ESTROGEN MODULATION

OF PHYSIOLOGICAL BODY FLUID CONTROL

DURING REPEATED SODIUM DEFICIENCY

By

EMILY EHRESMAN

Bachelor of Science in Biology
Oral Roberts University
Tulsa, Oklahoma
2015

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2021

ESTROGEN MODULATION OF

PHYSIOLOGICAL BODY FLUID CONTROL

DURING REPEATED SODIUM DEFICIENCY

Dissertation Approved:

Kathleen S. Curtis, PhD

Dissertation Adviser

Alexander J. Rouch, PhD

Randy S. Wymore, PhD

Jennifer L. Volberding, PhD

ACKNOWLEDGEMENTS

I cannot express the fullest extent of my thanks to my advisor, Dr. Kathleen Curtis. For the last four years, she has been a source of not only knowledge, but of support, assistance, and encouragement. Never have I felt like a burden as a student in her lab, but instead a valued colleague. I joined this lab feeling very unprepared, but I am leaving this lab feeling like a competent scientist. I also am thankful for the many times she and Dr. Tom Curtis played with my daughter so that I could complete experiments or check on rats. Without a supportive advisor, it would have been very difficult, if not impossible, to grow my family while also continuing my career in science.

I would also like to thank my committee, Drs. Kathleen Curtis, Alexander Rouch, Randy Wymore, and Jennifer Volberding. They have all been voices of reason and huge support during my time at OSU-CHS, especially in the face of COVID and pregnancy.

Thank you to Daniel Buck, Kelly McCracken for being wonderful teachers for techniques, helping me find needed equipment, and assisting me with sorting through data when I was lost. Without them, my research would not have been completed to the standard I have. Also thank you to Zinar Simsek, Stephanie Myers, and Amie Francis for various technical assistance, as well as letting me talk through issues. Thank you for also, at one time or another, holding my daughter so I could complete work.

I also could not have completed this research without the help of my family. My parents, Paul and Audrey Stanton, and grandparents, Tom and Chrissie Gray, provided

endless encouragement, support, and childcare. Especially during the pandemic, when childcare options were severely restricted, I was able to rely on them time and time again for help with my kids when I needed to be at school or writing. Special thanks to my dad for being the most frequent source of childcare, and my mom for directing me to this degree.

And finally, thank you to my children, Charlotte and Theodore Ehresman, for being sweet distractions when I needed a smile and the biggest blessings during otherwise stressful years of school. Also many thanks to my husband, Mark Ehresman, for always being present, keeping the children fed and happy, and bringing me ice cream when I needed a pick-me-up. Without the love and support, I could not have completed this dissertation.

Name: EMILY EHRESMAN

Date of Degree: MAY, 2021

Title of Study: ESTROGEN MODULATION OF PHYSIOLOGICAL BODY FLUID CONTROL DURING REPEATED SODIUM DEFICIENCY

Major Field: BIOMEDICAL SCIENCES

Abstract: Throughout much of life, women have lower blood pressures than men. However, after menopause, women's blood pressures surpass that of men, suggesting that estrogen plays a role in preventing hypertension. The low salt diet is a first line treatment for hypertension, but it is a difficult diet to maintain. Patients may alternate between periods of high and low salt intake, essentially "yo-yo" dieting. The effects of this are unknown, particularly in females. Additionally, it is unclear if any effects may be altered by the presence or absence of estrogen. Our goals were to determine the alterations in salt intake and body fluid hormones that occur with repeated dietary sodium deprivations. Using ovariectomized rats with (EB) and without (OIL) estrogen treatment, we subjected rats to one or two dietary sodium deprivations using low salt laboratory chow. 0.5 M NaCl and water intakes were recorded after each period of regular chow or deprivation. After deprivation, rats were sacrificed, and trunk blood was collected for analysis of vasopressin, norepinephrine, epinephrine, and aldosterone content. Plasma sodium concentration, plasma protein concentration, body weight, and uterine weight were also measured. There was no difference in the salt intakes of OIL- or EB-treated rats after one or two dietary sodium deprivations. However, EB-treated rats drank a less concentrated solution overall, suggesting less overcompensation after dietary sodium deprivation. Additionally, after a single episode of dietary sodium deprivation, EB-treated rats' consumption remained elevated above baseline even after returning to regular laboratory chow. These behavioral differences were not explained by alterations in vasopressin, norepinephrine, epinephrine, or aldosterone. Plasma sodium and plasma protein concentrations also did not show alterations related to the change in behavior. Further research is necessary to determine the mechanism behind these changes in intake in EB-treated rats, which may ultimately be clinically relevant for both pre- and postmenopausal women on the low salt diet.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Diagnoses of hypertension have increased over time, contributing to health consequences such as heart failure and renal injury (Lim et al., 2012). It is estimated that by 2030, hypertension will have grown 7.2% from the 2013 estimates (Go et al., 2013). It has become clear that blood pressure and hypertension are sexually dimorphic. While men have higher blood pressure throughout much of life, women's blood pressure increases after menopause (Reckelhoff, 2001; August & Oparil et al., 1999). Inexplicably, research into the mechanisms for these sex differences is lacking, despite the fact that women are at a greater risk of hypertension-related cardiovascular problems (Rosamond, 2007). The loss of ovarian hormones after menopause suggests estrogen may have a protective role against cardiovascular events, including hypertension. The mechanisms behind this remain to be determined. Given the aging population, better understanding of the control of blood pressure is critical to address this health problem.

Typically, hypertension is first treated with lifestyle changes, including adhering to a low salt diet. For some "salt-sensitive" individuals, blood pressure can fluctuate in relation to their salt intake (Weinberger et al., 1986). However, the low salt diet is difficult to maintain. Thus, patients may alternate between periods of high and low salt consumption, essentially "yo-yo" dieting. Home health visits, public education, and support of family members have limited success in increasing compliance to the diet (Cohen et al., 1991; Pietenin et al., 1984; Staessen et al., 1988). We are not aware of any studies that examined the possibility of long-term effects of yo-yo salt dieting, as could

occur in noncompliant human patients.

In contrast, a wealth of information about sodium depletion is available from animal studies, particularly about the behavioral consequences of sodium depletion. Repeated pharmacological treatments that deplete body sodium change the drinking behavior of rats, such that male rats drink increasingly more salt both during and between repeated furosemide-induced depletions, despite no greater sodium loss (Sakai et al., 1989). Similar effects in male rats have been seen using transperitoneal dialysis (Falk et al., 1966). Female rats tend to have higher salt intake than males in baseline conditions (i.e. with no manipulations of body sodium; Leshem et al., 2004), which persists in the period between multiple depletions.

However, the reasons for these behavioral differences are unclear. Sex-related changes in various body fluid hormones may be responsible. For example, it is known that vasopressin, an important hormone for controlling sodium and water balance via the kidneys, is influenced by salt intake (Ludwig et al., 1996), and may also play a role in salt appetite (Flynn 2002). The catecholamines epinephrine and norepinephrine are involved in vasoconstriction, kidney-mediated fluid balance, and are also altered by salt intake (Romoff et al., 1979). Additionally, aldosterone, which also affects the retention of salt in the kidneys, is increased by sodium depletion and decreased by salt consumption (Hall, 2015), but aldosterone is unchanged by multiple depletions despite increasing salt intake (Sakai et al., 1989). High doses of aldosterone also have been shown to increase salt intake in rats (Rice & Richter, 1943).

The difference in salt intake in males and females may be explained by these hormones, but the effects of estrogen on them require further study. Studies show conflicting results, with reports of an increase in baseline vasopressin with estrogen (Skowsky et al., 1979), or no change (Crofton et al., 1985). Similarly, norepinephrine's effects are reported to be augmented by estradiol treatment (Colucci et al., 1982), but acute injection of estradiol has no effect on the response to phenylephrine

(He et al., 1998). Estrogen treatment decreases aldosterone levels in the zona glomerulosa (Macova et al., 2008), but studies in humans have shown increases (Crane and Harris, 1969) or no change (Seely et al., 1999) in aldosterone with estrogen treatment. Any of these hormones may be playing a role in the observed sex differences in salt intake and the possible consequences for blood pressure control, but conflicting findings complicate our understanding and should be explored further.

Despite the knowledge gained from research in this field, there are still gaps in the literature. At present, the effects of estrogen on behavioral and physiological responses to repeated sodium deficiencies has yet to be elucidated. With few exceptions, studies examining repeated depletions have used only males. Studies that use both males and females are conflicted as to whether it is estrogen or testosterone responsible for the sex differences in intakes, with both testosterone (Chow et al., 1992) and estrogen (Scheidler et al., 1994) treatments causing decreases in stimulated salt intakes. More critically, few studies have used solely a low salt diet to induce sodium deficiency, as most use furosemide depletions (Sakai et al., 1987; Sakai et al., 1989; Leshem et al., 2004; Na et al., 2007) or a combination of furosemide and sodium deficient chow (Chow et al., 1992; Roitman et al., 2002). These studies focused on the behavioral consequences; thus, it is yet to be established whether vasopressin, norepinephrine, epinephrine, or aldosterone may be responsible for the observed sex differences in the responses to repeated deprivations.

Thus, we hypothesized that estrogen alters behavioral and hormonal responses during repeated episodes of NaD. This dissertation tested this hypothesis, addressing these specific aims:

Aim 1: Behavioral effects of repeated dietary sodium deficiency (rNaD) on female rats with and without estrogen. I will analyze salt and fluid intake to test the hypothesis that estrogen status changes compensatory behavioral responses to dietary sodium deficiency during repeated episodes of sodium deficiency.

Aim 2: Physiological effects of repeated dietary sodium deficiency (rNaD) on female rats with and without estrogen. I will use ELISA to measure plasma levels of vasopressin, catecholamines, and aldosterone, as well as using an ion selective electrode to measure plasma sodium, to test the hypothesis that estrogen status alters hormone concentrations and plasma sodium levels during repeated sodium deficiency.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Overview: Hypertension & Low Salt Diet

2.1.1 Hypertension

Hypertension has been increasing in recent years. 36% of Americans currently have hypertension, defined by the American Heart Association as blood pressure in excess of 130/80 (Whelton et al., 2018). In 2010, hypertension was listed as the leading cause of death and "disability-adjusted life years," or the number of years of life lost due to disability (Lim et al., 2012). Research from the American Heart Association predicts that by 2030 hypertension prevalence will increase 7.2% from the 2013 estimates (Go et al., 2013). Chronically elevated blood pressure can put a strain on numerous physiological systems. Patients with hypertension are more likely to have strokes, heart attacks, kidney injury, and other health consequences (Kotchen, 2018). Additionally, those who have high blood pressure are more likely to have comorbidities such as heart failure, cardiovascular disease, renal injury, diabetes, and obesity (Kotchen, 2018; Lim et al., 2012). Overall, it is estimated that hypertensive patients account for $131 billion more in healthcare costs than non-hypertensive patients (Kirkland et al., 2018).

While more research is necessary to address the public health crisis of hypertension, it is well known that blood pressure is sexually dimorphic (Crofton and Share, 1997; Khoury et al., 1992; Wiinberg et al., 1995). However, many of the studies performed on blood pressure and body fluid hormones have only included males (Cowley et al., 1981; Esler et al., 1977; Mohring

et al., 1977; Sakai et al., 1986; Sakai et al., 1987; Spielman and Davis, 1974). Regardless, research that has been performed on both sexes has provided valuable information. Before puberty, blood pressures in males and females are similar (Jackson et al., 2007; Rowland and Roberts, 1982). Throughout much of life, women have lower 24-hour ambulatory blood pressures than men (Khoury et al., 1992; Wiinberg et al., 1995); however, women's blood pressure increases after menopause, eventually surpassing men's (August & Oparil, 1999; Reckelhoff, 2001). A large cross-sectional study found that older women had significantly higher systolic and diastolic blood pressures regardless of smoking status, which was a possible confounding factor (Zanchetti, 2005). This study also stratified participants by age to reduce the blood pressure differences attributable to aging alone and found that while age influenced blood pressure independent of other factors, menopause was also an independent risk factor for increased blood pressure. Studies vary on the reported age at which women's blood pressure surpasses that of men, probably due to the varying age range at which individual women experience and complete menopause. Some report that age-matched men have higher 24-hour mean blood pressures until age 70 (Wiinberg et al., 1995), while others find that after 50 the incidence of hypertension increases in women (Hay et al., 2016). As shown, research has increasingly turned to studying hypertension in women, but this is still an area lacking in important research. It is imperative to expand this field, especially due to the increased risk of hypertension-related cardiovascular events in postmenopausal women compared to age-matched premenopausal women (Rosamond et al., 2007).

Sexual dimorphism related to hypertension is evident in animal studies as well. Multiple studies in different rat strains have shown higher blood pressures in males than in females (Crofton et al., 1993; Hinojosa-Laborde et al., 2000; Masubuchi et al., 1982). Research has pointed to estrogen playing a role in this dimorphism, but conflicting findings of the effect of estrogen have been reported. For example, when estrogen levels fall after menopause, blood

pressure increases (Staessen et al., 1989). Conflicting evidence for the use of hormone replacement therapy in postmenopausal women to decrease blood pressure has been reported. Some studies have found no benefit of oral hormone replacement therapy (Akkad et al., 1997), or a benefit only at certain times of day (Seely et al., 1999), while animal studies have found that administration of estrogen reverses hypertension (Crofton and Share, 1997). Clearly, estrogen's influence on many areas involved in body fluid control remains to be fully examined.

2.1.2 Salt Sensitivity

Treatment for hypertension includes medications such as thiazides, calcium channel blockers, and ACE inhibitors (James et al., 2014). However, lifestyle changes have increasingly been shown to provide benefit for many patients. These changes include exercising, losing weight, and decreasing salt intake. Salt consumption was first theorized to be a possible contributing factor to some health conditions in 1901 (DiNicolantonio and O'Keefe, 2017). As summarized in He and MacGregor's review (2002), in 1904, Ambard and Beaujard were the first to show a reduction in blood pressure through salt restriction in kidney disease patients. But in 1948, Kempner was the first to show a benefit of reducing salt specifically in hypertensive patients, though the diet proposed a salt restriction far beyond today's suggested amounts (Kempner, 1948). Since then, many studies have supported moderate reduction of dietary sodium for improving the blood pressure of the general population, even in normotensive patients (for review, see He and MacGregor 2010). One particularly successful intervention study performed in Portugal had participants lower salt intake but left the amount up to participants, and ultimately saw a 47% reduction in salt intake and a decrease in systolic blood pressure of 5.0 mmHg and in diastolic blood pressure of 5.1 mmHg after 2 years (Forte et al., 1989). Currently, JNC 8, the blood pressure management consensus guidelines, recommends that patients aiming for the "low salt diet" consume at most 2400 mg/day, but ideally even less (Arnette et al., 2019). Despite these

studies, Americans consume far more than the recommended amount of salt. The average American over the age of 2 consumes 3400 mg/day of salt ("What We Eat in America").

Despite the consequences of hypertension, many patients do not have it controlled. The CDC reported that, in 2015-2016, 30.2% of adults over 20 years of age had hypertension, and 59.7% of those patients were uncontrolled regardless of which intervention was employed (Fryar et al., 2017). For the low salt diet, controlling hypertension is particularly problematic. Patients advised to adhere to a low salt diet will often alternate between low and high consumptions of salt, essentially "yo-yo" salt dieting. This struggle with compliance has been shown in many intervention studies. For example, the North Karelia Project in Finland, which aimed to lower salt intake in the general population, used several methods including direct-to-patient nutrition education, press, and radio (Pietinen et al., 1984). A line of low salt products and a new mineral salt without sodium were released as well. After three years, the authors found that salt intake had not changed overall, and normotensive patients were more likely to have changed habits than hypertensive patients. Another intervention project used positive feedback through urine sodium monitoring to help their hypertensive subjects lower salt intake (Weinberger et al., 1988). Despite feedback, fewer than half of the patients were compliant with the diet by the authors' definition of a urinary sodium excretion of 80 mmol/day. Later studies recruited a household member to also go on the low salt diet, but this made no difference in compliance compared to instructing only the patient to change their diet (Cohen et al., 1991). These and many other studies indicate how difficult it can be for patients to maintain the low salt diet. Given how common such noncompliance is, it is crucial to understand the changes that happen with noncompliance in order to better educate patients and help healthcare providers make informed clinical decisions. Further research is necessary to determine what, if any, physiological or behavioral consequences occur with yo-yo salt dieting.

Though CDC recommends everyone lower their salt intake, some people are more sensitive to changes in dietary salt than others, a phenomenon termed salt sensitivity. However, results from studies on salt sensitivity vary widely depending on the duration of study and the amount of salt in the diets. Nevertheless, a meta-analysis of trials greater than a month in length supported a reduction in salt intake for both normotensive and hypertensive patients for the purpose of reducing blood pressure (He and MacGregor, 2002). This blood pressure reduction was predicted to reduce deaths from strokes and coronary events for both patient groups, which would also reduce healthcare costs and the burden on the healthcare system due to these complications.

While there are many studies on salt sensitivity in both humans and animals, the definition of salt sensitivity differs from study to study. For example, one paper defined it as more than a 10% increase in blood pressure when changing from a low salt diet to a high salt diet (Kawasaki *et al.* 1978), while another defined it as a decrease in 10 mm Hg in systolic blood pressure when salt depleted compared to salt loaded (Schulman et al., 2006). There are many other definitions, the discussion of which is beyond the scope of this dissertation. Researchers have struggled to determine the exact mechanisms of salt sensitivity. A study by Kawasaki *et al.* (1978) found that patients who were salt resistant excreted more sodium and gained less weight when on a high salt diet than salt sensitive patients did. However, what is clear is that salt sensitivity is sexually dimorphic.

The decrease in ovarian hormones that occurs during and after menopause does appear to be an independent risk factor for development of salt sensitivity, which may predispose women to develop hypertension. In support of this, Schulman *et al.* (2006) found that premenopausal women who underwent hysterectomy and ovariectomy were at risk of developing salt sensitivity four months post-surgery. In contrast, their baseline blood pressures did not increase in that time frame, indicating that the postmenopausal elevation takes longer than the time frame of this study.

However, it can take 5-20 years after menopause to develop elevations in blood pressure, so this result would not be surprising (Reckelhoff, 2001). Another study showed increased salt sensitivity in postmenopausal women compared to premenopausal women (Pechere-Bertschi, 2004). This study used dietary restriction, whereas the study by Schulman *et al.* used furosemide and saline for restriction and salt loading. With both methods, there is evidence that menopause and, therefore estrogen, may play a role in salt sensitivity. Additionally, animal studies have shown that salt sensitivity may increase in some rat strains after ovariectomy, as studies have found alterations in salt responsiveness in Dahl salt-sensitive rats (Zheng et al., 2008) and in spontaneously hypertensive rats (Fang et al., 2001).

While this sexual dimorphism is likely multifactorial, many theories have surfaced as to the causes. Reproductive steroid hormones like estrogen and testosterone are the primary suspects, with many studies attempting to identify how each affects blood pressure. Estrogen modulation of body fluid hormones likely occurs, but it is unclear what aspects of body fluid control are involved. The mechanisms of sex-related changes in animals or humans have yet to be elucidated. My goal is to address these gaps in our knowledge by investigating how estrogen alters physiological control of body fluid regulation during repeated episodes of dietary sodium deprivation.

2.2 Integrative Body Fluid Regulation

2.2.1 Physiology of Body Fluid and Blood Pressure Control

At its simplest, blood pressure is a function of body fluid control. Research in body fluid control can give insights into clinical applications such as blood pressure. Osmolarity of body fluid is tightly controlled, as it is important for optimal cellular functioning. The body fluid compartments include the intracellular space—which mostly contains the ions potassium and

phosphate—as well as the extracellular space (G&H Ch 25). The extracellular space is further divided into the interstitial fluid and plasma. These compartments contain much of the sodium found in the body. Sodium is the most important ion in the extracellular fluid, as water will quickly follow it through osmotic movement. In order to maintain homeostasis of all of these spaces, input of fluid through eating and drinking must be the same as the output. The body is finely tuned to keep osmolarity relatively constant. Too low of sodium concentration, or hyponatremia, can result in consequences such as hyponatremic encephalopathy, which is caused by osmotic swelling of brain cells (Giulani and Peri, 2014). Severe hypernatremia also has poor outcomes, including seizures and coma (Lewis 2018).

In normal kidneys, a phenomenon called pressure-natriuresis helps increase sodium excretion from the kidneys when there is increased blood pressure or increased salt intake. As a person consumes salt, their blood pressure begins to rise due to increased extracellular fluid volume, and the kidneys respond by increasing sodium and water excretion according to the pressure-natriuresis curve. This helps return blood pressure to normal. A defect in pressure-natriuresis due to a kidney disease or damage may lead to hypertension (Guyton et al., 1972).

In addition to the kidneys, other components of physiology work in concert to alter osmolarity and keep it within a normal limit. However, minimal research has been performed to determine how these processes may be altered during repeated sodium deprivations, such as when "cheating" on the low salt diet.

2.2.2 Hormones of Body Fluid Regulation

2.2.2a Vasopressin

Vasopressin is mainly secreted by the paraventricular nucleus (PVN) and supraoptic nucleus (SON) in the hypothalamus, then carried via axonal transport to the posterior pituitary to be released into the blood (Sherlock et al., 1975). When released, it causes water retention in the

kidneys, which helps normalize osmolarity. Conversely, when osmolarity is too low, vasopressin is decreased so that water can be excreted through the urine. During normal circumstances, vasopressin is present in very low levels in circulation (Robertson et al., 1976). Plasma ADH and plasma osmolality are closely correlated (Robertson et al., 1976). Osmotic threshold, or the osmolarity at which vasopressin is released, varies between species and even between humans. Studies in the US, France, and England found a threshold of approximately 280 mosmol/kg H2O (Beardwell, 1971; Fressinaud et al., 1974; Robertson et al., 1976), while a study in Japan found a threshold of approximately 265 mosml/kg H2O (Shimamoto and Miyahara, 1976). Stimuli for vasopressin release include decreased blood volume, such as hemorrhage and dehydration, or increased osmolarity in the extracellular fluid (Dunn et al., 1973). The osmotic threshold may be altered by these stimuli, such that vasopressin is released at lower or higher thresholds than in a normal state (Dunn et al., 1973; Robertson et al., 1976).

It is known that osmoreceptors found in circumventricular organs, mainly the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and area postrema (AP) are responsible for sensing osmolarity (G&H). The SFO and OVLT are found in the hypothalamus, while the AP is found in the hindbrain. When osmoreceptors sense increased osmolarity, a signal is sent to the SON and PVN via glutaminergic neurons to release vasopressin (Honda et al., 1992). Vasopressin then uses V2 receptors to influence the kidneys to retain water in the distal tubules and collecting ducts in order to bring osmolarity to normal, as well as the V1 receptors to increase blood pressure. During a hemorrhage, vasopressin can be responsible for increasing blood pressure as much as 60 mmHg (G&H). Vasopressin also plays a role in increasing water intake, further bringing osmolarity back to normal (Szczepańska-Sadowska et al., 1982).

Vasopressin is also influenced by the sympathetic nervous system, as decreased stretch of baroreceptors due to a drop in blood pressure also stimulates release of vasopressin. Normally, baroreceptor feedback acts to inhibit vasopressin release (Berl et al., 1974). Other studies have

found that stimulation of alpha receptors results in suppression of vasopressin (Liberman et al., 1970), while signals through the baroreceptors of the carotid sinus are necessary for blood pressure-mediated changes in vasopressin release (Berl et al., 1974). Furthermore, vasopressin acts centrally to affect the sympathetic nervous system and control blood pressure. This likely occurs through the CVOs, particularly the area postrema, which has connections to areas involved in body fluid control, such as the nucleus tractus solitarius (NTS). The presence of vasopressin also leads to a reflex sympathoinhibition (Hasser 2000). Sympathoinhibition causes a leftward shift in the baroreflex curve, meaning that drops in blood pressure cause less sympathetic activation than it would at lower levels of vasopressin. Administration of a V1 antagonist reduced the reflex sympathoinhibition caused by circulating vasopressin (Imai et al., 1983). This effect is probably mediated through the NTS pathway, since blockade of α_2 receptors abolishes sympathoinhibition.

The level of salt intake can alter vasopressin release. During periods of low salt intake, vasopressin is suppressed due to low osmolarity (G&H). Conversely, with high salt intake comes an increase in osmolarity, which will trigger a release of vasopressin into circulation in order to retain water in the kidneys and maintain fluid homeostasis (Cowley et al., 1981). However, it is unclear how long vasopressin remains elevated, as normotensive subjects may have normal plasma vasopressin despite elevated salt intake and plasma sodium concentration (Cowley et al., 1981). This may indicate that vasopressin fell quickly after salt loading. This study also showed that only hypertensive subjects had increased plasma vasopressin when sodium intake increased, though this relationship appeared only after age 50. A comparison was not reported between normotensive subjects of different ages, so this relationship may also be present in this group of subjects. There is also evidence that the baroreceptor inhibition of vasopressin through $GABA_A$ receptors is decreased by high salt intake, which may explain some cases of high vasopressin in hypertensive subjects if they are consuming large amounts of salt (Kim 2011)

Conversely, it has been suggested that vasopressin may also influence salt intake. Since vasopressin increases water intake, concurrent increases in salt intake would help prevent hypo-osmolarity. Intracerebroventricular administration of a V1 receptor antagonist decreased salt intake in rats which had been deprived of salt using furosemide and DOCA (Flynn et al., 2002). Their results are in disagreement with previous studies, which had reported vasopressin itself decreased salt intake (Sato et al., 1997); however, intracerebroventricular vasopressin causes seizure-like activity, which in Flynn's study corresponded with decreases in intake of both salt and sucrose. Other models also show evidence of vasopressin influence on intake. For example, adrenalectomized rats have elevated hypothalamic vasopressin, which may in part be influencing the large salt intakes observed in these animals (Franco-Bourland, 1998), though it would be expected to decrease intake given the results of Flynn *et al.* (2002). The differences may be due to differences in adrenalectomy and furosemide models, which stimulate salt through different mechanisms. Though much is known that vasopressin plays a role in body fluid control, the effects of vasopressin on repeated sodium intake has yet to be demonstrated. While studies have explored the effects of single deprivations, it is unclear if repeated sodium deprivations may alter levels of vasopressin over time. We are not aware of any research that has studied how vasopressin might be altered by repeated episodes of sodium deprivation.

2.2.2b Norepinephrine and Epinephrine

Catecholamines are another important hormone in body fluid control. The catecholamines norepinephrine and epinephrine play an important role in maintenance of blood pressure through the baroreflex arc. When blood pressure drops, signals are sent to the NTS, resulting in disinhibition of excitatory signals from the rostral ventrolateral medulla (RVLM). The RVLM sends excitatory signals to preganglionic sympathetic neurons in the interomediolateral cell column (IML) in the spinal cord. All of these areas in the brain and others, including the CVLM, IVLM, and locus ceruleus stain positive for enzymes involved in

catecholamine synthesis, as well as are fos positive during hypotension (Dampney et al., 2003). The preganglionic neurons release acetylcholine, while the postganglionic neurons are mostly adrenergic, secreting norepinephrine. Varicosities on the nerve fibers contain vesicles of norepinephrine, which is released to bind to alpha or beta receptors on the effector organ, such as the heart or blood vessels. The sympathetic receptors activate second messengers via these receptors to either excite or inhibit the cell. Within the cardiovascular system, alpha receptors are important for vasoconstriction of vessels, while beta-1 receptors increase heart rate and contractility.

The sympathetic system is always activated at a certain "tone" so that the tone can be increased or decreased as necessary to maintain homeostasis. The tone is due to a basal rate of epinephrine and norepinephrine release from the adrenal medulla, and may be responsible for some cases of hypertension. The sympathetic nervous system can experience "resetting" after long term increases in tone, where a higher sympathetic tone will be defended as a new baseline rather than attempting to bring it back down to the previous baseline. This resetting causes a new level of baseline epinephrine and norepinephrine to be released, which may ultimately result in hypertension. This has been backed up by evidence that hypertensive patients have decreased baroreflex sensitivity (Esler et al., 1977), indicating the normally inhibitory input from the baroreceptor is not responding to the increased pressures of hypertension.

Catecholamines may be influenced by salt intake. Prior research shows that plasma and urinary catecholamines increase with decreasing salt amounts in the diet (Luft et al., 1979; Romoff et al., 1979). On the other hand, other studies have shown no change in sympathetic nerve activity with the high salt diet (McBryde et al., 2009)., but these may not have taken into account the variability of individual response to salt Additionally, there are limitations to all methods of measuring sympathetic activity. Measuring sympathetic nerve activity can produce different results depending on the area measured, while measuring plasma catecholamines does

not provide measurement of minute by minute changes. Regardless of the method employed, past research suggests that sympathetic activity is altered by salt intake.

Salt consumption may also influence body fluid mechanics by altering the baroreflex response of the sympathetic nervous system. A study in salt-loaded dogs showed an increase in the pressor response to carotid occlusion (Brum et al., 1991). That lab saw the opposite effect in dogs deprived of salt, where sodium depletion blunted pressor response (Brum et al., 1991). This response was diminished by removal of vagal afferent, indicating this may be due to changes in baroreceptor sensitivity. Evidence points to a role for the sympathetic nervous system in salt sensitive patients as well. One study (Campese et al., 1982) found that patients who were salt sensitive did not have lowered norepinephrine when on a high salt (200 mEq) diet. The sympathetic nervous system and catecholamines may also play a role in salt sensitivity, as transection of the renal sympathetic nerves results in lower blood pressure in rats given deoxycorticosterone, which is a model for salt sensitivity (Jacob et al., 2005). A meta-analysis found that there was an overall increase in both norepinephrine and epinephrine in patients on a low salt diet compared to a high salt diet (Graudal et al., 2012). While these hormones are useful, it is difficult to determine the exact level of activation of the sympathetic nervous system, since much of the hormone action occurs within the neurons, target cells, and neuronal junctions. Regardless of the mechanism, it is clear that changes to sodium intake can cause alterations in the sympathetic nervous system which may affect body fluid regulation, including in salt sensitivity. But more research is to be done to determine if alterations in catecholamines are different with repeated sodium deprivations..

2.2.2c Aldosterone

The final hormone to be discussed is aldosterone. Release of aldosterone from the adrenal glands is stimulated by decreased blood pressure, which leads to an increase in renal sympathetic

activity. This activity causes a release of renin, which increases production of angiotensin II and aldosterone (G&H). Increased aldosterone promotes reabsorption of sodium and water from urine. Aldosterone also has important interconnections to other body fluid control systems, as central mineralocorticoid receptors are found in circumventricular organs and the paraventricular nucleus. These can bind aldosterone, which then increases vasopressin and central sympathetic tone (Gabor and Leenen, 2013). Additionally, some aldosterone is likely made in the brain, as one study found that adrenalectomy did not completely eliminate aldosterone presence in the brain after seven days (Gomez-Sanchez et al., 2005).

Several studies suggest that aldosterone increases salt intake. Administration of aldosterone or the precursor deoxycorticosterone acetate (DOCA) increases salt intake in sodium replete rats (Rice and Richter, 1943; Fluharty & Epstein, 1983; Wolf, 1965). This increase is dose-dependent (Wolf, 1965). Blocking aldosterone alone lowers salt intake (Sakai et al., 1986), but salt intake is virtually eliminated when both aldosterone and angiotensin II are antagonized (Sakai et al., 1986; Sakai et al., 1987), meaning that one alone is not responsible for stimulated salt intake. It has been theorized that both hormones work together to increase salt intake during periods of deficiency (Krause and Sakai, 2007). Aldosterone is one of the few hormones that has been studied during repeated sodium deprivations, but it has not been shown to be altered with repeated deprivations (Sakai et al., 1989). However, whether estrogen changes this result has yet to be determined.

2.2.3 Salt Intake & Water Intake in Animal Models

Regardless of the physiological data showing harmful effects of excess salt, animals—including both humans and rats—need salt in order to sustain life. Salt is required for growth and bone mineralization (Geerling & Loewy, 2008), as well as maintaining normal osmolality of plasma for optimal cellular functioning. Fluid volume and body homeostasis is also involved in

maintaining blood pressure. Without salt, a person will experience nausea, cramps, fatigue, and appetite loss, among other symptoms. However, there are clearly consequences to excessive intake. Therefore, research into the behavior of sodium appetite has provided valuable information. There have been numerous studies investigating unstimulated (i.e. baseline), stimulated, and "need-free" (i.e. post-deprivation) salt intakes.

Stimulated intake, or the intake in the period immediately after sodium deprivation, is also increased by repeated sodium deprivations (Chow et al., 1992; Falk 1966; Na 2007; Sakai 1986; Sakai 1987; Sakai 1989). When analyzing studies on stimulated salt appetite, it is necessary to consider the particular model used. Dietary deprivation is a technically easy method but short time periods on the diet do not elicit a salt appetite, which can confuse interpretations of data (Stricker 1991; Wolf 1982). Adrenalectomy, which eliminates endogenous aldosterone, is another method of stimulating salt intake. This causes the kidneys to excrete excessive salt, and the rats drink large amounts of salt to compensate for the loss. Adrenalectomized rats given no salt replacement in their diet die (Richter, 1936). If given exogenous aldosterone, however, they stop drinking salt (Wolf, 1965). This method is arguably most effective for specifically studying the renin-angiotensin-aldosterone system. Some studies use polyethylene glycol (PEG), which is a hyperoncotic colloid that draws water into other spaces. It causes a gradual local edema that does not have an impact on blood pressure unless combined with another method like sodium deficient chow (Stricker, 1981). Other studies use DOCA, which acts as a hormonal stimulus that signals to the body that there is a sodium deficiency in the absence of a true shortage. Finally, some studies use furosemide, which leads to an increase in the excretion of salt and water by the kidneys. Many studies dealing with furosemide deplete the animals for one day out of a week (Leshem et al., 2004; Sakai et al., 1986), which, while helpful for determining immediate depletion effects, is not similar enough to a long term low salt diet or long term diuretic use. Some studies also combine two or more methods, usually dietary sodium restriction and one other, to produce a

more robust salt intake. While all of these methods can provide useful information and insight into the physiology of body fluid balance, dietary sodium deprivation alone needs to be studied further due to its clinical implications for patients on the low salt diet.

In rats, the salt intake appears to also be influenced by prior episodes of sodium deprivation, as some studies have seen the post-deprivation unstimulated intake increase above baseline between deprivation periods (Chow et al., 1992; Leshem et al., 2004; Na et al., 2007; Sakai et al., 1989). This enhancement could not be explained by excess natriuresis, as urinary sodium concentration was normal, nor by hyponatremia on later depletions, since the plasma sodium concentration was also normal. This effect was also long-lasting, as the enhancement in intake amounts still occurred even when there were four months between depletions (Sakai et al., 1989). This phenomenon does not appear to be due to learning, as rats given a bolus of 3% saline into the stomach during their first dialysis still drink an increased amount of salt on their second (Falk, 1965). Research has also found rats more salt than was necessary to replenish their sodium levels, which the authors suggested was due to a delay on ingested fluids correcting sodium balance, or due to a delay in the signals to stop drinking (Falk, 1966). However, this effect has only been studied in furosemide and intraperitoneal dialysis, not a physiological model like dietary sodium deprivation. This could have clinical implications in patients on the low salt diet, but more research is needed to understand the mechanisms behind the responses to multiple depletions, since prior research has not pointed to a clear cause.

2.3 Estrogen & Body Fluid Regulation

2.3.1 Mechanism of Estrogen Actions

Estrogen has long been known to function as a steroid hormone, affecting the genome by binding to nuclear receptors and influencing gene transcription (Heldring et al., 2007). Initially,

only one estrogen receptor was known, described as an estrogen binding protein by Elwood Jensen in the 1960s (as summarized in Barton et al., 2018). This estrogen receptor (ER) was first sequenced and clone in the 1980s (Greene et al., 1986; Walter et al., 1985). In 1996, two studies simultaneously characterized a novel nuclear estrogen receptor, which became known as estrogen receptor beta (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996). Both ERβ and the previously known receptor, ERα, work through the classical or ERE pathway. When estrogen binds these receptors, they form dimers that bind to an estrogen response element (ERE), which is a section of DNA for ER binding. This is found in the promoter region of a target gene and will induce or inhibit transcription (Kushner et al., 2000). The ERs may alter transcription by recruiting activators or inhibitors. ERs may also function through the AP-1 pathway, where they interact with protein complexes in the fos/jun family and change transcription or cell activity. This pathway does not require DNA binding, and the ERs do not form a dimer (Paech et al., 1997).

Additionally, a G-coupled protein receptor, called GPER, was recently discovered (Filardo et al., 2007). It was first found in uterine and liver plasma membrane fractions in 1977 (Pietras and Szego, 1977), but was not cloned until the 1990s (Bonini 1997; Carmeci 1997; Owman et al., 1996). This membrane-associated receptor may be responsible for some of the rapid nongenomic effects observed in the presence of estrogen, since nuclear receptors act much more slowly. Nongenomic actions likely occur through protein-kinase cascades, such as adenylate cyclase, MAP kinase or PI3-kinase (Björnström and Sjöberg, 2005). One particular study (Filardo et al., 2007) found that GPER was associated with increased cAMP which led to downstream effects. The protein kinase pathways may also alter responses to neurotransmitters by altering the responsiveness of potassium and calcium channels in neurons (Malyala et al., 2005). Actions through plasma receptors like GPER may also indirectly lead to genomic effects by altering transcription factors such as c-fos (Albanito et al., 2007), which can then alter gene transcription as previously described (Björnström and Sjöberg, 2005).

Estrogen plays an important role in body fluid regulation, likely through both nuclear and plasma membrane receptors. Both during pregnancy and during normal menstrual cycles, body fluid balance changes drastically (for review, see Summy-Long and Kadekaro, 2002). Estrogen receptors are found throughout areas important to body fluid regulation, including the vessels, hypothalamus, and kidneys (Santollo and Daniels 2015; Sladek and Somponpun 2008). GPER is found in human blood vessels and plays a role in blood pressure (Haas et al., 2009). While it is likely that estrogen causes changes in body fluid regulation.

2.3.2 Estrogen Effects on Physiology of Body Fluid & Blood Pressure Control

As previously mentioned, throughout much of life, women have lower blood pressures than men. This may be due, at least in part, to the presence of estrogen. Estrogens have been found to increase vasodilators such as NO (Weiner et al., 1994). In fact, research has suggested that lack of modulation of the RAAS and NO system in postmenopause can lead to an increase in salt sensitivity, as summarized in Pilic *et al.* (2016). Pressure-natriuresis is also affected by estrogen, as females show a leftward shift in the pressure-natriuresis curve. Compared to males, they excrete more sodium at the same blood pressure (Hilliard et al., 2011). More research is necessary to determine the effects estrogen has on the physiology of body fluid balance, but growing research points to alterations in body fluid hormones.

2.3.3 Estrogen Effects on Hormones of Body Fluid Regulation

2.3.3a Vasopressin

While it appears that vasopressin is altered by estrogen, there is much disagreement among studies about estrogen's effects on vasopressin. In postmenopausal women and in ovariectomized rats, estradiol replacement increases vasopressin (Bossmar et al., 1995; Skowsky et al., 1979). Additionally, the threshold for sodium-induced vasopressin release appears to be reduced by estrogen (Ota et al., 1994), perhaps to defend low plasma sodium and high blood

volume for pregnancy and lactation. On the other hand, some research has shown decreases in vasopressin with high doses of estrogen and increases with low doses, which would point to the dosage of estrogen being at least partially responsible for differences in results between studies (Peysner & Forsling, 1990). Other studies have found no change in vasopressin related to the estrus cycle, nor when ovariectomized females were given supraphysiological doses of estrogen, even though ovariectomy increased plasma vasopressin (Crofton et al., 1985). This study also found that males had increased vasopressin compared to females, though it was unclear whether this was due to differences in renal clearance of vasopressin, sex steroids, or some other factor. However, other research only found estrogen-related differences in vasopressin during dehydration (Wang et al., 1996). Sladek *et al.* (2000) found that osmotically stimulated release of vasopressin was blocked by estradiol and decreased mRNA, but this study was done in explants and does not give the full picture of what is happening in a whole animal. All of these varying results may be due to methodological differences between studies, perhaps in the dosage or delivery method of estrogen. It is clear that more research needs to be done in this area.

Regardless of the direction of change, the literature does point to possible mechanisms for how estrogen may alter vasopressin. Some studies have theorized estrogen may alter vasopressin release and action through both genomic and nongenomic effects. It is known that ERβ is found in the supraoptic nucleus (SON) and paraventricular nucleus (PVN), areas involved in vasopressin release (Sladek et al., 2000). Wang *et al.* found that *in vitro* treatment of brain slices with 17β-estradiol induced exocytosis of vasopressin within 5 minutes, much faster than to be expected from genomic effects (1995). Given this information, estrogen may be working through nongenomic effects, such as G-coupled receptors, tyrosine phosphorylation, or interaction with other receptors like $GABA_A$ or glutamate, which would occur more quickly than classic estrogen receptors (Sladek et al., 2000).

Despite what is known about vasopressin, both with and without estrogen, we are not aware of any research investigating how repeated sodium deprivations affects vasopressin, and how estrogen may alter any response. This is an area that remains to be explored.

2.3.3b Norepinephrine and Epinephrine

Catecholamines are also altered by estrogen, but studies are conflicted as to how. Studies in brain tissue have shown estrogen-induced increases (Serova et al., 2002) and decreases (Arbogast and Hyde, 2000) of the mRNA for tyrosine hydroxylase, one enzyme in the biosynthetic pathway for catecholamines. A different study found no difference in levels of TH in the adrenal medulla (Macova *et al,,* 2008). Differences in studies may be due to age of animals, dosage of estradiol, or other methodological factors. However, tyrosine hydroxylase is also involved in dopamine synthesis, and therefore may not be directly indicative of levels of catecholamines such as norepinephrine and epinephrine, which play a more direct role in body fluid control. Unstimulated levels of norepinephrine are increased by estradiol treatment of bovine adrenal medullary cells (Yanagihara et al., 2006), but this may not be representative of what occurs in the body, nor what occurs during a challenge such as a low salt diet. It is also possible estrogen may modulate norepinephrine or epinephrine's effects at the organ level. Acute injection of estradiol has no effect on sympathetic response to phenylephrine, but chronic injection increases baroreflex sensitivity, in that sympathetic nerve activity still increased in response to increases in blood pressure despite a lower baseline (He et al., 1998). Some studies found the vasoconstrictor effect of norepinephrine increases with estradiol treatment (Colucci et al., 1982). While it seems contradictory to other studies which show decreases in components of the sympathetic nervous system, increased responsiveness to catecholamines could play a role in maintaining blood pressure while catecholamine levels are low. As can be seen, the research surrounding estrogen and catecholamines is conflicting. In addition, we are not aware of any studies relating repeated episodes of sodium deprivation to catecholamines and estrogen.

2.3.3c Aldosterone

Aldosterone is also known to be altered by estrogen. Aldosterone has been found to be higher in men than women (Miller et al., 1999). In animals, aldosterone secretion is increased after ovariectomy and blocked by estrogen replacement (Roesch et al., 2000). Peripherally, estrogen likely acts synergistically through the ERβ and GPER receptors, which are inhibitory and stimulatory, respectively (Caroccia et al., 2014). These are both found in the zona glomerulosa, and siRNA knockdown of ERβ in adrenal tissue resulted in increases in levels of the gene for aldosterone synthase, while silencing the GPER gene decreased it (Caroccia et al., 2014). This is corroborated by evidence that aldosterone synthesis is reduced by estrogen (Macova et al., 2008; Roesch et al., 2000). Additionally, levels of renin and aldosterone fluctuate with the cycle (Fommei et al., 2016).

Estrogen is likely playing a role in regulating aldosterone in the brain as well, as siRNA knockdown of ERβ in the RVLM and PVN increases aldosterone-induced hypertension. This implies that estrogen may have a protective role against aldosterone-induced hypertension. In fact, giving agonists of ERα or ERβ to ovariectomized rats reduces aldosterone-induced hypertension (Xue et al., 2013). Additionally, reactive oxygen species production from aldosterone is increased in the SFO and PVN by knockdown of both ERα and ERβ (Xue et al., 2013).

Minimal research has been done on aldosterone's response to repeated sodium deprivations, and what has been done used furosemide-induced deprivations. Sakai *et al.* reported no differences in levels of aldosterone between males and females, despite sexually dimorphic behavioral responses to the deprivations (1989). This research is instructive, but needs to be confirmed in dietary sodium deprivations. Additionally the research of Sakai *et al.* did not directly evaluate the role of estrogen, as they only used intact male and female rats. Research

needs to be done using both intact and ovariectomized female rats to determine if any differences occur.

2.3.4 Estrogen Effects on Salt Intake

There is some evidence for a sex difference in salt intake. Rats are prandial drinkers; therefore, it is hard to determine what effect estrogen has on intake when water and food are both *ad libitum.* Because of this there is conflicting reports on effects of estrogen on intake in sodium replete animals. Early studies do suggest that spontaneous salt ingestion is affected by estrogen in both males and females (Fregly, 1973). Unstimulated salt intake has also been shown to vary with the rat's estrus cycle, with decreased intake during high plasma estrogen, as summarized in Antunes-Rodrigues, *et al.* (2013). Seminal research in the field found that the sexual dimorphism in salt intake appears after 60 days of age, which is around when sexual maturity occurs in rats (Křeček *et al,*, 1972). In this study, they found that females ultimately drink a more concentrated solution than males at greater volumes per body weight. One study found a decreasing chorda tympani nerve response to salt in male, oil-treated females, and then EB-treated female rats (Curtis and Contreras, 2006). This correlated with an increasing intake of salt, meaning that estrogen may affect the palatability of salt, and therefore influence intake.

A critical question is whether salt intake varies when a salt appetite is stimulated in estrogen-treated rats. Much of the previous literature has compared intact males and females, rather than gonadectomized, which can better discern between the effects of testosterone and estrogen. Various studies have reported that intact female rats drink more salt immediately after one deprivation than males (Sakai et al., 1989). Some authors suggested that the stimulated intake after one deprivation is no different, as females drink larger amounts more quickly before males catch up (Chow et al., 1992). However, it is important to note that this study may ultimately demonstrate that females drink more than males if the data were expressed as per body weight,

but the authors did not record body weights for that particular experiment. Confusion still exists, however, as other authors have found that males drink more than females in various models of deprivation that stimulate robust sodium appetite (Scheidler et al., 1994; Stricker et al., 1991). A number of questions regarding the timing of gonadal hormone treatment remain to be answered. Some research has suggested that sex steroids like estrogen and testosterone may have an organizational effect in the brain. Females ovariectomized at one day old increase stimulated salt intake as adults compared to intact females, while castrated males increase intake to levels similar to intact females (Chow et al., 1992). However, the effects later in life has been drawn into question, as neither ovariectomy nor orchiectomy at 100 days of age changed salt intake in this study (Chow et al., 1992). However, other research found that ovariectomy in adulthood increased female stimulated salt intake, and was decreased again by physiological doses of estrogen (Stricker et al., 1991). Furthermore, it may be that estrogen leads to increased salt intake in females as an adaptation for pregnancy (Curtis, 2015; Kensicki et al., 2002), as this is a state of increased blood volume during which fluctuations in osmolarity may occur. Therefore, it is possible estrogen may prime for these responses as a reproductive advantage.

In addition to the question of salt intake during baseline and stimulated periods, an arguably more important question is whether salt intake after exposure to sodium deprivation is altered by estrogen. Most of the research regarding the consequences of exposure to sodium deprivation has been done in males (Falk et al., 1966; Na et al., 2007; Roitman et al., 2002; Sakai et al., 1986; Sakai et al., 1987). Additionally, research in this area has been limited to furosemide-induced deprivation and intraperitoneal dialysis, which, while instructive, does not provide the full picture for clinical application such as the low salt diet in humans. However, these studies still provide valuable information. In the time periods after deprivation, female rats show increased salt intake above that of male rats and above their baseline prior to deprivation (Chow et al., 1992; Sakai et al., 1989). In particular, unstimulated intake increased after successive

depletions for both males and females, but was 3% higher in females (Sakai et al., 1989). This indicates a possibility of a longer lasting change in sodium appetite, perhaps due to estrogen presence in the females. The differences in intake were not explained by net urinary sodium loss, nor by plasma angiotensin II or aldosterone levels, both of which rose after the first deprivation and did not increase further, as seen in other studies using only males (Sakai et al., 1986). Both aldosterone and angiotensin II returned to pre-deprivation levels in between deprivation episodes, so they are unlikely to be the explanation for the increased intake in either males or females. The effects were not affected by lapse of time, as rats depleted a second time four months after their first depletion drank comparable amounts to those which were depleted only a week later. Their plasma sodium was also no different than rats never depleted, and the urine sodium did not change in different depletions. These results indicate the change in intake is not due to a change in sodium handling by the kidney. Additionally, consuming high salt was not necessary to elicit a salt appetite, as rats who were not given salt to drink after depletion had no difference in their intake between depletions compared to rats that did drink salt.

Nonetheless conclusions about estrogen's relationship are limited in these studies, since estrogen was not controlled by administering it pharmacologically. Research needs to include specific experiments addressing what role, if any, estrogen is playing in these behavioral alterations in females. The question still remains, does estrogen increase or decrease sodium intake? If so, is this changed by multiple dietary deprivations? While it is likely that estrogen causes changes in body fluid regulation, more research is needed to determine the exact mechanisms behind its effects, which hormones estrogen influences, and how they relate to each other.

This dissertation aims to address this gap in the literature. Given what is known, we hypothesized that estrogen alters behavioral and hormonal responses during repeated episodes of dietary sodium deprivation. This dissertation tested this hypothesis, through these specific aims:

Aim 1: Behavioral effects of repeated dietary sodium deficiency (rNaD) on female rats with and without estrogen. I will analyze salt and fluid intake to test the hypothesis that estrogen status changes compensatory behavioral responses to dietary sodium deficiency during repeated episodes of sodium deficiency.

Aim 2: Physiological effects of repeated dietary sodium deficiency (rNaD) on female rats with and without estrogen. I will use ELISA to measure plasma levels of vasopressin, catecholamines, and aldosterone, as well as using an ion selective electrode to measure plasma sodium, to test the hypothesis that estrogen status alters hormone concentrations and plasma sodium levels during repeated sodium deficiency.

CHAPTER III

METHODOLOGY

3.1 General Methods

3.1.1 Animals, Ovariectomy, Hormone Replacement

All procedures were approved by Oklahoma State University Center for Health Sciences IACUC. Adult female Sprague-Dawley rats (85 days old) were purchased from Charles River Laboratories. Rats were housed two to a cage with *ad libitum* access to standard food and water for one week in a temperature and humidity controlled colony room on a 12:12 dark:light schedule (lights on at 07:00). After one week of acclimation, rats were pre-treated with oral meloxicam (0.15 mL of 1.5 mg/mL; io), weighed, and anesthetized using inhaled isoflurane. An incision was made on the abdomen, the uterus was exposed, and the uterine horns and ovaries identified. Each horn was tied with suture approximately 1 cm from the ovary, the arteries were cauterized, and the ovary was excised. Incisions were closed with suture in the muscle and staples in the skin. Rats were allowed to recover on heated pads, before being placed in individual cages and allowed to recover for one week with *ad libitum* access to standard rat chow (Harlan-#2018; 0.2% Na) and water. During the recovery week, rats were weighed daily and considered to be recovered when they regained pre-operative weight.

One week post-ovariectomy, rats were given injections of estradiol benzoate suspended

in sesame oil (EB; 0.1 μg/mL, s.c.) or of OIL vehicle (0.1 mL, s.c.). To mimic the pattern of estrogen fluctuations during the estrous cycle, injections were given on day one and day two each week throughout the experiment. Rats were weighed on injection days and again on day four each week of the injection protocol throughout the experiment (see Section 3.4 Verification of Estrogen below).

3.2 Single Sodium Deprivation Protocol

3.2.1 Testing

At the end of the recovery period, rats received a 50 mL bottle of 0.5 M NaCl overnight to adapt to the taste of salt. Twenty-four hours later, the OIL and EB injection protocol was begun as described in Section 3.1.1; OIL- and EB-treated rats were assigned to control or dietary sodium deficiency conditions. Control OIL- and EB-treated rats (sCon) were maintained on regular chow (NaR) throughout. Other OIL- and EB-treated rats were placed on a schedule of alternating weeks of NaR or sodium deficient chow (NaD; Harlan; 0.01-0.02% Na), as shown in Figure 1.

At the end of each diet period, rats were given a two-bottle test in which they received a graduated 50 mL bottle of 0.5 M NaCl (salt) and a 50 mL bottle of ddH_2O (water). Intakes of salt and water were measured after two hours. The 2-bottle test conducted after the first period of NaR was considered to be baseline intake. On the last day of NaD, some OIL- and EB-treated rats were randomly assigned to receive a final 2-hour, two-bottle test (sNaD+) and were sacrificed approximately 10-20 minutes later. Other OIL- and EB-treated rats did not receive a final two-bottle test prior to sacrifice (sNaD-, sCon).

The testing sequence is summarized as follows (see also Figure 1):

1. One week of NaR during recovery after ovariectomy (no injections)
    a. Overnight access to 50 mL bottle of NaCl at end of week (adapt to NaCl)
2. One week of NaR
    a. OIL and EB injections begin (see section 3.1.1 *Animals, Ovariectomy, Hormone Replacement*)
    b. Two-bottle test at end of week (Baseline intake)
3. 10 days of NaD
    a. Two-bottle test at end of 10 days (sNaD+) or no 2-bottle test (sNaD-, sCon)
    b. sNaD+/- and sCon rats sacrificed

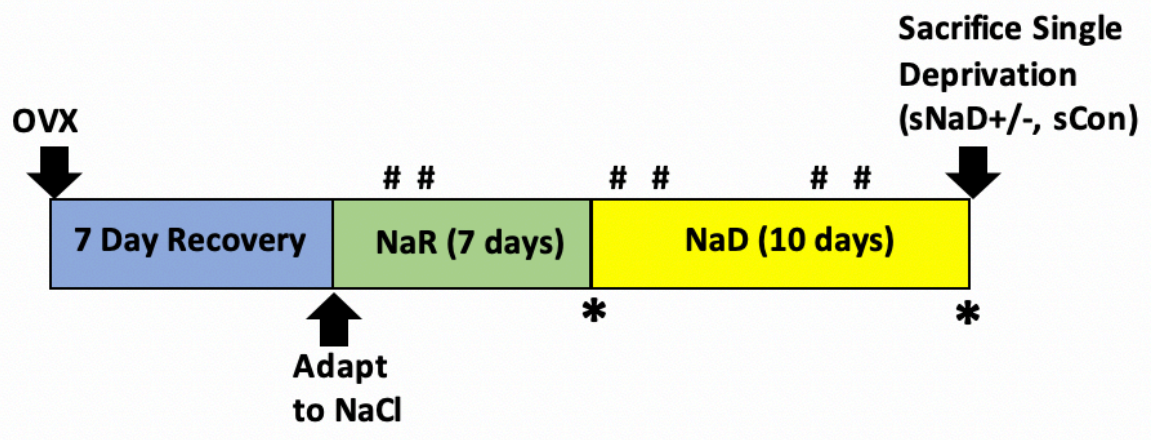

Figure 1. ***Timeline for single sodium deprivation protocol.*** *OVX = ovariectomy; NaR = regular chow; NaD = sodium deficient chow; # indicate days of OIL or EB injections; * indicate days of 2-hour, two-bottle tests (0.5 M NaCl and water). Control groups (sCon) received NaR throughout; some OIL- and EB-treated rats that underwent repeated episodes of NaD received a final two-bottle test prior to sacrifice (sNaD+), whereas others did not (sNaD-, sCon).*

At the end of the 10-day period of NaD (or comparable period of NaR for control rats), rats were rendered unconscious with CO_2, then decapitated to collect trunk blood into heparinized tubes. Brains were removed and stored at -80°C. Trunk blood was centrifuged and plasma sodium concentration (see Section 3.4 Verification of Estrogen); the remainder was stored in Eppendorf

tubes at -80°C. Uteri were also removed and stored in 0.15 M NaCl at 4°C prior to assessing uterine weights as described in Section 3.4.2 below.

The single deprivation groups are described in Table 1 below.

Table 1. **Summary of the single deprivation experimental groups**. Groups differ based on the exposure to sodium deficient chow (sNaD, sCon) and presence or absence (+, -) of final two-bottle test (Sodium Repletion) before being sacrificed.

Group	**sNaD+**	**sNaD-**	**sCon**
NaD Exposure	1 episode	1 episode	None
Sodium Repletion	Yes	No	No

3.2.2 Plasma Hormone Levels

Plasma was assayed for circulating levels of aldosterone, vasopressin, epinephrine, and norepinephrine using commercially available ELISA kits. Kits were purchased from Enzo (aldosterone), MyBioSource.com (vasopressin), Biomatik (epinephrine), and LSBio (norepinephrine) and were run according to the manufacturers' protocols.

3.3 Repeated Sodium Deprivation Protocol

3.3.1 Testing

At the end of recovery, a different group of ovariectomized rats received a 50 mL bottle of 0.5 M NaCl overnight to adapt to the taste of salt, 24-hours hours later the OIL and EB injection protocol was begun, and OIL- and EB-treated rats were assigned to control or NaD conditions as described in Section 3.2.1.

The testing sequence follow that detailed in Section 3.2.1., but with repeated episodes of NaD, as described below (see also Figure 2). Similar to procedures outlined in Section 3.2.1, on the day of sacrifice rats were randomly assigned to receive a final two-bottle test (rNaD+), or did not receive one (rNaD-, rCon).

1. One week of NaR post-ovariectomy (no injections)
    a. Overnight access to 50 mL bottle of NaCl at end of week (adapt to NaCl)
2. One week of NaR
    a. OIL and EB injections begin
    b. Two-bottle test at end of week (Baseline intake)
3. 10 days of NaD
    a. Two-bottle test at end of 10 days (all groups)
4. 18 days of NaR
    a. Two-bottle test at end of each week
5. 10 days of NaD
    a. Two-bottle test at end of 10 days (rNaD+) or no 2-bottle test (rNaD-, rCon)
    b. rNaD+/- and rCon rats sacrificed

Rats were sacrificed and brains, uteri, and blood were collected and stored as described in Section 3.2.1.

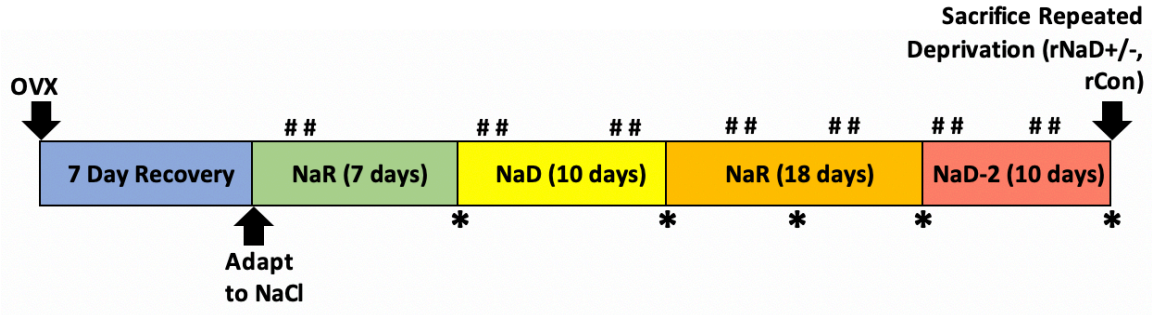

Figure 2. ***Timeline for repeated sodium deprivation experiment.*** *OVX = ovariectomy; NaR = regular chow; NaD = sodium deficient chow; # indicate days of OIL or EB injections; * indicate days of 2-hour, two-bottle tests (0.5 M NaCl and water). Control groups (rCon) received NaR throughout; some OIL- and EB-treated rats that underwent repeated episodes of NaD received a final two-bottle test prior to sacrifice (rNaD+), whereas others did not (sNaD-, sCon).*

The groups are summarized in Table 2.

Table 2. **Summary of the repeated deprivation experimental groups**. Groups differ based on exposure to sodium deficient chow (rNaD, rCon) and presence or absence (+, -) of final two-bottle test (Sodium Repletion) before being sacrificed.

Group	**rNaD+**	**rNaD-**	**rCon**
NaD Exposure	2 episodes	2 episodes	None
Sodium Repletion	Yes	No	No

3.3.2 Plasma Hormone Levels

Trunk blood was collected for analyses of aldosterone, vasopressin, epinephrine, and norepinephrine, as described in Section 3.2.2

3.4 Verification of Estrogen Treatment and Sodium-Deficient Diet Effectiveness

3.4.1 Body Weights

Rats were weighed on injection days (day 1 and 2) and two days after the second injection (day 4) each week.

3.4.2 Uterine Weights

Uteri were removed and stored in 0.15 M saline at 4°C as described in Section 3.1.1. Uteri were placed in fresh 0.15 M saline, one horn was isolated, and stripped of fat. A 1 cm section was cut and blotted, then weighed in a pre-weighed weigh boat.

3.4.3 Plasma Protein Concentration

After trunk blood was centrifuged as described in Section 3.1.1, a drop of plasma was used to analyze plasma protein for each rat with a refractometer (Leica).

3.4.4 Plasma Sodium Concentration

Approximately 100 µl of plasma was collected and frozen at -80°C until analyzed for sodium concentration using an ion selective electrode (Medica EasyLyte Na/K/Li analyzer).

3.5 Statistics

Data are presented as means ± SEM. All data were analyzed using ANOVAs as described in the sections below; significance was set as $p<0.05$. Statistically significant main effects or interactions were further analyzed using Fisher's LSD tests. Rats that did not consume any salt during the NaD-1 period were eliminated from all analyses.

3.5.1 Verification of Estrogen Treatment and Sodium-Deficient Diet Effectiveness

Body weights were analyzed as change in body weight from day 1 to day 4 of each week. Since the time courses for the experiments were different, we analyzed body weight changes separately for single and repeated deprivation protocols. Single deprivation protocols used a 2-way repeated measures ANOVA with hormone (OIL or EB) and days as factors, repeated for days. Con and NaD animals were analyzed using separate rmANOVAs. Repeated deprivation protocols were analyzed using a 3-way repeated measures ANOVA with hormone (OIL or EB), condition (control, NaD), and days as factors, repeated for days.

We compared uterine weights, plasma protein concentrations, and plasma sodium concentrations for rats that underwent single or repeated episodes of sodium deprivation using 3-way ANOVAs with hormone (OIL or EB), condition (control, NaD+, NaD-), and deprivations (single or repeated) as factors. Z-scores were calculated for uterine weights only in each group, but no outliers were found.

3.5.2 Salt and Water Intakes

All salt and water intakes were normalized to mL of fluid consumed per 100 g of body weight for each rat.

3.5.2a Baseline intakes

To ensure that there were no differences in intake prior to deprivation, baseline intakes were compared. NaD+ and NaD- groups were combined for analyses into a single NaD group. Baseline salt and water intakes for sNaD rats were analyzed using a 2-way ANOVA with hormone (OIL or EB) and diet (control or NaD) as the factors.

3.5.2b Stimulated and Unstimulated Intakes after Repeated Dietary Sodium Deprivation

rNaD rats were analyzed separately since the timeline was different. NaD+ and NaD- groups were combined for analyses into a single NaD group, and salt and water intakes during Baseline, NaD-1, Recovery-1, and Recovery-2 periods were analyzed using a 3-way repeated measures ANOVA with hormone (OIL or EB), diet (control or NaD), and day as the factors, repeated for day.

3.5.2c Test day intakes

These analyses compared intakes by sNaD+ and rNaD+ on the final day of NaD prior to sacrifice (because the NaD- and Con groups were sacrificed without a two-bottle test on the final day, these rats were not included in these analyses). Intakes by sNaD+ and rNaD+ rats on the test days were compared in a 3-way ANOVA with hormone (OIL or EB), diet (control or NaD), and number of deprivations (repeated or single) as the factors. Fluid concentrations consumed were analyzed by 3-way ANOVA with hormone (OIL or EB), diet (control or NaD), and number of deprivations (repeated or single) as the factors.

Concentrations of the ingested fluid were calculated as

$$Fluid\ concentration = 0.5\ x\ \frac{volume\ of\ salt\ consumed}{volume\ of\ water\ consumed}\ x\ 100$$

and were analyzed by 2-way ANOVA with hormone (OIL or EB) and number of deprivations (repeated or single) as the factors. Specific planned comparisons were made with Fisher's LSD using Bonferroni corrections.

3.5.3 Plasma Hormone Levels

Plasma hormone levels were normalized to protein concentration found via Bradford assay. Z-scores were calculated for each plasma hormone level and values more than two

standard deviations from the mean were eliminated from that particular analysis. We compared plasma aldosterone, norepinephrine, epinephrine, and vasopressin levels in rats that underwent single or repeated episodes of sodium deprivation using a 3-way ANOVA with hormone (OIL or EB), diet (control, NaD+, NaD-), and number of deprivations (single or repeated) as factors. Values from individual hormone assays greater than two standard deviations from the mean were not included in analyses for that hormone.

CHAPTER IV

FINDINGS

4.1 Verification of Estrogen Treatment and Sodium-Deficient Diet Effectiveness

4.1.1 Body Weights

Rats were comparable in weight during the post-OVX recovery period (OIL=285.6 g +/- 3.1 g; EB=281.5 g +/- 3.5 g). For the purposes of illustration, raw body weights are shown in Figures 3 and 4.

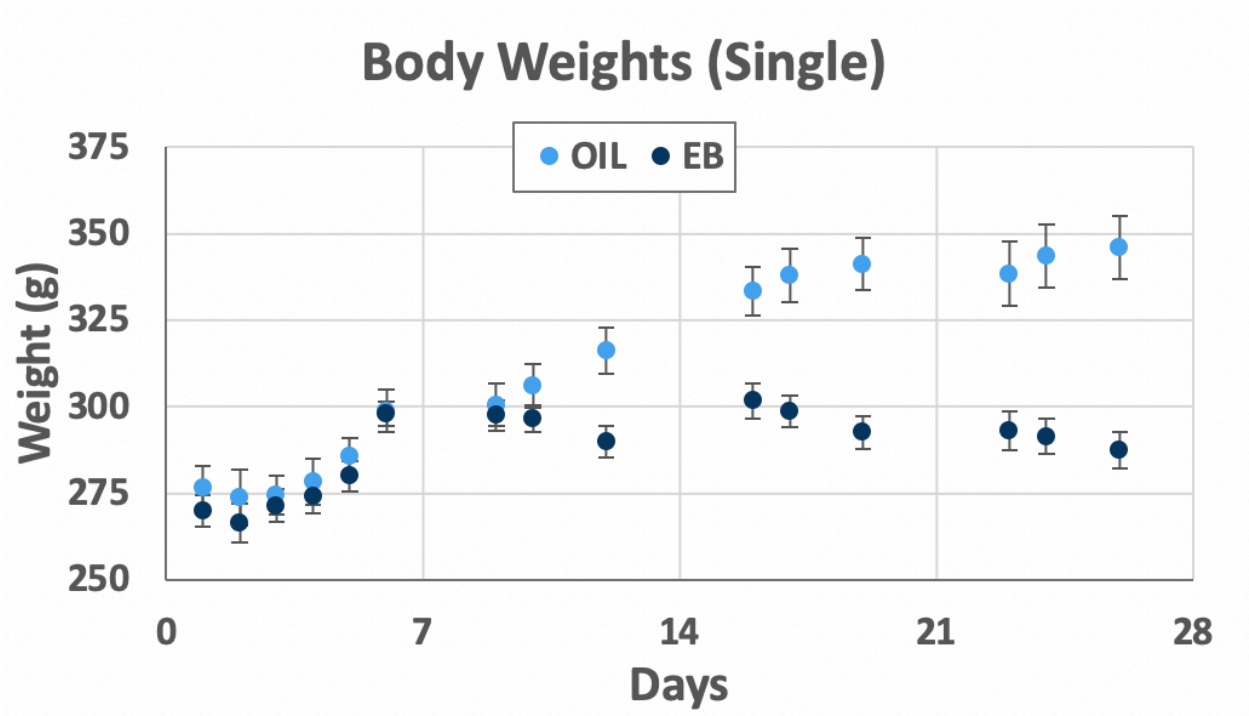

Figure 3. ***Raw body weights, single deprivation.*** *Raw body weights (g) of OIL- (light blue circles) and EB-treated (dark blue circles) rats during the single dietary sodium deprivation protocol.*

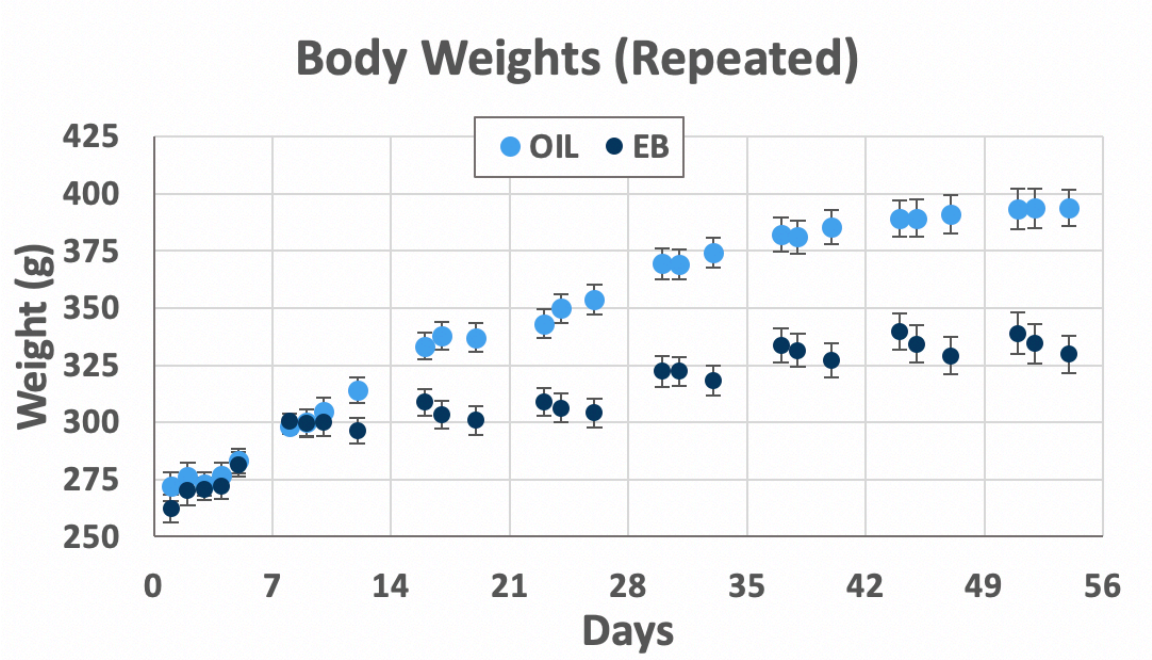

Figure 4. ***Raw body weights, repeated deprivation.*** *Raw body weights (g) of OIL- (light blue circles) and EB-treated (dark blue circles) rats during the repeated dietary sodium deprivation protocol.*

Tables 3 and 4 show the change in body weight between day 1 and day 4 each week for rats in the single and repeated deprivation protocols.

Table 3. **Change in body weight, single dietary sodium deprivation.** Change in body weight(g) between day 1 and day 4 during each week of the single dietary sodium deprivation protocol. NaR = regular diet; NaD = sodium deficient diet; Con = Control; OIL = Oil-treated; EB = Estrogen-treated. * = significantly less weight gain than OIL-treated animals overall; 1 = significantly more weight loss than all other weeks.

	Week 1	**Week 2**	**Week 3**
Con			
	NaR	**NaR**	
OIL	15.3 ± 2.4	17.3 ± 1.7	nd
EB*	-8.5 ± 1.8	-7.2 ± 2.9	nd
NaD			
	NaR	**NaD-1[1]**	**NaD-1**
OIL	15.8 ± 1.5	3.5 ± 2.4	8.2 ± 1.4
EB*	-7.2 ±1.4	-10.2 ± 2.9	-6.2 ± 1.9

nd = not done; these animals were sacrificed at the end of the second week of the experiment

Table 4. **Change in body weight, repeated dietary sodium deprivation.** Change in body weight (g) between day 1 and day 4 during each week of the repeated dietary sodium deprivation protocol. NaR = regular diet; NaD = sodium deficient diet; Con = Control; OIL = Oil-treated; EB = Estrogen-treated. * = significantly less weight gain than OIL-treated animals overall; ** = significantly greater weight gain than other groups; • = significantly greater weight loss than all other groups; 1 = significantly greater weight gain than all other time periods; 2 = significantly greater weight loss than Weeks 1, 3, 4; 3 = significantly greater weight loss than all other weeks.

	Week 1[1]	**Week 2[2]**	**Week 3**	**Week 4**	**Week 5**	**Week 6[3]**	**Week 7**
Con							
	NaR	**NaR**	**NaR**	**NaR**	**NaR**	**NaR**	**NaR**
OIL	14.5 ± 0.9 **	17.5 ± 1.1	10.2 ± 1.7	5.3 ± 1.1	4.2 ± 1.2	2.7 ± 0.9	0.8 ± 2.3
EB*	-3.7 ± 2.7	2.5 ± 1.7	-1.7 ± 1.9	-2.0 ± 1.1	-8.0 ± 1.2	-6.5 ± 0.9 •	-4.7 ± 2.3
NaD							
	NaR-1	**NaD-1**	**NaD-1**	**NaR-2**	**NaR-2**	**NaD-2**	**NaD-2**
OIL	14.1 ± 1.1 **	-1.7 ± 2.2	10.8 ± 1.8	4.9 ± 1.3	3.3 ± 1.2	-1.2 ± 1.9	2.4 ± 1.2
EB*	-3.3 ± 1.6	-12.8 ± 1.6	-6.5 ± 1.3	-4.8 ± 1.6	-5.6 ± 1.2	-16.8 ± 3.0 •	-5.8 ± 1.3

In single NaD protocol, a two-way rmANOVA of the NaD diet condition rats found a main effect of hormone [$F(1,46)=118.42$, $p<0.001$] on the change in body weight. There was also a main effect of time [$F(2,46)=6.93$, $p<0.01$]. The time by hormone interaction approached but did not reach significance [$F(2,46)=3.16$, $p=0.052$]. Pairwise comparisons of time using LSD showed that the NaD rats lost more weight on week 2 (NaD-1) than all other times.

In single NaD protocol, a two-way rmANOVA of the Con diet condition rats found a main effect of hormone [$F(1,10)=79.04$, $p<0.001$] on the change in body weight.

In repeated NaD protocol, a three-way rmANOVA found a main effect of hormone [$F(1,216)=178.91$, $p<0.001$] and of diet [$F(1,216)=18.71$, $p<0.001$] on the change in body weight. There was also a main effect of time [$F(6,216)=14.48$, $p<0.001$]. There was a significant time by hormone interaction [$F(6,216)=3.85$, $p<0.01$], and a time by diet interaction [$F(6,216)=11.80$,

$p<0.001$]. Pairwise comparisons of time using LSD showed that, overall rats gained significantly more weight gain on week 1 (NaR-1) than all other times, and lost significantly more weight loss on week 6 (NaD-2) than all other times.

In addition, there was a time by hormone interaction [$F(6,216)=3.85$, $p<0.01$]. Pairwise comparisons of this interaction found that, independent of diet condition, OIL-treated rats had greater weight gain during week 1 (NaR-1) than all other treatments and time points, whereas EB-treated rats had greater weight loss in week 6 (NaD-2) than all other treatments and time points, regardless of diet condition.

Finally, there was also a time by diet interaction [$F(6,216)=11.80$, $p<0.001$]. Pairwise comparisons of this interaction showed that, overall, the change in body weight was altered during NaD periods (greater weight loss in EB-treated rats, attenuated weight gain in OIL-treated rats), demonstrating efficacy of the sodium deficient chow.

4.1.2 Uterine Weights

Uterine weights are shown in Figure 5 below. Three-way ANOVA revealed a main effect of hormone [$F(1,64)=347.85$, $p<0.001$] and of the number of deprivations [$F(1,64)=7.15$, $p<0.01$]. Three-way ANOVA also showed an interaction between the number of deprivations and the diet condition [$F(2,64)=4.44$, $p<0.05$]. Pairwise comparisons of this interaction showed that independent of hormone, sCon rats had greater uterine weights than all others except sNaD- rats.

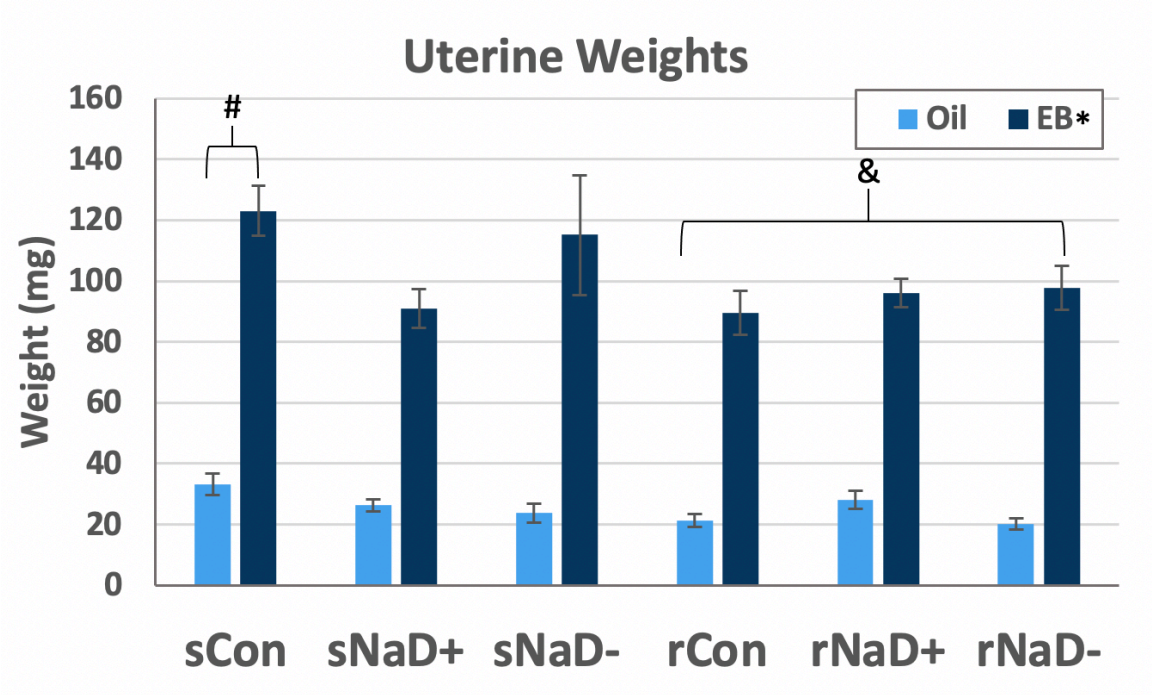

Figure 5. ***Uterine weights of OIL- and EB-treated rats.*** *Uterine weights (mg) in OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. # = significantly greater than all other rats except sNaD-; & = significantly less than single deprivation animals; * = significantly greater uterine weight in EB-treated rats, overall.*

4.1.3 Plasma Protein Concentration

Plasma protein concentration is shown in Figure 6. Three-way ANOVA showed a main effect of deprivation [$F(1,65)=16.39$, $p<0.0001$], condition [$F(2,65)=16.23$, $p<0.0001$] and hormone [$F(1,65)=210.76$, $p<0.0001$], with no interactions among the variables. Pairwise comparisons of the main effect of diet condition revealed that NaD+ rats had significantly lower plasma protein concentrations than all other rats, independent of hormone or number of deprivations.

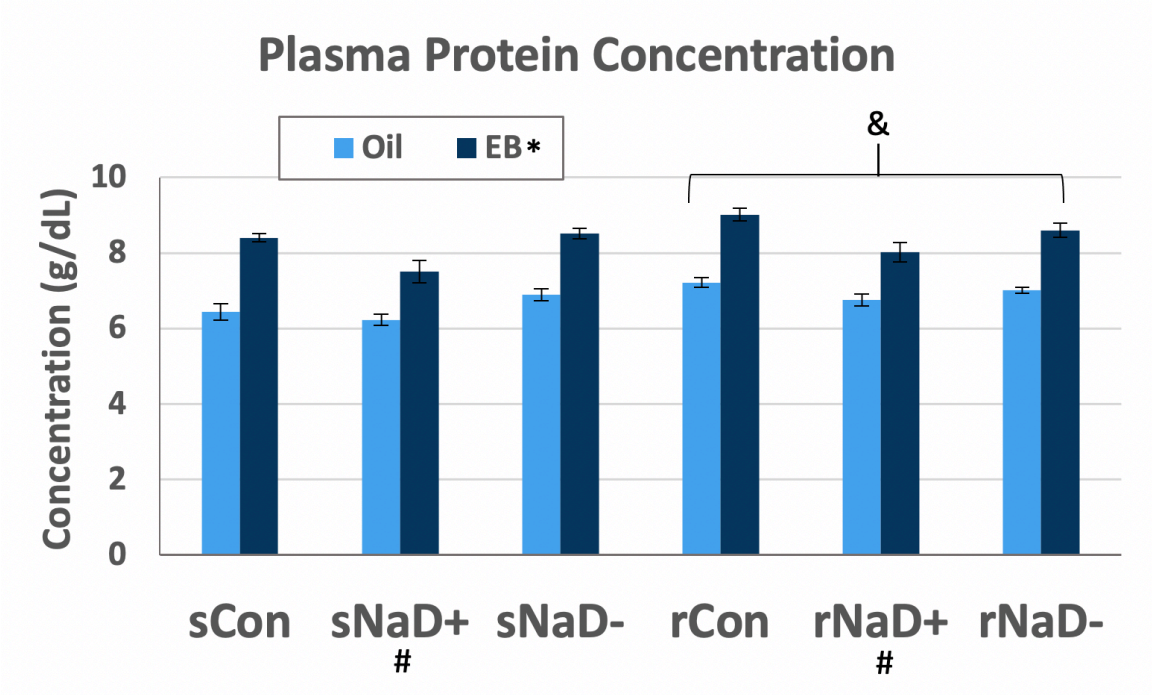

Figure 6. ***Plasma protein concentration in OIL- and EB-treated rats.*** *Plasma protein concentration (g/dL) in OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. * = significantly greater than OIL-treated rats; & = significantly greater than rats in single deprivation protocol; # = significantly less than NaD- or Con conditions.*

4.1.4 Plasma Sodium Concentration

Three-way ANOVA of plasma sodium concentrations (Figure 7) revealed a main effect of hormone [$F(1,61)=5.97$, $p<0.05$] and of diet condition [$F(2,61)=5.71$, $p<0.05$], with significantly greater sodium concentration in NaD rats that were allowed to replete their sodium. There was also an interaction between the number of deprivations and diet condition [$F(2,61)=3.49$, $p<0.05$]. Pairwise comparisons of this interaction showed that the rNaD+ group had a significantly greater plasma sodium than all other groups, independent of hormone.

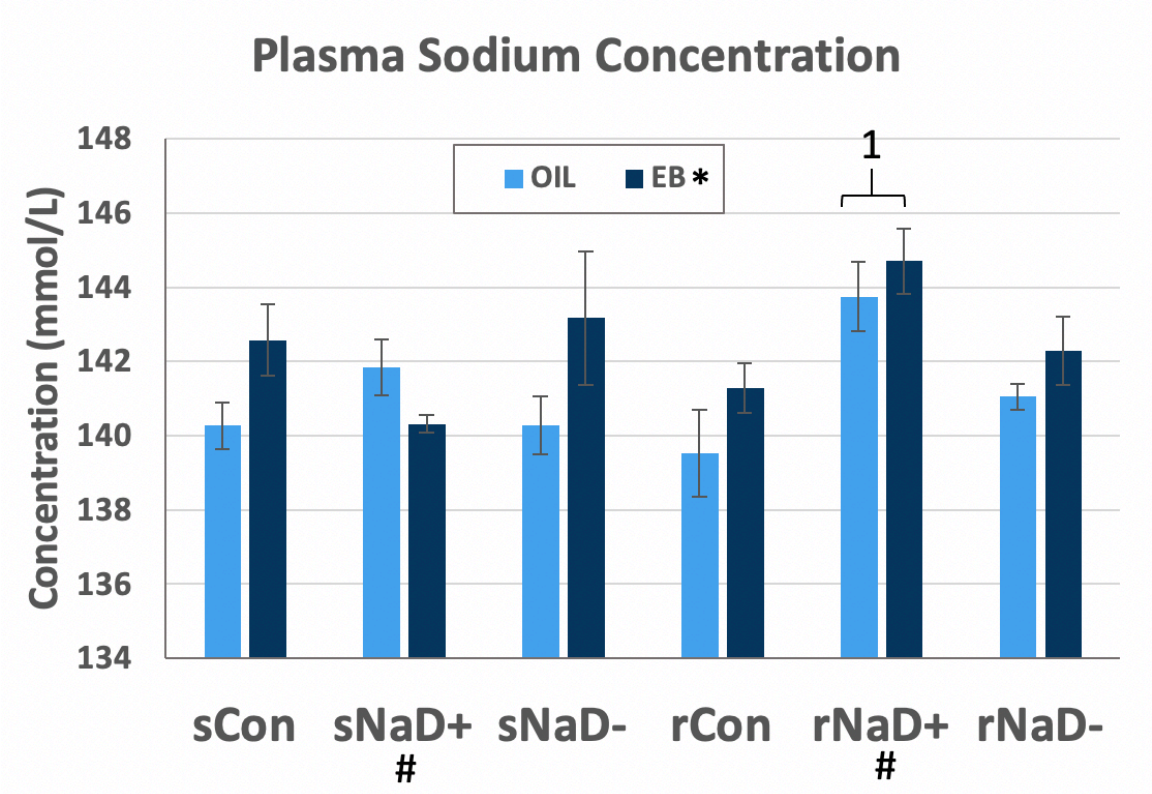

Figure 7. ***Plasma sodium concentration in OIL- and EB-treated rats.*** *Plasma sodium concentration (mmol/L) in OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. * = significantly greater than OIL-treated rats; # = NaD+ significantly greater than all other diet conditions; 1 = significantly greater than all other rats, independent of hormone.*

4.2 Salt and Water Intakes

4.2.1 Baseline Intakes

Two-way ANOVA comparing the baseline intakes of salt and water by rats in the single deprivation protocol showed no main effects or interactions of hormone or diet condition for salt or water intake at baseline (Figures 8 and 9).

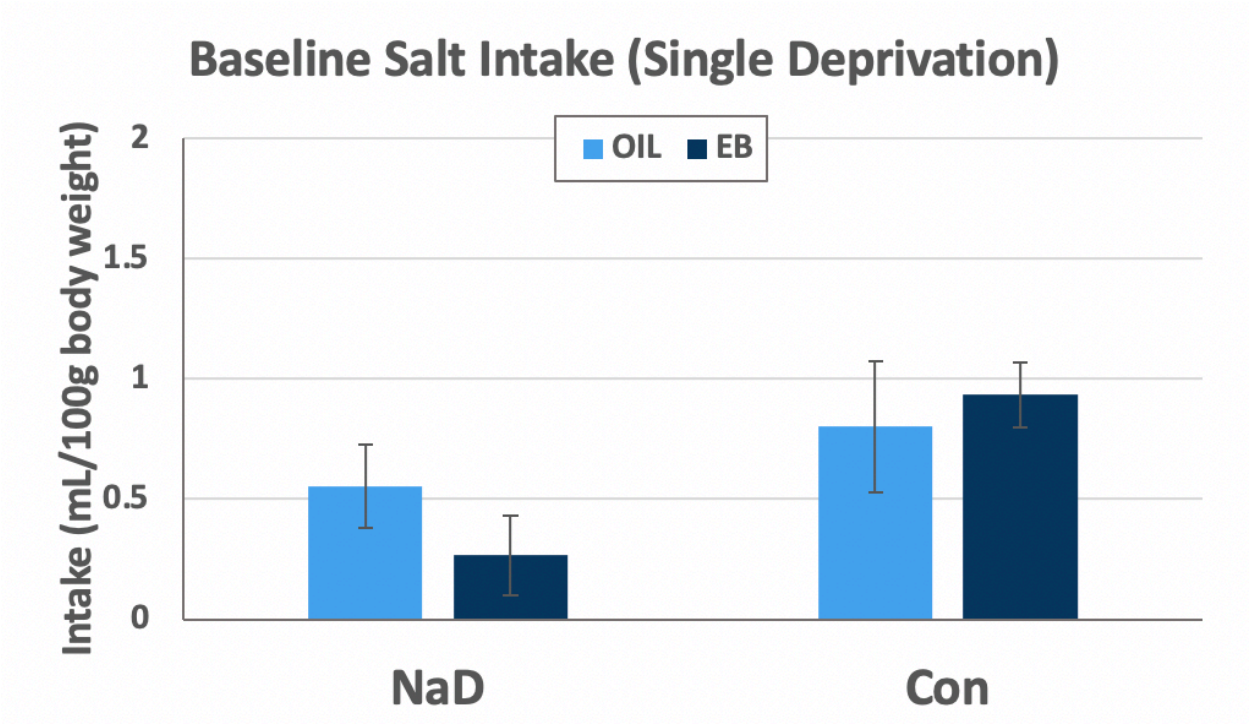

Figure 8. ***Baseline salt intakes by OIL- and EB-treated rats in the single dietary sodium deprivation protocol.*** *Baseline salt intakes (mL) by OIL- (light blue bars) and EB-treated (dark blue bars) rats. NaD = rats that underwent dietary sodium deprivation; Con = control rats that were not sodium deprived.*

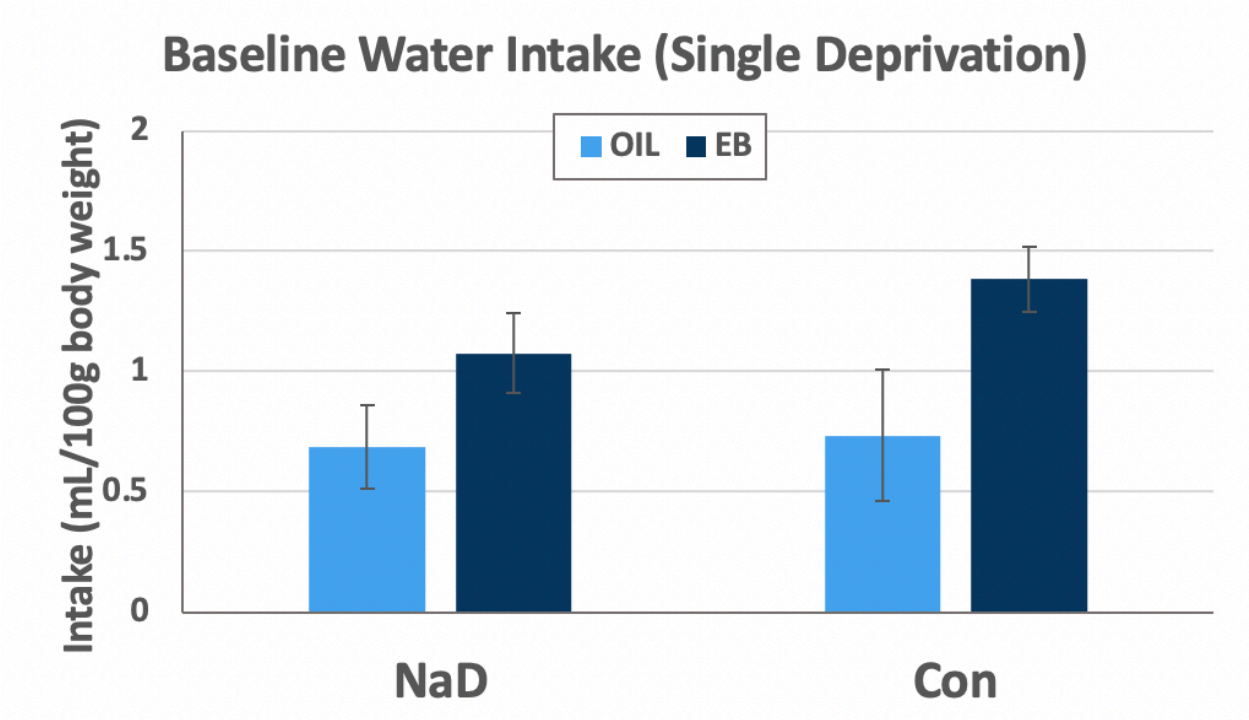

Figure 9. ***Baseline water intakes by OIL- and EB-treated rats in the single dietary sodium deprivation protocol.*** *Baseline water intakes (mL) by OIL- (light blue bars) and EB-treated (dark blue bars) rats. NaD = rats that underwent dietary sodium deprivation; Con = control rats that were not sodium deprived.*

4.2.2 Stimulated and Unstimulated Intakes after Repeated Dietary Sodium Deprivation

A 3-way repeated measures ANOVA of salt intake by rats in the repeated deprivation protocol (Figure 10) revealed a main effect of diet [$F(1,108)=5.28$, $p<0.05$] and of time [$F(3,108)=13.52$, $p<0.0005$]. There also was a time by diet interaction [$F(3,108)=12.47$, $p<0.0005$] and pairwise comparisons of the interaction showed that, independent of hormone, rats drank significantly more salt on the NaD diet during the NaD-1 period. In addition, there was a time by hormone interaction [$F(3,108)=4.76$, $p<0.005$]. Pairwise comparisons of this interaction showed that OIL-treated rats drank significantly greater amounts of salt water at the NaD-1 diet, regardless of the diet condition, and EB-treated rats drank less at baseline than they did at NaD-1, Recovery-1, or Recovery-2, independent of the diet condition. Specific planned comparisons using a Bonferroni correction of EB-treated rats on the NaD diet condition found that these rats drank more salt

during Recovery-1 than at baseline ($p<0.05$). Surprisingly, there was no difference in Recovery-2 intake ($p=0.07$), possibly due to variability in individual intakes. EB-treated rats during baseline also drank less than OIL-treated rats at baseline or NaD-1.

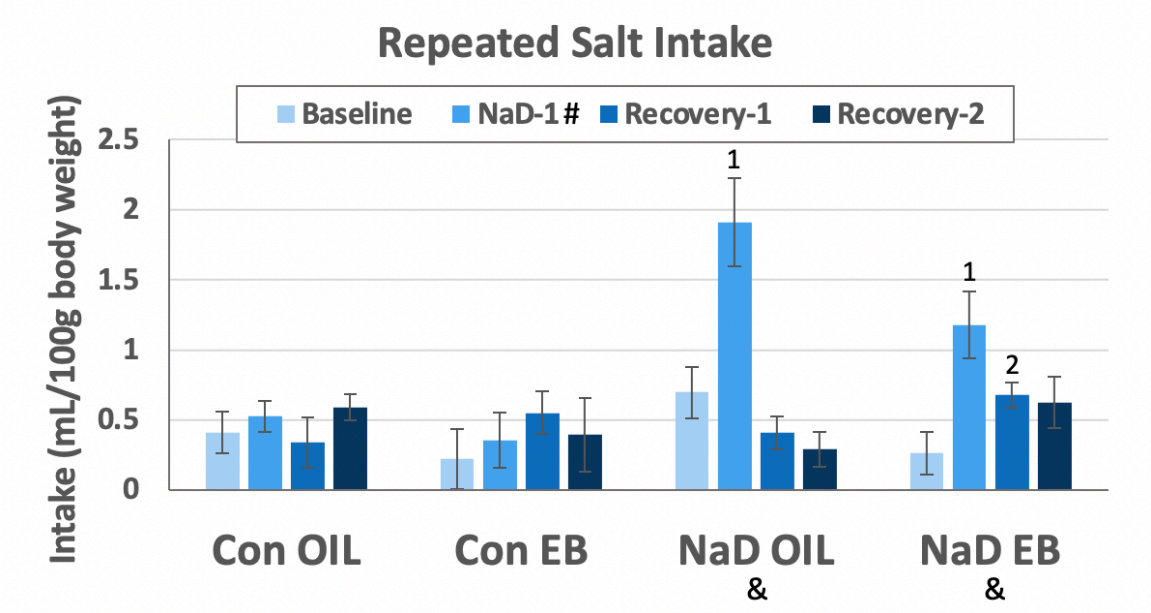

Figure 10. ***Salt intakes by OIL- and EB-treated rats during the repeated dietary sodium deprivation protocol.*** *Salt intakes (mL) by OIL- and EB- treated rats during the repeated dietary sodium deprivation protocol at baseline (first bar), first dietary sodium deprivation (second bar), first week of recovery (third bar), and second week of recovery (fourth bar). Con = control rats (never sodium deprived); NaD = rats that underwent two dietary sodium deprivations. Baseline = intake prior to NaD; NaD-1 = intake during first dietary sodium deprivation; Recovery 1, 2 = intake during NaR after the first dietary sodium deprivation. # = significantly greater intake than all other time points; & = significantly greater intake than Con rats; 1 = significantly greater intake than Con rats after NaD-1 diet period; 2 = significantly greater intake than EB-treated rats at baseline.*

A three-way repeated measures ANOVA of water intake by rats in the repeated deprivation protocol (Figure 11) revealed a main effect of time [$F(3,108)=4.79$, $p<0.005$] and a time by diet interaction [$F(3,108)=4.51$, $p<0.05$]. Pairwise comparisons of the interaction revealed that independent of hormone, rats drank more water during the NaD-1 period when they were on the NaD diet.

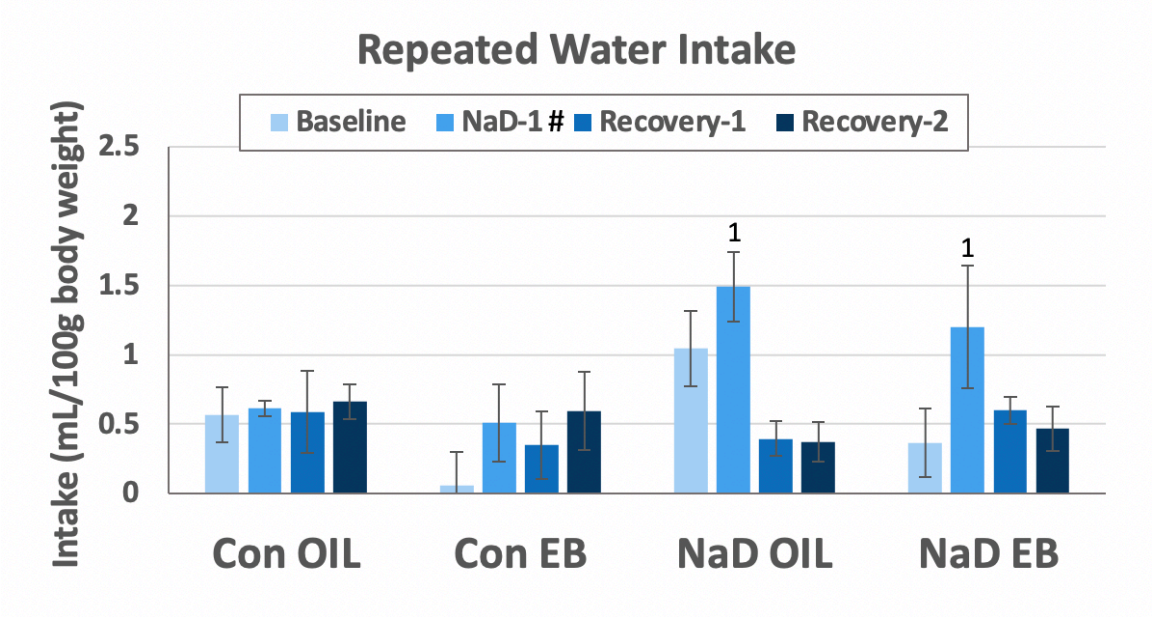

Figure 11. ***Water intakes by OIL- and EB-treated rats during the repeated dietary sodium deprivation protocol.*** *Water intakes (mL) by OIL- and EB- treated rats during the repeated dietary sodium deprivation protocol at baseline (first bar), first dietary sodium deprivation (second bar), first week of recovery (third bar), and second week of recovery (fourth bar). Con = control rats (never sodium deprived); NaD = rats that underwent two dietary sodium deprivations. Baseline = intake prior to NaD; NaD-1 = intake during first dietary sodium deprivation; Recovery 1, 2 = intake during NaR after the first dietary sodium deprivation.. # = significantly greater intake than all other time points; 1 = significantly greater intake than Con rats during NaD-1.*

4.2.3 Test Day Intakes

Two-way ANOVA of test day salt intakes by rats in single and repeated NaD protocol (Figure 12) revealed no main effects or interaction between hormone or number of deprivations.

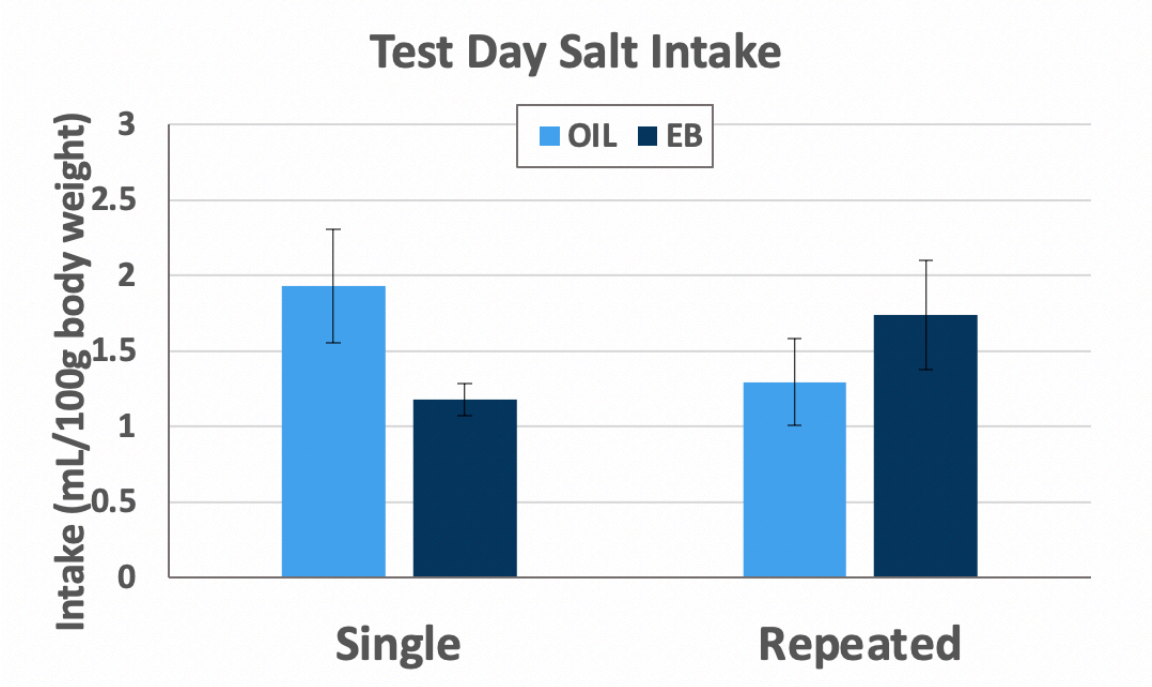

Figure 12. ***Test day salt intakes by OIL- and EB-treated rats in the single and repeated dietary sodium deprivation protocols.*** *Test day salt intakes (mL) by OIL- (light blue bars) and EB-treated (dark blue bars) rats that received single or repeated dietary sodium deprivations.*

Two-way ANOVA for the test day water intakes (Figure 13) showed no significant main effect or interaction.

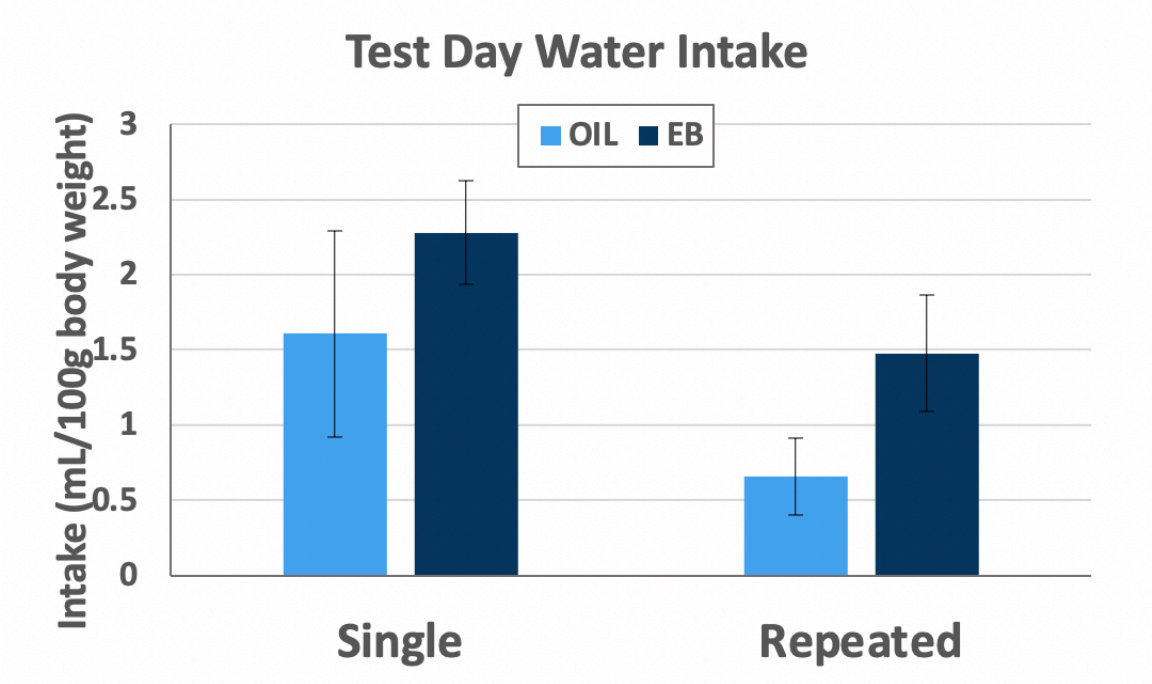

Figure 13. ***Test day water intakes by OIL- and EB-treated rats in the single and repeated dietary sodium deprivation protocols.*** *Test day water intakes by OIL- (light blue bars) and EB-treated (dark blue bars) rats that received single or repeated dietary sodium deprivations.*

Two-way ANOVA of the concentration of fluid consumed on test day (Figure 14) revealed a significant main effect of hormone [$F(1,24)=6.71$, $p<0.05$], with EB-treated rats drinking a significantly less concentrated fluid, independent of the number of deprivations. There were no other main effects or interactions.

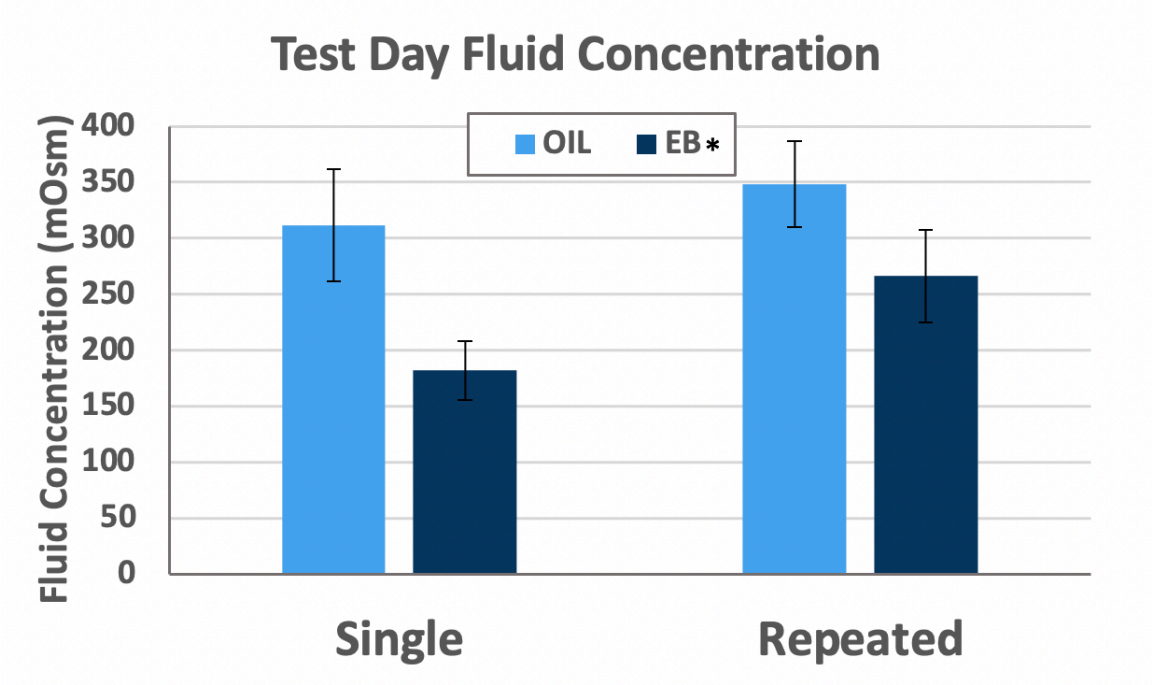

Figure 14. ***Concentration of fluid consumed on test day by OIL- and EB-treated rats in the single and repeated dietary sodium deprivation protocols.*** *Concentration (mOsm) of fluid consumed on test day by OIL- (light blue bars) and EB-treated (dark blue bars) rats that received single or repeated dietary sodium deprivations. * = significantly less than OIL-treated rats.*

4.3 Plasma Hormone Levels

Plasma norepinephrine concentration are shown in Figure 15. Three-way ANOVA revealed that there was a main effect of diet [$F(2,65)=5.31$, $p<0.05$], and pairwise comparisons showed that, independent of hormone, rCon rats had significantly lower plasma norepinephrine. There was also a main effect of hormone [$F(1,65)=6.81$, $p<0.05$]. EB-treated rats had significantly lower plasma norepinephrine, regardless of diet. Although it appears that norepinephrine levels in OIL- and EB-treated rats differed depending on the number of dietary sodium deprivations, this interaction failed to attain statistical significance.

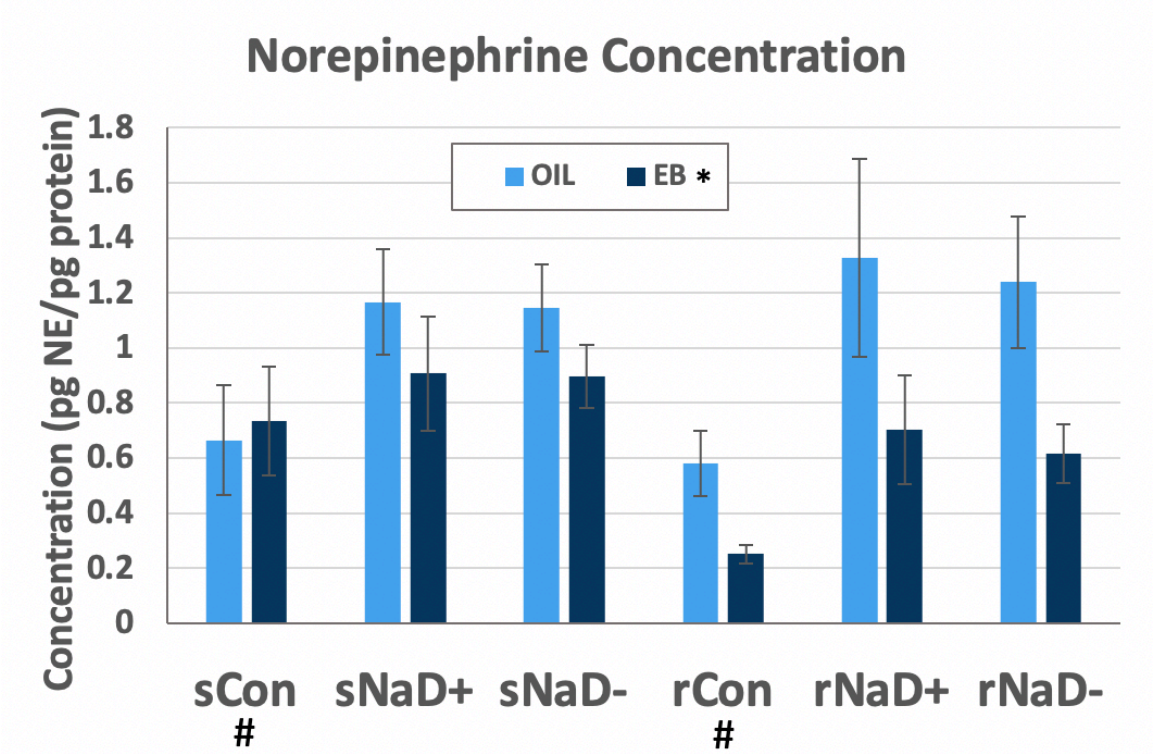

Figure 15. ***Plasma norepinephrine concentrations for OIL- and EB-treated rats.*** *Plasma norepinephrine concentrations (pg NE/pg protein) for OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. * = significantly less than OIL-treated rats; # = significantly less than all other groups.*

Plasma epinephrine concentration is shown in Figure 16. Three-way ANOVA showed no significant main effects or interactions.

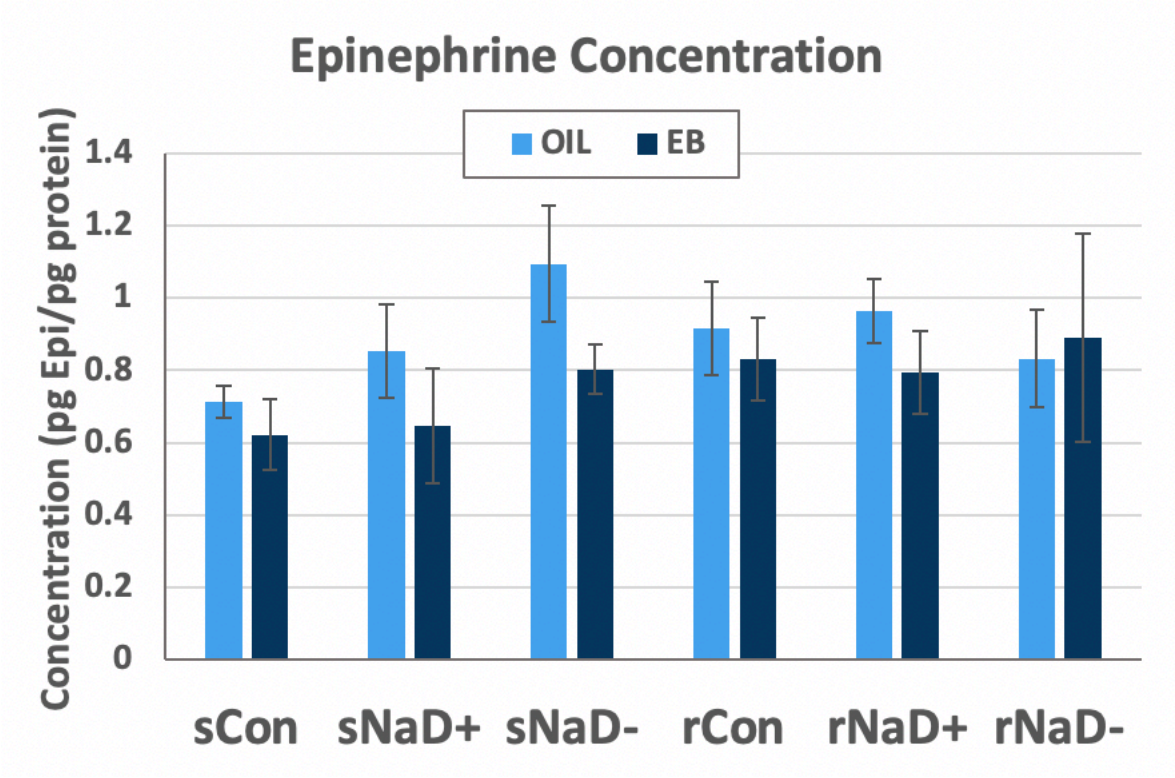

Figure 16. ***Plasma epinephrine concentrations for OIL- and EB-treated rats.*** *Plasma epinephrine concentrations (pg Epi/pg protein) for OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived.*

Plasma vasopressin concentration is shown in Figure 17. Three-way ANOVA showed a significant main effect of deprivations [$F(1,59)=4.41$, $p<0.05$], with rats in the repeated dietary sodium deprivations protocol having significantly greater vasopressin levels overall. There was also a main effect of diet [$F(2,59)=12.92$, $p<0.001$], with rats on the control (Con) diet having significantly greater vasopressin levels. In addition, the main effect of hormone [$F(1,59)=5.48$, $p<0.05$] showed significantly greater vasopressin levels for OIL-treated animals. Finally, there was an interaction between diet and hormone [$F(2,59)=3.42$, $p<0.05$]. Pairwise comparisons of the interaction showed that, independent of the number of deprivations, OIL-treated control rats had significantly greater vasopressin levels than all other conditions, including EB-treated controls.

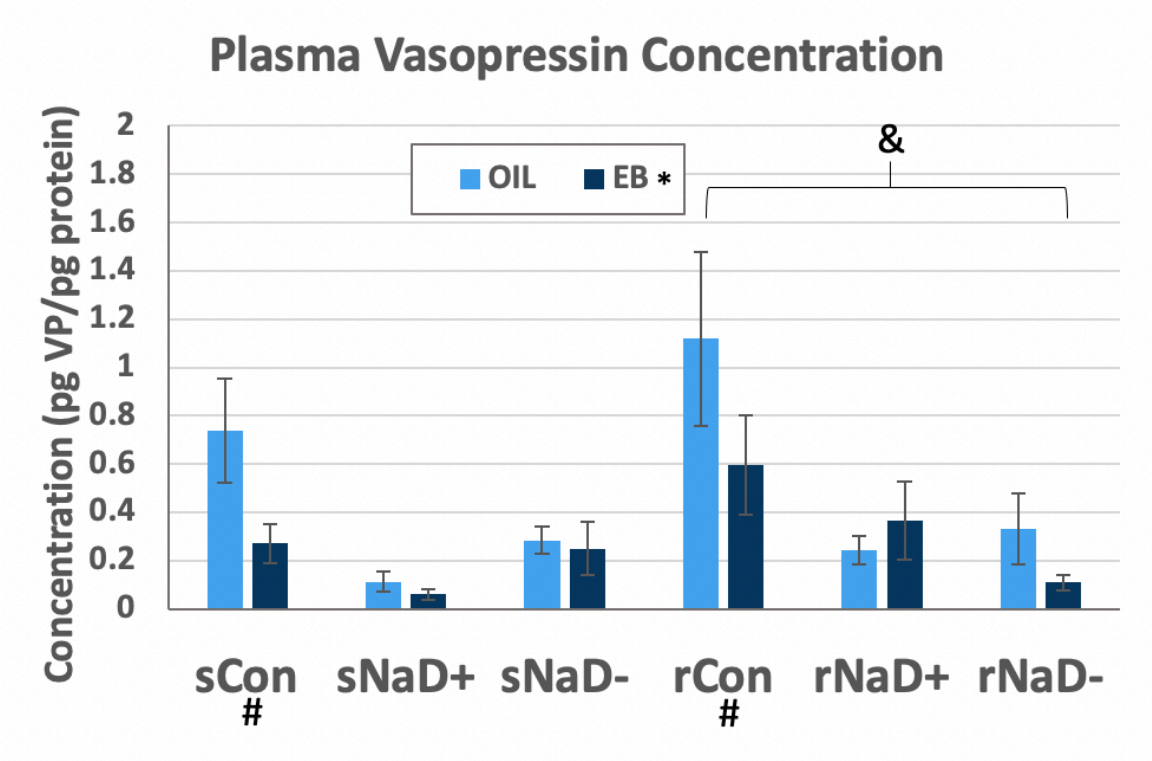

Figure 17. ***Plasma vasopressin concentrations for OIL- and EB-treated rats.*** *Plasma vasopressin concentrations (pg VP/pg protein) for OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. * = significantly less than OIL-treated animals; * = significantly less than OIL-treated animals; & = significantly greater than single deprivation animals; # = significantly greater than all other groups.*

Plasma aldosterone concentrations are shown in Figure 18. Three-way ANOVA showed a significant main effect of hormone [$F(1,54)=7.10$, $p<0.05$], with EB-treated rats having significantly lower plasma aldosterone overall. There were no other main effects or interactions.

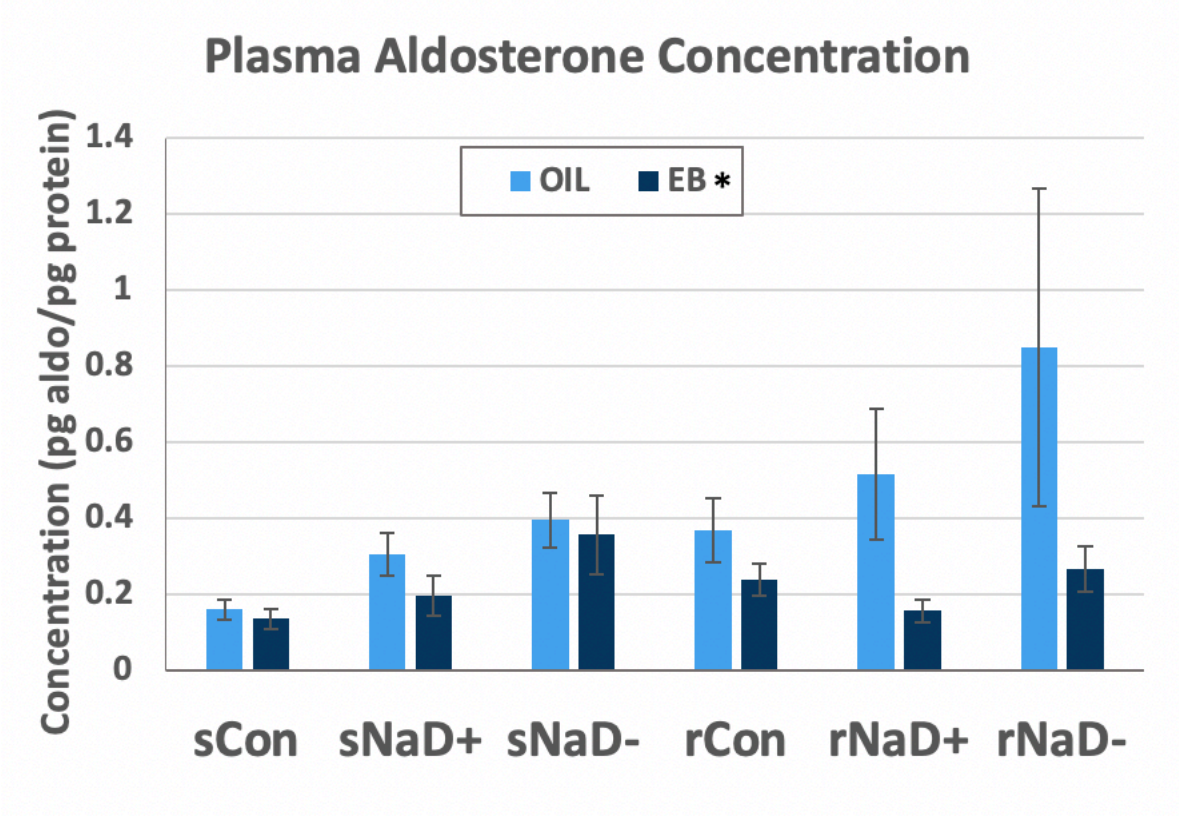

Figure 18. ***Plasma aldosterone concentrations for OIL- and EB-treated rats.*** *Plasma aldosterone concentrations (pg aldo/pg protein) for OIL- (light blue bars) and EB-treated (dark blue bars) rats. Some rats were given one dietary sodium deprivation with (sNaD+) or without (sNaD-) sodium repletion before sacrifice. Other rats had repeated dietary sodium deprivations with (rNaD+) or without (rNaD-) sodium repletion. Control rats (sCon and rCon) were not sodium deprived. * = significantly less than OIL-treated animals.*

CHAPTER V

CONCLUSION

Previous research has seen alterations in salt intake with repeated sodium deficiencies, but these mainly used a combined furosemide and low salt diet treatment. We aimed to determine the behavioral and physiological effects of repeated sodium deprivations in rats treated with estrogen and test the hypothesis that estrogen alters behavioral and hormonal responses during repeated episodes of dietary sodium deficiency. We tested this by observing the changes in salt and water intake after repeated sodium deprivations, then measured the plasma concentrations of norepinephrine, epinephrine, vasopressin, and aldosterone using ELISA.

5.1 Aim 1: Behavioral Changes

5.1.1 Unstimulated Intakes

At baseline, there was no difference in salt or water intake between OIL- and EB-treated rats (Figs. 9-11). Unsurprisingly, after sodium deficiency, NaD rats drank more salt to replenish that which was lost during deprivation. Water tended to mirror salt intake. Similar to the findings by Stricker *et al.* (1991), we saw that EB treatment resulted in a lower salt intake compared to the OIL-treated rats in the two-bottle test immediately following deprivation (NaD-1 intakes, Figure 11). In the Recovery period, where rats had access to regular chow, OIL- treated rats returned to

levels of salt intake similar to baseline. On the other hand, EB-treated rats had Recovery-1 intake that was significantly greater than baseline salt intake.

The increase in Recovery-1 salt intakes in EB-treated rats suggest that there are long-term changes where, despite the rats returning to a sodium replete status with access to regular chow, they continue to spontaneously consume salt without need, similar to the results seen by others such as Sakai *et al.* (1989). Our data adds to past research by extending this phenomenon to ovariectomized rats and further confirming a role for estrogen in this increase in post-deprivation unstimulated salt intake. This suggests estrogen is contributing to long term behavioral changes after repeated dietary sodium deficiency. Our study also extends the phenomenon to dietary sodium deprivation, as previous research had used furosemide to induce sodium deficiency.

5.1.2 Stimulated Intakes

Comparison of test day intakes immediately after NaD-1 or NaD-2 showed no significant difference in the salt or water intakes regardless of hormone treatment or number of deprivations, in contrast to previous research that showed increases in stimulated salt intake by intact females on subsequent sodium deprivations (Chow et al., 1992; Leshem et al., 2004; Sakai et al., 1987; Sakai et al., 1989). The observed differences between our study and previous research could be due to differences in sodium deficiency models (furosemide vs. dietary) or due to differences between endogenous or exogenous estrogen. Many previous studies also did not report the stage of estrus cycle during testing, which could obscure results. We chose to ovariectomize the rats and deliver estrogen on a four-day cycle to mimic the rat's natural estrus cycle, control the amount of estrogen received, and control the cycle stage at which we tested. Our dosage is meant to induce plasma concentrations similar to physiological levels. Regardless, we cannot rule out that stimulated salt and water intake vary with the estrus cycle and may be responsible for the differences between studies. It is also possible that further episodes of deficiency would increase

salt intake over time, as EB-treated rats appeared to drink more salt on the second deprivation, but the amount did not reach significance. Further research is needed to determine if repeated dietary sodium deprivations would increase stimulated salt intake after more than two deprivations and how the response may vary with different estrogen levels.

The lack of a difference in water intake was also somewhat surprising, as some previous research supports differences in water intake due to estrogen. Other studies had no differences in water intake between males and females (Sakai et al., 1989) and no effect of testosterone treatment (Chow et al., 1992), but we are not aware of previous studies that specifically studied estrogen's effects on water intake during repeated deprivations. While our data trend toward EB-treated rats drinking higher amounts of water, it failed to achieve significance.

Despite the non-significant results for salt and water intakes, when the concentration of ingested fluid was calculated, we found that EB-treated rats drank a less concentrated fluid during both deprivations. Previous studies have demonstrated an overcorrection of salt ingestion post-deficiency (Stricker et al., 1991), and our data suggest that perhaps the EB-treated rats are better at making adjustments to their water intake and minimizing this overcorrection. Post-ingestion signals from the stomach may be responsible for ceasing salt ingestion and switching to water, resulting in a less concentrated fluid overall. While we did not record which solution rats drank at particular times, other studies have seen that after experiencing sodium deficiency, rats drink large amounts of salt initially before alternating salt and water, which could be a means of adjusting the concentration of consumed fluid (Stricker et al., 1991). It is also possible that estrogen treatment alters taste perception, as it has previously been seen that chorda tympani responses to saline are altered by estrogen treatment (Curtis and Contreras, 2006). The chorda tympani has connections to gustatory centers in the brain, such as the NTS, which may be further altered by both EB and by repeated deprivations. While we did not see statistically significant differences in the concentrations of ingested fluid between sNaD and rNaD rats, the EB-treated

rats do appear to begin drinking a more concentrated fluid on the second depletion which approaches that consumed by the OIL-treated rats. It is possible that further depletions, such as the four-depletion protocol implemented by other labs (Sakai et al., 1989) would lead to further changes that are not evident after only two depletions, and that after a certain number of depletions, this effect of estrogen would diminish. Future studies should explore the effects of more depletions using dietary deprivation as in our protocol.

It is clear that the dietary sodium deprivation was sufficient to cause behavioral changes. EB-treated rats drink a less concentrated fluid immediately after deprivation, but drink more salt in the weeks following deprivation. This research must be expanded to determine if these behavioral phenomena continue with more episodes of deficiency.

5.2 Aim 2: Physiological Changes Part 1: Body Weight, Uterine Weight, Plasma Protein, and Plasma Sodium

5.2.1 Body Weight

EB-treated rats tended to experience decreases in body weight as we expected (Tables 1, 2). This suggests that our EB treatment protocol was effective. At the same time, both OIL- and EB-treated rats exhibited attenuated weight gain (OIL) or augmented weight loss (EB) during the weeks of NaD. This effect may indicate loss of extracellular fluid volume in urine as a result of the diet. Previous studies showing greater sodium loss in the urine in the initial days on a sodium deficient diet support this interpretation (Stricker et al., 1991). Additionally, we saw greater weight loss in EB-treated rats on week 6, regardless of the diet condition (NaD or Con). This appears to mainly be due to large weight loss in the EB-treated NaD rats, but the reasons for this are unclear. It is unlikely to be due to a greater natriuresis in the second deprivation, as this was not seen in other studies in furosemide (Sakai et al., 1989). It may be due to prolonged exposure

to estrogen, but further studies would be necessary to determine the mechanism behind the observed weight loss.

5.2.2 Uterine Weights

Uterine weights were measured as another means to assess EB treatment effectiveness. As expected, we saw greater uterine weight in rats that received EB treatment, which has been seen in other research (Peysner and Forsling, 1990). Additionally, rats in the repeated deprivation protocol had significantly decreased uterine weight regardless of hormone treatment. This is possibly due to age, as these rats were four weeks older than single deprivation rats at sacrifice. Our sCon group also had significantly greater uterine weight than all other groups, which we suspect is due to human error, as some uteri in that group were measured as much larger than all others.

5.2.3 Plasma Protein Concentration

Plasma proteins were increased in EB-treated rats as we expected. Previous studies have seen increases in plasma proteins with steroid treatments (Roesch et al., 2000), likely due to increases in carrier proteins for hormones such as estrogen (Raynaud et al., 1971). Additionally, we saw significantly decreased plasma protein in the sNaD+ and rNaD+ groups. Since these groups were allowed to consume salt and water within two hours prior to sacrifice, this is likely due to a small dilutional effect from consuming fluid. Finally, our repeated deprivation animals had significantly elevated plasma protein. Since rCon rats were also increased, it may not be due to an effect of the repeated episodes of NaD diet, but instead age. However, if true, it is unclear what proteins may be contributing to this effect

5.2.4 Plasma Sodium Concentration

Plasma sodium concentration has previously been shown to be unaltered in both dietary sodium deficiency (Stricker et al., 1991) and furosemide deprivation (Sakai et al., 1987). We observed an increase in plasma sodium in EB-treated rats, which is supported by previous research (Roesch et al., 2000). It has been suggested that sexually mature female rats may more closely guard a higher plasma sodium concentration as an adaptation for pregnancy (for review, see Weissgerber and Wolfe, 2011). Therefore, it is to be expected that the EB-treated rats have a slightly higher plasma sodium despite the low salt diet, and again suggests that our estrogen treatment is mimicking physiological estrogen levels. Additionally, increases in the plasma sodium concentration in sNaD+ and rNaD+ groups could indicate that these rats did not have time to return to baseline after consuming salt during the two-bottle test. Nonetheless, it is clear that any behavioral differences between single and repeated deprivations are not due to alterations in renal clearance of sodium, since there were no differences in plasma sodium between single and repeated deprivations.

5.3 Aim 2: Physiological Changes Part 2: Body Fluid Hormones

5.3.1 Vasopressin Concentration

We found a decrease in plasma vasopressin concentration with EB treatment, which supports previous data that found decreased vasopressin mRNA in the brain in the presence of estrogen (Sladek et al., 2000) and expands the findings to live animals. Previous data on the effects of estrogen on vasopressin secretion have been conflicting, with no change (Crofton et al., 1985), increases (Skowsky et al., 1979), and decreases (Sladek et al., 2000) all reported. However, these differences may be due to methodological differences, such as the delivery method or dosage of estrogen.

Estrogen receptor beta (ERβ) is expressed in the SON and PVN, areas responsible for release of vasopressin. It is believed that ERβ plays an inhibitory role on vasopressin release (Sladek and Somponpun, 2008). Therefore, stimulation of this receptor by estrogen circulating in the body would result in a decrease in estrogen release, which supports our findings.

The increase in vasopressin in repeated deprivation groups is interesting, but it is unclear whether this was a factor of the repeated sodium deprivations or age, since it appears that vasopressin trended toward increasing in the rCon group compared to sCon. The increase in this group may account for the main effect of repeated deprivations. As expected, the sCon and rCon groups had higher plasma vasopressin. These results indicate retention of water to maintain plasma osmolality while on standard laboratory chow, while the low salt chow suppresses vasopressin. Despite this suppression, we were still able to detect vasopressin levels with our assay, demonstrating its sensitivity. However, in order to determine whether age or number of deprivations are a factor in vasopressin, further studies with more deprivations need to be done. We did not see a difference in NaD+ and NaD- groups, despite the NaD+ rats consuming salt prior to sacrifice. It is possible that the time course between sodium repletion and sacrifice was not long enough for an increase in vasopressin to occur.

Our results highlight that vasopressin is affected by estrogen. In the case of subcutaneous administration of physiological levels of estrogen, our data support an inhibitory effect of estrogen on vasopressin. Further study is needed to evaluate the effect of repeated sodium deprivations on plasma vasopressin concentration. Measuring the alterations in ERβ in the SON and PVN after repeated deprivations may provide more insight as to whether the differences we observed were due to age or sodium deprivation.

5.3.2 Norepinephrine and Epinephrine Concentration

The consistent decrease in norepinephrine concentration in EB-treated rats suggests that these rats were not experiencing as much compensatory increase in the sympathetic nervous system in response to dietary sodium deprivation as OIL-treated rats. Over time, the difference between OIL- and EB-treated rats appears to increase, though neither group was statistically different from the levels observed after a single deprivation. It is possible that an effect of age or of repeated estrogen treatments over time may play a role in this growing difference, but longer term studies are needed to confirm this. More deprivations may lead to a greater rebound increase in norepinephrine in OIL-treated rats over time.

As expected, we saw increases in plasma norepinephrine concentration in rats in the NaD+ and NaD- groups compared to Con rats. This supports previous studies showing increases in catecholamines while on a low salt diet across a broad range of sodium concentrations (Luft et al., 1979; Romoff et al., 1979). However, it was somewhat surprising that norepinephrine was not altered by repeated deprivations. This suggests that norepinephrine and, therefore, the sympathetic nervous system, are not responsible for the alterations in salt and water intake observed in our study and previous studies (Chow et al., 1992; Sakai et al., 1987; Sakai et al., 1989;). Additionally, we did not see an effect of pre-sacrifice sodium repletion on the concentration of norepinephrine, as there was no difference between NaD+ and NaD- groups. The time between repletion and sacrifice may have been too short to show a measurable difference.

The lack of significant differences in hormone treatment or diet condition for epinephrine concentration requires further research. We expected that it would also be altered by dietary sodium deprivation, but it is possible that this is not a strong enough stimulus for epinephrine release. However, other research has found alterations in epinephrine on the low salt diet (Graudal et al., 2012). The reasons for this discrepancy are unclear. Future studies need to explore

the differential mechanisms behind norepinephrine and epinephrine release in the context of dietary sodium deprivation.

5.3.3 Aldosterone Concentration

Estrogen treatment showed a consistent suppression of plasma aldosterone across all groups, consistent with previous data (Macova et al., 2008). Previous research has demonstrated that aldosterone stimulated by dietary sodium deprivation alone is unchanged by estrogen treatment (Roesch et al., 2000). However, this research used a shorter time course of the sodium deficient diet than our protocol, which could explain the differences in results. It is possible that aldosterone may be decreased through suppression of the AT1 receptor, as estrogen treatment decreases expression of this receptor in the adrenals (Roesch et al., 2000).

A larger response to the repeated sodium deprivation was seen in OIL-treated rats than EB, though this effect failed to achieve significance. This could indicate an effect of repeated sodium deprivation that is obscured by a large amount of variability in our data, which may have been due to technical challenges in the aldosterone assay or due to a relatively small number of samples. However, previous studies have not seen alterations in aldosterone after repeated furosemide deprivations (Sakai et al., 1987; Sakai et al., 1989). This study also did not see a difference in aldosterone between males and females. While we did not use males in our experiment, it would be expected that there would be differences, since our EB-treated rats had consistently lower aldosterone. This could be due to a difference in intact females compared to females with estrogen replacement as in our study.

5.4 Behavioral and Physiological Integration

Taken together, the aforementioned results provide a clearer picture of the relationship between body fluid homeostasis and estrogen status. In Figure 19, we propose the following schematic to display the interrelationship between the hormones we measured and estrogen. Ultimately, the final result of treatment with estradiol benzoate achieved an end goal that, while we did not measure it, should decrease blood pressure through decreased renal sodium reabsorption, decreased water retention, and vasodilation. However, it still remains to be tested whether blood pressure is altered in female Sprague-Dawley rats during repeated episodes of sodium deprivation. While blood pressure can be altered with varying dietary salt in rats bred to be genetically prone to salt sensitivity (Fang et al., 2001; Zheng et al., 2008), less data is available on traditional strains such as we used, and we are not aware of any research that directly measured blood pressure alterations in relation to repeated episodes of sodium deprivation. Further research should also explore how repeated dietary sodium deprivations affect genetic models of hypertension and salt sensitivity, such as the Dahl salt sensitive and spontaneously hypertensive rat strains.

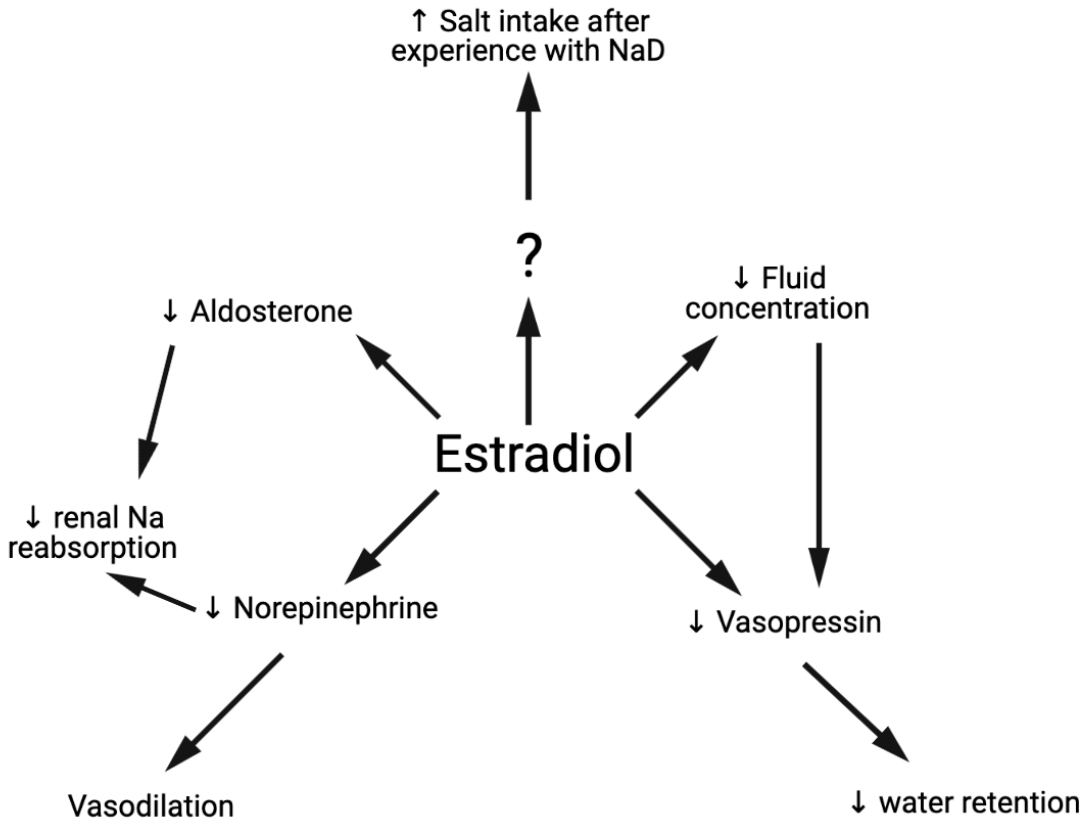

Figure 19. ***Proposed mechanistic schematic.*** *Estrogen decreases norepinephrine, which leads to vasodilation, as well as decreased renal sodium reabsorption. Decreases in aldosterone also affect the renal system. Estrogen also decreases fluid concentration consumed, which could influence vasopressin and lead to an overall reduction in water retention. All of these effects could be decreasing blood pressure. Additionally, unknown mechanisms lead to an increase in salt intake in the weeks after experience on a low salt diet. Created with BioRender.com.*

We also performed a semi-quantitative assessment to further examine the effects of estrogen and repeated dietary sodium deprivations. These are shown in Table 5 below.

Table 5. **Comparison of plasma hormone levels to sCon.** We compared group means to the control group means (sCon OIL or EB) based on Z scores. — = <0.5 standard deviations from control group; ↑/↓ = >0.0 to ≤ 1.0 standard deviations from control group; ↑↑/↓↓ = > 1.0 to ≤ 2.0 standard deviations from control group; ↑↑↑/↓↓↓ = > 2.0 to ≤ 3.0 standard deviations from control group; ↑↑↑↑/↓↓↓↓ = > 3.0 standard deviations from control group.

	sNaD+	**sNaD-**	**rCon**	**rNaD+**	**rNaD-**
Aldosterone					
vs. sCon OIL	↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
vs. sCon EB	—	↑↑↑	↑	—	↑↑
Vasopressin					
vs. sCon OIL	↓	—	—	—	—
vs. sCon EB	↓	—	↑↑	—	—
Norepinephrine					
vs. sCon OIL	↑	—	—	↑	↑
vs. sCon EB	—	—	↓	—	—
Epinephrine					
vs. sCon OIL	↑	↑↑↑	↑↑	↑↑↑	↑
vs. sCon EB	—	—	—	—	↑

This table compares rats within hormone treatment (OIL or EB). When viewed in this manner, it is clear that when on the sodium deficient diet, OIL-treated rats tend toward larger hormonal responses to the diet, as compared to sCon rats. While EB-treated rats do utilize compensatory mechanisms related to the sodium deficient diet, this response is not as

pronounced, and, in some cases, such as norepinephrine and epinephrine, largely no greater than the sCon rats, which were never exposed to low sodium chow.

The hormones we measured—norepinephrine, epinephrine, vasopressin, and aldosterone—do not explain the increased intake during recovery, as shown by both the data and Table 5. Increases in both vasopressin and aldosterone can increase sodium appetite. Yet we saw a sodium appetite that was increased above baseline, while these hormones were decreased. Therefore, it is unlikely either of these is responsible for the observed enhancement in EB-treated rats' sodium appetite after sodium deprivation. The mechanisms behind this increased intake remain to be determined. Nonetheless, our results indicate the post-deficiency enhancement in sodium appetite is present after dietary sodium deprivation only in EB-treated rats, indicating that estrogen is critical for this effect in females. Overall, it is clear that there were changes due to experience with estrogen treatment, others due to experience with salt, but it seems unlikely that the observed hormone changes are due to experiences with repeated dietary sodium deprivation. Whether further deprivations may alter this conclusion remains to be examined.

The increased salt intake occurs despite having consumed enough to restore salt lost from the low salt diet. This was not due to alterations in plasma sodium. Plasma sodium was no different in Con rats compared to NaD-, which did not obtain sodium repletion through a two-bottle test, nor is there a difference between OIL- and EB-treated NaD- rats. Other studies have compared plasma sodium concentration in males and females to determine the cause of the increased post-depletion intake in females, and have found no difference (Sakai et al., 1989). Our data extend this finding to OIL- vs. EB-treated rats, meaning that plasma sodium concentration is not altered by ovariectomy, despite the differences in salt intake in these groups.

Other studies have shown similar alterations in female rats' post-deprivation intakes, though this research used furosemide (Chow et al., 1992; Sakai et al., 1987; Sakai et al., 1989).

However, our data suggest a role for estrogen in the previously seen sex differences. Our study isolated estrogen as influencing this phenomenon by using ovariectomized rats given EB treatment. Additionally, our research extended the enhanced intake to females ovariectomized past sexual maturity. Previous studies have not compared ovariectomized females given estrogen replacement with those given vehicles. However, Chow *et al.* (1992) did find that ovariectomized females given testosterone had greatly decreased salt intake. Even though our results suggest estrogen is influencing the response to the low salt diet, we recognize it is possible the sex differences present in previous studies may also be influenced by testosterone. This will require further study to compare the effects of estrogen and testosterone when it comes to salt intake and the post-deprivation enhancement observed.

While the changes in post-deprivation intakes are not due to differences in plasma sodium concentration, nor due to alterations in the measured body fluid hormones, it is possible there are changes in the brain which may occur both in the presence of estrogen and after a perceived threat to body fluid homeostasis such as dietary sodium deprivation. Studies on brain changes after multiple deprivations are minimal. Na *et al.* (2007) found increased Fos-ir expression in the basolateral amygdala and shell of the nucleus accumbens, areas associated with the mesolimbic reward system. It is possible reward may be involved, however, we provided a 24-hour period of access to the 0.5 M NaCl prior to deprivations in an attempt to minimize reward responses. Regardless, the study by Na *et al.* was only done in males, using furosemide treatment, and needs to be extended to females.

Areas of the brain other than reward may also be involved and may be modulated by estrogen. Estrogen receptors, specifically ERβ, are present in the supraoptic nucleus (SON) and paraventricular nucleus (PVN), areas involved in release of vasopressin. We did see a significant difference in vasopressin levels in single vs. repeated deprivation animals, which may point to changes in one of these areas of the brain. The SON has been shown to have increases in Fos-ir

after repeated sodium deprivations (Na et al., 2007). We did find differences in vasopressin-treated animals, but rCon were also increased, meaning this difference could have been a factor of age. Regardless, the fact that vasopressin was elevated could mean that the estrogen modulated some component of the system that could play a role in changing salt appetite.

Estrogen receptor alpha (ERα) is also present in the nucleus of the solitary tract (NTS) and area postrema (AP) (Hay et al., 2014). These areas receive gustatory input from the chorda tympani, as well as baroreceptor input from glossopharyngeal and vagus nerves. ERα could be contributing to changes in taste perception after repeated sodium deprivations, which may ultimately be responsible for either the decreased sodium concentration consumed, the increased salt intake in the Recovery period, or both. Further studies are necessary to determine any alterations, if any, occur in these areas of the brain.

5.5 Clinical Implications

The low salt diet is usually one of the first line treatments for hypertension. However, it is clear from research that patients are often noncompliant despite interventions that attempt to increase compliance (Cohen et al., 1991; Pietenin et al., 1984; Staessen et al., 1988). Repeated dietary sodium deprivation in the rat functions as a physiological model for these specific patient populations who are noncompliant with their diet. Although none of the hormones we measured showed a definitive effect of multiple deprivations, behavior was affected by deprivations. While the immediate post-deprivation two-bottle tests did not have differences in salt intake between OIL- and EB-treated rats, EB-treated rats drank less concentrated solutions. This suggests that estrogen may cause less overcompensation in the immediate period following a time on the low salt diet. It is possible that postmenopausal women without hormone replacement therapy may consume higher amounts of salt when they “cheat” on their low salt diet. This phenomenon needs

to be verified in humans, but, if true, would require further counseling from the physician about the consequences of "cheating" for postmenopausal women.

Additionally, our data in the Recovery period suggest that estrogen results in long-lasting increases from baseline. Based on this, we can speculate that perhaps premenopausal women may unintentionally consume more salt after they have previously been on the low salt diet, despite having already replenished their salt after the time on the diet. For this reason, it may also be important to counsel premenopausal women on the consequences of "cheating" on the low salt diet, albeit for different reasons than postmenopausal women. Consuming more salt in the unstimulated period after the low salt diet may exacerbate hypertension in those who are salt sensitive. This may also apply to postmenopausal women on hormone replacement therapy, though the clinical applications in both populations will need to be explored further in future research.

It is already known that consuming salt can exacerbate hypertension and congestive heart failure, so individuals either with endogenous estrogen or on hormone replacement therapy should be aware of the possibility of greater salt cravings after exposure to the diet. It remains to be seen if estrogen status relates to greater incidence of exacerbations of salt sensitive hypertension and heart failure complications, but it is clear that this area must be explored further to better serve patients and provide appropriate education related to the low salt diet.

Our current study focused on females, which is an often overlooked population. This data and model could also be instructive in terms of broader applications. While we focused on the effects of estrogen, it also remains to be seen what effects are seen with repeated dietary sodium deprivations in males as well. While these two possible changes—increased intake immediately after deprivation in postmenopausal women, and increased intake for long periods after deprivation in premenopausal women—may not be a problem for many women, some may be

salt sensitive, and experience fluctuations in blood pressure with increasing salt consumption. Further research is needed on best methods for increasing compliance of this difficult diet, and how the animal studies apply to humans. Given the prevalence of salt in the American diet, it is imperative that this research expand so that physicians may counsel women on the importance of maintaining their diet, not just due to short term alterations, but because of possible long term changes that may have poor health consequences.

REFERENCES

Akkad, A. A., Halligan, A. W., Abrams, K., & al-Azzawi, F. (1997). Differing responses in blood pressure over 24 hours in normotensive women receiving oral or transdermal estrogen replacement therapy. *Obstetrics and Gynecology*, *89*(1), 97–103. https://doi.org/10.1016/s0029-7844(97)84258-5

Albanito, L., Madeo, A., Lappano, R., Vivacqua, A., Rago, V., Carpino, A., Oprea, T. I., Prossnitz, E. R., Musti, A. M., Andò, S., & Maggiolini, M. (2007). G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Research*, *67*(4), 1859–1866. https://doi.org/10.1158/0008-5472.CAN-06-2909

Antunes-Rodrigues, J., Ruginsk, S. G., Mecawi, A. S., Margatho, L. O., Cruz, J. C., Vilhena-Franco, T., Reis, W. L., Ventura, R. R., Reis, L. C., Vivas, L. M., & Elias, L. L. K. (2013). Mapping and signaling of neural pathways involved in the regulation of hydromineral homeostasis. *Brazilian Journal of Medical and Biological Research*, *46*(4), 327–338. https://doi.org/10.1590/1414-431X20132788

Arbogast, L. A., & Hyde, J. F. (2000). Estradiol attenuates the forskolin-induced increase in hypothalamic tyrosine hydroxylase activity. *Neuroendocrinology*, *71*(4), 219–227. https://doi.org/10.1159/000054539

Arnett, D. K., Blumenthal, R. S., Albert, M. A., Buroker, A. B., Goldberger, Z. D., Hahn, E. J., Himmelfarb, C. D., Khera, A., Lloyd-Jones, D., McEvoy, J. W., Michos, E. D., Miedema, M. D., Muñoz, D., Smith, S. C., Virani, S. S., Williams, K. A., Yeboah, J., & Ziaeian, B. (2019). 2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation*, *140*(11), e563–e595. https://doi.org/10.1161/CIR.0000000000000677

August, P., & Oparil, S. (1999). Hypertension in women. *J Clin Endocrinol Metab*, *84*(6), 1862–1866. https://doi.org/10.1210/jcem.84.6.5724

Barton, M., Filardo, E. J., Lolait, S. J., Thomas, P., Maggiolini, M., & Prossnitz, E. R. (2018). Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. *The Journal of Steroid Biochemistry and Molecular Biology*, *176*, 4–15. https://doi.org/10.1016/j.jsbmb.2017.03.021

Beardwell, C. G. (1971). Radioimmunoassay of Arginine Vasopressin in Human Plasma. *The Journal of Clinical Endocrinology & Metabolism*, *33*(2), 254–260. https://doi.org/10.1210/jcem-33-2-254

Berl, T., Cadnapaphornchai, P., Harbottle, J. A., & Schrier, R. W. (1974). Mechanism of Stimulation of Vasopressin Release during Beta Adrenergic Stimulation with Isoproterenol. *Journal of Clinical Investigation*, *53*(3), 857–867.

Björnström, L., & Sjöberg, M. (2005). Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Molecular Endocrinology*, *19*(4), 833–842. https://doi.org/10.1210/me.2004-0486

Bossmar, T., Forsling, M., & Akerlund, M. (1995). Circulating oxytocin and vasopressin is influenced by ovarian steroid replacement in women. *Acta Obstetricia Et Gynecologica Scandinavica*, *74*(7), 544–548. https://doi.org/10.3109/00016349509024387

Brum, J. M., Tramposch, A. F., & Ferrario, C. M. (1991). Neurovascular mechanisms and sodium balance in the pathogenesis of hypertension. *Hypertension*, *17*(1 Suppl), I45-51.

Campese, V. M., Romoff, M. S., Levitan, D., Saglikes, Y., Friedler, R. M., & Massry, S. G. (1982). Abnormal relationship between sodium intake and sympathetic nervous system activity in salt-sensitive patients with essential hypertension. *Kidney Int*, *21*(2), 371–378.

Caroccia, B., Seccia, T. M., Campos, A. G., Gioco, F., Kuppusamy, M., Ceolotto, G., Guerzoni, E., Simonato, F., Mareso, S., Lenzini, L., Fassina, A., & Rossi, G. P. (2014). GPER-1 and Estrogen Receptor-β Ligands Modulate Aldosterone Synthesis. *Endocrinology*, *155*(11), 4296–4304. https://doi.org/10.1210/en.2014-1416

Chow, S. Y., Sakai, R. R., Witcher, J. A., Adler, N. T., & Epstein, A. N. (1992). Sex and sodium intake in the rat. *Behav Neurosci*, *106*(1), 172–180.

Cohen, S. J., Weinberger, M. H., Fineberg, N. S., Miller, J. Z., Grim, C. E., & Luft, F. C. (1991). The effect of a household partner and home urine monitoring on adherence to a sodium restricted diet. *Social Science & Medicine (1982)*, *32*(9), 1057–1061. https://doi.org/10.1016/0277-9536(91)90163-7

Colucci, W. S., Gimbrone, M. A., McLaughlin, M. K., Halpern, W., & Alexander, R. W. (1982). Increased vascular catecholamine sensitivity and alpha-adrenergic receptor affinity in female and estrogen-treated male rats. *Circulation Research*, *50*(6), 805–811. https://doi.org/10.1161/01.RES.50.6.805

Cowley, A., Cushman, W., Quillen, E., Skelton, M., & Langford, H. (1981). Vasopressin elevation in essential hypertension and increased responsiveness to sodium intake. *Hypertension*. https://doi.org/10.1161/01.HYP.3.3_Pt_2.I93

Crane, M. G., & Harris, J. J. (1969). Plasma Renin Activity and Aldosterone Excretion Rate in Normal Subjects. I. Effect of Ethinyl Estradiol and Medroxyprogesterone Acetate. *The Journal of Clinical Endocrinology & Metabolism*, *29*(4), 550–557. https://doi.org/10.1210/jcem-29-4-550

Crofton, J. T., Baer, P. G., Share, L., & Brooks, D. P. (1985). Vasopressin release in male and female rats: Effects of gonadectomy and treatment with gonadal steroid hormones. *Endocrinology*, *117*(3), 1195–1200. https://doi.org/10.1210/endo-117-3-1195

Crofton, Joan T., Ota, M., & Share, L. (1993). Role of vasopressin, the renin-angiotensin system and sex in Dahl salt-sensitive hypertension. *Journal of Hypertension*, *11*(10), 1031–1038. https://doi.org/10.1097/00004872-199310000-00005

Crofton, Joan T, & Share, L. (1997). Gonadal Hormones Modulate Deoxycorticosterone-Salt Hypertension in Male and Female Rats | Hypertension. *Hypertension*, *29*(1), 494–499.

Curtis, K. S., & Contreras, R. J. (2006). Sex differences in electrophysiological and behavioral responses to NaCl taste. *Behavioral Neuroscience*, *120*(4), 917–924. https://doi.org/10.1037/0735-7044.120.4.917

Dampney, R. A. L., Polson, J. W., Potts, P. D., Hirooka, Y., & Horiuchi, J. (2003). Functional organization of brain pathways subserving the baroreceptor reflex: Studies in conscious animals using immediate early gene expression. *Cellular and Molecular Neurobiology*, *23*(4–5), 597–616.

DiNicolantonio, J., & O'Keefe, J. (2017). The History of the Salt Wars. *The American Journal of Medicine*, *130*(9), 1011–1014. https://doi.org/10.1016/j.amjmed.2017.04.040

Dunn, F. L., Brennan, T. J., Nelson, A. E., & Robertson, G. L. (1973). The role of blood osmolality and volume in regulating vasopressin secretion in the rat. *The Journal of Clinical Investigation*, *52*(12), 3212–3219. https://doi.org/10.1172/JCI107521

Esler, M., Julius, S., Zweifler, A., Randall, O., Harburg, E., Gardiner, H., & DeQuattro, V. (1977). Mild high-renin essential hypertension. Neurogenic human hypertension? *The New England Journal of Medicine*, *296*(8), 405–411. https://doi.org/10.1056/NEJM197702242960801

Falk, J. L. (1966). Serial sodium depletion and NaCl solution intake. *Physiology & Behavior*, *1*(1), 75–77. https://doi.org/10.1016/0031-9384(66)90044-8

Fang, Z., Carlson, S. H., Chen, Y. F., Oparil, S., & Wyss, J. M. (2001). Estrogen depletion induces NaCl-sensitive hypertension in female spontaneously hypertensive rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *281*(6), R1934–R1939. https://doi.org/10.1152/ajpregu.2001.281.6.R1934

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., & Thomas, P. (2007). Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology*, *148*(7), 3236–3245. https://doi.org/10.1210/en.2006-1605

Fluharty, S. J., & Epstein, A. N. (1983). Sodium appetite elicited by intracerebroventricular infusion of angiotensin II in the rat: II. Synergistic interaction with systemic mineralocorticoids. *Behavioral Neuroscience*, *97*(5), 746–758. https://doi.org/10.1037//0735-7044.97.5.746

Flynn, F. W., Kirchner, T. R., & Clinton, M. E. (2002). Brain vasopressin and sodium appetite. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *282*(4), R1236–R1244. https://doi.org/10.1152/ajpregu.00181.2001

Fommei, E., Chatzianagnostou, K., & Ghione, S. (2016). The importance of knowing the timing within the menstrual cycle in nonmenopausal hypertensive women in the diagnostic workup for primary aldosteronism. *Clinical Endocrinology*, *84*(5), 791–792. https://doi.org/10.1111/cen.13005

Forte, J. G., Miguel, J. M., Miguel, M. J., de Pádua, F., & Rose, G. (1989). Salt and blood pressure: A community trial. *Journal of Human Hypertension*, *3*(3), 179–184.

Franco-Bourland, R. E. (1998). Vasopressinergic, oxytocinergic, and somatostatinergic neuronal activity after adrenalectomy and immobilization stress. *Neurochemical Research*, *23*(5), 695–701. https://doi.org/10.1023/a:1022447023840

Fregly, M. J. (1973). Effect of an oral contraceptive on NaCl appetite and preference threshold in rats. *Pharmacology, Biochemistry, and Behavior*, *1*(1), 61–65. https://doi.org/10.1016/0091-3057(73)90056-7

Fressinaud, P., Corvol, P., Menard, J., & Allegrini, J. (1974). Radioimmunoassay of urinary antidiuretic hormone in man: Stimulation–suppression tests. *Kidney International*, *6*(3), 184–190. https://doi.org/10.1038/ki.1974.97

Fryar, C. D., Ostchega, Y., Hales, C. M., & Kruszon-Moran, D. (2017, December 20). *Hypertension prevalence and control among adults: United States, 2015-2016*. NCHS Data Brief, No 289. https://www.cdc.gov/nchs/products/databriefs/db289.htm

Gabor, A., & Leenen, F. H. H. (2013). Central Mineralocorticoid Receptors and the Role of Angiotensin II and Glutamate in the Paraventricular Nucleus of Rats With Angiotensin

II–Induced Hypertension. *Hypertension*, *61*(5), 1083–1090. https://doi.org/10.1161/HYPERTENSIONAHA.111.00797

Geerling, J. C., & Loewy, A. D. (2008). Central regulation of sodium appetite. *Experimental Physiology*, *93*(2), 177–209. https://doi.org/10.1113/expphysiol.2007.039891

Giuliani, C., & Peri, A. (2014). Effects of Hyponatremia on the Brain. *Journal of Clinical Medicine*, *3*(4), 1163–1177. https://doi.org/10.3390/jcm3041163

Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Borden, W. B., Bravata, D. M., Dai, S., Ford, E. S., Fox, C. S., Franco, S., Fullerton, H. J., Gillespie, C., Hailpern, S. M., Heit, J. A., Howard, V. J., Huffman, M. D., Kissela, B. M., Kittner, S. J., … Turner, M. B. (2013). Heart Disease and Stroke Statistics—2013 Update. *Circulation*, *127*(1), e6–e245. https://doi.org/10.1161/CIR.0b013e31828124ad

Gomez-Sanchez, E. P., Ahmad, N., Romero, D. G., & Gomez-Sanchez, C. E. (2005). Is aldosterone synthesized within the rat brain? *American Journal of Physiology. Endocrinology and Metabolism*, *288*(2), E342-346. https://doi.org/10.1152/ajpendo.00355.2004

Graudal, N. A., Hubeck-Graudal, T., & Jurgens, G. (2012). Effects of low-sodium diet vs. High-sodium diet on blood pressure, renin, aldosterone, catecholamines, cholesterol, and triglyceride (Cochrane Review). *Am J Hypertens*, *25*(1), 1–15. https://doi.org/10.1038/ajh.2011.210

Greene, G., Gilna, P., Waterfield, M., Baker, A., Hort, Y., & Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science*, *231*(4742), 1150–1154. https://doi.org/10.1126/science.3753802

Guyton, A. C., Coleman, T. G., Cowley, A. W., Scheel, K. W., Manning, R. D., & Norman, R. A. (1972). Arterial pressure regulation: Overriding dominance of the kidneys in long-term regulation and in hypertension. *The American Journal of Medicine*, *52*(5), 584–594. https://doi.org/10.1016/0002-9343(72)90050-2

Haas, E., Bhattacharya, I., Brailoiu, E., Damjanović, M., Brailoiu, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., Meyer, M. R., Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., Clegg, D. J., Dun, N. J., Resta, T. C., Prossnitz, E. R., & Barton, M. (2009). Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circulation Research*, *104*(3), 288–291. https://doi.org/10.1161/CIRCRESAHA.108.190892

Hall, J. E. (2015). *Guyton and Hall Textbook of Medical Physiology* (13th ed.). Saunders. https://www.elsevier.com/books/guyton-and-hall-textbook-of-medical-physiology/hall/978-1-4557-7005-2

Hay, M. (2016). Sex, the brain and hypertension: Brain oestrogen receptors and high blood pressure risk factors. *Clinical Science (London, England: 1979)*, *130*(1), 9–18. https://doi.org/10.1042/CS20150654

Hay, M., Xue, B., & Johnson, A. K. (2014). Yes! Sex Matters: Sex, The Brain and Blood Pressure. *Current Hypertension Reports*, *16*(8), 458. https://doi.org/10.1007/s11906-014-0458-4

He, F. J., & MacGregor, G. A. (2002). Effect of modest salt reduction on blood pressure: A meta-analysis of randomized trials. Implications for public health. *Journal of Human Hypertension*, *16*(11), 761–770. https://doi.org/10.1038/sj.jhh.1001459

He, Feng J., & MacGregor, G. A. (2010). Reducing Population Salt Intake Worldwide: From Evidence to Implementation. *Progress in Cardiovascular Diseases*, *52*(5), 363–382. https://doi.org/10.1016/j.pcad.2009.12.006

He, X.-R., Wang, W., Crofton, J. T., & Share, L. (1998). Effects of 17β-estradiol on sympathetic activity and pressor response to phenylephrine in ovariectomized rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *275*(4), R1202–R1208. https://doi.org/10.1152/ajpregu.1998.275.4.R1202

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., & Gustafsson, J.-Å. (2007). Estrogen Receptors: How Do They Signal and What Are Their Targets. *Physiological Reviews*, *87*(3), 905–931. https://doi.org/10.1152/physrev.00026.2006

Hilliard, L. M., Nematbakhsh, M., Kett, M. M., Teichman, E., Sampson, Widdop, R. E., Evans, R. G., & Denton, K. M. (2011). Gender Differences in Pressure-Natriuresis and Renal Autoregulation. *Hypertension*, *57*(2), 275–282. https://doi.org/10.1161/HYPERTENSIONAHA.110.166827

Hinojosa-Laborde, C., Lange, D. L., & Haywood, J. R. (2000). Role of female sex hormones in the development and reversal of dahl hypertension. *Hypertension*, *35*(1 Pt 2), 484–489.

Honda, K., Aradachi, H., Higuchi, T., Takano, S., & Negoro, H. (1992). Activation of paraventricular neurosecretory cells by local osmotic stimulation of the median preoptic nucleus. *Brain Research*, *594*(2), 335–338. https://doi.org/10.1016/0006-8993(92)91147-7

Imai, Y., Nolan, P. L., & Johnston, C. I. (1983). Restoration of suppressed baroreflex sensitivity in rats with hereditary diabetes insipidus (Brattleboro rats) by arginine-

vasopressin and DDAVP. *Circulation Research*, *53*(2), 140–149. https://doi.org/10.1161/01.res.53.2.140

Jackson, L. V., Thalange, N. K. S., & Cole, T. J. (2007). Blood pressure centiles for Great Britain. *Archives of Disease in Childhood*, *92*(4), 298–303. https://doi.org/10.1136/adc.2005.081216

Jacob, F., Clark, L. A., Guzman, P. A., & Osborn, J. W. (2005). Role of renal nerves in development of hypertension in DOCA-salt model in rats: A telemetric approach. *American Journal of Physiology-Heart and Circulatory Physiology*, *289*(4), H1519–H1529. https://doi.org/10.1152/ajpheart.00206.2005

James, P. A., Oparil, S., Carter, B. L., Cushman, W. C., Dennison-Himmelfarb, C., Handler, J., Lackland, D. T., LeFevre, M. L., MacKenzie, T. D., Ogedegbe, O., Smith, S. C., Svetkey, L. P., Taler, S. J., Townsend, R. R., Wright, J. T., Narva, A. S., & Ortiz, E. (2014). 2014 Evidence-Based Guideline for the Management of High Blood Pressure in Adults: Report From the Panel Members Appointed to the Eighth Joint National Committee (JNC 8). *JAMA*, *311*(5), 507–520. https://doi.org/10.1001/jama.2013.284427

Kawasaki, T., Delea, C. S., Bartter, F. C., & Smith, H. (1978). The effect of high-sodium and low-sodium intakes on blood pressure and other related variables in human subjects with idiopathic hypertension. *Am J Med*, *64*(2), 193–198.

Kempner, W. (1948). Treatment of hypertensive vascular disease with rice diet. *The American Journal of Medicine*, *4*(4), 545–577. https://doi.org/10.1016/0002-9343(48)90441-0

Khoury, S., Yarows, S. A., O'Brien, T. K., & Sowers, J. R. (1992). Ambulatory blood pressure monitoring in a nonacademic setting. Effects of age and sex. *American Journal of Hypertension*, *5*(9), 616–623. https://doi.org/10.1093/ajh/5.9.616

Kim, J. S., Kim, W. B., Kim, Y.-B., Lee, Y., Kim, Y. S., Shen, F.-Y., Lee, S. W., Park, D., Choi, H.-J., Hur, J., Park, J. J., Han, H. C., Colwell, C. S., Cho, Y.-W., & Kim, Y. I. (2011). Chronic Hyperosmotic Stress Converts GABAergic Inhibition into Excitation in Vasopressin and Oxytocin Neurons in the Rat. *Journal of Neuroscience*, *31*(37), 13312–13322. https://doi.org/10.1523/JNEUROSCI.1440-11.2011

Kirkland, E. B., Heincelman, M., Bishu, K. G., Schumann, S. O., Schreiner, A., Axon, R. N., Mauldin, P. D., & Moran, W. P. (2018). Trends in Healthcare Expenditures Among US Adults With Hypertension: National Estimates, 2003–2014. *Journal of the American Heart Association*, *7*(11). https://doi.org/10.1161/JAHA.118.008731

Kotchen, T. A. (2018). Chapter 271: Hypertensive Vascular Disease. In *Harrison's Principles of Internal Medicine* (20th ed., Vol. 1–2). McGraw-Hill Education / Medical.

Krause, E. G., & Sakai, R. R. (2007). Richter and sodium appetite: From adrenalectomy to molecular biology. *Appetite*, *49*(2), 353–367. https://doi.org/10.1016/j.appet.2007.01.015

Křeček, J., Nováková, V., & Stibral, K. (1972). Sex differences in the taste preference for a salt solution in the rat. *Physiology & Behavior*, *8*(2), 183–188. https://doi.org/10.1016/0031-9384(72)90358-7

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(12), 5925–5930.

Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., & Webb, P. (2000). Estrogen receptor pathways to AP-1. *The Journal of Steroid Biochemistry and Molecular Biology*, *74*(5), 311–317. https://doi.org/10.1016/S0960-0760(00)00108-4

Leshem, M., Kavushansky, A., Devys, J.-M., & Thornton, S. (2004). Enhancement revisited: The effects of multiple depletions on sodium intake in rats vary with strain, substrain, and gender. *Physiology & Behavior*, *82*(2–3), 571–580. https://doi.org/10.1016/j.physbeh.2004.05.003

Lewis, J. (2018). Hypernatremia. In *The Merck Manual of Diagnosis and Therapy* (20th ed.). Wiley-Blackwell.

Liberman, B., Klein, L. A., & Kleeman, C. R. (1970). Effect of Adrenergic Blocking Agents on the Vasopressin Inhibiting Action of Norepinephrine. *Proceedings of the Society for Experimental Biology and Medicine*, *133*(1), 131–134. https://doi.org/10.3181/00379727-133-34424

Lim, S. S., Vos, T., Flaxman, A. D., Danaei, G., Shibuya, K., Adair-Rohani, H., Amann, M., Anderson, H. R., Andrews, K. G., Aryee, M., Atkinson, C., Bacchus, L. J., Bahalim, A. N., Balakrishnan, K., Balmes, J., Barker-Collo, S., Baxter, A., Bell, M. L., Blore, J. D., … Memish, Z. A. (2012). A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, *380*(9859), 2224–2260. https://doi.org/10.1016/S0140-6736(12)61766-8

Ludwig, M., Williams, K., Callahan, M. F., & Morris, M. (1996). Salt loading abolishes osmotically stimulated vasopressin release within the supraoptic nucleus. *Neuroscience Letters*, *215*(1), 1–4. https://doi.org/10.1016/s0304-3940(96)12956-6

Macova, M., Armando, I., Zhou, J., Baiardi, G., Tyurmin, D., Larrayoz-Roldan, I. M., & Saavedra, J. M. (2008). Estrogen Reduces Aldosterone, Upregulates Adrenal Angiotensin

II AT2 Receptors and Normalizes Adrenomedullary Fra-2 in Ovariectomized Rats. *Neuroendocrinology*, *88*(4), 276–286. https://doi.org/10.1159/000150977

Malyala, A., Kelly, M. J., & Rønnekleiv, O. K. (2005). Estrogen modulation of hypothalamic neurons: Activation of multiple signaling pathways and gene expression changes. *Steroids*, *70*(5), 397–406. https://doi.org/10.1016/j.steroids.2005.03.004

Masubuchi, Y., Kumai, T., Uematsu, A., Komoriyama, K., & Hirai, M. (1982). Gonadectomy-induced reduction of blood pressure in adult spontaneously hypertensive rats. *Acta Endocrinologica*, *101*(1), 154–160. https://doi.org/10.1530/acta.0.1010154

McBryde, F. D., Malpas, S. C., Guild, S.-J., & Barrett, C. J. (2009). A high-salt diet does not influence renal sympathetic nerve activity: A direct telemetric investigation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *297*(2), R396-402. https://doi.org/10.1152/ajpregu.90741.2008

Miller, J. A., Anacta, L. A., & Cattran, D. C. (1999). Impact of gender on the renal response to angiotensin II. *Kidney International*, *55*(1), 278–285. https://doi.org/10.1046/j.1523-1755.1999.00260.x

Mohring, J., Mohring, B., Petri, M., & Haack, D. (1977). Vasopressor role of ADH in the pathogenesis of malignant DOC hypertension. *American Journal of Physiology-Renal Physiology*, *232*(3), F260–F269. https://doi.org/10.1152/ajprenal.1977.232.3.F260

Mosselman, S., Polman, J., & Dijkema, R. (1996). ER beta: Identification and characterization of a novel human estrogen receptor. *FEBS Letters*, *392*(1), 49–53. https://doi.org/10.1016/0014-5793(96)00782-x

Na, E. S., Morris, M. J., Johnson, R. F., Beltz, T. G., & Johnson, A. K. (2007). The neural substrates of enhanced salt appetite after repeated sodium depletions. *Brain Research*, *1171*, 104–110. https://doi.org/10.1016/j.brainres.2007.07.033

Ota, M., Crofton, J. T., Liu, H., Festavan, G., & Share, L. (1994). Increased plasma osmolality stimulates peripheral and central vasopressin release in male and female rats. *The American Journal of Physiology*, *267*(4 Pt 2), R923-928. https://doi.org/10.1152/ajpregu.1994.267.4.R923

Owman, C., Blay, P., Nilsson, C., & Lolait, S. J. (1996). Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochemical and Biophysical Research Communications*, *228*(2), 285–292. https://doi.org/10.1006/bbrc.1996.1654

Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J.-Å., Kushner, P. J., & Scanlan, T. S. (1997). Differential Ligand Activation of Estrogen Receptors ERα and ERβ at AP1 Sites. *Science*, *277*(5331), 1508–1510. https://doi.org/10.1126/science.277.5331.1508

Pechere-Bertschi, A., & Burnier, M. (2004). Female sex hormones, salt, and blood pressure regulation. *Am J Hypertens*, *17*(10), 994–1001. https://doi.org/10.1016/j.amjhyper.2004.08.009

Peysner, K., & Forsling, M. L. (1990). Effect of ovariectomy and treatment with ovarian steroids on vasopressin release and fluid balance in the rat. *The Journal of Endocrinology*, *124*(2), 277–284. https://doi.org/10.1677/joe.0.1240277

Pietinen, P., Tanskanen, A., Nissinen, A., Tuomilehto, J., & Puska, P. (1984). Changes in dietary habits and knowledge concerning salt during a community-based prevention programme for hypertension. *Annals of Clinical Research*, *16 Suppl 43*, 150–155.

Pietras, R. J., & Szego, C. M. (1977). Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature*, *265*(5589), 69–72. https://doi.org/10.1038/265069a0

Pilic, L., Pedlar, C. R., & Mavrommatis, Y. (2016). Salt-sensitive hypertension: Mechanisms and effects of dietary and other lifestyle factors. *Nutrition Reviews*, *74*(10), 645–658. https://doi.org/10.1093/nutrit/nuw028

Raynaud, J.-P., Mercier-Bodard, C., & Baulieu, E. E. (1971). Rat Estradiol Binding Plasma Protein (EBP). *Steroids*, *18*(6), 767–788. https://doi.org/10.1016/0039-128X(71)90035-3

Reckelhoff, J. F. (2001). Gender differences in the regulation of blood pressure. *Hypertension*, *37*(5), 1199–1208.

Rice, K. K., & Richter, C. P. (1943). Increased sodium chloride and water intake of normal rats treated with desoxycorticosterone acetate. *Endocrinology*, *33*, 106–115. https://doi.org/10.1210/endo-33-2-106

Richter, C. P. (1936). Increased salt appetite in adrenalectomized rats. *American Journal of Physiology-Legacy Content*, *115*(1), 155–161. https://doi.org/10.1152/ajplegacy.1936.115.1.155

Robertson, G. L., Shelton, R. L., & Athar, S. (1976). The osmoregulation of vasopressin. *Kidney International*, *10*(1), 25–37. https://doi.org/10.1038/ki.1976.76

Roesch, D. M., Tian, Y., Zheng, W., Shi, M., Verbalis, J. G., & Sandberg, K. (2000). Estradiol attenuates angiotensin-induced aldosterone secretion in ovariectomized rats. *Endocrinology*, *141*(12), 4629–4636. https://doi.org/10.1210/endo.141.12.7822

Roitman, M. F., Na, E., Anderson, G., Jones, T. A., & Bernstein, I. L. (2002). Induction of a Salt Appetite Alters Dendritic Morphology in Nucleus Accumbens and Sensitizes Rats to Amphetamine. *The Journal of Neuroscience*, *22*(11), RC225. https://doi.org/10.1523/JNEUROSCI.22-11-j0001.2002

Romoff, M. S., Keusch, G., Campese, V. M., Wang, M. S., Friedler, R. M., Weidmann, P., & Massry, S. G. (1979). Effect of sodium intake on plasma catecholamines in normal subjects. *J Clin Endocrinol Metab*, *48*(1), 26–31. https://doi.org/10.1210/jcem-48-1-26

Rosamond, W., Flegal, K., Friday, G., Furie, K., Go, A., Greenlund, K., Haase, N., Ho, M., Howard, V., Kissela, B., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C. J., Roger, V., … American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2007). Heart disease and stroke statistics--2007 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*, *115*(5), e69-171. https://doi.org/10.1161/CIRCULATIONAHA.106.179918

Rowland, M., & Roberts, J. (1982). Blood pressure levels and hypertension in persons ages 6-74 years: United States, 1976-80. *Advance Data*, *84*, 1–11.

Sakai, R. R., Fine, W. B., Epstein, A. N., & Frankmann, S. P. (1987). Salt appetite is enhanced by one prior episode of sodium depletion in the rat. *Behavioral Neuroscience*, *101*(5), 724–731.

Sakai, R. R., Frankmann, S. P., Fine, W. B., & Epstein, A. N. (1989). Prior episodes of sodium depletion increase the need-free sodium intake of the rat. *Behav Neurosci*, *103*(1), 186–192.

Sakai, R. R., Nicolaïdis, S., & Epstein, A. N. (1986). Salt appetite is suppressed by interference with angiotensin II and aldosterone. *The American Journal of Physiology*, *251*(4 Pt 2), R762-768. https://doi.org/10.1152/ajpregu.1986.251.4.R762

Santollo, J., & Daniels, D. (2015). Control of fluid intake by estrogens in the female rat: Role of the hypothalamus. *Frontiers in Systems Neuroscience*, *9*. https://doi.org/10.3389/fnsys.2015.00025

Sato, M. A., Sugawara, A. M., Menani, J. V., & De Luca, L. A. (1997). Idazoxan and the effect of intracerebroventricular oxytocin or vasopressin on sodium intake of sodium-depleted rats. *Regulatory Peptides*, *69*(3), 137–142. https://doi.org/10.1016/S0167-0115(97)00005-0

Schulman, I. H., Aranda, P., Raij, L., Veronesi, M., Aranda, F. J., & Martin, R. (2006). Surgical menopause increases salt sensitivity of blood pressure. *Hypertension*, *47*(6), 1168–1174. https://doi.org/10.1161/01.HYP.0000218857.67880.75

Seely, E. W., Walsh, B. W., Gerhard, M. D., & Williams, G. H. (1999). Estradiol With or Without Progesterone and Ambulatory Blood Pressure in Postmenopausal Women. *Hypertension*, *33*(5), 1190–1194. https://doi.org/10.1161/01.HYP.33.5.1190

Serova, L., Rivkin, M., Nakashima, A., & Sabban, E. L. (2002). Estradiol stimulates gene expression of norepinephrine biosynthetic enzymes in rat locus coeruleus. *Neuroendocrinology*, *75*(3), 193–200. https://doi.org/10.1159/000048237

Sherlock, D. A., Field, P. M., & Raisman, G. (1975). Retrograde transport of horseradish peroxidase in the magnocellular neurosecretory system of the rat. *Brain Research*, *88*(3), 403–414. https://doi.org/10.1016/0006-8993(75)90653-8

Shimamoto, K., & Miyahara, M. (1976). Effect of Norepinephrine Infusion on Plasma Vasopressin Levels in Normal Human Subjects. *The Journal of Clinical Endocrinology & Metabolism*, *43*(1), 201–204. https://doi.org/10.1210/jcem-43-1-201

Skowsky, W. R., Swan, L., & Smith, P. (1979). Effects of sex steroid hormones on arginine vasopressin in intact and castrated male and female rats. *Endocrinology*, *104*(1), 105–108. https://doi.org/10.1210/endo-104-1-105

Sladek, C. D., & Somponpun, S. J. (2008). Estrogen receptors: Their roles in regulation of vasopressin release for maintenance of fluid and electrolyte homeostasis. *Front Neuroendocrinol*, *29*(1), 114–127. https://doi.org/10.1016/j.yfrne.2007.08.005

Sladek, C. D., Swenson, K. L., Kapoor, R., & Sidorowicz, H. E. (2000). The role of steroid hormones in the regulation of vasopressin and oxytocin release and mRNA expression in hypothalamo-neurohypophysial explants from the rat. *Exp Physiol*, *85 Spec No*, 171S-177S.

Spielman, W. S., & Davis, J. O. (1974). The Renin-Angiotensin System and Aldosterone Secretion during Sodium Depletion in the Rat. *Circulation Research*, *35*(4), 615–624. https://doi.org/10.1161/01.RES.35.4.615

Staessen, J., Bulpitt, C. J., Fagard, R., Joossens, J. V., Lijnen, P., & Amery, A. (1988). Salt intake and blood pressure in the general population: A controlled intervention trial in two towns. *Journal of Hypertension*, *6*(12), 965–973. https://doi.org/10.1097/00004872-198812000-00003

Staessen, J., Bulpitt, C. J., Fagard, R., Lijnen, P., & Amery, A. (1989). The influence of menopause on blood pressure. *Journal of Human Hypertension*, *3*(6), 427–433.

Stricker, E. M. (1981). Thirst and sodium appetite after colloid treatment in rats. *Journal of Comparative and Physiological Psychology*, *95*(1), 1–25. https://doi.org/10.1037/h0077764

Stricker, E. M., Thiels, E., & Verbalis, J. G. (1991). Sodium appetite in rats after prolonged dietary sodium deprivation: A sexually dimorphic phenomenon. *Am J Physiol*, *260*(6 Pt 2), R1082-8. https://doi.org/10.1152/ajpregu.1991.260.6.R1082

Summy-Long, J. Y., & Kadekaro, M. (2001). Circumventricular Organs: Gateways to the Brain Role Of Circumventricular Organs (CVO) In Neuroendocrine Responses: Interactions Of Cvo And The Magnocellular Neuroendocrine System In Different Reproductive States. *Clinical and Experimental Pharmacology and Physiology*, *28*(7), 590–601. https://doi.org/10.1046/j.1440-1681.2001.03491.x

Szczepańska-Sadowska, E., Sobocińska, J., & Sadowski, B. (1982). Central dipsogenic effect of vasopressin. *The American Journal of Physiology*, *242*(3), R372-379. https://doi.org/10.1152/ajpregu.1982.242.3.R372

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., & Waterfield, M. (1985). Cloning of the human estrogen receptor cDNA. *Proceedings of the National Academy of Sciences*, *82*(23), 7889–7893. https://doi.org/10.1073/pnas.82.23.7889

Wang, Y. X., Crofton, J. T., Miller, J., Sigman, C. J., Liu, H., Huber, J. M., Brooks, D. P., & Share, L. (1996). Sex difference in urinary concentrating ability of rats with water

deprivation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *270*(3), R550–R555. https://doi.org/10.1152/ajpregu.1996.270.3.R550

Weinberger, M. H., Cohen, S. J., Miller, J. Z., Luft, F. C., Grim, C. E., & Fineberg, N. S. (1988). Dietary sodium restriction as adjunctive treatment of hypertension. *JAMA*, *259*(17), 2561–2565.

Weinberger, M. H., Miller, J. Z., Luft, F. C., Grim, C. E., & Fineberg, N. S. (1986). Definitions and characteristics of sodium sensitivity and blood pressure resistance. *Hypertension*, *8*(6), 127–134.

Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., & Moncada, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proceedings of the National Academy of Sciences*, *91*(11), 5212–5216. https://doi.org/10.1073/pnas.91.11.5212

Weissgerber, T. L., & Wolfe, L. A. (2011). Physiological adaptation in early human pregnancy: Adaptation to balance maternal-fetal demands. *Applied Physiology, Nutrition, and Metabolism*. https://doi.org/10.1139/h05-003

What We Eat in America. (n.d.). U.S. Department of Health and Human Services, U.S. Department of Agriculture. Retrieved March 24, 2021, from https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/1314/Table_1_NIN_GEN_13.pdf

Whelton, P. K., Carey, R. M., Aronow, W. S., Casey, D. E., Collins, Himmelfarb, C. D., DePalma, S. M., Gidding, S., Jamerson, K. A., Jones, D. W., MacLaughlin, E. J., Muntner, P., Ovbiagele, B., Smith, S. C., Spencer, C. C., Stafford, R. S., Taler, S. J., Thomas, R. J., Williams, K. A., … Wright, J. T. (2018). 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the

Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Hypertension*, *71*(6), e13–e115. https://doi.org/10.1161/HYP.0000000000000065

Wiinberg, N., Høegholm, A., Christensen, H. R., Bang, L. E., Mikkelsen, K. L., Nielsen, P. E., Svendsen, T. L., Kampmann, J. P., Madsen, N. H., & Bentzon, M. W. (1995). 24-h ambulatory blood pressure in 352 normal Danish subjects, related to age and gender. *American Journal of Hypertension*, *8*(10 Pt 1), 978–986. https://doi.org/10.1016/0895-7061(95)00216-2

Wolf, G. (1982). Refined salt appetite methodology for rats demonstrated by assessing sex differences. *J Comp Physiol Psychol*, *96*(6), 1016–1021.

Wolf, George. (1965). Effect of deoxycorticosterone on sodium appetite of intact and adrenalectomized rats. *American Journal of Physiology-Legacy Content*, *208*(6), 1281–1285. https://doi.org/10.1152/ajplegacy.1965.208.6.1281

Xue, B., Zhang, Z., Beltz, T. G., Johnson, R. F., Guo, F., Hay, M., & Johnson, A. K. (2013). Estrogen receptor-beta (ERβ) in the PVN and RVLM plays an essential protective role in aldosterone/salt-induced hypertension in female rats. *Hypertension*, *61*(6), 1255–1262. https://doi.org/10.1161/HYPERTENSIONAHA.111.00903

Yanagihara, N., Liu, M., Toyohira, Y., Tsutsui, M., Ueno, S., Shinohara, Y., Takahashi, K., & Tanaka, K. (2006). Stimulation of catecholamine synthesis through unique estrogen receptors in the bovine adrenomedullary plasma membrane by 17beta-estradiol. *Biochemical and Biophysical Research Communications*, *339*(2), 548–553. https://doi.org/10.1016/j.bbrc.2005.11.047

Zanchetti, A., Facchetti, R., Cesana, G. C., Modena, M. G., Pirrelli, A., Sega, R., & SIMONA participants. (2005). Menopause-related blood pressure increase and its relationship to age and body mass index: The SIMONA epidemiological study. *Journal of Hypertension*, *23*(12), 2269–2276. https://doi.org/10.1097/01.hjh.0000194118.35098.43

Zheng, W., Ji, H., Maric, C., Wu, X., & Sandberg, K. (2008). Effect of dietary sodium on estrogen regulation of blood pressure in Dahl salt-sensitive rats. *Am J Physiol Heart Circ Physiol*, *294*(4), H1508-13. https://doi.org/10.1152/ajpheart.01322.2007

VITA

Emily Morgan Ehresman

Candidate for the Degree of

Doctor of Philosophy

Dissertation: ESTROGEN MODULATION OF PHYSIOLOGICAL BODY FLUID CONTROL DURING REPEATED SODIUM DEFICIENCY

Major Field: Biomedical Sciences

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Biomedical Sciences at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in May, 2021.

Completed the requirements for the Bachelor of Science in Biology Pre-Medicine at Oral Roberts University, Tulsa, Oklahoma in 2015.

Professional Memberships: American Physiological Society, Society for the Study of Ingestive Behaviors