Johnson et al., 2016). These findings suggest copeptin, and therefore AVP, responds acutely and chronically to increased or decreased water intake.

Linking Elevated Copeptin and Impaired Glucose Regulation

Recent epidemiological studies show elevated copeptin levels are associated with hyperglycemia (Saleem et al., 2009). Roussel et al. studied the association of copeptin and allelic variations of the vasopressin gene with impaired fasting glucose and type 2 diabetes in 5,110 men and women. After stratifying participants into quartiles according to baseline copeptin values, the researchers found high copeptin was associated with reduced insulin sensitivity, increased risk of impaired fasting glucose, and type 2 diabetes. Although still associational, allelic variation data supported a role of the vasopressin gene in glucoregulatory impairments (Roussel et al., 2016). Similarly, in 2010 Enhorning et al. analyzed baseline and longitudinal copeptin levels as well as the development rate of diabetes and related insulin resistance in 4,377 adults. Cross-sectional data indicated increased copeptin levels were associated with higher prevalence of diabetes and insulin resistance. In the 12.6-year longitudinal follow-up, the odds of developing diabetes increased with higher levels of copeptin (odds ratio: 2.64) (Enhorning et al., 2010). As part of the prospective Malmo Diet and Cancer Study, the same group examined baseline copeptin values of 2,064 subjects. During a 15.8-year timespan, increasing quartiles of copeptin were associated with metabolic syndrome and diabetes (Enhorning et al., 2013).

This same trend was also observed in older populations. During the British Regional Heart study, Wannamethee et al. examined associations between plasma copeptin and risk of diabetes in 3,226 men aged 60 to 79 years during a 13-year timespan. Copeptin was positively and significantly associated with insulin resistance as measured using homeostatic model assessment. Furthermore, in men categorized into the top fifth of copeptin distribution (>6.79 $pmol \cdot L^{-1}$), risk of diabetes was significantly elevated even after adjustment for metabolic risk factors (hazard ratio: 1.78, CI: 1.34-2.37) (Wannamethee et al., 2015). In previous literature, hazard ratios for other factors linked to development of type 2 diabetes were similar to ratios found with elevated copeptin. In a 2005 study examining 41,372 men and women, researchers concluded the hazard ratios for incidence of diabetes with low physical activity was 1.26. In the same study the hazard ratio for incident diabetes in male smokers was 1.22 (CI: 1.04-1.43) if smoking less than 20 cigarettes per day and 1.57 (CI: 1.34-1.84) if smoking greater than 20 cigarettes per day. Corresponding ratios for women were 1.46 (CI: 1.21-1.76) and 1.87 (CI: 1.36-2.59) (Patja et al., 2005). Another study calculated hazard ratios for increases in BMI in ~ 25,000 men and women. The researchers found a 2.4-2.9 kg increase in bodyweight was associated with a hazard ratio for incident diabetes of 1.26 (CI: 1.24-1.29) for men and 1.24 (CI: 1.20-1.29) for women (Nagaya, Yoshida, Takahashi, & Kawai, 2005). Comparing these data to the hazard ratio of incident diabetes in the Wannamethee et al. study shows that elevated copeptin should be considered a risk factor at the very least from a correlational standpoint. In an additional epidemiological study, copeptin values of 6.1 $pmol \cdot L^{-1}$ in women and 10.7 $pmol \cdot L^{-1}$ in men were associated with a 3-4 time increased risk of diabetes (Enhorning et al., 2010; Melander, 2016). These findings suggest an associational relationship between elevated plasma copeptin and risk of impaired glucose regulation.

Additionally, previous epidemiological data supports an associational relationship between water-intake and markers of impaired glucose regulation. In a 9-year follow up study including 3,615 adults, Roussel et al. found that water-intake was inversely and independently associated with risk of new-onset hyperglycemia. Those who ingested <0.5 $L \cdot day^{-1}$ (n=677) had a higher risk of new-onset hyperglycemia as compared to those ingesting 0.5-1.0 $L \cdot day^{-1}$ or >1.0 $L \cdot day^{-1}$ (Roussel et al., 2011). In 2015 cross-sectional study, Carroll et al. observed water intake in 138 healthy individuals. There was a significant negative correlation between plain water and type 2 diabetes risk. Additionally, using multiple regression the researchers concluded for every 240 $mL \cdot d^{-1}$ of plain water ingested there was a 0.72% decrease in type 2 diabetes risk (β = -0.003, p = 0.014) (Carroll, Davis, & Papadaki, 2015). Similar results were observed by the same group when HbA1c was used as the main outcome measure for glucose regulation. In 456 healthy men there was a 0.04% decrease in HbA1c as water intake increased approximately 240 $mL \cdot d^{-1}$. However, no significant effects in HbA1c were found in 579 healthy women who were included in the same analysis (Carroll, Betts, & Johnson, 2016).

Associational studies have also implicated increased copeptin is linked to hyperglucagonemia. A recent cross-sectional study measured fasting plasma concentrations of copeptin and glucagon in 102 healthy obese men and a control group of 27 normal weight men. The obese men had significantly higher copeptin concentrations (6.6, IQR: 4.6-9.5 $pmol \cdot L^{-1}$) as compared to normal weight controls (4.9, IQR: 3.5-6.8 $pmol \cdot L^{-1}$; p = 0.04). Higher copeptin concentrations were accompanied by significantly higher plasma glucagon concentrations in obese men (8.5 ± 3.8 $pmol \cdot L^{-1}$) as compared to normal weight controls (5.3 ± 1.4 $pmol \cdot L^{-1}$; p < 0.001). After multiple linear regression analysis adjusting for age and weight status, there was a significant positive association between copeptin and glucagon (β = 1.35, 95% CI: 0.13-2.57; p =

0.031). However, increased glucagon did not translate into higher plasma glucose concentration (Lundegaard Asferg et al., 2019).

Not only has elevated copeptin been associated with diagnoses of diabetes, but also with the cardiovascular consequences of the disease. In a recent study, Potier et al. examined the association of copeptin with lower extremity leg amputations in type 1 and type 2 diabetic patients. Throughout a 5-10 y follow-up study, copeptin concentrations in 5,263 diabetic patients was divided into tertiles. With each increasing tertile there was a significant positive correlation in copeptin concentration (Tertile 1: 3.9%, Tertile 2: 3.3%, Tertile 3: 10.0%; P = 0.002). A Cox hazard ratio for leg amputations of 1.89 (95% CI: 1.28-2.82) was determined for each standard deviation increment of copeptin (Potier et al., 2019).

AVP and Impaired Glucose Regulation

When water intake is not sufficient to maintain plasma osmolality, AVP is released ultimately increasing the amount of water reabsorbed into the bloodstream by the kidney. Considering plasma concentration is narrowly maintained, this effect may not be observable by changes in plasma osmolality; however, urine osmolality will increase due to the anti-diuretic properties of AVP. As suggested by the aforementioned epidemiological data, this state of water conservation, termed underhydration by Kavouras, may contribute to glucoregulatory impairments (Kavouras, 2019). As outlined by DeFronzo and his colleagues, the pathogenesis of diabetes is a dynamic process involving multiple physiological systems. Distinctly, inappropriate increases in glucagon secretion and hepatic glucose production are major contributors to pathological hyperglycemia (Cersosimo, Triplitt, Solis-Herrera, Mandarino, & DeFronzo, 2000). Baron et al. showed that in type 2 diabetics increased plasma glucagon levels significantly contributed to increased hepatic glucose production as compared to normal controls. To further

emphasize this possible causal link, the researchers infused somatostatin, which inhibits pancreatic hormones. This resulted in a 44% decrease in plasma glucagon levels associated with a 58% decrease in hepatic glucose production (A. D. Baron, Schaeffer, Shragg, & Kolterman, 1987). Recent evidence suggests increased glucagon due to chronically elevated AVP may contribute to hyperglycemia. Interventions focusing on increasing water intake may potentially supplement primary prevention strategies of type 2 diabetes.

V2 receptors are responsible for the anti-diuretic effects of AVP in the kidney; however, stimulation of AVP receptors V1a and V1b result in actions with glucoregulatory implications. Through the activation of V1a receptors present in hepatic tissue, it has been suggested that glycogenolysis and gluconeogenesis increase ultimately contributing to hyperglycemia. A crucial series of animal models conducted by Taveau and colleagues in 2015 investigated this system. The researchers infused AVP or increased water intake in obese rats for four weeks. In a separate set of experiments also spanning four weeks, the researchers administered the V1a receptor antagonist Relcovaptan. After the treatment period, rats undergoing chronic infusion of AVP exhibited higher fasting glycemia. Rats treated with the V1a receptor antagonist demonstrated reduced glucose intolerance as compared to controls (Taveau et al., 2015). To investigate this effect further, the same group examined the acute and chronic (4 weeks) effects of increased vasopressin on glycemia. To investigate the acute effects, rats were pretreated with a V1a receptor antagonist for three days before intravenous injection of AVP. In rats not treated with the V1a antagonist there was a dose dependent increase in glycemia; however, treatment with 10 mg/kg or 30 mg/kg of the antagonist significantly inhibited the acute rise in glycemia by 25% and 50%, respectively. To examine the chronic effects of AVP, the researchers suppressed AVP levels by increasing water-intake in one group for four weeks while another group was infused

with AVP for the same time-period. After one week of treatment, half of the rats continuously received a V1a receptor antagonist. Chronically elevated AVP induced an increase in fasting glycemia; however, this effect was significantly lessened with two weeks of V1a receptor treatment (Taveau et al., 2017). These results suggest involvement of V1a receptor activation in acute and chronic hyperglycemia.

Previous studies have also implicated a potential physiological system independent of V1a receptor activation (Koshimizu et al., 2012; Nakamura et al., 2009; Tanoue, 2009). Pancreatic alpha cells contain V1b receptors, which are capable of stimulating glucagon secretion and indirectly increasing HGP. Evidence of this mechanism was observed in the work of Fujiwara and colleagues in 2007 in which researchers administered AVP to mice treated with a V1b receptor antagonist. Glucagon secretion was higher in mice not treated with the V1b receptor antagonist as compared to controls (Fujiwara et al., 2007). Nakamura et al. infused male Sprague-Dawley rats with AVP at a concentration of 30 and 300 $pmol \cdot L^{-1}$ for 30 minutes. Following a washout period of 10 minutes, the researchers observed a biphasic response in glucagon release after both of the different concentrations in AVP release. After the 30 $pmol \cdot L^{-1}$ infusion there was a 10-fold peak with a 5-fold sustained elevation of glucagon release as compared to baseline. After the 300 $pmol \cdot L^{-1}$ infusion there was a 35-fold peak followed by an 11-fold sustained elevation as compared to baseline. However, in this study glucagon release was dependent upon blood glucose concentration indicated by lower glucagon levels at higher blood glucose concentrations.

As another potential indirect contributing factor, stimulation of V1b receptors activates the hypothalamic-pituitary axis (HPA), thereby releasing adrenocorticotropin hormone and inherently cortisol (Koshimizu et al., 2012). Tanoue and colleagues created an animal model to

investigate the involvement of V1b receptors in regulation of the HPA axis. Utilizing gene targeting, the researchers created mouse models lacking V1b receptors. As compared to wild types, mice lacking V1b receptors had lower circulating concentrations of adrenocorticotropin hormone (ACTH) and corticosterone in resting states. Additionally, these mice exhibited a blunted response of circulating ACTH when administered exogenous AVP as compared to wild type mice (Tanoue et al., 2004).

Another proposed system independent of AVP suggests hepatocellular hydration state may influence carbohydrate metabolism potentially altering blood glucose levels. As extracellular fluid becomes more hypotonic, an osmotically stimulated shift of fluid from extracellular compartments to intracellular compartments occurs leading to cell swelling. When this happens in liver cells, inhibition of glycogenolysis and glycolysis simultaneously occurs with stimulation of glycogen synthesis potentially lowering blood glucose (al-Habori, Peak, Thomas, & Agius, 1992; Baquet, Hue, Meijer, van Woerkom, & Plomp, 1990; Meijer, Baquet, Gustafson, van Woerkom, & Hue, 1992). The opposite effect occurs when extracellular fluid becomes hypertonic, for instance when water-intake is low resulting in a net shift of intracellular fluid to extracellular compartments (Lang, Stehle, & Haussinger, 1989). In this situation, an increase in blood glucose would be expected (Graf, Haddad, Haeussinger, & Lang, 1988; Haussinger, Lang, & Gerok, 1994; Hussinger et al., 1994).

Water-intake and Glucose Regulation

Few studies have directly investigated the potential of decreased water-intake and/or increased AVP to deteriorate glucose regulation in humans. One of the earliest studies to do so was conducted by Bratusch-Marrain and DeFronzo in 1983. In this study, researchers induced hypertonicity via mannitol infusion in eight healthy participants across 2 hours. After induction

of hypertonicity, participants underwent a 2-hour euglycemic insulin clamp to measure the body's sensitivity to infused insulin. As compared to control experiments in which isotonic saline was infused, insulin-mediated glucose utilization was impaired by 22% in participants who underwent hypertonic mannitol infusion. In this study there was no between group differences in cortisol levels and glucagon was not measured. Furthermore, the use of mannitol to induce hypertonicity in this study is not congruent with increases in plasma sodium concentrations as occurs during periods of low water-intake (Bratusch-Marrain & DeFronzo, 1983). However, another early study conducted by Hensen et al. observed an acute increase in cortisol by infusing varying levels of AVP into six healthy men for a one-hour time-period. At the 50 and 60 minute time points, cortisol levels were significantly higher as compared to baseline values (Hensen, Hader, Bahr, & Oelkers, 1988). Although the aforementioned study utilized infusion of mannitol to induce hyperosmolality, an earlier study conducted by Spruce in 1985 utilized 210 minutes of AVP infusion in six male volunteers. Glucose production was increased after 30 minutes of AVP infusion and remained elevated for the remaining infusion period. Considering there was no effect in gluconeogenic precursors, this effect was most likely due to an increase in glycogenolysis, which is supported by increased glucagon levels during AVP infusion. However, the plasma AVP levels reached in this study would be considered supraphysiological in the context of low water intake (Spruce, McCulloch, et al., 1985).

Several studies have directly manipulated water-intake to explore this effect. Enhorning and her colleagues recruited 39 healthy participants to undergo one week of ingesting 3 $L \cdot day^{-1}$ of plain water in addition to habitual water-intake and subsequently 1 week of only habitual water-intake. In participants identified before the intervention as low drinkers, increased water-intake led to a significant reduction in fasting plasma glucagon. Participants with increased

water-intake had significantly lower copeptin values as compared to the control group. This study supports the potential physiological system of stimulated V1b receptors in the alpha cells of the pancreas leading to inappropriate secretion of glucagon in low drinkers (Enhorning et al., 2017). Despite a decrease in glucagon due to higher water-intake, there was no difference in glycemia. However, the same group investigated the effects of a six-week water intake intervention on fasting plasma copeptin and glucose in 31 participants. With the addition of 1.5 $L \cdot day^{-1}$ to habitual water intake, plasma copeptin decreased significantly from 12.9 $pmol \cdot L^{-1}$ to 7.8 $pmol \cdot L^{-1}$. During the same period, fasting plasma glucose also decreased significantly from 5.94 to 5.74 $mmol \cdot L^{-1}$ ($P = 0.04$)(Enhorning et al., 2018). Johnson et al. recently performed an intervention study in patients with type 2 diabetes. In a crossover manner, participants performed a 2-hour oral glucose tolerance test (OGTT) after three days of either low water-intake characterized by 1 $L \cdot day^{-1}$ for two days and 0.5 $L \cdot day^{-1}$ for one day or 3 $L \cdot day^{-1}$ as recommended by the Institute of Medicine. After ingesting a 75 g glucose load, participants in the low water-intake trial exhibited higher serum glucose levels across all time points during the OGTT. Homeostatic model assessment of insulin resistance after the low water-intake condition (6.10 ± 7.00) was significantly higher than after the high water-intake condition (4.50 ± 4.00) ($p = 0.021$). Furthermore, Matsuda whole-body glucose disposal during the OGTT was significantly higher in the high water-intake trial (4.10 ± 3.78) as compared to the low water-intake trial (3.59 ± 3.28) ($p = 0.011$) (Johnson, Bardis, et al., 2017).

Several studies have utilized infusion techniques as a model of underhydration. In 2003 Keller et al. induced hypo-osmolality via IV infusion of desmopressin, water intake, and infusion of 0.4% saline. Hyper-osmolality was induced via fluid restriction and IV infusion of hypertonic saline while iso-osmolality was induced via ad libitum water intake. To measure glucoregulatory

parameters the researchers used the gold standard method of euglycemic-hyperinsulinemic clamping. The results of this study included increased rate of appearance of endogenous glucose production during the hyper-osmolal trial indicating mainly hepatic glucose output. The researchers also reported increased plasma glucose concentrations during the hyper-osmolal trial (5.1 $mmol \cdot L^{-1}$) as compared to the iso- (4.9 $mmol \cdot L^{-1}$) and hypo-osmolal trials (4.7 $mmol \cdot L^{-1}$) likely a result of increases in endogenous glucose production. However, during the hypo-osmolal trial glycemic clamping data revealed a decreased glucose metabolic clearance rate indicating a glucose sparing effect. Jansen et al. recently investigated the effect of hypertonic saline infusion and increased copeptin on glucose handling. On two separate occasions, 60 adults underwent a 4-h oral glucose tolerance test after a 2-hour saline infusion of either 0.9% NaCl or 3.0% NaCl in a crossover manner. Considering intravenous hyper/isotonic saline infusions were used, this specific model suppressed the renin-angiotensin-aldosterone system via plasma volume expansion and hypernatremia to measure independently the effects of vasopressin on glucose regulation. Due to the increase in plasma osmolality, the researchers observed a steady increase in copeptin that lasted the entire 6-h protocol. In the hypertonic protocol, insulin responses were delayed and the area under the curve for serum glucose and glucagon were significantly greater as compared to the isotonic protocols (Jansen et al., 2019).

In type 1 diabetics, the effect of dehydration (4.1 ± 2.0% baseline bodyweight) on glycemic regulatory hormones was observed by Burge et al. in 2001. The participants of this study underwent a five hour insulin withdrawal protocol in a dehydrated state while deuterated glucose was continuously infused to calculate the rate of appearance and disappearance of endogenously released glucose. A control arm of the protocol during which participants were well hydrated was used as well as a fasted arm. Although a rise in plasma glucose concentrations

was expected considering type 1 diabetics were taken off insulin treatment, the increase was significantly higher in the dehydrated trial as compared to the control trial. As a possible cause for elevated plasma glucose concentration, glucagon area under the curve (AUC) was significantly higher in the dehydrated trial as compared to the control trial. Glucagon AUC in the dehydrated trials ultimately surpassed levels observed in the fasting trial at hour 4 of insulin withdrawal (Burge, Garcia, Qualls, & Schade, 2001). Since this study was conducted in type 1 diabetics, the glucagon counter regulatory hormone insulin significantly decreased during the five hour protocol. This created an increase in glucagon levels. Considering data indicating increased glucagon as responsible for hyperglycemia with low water intake, there may be an additive effect of low water intake in type 1 diabetics creating the significant increases in glucagon observed in this study.

Although several studies suggest impaired glucose regulation due to low water intake, one recent study conducted by Carroll and colleagues found contradictory results. Sixteen healthy adults under fluid restriction were dehydrated by remaining in a heat tent for one hour. On a separate occasion, participants were allowed to rehydrate after the same method of heat-induced dehydration. The following day, participants underwent an OGTT in either a hypohydrated or rehydrated state. Additionally, pre- and post-intervention computer tomography scans of thigh muscle were taken as an index of cell volume. In the hypohydrated trial pre-OGTT the researchers observed increased urinary hydration markers, increased copeptin values, and decreased fasted cell volume. Despite these interventions, no differences were found in serum glucose or insulin responses between trials. Although glucagon was not measured in this study, no differences in cortisol or ACTH were found between the hypohydrated and rehydrated trials (Carroll et al., 2018). In previous studies, short exposures to hot environments have been shown

to increase plasma vasopressin (Segar & Moore, 1968; Takamata, Mack, Stachenfeld, & Nadel, 1995). Considering in the study by Carroll et al. a heat tent was used to induce hypohydration, this may have affected vasopressin levels in the rehydrated group ultimately minimizing between trial differences in vasopressin. Although the effect of temperature on AVP levels is not well understood, previous literature indicates a significant effect that should be controlled by researchers (Robertson, 2013; Segar & Moore, 1968; Takamata et al., 1995).

Although theoretical, a contradictory system has been hypothesized to explain data suggesting hyperglycemia due to increased AVP (Carroll & James, 2019). This theory suggests a temporally opposite effect in which hyperglycemia drives the increase in AVP as opposed to a vice versa relationship. This hypothesis suggests hyperglycemia increases plasma osmolality causing chronic AVP elevation. This may explain results of studies showing a correlation between increased vasopressin and hyperglycemia as well as in experiments performed in diabetic subjects. However, the recent study by Jansen et al. was performed in fasted subjects with HbA1c levels less than 6.5%. Results showed the osmotic stimulation of vasopressin via hypertonic saline infusion produced increased area under the glucose curve as compared to isotonic saline infusion. This result suggests hyperglycemia is driven by increased vasopressin due to hyperosmolality (Jansen et al., 2019). Furthermore, decreases in plasma glucose with higher water intake could be attributed to increased urine production, and therefore, more glucose being dumped by the kidney. The kidney filters approximately 160 g of glucose every day, however, in diabetics the amount of sodium-glucose transporters in the kidney is increased leading to inappropriate increases in glucose reabsorption (Abdul-Ghani & DeFronzo, 2008). Considering some studies suggesting increased water intake could effect glucose regulation were

performed in diabetic individuals (Johnson, Bardis, et al., 2017), it is important to investigate the potential role of urinary glucose output.

Another possible link between the AVP system and metabolism is the incretin hormone glucagon-like-peptide-1 (GLP-1), which is secreted from intestinal L-cells and is facilitated by V1b receptors. Intestinal water balance is largely hormonally controlled, partly due to GLP-1 which has been shown to increase plasma glucocorticoid concentration and thereby stimulate intestinal sodium and water absorption (Enhorning & Melander, 2018; Pais et al., 2016).

Previous evidence suggests adequate water intake may have a significant relationship with glucose regulation, particularly in low drinkers. However, the physiological system by which this effect occurs is complex and remains unclear. Furthermore, previous literature does not explore the acute effect of water intake on glucose regulation. Developing a further understanding may lead to interventions that remove a contributing factor to type 2 diabetes. Considering the deleterious health consequences coupled with substantial economic burdens associated with diabetes, a low-cost supplemental intervention could be important. Therefore, the purpose of this study is to examine the acute effect of low water intake on glucose regulation in low drinkers and identify the effect of water intake on potentially related glucoregulatory hormones.

METHODS

Participants & Screening Process

7 healthy (5 male, 2 female) participants aged 30-55 years with a body mass ranging from 27.5-35.0 $kg \cdot m^2$ were recruited from the larger Phoenix metropolitan area via Arizona State University's campus news medium and social media. All procedures were approved by the University Institutional Review Board and written informed consent was obtained from all

individuals prior to participation. The range of BMI selected was to target individuals not overly sensitive to insulin. Only individuals exhibiting low total water intake (low drinkers) were included in this study. Specific parameters for identifying low drinkers were observed between two screening visits before any experimental visit occurred.

Before the first visit, participants were provided a medical history questionnaire to identify any potential exclusion criteria including people with diabetes, impaired liver or kidney function, cardiovascular disease, weight change of more than 3 kg in the past month, pregnancy, or previous surgery on digestive tract. Before the first visit, participants were also asked to complete a validated water intake questionnaire (Johnson, Peronnet, et al., 2017). To be classified as a low drinker, a fluid intake (water and other beverages) less than or equal to 1.5 $L \cdot day^{-1}$ in males or 1.0 $L \cdot day^{-1}$ in females was required. During the first screening visit, participant's body weight and height were measured (Seca 284, Hamburg, Germany). Participants then provided a spot urine sample that was analyzed for USG and osmolality. In order to be categorized as a low drinker, a urine specific gravity (USG) greater than 1.025 and urine osmolality (UOsm) greater than 800 $mmol \cdot kg^{-1}$ was required. At the end of the first screening visit, participants were provided a container to collect a 24 h urine sample and then return for a second screening visit. To meet the criteria of a low drinker, an osmolality of greater than 800 $mmol \cdot kg^{-1}$ was required for the 24 h urine sample. A blood sample was then drawn and analyzed for copeptin and glycosylated hemoglobin (HbA1c) (Siemens, DCA Vantage Analyzer) in order to assess undiagnosed cases of diabetes. An HbA1c greater than 6.5% was considered exclusionary.

Experimental Visits

This study was registered as a clinical trial at clincialtrials.gov (NCT04076995) prior to enrolling any subject. Participants were randomly assigned in a counterbalanced fashion to the order in which they completed both trials (www.randomization.com). Study visits for women were scheduled during the early follicular phase of the menstrual cycle to control for sex hormone confounding effects on metabolism and fluid balance (Stachenfeld & Keefe, 2002; Yeung et al., 2010). At least two days before each participant's experimental visit a continuous glucose monitoring system (CGMS) (FreeStyle Libre Pro) was placed on the right dorsal upper arm. This system sampled interstitial glucose concentrations every 15 minutes until removed or until two weeks had passed. This system imputes interstitial glucose to an algorithm that predicts blood glucose values (Ghane et al., 2019; Yajima, Takahashi, & Yasuda, 2019). The CGMS remained on participants until their first experimental visit was concluded. Before the second experimental visit, the same CGMS process occurred. The day before each experimental visit, participants recorded their food intake in a provided diary. In a crossover design with at least 3 days between each trial, participants reported to the laboratory to undergo a high water intake trial of 3 $L \cdot day^{-1}$ for men and 2 $L \cdot day^{-1}$ for women (HWI) or low water intake trial of 0.5 $L \cdot day^{-1}$ for men and 0.4 $L \cdot day^{-1}$ for women (LWI). The intake values in HWI were determined using the Institute of Medicine's recommendations for total water intake of 3.7 $L \cdot day^{-1}$ for men and 2.7 $L \cdot day^{-1}$ for women. Intake values selected represent total water intake without accounting for water from food assuming 20% of total water intake comes from the moisture of foods as previously described from analysis of National Health and Nutrition Examination Survey data (NHANES). The intake values for LWI represent the bottom quartiles of NHANES water intake for men and women (Drewnowski, Rehm, & Constant, 2013). Water was ingested at identical

times for each trial and only varied by amount between trials. Participants remained in the laboratory from 6:30 am until after the last blood draw at 6:00 pm. All participants were asked to remain seated as much as possible during the entire protocol and were not allowed to sleep to avoid shifts in body fluid associated with changes in posture (Hagan, Diaz, & Horvath, 1978). Participants were asked to refrain from using the restroom within 20 minutes of any blood draw. An intravenous catheter was placed followed by a 20-minute wait time. During this time, participants remained seated in a phlebotomy chair. At 7:00 am, the first baseline blood draw was taken. Subsequent blood draws were performed immediately before each meal, 60 minutes after each meal, and 120 minutes after each meal (breakfast = 7:00 am, lunch = 12:00 pm, dinner = 4:00 pm). All urine voids that occurred while the participant was in the laboratory were collected and pooled. Before each blood draw, participants completed a visual analog scale indicating their level of thirst and nausea with anchors at 0 mm (not at all) and 125 mm (extremely). An outline of water intake amounts, blood draws, and meal scheduling is outlined in Figure 1 below. One hour after each meal (8:00 am, 1:00 pm, and 4:00 pm) participants were provided a profile of mood state (POMS) questionnaire (Heuchert, 2012).

Figure 1: Experimental visit water amount, blood draw time, and meal schedule.

Physical Activity and Meal Standardization

Participants were instructed to avoid exercise training and/or heavy physical labor for at least three days prior and including the day of each experimental protocol. Participants were instructed to keep a comprehensive record of physical activity for the three days leading up to each experimental protocol including duration of exercise and/or physical labor to the nearest ten minutes. Only activity described as moderate or vigorous was included. Participants were instructed to record a comprehensive record of their diet the day before the first experimental protocol. This diet was repeated exactly before the second experimental protocol. A standardized dinner was given to each participant that was consumed the night before each experimental visit. This meal consisted of 44 $kJ \cdot kg^{-1}$ of bodyweight composed of 64% carbohydrate, 25% fat, and 11% protein. After consuming the standardized dinner, participants remained fasted until breakfast was provided in the experimental protocol During both 11 h intervention periods, participants remained in the laboratory and were given three identical meals and two identical snacks totaling 100 $kJ \cdot kg^{-1}$ of bodyweight composed of 64% carbohydrate, 25% fat, and 11% protein at set time points. This amount of energy intake and macronutrient composition has been used in similar previous studies in obese and diabetic patients under continuous glucose monitoring for 24 h or greater. However, the caloric intake for the current study has been adapted for an 11 h timeframe (Manders, Pennings, Beckers, Aipassa, & van Loon, 2009; Praet et al., 2006).

Biochemical Analysis

Blood Analysis

Blood was collected in tubes treated with sodium fluoride for plasma glucose measurement, lithium heparin for plasma osmolality measurement, and serum separator tubes.

Serum samples were used to analyze insulin, glucagon, cortisol, and copeptin. These samples were centrifuged for 15 minutes at 3,000 RPM. Blood was collected in EDTA treated tubes with DPP-IV inhibitor (10 µL per mL whole blood) and aprotinin (8.3 µL per mL whole blood) for plasma GLP-1 measurement. These samples were centrifuged for ten minutes at 2,200 RPM. Aliquots of samples were immediately frozen at -80˚ C until time of analysis. Untreated whole blood was analyzed in triplicate for hematocrit using microcentrifugation. Hemoglobin was quantified in triplicate via colorimetric analysis by aliquoting 20 µL whole blood into 5 mL of Drabkin's reagent (Drabkin's Reagent; RICCA Chemical Laboratories, Arlington, TX, USA). After a minimum of 30 minutes incubation away from any light sources, absorbance of the solution was quantified using a spectrophotometer at 540 nm against a standard curve created with cyanmethemoglobin (Genesys 180 UV-Vis, ThermoFisher, Waltham, MA). Plasma volume changes were calculated using hematocrit and hemoglobin based off the equation from Dill and Costill (Dill & Costill, 1974). Total plasma protein was measured via refractometry. Plasma glucose was analyzed using a spectrophotometric enzymatic glucose oxidase method (Randox Daytona+, Randox Laboratories Ltd., Crumlin, UK). GLP-1 (Millipore Sigma Inc., Darmstadt, Germany), glucagon, and cortisol (R&D systems, Minneapolis, MN, USA) were analyzed using enzyme-linked immunosorbent assay. Insulin was measured via radioimmunoassay (Millipore Sigma Inc., Darmstadt, Germany). Copeptin was measured using random access immunoanalysis (B.R.A.H.M.S Kryptor, ThermoFisher; Berlin, Germany).

Urine Analysis

During each experimental visit a pooled 11 h urine sample was collected. Urine osmolality was analyzed fresh via freezing point depression (A_20, Advanced Instruments, Norwood, MA) and urine specific gravity (USG) was measured via refractometry. Aliquots of 11

h pooled samples were frozen at -80˚ C. Urinary glucose output during experiments was measured from frozen samples using enzyme linked immunosorbent assay (Boehringer Mannheim, Mannheim, Germany).

Statistical Analysis

All statistical analyses were performed using SPSS version 24 (IBM Corporation, Somers, NY). A two-way (water intake x time) repeated measures analysis of variance was used to determine differences in hormones and perceptual measures. Dependent t-tests were used to measure between trial differences in plasma glucose area under the curve (AUC), 11 h urine volume, osmolality, and total urinary glucose output. Incremental AUC was calculated as described previously (Brouns et al., 2005). Single linear regression analysis was used to define relationships between hydration and glucoregulatory parameters. Outliers were identified using 3 x interquartile range. Statistical analysis was completed initially including outliers and analysis was re-run excluding outliers. If no differences in statistical conclusions were found the results were reported without exclusion. Tukey's HSD post-hoc test was used for pairwise comparisons. For each trial, one glucose measurement before 7:00 am was included in the CGMS data set. CGMS data was grouped according to meal/snack periods creating a time variant factor (meal) that was included in the analysis (Meal 1: Breakfast, Meal 2: Snack 1, Meal 3: Lunch, Meal 4: Snack 2, Meal 5: Dinner). A meal group was measured from time of meal (time 0) until the next meal began. Statistical significance was set *a priori* at an alpha of 0.05.

Considering a Cohen's d effect size in previous literature of ~ 0.3 (plasma glucose AUC), to achieve a power of 0.8 with a type 1 error rate of 0.05 would require a sample size of 12 participants in the current study. This study incorporates 7 subjects with the aim of providing

pilot data for future investigation. The methods can be utilized as a measure of feasibility and results/conclusions can be used to guide future studies in sample size calculation.

RESULTS

Low Drinker Characteristics

All results are reported as mean ± SD unless otherwise stated. Subject characteristics are included in Table 1. Total fluid intake estimated from the WFQ was 823±403 $mL \cdot d^{-1}$. Baseline spot and 24 h UOsm measured during screening for all participants was 973±118 and 961±105 $mmol \cdot kg^{-1}$, respectively, while spot USG was 1.028±0.003. Copeptin from screening blood samples was 8.17±3.05 $pmol \cdot L^{-1}$ (Table 1). HbA1c measured during screening process was 5.5±0.5 %.

Table 1: Baseline characteristics of study participants

	Male	Female	Total
N	5	2	7
Age (y)	42±7	47±1	43±6
BMI ($kg \cdot m^{-2}$)	30±3	33±0	30.9±3
Spot USG	1.028±0.004	1.027±0.001	1.028±0.003
Spot UOsm ($mmol \cdot kg^{-1}$)	995±125	919±114	973±118
WFQ Volume (mL)	922±445	574±92	823±403
24 h UOsm ($mmol \cdot kg^{-1}$)	1000±90	862±81	961±105
HbA1c (%)	5.3±0.3	5.9±0.7	5.5±0.5
Copeptin ($pmol \cdot L^{-1}$)	8.83±3.31	6.53±2.15	8.17±3.05

Data expressed as mean ± SD; BMI = body mass index
HbA1c = glycated hemoglobin; USG = urine specific gravity
WFQ = Water frequency questionnaire

Perceptual Measures

There was a significant main effect of water intake on thirst responses (F = 17.253, P = 0.006), but no difference across time (P = 0.691). There was a significant interaction effect (P = 0.001). Pairwise comparisons revealed thirst was significantly higher in the low water intake trial at 0800 (HWI: 10±12; LWI: 43±26, P = 0.007), 0900 (HWI: 10±11 mm; LWI: 49±34 mm, P = 0.006), 1200 (HWI: 13±20 mm; LWI: 49±41 mm, P = 0.014), 1300 (HWI: 13±16 mm; LWI: 53±40 mm, P = 0.009), 1400 (HWI: 22±33 mm; LWI: 56±40 mm, P = 0.013), 1600 (HWI:14±17 mm; LWI: 48±39 mm, P = 0.017), 1700 (HWI: 10±20 mm; LWI: 59±38 mm, P =0.002), and 1800 (HWI: 17±28 mm; LWI:45±44 mm, P = 0.045) (Figure 2). There were no main effects of time or water intake on nausea (P = 0.94). Total mood disturbance calculated from the POMS questionnaire was similar between trials (P = 0.98).

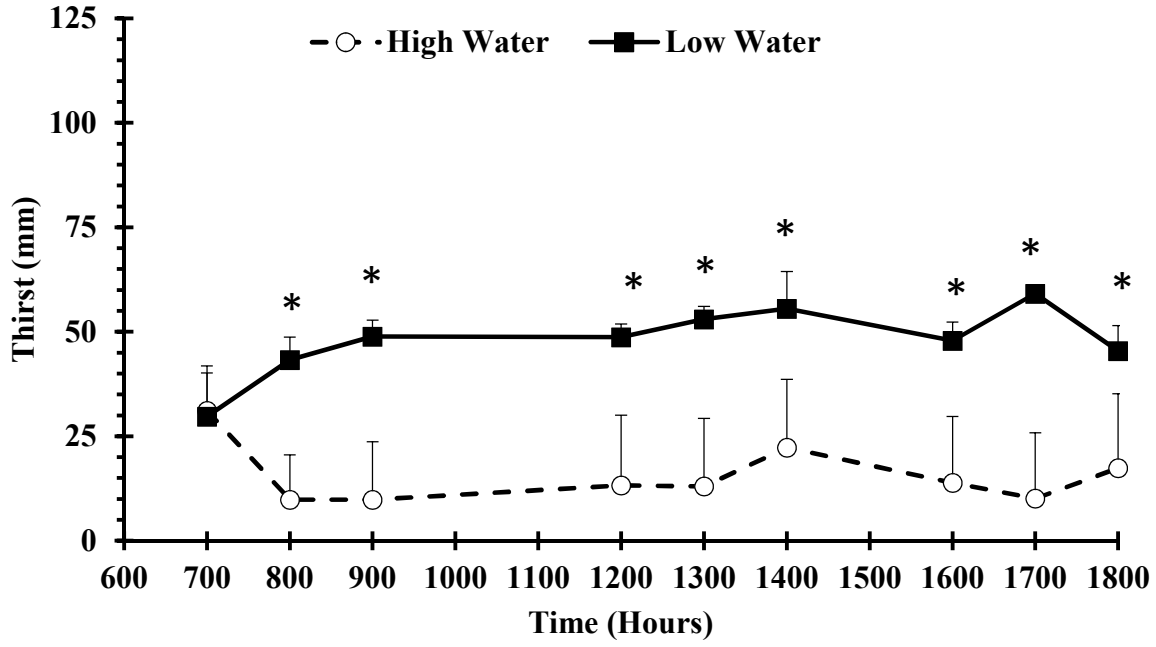

Figure 2: Thirst during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. • represents significantly different from HWI for each time point ($p < 0.05$). Error bars = SE.

Hydration Markers

11 h urine measures during each trial are outlined in Table 2. 11 h urine volume was significantly higher during HWI as compared to LWI (t = 10.607, $P < 0.001$). 11 h UOsm was significantly higher in LWI as compared to HWI (t = 12.040, $P < 0.001$). There were no significant differences in pre-trial USG ($P = 0.153$) (Table 2).

Table 2: In trial (11 h) urine measures

	High Water	Low Water	p
Number of voids	10±4	4±2	-
11 h Urine Volume (mL)	2233±372*	445±203	< 0.001
11 h UOsm (mmol·kg^{-1})	224±48*	956±120	< 0.001
Pre-Trial USG	1.022±0.006	1.027±0.004	0.153

Data expressed as mean ± SD; * indicates significantly different from LWI.

There was a significant main effect of water intake on plasma osmolality (F = 15.967, $P = 0.007$) as well as a main effect of time (F = 2.621, $P = 0.003$), but no interaction effect was observed (F = 1.897, $P = 0.082$) (Figure 3).

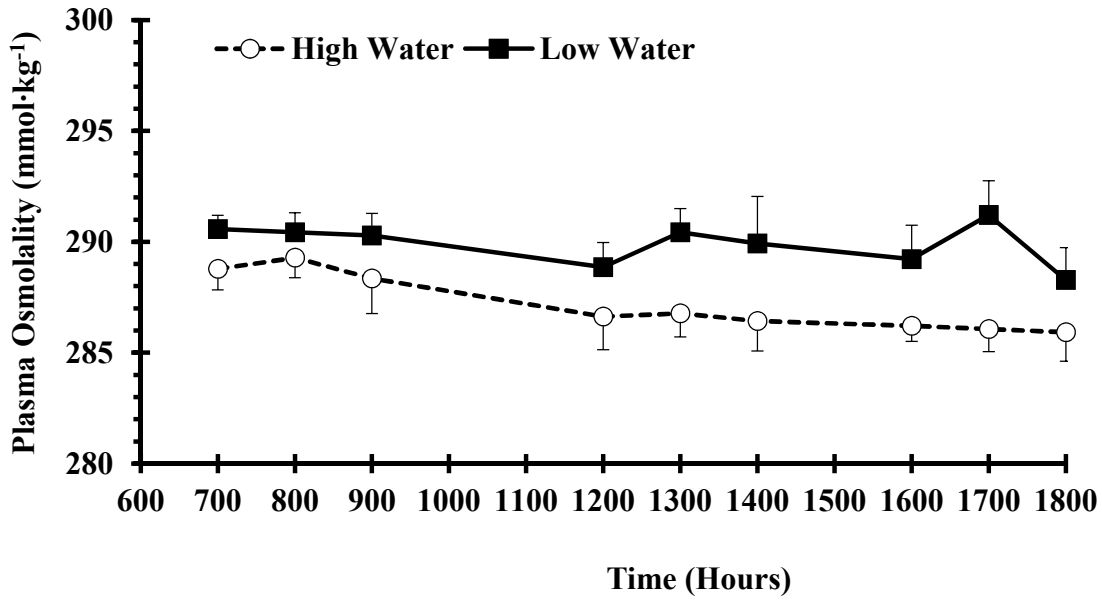

Figure 3: Plasma osmolality during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. represents significantly different from HWI for each time point ($p < 0.05$). Error bars = SE.

There was a significant main effect of water intake on copeptin (F = 10.008, *P* = 0.019), however, there was no interaction effect between water intake and time (F = 0.989, *P* = 0.358) (Figure 4).

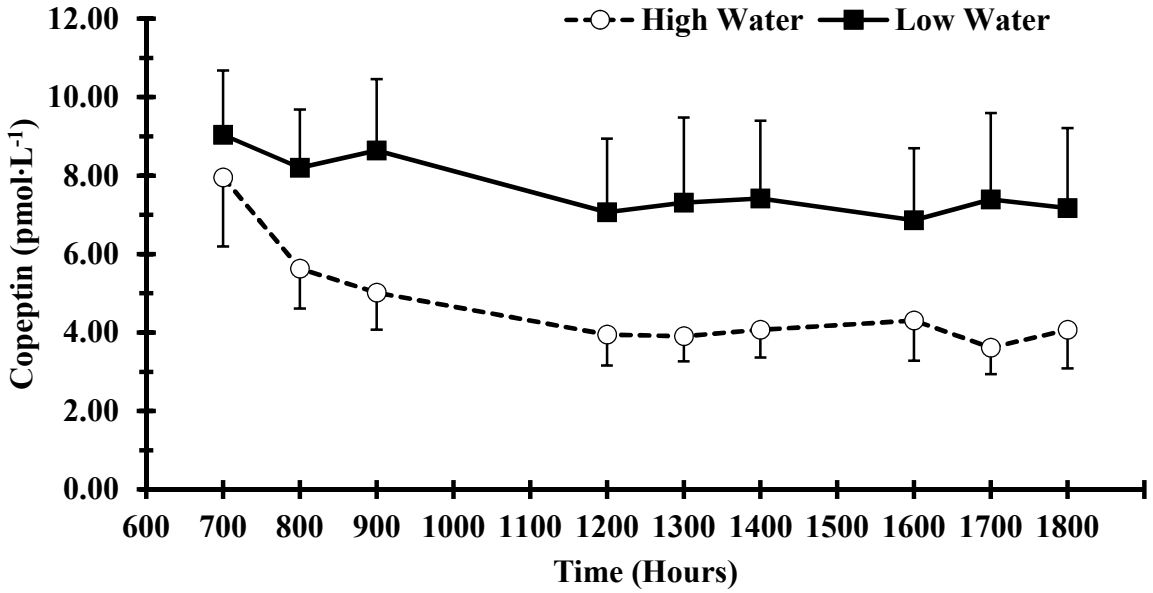

Figure 4: Copeptin during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. represents significantly different from HWI for each time point ($p < 0.05$). Error bars = SE.

There was no significant main effect of water intake (*P* = 0.145) on change in plasma volume, however there was a main effect of time (F = 32.227, *P* = 0.001). No significant main effect of water intake or time on total plasma protein was observed.

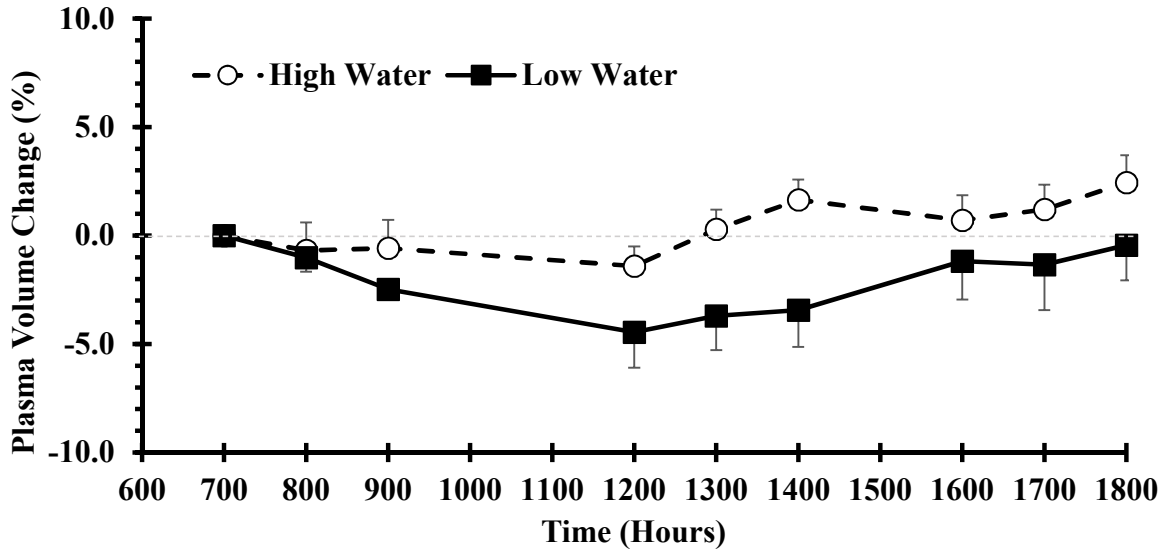

Figure 5: Plasma volume during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant differences between HWI and LWI ($P = 0.214$). Error bars = SE.

Glucoregulatory Markers

There was no significant main effect of water intake on glucagon ($P = 0.372$), however there was a significant effect of time ($F = 2.466$, $P = 0.025$) (Figure 8).

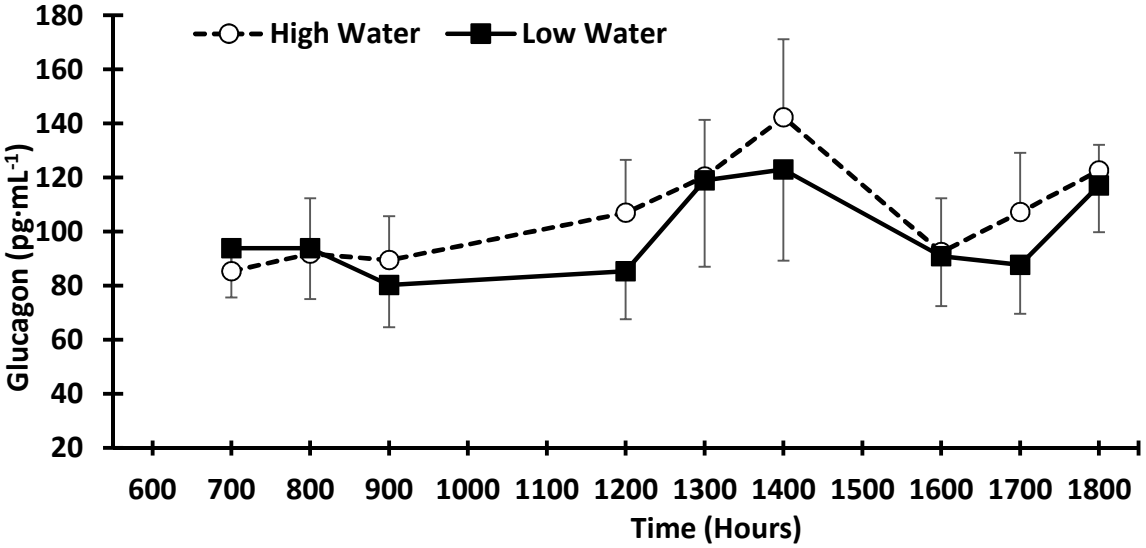

Figure 8: Glucagon during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant main effect of water intake. Error bars = SE.

There was a significant main effect of water intake (F = 14.375, P = 0.009) and time (F = 6.072, P = < 0.001, but no interaction effect was observed (P = 0.581, Figure 9). Additionally, copeptin was a significant predictor of cortisol concentration (R^2 = 0.520, P = 0.001). There was no main effect of water intake on GLP-1 (P = 0.251), however there was a main effect of time (F = 7.901, P = < 0.001, Figure 10).

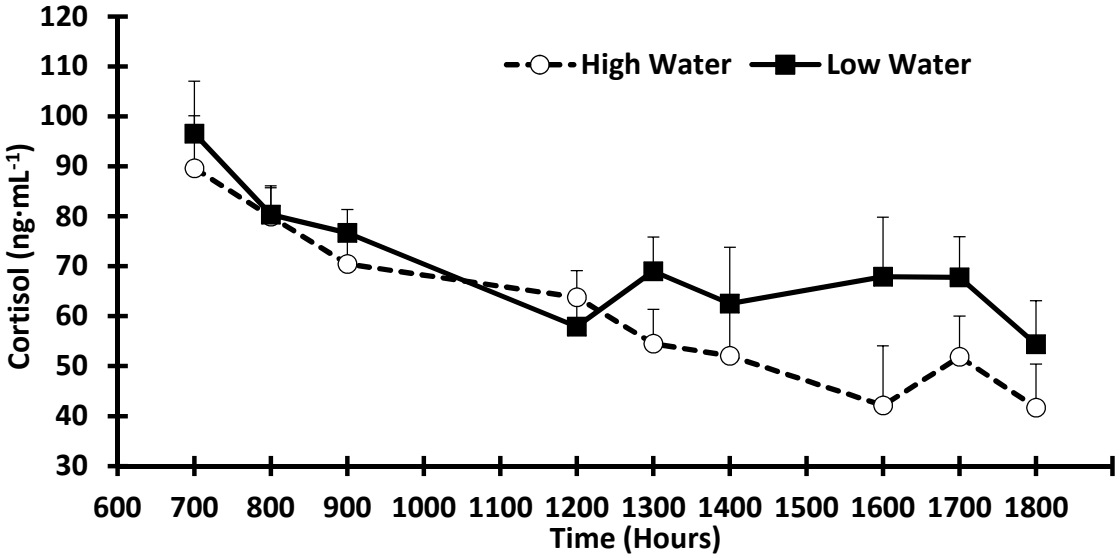

Figure 9: Cortisol during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. Significant main effect of water intake (P = 0.009). No significant pairwise differences. Error bars = SE.

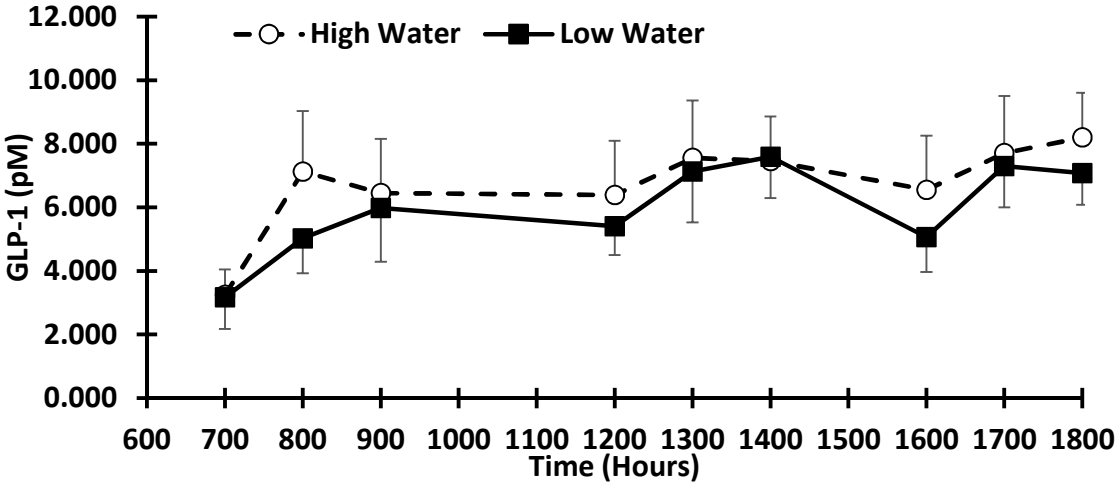

Figure 10: GLP-1 during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant differences between HWI and LWI (P = 0.251). Error bars = SE.

No main effect of water intake on insulin was observed ($P = 0.229$), however, there was a main effect of time ($F = 6.309$, $P < 0.001$, Figure 11).

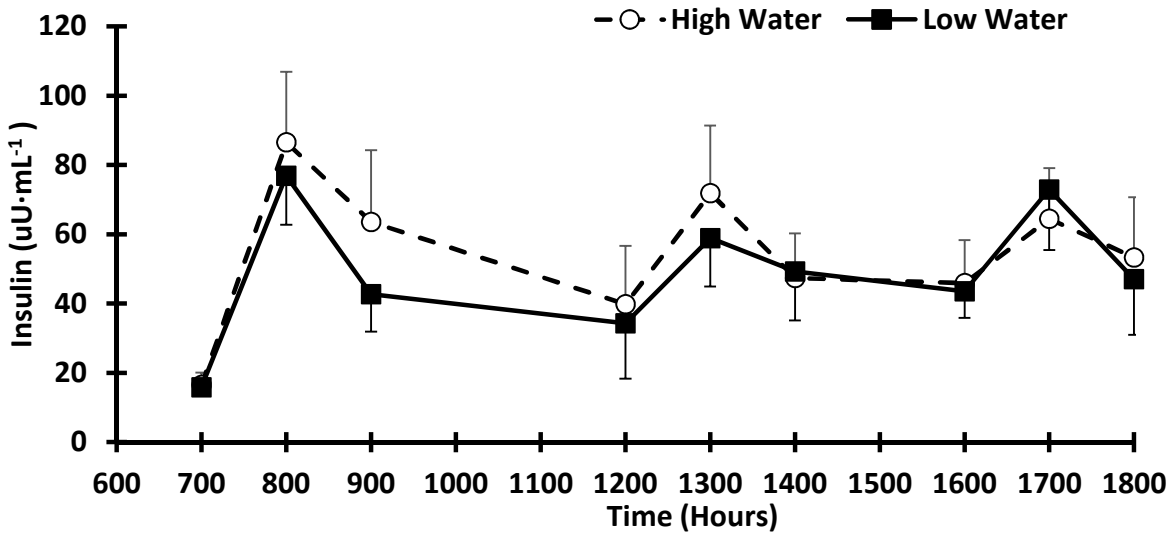

Figure 11: Insulin during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant main effect of water intake. Error bars = SE.

No significant differences in plasma glucose were found due to water intake ($P = 0.07$), however, there was a main effect of time ($F = 3.656$, $P = 0.005$, Figure 12).

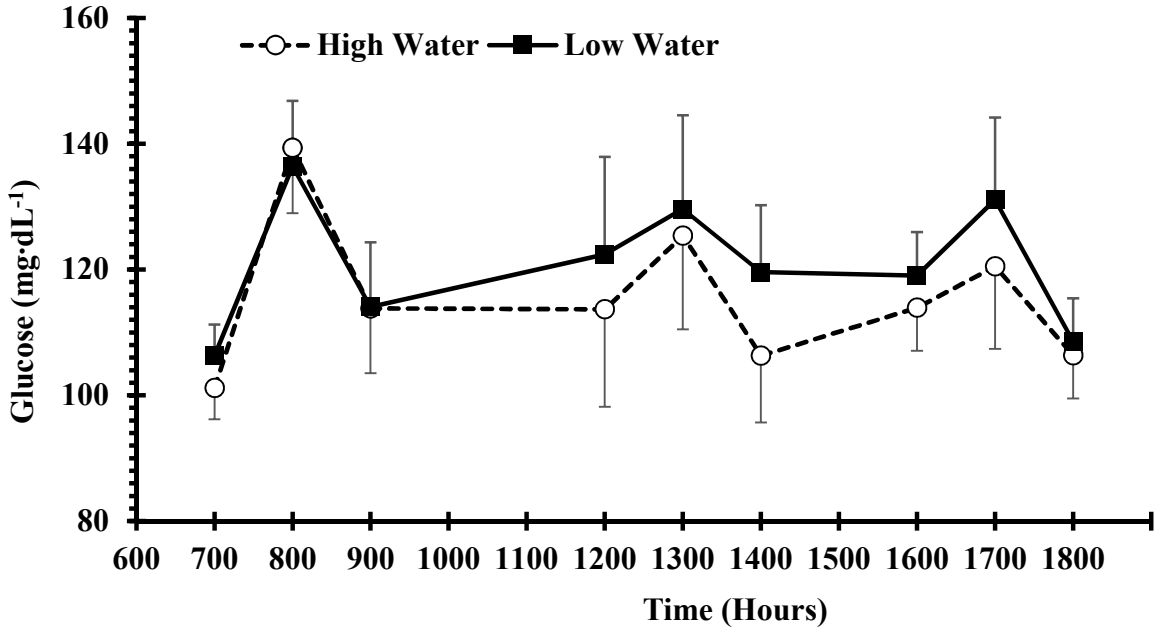

Figure 12: Plasma glucose during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant differences between HWI and LWI ($P = 0.07$). Error bars = SE.

There were no differences in plasma glucose incremental AUC between HWI and LWI (P = 0.626, Figure 14) nor was there a significant difference in total urinary glucose output between HWI and LWI (P = 0.133, Figure 15).

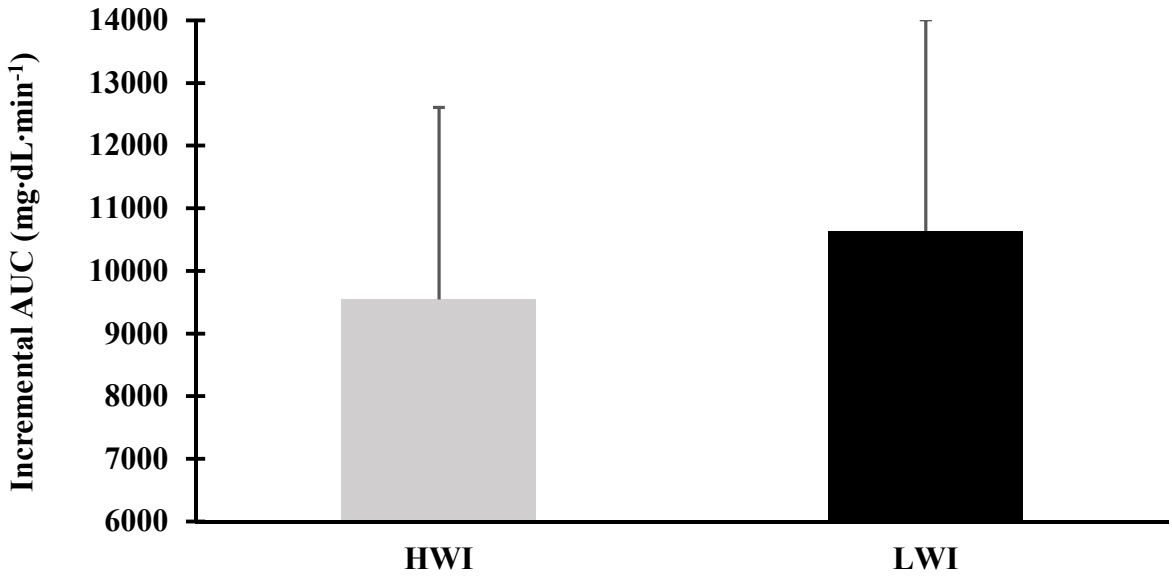

Figure 14: Incremental AUC for plasma glucose in HWI and LWI trials. No significant difference between trials. P = 0.626. Error bars = SE

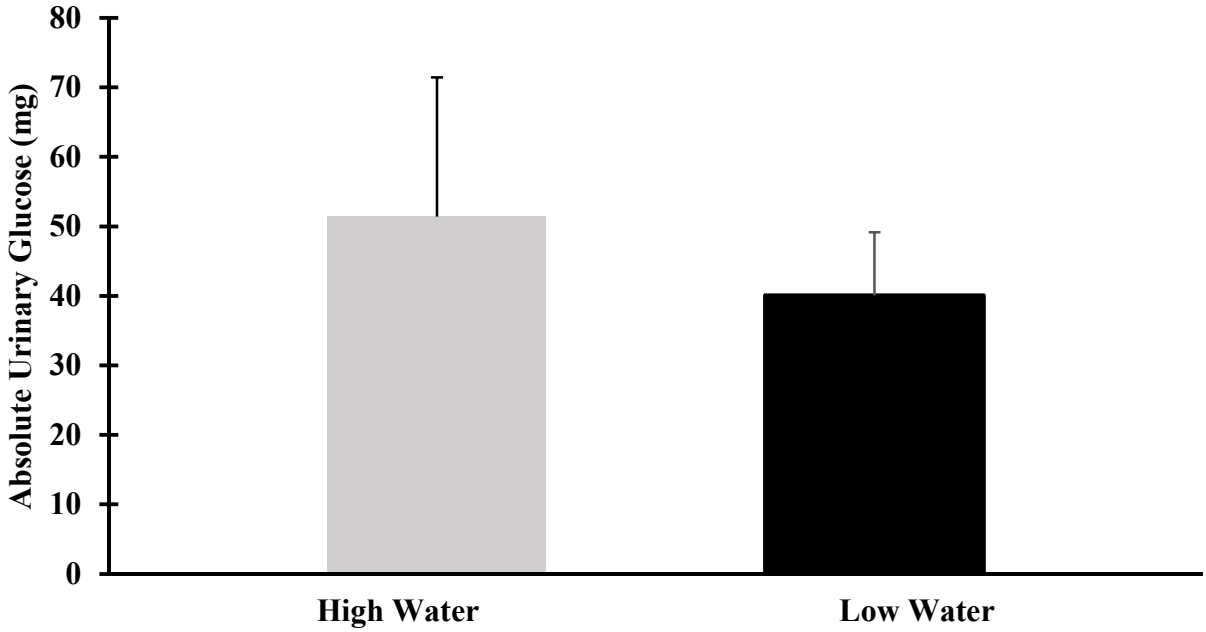

Figure 15: Total urinary glucose excretion during high (males: 3 L; females: 2 L) and low (males: 1 L; females: 0.5 L) water intake trials. No significant difference between HWI and LWI (P = 0.133). Error bars = SE.

During continuous glucose monitoring three measurement sensors fell off participants in the days leading up to experimental protocols. For this reason, complete data sets for only 4 of the 7 participants were collected and included in the analysis. Mean estimates of blood glucose measured at 44 time points are included in Figure 16.

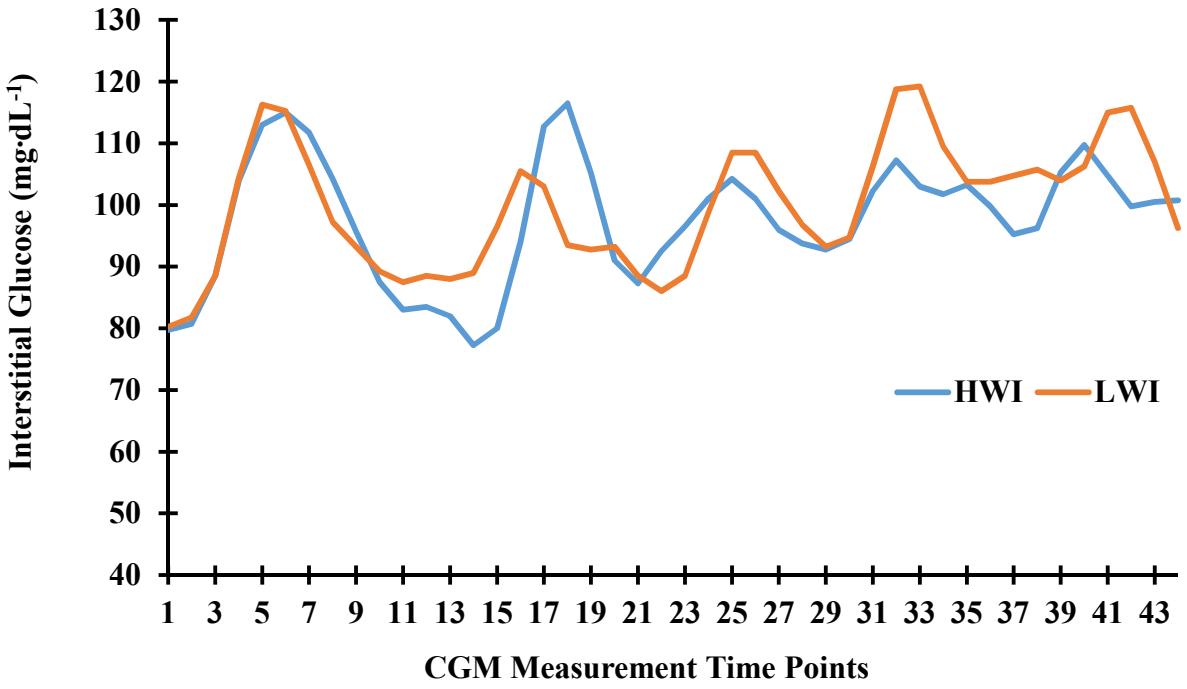

Figure 16: Mean blood glucose estimated by continuous glucose monitoring system during HWI and LWI. Breakfast = 2 (7 am), Snack 1 = 12 (10 am), Lunch = 20 (noon), Snack 2 = 28 (2 pm), Dinner = 36 (4 pm). 1 tick mark on x-axis represents 30 minutes. N = 4.

Regression equations revealed the effect of plasma osmolality on cortisol levels could be partially mediated by copeptin. This mediation effect was tested as described by Baron and Kenney (R. M. Baron & Kenny, 1986). A post-hoc Sobel analysis was used as an approximate significance test to describe indirect effects of plasma osmolality on cortisol as mediated by copeptin (Sobel, 1982).

There was a significant relationship between plasma osmolality and cortisol ($R^2 = 0.536$, $\beta = 0.086$, SE = 0.020 $P = 0.001$). There was also a significant relationship between plasma

osmolality and copeptin ($R^2 = 0.805$, $\beta = 0.861$, SE = .106, $P < 0.001$). Finally, there was a significant relationship between copeptin and cortisol ($R^2 = 0.523$, $\beta = 0.088$, SE = 0.021, $P = 0.001$). Despite these regression analyses, copeptin was not a significant mediator of the relationship between plasma osmolality and cortisol (Sobel test statistic: 0.88, $P = 0.38$).

DISCUSSION

The purpose of this study was to examine the acute effect of water intake on glucose regulation in low water drinkers. Furthermore, the study sought to investigate the effect of water intake on glucoregulatory hormones possibly involved in altered glucose metabolism as demonstrated in previous studies. In current literature, no study has acutely controlled water intake and diet in a laboratory setting for up to 11 hours to investigate this effect. The main finding of this study is that no differences were observed in plasma glucose during the high water intake trial as compared to low water intake trial. Additionally, cortisol may be effected by water intake as indicated by significant differences between the low and high water intake trials.

By design, blood and urine measures throughout the 11 h protocol indicate hydration status was acutely manipulated between trials. Additionally, thirst rapidly declined in HWI and was subsequently suppressed throughout the 11 hour protocol as compared to LWI. The end goal of acutely decreasing vasopressin levels was accomplished as evident by the significantly lower levels of copeptin in the high water intake trial independent of baseline copeptin levels for each trial (0700 = HWI: 8±4; LWI: 9±4 $pmol \cdot L^{-1}$). Several factors may have played a role in these differences. To begin with, plasma osmolality was significantly higher in LWI as compared to HWI. The main influencer of vasopressin levels is tonicity of body fluid, which is tightly regulated as osmoreceptors detect small fluctuations in body water concentration. Previous literature has identified a direct positive relationship between vasopressin levels and plasma

osmolality. In the current study, plasma osmolality was a significant predictor of copeptin concentration ($R^2 = 0.805$, $P < 0.001$). Considering plasma osmolality was significantly higher during LWI, this was likely the main driver of the observed elevations in copeptin. This is supported by the large difference in 11 hour urine osmolality observed between HWI and LWI. This difference indicates vasopressin was working at the level of the kidney to prevent further rises in plasma osmolality. Urine volume has also been shown to be highly negatively correlated with vasopressin levels (Armstrong & Johnson, 2018). In the current study urine volume was significantly increased in the HWI as compared to the LWI, further supporting vasopressin was successfully attenuated in HWI.

Although not a statistically significant difference, baseline plasma osmolality was 291 $mmol \cdot kg^{-1}$ in LWI as compared to 289 $mmol \cdot kg^{-1}$ in HWI. As outlined previously, small fluctuations in plasma osmolality influence vasopressin levels. Closer examination of the data revealed that one subject was the main driver behind this difference. Baseline plasma osmolality for HWI in this subject was 287 $mmol \cdot kg^{-1}$ as compared to 291 $mmol \cdot kg^{-1}$ for LWI, which represents the largest baseline difference for any of the participants. This difference is supported by pre-trial USG in this individual (HWI: 1.015; LWI: 1.033). If the baseline values of this participant are excluded from calculation of baseline values across all participants, the means for HWI and LWI become 289 $mmol \cdot kg^{-1}$ and 290 $mmol \cdot kg^{-1}$, respectively. Although participants were instructed to avoid drinking or eating before arrival to the laboratory, these differences may have been caused by a compliance issue with the participant described above.

In previous studies, copeptin concentrations in low drinkers have been shown to have a greater response to increased water intake as compared to those consuming adequate water (Enhorning et al., 2018; Johnson et al., 2016). As stated previously, the relationship between

AVP and plasma osmolality could be characterized by a "set-point" of osmolality that once reached produces a steep curvilinear response in vasopressin. Individual differences in this threshold could be genetically influenced (Zerbe, Miller, & Robertson, 1991), and therefore, contribute to large decreases in copeptin levels of low drinkers who acutely increase water intake. This relationship between water intake and copeptin in low drinkers is consistent with the results of the current study, which demonstrated a rapid and sustained suppression of copeptin during HWI.

Hemodynamic properties also influence vasopressin levels as demonstrated in previous literature (G. L. Robertson, 1983; Zerbe, Henry, & Robertson, 1983). However, in order to produce a meaningful effect on vasopressin levels, blood volume changes of 20 to 30% are required as smaller fluctuations (~ 5 to 10%) have little to no effect (Robertson, 2013). In the current study there were no significant differences between HWI and LWI in change in plasma volume. The largest pairwise difference in change in plasma volume occurred at 1400 and was a mean difference of 5.1%. Therefore, any fluctuation in plasma volume likely had no effect on vasopressin levels in the current study.

Other factors potentially effecting vasopressin levels including nausea and stress were measured throughout the 11 hour protocol. There were no significant differences between trials in nausea (visual analog scale) or total mood disturbance (profile of mood state questionnaire), which incorporates stress and anxiety into its measurement. Additionally, in female participants menstrual cycle was controlled in the current study mitigating confounding sex hormone effects on osmoregulation. Therefore, these factors likely did not play a role in increases in copeptin observed during LWI. Another possible contributing factor of decreased copeptin in HWI was the physical act of drinking. Previous literature shows oropharyngeal receptor stimulation may

cause an acute transient suppression of vasopressin (Davison, Shiells, Philips, & Lindheimer, 1988; Geelen et al., 1984; Thompson, Burd, & Baylis, 1987). Considering the large volume of water consumed during HWI, this effect may have been continually stimulated throughout the 11 hour protocol.

Several previous studies indicate increased vasopressin/copeptin concentrations may result in elevated plasma glucose (Jansen et al., 2019; Spruce, McCulloch, et al., 1985). Similar studies manipulating water intake in order to increase copeptin have identified glucagon as a possible pathway contributing to increased glucose (Enhorning et al., 2017). This theory was developed in response to epidemiological studies indicating an association between water intake and glucagon (Lundegaard Asferg et al., 2019) as well as animal models indicating pancreatic V1b receptor involvement (Fujiwara et al., 2007; Koshimizu et al., 2012; Tanoue et al., 2004). These receptors are capable of stimulating glucagon secretion leading to an indirect increase in hepatic glucose production. In the current study there were no significant differences in glucagon concentrations between HWI and LWI. Perhaps elevated glucagon seen in previous studies does not occur acutely as was measured in the current study.

One alternative theory proposes increases in urine production due to increased water intake could result in higher total urinary glucose excretion. Due to the greater amount of glucose excreted by the kidney, this could result in lowered plasma glucose as water intake is increased. In the current study, there was no difference in total urinary glucose production between HWI and LWI. Concentration of urinary glucose was higher in LWI, but when adjusted for the significantly higher volume observed in HWI, the absolute value of urinary glucose excretion was similar between both trials.

Another proposed system potentially responsible for increased plasma glucose in previous studies involves the hypothalamic-pituitary-axis. AVP stimulates hypothalamic V1b receptors, thereby mediating secretion of ACTH from the anterior pituitary (Baertschi & Friedli, 1985). In turn, ACTH stimulates the release of glucocorticoids such as cortisol from the adrenal cortex leading to gluconeogenesis in hepatic tissue (Geer, Islam, & Buettner, 2014; Koshimizu et al., 2012). In the current study, there was a significant main effect of water intake on cortisol, which was significantly higher in LWI (grand mean: 71.92 $ng \cdot mL^{-1}$, 95% CI: 66.448, 77.395) as compared to HWI (grand mean: 61.046 $ng \cdot mL^{-1}$, 95% CI: 50.863, 71.228) ($P = 0.009$).

Regression analysis demonstrated that plasma osmolality was a significant predictor of cortisol in the current study. Mediation testing showed that the relationship between plasma osmolality and cortisol was partially mediated by copeptin. Considering plasma osmolality was a significant predictor of copeptin, this model supports analysis of variance results that water intake has a significant effect on cortisol concentration. However, this effect did not result in any differences in plasma glucose between trials. The differences in cortisol may partially be due to differences in copeptin, and therefore vasopressin, between trials. One previous study conducted by Johnson et al. also observed significantly increased cortisol concentration as a result of manipulated water intake. In this study, plasma glucose area under the curve was significantly higher in a hypohydrated trial as compared to a euhydrated trial. Additionally, the Matsuda index, an indication of whole body glucose disposal was reduced in the hypohydrated trial indicating increased gluconeogenesis. However, manipulation of water intake in this study occurred for three days prior to an oral glucose tolerance test as compared to the current study which examined acute effects of water intake (Johnson, Bardis, et al., 2017). These conflicting results may indicate the effect of water intake on glucose regulation does not occur acutely, but

instead manifests on a chronic basis. Although the current study was performed in participants defined as low drinkers, the amounts provided during LWI were lower than the average total fluid intake observed at baseline for each participant. Other studies observing a decrease in plasma glucose as a result of adequate hydration have utilized longer periods of water intake manipulation (Enhorning et al., 2018).

Congruent with results of the current study, acute hypohydration induced from fluid restriction and 1 hour of heat-tent exposure also had no effect on glycemic regulation (Carroll et al., 2018). Although in this study the use of temperature to induce hypohydration could have effected vasopressin levels independent of osmotic variation, perhaps the absence of glucoregulatory impairments was also due to the acute rather than chronic manipulation of water intake.

The strength of the current study is the acute level of control over water intake and diet to create a valid context to investigate glucose metabolism in different hydration states. To the author's knowledge no study in current literature has investigated these effects with participants remaining in a laboratory setting for the duration of data collection. Furthermore, the stringent recruiting criteria of low drinkers ensured enrollment of true "water responders" as evident by elevated baseline copeptin and urinary hydration measures. Despite these strengths, there are several limitations the author concedes. To begin with, this study is under powered considering a sample size of only 7. Results of this study should be interpreted understanding effect size in previous literature necessitates a sample size of 12 to provide sufficient power. The Sobel test used as a significance test of copeptin mediation between plasma osmolality and cortisol is significantly more liberal with lower sample size. However, the current study does provide information on the feasibility of the protocol as well as effect size estimations to guide future

research. Furthermore, future studies should include analysis of other variables, particularly gluconeogenic precursors to further explore the role of cortisol. Also, hepatocellular hydration state has been proposed as an alternative explanation for altered glucose metabolism in varying hydration states (Graf et al., 1988; Haussinger et al., 1994). The current study fails to provide a direct measure of this variable, which should be considered in future research.

CONCLUSIONS

In individuals identified as low drinkers defined by elevated copeptin and increased urine concentration, acute administration of adequate water intake successfully suppresses copeptin. When these individuals are provided the IOM recommended amount of total fluid intake (males: 3 $L \cdot d^{-1}$; females 2 $L \cdot d^{-1}$), hydration measures are significantly improved. Interestingly, plasma osmolality seems to respond acutely to increased water intake in low drinkers, which may explain the large rapid response of copeptin observed in previous studies.

Increased cortisol concentration as a result of vasopressin mediated ACTH secretion should be investigated further as a possible system explaining increased plasma glucose in previous studies. However, acute manipulation of water intake in low drinkers does not seem to effect glucose regulation. Although the literature is limited in randomized controlled trials investigating this effect, other acute interventions have shown similar results. Despite these results, chronic manipulation of water intake, especially in low drinkers, remains a potential contributory variable for lifestyle modifications in at risk individuals as demonstrated in previous literature.

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APPENDIX

ASU Knowledge Enterprise Development

APPROVAL FULL BOARD

Stavros Kavouras
Nutrition
(602) 827-2265
Stavros.Kavouras@asu.edu

Dear Stavros Kavouras:

On 7/9/2019 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Effect of low water intake on glucose regulation
Investigator:	Stavros Kavouras
IRB ID:	STUDY00010276
Funding:	None
Grant Title:	None
Grant ID:	None
Documents Reviewed:	• Form-Bioscience-Protocol - INDIGO.docx, Category: IRB Protocol; • CRF INDIGO.pdf, Category: Other (to reflect anything not captured above); • VAS thirst.pdf, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • Flyer INDIGO.pdf, Category: Recruitment Materials; • Consent form INDIGO.pdf, Category: Consent Form; • Health History Questionnaire INDIGO.pdf, Category: Screening forms;

The IRB approved the protocol from 6/19/2019 to 6/18/2020 inclusive. Before 6/18/2020, you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 6/18/2020 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc: Adam Seal
Abigail Colburn
Selin Aksoy
HyunGyu Suh
Adam Seal
Justin Huynh